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(*S*)-(+)-LINALOOL, A MATE ATTRACTANT PHEROMONE COMPONENT IN THE BEE *Colletes cunicularius*

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Abstract—Enantiomerically pure (*S*)-(+)-linalool was the main constituent in the extracts of the cephalic secretions of virgin females, mated females, freshly emerged males, and patrolling males of the solitary bee *Colletes cunicularius*. After copulation, the content of (*S*)-(+)-linalool emitted by the female was strongly reduced. Electrophysiological experiments revealed that both enantiomers of linalool elicited responses from the antennae of the males. Field tests using the pure enantiomers and the racemate of linalool showed that the number of male bees attracted was highest for (*S*)-(+)-linalool. The search flight activity in the mating flight area increased dramatically when patrolling males were presented with (*S*)-(+)-linalool *vs* (*R*)-(−)-linalool. Taken together, these data indicate a mate attractant pheromone function of (*S*)-(+)-linalool.

Key Words—Hymenoptera, *Colletes*, solitary bee, (*S*)-(+)-linalool, chirality, pheromone, sex attraction, behavior, pollination.

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INTRODUCTION

The specificity and the degree of complexity of a chemical signal are expected to reflect the strength of environmental constraints during the evolutionary process, having their backgrounds in intra- and/or interspecific competition. Species and sex recognition, as well as female choice and antagonistic sexual selection, play important roles in shaping the acuity of a mate attraction signal (West-Eberhard, 1984; Tengö et al., 1989; Ayasse et al., 2001).

The solitary bee, *Colletes cunicularius* (L.) (Hymenoptera: Colletidae), is a species that flies in early spring. Few bees or other insect species are in flight during the short mating season of *C. cunicularius* (normally 3–10 days), possibly indicating weak competition with other species in the evolutionary establishment of signal specificity. *C. cunicularius* has to cope with an often harsh meteorological situation, possibly entailing a need for parsimony of the investment in the production of signal substances. The male-attracting female mandibular gland secretion is, in fact, composed of only few volatile compounds as compared with the secretion of other solitary or social bee species (cf. Tengö and Bergström, 1977; Tengö, 1979; Bergström et al., 1982; Hefetz et al., 1996; Francke et al., 2000), and linalool is the dominant compound in the secretion of receptive females. Earlier studies revealed that a mixture of linalool enantiomers presented to *C. cunicularius* males patrolling over a nest aggregation in search of mates releases an immediate increase in flight activity (Bergström and Tengö, 1978), as well as in other *Colletes* species (Lindsley and Zavortnik, 1977). Higher doses elicited both a more local search behavior and a higher general activity (Bergström and Tengö, 1978; Cane and Tengö, 1981), but did not release as many approach flights directed to the odor source as did extracts of male or female bees. In addition, extracts of heads attracted more patrolling males, caused more local searching, and elicited more copulatory pouncing than linalool alone (Cane and Tengö, 1981). Since the enantiomeric composition of chiral semiochemicals may play a crucial role in the biological activity of a signal (Mori, 1998), the surplus of an enantiomer of linalool not produced/emitted by the bee species may explain the low degree of close attraction in earlier tests with the synthetic blend of linalool.

Patrolling males locate the spot where a virgin female will emerge from her subterranean natal cell, even before she reaches the soil surface. The aggregated males dig competitively to mate with the monandrous female (Bergström and Tengö, 1978). Larsen et al. (1986) have found that, in addition to the odor, the buzzing of the emerging female may function as an auxiliary signal in facilitating the pinpointing of emerging females.

The development of chiral stationary phases in gas chromatography (Schurig and Novotny, 1990; König, 1992) has simplified the determination of the enantiomeric compositions of chiral compounds, especially when applied in a two-dimensional GC system for separating complex mixtures (Borg-Karlson et al.,

1993 and references therein). Here, we report on the determination of the enantiomeric composition of the linalool present in the cephalic secretions of virgin females, nesting females, and patrolling males of *C. cunicularius*. The attractiveness of the linalool enantiomers and the racemate to patrolling males was evaluated in field tests.

METHODS AND MATERIALS

Biological Samples. For extraction of volatiles, bees were collected at different stages during the life cycle: 22 virgin females (excavated during emergence from their natal nest); 16 freshly mated females (collected immediately after completing copulation); 5 nesting females; 10 emerging males; and 15 patrolling males. The bees were kept in chilled boxes and freeze-killed before dissection. Excised heads, with mandibles loosened for exposure of the mandibular glands, were immediately extracted in 200 μl of pentane (Merck, Uvasol) for 18 hr. Each head was extracted separately.

For studies on the release of linalool, males and females were excavated during emergence from the natal nests, and each bee was kept separately in an entrainment chamber for 3 hr. In addition, pairs *in copula* initiated at female emergence were immediately transferred to an entrainment chamber, consisting of two glass cone fittings with a volume of ca. 30 ml. The outlets of the cones were connected to 1.5-mg charcoal filters (Brechbühler, Schlieren, Switzerland). When air was drawn through the entrainment chamber (ca. 45 ml/min, Brey-pump, Memmingen, Germany), one filter served to clean the incoming air, while volatiles emitted by the bees were collected on the other. After completion of an entrainment run, the collection filter was extracted with 30 μl of pentane (Merck, Uvasol). In total, 32 pairs were studied. For each pair, first collection was made between 0 and 2 min after the copulation began, a second was made between 4 and 6 min, and a third 2 min after copulation had ceased. The copulation usually lasted for 6–8 min. Extracts obtained from three to five pairs during the same periods were pooled, concentrated (Klimetzek et al., 1989), and analyzed. Geraniol served as the internal standard.

Chemical Analyses. Extracts of the bee heads were analyzed on a Varian 3400 gas chromatograph using a DB-Wax fused silica capillary column (J&W, 30 m $\times$ 0.25 mm, film thickness 0.25 μm). After splitless injection (injector temperature 150°C), the GC temperature program started at 40°C (1 min), followed by an increase of 50°C/min to 60°C (6 min hold), increase of 1°C/min to 90°C (0.5 min hold), increase of 20°C/min to 110°C (0.5 min hold), increase of 7°C/min to 180°C (15 min hold). The enantiomeric composition of the linalool present in each sample was determined by means of a two-dimensional Varian 3400 GC system using a DB-Wax column and a permethylated β-cyclodextrin column (Chrompack,

50 m × 0.25 mm, film thickness 0.25 μm) operated at 100°C. The mass spectral identifications were carried out under the same temperature conditions as those mentioned above but using a Finnigan SSQ 7000 GC-MS instrument linked to a Varian 3400 gas chromatograph.

A second set of analyses was carried out by using an HP 5890 gas chromatograph equipped with a 50-m fused silica capillary column (50 m × 0.25 mm ID, film thickness 0.25 μm; Permabond, Macherey & Nagel, Düren, Germany) coated with FFAP. Conditions were: splitless injection, temperature program starting at 60°C and increasing at 8°C/min to 220°C. The linalool enantiomers were separated with a glass capillary column coated with heptakis-(2,3,6-tri-*O*-pentyl)-β-cyclodextrin (25 m × 0.25 mm ID, film thickness 0.25 μm; Lipodex C, Macherey & Nagel) operated at 80°C (helium flow 3 ml/min) or with a fused silica capillary coated with 2,3-diethyl-6-*tert*-butyldimethylsilyl-β-cyclodextrin (25 m × 0.25 mm ID, film thickness 0.25 μm; PS 086, Mega, Legnano, Italy) operated at 100°C (helium flow 2.5 ml/min). Mass spectra (70 eV) were obtained with a double-focusing instrument VG 7070/250 SE (Vacuum Generators, Manchester, England) linked to an HP 5890 gas chromatograph operated under the conditions mentioned above.

Synthesis of (S)-*(+)-Linalool*. (*R*)-(−)-Linalool (Dragoco, Holzminden, Germany) (Cornforth et al., 1960) was subjected to the following modified Mitsunobu inversion reaction (Martin and Dodge, 1991) (Figure 1). (*R*)-(−)-Linalool (2.6 g, 17 mmol) was mixed with triphenylphosphine (21.7 g, 83 mmol) and *p*-nitrobenzoic acid (12.3 g, 74 mmol) in 300 ml of dry toluene. Diethyl azodicarboxylate (DEAD, 13 ml, 83 mmol) dissolved in 20 ml of toluene was added dropwise at ambient temperature. After stirring over night, the solvent was evaporated, and the reaction product was purified by liquid chromatography (SiO_2). The conversion was not complete. The yield of *p*-nitrobenzoyl esters was 317 mg (6.2%). NMR analysis revealed that the reaction product was a mixture of linaloyl *p*-nitrobenzoate and the two rearranged products, neryl and geranyl *p*-nitrobenzoate. The benzoates were hydrolyzed (5% KOH in 1:1 MeOH/THF) to yield (*S*)-(+)-linalool, geraniol, and nerol. The tertiary alcohol, linalool, was easily separated from the two primary alcohols by medium-pressure liquid chromatography, using SiO_2 as adsorbent in a 25-mm-diameter column [for chromatography

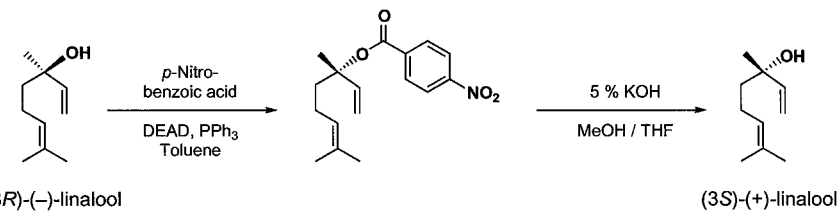

FIG. 1. Conversion of (*R*)-(−)-linalool into (*S*)-(+)-linalool.

details see Baeckström (1987)]. The eluent was ethyl acetate in hexane, starting with 100 ml hexane in the mixing chamber, followed by 50 ml portions of solvent with increasing concentrations of ethyl acetate in hexane: 0.625, 1.25, 2.55, 7.5, 10, 15, 20, 30, 40, 60, 80%, and finally 50 ml of 100% ethyl acetate. The eluent was collected in 15-ml fractions and pooled, based on the purity visualized on TLC plates after elution with 20% ethyl acetate in hexane, checked under UV irradiation, and developed with a vanillin/H_2SO_4/EtOH spray. The enantiomeric purity of the (*S*)-(+)-linalool obtained was 98.5% (i.e., 97% ee), as determined by GC using the two-dimensional system mentioned earlier. NMR spectra were recorded in $CDCl_3$ using Bruker WP 200 and AM 400 spectrometers.

Test Samples. (*R*)-(−)-Linalool had 97% chemical purity, >99.9% enantiomeric purity (Dragoco); racemic linalool, tech. grade, 90%; (*S*)-(+)-linalool, 98.5% enantiomeric purity (for the synthesis see above). Pentane and hexane (Merck, p.a., redistilled) were used as solvents.

Electrophysiology. Electrophysiological investigations were performed with a commercially available instrument (Syntec, Hilversum, The Netherlands) using a conventional experimental set-up (Bjostad, 1998). Electroantennographic studies (EAD) of linalool were carried out with the racemate, which was resolved on-line by GC with a chiral stationary phase (PS 086, see above).

Behavioral Experiments. Field tests were performed from the middle of April to the beginning of May at three localities: (1) Central Öland, an island located southeast of the Swedish mainland in the southern Baltic, (2) Bogesund, and (3) Margretelund, both located 35 km northeast of Stockholm. Test samples of linalool (50 μl or 100 μl) in hexane (dilutions of 1/100 and 1/10,000 v/v), i.e., doses of 0.005 μl (ca. 4.5 ng) and 1 μl (ca. 900 ng) of linalool, were applied to pieces of black velvet (5 × 8 mm) that were fixed on insect pins. Immediately after application of the sample and evaporation of the solvent, the scented velvet was placed in one of the mating flight areas of the male bees, 10–20 mm above the ground. Samples were tested one by one in random order. Two series of tests were performed in parallel; the trials in each were alternated between two spots separated by 6–8 m to minimize the effects that might result from behavioral adaptation of the bees to the location of the experimental set-up. The test series included the samples (*S*)-(+)-, (*R*)-(−)-, and racemic linalool, as well as the solvent, hexane or pentane, for control. Each sample was presented for 5 min.

The flight behavior of each approaching male was classified according to the minimum distance to the odor source when the approach flight ceased, the duration of the approach flight, and the manner of the approach (Table 1). The intensity of the oscillation flight, occurrence of the male's contact with the piece of velvet (pounce), as well as unaffected passages were recorded. The behavior types described in Table 1 were pooled (shown in Figure 3 below) into two types: attraction and passages (the sum of the behavior types 2long, 2, and 1) and no activity (the passages). In total, 23 test series were performed, including 12 at

TABLE 1. CLASSIFICATION OF TYPES OF BEHAVIOR OF *Colletes cunicularius* MALES IN FLIGHTS TOWARDS ODOR SOURCES USED IN FIGURES 2–6

Event	Description
passage (p)	Passing the scented source within a distance of 100 mm without any change in behavior
1	Quick change of flight direction towards the scented source
2	Zigzagging/pendulating approach upwind and directed towards the scented source
2long	Duration >2 sec, normally 5–10 sec
2−−	Flight approach ending at a distance of ≥1 m
2−	Flight approach ending at a distance between 10 cm and 1 m
2+	Flight approach ending at a distance of 5–10 cm
2++	Flight approach ending at a distance <5 cm from the odor source, without any contact
3	Circling inspection, wide sideway movements to and fro along a circle line at approximately the same distance from the odor source, indicating an intensive reaction
pounce	Short contact with the scented source

Öland, 5 at Bogesund, and 6 at Margretelund. Statistical evaluations of the test results were made using the χ^2 test for pairwise comparison of the behavior types.

RESULTS

Independently of sex and life stage, *Colletes cunicularius* produced (*S*)-(+)-linalool of >99.9% enantiomeric purity (constituting 30–90% of the total amount of volatiles in the head extract). The relative amounts of linalool in the pentane extracts of the cephalic secretion differed slightly among patrolling males (62–81%, average 71%), mated females (34–58%, average 47%), and virgin females (44–91%, average 71%). Entrainment studies of copulating pairs showed that 1–3 ng of (*S*)-(+)-linalool were released by the pair during the first 2 min. During the middle of the copulation, the amount dropped to ca. 10% of its initial value and remained at this level during a short period after the copulation.

Electrophysiology (GC/EAD), using on-line separation of racemic linalool on the PS 086 column (baseline separation, α value = 1.04) and antennae of males as detectors, revealed that both enantiomers elicited antennal responses (Figure 2). The elution order of the enantiomers was (*R*)-(−)-linalool (4.3 min) and (*S*)-(+)-linalool (4.6 min).

In the behavioral tests at central Öland with a high-density bee population, the attraction to (*S*)-(+)-linalool and to the racemate was not significantly different when all events were measured (Figure 3). When the unaffected passages are compared with all other behavior types, the racemic and (+)-linalool samples elicited significantly more approaches than the (−)-linalool and pentane samples. When we consider the different types of behavior separately (Figure 4), the

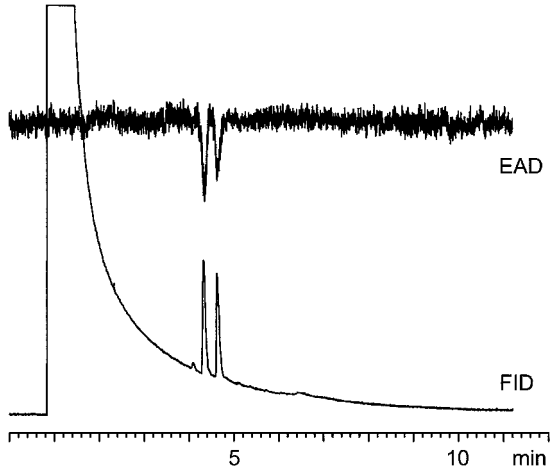

FIG. 2. Separation of linalool enantiomers by enantioselective gas chromatography recorded by flame ionization detection [FID trace, elution order (*R*)-(−)-linalool/(S)-(+)-linalool] and electroantennographic detection (EAD trace). For conditions see text.

numbers of highly excited bees (2++long and pounce) are significantly higher for the racemic and the (+)-linalool than for the pentane and (−)-linalool samples. The proportions of strongly attracted bees and weakly attracted bees differ between (−)-linalool and (+)-linalool. The number of long-lasting behavior types around the dummies (2long) were higher towards (*S*)-(+)-linalool than towards the racemate ($P < 0.05$). The (+)- enantiomer also differed from the racemate by releasing more males pouncing the velvet dummy and the soil under or close to the odor dummy (Figure 4).

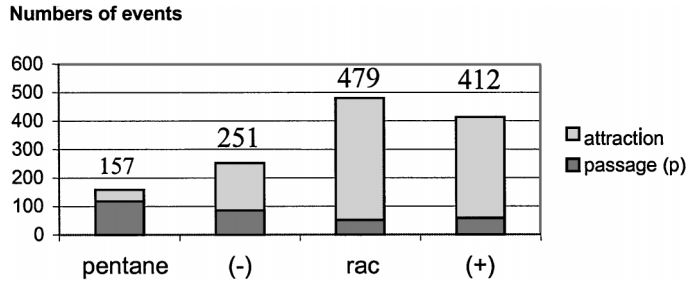

FIG. 3. Behavior of *C. cunicularius* males in response to pentane and different linalool samples. The data are based on 12 test series performed on Öland (high-density population) from which all attraction associated-behavioral events were pooled. (−) = (−)-linalool; (+) = (+)-linalool; rac = racemic linalool. For classification of the behavior types, see Table 1.

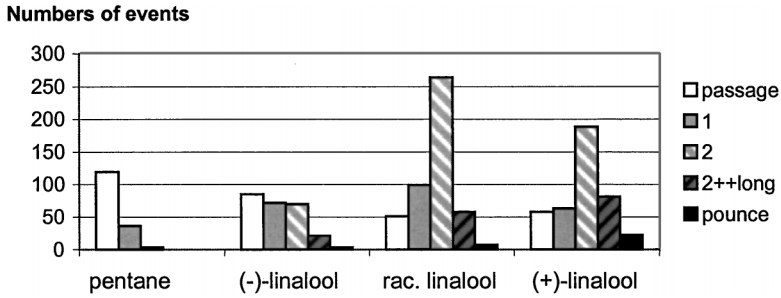

FIG. 4. Distribution of behavior types among the odor samples tested. The data are based on 12 test series performed on Öland (high-density population). For classification of the behavior types, see Table 1.

At Bogesund, where the bee population was also dense, we divided the approach flights into zigzagging and the more intense circulating behavior, thus reflecting two excitation levels (Figure 5A–C). Distinct differences between the activities towards the racemate and towards the (+)- and (−)- enantiomers were found, the (+)-form releasing the highest activity (Figure 5C). The data also show that the attraction is short-lived. The scent elicits the most intensive behavior for the first 3 min. The high attraction to the scented dummies decreased after 3 min during the 5-min tests (Figure 6). The differences in behavior on attraction to the odor source appear most clearly in the events of type 3 (circling inspections).

In the low-density bee population at Margretelund, the number of bees and the field conditions allowed longer observations of the approach flights of separate bees than did the conditions at Öland and Bogesund. The males started the approach flights at a distance of 5–10 m upwind of the odor source. Their approach flights of type 2 ended closest to the test dummy when the (*S*)-(+)-enantiomer was presented (Figure 7). The low-density population showed a difference in reactions towards (+)- and (−)- enantiomers that was similar to the one found in the high-density populations, and furthermore, an increase in behavior type 2 for (+)- over that of the (−)- enantiomer. The total numbers of bees flying in the test area were strongly influenced by the presence of the (*S*)-(+)- enantiomer. Results from the different localities indicated the same overall pattern.

Considering the behavior of type 2, the patrolling bees approach the (+)-linalool dummy more closely (<5 cm, ++ distance) than (−)-linalool or hexane dummies (Figure 8). The numbers of bees attracted (121) confirm the strong attraction effect. The flight intensity, the duration of the approaching behavior, and the number of individuals within a distance of 50 cm of the odor source were significantly larger for (*S*)-(+)-linalool than for the racemic mixture and for (*R*)-(−)-linalool ($P < 0.05$). In addition, the proportion of the "close attraction" (++ and + distance of the odor source) to "distant attraction"

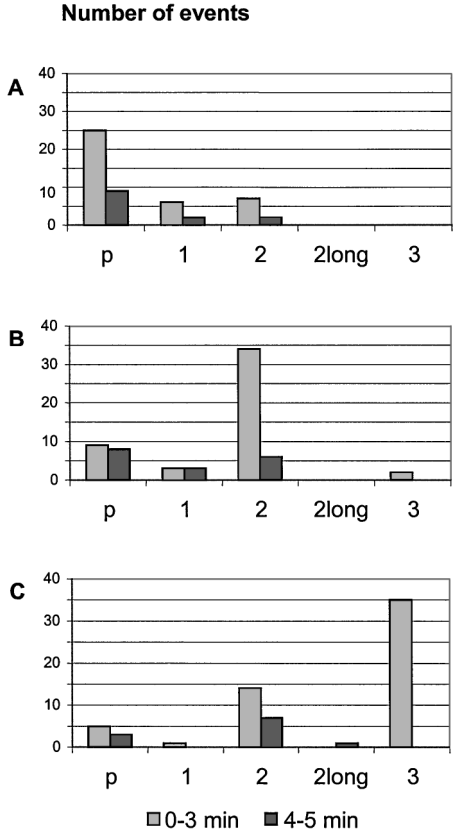

FIG. 5. Behavior of *C. cunicularius* males in response to the pure enantiomers and the racemate of linalool (high-density population). The most intensive behavior has been separated from the zigzagging/pendulation approach behavior. The data are based on five test series performed at Bogesund. (A) (−)-linalool; (B) racemic linalool; (C) (+)-linalool. For classification of the behavior types, see Table 1.

(− and −− distance of the odor source) was significantly higher for the (*S*)-(+)-enantiomer (++, +/−, −− = 3.1) than for the (*R*)-(−)-enantiomer (++, +/−, −− = 1.4) or for the hexane (++, +/−, −− = 0.88).

DISCUSSION

All species of the genus *Colletes* hitherto analyzed produce large amounts of linalool in their mandibular glands (Bergström and Tengö, 1978; Hefetz et al., 1979). Mandibular gland secretions of *C. thoracicus* elicit general attraction and

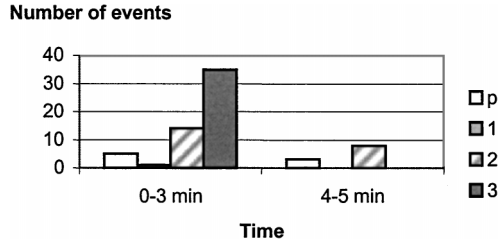

FIG. 6. Duration of attraction to (+)-linalool in the high-density population at Bogesund. For classification of the behavior types, see Table 1.

increase flight activity (Batra, 1980). The males of *C. thoracicus* are not attracted to racemic linalool alone but to a mixture of racemic linalool and citral in the ratio 3:2 (Hefetz et al., 1979). When compared with data presented in this paper, earlier results of behavioral tests obtained with *C. cunicularius* and linalool (Bergström and Tengö, 1978; Cane and Tengö, 1981) may be explained by the use of test samples containing undefined ratios of linalool enantiomers. Although linalool has been reported to enhance attraction and local search behavior in *C. cunicularius*, it has rarely elicited pouncing in those studies. Likewise, discrimination between (*S*)-(+)-linalool and the racemate was not immediately obvious in our experiments with high-density populations. The responses towards the racemate may have been influenced by the intensity of male swarming.

The males are exposed to a strong intrasexual competition, as the females are monandrous and mate only once. In the dense population at Öland, the competing males might have triggered a high level of excitement in each other. However, when the movements were analyzed with respect to circling and zigzagging flights (Bogesund population), which represented typical search behavior, differences

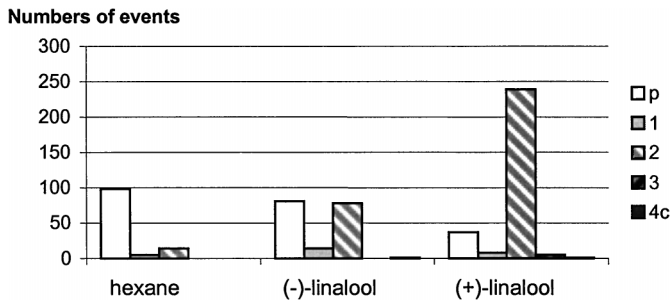

FIG. 7. Behavior of *C. cunicularius* males in response to the pure enantiomers of linalool (low-density population). The data are based on the sum of events observed at 6 test series at Margretelund. For classification of the behavior types, see Table 1.

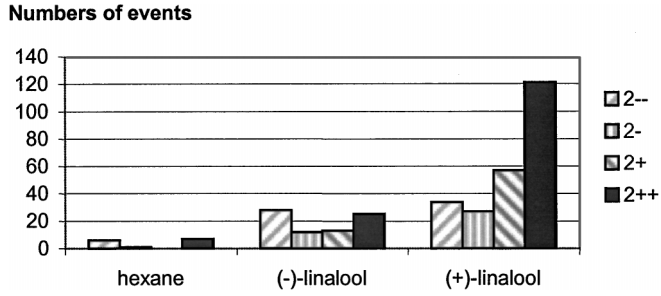

FIG. 8. Behavior of *C. cunicularius* males towards the enantiomers of linalool (distribution of the attractive behavior within type 2). The data are based on six test series performed at Margretelund (low-density population). For classification of the behavior types, see Table 1.

between the influence of the racemate and that of the (*S*)-(+)-enantiomer were also obvious in a high density population (Figure 4).

Margretelund, which was inhabited by a small bee population flying over a large area, the response to (*S*)-(+)-linalool was significantly more intense than the responses to its enantiomer. The intensity of the behavior could be observed up to 2 m from the odor source, which facilitated the evaluation of the duration and quality of the response. Excited and attracted individuals were observed even after several minutes of testing.

In the high-density population at Bogesund, which was similar to the one at Öland, the frequency of the visits was highest within the first 3 min of the tests. The numbers of events with different behavior types during minutes 0–3 and 4–5 show that the attraction is highest during the first 3 min (Figure 6), possibly indicating that the male excitement increases with a higher dose of odor evaporated from the test dummy during the first minutes, or simply that the male adapts to the odor site.

The receptive female emerging from the ground might be covered by tens of males, but only a few minutes after initiation of the copulation, the pair is left unattended by other males (Tengö, unpublished), as has also been observed in *C. thoracicus* (Rajotte and Roberts, 1980). The decrease in the release of linalool during and after copulation might explain the fact that mated females do not release copulation attempts in patrolling males. In our tests, we used biologically relevant concentrations, as we applied around 4.5 ng/dummy.

Linalool may act both as a sex attractant and a food attractant. A number of spring flowering plants emit one or both of the linalool enantiomers. The (+)-enantiomer is present in the European deceit-pollinated shrub *Daphne mezereum*, and the volatiles from its flowers attract males of *C. cunicularius* in field experiments (Borg-Karlson et al., 1996). The nectarless shrub is in blossom at

the time when *C. cunicularius* are patrolling in search of females. Although the shrubs grow in adjacent habitats, *C. cunicularius* are relatively large and fast-flying insects and capable of flying long distances. In their search for food, they may be attracted by the (+)- enantiomer, visit the flower, and to a certain degree act as pollen vectors for *Daphne mezereum* (Borg-Karlson et al., 1996). However, data on insect visitors to *D. mezereum* flowers are rather scarce, and it has not yet been shown clearly which insects are legitimate pollinators of this shrub (Manktelow, 1991). In *Salix*, which is a main nectar and pollen source for the bees, linalool is also present, especially in the male flowers (Tollsten and Knudsen, 1992), which might strengthen the attraction of bees to the flowers. *Primula veris* is in bloom at the end of the *C. cunicularius* flight period and emits large amounts of linalool with the (+)- enantiomer prevailing (Borg-Karlson, unpublished results).

From the biosynthetic point of view, the presence of pure (+)-linalool in the secretion of *C. cunicularius* may mean that the gene for the enzyme producing the (−)- enantiomer is not expressed or it might be absent. With respect to insect plant interactions and aspects of coevolution, however, it is interesting that the antennae of *C. cunicularius* respond equally to both linalool enantiomers. If the EAG response elicited depended on the responses from two receptor neurons, each tuned for one of the enantiomers, the insect would be able to distinguish between the linalool enantiomers.

The behavioral test results with all three populations tested clearly indicate that (*S*)-(+)-linalool is an important component in the mate attractant pheromone of the solitary bee *Colletes cunicularius*.

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SEX PHEROMONE OF THE SCARAB BEETLE *Phyllophaga elenans* AND SOME INTRIGUING MINOR COMPONENTS

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Abstract—Three amino acid-derived compounds were identified in extracts from the pheromone glands of the scarab beetle *Phyllophaga elenans*, i.e., L-isoleucine methyl ester (LIME), *N*-formyl L-isoleucine methyl ester (For-LIME), and *N*-acetyl L-isoleucine methyl ester (Ac-LIME). The compounds were characterized from their spectral data (MS and IR), confirmed by synthesis, and their absolute configurations were assigned by gas chromatography with a chiral phase column. The amount of LIME in calling females was ca. 2 μg/gland, whereas only traces of For-LIME and Ac-LIME (0.005% of LIME) were detected in fresh extracts. Regardless of the storage temperature, the amounts of the minor constituents in the extracts increased over time. Field tests showed that traps baited with For-LIME captured more beetles than control traps. Ac-LIME *per se* was not attractive, and it did not increase trap catches when combined with For-LIME. Traps baited with LIME caught ca. 150 beetles/trap/day, but catches

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did not increase with the addition of For-LIME and/or Ac-LIME in binary or tertiary blends.

Key Words—*Phyllophaga elenans*, *Holotrichia parallela*, Scarabaeidae, Melolonthinae, L-isoleucine methyl ester, *N*-formyl L-isoleucine methyl ester, *N*-acetyl L-isoleucine methyl ester, chiral resolution.

INTRODUCTION

We have been studying chemical communication in scarab beetles (Coleoptera: Scarabaeidae), which utilize a wide variety of chemical compounds as pheromones (Leal, 1998a, 1999; Ruther et al., 2001). While pheromones of rutelines (subfamily Rutelinae) arise largely by transformation of fatty acids (Leal et al., 1999), melolonthine (Melolonthinae) pheromones are derived from fatty acids and other precursors, including isoprenoids and even amino acids (Leal, 1999). An amino acid-derived pheromone, L-isoleucine methyl ester (LIME), was first isolated and identified in the large black chafer, *Holotrichia parallela* (Motschulsky) (Leal et al., 1992, 1993). Later, L-isoleucine and L-valine methyl esters were identified in the cranberry white grub, *Phyllophaga anxia* (Zhang et al., 1997).

The genera *Holotrichia* and *Phyllophaga* belong to the same tribe Melolonthini; their separation is based on a geographical division where species from Asia are placed in the former and species from South, Central, and North America in the latter (Mila Coca Abia, personal communication). We envisaged that other *Holotrichia* species would utilize amino acid-derived pheromones, but to our surprise the pheromone of a species native to India, *H. consanguinea* Blanchard, was identified as anisole (Leal et al., 1996). This finding prompted us to analyze the pheromone chemistry of *Phyllophaga* species from South and Central America to compare their pheromones. Here, we report the identification of the sex pheromone of *P. elenans* (Saylor), one of the most damaging pests of grain crops and sugarcane in Central America (King, 1984).

METHODS AND MATERIALS

Pheromone Extraction. Insects were collected in Turrialba and Guanacaste, Costa Rica, or obtained from laboratory cultures maintained in Turrialba. Abdominal tips of calling females were excised with forceps, and the material was cleaned to remove tissues other than the ball-shaped sac housing the pheromone glands (Kim and Leal, 1999). Glands were extracted for 3 min either with diethyl ether or methanol. After removing the pheromone glands, samples were transferred to glass ampoules, sealed, and shipped to Tsukuba, Japan, or Davis, California. Diethyl ether was distilled in an all-glass distillation apparatus, and methanol was HPLC grade (Merck). Distilled diethyl ether was sent to Costa Rica

in flame-sealed ampoules; some ampoules were returned with the samples for blank analyses. A whole body extract of a scarab beetle, *Cyclocephala amazonica* (Dynastinae: Cetoniinae), also was obtained so as to detect possible contaminants of the extraction procedure. In order to investigate possible artifacts in the samples shipped from Costa Rica, two series of experiments were conducted there (ChemTica Lab). First, calling females were collected in the field, brought to the lab, and extracted within 2 hr of collection. Extracts were made with diethyl ether (Merck analytical grade), flame-sealed, and kept at -20°C (hereafter referred to as the -20°C protocol). Aliquots were analyzed after 30 hr and 7 days. In another procedure, glands were extracted with diethyl ether at -78°C (referred to as the -78°C protocol), warmed to room temperature for 2 min, and then an aliquot was taken for analysis. Samples were stored at -20°C and analyzed again after 3 days. The stability of LIME in diethyl ether was studied by preparing fresh samples (10 μg/ml) and analyzing them by GC-MS soon after their preparations and every other day up to 15 days. In addition, gland extracts from calling females of the large black chafer were also obtained in Tsukuba. The extracts were kept at room temperature and analyzed immediately after extraction and 3 days later.

Analytical Procedures. Low-resolution electron impact mass spectrometry (EI-MS) was carried out with an HP 6890 gas chromatograph (GC; Agilent, formerly Hewlett-Packard, Palo Alto, California, USA) linked to a mass selective detector (MSD 5973; Agilent). Chromatographic resolution was done on an HP-5MS column (30 m $\times$ 0.25 mm; 0.25 μm; Agilent) that was operated at 70°C for 1 min, increased to 230°C at a rate of 10°C/min, and finally held at this temperature for 10 min. GC-MS analyses in Costa Rica were performed on a similar instrument equipped with a DB-5 column (30 m $\times$ 0.25 mm; 0.25 μm; J&W Scientific, Folsom, California, USA). The oven was operated at 50°C, increased to 70°C at a rate of 25°C/min, held at 70°C for 2 min, increased again at 10°C/min to 200°C, and finally held at this temperature for 2 min. Helium was used as the carrier gas at 1 ml/min (analyses in Japan and the USA) and 0.7 ml/min (analyses in Costa Rica). GC analyses with a chiral stationary phase were performed on an HP6890 gas chromatograph with a CP-Chirasil-DEX CB column (30 m $\times$ 0.25 mm; 0.25 μm; Chrompack, Middelburg, The Netherlands). The oven temperature was programmed initially 75°C, held for 25 min, increased at a rate of 1°C/min to 85°C, held for 25 min, and increased again at 1°C/min to 95°C, held for 25 min, and finally increased at a rate of 10°C/min to 120°C, and held at this final temperature for 10 min. Vapor-phase Fourier transform infrared (FTIR) spectra were recorded with an HP 6890 GC coupled to a FTIR system equipped with a light pipe interface, FTS-40A, GC/C32 (BioRad, Cambridge, Massachusetts, USA). The GC was equipped with an HP-5 column (30 m $\times$ 0.32 mm; 0.25 μm) operated at 70°C for 1 min, increased to 250°C at a rate of 10°C/min, and finally held at this temperature for 10 min. The light pipe and the transfer line were set at 230°C and 250°C, respectively. Helium was used as carrier gas for GC-FTIR at 2 ml/min.

Syntheses. LIME was obtained as previously described (Leal et al., 1993). *N*-Acetyl- and *N*-formyl-isoleucine methyl ester were prepared by acetylation and formylation with acetic anhydride and formic acid, respectively. Acetic anhydride (3 ml) was added to a solution of LIME (0.5 g, 3.45 mmol) in dry ether (10 ml) at 40°C. After stirring for 15 min, diethyl ether was added (15 ml), and the mixture was washed with cold water and then sodium bicarbonate (10%). The organic phase was dried (anhydrous magnesium sulfate), concentrated, and purified by flash chromatography with Wakogel C-200 (Wako, Tokyo, Japan) to give *N*-acetyl L-isoleucine methyl ester (484 mg, 75%). mp 54°C. MS data:128 (100%), 41 (21), 43 (54), 44 (27), 57 (30), 60 (13), 69 (13.1), 74 (8.3), 86 (85), 88 (53), 89 (23), 99 (62), and 131 (13). Vapor-phase IR data (cm^{-1}): νN-H, 3456; νC-H, 2974; νC = O (methyl ester) 1755; νC = O, 1717 (acetate); νC-O (acetate), 1492; νC-O (ester) 1202. Similarly, *N*-formyl L-isoleucine methyl ester was prepared from LIME (0.3 g, 2.1 mmol) and formic acid (5 ml, 90%). The reaction mixture was refluxed for 15 min before extraction and washed with a saturated solution of sodium bicarbonate. After drying, concentration, and chromatographic purification, *N*-formyl L-isoleucine methyl ester was obtained (243 mg, 67%). bp 112°C/mm Hg. MS data: 85 (100%), 41 (24), 46 (19), 57 (16), 69 (28), 88(8), 96 (3), 114 (67), 117 (20), 128 (10). Vapor phase IR data (cm^{-1}): νN-H, 3447; νC-H, 2974; νC-H (aldehydic), 2851, νC = O (methyl ester) 1755; νC = O, 1728 (formate); νC-O (formate), 1487; νC-O (ester) 1186. Similar procedures were done for the synthesis of the other stereoisomers starting with the synthesis of the methyl esters of D-, L-*allo*, and D-*allo*-isoleucine methyl esters.

Field Experiments. Tests were conducted in a sugarcane plantation in Guanacaste, Costa Rica. Traps were 20-liter white plastic buckets with crossed white plastic vanes (75 × 35 cm). Detergent-laced water was added to the bottom to retain insects. Test compounds were released from membrane devices at a rate of ca. 1 mg/day. Traps were placed in a randomized block, with the intertrap distance of 35 m. Five replicates were placed in the field, and the treatments were rerandomized after daily capture counts. Capture data were transformed to $\log(x + 0.5)$ and analyzed by ANOVA.

RESULTS AND DISCUSSION

Analyses of the gland extracts from samples collected in Costa Rica and shipped to Japan showed three major peaks (Figure 1A). Other longer retention time peaks were ruled out on the basis of the profiles obtained with a blank (solvent) and a whole-body extract from *C. amazonica*, particularly hydrocarbons and phthalate esters. The mass spectrum profile of peak 1 (data not shown) was indistinguishable from LIME, previously identified from *H. parallela* (Leal et al., 1992, 1993). The base peak at *m/z* 86 is due to fragmentation

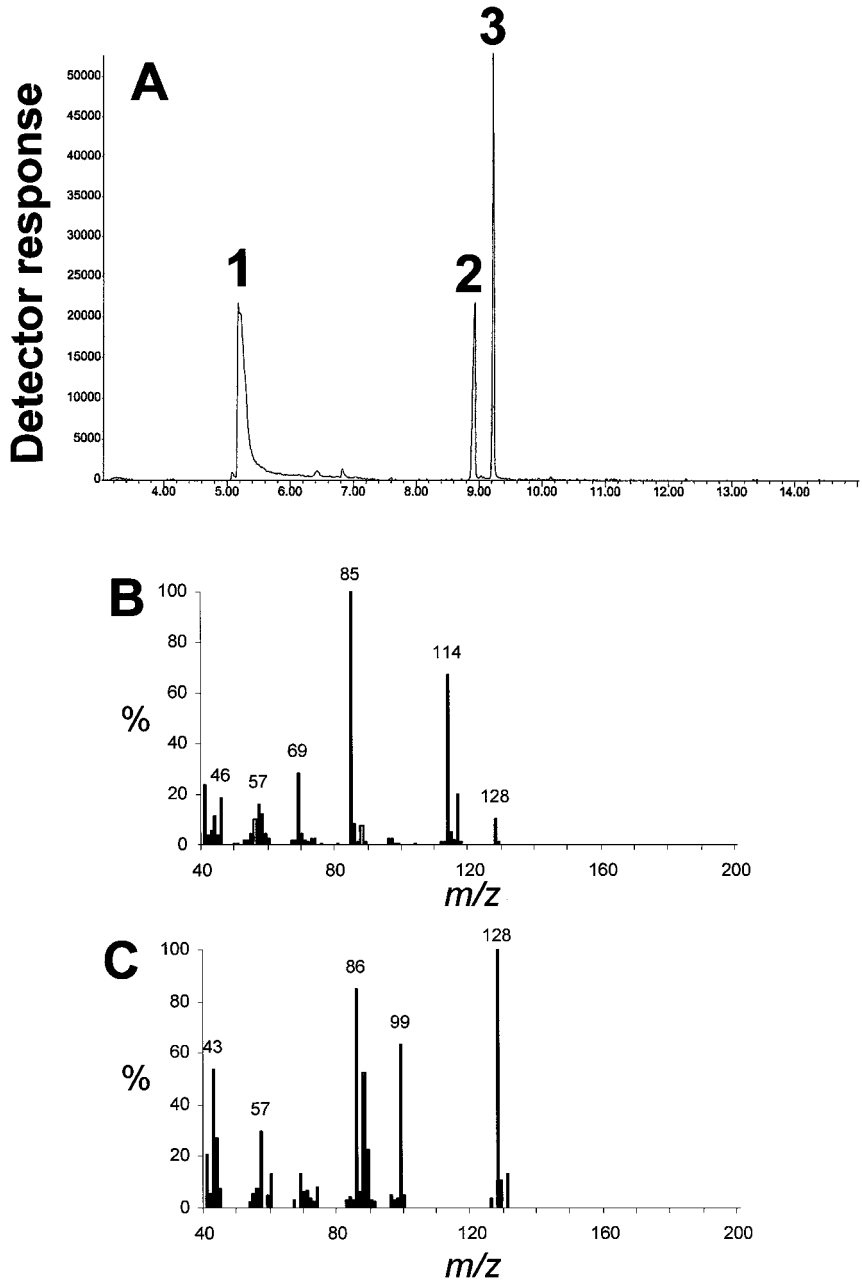

FIG. 1. GC-MS profile of a gland extract from *P. elenans* analyzed on a nonpolar HP-5MS column (A). The mass spectrum profile of compound 1 was identical to that of L-isoleucine methyl esters. Compounds 2 and 3 generated the MS displayed in B and C.

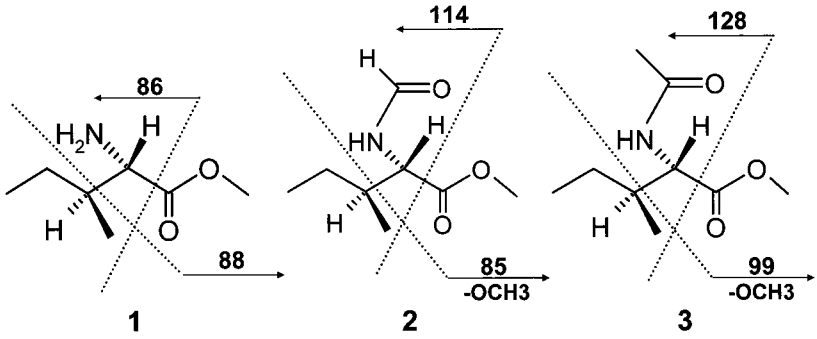

FIG. 2. Structures of compounds **1**, **2**, and **3** and proposed fragmentation patterns. The stereochemistry of displayed structures is consistent with the assignment made by chromatography with a chiral stationary phase.

of the C-2–C-3 bond. The vapor phase IR spectrum of 1 (data not shown) and its retention time ($t_R = 5.14$ min) were indistinguishable from those of authentic LIME. The mass spectral data for peaks 2 and 3 (Figure 1B and C, respectively) indicated that they were derived from LIME. Fragmentations of the C-1–C-2 bonds in the two compounds (Figure 2) give rise to the peaks at *m/z* 114 and 128 in the MS of compounds 2 and 3, respectively. Fragmentation of the C-2–C-3 bond seems to be followed by loss of OCH_3 to generate peaks at *m/z* 85 and 99 from compounds 2 and 3, respectively. Vapor phase IR spectra of these compounds (Figure 3) confirmed that 2 and 3 were formyl and acetyl derivatives of LIME, respectively. First, the two carbonyl bands were observed in the IR spectrum of each compound. A carbonyl band of the methyl ester moiety appeared at 1755 cm^{-1}, whereas the carbonyl bands of the formyl and acetyl moieties appeared at 1728 and 1717 cm^{-1}, respectively (Leal, 1998b). In addition, N–H stretching bands appeared at 3447 and 3456 cm^{-1} along with stretching bands of the formyl and acetyl groups at 1487 and 1492 cm^{-1}, respectively. Furthermore, an aldehydic stretching band at 2851 cm^{-1} confirmed the presence of a formyl group (Leal, 1998b).

Synthetic *N*-formyl L-isoleucine methyl ester (hereafter referred to as For-LIME) and *N*-acetyl L-isoleucine methyl ester (hereafter referred to as Ac-LIME) gave retention times identical to the natural compounds (For-LIME, $t_R = 8.93$ min; Ac-LIME, $t_R = 9.23$ min). Next, we investigated the stereochemistry of the formyl and acetyl derivatives of LIME. Chiral resolution of the four possible stereoisomers was obtained with a cyclodextrin-based column, CP-Chirasil-DEX CB. Separation of the four isomers of For-LIME was achieved (Figure 4D), with *N*-formyl D-allo-, L-allo-, D-, and L-isoleucine methyl ester appearing at 108.1, 109.3, 110.0, and 112.0 min, respectively. The diastereomers of Ac-LIME were well separated, but the enantiomers were not baseline separated (Figure 4B). The four stereoisomers, *N*-acetyl D-allo-, L-allo-, D-, and L-isoleucine methyl ester, appeared at 87.4, 88.1,

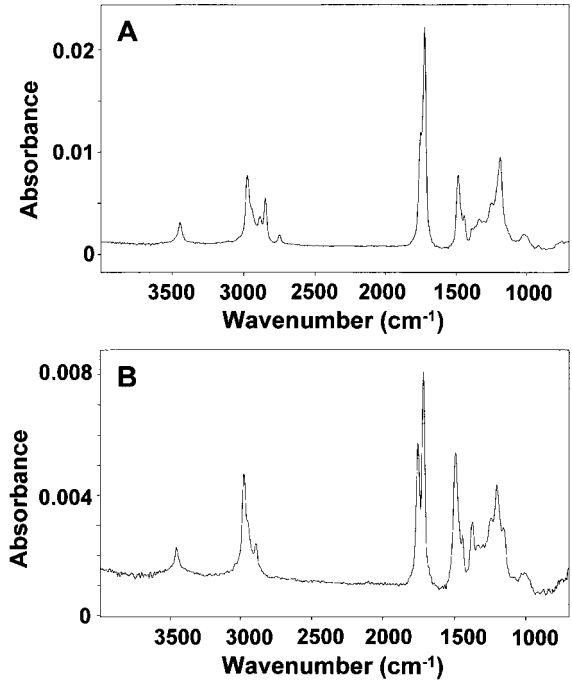

FIG. 3. Vapor-phase IR spectrum of compounds 2 (A) and 3 (B), showing two carbonyl bands, C-O and N-H stretching bands, and other identifying features. A major difference between the two spectra is the aldehydic C-H stretching band at 2851 cm^{-1} (A) in the formyl compound.

93.6, and 94.4 min, respectively. Injection of the natural products under the same conditions showed that peaks 2 and 3 eluted with the same retention times as *N*-formyl L-isoleucine methyl ester and *N*-acetyl L-isoleucine methyl ester at $t_R = 94.4$ min and 111.9 min, respectively (Figure 4A,C). The stereochemistry of the two compounds was, therefore, unambiguously identified as L, i.e., in the two compounds the carbons at positions 2 and 3 have the *S* configuration as in the natural amino acid isoleucine.

Initial attempts to determine the ratio of the two compounds in the pheromone glands failed, as the ratio varied dramatically from sample to sample, with some being even completely devoid of LIME (peak 1). We suspected that extraction artifacts could account for this variation. A series of experiments was conducted to clarify this. Analysis of gland extracts (-20°C protocol) showed that LIME was the major constituent of fresh samples (Figure 5A). When analyzed 7 days later (stored at -20°C) (Figure 5B), the profile changed dramatically with content of LIME decreasing and For-LIME and Ac-LIME increasing.

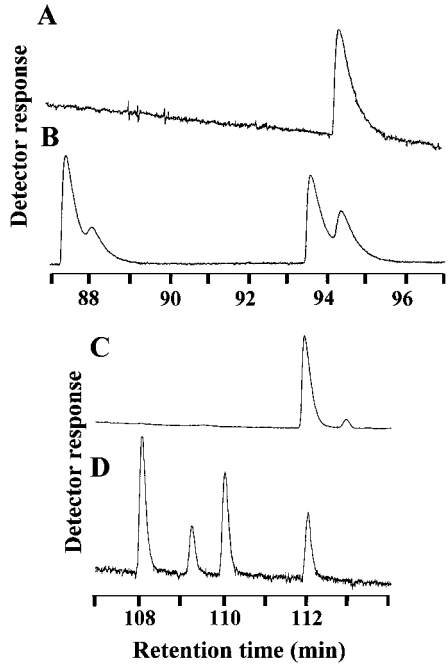

FIG. 4. Chiral resolution of the stereoisomers of the two compounds derived from LIME. Analysis of gland extracts (A and C). The *N*-acetyl isoleucine methyl esters showed shorter retention times (B) than the corresponding formyl compounds (D). Furthermore, the four peaks of the stereoisomers of *N*-formyl isoleucine methyl esters had higher resolution. The four stereoisomers for the two compounds appeared in the following order: D-*allo*, L-*allo*, D, and L.

We made an in-house comparison by analyzing fresh ether extracts from the pheromone glands of calling females of the large black chafer, *H. parallela*. Although trace amounts of For-LIME and Ac-LIME were detected in these fresh extracts (obtained with freshly distilled ether), there was no significant change in their contents over time. We observed, however, that the contents of LIME decreased dramatically over time when old ether was used for gland extracts, but this decrease was not associated with an increase in the trace amounts of For-LIME and Ac-LIME. Degradation of synthetic LIME in old ether solutions (10 μg/ml) was also observed, but this could not be correlated with formation of the acetyl and formyl derivatives.

Finally, analyses of fresh gland extracts obtained at low temperature (-78°C protocol) showed (Figure 5C) that LIME is the major constituent of the pheromone gland (ca. 2 μg/female gland), whereas For-LIME and Ac-LIME were detected in trace amounts (0.005% of LIME). These trace constituents increased over time

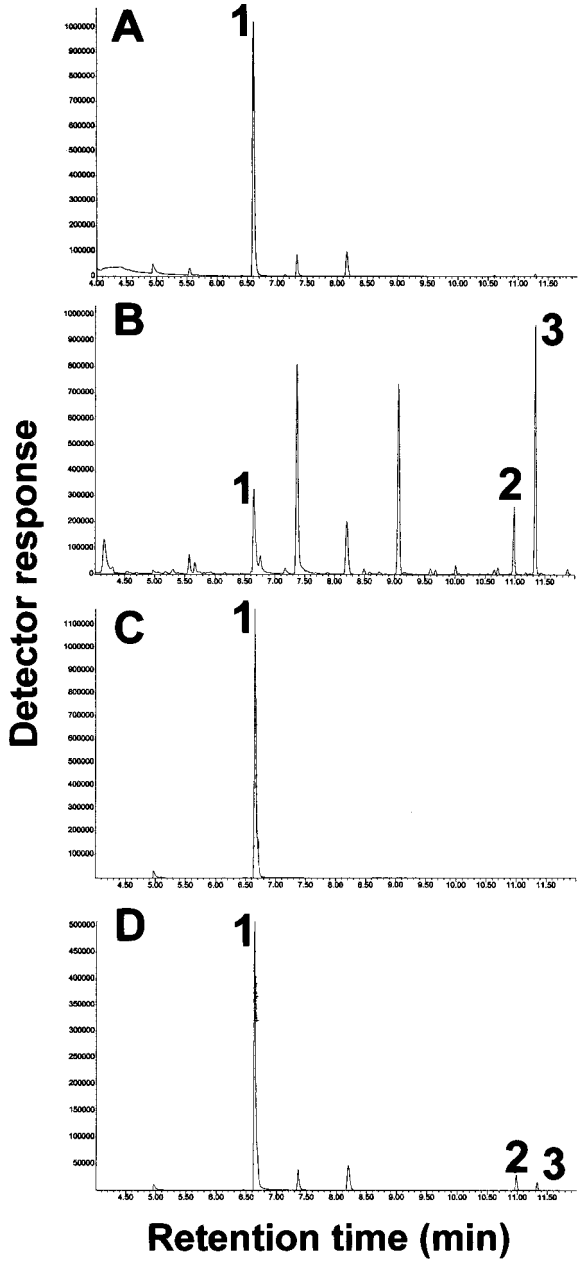

FIG. 5. Chemical analyses confirming the occurrence of trace amount of For-LIME (peak 2) and Ac-LIME (peak 3) in the glands of *P. elenans*. Sample obtained by the -20°C protocol analyzed soon after extraction (A) and 7 days later (B). Sample prepared by the -78°C protocol showed trace amounts of For-LIME and Ac-LIME (0.005% of LIME) (C); the amounts increases over time even in samples kept at -20°C (D).

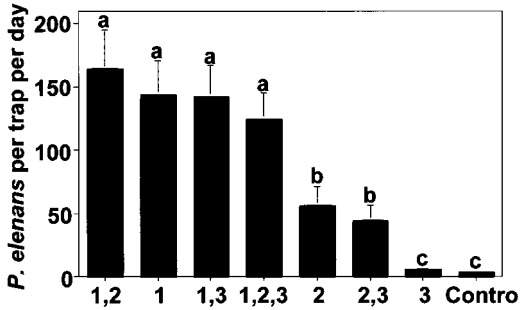

FIG. 6. Results of field tests conducted in a sugarcane plantation in Costa Rica. There is no difference in catches by traps baited with L-isoleucine methyl ester (1) alone, or in combination with For-LIME (2) and Ac-LIME (**3**). ANOVA on log $(x + 5)$ transformed data gave $F = 31.35$, $df = 7.63$, $P < 0.05$. Means followed by different letters are different by Bonferonni t test ($P > 0.95$).

(Figure 5D). We, therefore, conclude that the putative pheromone system of *P. elenans* comprises a major constituent (LIME) and two secondary compounds (For-LIME and Ac-LIME).

Field tests showed that traps baited with For-LIME alone caught more beetles than control traps, whereas captures in Ac-LIME traps were not different from the control (Figure 6). On the other hand, LIME–baited traps captured over 150 beetles/trap/day. There was no significant increase in trap catches when the secondary constituents, For-LIME and Ac-LIME, were added. However, catches varied with trap design and location within field (Oehschlager et al., 2003). In conclusion, LIME has been identified as a sex pheromone of *P. elenans*. Two minor constituents of the pheromone gland, For-LIME and Ac-LIME, are not essential for practical applications.

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TRAPPING OF *Phyllophaga elenans* WITH A FEMALE-PRODUCED PHEROMONE

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Abstract—Attraction of *Phyllophaga elenas* to vaned bucket traps baited with the recently identified female-produced pheromone, L-isoleucine methyl ester (LIME), is efficient. Pheromone-baited vaned buckets with water to retain insects were more effective than buckets without vanes or plastic containers with the sides cut out. Pheromone-baited vaned bucket traps from which water was omitted required the addition of a funnel below the vanes to retain insects. Normally used light traps were about 10 times more effective than pheromone-baited vane bucket traps in capturing *P. elenans*. Over 95% of *P. elenans* were captured between 6:00 and 9:00 PM. The male–female ratio was ~3–4:1 in both light and pheromone traps, and the ratio was relatively unchanged throughout the capture period. Most *P. elenans* were captured in the treed areas surrounding sugarcane fields. More *P. elenans* were captured in treed borders than in grassy borders of sugarcane fields. The effective radius of the pheromone-baited vaned bucket trap is between 5 and 15 m.

Key Words—*Phyllophaga elenans*, May and June beetles, pheromone trapping, sugarcane.

INTRODUCTION

Scarabaeidae in the genus *Phyllophaga* are commonly known as May or June beetles and have worldwide distribution (Westcott, 1964). In Central America, several *Phyllophaga* spp. are important pests of food crops (King, 1984). *Phyllophaga*

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larvae damage roots during their long life. *P. elenans* and *P. vincina* are economically important species from Guatemala to Costa Rica, and *P. elenans* is the most common destructive species in El Salvador and Honduras (King, 1984). In the dry tropical zone of northwestern Costa Rica, *P. elenans* is a major pest of several crops, the most important of which is sugarcane. This species is typical of other *Phyllophaga* spp. with a 2-year life cycle in which adults emerge just after moderate rains following annual dry periods (King, 1984). The major flight period is usually less than a month. During their short lives as adults, *P. elenans* emerge from the soil at dusk and fly to nearby plants that were either the crop that acted as a food source for larvae or other plants in the area. In related *Phyllophaga* spp., females emerge and fly first (King, 1984). Upon arrival at a suitable site, the female raises her abdomen, exposing the pheromone gland, and begins calling. Males arrive, and mating occurs. At daybreak, *P. elenans* return to the ground. A technique to manage the large populations of *P. elenans* that build up in sugarcane involves planting attractive trap trees, such as guacimo (*Guajunia ulmifolia*) and malinche (*Delonix regia*), around infested fields. Insecticide treatment of the trap trees during the flight season is used to kill adult *P. elenans*. Another commonly used technique is to trap adults with light traps.

Isoleucine methyl ester (LIME) has recently been reported as the female-produced pheromone for *P. anxia* by Zhang et al. (1997). Recent work (Leal et al., 2003) showed that LIME is the female-produced pheromone for *P. elenans*. In this work, we examined attraction of *P. elenans* to traps baited with LIME. We also examined the design and positional factors that affect the ability of pheromone-baited traps to capture *P. elenans*.

METHODS AND MATERIALS

Field Site. Experiments were carried out in the 3700-ha Central Azucarera Tempisque sugarcane plantation in the Guanacaste Province of Costa Rica. This is a dry tropical region in the northwest part of Costa Rica. The plantation has sugarcane of all ages with many plantings bordered by guacimo and malinche trees that are attractive food for adult *P. elenans* (King, 1984). Except for test 8, experiments were positioned in treed or grassy borders of sugarcane fields with intertrap distance of 10–50 m (Table 1). For test 8, each replicate (4 replicates/night) consisted of a set of equivalently baited traps in a line perpendicular to the border of a field and 30 m from adjacent replicates.

Traps. Trap A consisted of a 20-liter white plastic bucket containing 3–5 cm of detergent-laced (1–2%) water. Trap B was like trap A additionally topped by two intersecting 75-cm-high $\times$ 33-cm-wide vanes of galvanized metal. The vanes contained a 5-cm-diameter hole at the center of each vane to allow air to pass through the vanes freely. Pheromone lures were hung from wires so that their release area

TABLE 1. EXPERIMENTS

Test	Treatments	Date 1997	LIME (mg/day)	Trap Dist	SC Age Months	Statistics
1 (Fig. 2)	Trap A with no vane & trap B with different vane materials	May 2–5	2	35 m	2	3 Replicates/night, traps rerandomized nightly ANOVA ($N = 12$) log ($x + 0.5$) transformed pooled data $F = 4.72$, $df = 3, 42$, $P < 0.05$
2	Trap B vs D both LIME-baited and with galvanized metal vanes	May 26	8	35 m	9	ANOVA ($N = 5$), $F = 0.26$ $df = 1, 8$, $P \sim 0.662$
3	Trap B with galvanized metal vanes vs. container trap C	May 24	8	25 m	6	ANOVA ($N = 10$), $F = 38.62$ $df = 1, 18$, $P < 0.05$).
4	Trap B with white plastic vanes with 3–5 cm 3% detergent laced water vs. dry	May 6	11	50 m	2	ANOVA ($N = 8$), $F = 1.35$ $df = 1, 14$, $P \sim 0.263$
5	Trap B with white plastic vanes with cardboard funnel in bucket or 4 cm 3% detergent laced water in bucket vs. light trap (D)	May 14	8	35–50 m	9	ANOVA ($N = 8$), $F = 13.87$ $df = 2$, 22; $P < 0.05$
6 (Fig. 3)	Trap B with white plastic vanes with three different LIME release rates	May 2–3	2, 8, 11	35 m	2	Traps rerandomized nightly ANOVA log ($x + 1$) transformed pooled data ($N = 20$) $F = 38.12$, $df = 2, 55$, $P < 0.05$
7 (Fig. 4)	Trap B with white plastic vanes, 6 traps placed along treed border	May 14	8	10–20 m	2	Captured insects removed at 30-min intervals from 5:00 to 9:00 PM and 4:00 to 5:30 AM
8 (Fig. 5)	Trap B with white plastic vanes placed along border and in fields	May 4–5	8	30 m betw rep	8	Insects collected nightly, 4 replicates/night ANOVA ($N = 8$) square ($X + 1$) transformed pooled data $F = 11.95$, $P < 0.05$
9	Trap B with white plastic vanes placed along grassy or treed borders	May 18	8	50	9	ANOVA ($N = 9$), $F = 8.69$ $df = 1, 20$, $P < 0.05$).
10 (Fig. 6)	Trap B with galvanized metal vanes	May 23–24	8	Var	9	ANOVA ($N = 10$) 50-m vs. 15-m distance, NS, ANOVA on 50-m vs. 5-m dist, $F = 4.88$ $df = 1, 19$, $P < 0.05$.

was over these holes. Vanes protuded 20 cm into the bucket. Trap C consisted of a rectangular 20-liter plastic container with windows 16 × 23 cm or 20 × 23 cm cut into the sides. This trap contained 4 cm of detergent-laced (3%) water, and the pheromone lure was suspended from the top of the container near the center of the trap. Trap D consisted of two crossed 20-cm-wide × 35-cm-high galvanized metal vanes with a 10-cm open space at the center into which a portable (23-cm-long, 8-W, 12-V) florescent light was placed. Below the vanes was a galvanized metal funnel (50 cm diameter at top) that directed captured insects into a wire mesh basket (40 cm diameter × 80 cm deep) that retained insects. The entire assembly was mounted on a metal frame so that the light was approximately 1.3 m above the ground.

Lures. Pheromone lures were membrane release devices (ChemTica International, San Jose, Costa Rica) that released the described amounts of LIME under a daily temperature regime of $26 \pm 2^{\circ}$C maximum and $17 \pm 2^{\circ}$C minimum.

Statistics. Where experiments were conducted for more than one night, traps were rerandomized nightly and captures analyzed for date effect. In no case was a date effect detected, and capture data were pooled for comparison of treatments. Capture data were analyzed for normal distribution and, where necessary, transformed as indicated to achieve homogeniety. ANOVA (fully factorial routine) was conducted using Systat 5.2.1. Means are always presented untransformed and when topped or followed by different letters are significantly different by Bonferonni *t* test ($P > 0.95$).

RESULTS AND DISCUSSION

All experiments were conducted with LIME, identified as the female-produced pheromone of *P. elenans* (Leal et al., 2003). Initially, we examined the effect of trap design on capture rates of *P. elenans*. Several designs were examined (Figure 1). Bucket traps (A) were less efficient than bucket traps with vanes (B), and several vane materials gave statistically equivalent capture rates (Figure 2). During this experiment, we observed most *P. elenans* approached traps in the area of the buckets. Those that approached galvanized metal and white plastic vanes hovered near the vane and then dropped, while flying, into the traps. A higher proportion of insects that approached Plexiglas vanes flew into the vanes before dropping into the trap. Although traps with Plexiglas vanes captured numerically more insects, we did not observe statistical differences between capture rates of traps with different vane materials.

The bucket trap with galvanized metal vanes (B) is a variant of the vaned metal light trap (D) that is commonly used to capture *Phyllophaga* spp. We found that (test 2) LIME-baited vaned bucket traps (B) captured 40.8 ± 11.9 *P. elenans*/trap/night compared to 53.2 ± 21.0 for light traps (no light, D). Although LIME-baited light

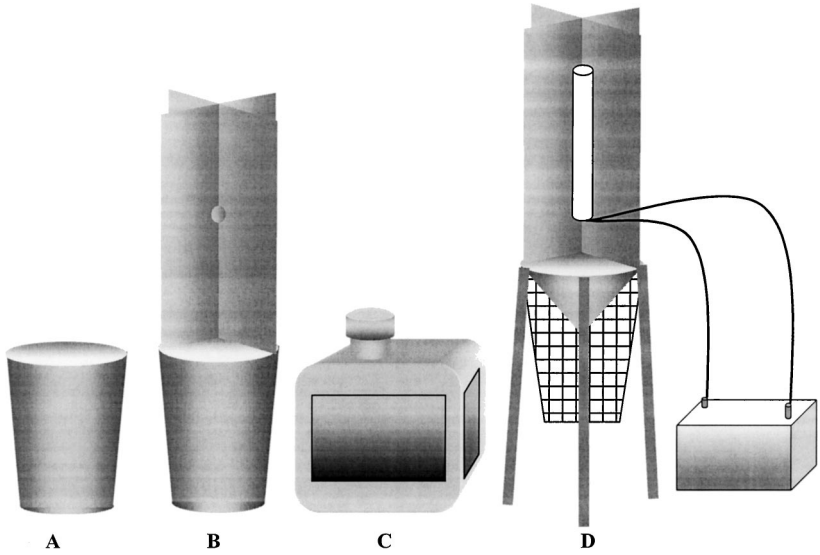

FIG. 1. Trap types examined for efficiency in capture of *P. elenans*.

traps captured numerically more *P. elenans*, no statistical difference was observed between the two vane trap designs.

Since *P. elenans* were observed to approach bucket traps with vanes in the area of the bucket tops, we examined the open bucket trap design that is readily available in the form of used insecticide containers. This design is based on 20-liter rectangular containers into which are cut large windows (C). We observed (test 3)

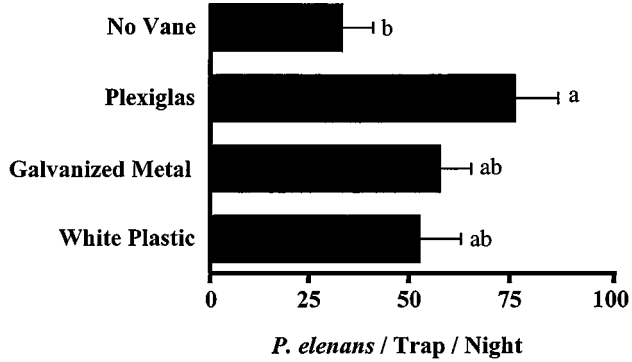

FIG. 2. Effect of no vane vs. different vane materials on *P. elenans* capture rates in bucket LIME-baited bucket traps (test 1). Bars followed by different letters are statistically significant at $P < 0.05$.

that LIME-baited vaned bucket traps (B) captured significantly more *P. elenans* (94.6 ± 6.0/trap/night) than container traps (C, 49.6 ± 4.0/trap/night).

The normal flight period of *P. elenans* spans the months of April, May, and June in the Guanacaste region of Costa Rica. This period is usually dry and windy but punctuated with periodic heavy rain. Since drowning is an easy way to retain captured insects, we compared (test 4) LIME-baited vaned bucket traps (B) that contained water with dry traps of the same design. We observed that traps containing detergent-laced water captured 942 ± 222.8 *P. elenans*/trap/night compared to 669.6 ± 70.0/trap/night for dry traps. Although traps containing water captured numerically more *P. elenans*, the differences were not statistically significant.

We attributed the above result to escape, and in a subsequent experiment (test 5), we examined the effect of adding a funnel under the vanes to retain captured insects. This test was conducted using LIME-baited vaned traps that had either a white cardboard funnel inserted into the bucket just below the vanes or detergent-laced water. In this test, the funnel-containing traps and the water-containing traps captured similar numbers of *P. elenans* (80.1 ± 27.6 and 84.7 ± 16.9 *P. elenans*/trap/night, respectively). In the same test, we found that these pheromone-baited vane traps (B) were significantly less efficient than light traps (D, 858.2 ± 176.7 *P. elenans*/trap/night). In a subsequent test, we determined that the release of LIME at the rate of 16 mg/day from light traps (D) numerically increased capture rates (∼65%) compared to unbaited light traps but that the increase was not statistically significant.

We examined the effect of LIME release rate on capture rate for bucket traps (B). We observed (test 6) that increasing LIME release rates between 2 and 11 mg/day increased capture rates (Figure 3). The observation that increasing pheromone release rate increases capture rates parallels observations for another economically important scarab, *Oryctes rhinoceros*. In the later case, increasing capture rates were found up to the highest release rate studied, 30 mg/day (Hallet et al., 1995). The above indicate that a bucket trap with white plastic vanes (B) with a lure releasing 11 mg/day of LIME is an efficient trap for *P. elenans*.

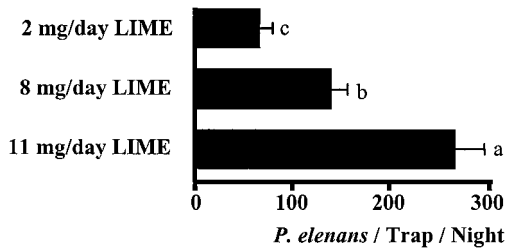

FIG. 3. Effect of different LIME release rates on *P. elenans* capture (test 6) in vaned bucket traps. Bars followed by different letters are statistically significant at $P < 0.05$.

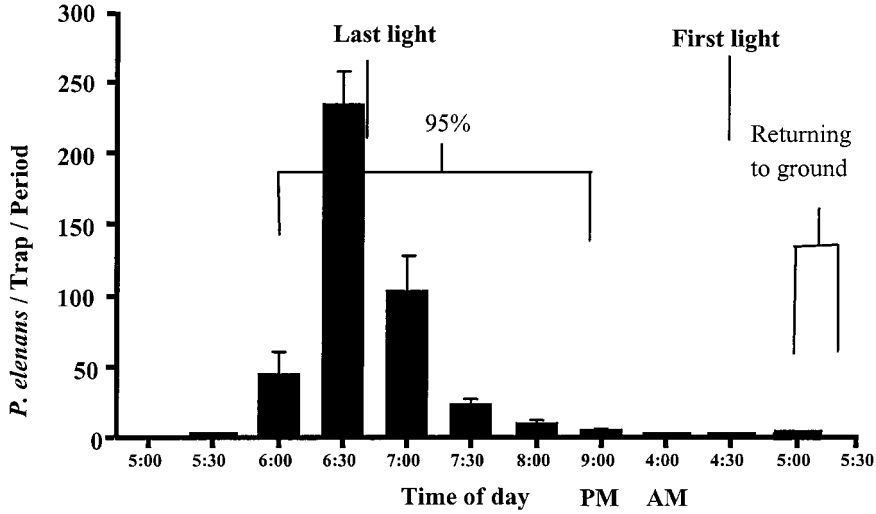

FIG. 4. Determination of time of day of flight of *P. elenans* (test 7).

Adult *P. elenans* are in the soil until sunset (~6:00 PM), at which time they fly within 2 hr to plants on which they feed and mate. At first light (4:30 AM), they become active again and begin to fly from feeding locations to the ground. This process is complete by 5:30 AM. The time-dependent response of *P. elenans* to LIME-baited bucket traps with white plastic vanes (B) was examined, and it revealed that 75% of all captures occurred between 6:00 and 7:00 PM and that this increased to 95% by 9:00 PM (Figure 4). During their return from feeding and mating sites to the ground, *P. elenans* are not attracted to LIME-baited traps.

The tendency of *P. elenans* to be more responsive to LIME-baited traps placed near food sources was observed in a comparison of capture rates of traps placed in the fields compared to traps placed at the borders of fields. Capture rates are significantly higher in traps along borders of fields containing trees than in the fields from which they emerge but in which they do not feed (Figure 5).

A further experiment (test 9) was conducted to determine if placement of traps on treed borders was more effective than placement on grassy borders. Traps in borders containing trees fed upon by *P. elenans* captured significantly more *P. elenans* (375.5 ± 53.2/trap/night) than traps in grassy borders (177.0 ± 23.6/trap/night).

During the flight season (late April to late May), we found the ratio of male to female *P. elenans* captured in LIME-baited vane bucket traps (B) to be rather constant at 4:1. This ratio was similar to that (3.3:1) obtained in light traps in the same sugarcane plantation during the same time period. Additionally, the sex ratio of *P. elenans* captured in LIME-baited traps within fields was the same as along borders. The similar sex ratio of *P. elenans* captured in LIME-baited and light

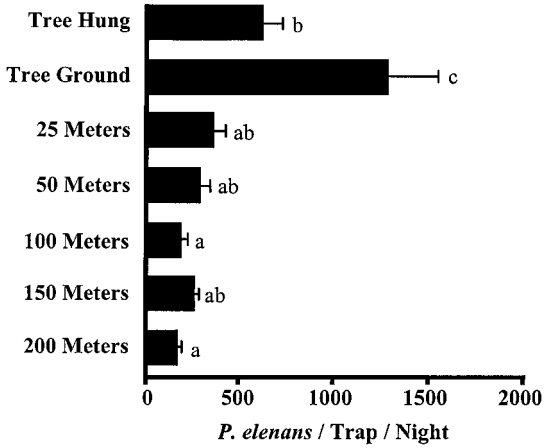

FIG. 5. Effect of trap location of capture rate of *P. elenans* (test 8). Traps (B) were placed under trees in which *P. elenans* feed and 25, 50, 100, 150, and 200 m from treed border of field. Bars followed by different letters are statistically significant at $P < 0.05$.

traps suggests LIME functions as an aggregation pheromone for *P. elenans*. In the case of *Oryctes rhinoceros*, male-produced ethy1-4-methyloctanoate attracts more females than males (Hallett et al., 1995). The ability of LIME to attract both male and female *P. elenans* increases its utility in reducing populations in mass trapping strategies.

Since it is likely that mass trapping *P. elenans* in mature sugarcane would involve placement of traps around the periphery of fields, we examined the effective radius of pheromone-baited traps in this configuration (Figure 6). Thus, we compared capture rates of traps in the center of an array of three traps in which the distances between the center and distal traps were 5, 15, or 50 m. There was no statistical difference in capture rates between traps possessing adjacent traps 15 or 50 m away, but when traps possessing adjacent traps 50 m apart were compared with those possessing adjacent traps 5 m away, the latter captured significantly fewer *P. elenans* (Figure 6).

This work has resulted in the development of an efficient pheromone-baited trap for *P. elenans* that is more economical than a light trap in cost per beetle captured. Light traps cost USD200, while vane traps with pheromone lures cost $\approx$USD10. Although light traps are $10\times$ more efficient than pheromone traps, they require the nightly servicing of battery retrieval to prevent theft. Thus, pheromone traps seem more economical. Since the completion of this work, major sugarcane plantations in Guancaste province of Costa Rica have adopted the strategy of trapping adult *P. elenans* during May and June. To date no data are available relating to the effect of trapping *P. elenans* on damage to sugarcane.

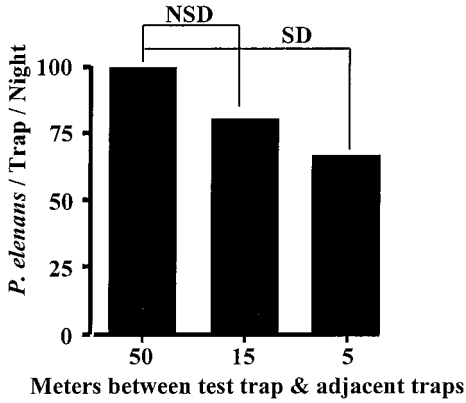

FIG. 6. Effect of proximity of adjacent traps on capture of *P. elenans* in LIME-baited vaned bucket traps (test 10). Traps were placed at 50-, 15-, or 5-m intervals along a road at the edge of 6-month-old sugarcane. On the first day, traps having adjacent traps 50 and 15 m away were compared, while on the second day traps having adjacent traps 50 and 5 m away were compared. Captures for both tests are scaled so that captures in traps with adjacent traps 50 m away equal 100 for both tests. SD = significant at $P < 0.05$, NSD = no significant differences.

One can imagine the use of a modification of this trap to allow use in pathogen deliverly. A combination of pheromone-based mass trapping, *Metarhizium anisopliae*, and baculovirus delivery has been shown to be an effective management system for high populations of *Oryctes rhinoceros* in which pheromone trapping alone is not sufficient to lower populations to economically acceptable levels (Tuck, 1996).

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL EVIDENCE FOR VOLATILE SEX PHEROMONES IN *Parcoblatta* WOOD COCKROACHES

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Abstract—Species within the cockroach genus *Parcoblatta* are sexually dimorphic for wing length; females have reduced wings and are flightless, while males have long wings that are used in flight. We predicted that *Parcoblatta* females would release a volatile sex pheromone to attract the more mobile males. Nymphs of the broad wood cockroach, *P. lata*, and the Caudell's wood cockroach, *P. caudelli*, were collected in forested areas in North Carolina, USA, and reared in the laboratory for observations of sexual behavior and for pheromone analysis. After several days of sexual maturation, virgin females of both species exhibited distinct calling behaviors. In females of *P. lata*, calling commenced 6 days after adult emergence. Under a light–dark photoperiod regime, calling behavior in both species was restricted to the scotophase. Calling consisted of a repeated pattern of raising and lowering the abdomen with occasional exposure of the genital vestibulum. To test whether calling behavior is associated with the release of pheromone, volatiles from calling and noncalling females were collected on Super-Q and tested by electroantennogram (EAG) and behavioral assays. Volatile collections from calling females elicited higher male-specific EAG responses than collections from noncalling females of the same physiological stage. In an olfactometer choice test (Y-tube), males preferred volatiles from calling females over those from noncalling females. To determine the anatomical source of the pheromone, solvent extracts of various body parts were analyzed by EAG. The first through seventh tergites were the only body parts that elicited male-specific EAG responses in both species. In *P. lata*, the activity of the extract increased from 1- to 7-day-old females, but was lower in mated than in virgin

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females of the same age. The putative pheromone gland appears to consist of numerous class-3 secretory units, each composed of a secretory cell connected to a cuticular pore via a tubular duct. We conclude that female *P. lata* and *P. caudelli* produce sex-specific volatile pheromones that are emitted during calling behavior.

Key Words—*Parcoblatta caudelli*, *Parcoblatta lata*, wood cockroach, sex pheromone, calling, male response, volatiles, pheromone gland, electroantennogram.

INTRODUCTION

Sex attraction and courtship behavior in cockroaches are mediated primarily by sex pheromones (Roth and Barth, 1967; Schal et al., 1984; Sreng, 1993; Gemeno and Schal, 2003). Depending on the species, males or females may release the pheromone that attracts the opposite sex from a distance. Once the sexes contact each other, a cuticular female-produced sex pheromone elicits wing-raising courtship behavior in males and the release of short-range volatile signals from glands located on the male's tergites. This pheromone, in turn, stimulates female feeding on male tergal secretions and facilitates orientation of the sexes for mating. Release of the volatile pheromones is accompanied by specific body postures and movements, termed calling behavior, which vary among species, depending on where the pheromone is produced.

Generally, but with some exceptions, in those species in which females release the sex attractant, either the males or both sexes are good flyers. In species with flightless males, on the other hand, the males produce the long-range attractants (Gemeno and Schal, 2003). Ultimately, the ability to fly, and hence the nature of the mating system, may be related to resource availability (Breed, 1983; Schal et al., 1984; Gautier et al., 1988). In species that aggregate and feed on concentrated food sources, such as guano in caves, it may be adaptive for the males not to fly and instead evolve male–male hierarchies and defend territories to monopolize mates through intrasexual competition. Because searching for mates is an energetically expensive undertaking that also exposes the searcher to increased predation risk, in most chemical communication systems, females emit attractant pheromones, whereas males are exposed to the risks associated with searching. Females, then, can adopt a flightless larviform strategy, feeding in the leaf-litter and calling males with volatile attractants. Whatever the evolutionary explanation, the ability of males to fly results in dispersion of individuals and consequently a need for a long-range volatile sex pheromone to bring the sexes together. Relative mobility of the sexes, therefore, appears to have predictive power as to which sex produces the long-range attractant.

We tested this prediction using two common forest cockroach species that exhibit strong wing dimorphism. Wood cockroaches of the genus *Parcoblatta*

comprise 12 species native to North America (Hebard, 1917; Atkinson et al., 1991). Although some of the species are abundant in wooded urban areas as well as in natural areas, little is known about their behavior and ecology (Gorton, 1980, 1981). *Parcoblatta* species are highly dimorphic for wing length: males have well-developed wings and can fly, and females have short wings and cannot fly. We hypothesized that females release a volatile sex pheromone that attracts the males. We focused on two *Parcoblatta* species, *P. lata* (Brunner) and *P. caudelli* (Hebard), which are common in forested areas in eastern North America. Nymphs were collected in the wild and reared in the laboratory to observe calling behavior and to perform pheromone analysis on the adults.

Parcoblatta cockroaches represent an important ecological indicator because recent studies show that 50% of the diet of the endangered red-cockaded woodpecker, *Picoides borealis*, consists of wood cockroaches (Hanula and Engstrom, 2000). The information presented in this paper will be used in the identification of the sex pheromone of *P. lata*, which can then be used to monitor populations of this cockroach in relation to those of *P. borealis*.

METHODS AND MATERIALS

Insects. Late-instar nymphs of *P. lata* were collected from under the bark of fallen, decomposing pine trees and with pitfall traps in Raleigh, Wake County, North Carolina, USA, in the spring of 2001 and reared in the laboratory under a 12L:12D photoperiod. Females of *P. caudelli* were collected with pitfall traps in the fall of 1999 and their progeny were reared in the laboratory under a 16L:8D photoperiod and tested when they became adults one year later. We chose a longer day length for *P. caudelli* because under 12L:12D their development was slow. It is likely that *Parcoblatta* requires a chilling period and longer day length in order to develop at a normal rate. Insects were placed in plastic cages with the inner walls covered with a light layer of petroleum jelly to prevent escape; paper towels or a cardboard egg carton were provided as shelter, and Purina Rat Chow # 5012 or Purina Dog Chow Nutritional Excellence Formula (Purina Mills, St. Louis, Missouri, USA) and water were provided *ad libitum*. The temperature was maintained at $27 \pm 1^{\circ}$C. Nymphs were checked daily, and adult males and females were separated in different containers.

Calling Behavior. Females were kept individually in 250-ml clear plastic cages with food, water, and a white filter paper disk on the floor of the cage to maximize contrast during observations. Illumination was provided with two fluorescent light bulbs covered with red photographic gel filters placed 20 cm above the females. Additional illumination was provided with a flashlight covered with a red photographic filter (Kodak Wratten Gelatin #29, Rochester, New York, USA).

Few females of the same age were available on the same day, and the number of females observed on a given day varied as a consequence of individuals being introduced (adult emergence) or removed (females used in experiments or for mating). In addition, not all of the females in our pool of available females were in the proper stage for calling (some were sexually immature and some were near to or ovipositing), and for every hour of the scotophase, we had a variable number of observations accumulated over several days. We did not separate these different types of females and, therefore, it was not appropriate to calculate the percentage of females calling for each hour of the scotophase. To estimate the amount of calling during each hour, we divided the total number of females calling each hour by the number of observations accumulated for that hour over several days. For example, in the first hour of the scotophase of day 1, there may be 20 females available, 3 of which are observed calling. In the first hour of the next scotophase, there may be 15 females available, of which 5 and 2 are scored as calling on two separate observations during the hour. The calling index derived for the first hour of the scotophase is 3.33 $[(3 + 5 + 2)/3]$. Because the number of observations varied greatly within the scotophase, while the relative number of females and their physiological stage varied less, this calling index is a reasonable measure of calling periodicity. Nevertheless, we did not attempt to analyze these data statistically or to compare the calling periods of the two species, but rather to provide information relevant to the periodicity of calling behavior in *Parcoblatta*. Observations on *P. lata* and *P. caudelli* were performed on 15 and 13 days, respectively. No observations were made during the first two hour of the scotophase of *P. lata* due to time constraints.

To calculate the age of first calling in *P. lata*, we conducted one or more observations per night on a cohort of females from emergence until they called for the first time. In addition we recorded the age at which the first egg case was produced.

Volatiles Collections. To determine if pheromone was released during calling behavior, we collected volatiles from calling and noncalling females. Collections were made from 8- and 9-day-old calling females during the scotophase and from the same females during the photophase while they were resting. Females were placed into 15-cm-high $\times$ 8-cm-diameter glass jars that had been thoroughly washed with detergent and water and rinsed with acetone. Food, shelter, and water were excluded from the jars, but petroleum jelly was applied to the inner wall to prevent cockroaches from escaping. Volatiles were collected in a 3-cm-long Pasteur pipet packed with 8–10 mg of 80–100 mesh Super Q polymer (Alltech, Deerfield, Illinois, USA), held in place between two beds of silanized glass wool. The Super Q collection traps were held directly above the female. A 2 liter/min airflow was generated with a vacuum pump when the female called, and the collection was interrupted between calling bouts. We accumulated $\sim$20 min of collection from each female ($N = 8$). The pheromone was extracted immediately after collection with 1.5 ml of hexane followed by 1.5 ml of CH_2Cl_2. The traps were rinsed

again, dried, and reused. The same collection trap was used for the day and night collections from a given female. Photophase and scotophase control collections were done on empty jars during both times.

Electroantennograms (EAGs). Males and females were taken out of the rearing chamber during the scotophase shortly before being tested. They were anesthetized briefly with CO_2, and one antenna was excised with fine forceps. The proximal end of the antenna was placed in the narrow end of a Pasteur pipet filled with cockroach saline BG-SSA (Kurtti and Brooks, 1976). Several terminal segments of the distal end of the antenna were excised, and the distal end of the antenna was placed in a second glass capillary. Ag–AgCl wires, 0.5 mm in diameter, connected the saline-filled capillaries to a Grass P16 amplifier (Astro-Med, West Warwick, Rhode Island) with coaxial wire and BNC connectors. With this set-up, and a small, grounded wire screen around the preparation, we experienced little environmental noise, and no further shielding was necessary around the EAG preparation. The antenna was slightly curved between the electrodes, forming a horizontal arch, and introduced into a 1-cm-diameter glass tube, which carried clean humidified air continuously over the antenna at 1.5 liters/min. The test sample was delivered through a rubber septum at the end of a lateral branch of the air delivery tube, 8 cm upwind from the antenna. The signal was acquired through an A/D board installed in an HP5890 GC and recorded and analyzed with ChemStation software (Agilent Technologies, Palo Alto, California, USA).

The Super Q or tissue extract was absorbed onto rectangular filter paper (1×1.5 cm) that was air dried and placed into a Pasteur pipet. Two milliliters of room air were delivered to the antenna as a rapid puff from a calibrated glass syringe (Perfektum, New York) and through the pipet containing the test extract. Each sample was puffed three times, and the average constituted the experimental unit. Solvent (CH_2Cl_2) alone served as negative control, whereas the positive control was 10 μg of (−)-bornyl acetate, a monoterpenoid constituent of conifer trees that elicits similar responses in female and male antennae in both the American cockroach, *Periplaneta americana* (L.) (Nishino et al., 1977) and in *Parcoblatta* (this paper). To control for variation in response among antennae, the average amplitude of each set of three EAG responses was divided by the average EAG amplitude in response to the nearest set of three puffs of bornyl acetate, either preceding or following the stimulus of interest. Each sample was tested on a male and a female antenna. To test for differences between volatiles collected from calling and noncalling females, we performed ANOVA on log-transformed EAG amplitudes of male and female antennae.

Behavioral Assays. Six olfactometer tubes consisted of 2.7-cm-diameter glass Y tubes with a 45-cm-long common section and 10-cm-long branches. The branches were extended with 8-cm-long Plexiglas tubes sealed with 2-mm^2-mesh metal screen at the ends. A 12-cm-long Plexiglas tube with a rotating gate made of 1-mm^2-mesh metal screen connected to the downwind end of the common arm

of the Y tube and served to cage the insect at the start of the trial. The olfactometer tubes were connected to an exhaust fan that generated a constant airflow of 20 cm/sec in the common arm of the olfactometer. Males used in the test were ~30 days old. *P. lata* males were tested 4–10 hr into the 12 hr scotophase, and *P. caudelli* males were tested in the last 3 hr of the 8-hr scotophase. Males were introduced into the olfactometer cage and allowed to acclimate in the airflow for 10–60 min before being tested. In one branch of the olfactometer, we placed a nighttime volatiles collection (i.e., from a calling female), and in the other a daytime collection from the same female when she was not calling. The males were allowed to sample the air for a few seconds, and then the gate was opened. We recorded the branch of the olfactometer that the male chose. If there was no response within 2 min, the male was scored as nonresponsive. Males were used only once. For *P. lata*, volatiles collections from 8 females were used, and 5 males were tested for each calling versus noncalling female pair. For *P. caudelli*, volatiles collections from 8 females were tested on 4–5 males each. A goodness-of-fit test was used to compare the percentage of males responding to volatiles from calling and noncalling females, out of the total number of males that responded.

Anatomical Site of Pheromone Production. To determine which part of the body produces the pheromone, 5- to 9-day-old *P. lata* and 9- to 15-day-old *P. caudelli* virgin and non-egg-carrying females were anesthetized with CO_2 and dissected during the photophase as follows: individual tergites 1–10, sternites (*P. lata* only), digestive tract, and the rest of the body (*P. lata* $N = 5$, *P. caudelli* $N = 6$). Dissection instruments were cleaned between dissections of different body parts. Each body part was vortexed gently in 0.5 or 1 ml of CH_2Cl_2, left at room temperature overnight, and stored at -20°C. For EAG analysis, the tissues were removed, the solvent reduced under N_2 to a few microliters, which were transferred to a filter paper. Overall differences in male and female EAGs to the body parts and differences between the male and female antennae for each body part were analyzed with an ANOVA on log-transformed data.

Effect of Age and Mating on Pheromone Production. To determine the effect of age on pheromone quantity, *P. lata* females were caged in pairs from emergence, and tergites 1–10 were extracted together at 1, 3, 5, 6, and 7 days of age ($N = 4$). Differences in the EAG responses of male antennae were analyzed with an ANOVA on log-transformed data.

To determine the effect of mating on calling and pheromone quantity, same-age *P. lata* females were paired; one pair was mated during the first gonotrophic cycle, and the second pair was left unmated ($N = 4$). Tergites of female pairs were then extracted either one day after mating or one day after the second egg case had been dropped. Differences in male EAG responses between the mated and virgin females were analyzed with Wilcoxon's two sample test.

Morphological Studies. For scanning electron microscopy (SEM), tergites 1, 4, and 7 of *P. lata* were removed, sonicated briefly, and dried. For observation

of the tergal integumental glands, the tergites were fixed in 3% glutaraldehyde in 0.1 M Dulbecco's phosphate-buffered saline and dehydrated in an ethanol series. After critical-point drying, they were sputter-coated with gold and observed in an Hitachi S450 SEM.

For counting glands in the integument, abdominal tergites 1, 4, and 7 were removed, freed from underlying tissues, and viewed under a coverslip on a microscope slide. Cuticular pores were counted in the middle and lateral portions of the segment by using a light microscope at 1000× magnification.

RESULTS

Calling Behavior. Virgin *P. lata* and *P. caudelli* females produced nonviable egg cases 10.4 ± 0.39 (mean $\pm$ SE, $N = 30$) and 10.8 ± 0.38 days ($N = 28$), respectively, after adult emergence. Female *P. lata* called for the first time 5.8 ± 0.35 days after adult emergence ($N = 17$). Calling females extended their legs, arching and raising the abdomen above the ground, while performing repeated movements of the abdomen between two positions: upward and longitudinal compression (Figure 1A), and downward and longitudinal extension (Figure 1B). In general, each raising and lowering sequence lasted a few seconds and was repeated in bouts, each lasting several seconds or minutes. On one occasion, we recorded a continuous bout of calling that lasted over 2 hr. Females did not walk extensively while calling, but they normally moved about the cage between calling bouts. They were observed calling on the floor and on the walls of the cage, and they

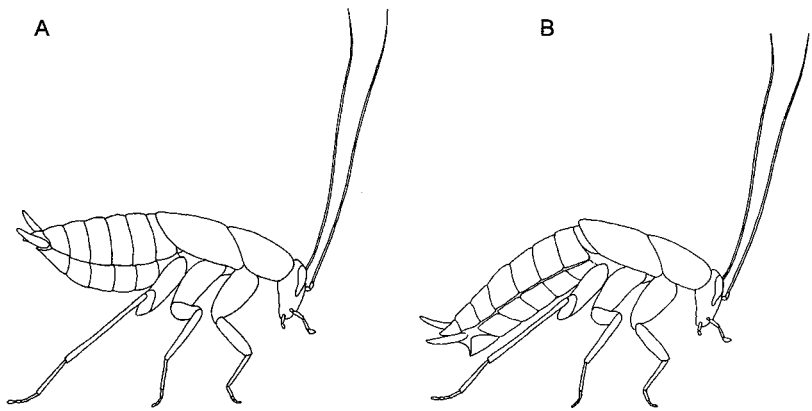

FIG. 1. Calling behavior of female *Parcoblatta lata*. Females in the calling posture raise the body from the substrate while performing repeated movements of the abdomen between two positions: (A) upward and longitudinal compression, and (B) downward and longitudinal extension. Occasionally the genital vestibulum is exposed during calling, as shown in (B).

occasionally performed other activities while calling, such as grooming the antennae and eating. The intensity of calling varied among females, so that in some females it could be recognized easily, whereas in others it was difficult to distinguish, except at close range. The calling posture was more pronounced in *P. lata* than in *P. caudelli* females. In general, fewer *P. caudelli* than *P. lata* called, and when calling, *P. caudelli* were more easily disturbed during observations and volatiles collections. Occasionally, females of both species exposed the genital vestibulum at the time of calling and maintained it open for a few seconds.

Calling was restricted to the scotophase and showed a marked periodicity; few females called at the beginning and end of the scotophase, and many called in the middle (Figure 2). Observations made during the photophase indicated that the females remained motionless on the floor of the cage and never called during the day. For *P. lata*, the number of observations was low at the beginning and end of the scotophase (Figure 2A). In *P. caudelli*, the number of observations was similar throughout, and therefore the calling index in this species is a more reliable indicator of actual calling periodicity. The calling index shows that females start calling at lights-off and continue calling, with a peak 4 hr into the scotophase, after which calling declines and is not observed during the last 2 hr of an 8-hr scotophase (Figure 2B).

EAG Responses to Female Volatiles. *Parcoblatta lata* females called during the entire time of the volatiles collection during the scotophase (182 of 182 min). In contrast, female *P. caudelli* called only for a fraction of the total time (23 of 150 min). During the photophase, females were quiescent, and no calling was observed. Male antennae were much more responsive to volatiles collected from calling *P. lata* and *P. caudelli* females during the scotophase than to volatiles collected from the same females during the photophase (Figure 3; $F_{1,14} = 15.52$, $P < 0.01$ and $F_{1,14} = 12.23$, $P < 0.01$, respectively). Female antennae of both species, on the other hand, responded nearly equally to volatiles collected from calling and noncalling females ($F_{1,14} = 1.27$, $P = 0.28$ in *P. lata* and $F_{1,14} = 5.11$, $P = 0.04$ in *P. caudelli*). Control collections elicited weak and similar EAG responses in both the male and the female antennae.

Behavioral Assays. Thirty-five percent (14/40) of the *P. lata* males tested in the olfactometer did not leave the cage or reach the branch point of the Y tube. Of the 65% that responded, 85% (22/26) chose volatiles from calling females, and 15% (4/26) chose volatiles from noncalling females ($\chi^2 = 12.5$, $df = 1$, $P < 0.01$). All of the *P. caudelli* males tested in the olfactometer responded, 75% (27/36) to the volatiles from calling females and 25% (9/36) to the volatiles from non-calling females ($\chi^2 = 9$, $df = 1$, $P < 0.01$).

Pheromone Source and Morphological Studies. Male EAG responses were significantly affected in both species by the tissue from which the extract was obtained (Figure 4; *P. lata* $F_{12,52} = 7.16$, $P < 0.01$ and *P. caudelli* $F_{11,60} = 4.25$, $P < 0.01$). *P. caudelli* females, on the other hand, showed a similar low level

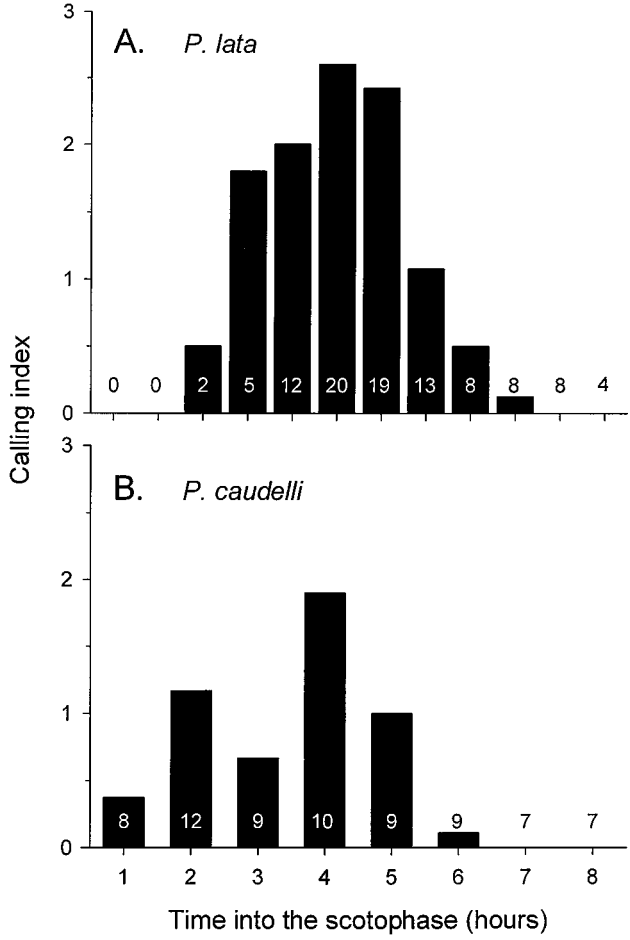

FIG. 2. Calling periodicity of *Parcoblatta lata* (A) and *P. caudelli* (B) virgin females. For every hour of the scotophase, we recorded the number of females calling. The calling index was obtained by dividing the number of calling females by the number of observations (shown above the *x* axis) for each hour of the scotophase. No observations of *P. lata* were made during the first 2 hr of the scotophase.

of EAG responses to extracts from all of the body parts ($F_{11,60} = 0.33$, $P = 0.97$). EAG responses of *P. lata* females varied somewhat with the tissue extracted ($F_{12,52} = 2.03$, $P = 0.4$), but this was caused by heightened non-sex-specific responses to digestive track extracts (see below). Male-specific EAG responses were elicited exclusively by the tergites in both species, and no other part of the body elicited sex-specific EAG responses. Only tergites 1–6 produced

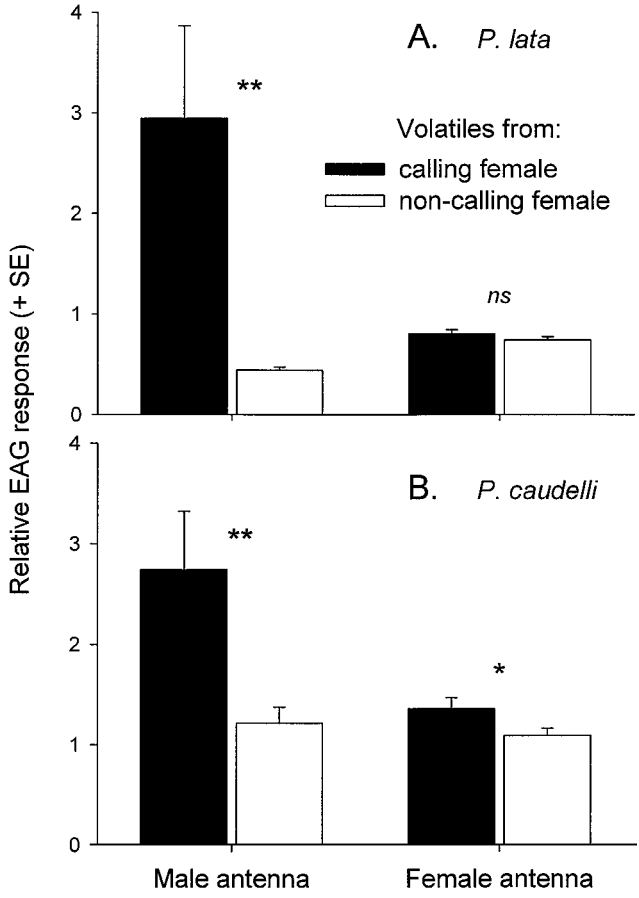

FIG. 3. EAG responses of *Parcoblatta lata* (A) and *P. caudelli* (B) male and female antennae to volatiles collected from calling and noncalling females. The EAG amplitude in response to the test sample was divided by the EAG elicited by a general odor (bornyl acetate) to control for variation in antennal response. Significant differences in EAG responses to volatiles from calling and noncalling females are indicated (*ns* = not significant; $^{*}P < 0.05$; $^{**}P < 0.01$).

significantly higher male than female EAGs in *P. lata* (Figure 4A; $F_{1,8} = 8.24$, 26.35, 30.47, 32.57, 38.51, 5.63, 0.20, 0.15, 0.13, 0.61, 0.13, 0.99, 0.80, and $P = 0.02$, <0.01, <0.01, <0.01, <0.01, 0.04, 0.67, 0.71, 0.73, 0.46, 0.73, 0.35, 0.80 for tergites 1–10, sternites, digestive tract, and rest of the body, respectively) and tergites 1–7 in *P. caudelli* (Figure 4B; $F_{1,10} = 6.40, 14.21, 13.25, 19.11, 12.46$, 11.26, 12.9, 1.56, 0.10, 0.05, 0.03, 0.35, and $P = 0.03$, <0.01, <0.01, <0.01,

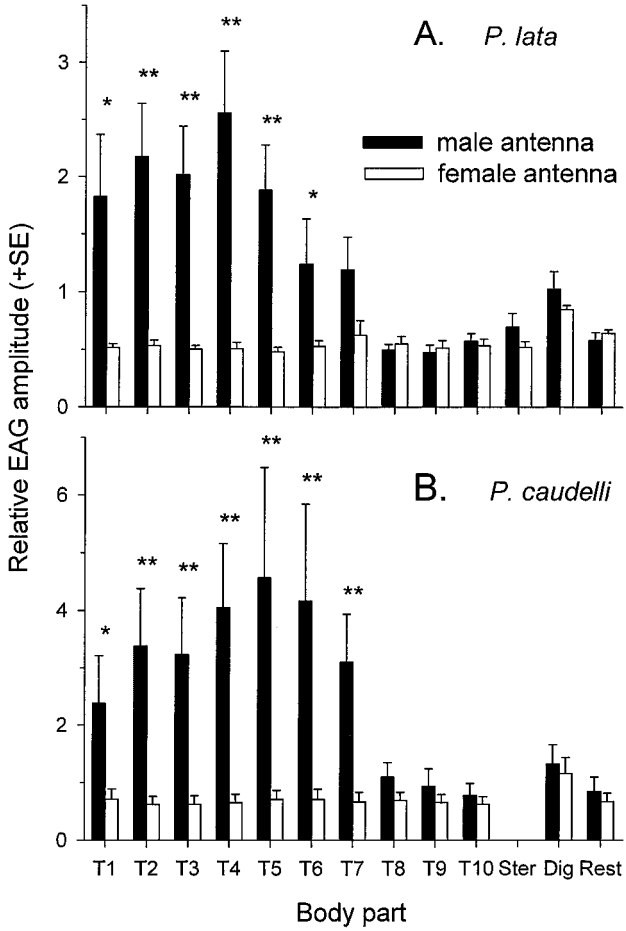

FIG. 4. EAG responses of *Parcoblatta lata* (A) and *P. caudelli* (B) male and female antennae to extracts of female tissues. T*n* = tergite number, Ster = all sternites (only in *P. lata*), Dig = digestive tract, Rest = rest of the body. Significant differences in EAG amplitudes of male and the female antennae are indicated by asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$). Most of the male-specific EAG response is to tergites 1–7.

<0.01, <0.01, <0.01, 0.24, 0.50, 0.83, 0.86, 0.56 for tergites 1–10, digestive tract, and rest of the body, respectively). The highest male-specific EAGs were recorded from tergite 4 of *P. lata* and tergite 5 of *P. caudelli*. The last three tergites (8–10) elicited low and similar EAG responses in male and female antennae. Similarly, although the digestive tract produced larger EAG responses, these were not sex-specific and can be accounted for by the presence of food odors in these samples.

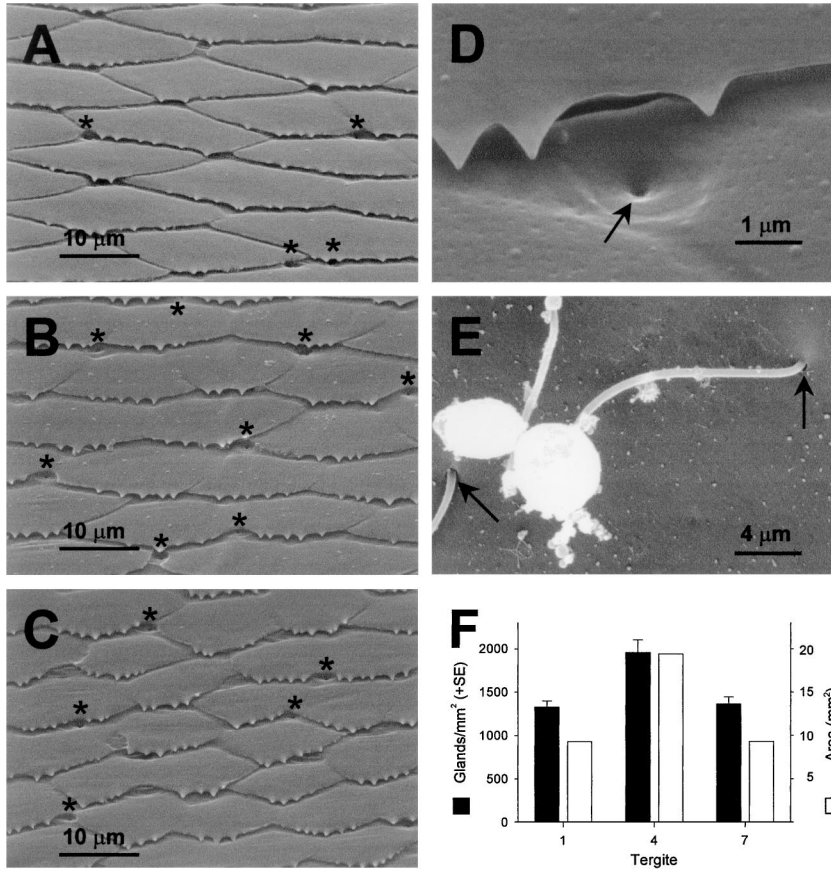

FIG. 5. SEM photographs of *Parcoblatta lata* tergites 1 (A), 4 (B), and 7 (C) showing the pheromone gland pores between the cuticular scales (asterisks). Close-up of one of the cuticular pores (arrow) from the fourth tergite (D) and the internal putative pheromone gland of the fourth tergite consisting of a tubular structure emerging from a cuticular pore (arrows) and terminating in cellular tissue (E). The number of glands per square millimeter and the area of tergites 1, 4, and 7 are also shown (F).

Under the light microscope, many filaments were visible in the cellular underside of *P. lata* tergites. These structures, which we later identified as the potential sex pheromone-producing glands, were present in all the tergites but were more numerous in tergite 4 (Figure 5F). Under the SEM, tergites 1, 4, and 7 had numerous ~0.25-μm-diameter pores between the scales, and these pores were more abundant in tergite 4 than in tergites 1 and 7 (Figure 5A–D). On the underside of the tergites, we observed by SEM tubular structures that appeared to connect

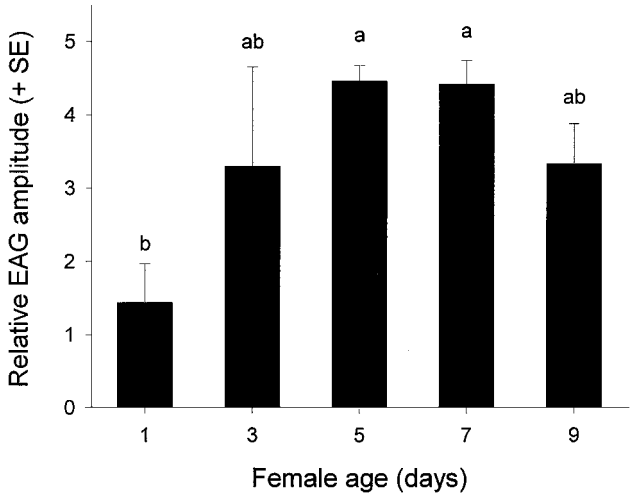

FIG. 6. Effect of age of *Parcoblatta lata* on pheromone production. Differences in male EAG responses to tergite extracts from females of various ages are indicated by different letters (Tukey's test, $P < 0.05$).

to the exterior of the tergite through a pore and terminated in a globular cellular structure at its distal end (Figure 5E).

Effect of Age and Mating on Pheromone Gland Content. The male response to extracts of female tergites increased with age of the female, from day 1 to day 7 and then declined slightly on day 9 (Figure 6; $F_{4,15} = 3.96$, $P = 0.02$). Mating suppressed calling in females: None of the 12 females that were mated called the day following mating, whereas 9 of the 12 unmated females of the same age group that had called, also called on the following day. The quantity of pheromone in pairs of mated and virgin females remained the same one day after mating (Student's *t* test, $T = 0.25$, $df = 10$, $P = 0.404$), and also did not decrease in mated females immediately after oviposition ($T = 1.41$, $df = 5$, $P = 0.108$).

DISCUSSION

Based on sexual dimorphism in wing length, we hypothesized that female *P. lata* and *P. caudelli*, which are flightless, produce long-range volatile sex pheromones to which the fully-winged males respond from a distance, as in the cockroach *S. longipalpa* (Charlton et al., 1993). Our results confirm this prediction and further demonstrate that females engage in a calling behavior similar to that observed in other cockroach species, and that a sex-specific attractant is emitted when females call but not when they rest. Besides this report, the only other

empirical association between calling and pheromone release in cockroaches is in *S. longipalpa*. Significantly more male *S. longipalpa* are attracted to calling females than to noncalling females of the same physiological stage, but on a reversed photocycle (Smith and Schal, 1990b). Furthermore, in *Blattel germanica* L. female headspace collections from virgins are significantly more attractive to males than collections obtained from mated females, suggesting that virgin females emit a sex pheromone during calling behavior (Liang and Schal, 1993a). In addition to the two *Parcoblatta* species studied here, we have observed a similar calling behavior in *P. virginica* (Brunner), and other studies indicate that female long-range volatiles are also used by *P. fulvescens* (Sausure & Zehntner) (Wendelken and Barth, 1971). It is likely that other species of the *Parcoblatta* group employ a similar mode of sexual communication.

Calling behavior has now been reported in three of the five cockroach families—Blattidae, Blattellidae, and Blaberidae (Schal and Bell, 1985). The calling behaviors of *P. lata* and *P. caudelli* are similar to those observed in females of other cockroach species, where the body is elevated and the posterior of the abdomen is lowered toward the substrate. Observations of *P. lata* and *P. caudelli* calling periodicity were constrained by the availability of individuals, but even so, it is clear that calling is restricted to a specific portion of the scotophase, as in most other cockroach specied studied, including species in which the males call [e.g., *Eurycotis floridana* (Walker)] (Schal and Bell, 1985; Smith and Schal, 1991; Farine et al., 1996). In some other species, however, the periodicity of calling is not as well defined. For example, *B. germanica* females call almost continuously, both during the photophase and the scotophase, with a broad peak occurring before the end of the scotophase (Liang and Schal, 1993a).

We have shown that the sex pheromones of *P. lata* and *P. caudelli* are released only during calling. This was demonstrated by two independent observations. First, volatile collections from calling females produced higher male-specific EAG responses and were preferred by males over volatile collections from non-calling females. Second, as measured by EAG responses to tergite extracts, females have sufficient pheromone in their glands during the photophase to elicit strong male-specific antennal responses. Therefore, the lack of sexual activity of the diurnal volatile collections is probably caused by the shutdown of the pheromone releasing mechanism and not solely by a decrease in pheromone quantity. This is probably because pheromone production in cockroaches depends, at least in part, on slow developmental changes in the pheromone glands (Schal et al., 1996; Sreng, 1998), and rapid diel changes in pheromone quantity are not expected in cockroaches. This may also explain why after mating the calling behavior was completely suppressed, but the quantity of pheromone declined slowly through the completion of the first gonotrophic cycle. Only after the second egg case was deposited did we see a marked decrease in the quantity of pheromone. This could be related to a slow, gradual process of turning off pheromone production, possibly involving

developmental changes in the pheromone gland cells. It is plausible, however, that pheromone may remain in the glands of mated females because after several ovarian cycles these females may regain sexual receptivity and resume calling. Because noncalling females do not release pheromone, there is minimal cost for mated females to maintain pheromone in their glands, in terms of attraction of males or natural enemies.

As with *P. lata*, mating in *S. longipalpa* also suppressed calling behavior, and in this species, too, it remains to be determined how mating affects pheromone production (Smith and Schal, 1990a). Females of the German cockroach, *B. germanica*, undergo a 3-week pregnancy after the egg case is formed. Pheromone production in mated females is greatly reduced and does not return to the high levels observed in virgin females until gestation is completed (Schal et al., 1996). This pattern of pheromone production is probably unusual among oviparous cockroaches and is likely related to the protracted pregnancy in *B. germanica*. During this period, the females remain sexually unreceptive and, therefore, have no need to attract males. Mated *Parcoblatta* and *S. longipalpa*, on the other hand, drop the egg case soon after it is produced (*P. lata*: 4.2 ± 0.40 days, $N = 6$; *P. caudelli*: 2.7 ± 0.17 days, $N = 21$; *S. longipalpa*: 0.99 ± 0.024 days, $N = 84$), and they experience relatively rapid reproductive cycles. In these species, some pheromone may be retained in the pheromone gland for use in a subsequent reproductive cycle when the female needs to remate.

In cockroaches, the site of pheromone production is species-specific. In the blattid *P. americana*, the female sex pheromone is produced in the digestive tract, associated tissues, or in the atrial glands (Abed et al., 1993b; Yang et al., 1998). In *Nauphoeta cinerea* (Olivier), a blaberid within the Oxyhaloinae, the male pheromone gland is localized in sternites 3–7 (Sreng, 1985). In the few blattellids that have been studied, the female sex pheromone is produced in the tergites. *S. longipalpa* females produce a sex pheromone in tergites 4 and 5 (Schal et al., 1992), whereas in *B. germanica* females the pheromone is made in the 10th tergite, or pygidium (Liang and Schal, 1993b; Tokro et al., 1993). Both *P. lata* and *P. caudelli* produce the female sex pheromone in the anterior tergites, and there is a clear correlation between the density of cuticular pores, presumably representing the openings of pheromone glands, and the quantity of pheromone produced by tergites. The morphology of their pheromone gland appears consistent with that of the class-3 exocrine gland (Noirot and Quennedey, 1974). Each 0.25-μm-diameter cuticular pore is connected via a duct to a cellular structure, which probably consists of a large secretory cell, as in other species. The pheromone glands of *S. longipalpa* and *B. germanica* contain similar cell types, and the secretory cell appears to transfer its excretions through an extensive array of microvilli into the duct and then to the exterior (Schal et al., 1992; Liang and Schal, 1993b; Tokro et al., 1993). Unlike *S. longipalpa*, however, in which the glands are more abundant on the lateral regions of the tergites, the glands of *P. lata* show a uniform

distribution throughout the tergal surface (data not shown). Furthermore, the density of pores in tergites 1, 4, and 7 of *P. lata* (Figure 5F) is much lower than in *S. longipalpa*. However, because pheromone production is only one of many functions of class-3 integumental glands (Noirot and Quennedey, 1974), some of the glands counted in this and previous studies may represent other functional units. Indeed, glandular structures similar to those found in the tergites of *Parcoblatta* females were also observed in the sternites, which do not produce pheromone. Likewise, tergites 1 and 7 of *S. longipalpa* have cuticular pores, but they do not produce pheromone (Schal et al., 1992). All the same, the positive correlation between gland density (Figure 5F) and the quantity of pheromone produced (Figure 4A) indicates that a significant fraction of the glands observed in tergites 1, 4, and 7 are involved in pheromone production, and this is probably also true for the other tergites that produce pheromone.

During calling, *P. lata* and other cockroach species periodically expose the genital vestibulum, but the function of this behavior is unknown. It is not performed predictably during each calling bout and, unlike calling, its occurrence is erratic. We observed this on several occasions immediately after a female contacted a male with her antennae, but most times it occurred in solitary calling females, as also reported in other species (Schal and Bell, 1985). In the Oriental cockroach, *Blatta orientalis* L., the presence of calling males or their extracts stimulates females to perform this behavior, which "triggers an immediate response from the male" (Abed et al., 1993a). *Periplaneta americana* females, on the other hand, expose their atrial glands in the absence of males. Extracts from atrial glands of *P. americana* and *B. orientalis* have sex-specific activity, and it has been suggested that the sex pheromone of these species is synthesized in these glands (Abed et al., 1993a,b). However, empirical demonstration that a volatile attractant is released during opening of the vestibulum is lacking. Different signals may be released during this behavior and during calling, and male attraction under natural conditions may require a combination of these or even more chemical signals.

In some species, there is a causal relationship between calling behavior and pheromone emission, because the pheromone gland, which is normally covered, becomes exposed during calling. In other species, namely *B. germanica* and *S. longipalpa*, the glandular area does not seem to become appreciably more exposed during calling. In *P. lata*, calling exposes the anterior region of each tergite, which is normally covered by the adjacent tergite. Yet, the pheromone glands seem to be as numerous in the posterior as in the anterior and lateral areas of the tergites. Minimally, the behavioral postures employed by calling females might expose additional cuticular pores in the anterior regions of tergites 1–7. More likely, however, calling directly effects the release of the pheromone by facilitating its secretion. The exact mechanism by which the pheromone in these cockroaches is transported from the secretory cells, through the ducts, and to the exterior is unknown, however, and deserves further study.

Roughly <1% of the >4000 described cockroach species are associated with human structures, yet most of the studies on cockroach biology and reproduction have focused on these species. Because the pest species represent three of the five cockroach families, they have provided material for comparative studies of reproductive physiology and chemical ecology. Interestingly, even within this small group, a great deal of diversity has been revealed by the patterns of sexual behavior and the chemical structures of the identified pheromones (Gemeno and Schal, 2003). However, because pest species generally live in aggregations, relying only on pest species for studies of chemical ecology can bias our general understanding of reproductive strategies in cockroaches as a whole. *Parcoblatta* species offer an excellent opportunity to study the mating behavior and chemical ecology of a group of endemic, closely related cockroach species under both natural and laboratory conditions.

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FEMALE SEX PHEROMONE-MEDIATED EFFECTS ON BEHAVIOR AND CONSEQUENCES OF MALE COMPETITION IN THE SHORE CRAB (*Carcinus maenas*)

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Abstract—Exposure to receptive female pheromone elicits guarding behavior in shore crab males (*Carcinus maenas*), but little is known about the effects of sex pheromone on male competition or if the female plays an active role in mate choice. This study examined whether female pheromone enhanced agonistic behavior between males and what effects visual and chemical cues had on the rules and costs of such contests. We also investigated whether females exhibit a preference for males in terms of size. Under laboratory conditions, solitary male shore crabs engaged males who already had possession of a female. The visual and chemical presence of a receptive female had an impact on contest rules and costs. Fights were costly in terms of duration and of sustaining injury with either one or both crabs incurring injury in 40% of fights. To investigate the metabolic consequences of fighting over a perceived sexual resource (chemical cue only), fights were staged between pairs of size-matched males in the presence of water containing the female sex pheromone, water in which males had been kept, and untreated seawater. The duration and intensity of contests were greater when staged in the presence of the female pheromone compared with the two other treatments. Crabs that fought in the presence of female sex pheromone also had a greater accumulation of L-lactate and a reduction of glycogen stores. Fights were less costly in terms of injury with a single chemical cue compared with enhanced costs with a multiple sexual cue. The importance of female choice was determined by presenting postmolt females with different sized males. Males were kept in a fixed position, and the majority of females approached and

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performed courtship behavior to the largest males, demonstrating that females may be selective in terms of size.

Key Words—*Carcinus maenas*, shore crab, sex pheromone, male competition, female choice, agonistic behavior, relative size.

INTRODUCTION

Chemical communication is important in many behavioral and ecological interactions. Research has demonstrated the importance of chemical signals in attracting sexual partners [e.g., shore crab (*Carcinus maenas*) (Bamber and Naylor, 1997), American lobster (*Homarus americanus*) (Bushmann and Atema, 2000), rainbow trout (*Oncorynchus mykiss*) (Yambe and Yamakazi, 2001)], signaling aggressive motivation [e.g., brown bullhead (*Ictalurus nebulosus*) (Carr and Carr, 1986), sticklebacks (*Gasterosteus aculeatus*) (Waas and Colgan, 1992), American lobster (*H. americanus*) (Bushmann and Atema, 1997), lizards (*Podarcis hispanica*) (López and Martín, 2001)], alerting conspecifics to danger [e.g., large-mouth bass (*Micropterus salmoides*) (Brown et al., 2001), slimy sculpins (*Cottus cognatus*) (Bryer et al., 2001)], locating prey [e.g., American lobster (*H. americanus*) (Scholz et al., 1989)], and for individual recognition [e.g., rock crabs (*Cancer irroratus*) (Ristvey and Rebach, 1999), Atlantic salmon (*Salmo salar*) (Griffiths and Armstrong, 2000), Eurasian deer (*Cervus elaphus*) (Lawson et al., 2001)]. Chemical cues in the urine are a source of information regarding identification, sex, aggressive intentions, and physiological status (Bushmann and Atema, 1996, 1997, 2000; Bamber and Naylor, 1997; Beithaupt and Atema, 2000; Zulandt Schneider and Moore, 2000; Yambe and Yamazaki, 2001; Zhang et al., 2001). Reception of chemical stimuli in urine is critical for the formation and stability of dominance hierarchies in some species (e.g., Karavanich and Atema, 1998). This importance of urine signaling components has been demonstrated in the social interactions of crustacean species (Berkey and Atema, 1999; Breithaupt and Atema, 2000; Zulandt Schneider and Moore, 2000) and, indeed, female American lobsters (*H. americanus*) can discriminate between males of differing dominance status without prior contact with these males (Bushmann and Atema, 2000). However little attention has been paid to the impact of female sex pheromones on the interactions among competing males.

Male shore crabs (*C. maenas*) fight readily over resources such as food and individual space (Sneddon et al., 1997a), but relatively little is known about male competition for sexually receptive females. The breeding season of this species begins in May and ends in September, during which time females can mate once during the short postmolt period when the exoskeleton is soft and the female releases a stimulating pheromone (Crothers, 1964). Prior to molting, females release a potent pheromone that elicits stereotypical mating behavior in responsive males (Bamber and Naylor, 1996a,b; Williams et al., 1998). Mating behavior includes

precopulatory guarding (the female shore crab is cradled beneath the male before the female molts), followed by copulation (after molting) and postcopulatory guarding (Bamber and Naylor, 1996a). Since shore crab populations are heavily male-biased (Sekkelsten, 1988; Hunter and Naylor, 1993), it can be expected that competition between males for receptive females will be fierce. In the hermit crab, *Pagurus middendorffii*, males guard their mates longer when the population is more male-biased (Wada et al., 1999). The principal objective of this study was to determine whether male crabs actually do fight over receptive females under laboratory conditions.

Engaging in fights is costly, in the sense that crabs engaged in contests risk injury (Smith et al., 1994) and experience various adverse metabolic consequences (Sneddon et al., 1999). Some of the costs of fighting can be considered in terms of loss of energy (Sneddon et al., 1999), accumulation of an anaerobic debt (Neat et al., 1998), increased exposure to predation (Ellington, 1983), risk of injury or death (Shuster and Caldwell, 1989), and loss of time that could have been allocated to other activities such as reproduction and foraging (Glass and Huntingford, 1988). In the shore crab, studies have shown that crabs incur a significant anaerobic debt (accumulation of L-lactate in the blood and tissues) and loss of energy stores (glycogen in the tissue is broken down into glucose to enter metabolic pathways) during fights as well as the loss of time due to the lengthy duration of fights (Sneddon et al., 1997a, 1998, 1999). Therefore, the second objective of this study was to examine what costs male crabs sustain when fighting over receptive females and whether multiple cues (visual, tactile, and chemical and also possibly hydrodynamic) had a greater impact on male agonistic behavior.

If individuals of one sex actively choose specific characteristics in a sexual partner, then intrasexual competition for mates is important, and such selective mating may influence the differential mating success of the other sex. Usually females are the limiting sex, and this results in choosiness in females and competition among males (Atema, 1986). Female choice may influence mating success in male shore crabs, and it has been suggested that pheromone release is pulsatile and may be under the control of the female (Cheung, 1966; Bamber and Naylor, 1997). Therefore, females may be actively choosing males by releasing pheromone only in the presence of suitable or attractive males. Bamber and Naylor (1997) presented an individual male *C. maenas* to sexually receptive females and observed that females directly approached the male and performed flapping movements of the abdomen. This suggested that the behavior of female *C. maenas* may directly influence male mating success, but it did not demonstrate that females could discriminate among males. A third aim of this study was to investigate if females prefer smaller or larger mates, by offering newly molted females a choice of tethered male crabs of different sizes.

This study explored whether multiple cues of a sexual resource (visual, tactile, and chemical presence) had a greater effect on male competition than a single

chemical cue. Three specific experiments were conducted on the mating behavior of the shore crab, *Carcinus maenas*. (1) The rules and costs of contest behavior were examined between a single male and a paired male with multiple sexual cues present. (2) The importance of chemical cues on the rules and metabolic consequences of fights between male crabs contesting a perceived sexual resource were determined. (3) It was investigated whether female shore crabs exhibit a preference for males in terms of size.

METHODS AND MATERIALS

Collection and Husbandry. Pairs of male and female crabs ($N = 70$) were collected from the shores of Forder, Cornwall, UK, in July. Crabs were found in mud-filled rock crevices beneath seaweed with only the eyes of the male crab visible and the female found beneath the male. Each pair was placed into a plastic bag that was loosely tied to allow aeration and then kept in continued isolation within cylindrical cages (10 cm diameter, 25 cm long) made of plastic mesh (0.5 mm mesh size). Cages were large enough to allow the crabs restricted movement and placed into 15 circular tanks (57 cm diameter, 33 cm deep) that had been partitioned by perforated plastic dividers to reduce visual and chemical contact. These tanks were supplied with a continuous flow of seawater and kept under ambient temperature (13–14°C) and light conditions. Crabs were kept in pairs until they were assigned to an experimental treatment. They were held in captivity for approximately 48 hr. Male and female crabs do not feed when in breeding condition, so they were not supplied with food (Bamber and Naylor, 1996a). Crabs were used once in an experiment and then they were released them into Plymouth Sound.

Experiment 1: Do Solitary Males Engage Paired Males in Contest Behavior? Fifteen pairs were chosen at random, and the females were removed from the pairs. The male was left overnight without the female and became the single male. A single male was placed into a glass observation tank ($50 \times 30 \times 45$ cm) with a gravel substratum and seawater and separated from an unfamiliar paired male (with female) with an opaque partition dividing the tank in two. Special sealant rims holding the partition in place ensured chemical contact between the contestants was kept to a minimum. Both crabs were measured (carapace width and claw length) with callipers (to 0.1 mm) prior to being introduced into the tank. The crabs were left to settle for 15 min, then the partition was raised and the behavior of the males was recorded verbally onto audiotapes using a dictaphone. Tapes were played back, and the behavior, duration, and outcome of the interactions ($N = 15$) were logged onto an event recorder. In this species, fights only begin when one crab directly approaches the other crab and engages in aggressive behavior. There is no initial display, and it appears as if shore crabs assess their opponent by direct physical

contact (see Sneddon et al., 1997a for details). Contests can be brief interactions, where the loser quickly disengages from his opponent, or longer fights, where both crabs escalate to potentially injurious behavior. At any point during the fight a crab can retreat from his opponent and no longer participate in aggressive behavior. The intensity of each fight was assessed by behavioral actions. Intensity was divided into seven types with type 1 being the least intense and type 7 the most intense (Sneddon et al., 1997a). The duration of each contest was calculated from when the crabs first engaged one another in aggressive behavior to when the loser retreated from the winner and behaved submissively.

Spearman rank tests were used to determine if there was a correlation between fight intensity and duration and also between the relative size of opponents and either intensity or duration of contests. The relative size of the interactants in a contest was calculated as the percentage size difference in carapace width between the two crabs in each fighting pair.

Experiment 2: Are There Metabolic Consequences of Fighting over a Perceived Sexual Resource? Eighty-five pairs of crabs were selected, and the males and females separated. Fights were staged between two single size-matched (carapace width and claw length to 1%) males by placing them into one of three glass observation tanks as described before. After 15 min, the partition was removed and water of one of three types was pumped through plastic tubing into the center of the tank (100 ml/liter of tank water). The three types were seawater ($N = 10$ fights); male-conditioned seawater ($N = 10$), or female-conditioned seawater ($N = 10$). Conditioned water was obtained by placing 15 males, or 15 receptive females into 2 liters of seawater in a tank for 2 hr. The female sex pheromone has been isolated and partially purified using this method (Williams et al., 1998). A further 10 males were used as controls and were not fought. The behavioral data (outcome, duration, and intensity) of each contest were recorded as before. The duration of fights staged under the three different treatments was compared using ANOVA, and to establish whether there was a difference in the intensity of contests, the three treatments were compared using chi square.

After the resolution of five contests of each of the three types, each crab was removed immediately from the observation tank and a blood sample taken from the arthrodial membrane at the base of the fourth pereiopod using a hypodermic needle and syringe. In another five contests of each of the three types, crabs were removed and killed by rapid immersion in liquid N_2 to obtain tissue samples. Ten crabs, used as controls, were placed individually into the experimental arena, left for the settling period, and then the partition was raised. Control crabs were left in the tank for 504 sec (overall mean of the 30 contests in experiment 2), and then either a blood sample ($N = 5$) was taken or the crabs were killed for tissue sampling ($N = 5$).

The blood samples (0.5 ml) were added to 0.5 ml of chilled 0.6 M perchloric acid (PCA) to halt all enzymatic activity and to precipitate proteins. Samples were

centrifuged (10,000 *g* for 10 min), and the supernatant removed. The supernatant was neutralized by dropwise addition of potassium bicarbonate (0.2 M) and stored at −20°C prior to analysis. Muscle tissue was removed from the legs and homogenized with liquid N_2 in a mortar and pestle. The resultant powder (50 μg) was weighed into an Eppendorf reaction tube containing 0.25 ml of 0.3 M PCA. The tube was vortex mixed, centrifuged, and the supernatant removed and retained. Another 0.25 ml of 0.3 M PCA was added to the precipitate in the tube, which was again centrifuged, and the supernatant was removed and added to the original supernatant. The pooled supernatant was neutralized and stored at −20°C for L-lactate analysis. Further samples of muscle tissue (50 μg) from each crab were weighed into tubes containing chilled 0.4 ml potassium hydroxide (2 M KOH) and were placed into a boiling water bath for 2 hr. A small hole was made in the lids of the Eppendorf tubes to release gaseous material. After removal from the water bath and having cooled to room temperature, 0.5 ml of ethanol was added to each tube. After 1 hr, the supernatant was removed and discarded, and the precipitate was washed in ethanol five times to remove glucose from the muscle. The precipitate was analysed for glycogen content (Keppler and Decker, 1974). The L-lactate concentrations in hemolymph and muscle tissue were determined using the method of Gutmann and Wahlefeld (1974). Glycogen content was analyzed using the method of Keppler and Decker (1974).

The metabolite data were not normally distributed, so the residuals were transformed ($\ln + 1$), and ANOVA was used to determine any differences in L-lactate and glycogen concentrations among crabs that fought under the three treatments. The data for crabs fighting under each treatment were pooled, since in previous studies there was no difference between winners and losers, and no difference was found in this study (paired *t* test $t = 1.27$, $P = 0.213$) (Sneddon et al., 1998, 1999). ANOVA was used to compare the metabolite data of crabs that fought with control crabs to show whether fighting exerted an increased anaerobic debt and reduction in energy reserves. Two-tailed tests of significance were used throughout, and significance was accepted at the 5% level.

Experiment 3: Do Female Shore Crabs Choose Mates by Size? Precopula pairs containing a hard-bodied female were kept together until they had molted in the laboratory aquarium ($N = 7$). Then, females were removed from their pairs, and an individual female was placed in one half of the experimental tank separated by the opaque partition from three male crabs of differing size. Males were either small (carapace width 55–60 mm), medium (61–65 mm), or large (65–70 mm), and they were tethered to three different plastic pipes (diameter 2 cm) attached to the sides of the tank. Crabs were tethered by elastic bands placed under the abdomen between walking legs 2 and 3, and between legs 3 and 4. This allowed some vertical movement but prevented them from moving away from the pipes. Males were kept under water while the bands were gently positioned between their legs, and they were generally quiescent during this procedure. Once placed at the

pipes, the crabs initially attempted to move away but within a few minutes they became quiescent and remained motionless until the introduction of the female. This method of tethering was also used by Bamber and Naylor (1997). Different male crabs were used in each trial, and the females had no prior contact with the males. Males were placed in a random order at the pipes to eliminate preference for position within the arena. After a settling period of 1 hr, the partition was raised and the female allowed to wander freely. The size of the male crab that the female approached first and then performed abdomen flapping to was noted (as described by Cheung, 1966; Bamber and Naylor 1997).

RESULTS

Experiment 1: Do Solitary Males Engage Paired Males in Contest Behavior? In all 15 fights, the single male engaged the paired male in agonistic behavior, and the behavioral acts performed by the fighting crabs were similar to those recorded in previous studies of this species (Sneddon et al., 1997a, 1999). During these fights, both crabs faced up to one another and engaged in wrestling activity. They stood high on the ambulatory limbs with chelipeds outstretched and then embarked on a pushing contest during which the fights could escalate with crabs using claws to grasp or strike. At any point, the crabs could disengage and one male could retreat and no longer participate in aggression. Fight duration ranged from 80 to 2675 sec, with a mean fight duration of 753 ($\pm$197 SEM) sec. Fights ended early if the single male was smaller ($>$15% difference in body size) than the paired male, with the single male retreating from the pair (Figure 1). The paired male never disengaged from the single male until the fight had escalated and some form of injury had occurred. The paired male only initiated fights if the female was soft bodied in five of seven contests (postmolt, $N = 7$), whereas the paired male did not initiate if the female was hard bodied ($N = 8$). The single male was only successful in obtaining a female if it was 9% larger in body size and 4% larger in claw size, and this was observed in only three contests. In 40% of these fights, injuries (cracks in claws, legs, and carapace) occurred to one or both crabs. These injuries were quite common in males that we found at the sampling site. Most of these males were not paired, however, it cannot be ascertained if these injuries were sustained through competition for receptive females.

Intensity and duration of fights were positively correlated ($R_s = 0.763$, $P = 0.001$, $N = 15$). As the male crabs became more evenly matched in size, the intensity ($R_s = 0.814$, $P < 0.001$, $N = 15$) and duration ($R_s = 0.869$, $P < 0.001$, $N = 15$) of these contests increased (Figure 1).

Experiment 2: Are There Metabolic Consequences of Fighting over a Perceived Sexual Resource? Fights between males in the presence of female water were longer in duration (mean 779 ± 95.7 sec) than those fights staged in the

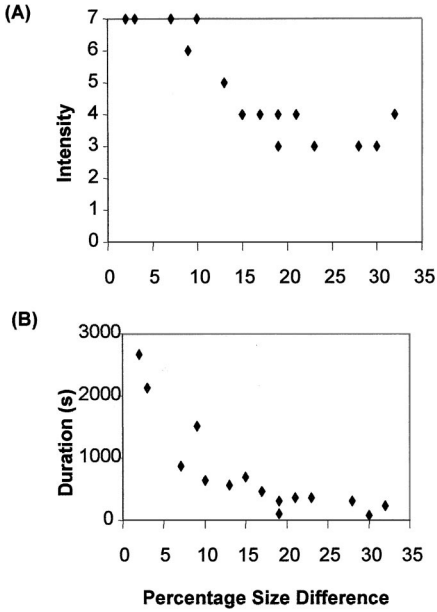

FIG. 1. (A) The influence of relative size on the intensity of contests staged between "single" male crabs and paired male crabs ($N = 15$). Relative size was calculated as the percentage size difference in carapace width between the two opponents. (B) The influence of relative size on the duration of contests staged between single male crabs and paired male crabs ($N = 15$).

presence of untreated seawater (327 ± 61.2 sec, $F_{1,15} = 12.98$, $P = 0.001$) and male water (408 ± 61.3 s, $F_{1,15} = 10.26$, $P = 0.005$; Figure 2). Fights between males in tanks to which male water and seawater had been added were similar in duration ($F_{1,15} = 2.94$, $P = 0.36$). Contests were divided into low (types 1–4) and high-intensity (types 5–7) fights. In the female water treatment, the majority of contests were of high intensity (80%), whereas in the other two treatments the majority were of low intensity (60% in both seawater and male water). However, the intensity of the contests in the three different treatments was not significantly different ($\chi^2 = 4.29$, $0.25 \langle P \rangle 0.1$, $df = 3$). The crabs did not sustain any injuries in these fights.

Crabs exposed to female water had a greater accumulation of L-lactate (ANOVA: blood $F_{3,16} = 48.3$, $P < 0.001$; tissue $F_{3,16} = 13.8$, $P < 0.001$) and lower glycogen stores ($F_{3,16} = 15.5$, $P < 0.001$) than crabs in the two other treatments (Figure 3A–C). After fighting, crabs had significantly higher concentrations of L-lactate in the blood ($F_{3,16} = 48.33$, $P < 0.001$) and tissues ($F_{3,16} = 13.78$, $P < 0.001$) and significantly lower concentrations of glycogen ($F_{3,16} = 15.50$, $P < 0.001$) in the muscle compared with control crabs (Figure 3).

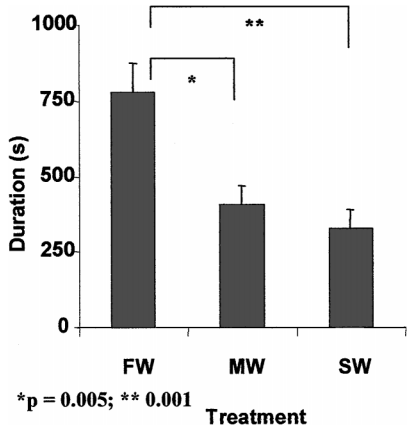

FIG. 2. The mean (±SE) duration of contests between pairs of male crabs staged in the presence of female-conditioned water (FW), male-conditioned water (MW), and seawater ((SW); $N = 10$ contests of each treatment).

Experiment 3: Do Female Shore Crabs Choose Mates by Size? In these choice experiments, the majority of females approached the largest male ($N = 5$) first, with two females approaching the medium-sized male and no females approaching the smallest male. After approaching the males, females fell backwards, exposing the abdomen to the male, and performed abdomen flapping in front of then for time periods ranging from 47 sec to 143 sec. Males responded stereotypically by standing high on the fully extended walking legs. Eventually, females either approached the male and crouched beneath his abdomen, or approached other males ($N = 1$ large and 2 medium).

DISCUSSION

Solitary male shore crabs that were sexually responsive approached males paired with receptive females and engaged in agonistic interactions. The behavioral content of these fights was similar to previous studies describing shore crab aggression (Sneddon et al., 1997a) and to male competition of other crabs (Jachowski, 1974; Berrill and Arsenault, 1982; Smith, 1992; Smith et al., 1994; Jivoff, 1997). Solitary males were only successful in usurping a paired male and obtaining the female if they were at least 9% larger in body size and 4% larger in claw length. In comparison with fights over food, shore crabs had to be at least 30% larger in body size and 18% larger in claw size (Sneddon et al., 1997b). Thus, the effect of being larger in size and winning fights is enhanced when fighting is over direct access to sexually receptive females. In fights with the female present, these interactions were short and of low intensity if the size difference between two males was large.

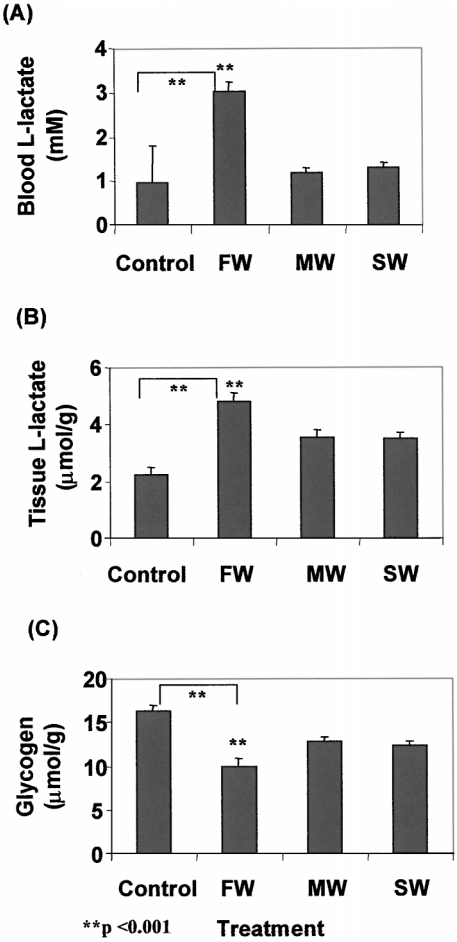

FIG. 3. (A) The mean (±SE) concentrations of L-lactate in the blood of control crabs (nonfought; $N = 5$) and in the blood of crabs fought in the presence of female-conditioned water (FW), male-conditioned water (MW), and seawater (SW) ($N = 10$ crabs for each treatment). (B) The mean (±SE) concentrations of L-lactate in the muscle tissues of control crabs (nonfought; $N = 5$) and in the tissues of crabs fought in the presence of FW, MW, and SW ($N = 10$ crabs for each treatment). (C) The mean (±SE) concentrations of glycogen in the muscle tissues of control crabs (nonfought; $N = 5$) and in the tissues of crabs fought in the presence of FW, MW, and SW ($N = 10$ crabs for each treatment).

As the size discrepancy decreased, contests became intense, and they lasted much longer than in a previous study that had examined contests over food (mean duration: female 753 sec; food 559 sec (Sneddon et al., 1997a). Males appeared to be prepared to fight longer over receptive females, which are perhaps a more highly

valued resource than food (Sneddon et al., 1997a). However, it may be that it takes a much larger and not a slightly larger opponent to steal a paired female since they are such a valued resource. When there is smaller competing male, the fights are short since the competitor quickly ascertains that it is not likely to obtain the paired female. When the unpaired male is much larger, the paired male may give up the female more quickly to avoid the costs of fighting a losing battle. Males also stood a 40% chance of being injured in these contests, which is a cost they do not incur fighting over food or individual space (Sneddon et al., 1997a).

The incidence of injury in fights with a female present was high, yet crabs fighting in the presence of a single cue, female pheromone, in experiment 2 did not sustain any injuries and neither do crabs fighting over other resources (Sneddon et al., 1997a). Crabs that do suffer injuries probably have their reproductive performance impaired, since crabs that suffer the loss of a claw are less able to acquire and defend females (Abello et al., 1994). Injured limbs are usually autotomized, which accelerates the onset of the molt cycle. After the molt, the regenerated limb is smaller than the other limbs, and the size increment (increase in body size) that the crab gains is lower than if no limbs were lost and the onset of molt was not induced by a lost limb (McVean, 1982; Skinner, 1985; Smith, 1990; Norman and Jones, 1991). Similar injuries were seen on solitary males at the sample site and have been observed in other C. maenas populations during the breeding season (Abello et al., 1994; Reid et al., 1994). Although limb loss is highest in males and studies have shown that limbs are lost in intraspecific interactions in many crustaceans (McVean, 1982; Norman and Jones, 1991; Smith and Hines, 1991), predation and cannibalism can also be responsible for injury.

Single males initiated the majority of fights (66%), and paired males only initiated them if their female was soft bodied, in the vulnerable postmolt period. Presumably, the paired male had mated or was about to mate with the female after the molt protects his investment, whereas a male may not initiate a contest over a hard female he has not yet mated with, as the costs of fighting over females are high. After molting, females can remate for at least 8 days (Bamber and Naylor, 1996b), and it may be in the male crab's interest to aggressively guard a postmolt female to ensure that he is the only male to mate with her and all offspring will be his. An alternative explanation is that the female sex pheromone is under the female's control and as such, a postmolt female may release more pheromone so that the male will continue to protect her during this vulnerable period.

Previous studies in shore crabs have shown no relationship between relative size and either fight duration or intensity (Sneddon et al., 1997b, 1998). It appears as if in fighting over a highly valuable resource, the chance of reproducing and passing on genetic material to offspring alters the rules of contest behavior in this species and increases the motivation to fight longer when the size disparity is small. This supports the hypothesis that intensity or duration of fighting should be adjusted according to the value of the disputed resource (Bishop et al., 1978;

Hammerstein and Parker, 1982; Enquist and Leimar, 1987). The mechanism by which males judge resource value may be by smelling the female sex pheromone, leading to increased aggression.

In the second experiment, crabs were used that were evenly matched in size, and only the effects of the odor treatment on metabolic consequences and behavioral content of fights were examined. The female-conditioned treatment increased the duration and intensity of contests compared with the seawater and male water treatments. This agrees with a similar study on velvet swimming crabs, *Necora puber* (Smith et al., 1994). The potency and specificity of the female sex pheromone on the male has already been demonstrated in *C. maenas* (Eales, 1974; Bamber and Naylor, 1996b, 1997; Williams et al., 1998) and other crustaceans (review in Dunham, 1988). Responsive male shore crabs can detect a dilution of 1 μl of urine containing female pheromone in 10 ml of seawater (Bamber and Naylor, 1997). Therefore, fighting over this perceived sexual resource may increase the motivation of the male crabs to fight longer and perform a greater intensity of aggression. No injuries were sustained in the second experiment, and the maximum fight duration was less than half of the maximum duration when the female was present (maximum duration in experiment 1, 2675 sec; experiment 2, 1276 sec). It may be that the tangible, visible presence of a female increases the benefits of fighting (i.e., direct access to reproduction), and, thus, crabs fight longer and more intensely (experiment 1), whereas merely the smell of a female may not justify the costs of potentially injurious, lengthy fighting behavior.

The accumulation of L-lactate, creating a metabolic debt, was higher in the female water treatment compared with the seawater and male water treatments, and there was also a greater reduction in the storage compound, glycogen, which is broken down into glucose to enter the respiratory pathways. This may be due to the fact that fights were significantly longer in the female water treatment, and crabs utilized more energy than crabs in the other two treatments. Again, this supports the idea that crabs are more motivated to fight over the perceived sexual resource and are willing to accrue greater costs in its presence. Alternatively, detecting the female pheromone may excite the male and increase energy turnover in preparation for mate-guarding behavior and sperm production. Crabs after a fight had higher L-lactate concentrations and lower glycogen reserves than control crabs, and this agrees with the results of a previous study that examined metabolic consequences of *C. maenas* fighting over food (Sneddon et al., 1999).

The newly molted females in experiment 3 behaved in a similar fashion to those described by Cheung (1966) and Bamber and Naylor (1997). They approached the tethered male crabs directly. The majority of females approached the largest male, with no females approaching the smallest males, suggesting that females are able to distinguish among males of different size and prefer larger males. Recognition of individuals has been demonstrated in crustaceans (Vannini and Gherardi, 1981; Bushmann and Atema, 1997; Karavanich and Atema, 1998),

and female lobsters (*H. americanus*) can differentiate among males of differing dominance status, choosing more dominant males to copulate with (Bushmann and Atema, 2000). Choosing the largest or most dominant males would be advantageous to females since these males may be more able to protect her during the vulnerable postmolt period. Moreover, large males are more successful competitively (Reid et al., 1994; Sneddon et al., 1997b), and, thus, the females' resultant offspring may be of better genetic quality. Few studies have investigated the interaction between male competition and female choice (although see Cunningham and Birkhead, 1998; Kendall and Wolcott, 1999; White and Galef, 1999), and in shore crabs it appears that females are selective in response to males of different sizes. Signaling sexual receptivity to only large males may explain why Reid et al. (1994) found that only the largest males of a population of shore crabs were paired with females during the mating season. However, male agonistic behavior seems to be a more likely reason for the largest males obtaining females. The male response to a receptive female is extremely swift, and perhaps that is why female choice in this species has been largely ignored. It may be that, since pheromone release is pulsatile (Bamber and Naylor, 1996b, 1997; Breithaupt et al., 1999) and possibly controlled by the female, a female in a pair may only release large amounts of pheromone in the presence of a solitary, larger, more attractive male. This may result in aggression between the males with the new larger male more likely to obtain the female. In this way, female choice may mediate male mating success. However, this subject must be studied in more detail to understand fully the precise nature of mating behavior in the shore crab. Another study has shown that female blue crabs (*Callinectes sapidus*) are not choosy with regards to sperm quantity when choosing between similar sized males that have high or low sperm reserves, and, thus male mating success depends upon male–male competition (Kendall and Wolcott, 1999).

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QUANTITATIVE ANALYSIS OF THE EFFECTS OF ULTRASOUND FROM AN ODOR SPRAYER ON MOTH FLIGHT BEHAVIOR

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Abstract—A piezoelectric sprayer was recently developed for precision release of odor stimuli in olfactory research. The device replaces conventional dispensers used to release semiochemicals in studies of moth flight toward odor sources. However, the device generates high-frequency sounds in the range that some moths can hear. Ultrasound from the standard set-up sprayer had a considerable impact on flight behavior of the silver Y moth, *Autographa gamma*, tested in a flight tunnel. It was affected at all behavioral stages when the dispenser was driven at 120 kHz. Only 5% of the moths reached the source when exposed to 120-kHz sound from the dispenser compared to 65% in the control group without sound. The proportion taking flight was also reduced. Hearing threshold curves obtained electrophysiologically revealed that moths were sensitive to the frequency range at which the sprayer was operated and that sound intensity from the sprayer was up to 40 dB above the moths' electrophysiological hearing threshold. The audiogram for *A. gamma* was similar to audiograms obtained for other noctuids. Hearing sensitivity was highest at around 15 kHz, where the threshold was 35 dB SPL (sound pressure level). The threshold increased with frequency up to 94 dB SPL at 160 kHz. We improved the sprayer to operate at 300 kHz, which is beyond the hearing ability of most insects with ears. At this high frequency, the moths' sensitivity to ultrasound is reduced considerably, and we did not observe any effect on flight behavior compared to a control group

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without sound. Accordingly, this new piezoelectric sprayer can be used with ultrasound-sensitive insects and insensitive insects alike.

Key Words—olfactory stimulation, odor application, dispenser, odor quantification, ultrasound, moth hearing, piezoelectric sprayer, *Autographa gamma*, silver Y moth.

INTRODUCTION

El-Sayed et al. (1999) recently developed a novel device for quantitative application of odor stimuli. The device makes use of high-frequency sound to disperse an aerosol of a chemical solution. An oscillator drives a disk made of a piezoelectric ceramic crystal attached to a glass capillary that in turn vibrates at ultrasonic frequencies. When a solution of chemicals is passed through the capillary, the high-frequency vibration results in the formation of an aerosol from the capillary tip. The sprayer has the advantage of releasing chemical compounds at a precise rate and proportion of blends, facilitating precise determination of effects of sex pheromones or plant volatiles on insects. Usually, in assays testing compounds or mixtures for their effect on behavior, chemical compounds are applied on filter paper or rubber septa. The disadvantages of these methods are that the odor release rate is unknown and the ratios of compounds in a formulated blend and in the air usually are distorted. Accurate information about the release rate of a substance makes comparisons with the natural odor source possible. One disadvantage of the sprayer is that it emits intense sounds at ultrasonic frequencies, which many moths can hear. Most moth species have ultrasound-sensitive hearing organs (tympanal organs), that are used to detect the ultrasonic cries of insectivorous echolocating bats (Hoy, 1992; Miller and Surlykke, 2001). Moths respond to ultrasound with a variety of behavioral escape responses, such as turns, spirals, loops, dives, and zigzag flight, to avoid being caught by an approaching bat (Roeder, 1967). El-Sayed et al. (1999) conducted a control experiment to study the effect of ultrasound on male response and concluded that the sound from the sprayer did not cause an effect on the upwind flight of the moth *Lobesia botrana* Denis & Schiffermüller (Tortricidae). However, the Tortricidae is one of the moth families where tympanic organs have not been found and, therefore, it is most likely that *L. botrana* is insensitive to ultrasound (Scoble, 1992). El-Sayed et al. (1999) stressed that it could not be concluded that other moth species would not be affected by sounds from the sprayer. Since most macrolepidoptera have ultrasound-sensitive ears and many of them are economically important pests, we decided to investigate the hearing in a moth species from this group, i.e., the silver Y moth, *Autographa gamma* (Noctuidae), and analyze the acoustic effects of the sprayer device. Based on these results, we suggest an improvement of the sprayer device, so that it can be used on ultrasound-sensitive moth species as well.

METHODS AND MATERIALS

Insects. We used males and females of the noctuid silver Y moth (*Autographa gamma*). Moths were obtained from a laboratory culture that was started from field-caught insects in 1997. Larvae were reared on a semisynthetic bean diet (Zhu et al., 1996) and kept at 24°C on a reversed 17L:7D light cycle. Pupae were separated according to sex. Emerged males and females were kept in separate climate chambers at the above-mentioned conditions.

The Sprayer. We used a similar sprayer set-up as described in El-Sayed et al. (1999). A custom-built disk made of a piezoelectric ceramic crystal (16 mm diam.) attached to a glass capillary tube was set in motion by a custom-built oscillator, which operated between 90 and 160 kHz. The piezoelectric ceramic disk attached to the glass capillary was placed inside a cylinder with a distal mesh to protect it from approaching insects.

In our set-up, the relationship between the frequency applied to the piezoelectric ceramic crystal and the resulting response depends upon the piezoelectric properties of the ceramic crystal, the size and the shape of the crystal, the direction of the electrical and mechanical excitation, the thickness of the attached U-shaped wire, and the geometry and glass type of the capillary tube (Jaffe et al., 1989). The most important factor that affects the resonance frequency of a piezoelectric ceramic crystal is the thickness of the disk. The resonance frequency is directly proportional to the thickness (Kinsler et al., 2000). Accordingly, we carried out an experiment in which an identical U-shaped wire was soldered to four piezoelectric ceramic disks; the diameter of the four piezoelectric ceramic disks was kept constant, i.e., 7 mm, while the thickness was varied, i.e., 1, 2, 3, or 4 mm. The resonance frequency at which vaporization of the solution of semiochemicals occurs was estimated for each piezoelectric ceramic disk using the same glass capillary tube. We found a linear relationship ($r^2 = 0.99$) between the driving frequency (i.e., resonance frequency) that gave rise to an aerosol and the thickness (millimeters) of the piezoelectric ceramic disk (Figure 1). A higher frequency was required to atomize semiochemicals as the thickness increased and the piezo behaved like a rod. Therefore, reducing the diameter of the piezo disk and increasing its thickness can provide a solution to the problem associated with ultrasound-induced behavior in insects. From these experiments, we developed an alternative ultrasonic sprayer, which was driven at higher frequencies, i.e., by 307 kHz square wave signals from a Tektronix function generator (CFG 253).

Behavioral Experiments. We used a 0.7 m × 0.7 × 2 m Plexiglas flight tunnel at Lund University, Sweden. Air is pushed into the tunnel at one end by a fan, and the pheromone plume is pulled out by an exhaust fume at the other. This creates a steady laminar flow of air in the middle of the tunnel. The shape and position of the pheromone plume were visualized with titanium tetrachloride, which forms smoke upon reaction with humidified air. The flight-tunnel

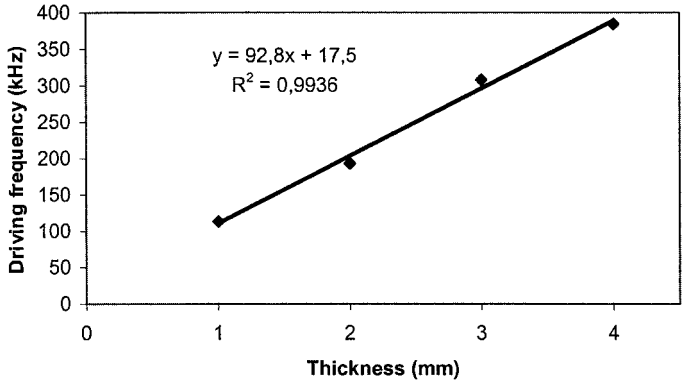


FIG. 1. The driving frequency at which vaporization of semiochemicals occurred is related to disk thickness in a linear manner.

conditions included a 2-lux red light, 48–70% relative humidity, and temperature of 21–23°C.

In order to test both males and females, we used a solution of a plant odor, phenyl acetaldehyde (PAA), which attracts both sexes. A rubber septum dispenser containing 1 mg PAA was placed in the upwind end just beside the vibrating capillary. A metal mesh in front of the rubber septum allowed the moths to land "at the source" without touching the devices.

Two- to 4-day-old moths were tested 0–4 hr after the start of their scotophase. They were transferred to the flight-tunnel room approximately 1 hr before initiation of the dark period, which allowed them to acclimate prior to experiments. They were released individually from screen cages into the plume at the downwind end. Five behavioral steps were scored: take flight (TF), orientation (OR), half way (HW), approach source (AS): 0–10 cm, and source contact (SC). Each individual was tested once and then discarded.

We tested the moths with the ultrasonic emitter turned off (no sound = control) and at four different frequencies: 90 kHz (the lowest test frequency), 120 kHz (the frequency with highest sound pressure), 160 kHz (the highest test frequency for the conventional system), and 307 kHz (with the newly built piezoelectric ceramic disk). When running the experiments with sound, the ultrasonic emitter was turned on continuously. Every test day included experiments with at least two frequencies and control. At the end, we pooled data from different days reducing possible day effects.

Sound Recording. Sounds from the sprayer were recorded at a distance of 20 cm from the piezoelectric ceramic crystal by a 0.25-in. G.R.A.S. (Vedbæk, Denmark) microphone (type 40 BF) without grid, a G.R.A.S. preamplifier (type 26AC), and a measuring amplifier (type 12 AK). Signals were read from the

oscilloscope screen. In order to calculate the intensity of the sounds, the microphone (with grid on) was calibrated against a G.R.A.S. sound calibrator (type 42AB). The frequency response of the microphone is relatively flat ($\pm$1 dB) up to 100 kHz. For measurements between 100 kHz and 170 kHz, we adjusted the values according to the microphone's frequency response curve. For power spectrum analysis, sounds were digitized online (Iotech Wavebook 512 A/D converter, clock rate of either 500 or 800 kHz) and stored directly on an IBM laptop computer. This recording chain is linear up to ca. 120 kHz, above which the microphone starts falling off. We analyzed the sounds using commercial software: BatSound (Pettersson Elektronik AB). Power spectra were calculated using an 8192-point Hanning window.

The sensitivity range of our microphone did not allow us to measure sound intensities at frequencies above 200 kHz. Therefore, when driving the piezoelectric disk at 307 kHz, we could only measure the intensity of subharmonics, which is also more relevant for the moths' hearing. The sound intensity was measured at several distances downwind from the dispenser. The inverse square law was valid down to 10 cm (the shortest distance tested) at all tested frequencies, meaning that the sound pressure level decreased by 6 dB every time the distance doubled. Hence, our measurements were done in the acoustic far-field.

Hearing. We determined the audiograms of the moths by recording the electrophysiological response of the hearing nerve when the moth ears were exposed to sound at different frequencies. Ultrasonic sound pulses were generated with a Wavetek oscillator (model 186) controlled by a custom-built pulse generator that gave shaped pulses with linear rise and fall times. The stimulus was amplified and broadcast through a custom-built electrostatic loudspeaker (6 cm diam.). The loudspeaker was calibrated before and after the experiments by means of a Brüel & Kjær type 4135 0.25-in. microphone (grid off), a Brüel & Kjær type 2619 preamplifier and a (type 2607 measuring amplifier). The frequency response of the loudspeaker was flat ($\pm$3 dB) from 25 to 130 kHz. The frequency response of the loudspeaker and microphone was taken into account when calculating the thresholds. Sound levels are given as dB SPL, i.e., relative to 20 μPa rms.

The tympanic nerve in *A. gamma* was exposed from a dorsal approach (Roeder, 1966) and hooked onto an extracellular tungsten electrode. The preparation was placed 40 cm from the loudspeaker with the ear facing the loudspeaker. The tympanic nerve activity was bandpass filtered, amplified (custom built equipment), and displayed on an oscilloscope and through an audio monitor. Sound stimuli were 10 msec long pulses with a rise/fall time of 0.5 msec repeated at 1 Hz. Threshold was defined as the SPL necessary to elicit one to two spikes in at least 8 of 10 stimulations. Hearing sensitivity was tested in 10-kHz steps in the frequency range from 5 to 160 kHz.

RESULTS

All three frequencies (90 kHz, 120 kHz, and 160 kHz) that we tested from the standard set-up had a significant effect on the moths' flight behavior close to the scent source compared to the control situation without sound (Figure 2) (χ^2 test, $P < 0.05$). Moreover, driving the sprayer with 120 kHz affected moth flight behavior at all distances. At this frequency, the moths' early flight behaviors, such as upwind flight in the plume (OR), were affected significantly more than at the other frequencies (Figure 2). Furthermore the proportion taking flight (TF) was reduced when compared to the control. Only 82% of moths left the release cage in the downwind end when the transducer was driven at 120 kHz compared to 97% in the control group without sound. Males and females generally responded in the

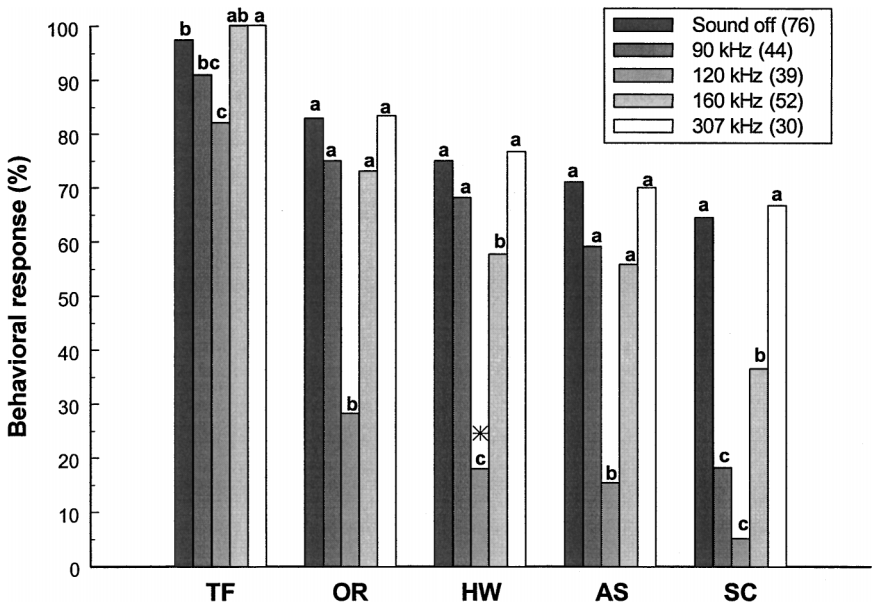


FIG. 2. The average behavioral response frequency of male and female *A. gamma* to 1 mg of PAA, while driving the sprayer at four different frequencies in relation to the control without sound: TF (take flight), OR (orientation), HW (half way), AS (approach source), SC (source contact). Treatments are compared within each behavioral step. Bars with the same letter are not significantly different (χ^2 test, $P \leq 0.05$). The asterisk indicates that there was significant difference between male (47%) and female (5%) response frequency. Numbers of animals are given in parentheses.

same way to sound. We only found statistically significant differences between male and female behavioral responses in one case: When the sprayer device was driven at 120 kHz, significantly more males (47%) than females (5%) made orienting flight toward the odor source (χ^2 test, $P < 0.05$). For practical reasons, we pooled data from males and females. Moths that flew all the way to the odor source and landed when the transducer was driven at 120 kHz generally needed more approaches before landing than at other frequencies. Often their first and/or second approach was disrupted by an evasive reaction. At 90 and 160 kHz, the moths did not seem to be affected at the farthest distance. However, when they approached the dispenser, we observed that they often disrupted their forward flight by making rapid evasive maneuvers away from it. The newly developed piezoelectric ceramic disk driven at 307 kHz did not evoke behavioral responses at any distances in the tunnel compared with the control (Figure 2).

Intensity measurements are given for the fundamental frequency measured at a distance of 20 cm from the dispenser. The highest sound intensity from the sprayer was measured at around 120 kHz (116–119 kHz), where the intensity was between 94 and 108 dB SPL at 20 cm. The intensity was a bit lower around 90 kHz (88–93 kHz) and 160 kHz (156–167 kHz), 78–90 dB SPL and 80–91 dB SPL, respectively. From these values, we calculated intensities at the source (i.e., 10 cm from dispenser) and at the downwind end (150 cm), taking the geometrical spreading loss and atmospheric attenuation into consideration. The calculated values are shown on Figure 3. The harmonics of the sound emitted from the disks were attenuated by more than 30 dB relative to the intensity of the fundamental frequency (i.e., the driving frequency) (Figure 4). The most intense harmonic was found at 42 kHz. Driving the dispenser at 120 kHz resulted in a harmonic at 42 kHz with an intensity of 52–66 dB SPL at 10 cm. A similar intensity at the same harmonic frequency occurred as a result of driving the dispenser at 90 kHz. When driving the smaller piezoelectric disk at 300 kHz, a subharmonic occurred at 42 kHz. The intensity of this harmonic could not be related to the intensity of 300 kHz for technical reasons mentioned in the methods. Hence, we used an indirect measure to estimate the intensity of the 42-kHz harmonic. By driving the smaller piezo at the 42-kHz harmonic frequency, we calculated an intensity of 51 dB SPL. This value might be overestimated, since we used the harmonic frequency to excite the piezoelectric disk instead of the 300 kHz signal.

The frequency and intensity ranges displayed in Figure 3 illustrate the minimum and maximum values of the fundamental frequencies and the most relevant subharmonics emitted by the sprayer. The audiogram shows that moths' acoustic receptor cells respond across the whole operating range of the standard sprayer. They are most sensitive at around 15 kHz where the threshold intensity is 34 dB SPL on average. The threshold increased with frequency up to around 95 dB SPL at 160 kHz, which was the highest frequency we tested.

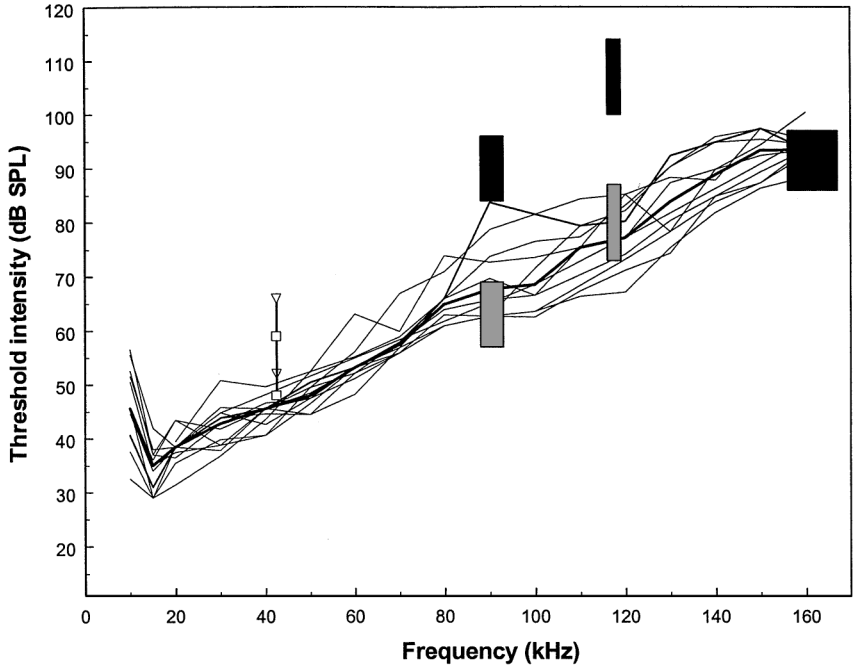


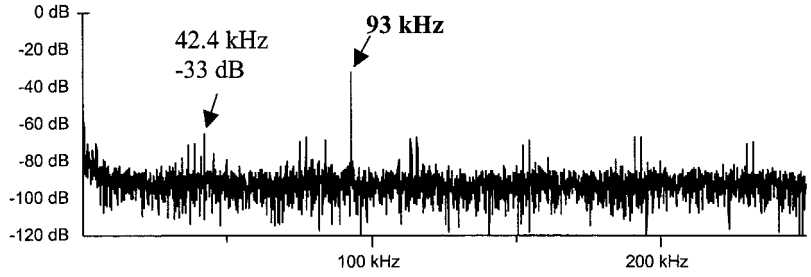
FIG. 3. Hearing threshold curves for *A. gamma*. The thin lines show individual hearing threshold curves, and the median audiogram is represented by a thick line. The rectangles show the maximum and minimum frequencies and maximum and minimum intensities from the dispenser at two different distances from the sprayer: at 10 cm (dark), which corresponds to the position for source contact (SC) and at 150 cm (gray), which corresponds to the position where moths take flight (TF). The intensity range of the dominant subharmonic (42 kHz) at 10 cm is shown for the 120 kHz (∇) and 165 kHz (□) signals. The values are calculated from intensity measurements taken at a distance of 20 cm.

DISCUSSION

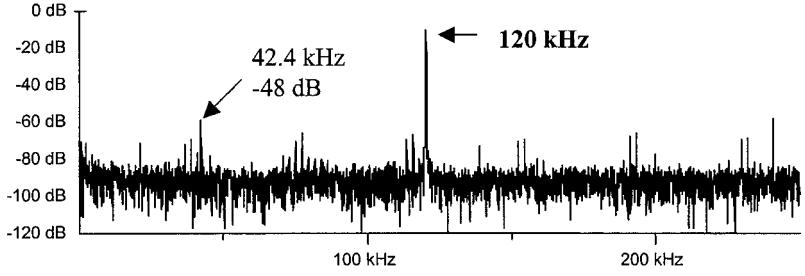
This study shows that flight behavior in ultrasonically sensitive moths is affected by ultrasound generated by the standard set-up of the odor sprayer. The

FIG. 4. Power spectra of sound emitted form the ultrasonic sprayer. (**A–C**) Spectra of the conventional piezoelectric ceramic disk when driven at 93, 120, and 165 kHz, respectively. (**D**) Spectrum of the new piezoelectric ceramic disk driven at 300 kHz. Arrows point to the fundamental frequency (in bold) and to the next highest peak, which is the most intense of the harmonics. In A–C, the intensity of the harmonics is indicated in dB relative to the peak intensity of the fundamental frequency. In D, the intensity of the subharmonic is calculated in dB SPL, since it was not possible to measure the intensity at 300 kHz.

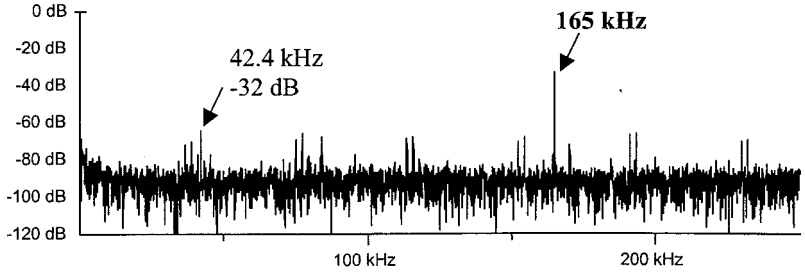
A.


0 dB
-20 dB
-40 dB
-60 dB
-80 dB
-100 dB
-120 dB
42.4 kHz
-33 dB
93 kHz
100 kHz
200 kHz

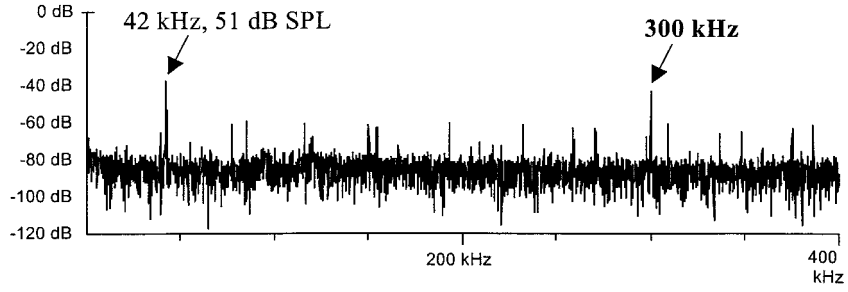
B.


0 dB
-20 dB
-40 dB
-60 dB
-80 dB
-100 dB
-120 dB
42.4 kHz
-48 dB
120 kHz
100 kHz
200 kHz

C.


0 dB
-20 dB
-40 dB
-60 dB
-80 dB
-100 dB
-120 dB
42.4 kHz
-32 dB
165 kHz
100 kHz
200 kHz

D.


0 dB
-20 dB
-40 dB
-60 dB
-80 dB
-100 dB
-120 dB
42 kHz, 51 dB SPL
300 kHz
200 kHz
400
kHz

effect depends on intensity of the sound. Flight behaviors close to the source were generally more affected than behavior further downwind, where the sound intensity was lower. The degree of disturbance is related to the difference in dB between the hearing threshold and the intensity of the dispenser. When the dispenser was driven at 120 kHz, which gave the highest sound intensity, the proportion of moths taking flight (TF) was reduced (Figure 2). This indicated that the moths could hear the sound from the other end of the tunnel. Examination of the hearing threshold curves indicates that the sound intensity at 120 kHz at the downwind end was above the neurophysiological threshold for some moths but not for all. This is probably the reason that only some of the moths (those that do not experience a high sound pressure) leave their flight cage. Remaining stationary on the ground when detecting ultrasound is most likely an adaptive defense behavior against substrate gleaning bats, as suggested by Werner (1981).

The sound pressure level 10 cm from the sprayer at 120 kHz corresponded to a sound pressure level 25–40 dB above the sensory threshold (Figure 3) and caused the most severe effects (Figure 2). Driving the sprayer at 120 kHz affected moths even at the downwind end where they were released.

At 90 and 160 kHz, where the intensity was lower, the moths did not seem to be affected at the farthermost distance. However, when they were closing in on the dispenser, we observed that they often disrupted their forward flight by making rapid evasive maneuvers away from the dispenser. These reactions were similar to evasive reactions observed by other noctuid moths when stimulated with sounds from an ultrasonic loudspeaker (Skals, unpublished observation). The behavioral effects caused by driving the emitter at 90 and 160 kHz, as shown on Figure 2, might be underestimated compared to the more intense sounds at 120 kHz, since the moths might become habituated to a higher degree at the low-intense signals than to the more intense signals at 120 kHz.

It is not necessarily the fundamental frequency that causes the negative effects on the moths' flight behavior. At 160 kHz, the sound intensity of the fundamental frequency is only above the sensory threshold in some cases (Figure 3). However, the behavioral data show that moths' flight behavior is affected at close range, since only 37% of the moths land on the source compared to 64% in the control group. This indicates the presence of lower frequency harmonics (i.e., subharmonics). The most intense of these subharmonics were found at 42 kHz (Figure 4), close to the moths' most sensitive frequency range. Although the intensity was 33–48 dB lower than for the fundamental frequency, it was still above the hearing threshold of the moth (Figure 3). Since the intensity of harmonics of the sprayer are above the hearing threshold of the moths, these subharmonic frequencies might also affect their behavior. Driving the new piezoelectric ceramic disk at 300 kHz gave rise to a 42-kHz subharmonic that had an intensity that is maximally 6 dB higher than the physiological hearing threshold. However, we found no effects on flight behavior towards the odor plume, even at close range to the transducer when driven

at 307 kHz. We could not measure auditory threshold at this high frequency, but the steady monotonic increase from 20 kHz to the maximal frequency that we checked, 160 kHz, makes it unlikely that gamma moths can hear 307 kHz at the intensity emitted.

The audiogram of *A. gamma* (Figure 3) resembles that of other similar-sized noctuid species (Surlykke et al., 1999), both in relation to best frequency, which is the frequency where threshold is lowest, and also with respect to threshold intensity. Hearing capabilities differ among moth species, for example, large noctuids appear to have more sensitive auditory organs than small noctuids (Surlykke et al., 1999), and pyralids seem to be relatively more sensitive to high ultrasonic frequencies than noctuids (Skals and Surlykke, 2000). Such differences will affect the behavioral reactions of different species. Thus, it is important to consider the hearing ability of the species before choosing the type of dispenser for testing olfactory responses. However, it is likely that the new 300 kHz model will be applicable for most, if not all, moth species, since it is likely that no species can hear sound at this frequency. We found that a relatively small modification of the sprayer developed by El-Sayed et al. (1999) was sufficient to avoid the negative effects of the apparatus without forsaking all the advantages for studying attraction to odors in insects. We conclude that the sprayer can be used on insects with ears by using high ultrasonic frequencies to which insects are less sensitive.

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DIFFERENTIAL METABOLISM OF 1,8–CINEOLE IN INSECTS

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Abstract—In order to compare the metabolism of 1,8-cineole in the pyrgo beetle, *Paropsisterna tigrina*, three other herbivorous insect species, *Faex nigroconspersa*, *Chrysophtharta bimaculata*, and *Oxyops vitiosa*, were fed 1,8-cineole leaf diets. *F. nigroconspersa* adults excreted predominantly 9-hydroxy-1,8-cineole (36.2% of the volatile constituents) with some 2α-hydroxy-1,8-cineole (11.4%). In contrast, larvae excreted predominantly 2α-hydroxy-1,8-cineole (27.4%) and smaller proportions of 9-hydroxy-1,8-cineole (5.2%) and 3α-hydroxy-1,8-cineole (4.3%). *C. bimaculata* adults excreted predominantly 3α-hydroxy-1,8-cineole (16.5%). *Oxyops vitiosa* adults, on a lower 1,8-cineole diet, excreted predominantly 2α,9-dihydroxy-1,8-cineole (4.2%) and 2α-hydroxy-1,8-cineole (3.5%), with smaller proportions of 3α-hydroxy-1,8-cineole (1.1%) and 9-hydroxy-1,8-cineole (0.5%). This is the first reported occurrence of a dihydroxycineole as an insect metabolite. Gas chromatographic and mass spectral data for hydroxycineoles are recorded and interspecific metabolite variation discussed.

Key Words—*Faex nigroconspersa, Chrysophtharta bimaculata, Oxyops vitiosa, Pergagrapta,* terpenoid metabolites, 1,8-cineole, hydroxy-1,8-cineole metabolites, gas chromatography–mass spectrometry.

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INTRODUCTION

Trees and shrubs of the family Myrtaceae are abundant food sources for numerous herbivores throughout Australia. Many species of the common *Eucalyptus* and *Melaleuca* genera contain an essential oil that frequently comprises substantial concentrations of the cyclic monoterpenoid ether 1,8-cineole (Figure 1, structure **1**) (Boland et al., 1991; Southwell, 1999a). Although the ingestion of neat eucalyptus oil (presumably 1,8-cineole rich oils) has caused death in humans (Tisserand and Balacs, 1995), herbivorous marsupials, such as the possum (Flynn and Southwell, 1979; Southwell et al., 1980; Carman and Klika, 1992; Bull et al., 1993; Carman et al., 1994; Boyle et al., 2000) and koala (Southwell, 1975; Eberhard et al., 1975), mammals, such as the rabbit (Miyazawa et al., 1989) and rat (Madyastha and Chadha, 1986), and invertebrate insects (Morrow and Fox, 1980; Ohmart and Larsson, 1989; Southwell et al., 1995; Schmidt et al., 2000; Fletcher et al., 2000) and microorganisms (MacRae et al., 1979; Nishimura et al., 1982; Carman et al., 1986; Williams et al., 1989; Liu and Rosazza, 1990;

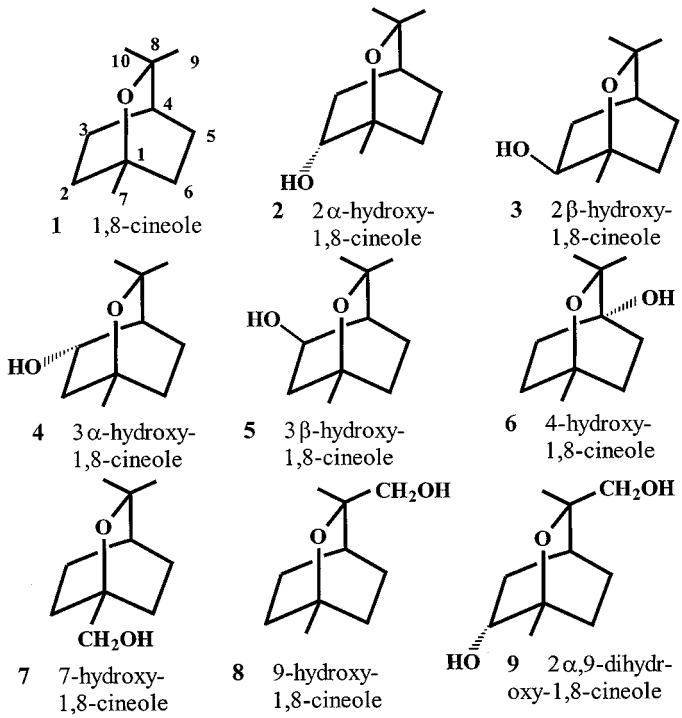

FIG. 1. Structures of 1,8-Cineole and metabolites.

Miyazawa et al., 1991) are able to cope with substantial quantities of this potential toxin.

In addition, 1,8-cineole has significant bioactivity as a mosquito feeding deterrent and ovipositional repellent (Klocke et al., 1987) and repellent and toxicant against stored-grain beetles (Obeng-Ofori et al., 1997). The use of terpenoids, including trace quantities of 1,8-cineole in the fecal shields of the tortoise beetle, *Eurypedus nigrosignata*, for chemical defense also has been demonstrated (Gomez et al., 1999).

Identification of 1,8-cineole metabolites (+)-2β-hydroxycineole **(3)** and 2α-hydroxycineole **(2)** in the frass of pyrgo beetle, *Paropsisterna tigrina* when fed 1,8-cineole-rich *Melaleuca* leaves (Southwell et al., 1995) raised the question: do other insect species metabolize 1,8-cineole in an identical manner? With *P. tigrina*, frass analysis indicated that all terpenoids except 1,8-cineole were being excreted unchanged. In an attempt to acquire evidence more general for insects, the cineole metabolites of other closely and distantly related species were examined, and details of this investigation provided support for a preliminary communication (Southwell, 1999b).

This paper reports the metabolite structures of 1,8-cineole in the frass of chrysomelid beetles, *Faex nigroconspersa* and *Chrysophtharta bimaculata*, and compares the structures with those previously reported for pyrgo beetle, *Paropsisterna tigrina* (Southwell et al., 1995). Recently published 1,8-cineole metabolites reported from both Leichhardt's grasshopper, *Petasida ephippigera* (Fletcher et al., 2000), and from the larvae of *Pergagrapta*, a Pergid sawfly (Schmidt et al., 2000), also are discussed and compared. In addition, the 1,8-cineole frass metabolites of the weevil *Oxyops vitiosa* are described. We recorded chromatographic (GC) and spectral (MS) data useful for identifying 1,8-cineole metabolites.

METHODS AND MATERIALS

Instrumentation. Analytical chromatography was performed on the following: (A) an Hewlett Packard 5890 chromatograph, 3393A Integrator, 7673A autosampler and an Alltech AT35 60-m $\times$ 0.25-mm, 0.2-μm film thickness, midpolarity FSOT column with hydrogen (45 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, flame ionization detector at 300°C and temperature program from 60°C (1 min) to 250°C at 10°C/min; (B) an Hewlett Packard 6890 gas chromatograph, HP Chemstation Rev A.06.03 (509) and autosampler using an Alltech AT35 60-m $\times$ 0.25-mm, 0.25-μm film thickness, midpolarity FSOT column with hydrogen (55 cm/sec) as carrier gas, injection port (split 1:50) at 200°C, flame ionization detector at 300°C and temperature program from 60°C (3 min) to 240°C at 9°C/min; and (C) a Shimadzu GC14B gas chromatograph with AOC 14 autoinjector, AOC 1400 autosampler using a BP1 50-m $\times$ 0.22-mm, 0.25-μm film thickness, FSOT

column with hydrogen (35 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, flame ionization detector at 300°C and temperature program from 50°C (1 min) to 240°C at 6°C/min, and a DB WaxETR 60-m × 0.25-mm, 0.25-μm film thickness, FSOT column with hydrogen (35 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, flame ionization detector at 300°C and temperature program from 50°C (1 min) to 240°C at 6°C/min. Retention indices were measured against *n*-alkanes. For constituent identification, GC-MS investigations were performed similarly using (D) a Hewlett Packard 6890 instrument using an HP5-MS 30.3-m × 0.25-mm, 0.25-μm film thickness, FSOT column with helium (36 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, mass selective detector (HP 5973) at 250°C (source) and 150°C (quad) with transfer line 280°C, and ion source filament voltage of 69.9 eV. Component identification was made on the basis of mass spectral fragmentation, retention time comparison with authentic constituents, and mass spectral and retention matching with commercial (NIST, Wiley and Adams) libraries. Authentic components were donated by Dr. Ray Carman, Department of Chemistry, University of Queensland, and 4-hydroxy-1,8-cineole was synthesized from terpinolene via the 4,8-epoxide, which, after ring opening with acid and oxymercuration, yielded the desired isomer as previously reported (Carman and Rayner, 1994).

Insect Species and Diets. Faex nigroconspersa were collected from a New South Wales (NSW) Agriculture planting of *Melaleuca alternifolia* at Duck Creek, Ballina, NSW, transferred to the Tropical Fruits Research Station, Alstonville, NSW, and maintained on a foliage diet gathered from mature high cineole (59.8%) *M. linariifolia* trees cultivated at Richmond Hill, Lismore.

Chrysophtharta bimaculata adults were obtained as a small population (<50) from a laboratory colony at the Cooperative Research Centre for Temperate Hardwood Forestry at Sandy Bay in Tasmania and prefed on cuttings from preferred field host species *Eucalyptus nitens* and *E. regnans*. Following arrival at the Tropical Fruits Research Station, Alstonville, NSW, the foliage diet was changed to mature high cineole (59.8%) *M. linariifolia* trees cultivated at Richmond Hill, Lismore.

Oxyops vitiosa adults were collected from *M. quinquenervia* trees growing in an abandoned nursery in Fort Lauderdale, Florida, USA, and fed fresh leaves of an intermediate cineole (22.7%) variety of *M. quinquenervia* collected from a field site also in Fort Lauderdale.

Pterygophorus sp. sawfly larvae were investigated in a commercial low cineole (1.6%) *M. alternifolia* natural stand at Greenridge, NSW, where frass was collected during heavy infestation.

Frass Collection, Extraction, and Replication. Samples of frass (10–100 mg) were collected, extracted with absolute ethanol while moist, exposed to microwave irradiation (700 W) (Southwell et al., 1995), and analyzed by GC and GC-MS. In these qualitative investigations, sample size was too small to quantify the amounts

of metabolite excreted with respect to the quantity of cineole ingested, and enantiomeric ratios of the hydroxycineole metabolites were not measured. For all frass samples, the best of several replicates, similar by GC, was used for detailed GC-MS analysis and compared with both the leaf ingesta volatiles and frass volatiles from low to zero cineole diets.

RESULTS

The regiospecific and stereospecific nature of the hydroxylation of 1,8-cineole **(1)** to predominantly (+)-2β-hydroxy-1,8-cineole **(3)** by the pyrgo beetle (*Paropsisterna tigrina*) feeding on 1,8-cineole-rich *M. linariifolia* leaf (Southwell et al., 1995) raised the question of whether the function of this conversion was detoxification or metabolism for the production of semiochemicals. As a first step in seeking clarification of this issue, the *in vivo* conversion of 1,8-cineole in a number of other insects was investigated and compared with that of pyrgo beetle (Table 1).

F. nigroconspersa (Clark) is a chrysomelid beetle that occupies a similar niche to *P. tigrina* (Southwell et al., 1995) among *M. alternifolia/linariifolia* herbivores. Larvae and adults of both species preferentially attack the terminal foliage and young flush leaf, thus restricting plant growth and regrowth. *F. nigroconspersa* is normally more abundant in drier seasons (Maddox 1996).

When fed 1,8-cineole-rich (60.0%) leaf from *M. linariifolia*, the larvae excreted frass containing volatile constituents that were found, by GC-MS, GC coinjection, and retention index comparison with authentic standards, to consist of 27.4% 2α-hydroxy-1,8-cineole **(2)**, smaller proportions of the 3α- **(4)** (4.3%) and 9- **(8)** (5.2%) hydroxyl isomers, and a trace of the 2β-**(3)** (0.5%) hydroxyl isomer. In contrast, the adult insect frass volatile constituents contained the 9-hydroxyl isomer **(8)** as the major component (36.2%), smaller proportions of the 2α- **(2)** (11.4%) and 3α- **(4)** (6.7%) hydroxyl isomers, and again a trace of the 2β- **(3)** (0.5%) hydroxyl isomer. Hence, the *F. nigroconspersa* frass volatile components contrast with *P. tigrina* frass volatile components by varying not only in chemical content, but also in larvae and adults (Table 1).

With *F. nigroconspersa* and the other species investigated, the enantiomeric excess of the metabolites derived from achiral 1,8-cineole was not investigated. Even though chiral standards of the synthetic hydroxycineole metabolites were available, they could not be separated on two different chiral GC columns. The chirality of the major *P. tigrina* metabolite was determined by optical rotation measurement of a substantial quantity (57.4 mg) of (+)-2β-hydroxy-1,8-cineole (Southwell et al., 1995).

C. bimaculata (Olivier) is another paropsine chrysomelid beetle and a major flush leaf defoliator in *Eucalyptus* plantations and native regrowth forests of Tasmania (Greaves 1966; De Little, 1983). When fed 1,8-cineole-rich (59.8%)

TABLE 1. PROPORTIONS (%) OF 2α,- 2β,- 3α,- 3β-, AND 9-HYDROXY AND 2α, 9-DIHYDROXY METABOLITES OF 1,8-CINEOLE DETECTED IN FRASS VOLATILES OF INSECTS FEEDING ON 1,8-CINEOLE-RICH *Eucalyptus* OR *Melaleuca* LEAF

	Coleoptera						Orthoptera	Hymenoptera	
	Paropsisterna tigrina (pyrgo beetle)[a]		*Faex nigroconspersa*[b,c]						
Hydroxy-1,8-cineole isomer	Adults	Larvae	Adults	Larvae	*Chrysophtharta bimaculata* adults[b,c]	*Oxyops vitiosa*[c] (melaleuca snout beetle adults)	*Petasida ephippigera*[d] (Leichhardt's grasshopper adults)[d]	*Perga-grapta* sp.[e,f] (Sawfly larvae)	*Pterygo-phorus* sp.[c] (sawfly larvae)
2α-OH **(2)**	1.7	2.0	11.4	27.4	0.6	3.5	5	tr	
2β- OH **(3)**	38.7	39.8	0.5	0.5	0.8		0.5		
3α- OH **(4)**	1.6	1.9	6.7	4.3	16.5	1.1	34		tr
3β- OH **(5)**							3.5		
9- OH **(8)**	1.7	0.7	36.2	5.2	3.4	0.5	7		
2α,9-di OH **(9)**						4.2			
1,8-cineole (unreacted)	10.1	7.4	6.9	19.2	28.0	5.8	0		0.2

[a] Southwell et al. (1995).
[b] Preliminary communication, Southwell (1999b).
[c] This communication.
[d] Extrapolated from Fletcher et al. (2000).
[e] Reexamination of Schmidt et al. (2000) data.
[f] Moore (2002, personal communication).

leaf from *M. linariifolia*, the adults excreted frass containing volatile constituents found, by GC-MS, GC coinjection, and retention index comparison with authentic standards, to consist of 16.5% 3α-hydroxy-1,8-cineole **(4)**, a smaller proportion of the 9- **(8)** (3.4%) hydroxyl isomer, and traces of the 2β- **(3)** (0.8%) and 2α- **(2)** (0.6%) hydroxyl isomers (Table 1). The larvae of this species were unavailable for investigation.

O. vitiosa is an Australian weevil species that has become widely established in Florida, USA, since its release in 1997 for the biological control of *Melaleuca quinquenervia* (Center et al., 2000). Invasive populations of *M. quinquenervia* threaten the biodiversity of the Florida Everglades (Turner et al., 1998). Both the larvae and adults of *O. vitiosa* feed on the emerging flush foliage of *M. quinquenervia* and a few related taxa (Wheeler, 2001). When fed *M. quinquenervia* leaves with intermediate levels of 1,8-cineole, the adults excreted frass containing volatile constituents found, by GC-MS, GC coinjection, and retention index comparison with authentic standards, to consist of 4.2% of 2α,9-dihydroxy-1,8-cineole **(9).** This is, to the best of our knowledge, the first report of a dihydroxycineole from insect frass. This diol has been identified previously as a 1,8-cineole metabolite of the brushtail possum, *Trichosurus vulpecula*, and subsequently synthesized (Carman et al., 1994). Other hydroxylated cineoles detected were 2α-hydroxy-1,8-cineole **(2)** (3.5%), a smaller proportion of 3α-hydroxy-1,8-cineole **(4)** (1.1%), and a trace (0.5%) of the 9-hydroxyl isomer **(8)** (Table 1). Although the larval frass of this weevil was unavailable, a shiny oil secretion from the cuticle has been the subject of a separate investigation (Wheeler et al., 2002) and was also available for hydroxycineole determination. This analysis showed the presence of trace (0.1%) quantities of 2α-hydroxy-1,8-cineole **(2)** and none of the other hydroxyl cineoles.

No further investigations of the *Petasida ephippigera*, Leichhardt's grasshopper, were undertaken (Fletcher et al., 2000) other than an extrapolation of the contribution of the hydroxycineoles to the total frass volatiles, given that the hydroxycineoles contributed 50% (Fletcher, personal communication, 2001). Hence, the contribution to the total frass volatile fraction from each of the hydroxycineoles identified is shown in Table 1 for comparison. Fletcher et al. (2000) were incorrect in claiming "the first occurrence in which the 3α-isomer (9) [our **(4)**] is the predominant isomer formed and only the second reported occurrence of cineole oxidation in insects." We had described cineole metabolites from two other species in a preliminary report (Southwell, 1999b) (including the 3α-isomer as the predominant isomer from *C. bimaculata* adults) published prior to the submission of their report.

Two other earlier investigations reported, but did not identify, cineole metabolites from insect frass. The published chromatograms (Ohmart and Larsson, 1989) for the larval frass oils of *Paropsis atomaria* indicate substantial modification of the 1,8-cineole in their *Eucalyptus blakelyi* diet to compounds with similar

retention times to the above hydroxycineoles. 1,8-Cineole from *M. quinquenervia* was modified by *Pergagrapta* sawfly larvae (Hymenoptera, Symphyta, Pergidae) to an unassigned hydroxycineole (Schmidt et al., 2000). The published mass spectrum indicates one of the 2-hydroxy-1,8-cineoles but does not distinguish between the α- **(2)** and β- **(3)** isomers. Coinjection of 2β-hydroxy-1,8-cineole **(3)** did not give peak enhancement, but showed that the sawfly metabolite eluted 10 sec. later when injected on a HP5 stationary phase column (Moore, personal communication, 2001), indicating that the metabolite was almost certainly 2α-hydroxy-1,8-cineole **(2)**.

The usefulness of this GC-MS method for the determination of cineole metabolism in insects was established by detecting as little as 0.09% of 3α-hydroxy-1,8-cineole **(4)** as the only 1,8-cineole metabolite from a different sawfly species, *Pterygophorus* sp. (also Hymenoptera, Symphyta, Pergidae), feeding on the terpinen-4-ol chemotype of *M. alternifolia* containing only 1.6% 1,8-cineole. This is a relative of *Pergagrapta* sp. (Schmidt et al., 2000) and is also known to defoliate *Eucalyptus.* This is consistent with the detection of trace quantities of 2β-hydroxycineole in the frass of the pyrgo beetle, *P. tigrina*, adults and larvae fed *M. alternifolia* leaf containing 2% 1,8-cineole in the volatile extract (Southwell et al., 1995). The major component of the *Pterygophorus* frass volatiles was viridiflorol (24.6%). This sesquiterpene alcohol was present in only trace quantities in the *M. alternifolia* on which the larvae were feeding and, hence, accumulated from either this food source or another local food source, such as *M. quinquenervia* that can contain higher concentrations of viridiflorol (Brophy, 1999). The *Pergagrapta* sawfly was not seen as accumulating viridiflorol in frass (Schmidt et al., 2000) when feeding on *M. quinquenervia*, whereas the weevil, *O. vitiosa*, does seem to sequester this sesquiterpenoid in cuticular secretions (Wheeler et al., 2002).

With metabolites of 1,8-cineole becoming increasingly significant in chemical ecology, ease of detection is imperative. NMR assignments of both ^{1}H and ^{13}C signals are available (Carman et al., 1994). A comprehensive investigation into the biotransformation of 1,8-cineole in the brushtail possum, *Trichosurus vulpecula*, (Boyle et al., 2000), has recorded MS and GC retention data for the trimethylsilyl ethers of metabolites of undefined stereochemistry. Consequently, for the ease of identification of small quantities of metabolites in mixtures, we now summarize the GC retention indices on four stationary phases, along with the MS data of the eight most significant ions from eight known hydroxycineole isomer metabolites from insects (Table 2). These data are based on the authentic, synthetic hydroxycineoles as measured in our laboratory and are identical to the insect metabolites where identified.

DISCUSSION

This investigation has shown that the metabolism of 1,8-cineole in insects is extremely variable, with variation occurring both between species and, in at

TABLE 2. GAS CHROMATOGRAPHIC RETENTION INDEX AND MASS SPECTRAL DATA FOR 1,8-CINEOLE METABOLITES

	GC retention index				Mass Spectral m/z (*abundance %*)							
1,8-Cineole metabolite	RI_1^a	RI_2^b	RI_3^c	RI_4^d	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8
2α-OH **(2)**	1300	1228	1359	2081	170(15)	155(2)	137(3)	126(70)	111(53)	108(100)	71(70)	43(75)
2β-OH **(3)**	1288	1217	1345	1951	170(12)	155(2)	137(3)	126(78)	111(52)	108(100)	71(68)	43(78)
3α-OH **(4)**	1317	1246	1381	2133	170(26)	155(27)	137(25)	127(28)	108(48)	93(62)	87(53)	43(100)
3β-OH **(5)**	1327	1259	1400	2160	170(5)	155(100)	137(28)	127(30)	108(36)	93(80)	85(48)	43(91)
4-OH **(6)**	1258	1187	1316	1993	170(5)	155(8)	142(10)	124(5)	112(100)	97(67)	69(58)	43(100)
7-OH **(7)**		1263			170(10)	155(59)	137(18)	111(100)	93(66)	79(47)	69(70)	43(74)
9-OH **(8)**	1340	1267	1400	2091	170(0)	155(0)	139(100)	121(6)	95(35)	81(13)	71(21)	43(67)
2α,9-diOH **(9)**	1692	1470	1688	2801	186(0)	170(0)	155(83)	124(18)	109(22)	93(28)	84(25)	43(100)

[a] BP1 50-m column.
[b] HP5 30-m column.
[c] AT35 60-m column.
[d] DB WaxETR 60-m column.

least one case (*F. nigroconspersa*), between the larval and adult forms. In contrast, however, the adult frass from another species (*O. vitiosa*) was found to contain the same major cineole metabolite, 2α-hydroxy-1,8-cineole, as the cuticular secretion of the larvae of the same species. Hydroxylation, presumably catalyzed by microsomal, cytochrome P-450-dependent enzymes (Gershenzon and Croteau, 1991; Miyazawa et al., 2001) favored, with the possible exception of *F. nigroconspersa* and *O. vitiosa* adults, ring carbon oxidation rather than the exposed methyl oxidation more common with vertebrates (Carman and Klika, 1992; Bull et al., 1993; Carman et al., 1994; Southwell et al., 1995).

Although these hydroxycineole metabolites have been positively identified as metabolites of the stated species, and dual oxygenase roles of both detoxification and semiochemical production are possible (Gershenzon and Croteau, 1991), bioassay investigations are needed to test the activity of these compounds before it is known whether they actually induce a response in the insect.

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COMPARATIVE SURVEY OF ABDOMINAL GLAND SECRETIONS OF THE ANT SUBFAMILY PONERINAE

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Abstract—The chemical contents of three abdominal glands were investigated in representative species of the ponerine ants. The Dufour glands of 14 species show a wide variety of contents. In *Mystrium camillae* and *Proceratium itoi*, no volatile substances were found in either the Dufour or venom glands. In *Ectatomma* sp., *Diacamma ceylonense, Diacamma indicum, Pachycondyla obscuricornis,* and *Pachycondyla striata,* volatile chemicals were found in the venom glands as well as in the Dufour glands. *Platythyrea punctata* was examined, but unusually it does not have a Dufour gland and its venom gland contained no volatile substances. Epoxides were found in ants for the first time in the Dufour glands of *Amblyopone reclinata*. Venom glands of *Pachycondyla tarsata* were also found to contain volatile material, including bitter-tasting cyclic dipeptides. In all, 16 species have been added to the list of those examined. All of the 27 known analyses of Dufour glands, 21 analyses of venom glands, and 4 of pygidial glands of workers of ponerine ant species have been brought together in order to seek some pattern in the type of glandular contents. Although the great majority of species produce hydrocarbons in their worker Dufour glands, and some have terpenes, there is no observable pattern for this gland on a tribe or genus level. Volatile compounds have been found in the venom glands of some

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species of the tribe Ponerini only. The information on pygidial glands is still too fragmentary for any conclusions.

Key Words—Hymenoptera, Formicidae, Ponerinae, Dufour gland, venom gland, pygidial gland, exocrine secretion, chemical analysis.

INTRODUCTION

Chemical communication is important in the social organization of ants. The use of pheromones as transmitters of information allows for efficient functioning of colonies made up of tens of thousands of individuals. These pheromones serve a variety of functions, such as attracting mates, recruiting nestmates to food sources, alarm, defense, and other actions (Hölldobler and Wilson, 1990). These functions correspond to the huge variety of exocrine glands found in ants (Billen and Morgan, 1998). Pheromones with different functions originate from different glands, although one gland may have entirely different functions in separate species. The chemical nature of glandular secretions is indeed often species-specific (Blum and Hermann, 1978; Morgan et al., 1999). This fertile complexity of glands and pheromones ensures smooth social organization in the ant societies.

Ants of the subfamily Ponerinae are often considered "primitive" since they show many of the putative ancestral traits (Peeters, 1997). These comprise low queen–worker dimorphism, small colony sizes, and predatory behavior, all of which may account for simple social structures and simple communicative needs. Nevertheless, recent studies provide ample evidence that ponerine societies are more complex than previously thought (Peeters, 1997). Ponerine ants of all tribes show levels of social organization that are comparable to those in "advanced" ant subfamilies, coupled with elaborate chemical communication. Some species use complex chemical recruitment of nestmates to be able to predate other social insects (Duncan and Crewe, 1994; Hölldobler et al., 1994; Leal and Oliveira, 1995). Some even use army ant behavior in which entire colonies move along chemical trails when active (Maschwitz et al., 1989; Witte and Maschwitz, 2000). Recently, more subtle forms of social communication have been demonstrated: workers can accurately estimate levels of ovary development in their nestmates (Gobin et al., 1999; Kikuta and Tsuji, 1999), and this information seems to be contained in their cuticular hydrocarbons (Peeters et al., 1999; Liebig et al., 2000). Furthermore, these chemicals can be transferred to eggs, enabling policing of any subordinate eggs (Monnin and Peeters, 1997). Although these hydrocarbons presumably originate from abdominal exocrine glands or abdominal oenocytes, the exact glandular source remains elusive.

Because of their diversity, the Ponerinae have been systematized into eight tribes according to Bolton (1995). These are Amblyoponini (8 extant genera), Ectatommini (6 extant genera), Paraponerini (1 genus), Platythyreini (2 genera),

Ponerini (22 extant genera), Proceratiini (2 genera), and Thaumatomyrmecini and Typhlomyrmecini (1 genus each). The genera *Cerapachys* and *Cylindromyrmex* have been placed in a separate subfamily (Cerapachyinae) by Bolton (1994) but have been included in the Ponerinae by others (e.g., Hölldobler and Wilson, 1990).

Ponerine ants appear to be quite diverse in the products of their exocrine secretions. We have recently published analyses of mandibular glands of a number of ponerine species and gathered together the information on mandibular glands then available (Morgan et al., 1999). The information on abdominal exocrine glands is more fragmentary. Analyses of Dufour, poison, and pygidial glands have been made, although the information is sparse on the latter two. The pygidial gland is the putative origin of sex attractants in some species that show sexual calling behavior (i.e., lifting the gaster and exposing intersegmental membranes), but the precise origin and chemistry have never been elucidated (Hölldobler and Haskins, 1977). The position is similar with tandem running. While its existence is recognized and its nature is chemical, the identification and origin of the pheromone remains unknown, although Hölldobler and Traniello (1980) showed that the pygidial gland secertion is necessary to cause *Pachycondyla obscuricornis* to perform tandem running behind a dummy.

With our study, we aim to fill the gap between increasing biological evidence for complex chemical communication and the source of the putative pheromones. We have attempted to obtain at least one species from each of the six common tribes in the system of Bolton (1995), excluding only the two monogeneric and cryptic tribes Thaumatomyrmecini and Typhlomyrmecini. We expand and review the general knowledge of the chemical nature of abdominal gland secretions in the Ponerinae and show the diversity of chemical components within and across genera. The diversity of compounds documented will allow testing of various behavioral functions that were previously attributed to chemistry, without pheromonal identification. Our data should permit quick testing of putative pheromones in functional studies.

METHODS AND MATERIALS

In general, foraging workers were collected for dissection and analysis (Table 1). Dufour glands, pygidial glands, and poison glands were dissected separately, and the dissected glands immediately sealed in thin-walled glass capillaries according to the solid-sampling technique (Morgan and Wadhams, 1972; Morgan, 1990).

Gas chromatography–mass spectrometry (GC-MS) was carried out on an Hewlett Packard 5890 gas chromatograph and 5970 mass selective detector quadrupole mass spectrometer with 70 eV ionization. Chromatography was carried out on a fused silica capillary column (12 m $\times$ 0.32 mm with a 0.3-μm coating of

TABLE 1. PONERINE SPECIES STUDIED AND COLLECTION SITES

Tribe	Species, authority	Location, Country[a]
Amblyoponini	*Amblyopone reclinata* Mayr, 1879	Bogor
	Mystrium camillae Emery, 1889	Kebun Raya, Bogor, Indonesia
Ectatommini	*Ectatomma sp.*	Maceió, AL, Brazil
	Gnamptogenys menadensis Mayr, 1887	Karaenta, Sulawesi, Indonesia
Platythyreini	*Platythyrea cribinodis* (Gerstäcker, 1859)	Hunter's Lodge, Nairobi, Kenya
	Platythyrea punctata Smith, 1858	Florida, USA
Ponerini	*Diacamma celonense* Emery, 1897	Masinagudi, TN, India
	Diacamma indicum (Santschi, 1920)	Masinagudi, TN, India
	Dinoponera australis Emery, 1901	Itirapina, SP, Brazil
	Odontomachus bauri Emery, 1892	Maceió, AL, Brazil
	Odontoponera transversa (F. Smith, 1857)	Singapore
	Pachycondyla indica (Emery, 1899)	Bangalore, KT, India
	Pachycondyla obscuricornis (Emery, 1890)	Viçosa, MG, Brazil
	Pachycondyla striata (Santschi, 1930)	Rio Claro, SP, Brazil
	Pachycondyla tarsata (Fabricius, 1789)	Ellisras, Northern Province, South Africa
Proceratiini	*Proceratium itoi* (Forel, 1918)	Takamatsu, Japan

[a] AL = Alagoas, KT = Karnataka, MG = Minas Gerais, SP = São Paulo, TN = Tamil Nadu.

polydimethylsiloxane; SGE, Milton Keynes, UK). Samples were heated in the injector to 150°C before crushing in a Keele solid-sample injector (Morgan, 1990). The oven was programmed from 30°C to 150°C at 8°C/min and then held isothermally. The carrier gas was helium at 1 ml/min.

Mass spectral identifications were confirmed wherever possible by comparison of retention times and mass spectra with those of synthetic standards. Where pure standards were not available, identification was based on comparison with spectra in our own collection and in computer libraries (NIST Library, 2001).

RESULTS

The species of Ponerinae examined in this study and their place of collection are listed in Table 1.

Amblyoponini. Two examples of the tribe Amblyoponini were available. *Amblyopone reclinata* had a simple mixture of four related epoxides in its Dufour glands, their structures were confirmed with the help of mass determinations on the molecular ions and the principal fragment ions. The first substance, 9,10-epoxyoctadecane (M^+ 250) formed only 0.5% of the total, the second,

9,10-epoxy-6-nonadecene (M^+ 280) was 0.9%. The third, the major component, *cis*-9,10-epoxynonadecane (M^+ 282) (*m/z* 264, 253, 239, 225, 211, 197, 183 (all weak), 169 (26%), 155 (30), 138 (10), 124 (15), 109 (12), 97 (39), 83 (82), 96 (92), 69 (92), 55 (100), 43 (95), 41 (92), represented over 98% of the total (the results are, therefore, not tabulated). The fourth compound, *trans*-9,10-epoxynonadecane (M^+ 282) is less than 0.05% of the total. This is the first report of epoxides in the exocrine glands of ants. We found no volatiles in either the Dufour or venom glands of *Mystrium camillae.* There have been earlier examples of species without volatile substances in the Dufour glands, but it is not a frequent phenomenon.

Ectatommini. The *Ectatomma* species from Brazil, probably a new species, had a simple mixture of hydrocarbons in its Dufour glands, with nonadecene the major component (Table 2). Examination of the venom glands revealed no volatile compounds. In *Gnamptogenys menadensis*, we found a group of unidentified δ-lactones and methyl 6-methylsalicylate. The sticky and smelly hydrophobic mixture of these compounds was apparent each time the ants were handled with forceps.

Platythyreini. One unidentified species from Kenya contained heptadecene with small amounts of related alkenes and alkanes (Table 2). The venom glands were empty of volatile components. *Platythyrea punctata* from Florida was also

TABLE 2. COMPOSITION OF DUFOUR GLANDS OF *Ectatomma* SP.($N = 10$) AND *Platythyrea cribinodis* WORKERS

Component	Compound	Method of identification	Proportion (%, mean ± SD)	
			Ectatomma sp.	*P. cribinodis*
1	Pentadecene	RT, MS		0.67 ± 0.95
2	Pentadecane	RT, MS		2.98 ± 2.01
3	Hexadecene	RT, MS		1.19 ± 0.59
4	Hexadecane	RT, MS		0.18 ± 0.25
5	Heptadecadiene	MS		12.35 ± 0.15
6	Heptadecene	MS	4.4 ± 2.6	55.23 ± 4.54
7	Heptadecane	RT, MS	1.7 ± 1.4	3.00 ± 0.53
8	Octadecene	MS	2.4 ± 1.8	0.22 ± 0.30
9	Nonadecadiene	MS	3.1 ± 2.2	2.11 ± 0.27
10	Nonadecene	MS	88.9 ± 6.9	11.02 ± 2.39
11	Nonadecane	RT, MS	<0.1	0.25 ± 0.35
12	Heneicosene	MS		4.91 ± 0.42
13	Heneicosane	RT, MS		0.39 ± 0.55
14	Tricosene	RT, MS		0.96 ± 0.44
15	Tricosane	RT, MS		3.31 ± 0.75
16	Pentacosane	RT		0.70 ± 0.81
17	Heptacosane	RT		0.57 ± 0.81
Mean total amount			1.4 ± 1.3 μg	230 ng

TABLE 3. COMPOSITION OF DUFOUR GLANDS OF *Diacamma ceylonense* WORKERS ($N = 15$) AND *D. indicum* ($N = 4$)

Component	Compound	Method of identification	Proportion (%, mean ± SD)	
			D. ceylonense	*D. indicum*
1	Tridecane	RT, MS		0.9 ± 0.3
2	Tetradecane	RT, MS	0.8 ± 0.2	1.1 ± 0.5
3	7-Methyltetradecane	MS	0.6 ± 0.2	1.4 ± 0.8
4	Pentadecadiene	MS	1.1 ± 0.5	2.7 ± 3.4
5	Pentadecene	MS	8.0 ± 1.6	8.7 ± 4.3
6	Pentadecane	RT, MS	11.9 ± 3.1	16.6 ± 3.7
7	Hexadecene	MS	0.6 ± 0.4	0.8 ± 0.9
8	Hexadecane	RT, MS	1.8 ± 0.2	4.3 ± 7.8
9	Heptadecadiene	MS	7.4 ± 2.0	7.1 ± 0.6
10	Heptadecene	MS	30.7 ± 4.8	21.2 ± 8.7
11	Heptadecane	RT, MS	1.6 ± 0.3	2.9 ± 1.1
12	Octadecene	MS	0.3	0.8 ± 0.7
13	Octadecane	RT, MS	0.4 ± 0.1	0.9 ± 0.5
14	Nonadecadiene	MS	1.6 ± 0.5	0.5 ± 0.4
15	Nonadecene	MS	2.8 ± 0.6	3.6 ± 0.7
16	Nonadecane	RT, MS	4.5 ± 1.1	4.5 ± 1.2
17	Eicosane	RT, MS	1.6 ± 0.5	1.3 ± 0.3
18	Heneicosene	MS	3.0 ± 0.8	2.1 ± 6.2
19	Heneicosane	RT, MS	5.6 ± 0.7	4.4 ± 0.3
20	Tricosene	RT, MS	2.6 ± 0.4	2.1 ± 0.1
21	Tricosane	RT, MS	1.4 ± 1.0	0.9 ± 0.4
22	Tetracosene	MS	0.4 ± 0.5	0.4 ± 1.3
23	Pentacosene	MS	4.1 ± 6.4	3.3 ± 1.0
24	Pentacosane	RT, MS	2.0 ± 0.9	2.1 ± 1.3
25	Hexacosene	MS	0.5 ± 0.5	0.2
26	Hexacosane	RT, MS	0.3 ± 0.4	1.0 ± 0.4
27	Heptacosene	MS	4.4 ± 5.9	4.2 ± 1.1
Mean total amount (ng)			992	1013

examined, but this species is unusual in that it does not possess a Dufour gland and its venom glands and sternal glands did not contain any volatile material.

Ponerini. The Dufour glands of the two species investigated had similar mixtures of linear alkenes and alkanes. *Diacamma ceylonense* had heptadecene as the major compound, and *Diacamma indicum* from India had almost equal proportions of pentadecane and heptadecene (Table 3). No oxygenated compounds or terpenes were detected. The venom glands contained small but significant amounts of volatiles, dominated in both by up to 200 ng of 2-undecanone but averaging less than that (Table 4).

Distinct differences were found between the workers and a single sample of the gamergate of *Dinoponera australis* from a single nest. The workers had

TABLE 4. COMPOSITION OF VENOM GLAND VOLATILE SUBSTANCES OF *Diacamma ceylonense* ($N = 13$) AND *D. indicum* ($N = 3$) WORKERS[a]

Component	Compound	Mean proportion (%)	
		D. ceylonense	*D. indicum*
1	Methyl butanoate	2.07	21.60
2	2-Heptanone		1.30
3	Heptanoic acid	0.57	6.80
4	2,4,6-Trimethylpyrazine		3.53
5	2-Undecanone	96.7	60.63
6	Indole	0.63	6.10
Total amount (ng)		42.2	29.6

[a] All compounds were identified by mass spectra and comparison of retention times with authentic samples.

a complex mixture of up to 60 components, with the hydrocarbons nonadecene, heneicosene, octadecene, heptadecene, and heptadecadiene, in decreasing order, as the most abundant. Nonadecene was by far the most abundant. There was considerably less of the hydrocarbons in the gamergate, but nonadecene, heneicosene, and heneicosadiene were the important ones. Both workers and gamergate contained docosanol, the proportion of the alcohol being much greater in the gamergate. These results have not been tabulated because the amounts varied considerably among individuals in the one colony, and only one gamergate was analyzed. Attempts to obtain further samples of this species were unsuccessful.

The Dufour gland of *Odontomachus bauri* contained a relatively simple mixture of hydrocarbons with 8-heptadecene the major one, closely followed by heptadecadiene (Table 5). There were also three acyclic diterpenes. The first was a compound first identified in the myrmeciine ant *Myrmecia nigriceps* and there called T_1 (Jackson et al., 1989). It had a retention index of 1770, a molecular mass of 262, and a mass spectrum similar to that of geranylgeraniol. Its molecular formula appears to be $C_{19}H_{34}$, and is, therefore, a partly reduced and degraded diterpene. It has also been found in *Pachycondyla apicalis* (Cruz Lopez and Morgan, 1997), and in this work also in *Odontoponera transversa* and *Pachycondyla obscuricornis*. The second compound has a similar mass spectrum to the first (base peak at *m/z* 69, but with a strong ion at *m/z* 95, 30% intensity) and a molecular mass of 278. Its molecular formula is apparently $C_{20}H_{38}$. This compound was also found in *Pachycondyla obscuricornis*. The third diterpene (probable molecular mass 294, mass spectrum similar to the second, suggested molecular formula $C_{20}H_{38}O$) was also found in *Pachycondyla apicalis*. and *P. obscuricornis*, This third compound is thought to be an alcohol derivative of the second compound. The venom glands were examined but no volatile substances were found.

TABLE 5. COMPOSITION OF DUFOUR GLAND SECRETION OF *Odontomachus bauri* WORKERS ($N = 10$)

Component	Compound	Method of identification	Proportion (%, mean ± SD)
1	Tetradecane	RT, MS	0.7 ± 0.3
2	Pentadecene	RT, MS	2.9 ± 0.9
3	Pentadecane	RT, MS	15.1 ± 2.9
4	Hexadecadiene	MS	1.2 ± 0.2
5	Hexadecene	RT, MS	2.0 ± 0.4
6	Hexadecane	RT, MS	0.7 ± 0.4
7	Heptadecadiene	MS	26.0 ± 2.9
8	8-Heptadecene	RT, MS	32.9 ± 3.5
9	Heptadecane	RT, MS	2.7 ± 0.8
10	Acyclic terpene $C_{19}H_{34}$	MS	5.1 ± 2.8
11	Geranylgeraniol	MS	9.1 ± 6.4
12	Acyclic terpene $C_{20}H_{38}O$	MS	1.1 ± 1.2
Mean total amount (μg)			1.47 ± 1.25

The Dufour glands of *Odontoponera transversa* contained a mixture of alkanes; branched alkanes and alkenes; the linear terpene $C_{19}H_{34}$ also found in *Odontomachus bauri*, *Pachycondyla apicalis*, and *Pachycondyla obscuricornis*; and a small amount of geranylgeraniol. The most abundant substance was pentadecane (Table 6). Geranylgeraniol has also been found in Dufour glands of *Ectatomma ruidum* (Bestmann et al., 1995) as well as in the doryline ant *Aenictus rotundatus* (Oldham et al., 1994) and the formicine ants *Formica fusca*, *F. nigricans*, and *F. polyctena* (Bergström and Löfqvist, 1973), and in the labial glands of several species of bumblebees.

Four species of *Pachycondyla* (*P. indica*, *P. obscuricornis*, *P. striata*, and *P. tarsatus*) have been added to the one species (*P. apicalis*) from Mexico already examined (Cruz Lopez and Morgan, 1997). *P. indica* contained a complex mixture of linear and methyl-branched hydrocarbons, with pentadecane dominant (Table 7). *P. striata* contained a complex mixture of hydrocarbons, with heptadecane most abundant, but with five unidentified sesterterpenes (C_{25} compounds) (Table 7) with mass spectra similar to, but not the same as, that of geranylfarnesol found in the Dufour gland of the stingless bee *Frieseomelitta languida* (Cruz López et al., in preparation). Such compounds are unusual and need further chemical examination. *P. obscuricornis* contained no linear hydrocarbons but a mixture of two groups of diterpene homolog, one group of four compounds with base peak at *m/z* 69, exemplified by the substance $C_{19}H_{34}$ of mass 262 (Table 8), also found in *Myrmecia nigriceps*, *Pachycondyla apicalis*, *Odontomachus bauri*, and *Odontoponera transversa*. The second and third of this series were the same as the second and third in *Odontomachus bauri*. The second series of four diterpenes apparently

TABLE 6. COMPOSITION OF THE SECRETION OF DUFOUR GLANDS OF WORKERS OF *Odontoponera transversa* ($N = 10$)

Component	Compound	Method of identification	Proportion (%, mean ± SD)
1	Tridecane	RT, MS	2.3 ± 1.4
2	3-Methyltridecane	MS	0.04 ± 0.09
3	Tetradecane	RT, MS	3.3 ± 1.5
4	3-Methyltetradecane	MS	0.08 ± 0.2
5	Pentadecadiene	MS	0.2 ± 0.2
6	Pentadecene	RT, MS	3.2 ± 1.1
7	Pentadecane	RT, MS	38.0 ± 7.4
8	7-Methylpentadecane	MS	0.01 ± 0.02
9	5-Methylpentadecane	MS	0.03 ± 0.05
10	Hexadecadiene	MS	1.4 ± 0.4
11	Hexadecene	RT, MS	1.4 ± 0.2
12	Hexadecane	RT, MS	1.2 ± 0.2
13	Heptadecadiene	MS	19.5 ± 5.6
14	Heptadecene	MS	19.6 ± 3.9
15	Heptadecane	RT, MS	4.0 ± 1.3
16	Nonadecadiene	MS	0.4 ± 0.7
17	Nonadecene	MS	1.9 ± 2.1
18	Nonadecane	RT, MS	0.6 ± 1.1
19	Heneicosene	MS	0.4 ± 0.5
20	Tricosene	RT, MS	0.4 ± 0.4
21	Acyclic terpene $C_{19}H_{34}$	MS	1.0 ± 0.5
22	Geranylgeraniol	MS	0.8 ± 1.0
23	Geranylcitronellol (?)	MS	0.2 ± 0.1
Mean total amount (μg)			29.4 ± 29.8

contained a homomevalonate unit since their mass spectra showed strong ions at *m/z* 55 and 83 instead of *m/z* 41 and 69. These substances are all derived from acyclic diterpenes, some of them having lost one carbon atom, but their structures remain unsolved. The venom glands of *P. tarsatus* were also examined. The samples were divided into those in which the venom had a bitter taste and those that were not bitter. The samples were variable in their content, but most contained a rich mixture of nitrogenous compounds. The greatest part consisted of dimethylaminoethanol, 2,6-dimethylpiperidine (two isomers, presumably *cis* and *trans*), indole, cycloprolylleucine, and a compound with a mass spectrum corresponding closely to that of *N*-2-methylbutyl-*N*-2-methylbutylideneamine (otherwise, the aldimine or Schiff's base produced from 2-methylbutylamine and 2-methylbutanal). The 11 samples of bitter-tasting venom were variable, but 8 of them contained, in addition to cyclo-leucylproline (1,4-diaza-2,5-dioxo-3-isobutylbicyclo[4.3.0]nonane) smaller amounts of cycloglycylproline 1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane, and cycloalanylleucine.

TABLE 7. COMPOSITION OF DUFOUR GLAND VOLATILE SUBSTANCES OF *Pachycondyla striata* ($N = 5$) AND *P. indica* ($N = 2$) WORKERS

Component	Compound	Method of identification	Proportion (%, mean ± SD)	
			P. striatas	*P. indica*
1	Limonene	RT, MS	0.7	
2	2,5-Dimethyl-3-butylpyrazine	RT, MS	1.3	
3	Tridecene	MS		0.2
4	Tridecane	RT, MS		8.6
5	7-Methyltridecane	MS		0.2
6	3-Methyltridecane	MS		0.7
7	Tetradecane	RT, MS		4.0
8	7-Methyltetradecane	MS	0.6	2.8
9	5-Methyltetradecane	MS		4.6
10	3-Methyltetradecane	MS		1.8
11	Pentadecene	RT, MS		0.1
12	Pentadecane	RT, MS	4.9	38.0
13	12-Methylpentadecane	MS	1.0	
14	7-Methylpentadecene	MS	0.5	
15	7-Methylpentadecane	MS		0.6
16	Hexadecene	MS	0.3	0.1
17	Hexadecane	RT, MS	2.9	2.2
18	Heptadecadiene	MS	1.9	1.7
19	Heptadecene	RT, MS	5.3	7.0
20	Heptadecane	RT, MS	34.6	11.2
21	Octadecadiene	MS	1.3	
22	Octadecene	MS	3.4	
23	Octadecane	RT, MS	0.5	
24	Acyclic terpene $C_{19}H_{34}$	MS	2.3	0.5
25	Nonadecadiene	MS	1.0	
26	Nonadecene	RT, MS	11.9	2.5
27	Nonadecane	RT, MS	0.5	2.0
28	Heneicosene	MS		0.5
29	Heneicosane	MS		0.3
30	Tricosene	RT, MS		7.8
31	Tricosane	RT, MS		2.3
32	Acyclic terpene $C_{19}H_{36}O$	MS	1.6	
33	Terpene 2	MS	24.3	
34	Terpene 3	MS	0.6	
35	Terpene 4	MS	1.1	
36	Terpene 5	MS	1.7	
Mean total amount (μg)			1.8	1.0

Proceratiini. We found no volatile substances in the Dufour glands, venom glands, or pygidial glands of *Proceratium itoi*. This was the second species in this work without volatile material in the Dufour glands.

TABLE 8. COMPOSITION OF DUFOUR GLAND VOLATILE SUBSTANCES OF *Pachycondyla obscuricornis* ($N = 3$) WORKERS[a]

Component	Retention time (min)	Mass spectral ions (with relative intensity, %).[b]	Proportion (%, mean ± SD)
1	19.67	M^+ 280, 195, 193, 178, 151, 137, 136, 123, 121, 109, 95, 81 (30), 69 (100), 55, 41 (35)	0.9
2	20.19	M^+ 294, m/z 207, 150, 137, 123, 109, 95 (30), 83, 81, 69 (100), 55, 41 (35)	0.6
3	20.5	M^+ 294, m/z 247, 233, 207, 192, 150, 137, 123, 109, 95 (45), 81, 69 (100), 55, 41 (45)	17.4
4	20.64		0.5
5	20.7	M^+ 294, m/z 219, 193, 150, 137, 125, 109, 95 (25), 83 (80), 81 (35), 69 (40), 55 (100), 41 (40)	5.5
6	20.8		0.9
7	21.3	M^+ 294, 261, 221, 219, 151, 137, 123, 109, 95 (55), 83, 81, 69 (100), 55, 41 (55)	1.6
8	21.58	M^+ 294, 261, 233, 207, 191, 177, 164, 151, 135, 123, 109 (20), 95 (55), 83 (65), 69 (40), 67 (30), 55 (100), 41 (50)	67.2
9	21.68	M^+ 294, 259, 231, 205, 177, 149, 135, 123, 121, 109 (25), 95 (35), 83 (60), 69, 67 (35), 55 (100), 41 (45)	1.9
10	22.3	(M^+ 298 ?), 275, 247, 233, 221, 165, 151, 135, 123, 109 (25), 95 (50), 83 (70), 69 (35), 67 (32), 55 (100), 41 (45)	3.2
11	22.5		0.4
Mean total amount (μg)			1.2

[a] The secretion consisted of unidentified terpenes, so their relative retention times and principal mass spectral ions are given.
[b] No intensity recorded means a weak ion.

DISCUSSION

The tribe Amblyoponini contains eight living genera, *Adetomyrma*, *Amblyopone*, *Concoctio*, *Myopone*, *Mystrium*, *Onychomyrmex*, *Prionopelta*, and *Paraprionopelta*. Table 9 brings together our work here with earlier reports on Dufour glands. One species of *Amblyopone* has hydrocarbons like many other ponerines, but *A. reclinata* introduces a previously unencountered theme of essentially one substance, an epoxide. *Mystrium camillae* contained no volatile substances in Dufour or venom glands and shares this trait with one other species. Table 10 summarizes our knowledge on ponerine venom glands. The information here is fragmentary. It is not common to find volatile materials in venom glands of ants, and there has been little study of their protein venoms. Table 11, which summarizes our knowledge of pygidial glands, contains even less information.

TABLE 9. SUMMARY OF RESULTS OF INVESTIGATIONS OF DUFOUR GLANDS OF PONERINE ANTS

Tribe, genus, species	Chemicals	Reference
Amblyoponini		
Amblyopone australis	Hydrocarbons	Cavill and Williams (1967)
Amblyopone reclinata	9,10-Epoxynonadecane	This work
Mystrium camillae	Nothing volatile	This work
Ectatommini		
Ectatomma ruidum	Geranylgeranyl acetate and geranylgeraniol	Bestmann et al. (1995)
Ectatomma sp.	C_{17}–C_{19} alkanes and alkenes, about 90% nonadecene	This work
Gnamptogenys menadensis	C_{13}–C_{19} alkanes and alkenes and farnesenes	Gobin et al. (1998)
Rhytidoponera aciculata[a]	Hydrocarbons, 2-hydroxy-6-methylaceto phenone	Brophy et al. (1983)
Rhytidoponera chalybaea[a,b]	Mellein, C_{13}–C_{19} alkanes, methyl-branched alkanes and alkenes, 3-methyl-2-cyclo hexenone; 2,4-dihydroxyacetophenone	Brophy et al. (1981), Sun and Toia (1993)
Rhytidoponera metallica	C_{13}–C_{17} alkanes, 2,5-dimethylchromone, isogeraniol	Brophy et al. (1988)
Paraponerini		
Paraponera clavata	C_{16}–C_{25} alkanes and alkenes[c]	Hermann et al. (1984)
Platythyreini		
Platythyrea cribinodis	Nothing volatile	This work
Platythyrea punctata	No Dufour gland	This work
Ponerini		
Diacamma ceylonense	C_{15}–C_{27} alkanes and alkenes	This work
Diacamma indicum	C_{13}–C_{27} alkanes and alkenes	This work
Dinoponera australis	C_{15}–C_{21} alkanes and alkenes, docosanol	This work
Dinoponera grandis	C_{15}–C_{19} alkanes and alkenes	Hermann et al. (1984)
Harpegnathos saltator	C_{15}–C_{25} alkanes and alkenes, tetradecyl propionate	do Nascimento et al. (1993)
Leptogenys diminuta	C_{23}–C_{27} hydrocarbons and farnesenes	Maile et al. (2000)
Odontomachus bauri	C_{15}–C_{17} alkanes and alkenes and three partially identified diterpenes	This work
Odontoponera transversa	C_{13}–C_{19} alkanes, methyl-branched alkanes, alkenes, a diterpene and geranylgeraniol	This work
Pachycondyla apicalis[e]	$C_{19}H_{34}$, $C_{20}H_{38}O$[d] (revised formulae), also in *O. bauri* and *P. obscuricornis*	Cruz Lopez and Morgan, 1997
Pachycondyla castanea[e]	Nonanal, undecanal, isovaleric, nonanoic and phenylacetic acids, three amides and four amines	Fales et al. (1984)
Pachycondyla castaneicolor[5]	The same three amides and four amines as found in *P. castanea*	Fales et al. (1984)
Pachycondyla indica	C_{13}–C_{23} alkanes, methyl-branched alkanes and alkenes,	This work

TABLE 9. CONTINUED

Tribe, genus, species	Chemicals	Reference
Pachycondyla insularis	Gland atrophied	Maschwitz et al. (1981)
Pachycondyla obscuricornis	8 unidentified linear diterpenes	This work
Pachycondyla striata	C_{15}–C_{19} alkanes, methyl-branched alkanes and alkenes, and five linear C_{25} terpenes	This work
Pachycondyla tridentata	Gland atrophied	Maschwitz et al. (1981)
Proceratiini		
Proceratium itoi	No volatiles found	This work

[a] Body extracted, but probably from the Dufour gland.
[b] Originally called *Rhytidoponera metallica* but corrected to *R. chalybaea* by Brophy et al. (1983).
[c] Information is unreliable and probably refers to cuticle.
[d] Unidentified terpenes.
[e] *Mesoponera* when published, the substances were found in total extracts of abdomen and thorax.

The tribe Ectatommini, according to Bolton's (1995) system, consists of six living genera: *Acanthoponera, Aulacopone, Ectatomma, Gnamptogenys, Heteroponera,* and *Rhytidoponera. Ectatomma* contains four known Neotropical species (*planidens, quadridens, ruidum,* and *tuberculatum*). The one we sampled is not one of these and must be a new species. There was nothing about its Dufour gland or mandibular glands that distinguished it chemically from examples from the large tribe of Ponerini, but *E. ruidum* belongs to the increasing number of ponerines that produce diterpenes. The three species of *Rhytidoponera* show some homogeneity by all producing in their Dufour glands acetophenone derivatives that are derived from a polyketide (Sun and Toia, 1993). The Asian species *Gnamptogenys menadensis* secretes a characteristic sticky substance from its pygidial gland, presumably serving as a repellent for potential predators. It also has some alarming and attractant properties (Gobin et al., 1998). Methyl 6-methylsalicylate, which is present, is a known alarm pheromone in the South American species *G. pleurodon* (Duffield and Blum, 1975), where it is found in the mandibular glands, but it has also been identified as a trail pheromone in the venom gland of the myrmicine ant *Tetramorium impurum* (Morgan and Ollett, 1987).

The tribe Paraponerini contains only the genus *Paraponera.* The Dufour glands of *Paraponera clavata* have been examined by Hermann et al. (1984) and the venom glands by Piek et al. (1991a,b). Their results, included in Tables 9 and 10, show similarities to some other ponerines.

The tribe Platythyreini contains only *Platythyrea* and *Probolomyrmex*. The Dufour glands of an unidentified species of *Platythyrea* from Kenya contain a series of alkenes and alkanes, with heptadecene providing over 50% of the total. *Platythyrea punctata* from Costa Rica is unusual in not possessing Dufour glands.

TABLE 10. SUMMARY OF KNOWN COMPOUNDS FOUND IN VENOM GLANDS OF PONERINE ANTS

Tribe, genus, species	Chemicals	Reference
Amblyoponini		
Mystrium camillae	Nothing volatile	This work
Ectatommini		
Ectatomma tuberculatum	More than 15 polypeptides; complete sequence of ectatomin (molecular mass 7928 Da) has been solved	Pluzhnikov et al. (1994), Zaitseva et al. (1995)
Ectatomma sp.	Nothing volatile	This work
Gnamptogenys menadensis	Nothing volatile	This work
Paraponerini		
Paraponera clavata	Poneratoxin, at least 6 proteins[a]; acidic proteins, some sequences determined	Zaitseva et al. (1995)
Platythyreini		
Platythyrea cribinodis	Nothing volatile	This work
Platythyrea punctata	Nothing volatile	This work
Ponerini		
Diacamma ceylonense	Undecanone	This work
Diacamma indicum	Undecanone, methyl butanoate, 2-heptanone, heptanoic acid, 2,4,6-trimethylpyrazine, indole	This work
Dinoponera australis	Nothing volatile, five proteins very similar to those of *Pachycondyla apicalis*	Cruz López (1994)
Dinoponera grandis	At least three proteins and glycoproteins by electrophoresis	Leluk et al. (1989)
Harpegnathos saltator	Nothing volatile	do Nascimento et al. (1993)
Leptogenys diminuta	(3*R*,4*S*)-4-Methyl-3-heptanol	Attygalle et al. (1988)
Leptogenys peuqueti	4-Methyl-7-tetradecanol and acetate, 4,10-dimethyl-7-tridecanol and acetate, 2,6-dimethyl-3-nonanyl acetate, 3,9-dimethyl-6-dodecanol, 6-methyl-3-nonanyl acetate, 4-methyl-7-dodecanol and acetate, 4-methyl-7-hexadecanol and acetate and two other related acetates and one alcohol	Janssen et al. (1997)
Odontomachus bauri	Nothing volatile	This work
Odontoponera transversa	Many proteins and glycoproteins	Leluk et al. (1989)
Pachycondyla apicalis	3-Benzyl-6-(2-methylpropyl)-2,5-piperazinedione, and a mixture of 5 proteins, very similar to those of *Dinoponera australis*	Cruz López and Morgan (1997), Cruz López (1994)
Pachycondyla foetens	*N*,*N*-Dimethyluracil	Janssen et al. (1995)
Pachycondyla insularis	Two major and seven minor proteins	Maschwitz et al. (1981)

TABLE 10. CONTINUED

Tribe, genus, species	Chemicals	Reference
Pachycondyla obscuricornis	Benzaldehyde,2,5-dimethylpyrazine, limonene, trimethylpyrazine, 2,5-dimethyl-3-ethylpyrazine, indole	This work
Pachycondyla striata	Nonanal, decanal, and in some limonene	This work
Pachycondyla tarsata	Dimethylaminoethanol, 2,6-dimethyl piperidine (two isomers), an amine, indole, *cyclo*-prolylleucine and sometimes other dipeptides	This work
Pachycondyla tridentata	Two major and seven minor proteins	Maschwitz et al. (1981)
Pachycondyla villosa	Rich in proteins[a]	Blum and Hermann (1978)
Proceratiini		
Proceratium itoi	Nothing volatile	This work

[a] Unpublished results quoted by Blum and Hermann (1978).

The tribe Ponerini contains by far the most of the ponerine genera, 22 in all, of which we have analyzed 11 species. *Diacamma* is one of the ponerine genera that produce no true queens but have a reproductive system in which gamergates play a central role (Peeters and Crewe, 1986). Both *Diacamma ceylonense* and *Diacamma indicum* contained a complex mixture of linear hydrocarbons with just one methyl-branched alkane (Table 3). They were both unusual in having volatile compounds in their venom glands, and in both, 2-undecanone was the major substance (Table 10). This seemed particularly interesting since tandem recruitment has been demonstrated in a *Diacamma* species (Maschwitz et al., 1986). Of *Odontomachus*, with over 100 species scattered throughout the tropics, *O. bauri* is the

TABLE 11. SUMMARY KNOWN COMPOUNDS FOUND IN PYGIDAL GLANDS OF PONERINE ANTS

Tribe, genus, species	Chemicals	Reference
Amblyoponini	No reports	
Ectatommini		
Rhytidoponera metallica	Isogeraniol, *m*-hydroxybenzaldehyde, heptadecene and heptadecadiene	Meinwald et al. (1981)
Gnamptogenys menadensis	Methyl 6-methylsalicylate and unidentified δ-lactones	This work
Paraponerini	No reports	
Platythyreini	No reports	
Ponerini		
Pachycondyla foetens	Actinidine	Janssen et al. (1995)
Pachycondyla marginata	Citronellal, isopulegol	Hölldobler et al. (1996)
Proceratiini		
Proceratium itoi	Nothing volatile	This work

only example of the genus yet examined. It had a mixture of hydrocarbons and diterpenes (Table 5). *Odontoponera transversa*, the only species in this genus, is found throughout the Indo-Malayan region. Its Dufour glands contain a complex mixture of hydrocarbons and three diterpenes (Table 6). Three species of *Pachycondyla*, all collected in India, were examined. They showed no great similarity in their Dufour gland chemistry. *P. indica* had only hydrocarbons, *P. striatus* had a mixture of hydrocarbons and diterpenes, and *P. obscuricornis* had only the complex mixture of diterpenes (Table 8). *P. obscuricornis* is recorded as another of the species that practices recruitment by tandem running (Hölldobler and Traniello, 1980). A curious form of defense has been recorded for *Pachycondyla*, whereby they produce a foam from the venom gland (Maschwitz et al., 1981). We looked particularly at *Pachycondyla tarsatus* venom glands because the venom of some of the ants was bitter-tasting, but not of others. All samples contained the cyclic dipeptide cycloleucylproline. This has been isolated from a number of sources, including the chrysalis of the silkworm moth *Bombyx mori* (Butenandt et al., 1951), together with cycloalanylleucine, cycloalanylproline, and cycloleucylvaline. No physiological role for them was stated. Cycloleucylproline was also isolated from the culture filtrate of a *Streotomyces* bacterium. The bitter tasting samples also contained cycloglycylproline and cycloalanylleucine. We could not relate the bitterness to any particular compound. All dipeptides with hydrophobic side chains are said to be bitter-tasting (Matoba and Hata, 1972). Cycloleucylproline has been shown to be a bitter compound formed by ageing in Japanese sake (Takahashi et al., 1974). From our data it is not possible to calculate whether the concentration of cycloleucylproline was above the taste threshold of 1.3 mM (Takahashi et al., 1974) in the venom. The bitter dipetide cycloleucylphenylalanine was earlier found in the venom of *P. apicalis* (Cruz López and Morgan, 1997).

The tribe Ponerini share a mixture of hydrocarbons and diterpenes in their Dufour glands. Chemically, the most interesting point is the frequent occurrence of diterpenes, rare in other subfamilies and best exemplified by *Pachycondyla obscuricornis*. The Ponerini are also noteworthy as the only tribe known so far to have volatile compounds in their venom glands, but this is not true of all species.

The tribe Proceratiini contains just *Discothyrea* and *Proceratium*, for which we have one example of the latter, but it was another species of ponerines that had no volatile compounds in the Dufour glands or venom glands.

Finally there are two monogeneric tribes, Thaumatomyrmecini containing the genus *Thaumatomyrmex*, and the tribe Typhlomyrmecini with the one genus *Typhlomyrmex*. Both of these are cryptic, and there are few data on them.

The contents of the Dufour glands of 26 species of ponerines have now been examined (Table 9), and we have been able to gather information on six of the eight tribes with 15 genera represented in this table. These examinations of the Dufour gland bring us no closer to an understanding of its possible functions. Some species contain compounds that could be considered as candidate pheromones, such as the

diterpenes of *Ectatomma ruidum*, *Pachycondyla apicalis*, and *P. obscuricornis*, or the acetophenone derivatives found in *Rhytidoponera* species. Others, such as the *Diacamma* and *Platythyrea* species, contain only alkanes and alkenes, which seem less likely potential pheromones because they are found so widely distributed among the Hymenoptera and insects generally, while others, like *Mystrium camillae* and *Proceratium itoi* have nothing volatile in the gland. One wonders what is different about *Platythyrea punctata* that it does not need this gland. Except for the epoxides of *Amblyopone reclinata*, the same or similar substances listed here have been found elsewhere among the Hymenoptera. All of the substances in Table 9 can be biosynthesized in the ant from acetate units, either through the fatty acid pathway for hydrocarbons, alcohols, and esters; through polyketides for the acetophenones of *Rhytidoponera* (Brophy et al., 1983; Sun and Toia, 1993); or through mevalonate or homomevalonate units for the terpenes. There is nothing to suggest from the structures that they are sequestered from plants or other invertebrates.

Since hymenopteran venom is usually an aqueous protein solution, it is more appropriate to ask why the venom glands should have any volatile substances at all. Some ant venom glands contain trail pheromones (Billen and Morgan, 1998), but these are usually just trace amounts. Many of the substances listed in Table 10, for example the pyrazines (Morgan, 1984), indole, and certainly the piperazinedione of *Pachycondyla apicalis* are made from aminoacids. This is as expected for a gland primarily designed for producing protein venoms. Analyses of pygidial gland are at too elementary a stage to draw any conclusions.

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CHEMICAL CUES FROM *Murgantia histrionica* ELICITING HOST LOCATION AND RECOGNITION IN THE EGG PARASITOID *Trissolcus brochymenae*

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Abstract—Host location and recognition by the egg parasitoid *Trissolcus brochymenae* were analyzed in terms of response to kairomones from several stages of its host, *Murgantia histrionica*. In a Y-tube olfactometer, parasitoid females responded by increasing residence time and/or reducing linear speed to chemical cues from gravid females, virgin females and males, fifth and third instars, and eggs. In an open arena, *T. brochymenae* females also responded to patches contaminated by chemicals from the host in the same stages, sexes, and/or physiological conditions as those tested in the olfactometer. The parasitoid displayed arrestment behavior, increased residence time, changed walking pattern, and intense substrate examination. When host egg clusters or glass dummies with a chemical egg extract were placed on the host-contaminated open arena, these elicited an orientation response in the parasitoid. In addition, the chemical egg extract without dummies elicited the same response, whereas dummies without extract did not influence parasitoid behavior. In a closed arena, the parasitoid females recognized and attempted to probe glass beads treated with chemical extracts of host eggs. There were no significant differences compared with their response to the host eggs, and they did not respond to untreated beads. Host recognition was elicited by chemicals from the follicular secretion used by

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the host to glue the eggs on the substrate. These results are discussed in relation to the level of the host selection sequence influenced by these cues.

Key Words—*Murgantia histrionica*, Pentatomidae, *Trissolcus brochymenae*, Scelionidae, egg parasitoid, kairomone, host selection behavior, host location, host recognition.

INTRODUCTION

When seeking hosts for reproduction, insect parasitoids follow a hierarchical sequence of phases that, generally through habitat and microhabitat preference, host community location, host location, and recognition, eventually lead to host acceptance and oviposition (Vinson, 1984, 1985). Chemical and/or physical cues from the host, the substrate, and associated material and/or organisms play a fundamental role in mediating the different steps of host selection (Vinson, 1984, 1985, 1991, 1998; Schmidt, 1991; Vet and Dicke, 1992; Vet et al., 1995), and the ensemble of such cues for a given host-parasitoid association has been defined as a "host unit" (Conti et al., 2000). When dealing with *Trissolcus* spp. (Hymenoptera: Scelionidae), which attack pentatomid bugs (Heteroptera: Pentatomidae), an "egg host unit" may be represented by the characteristics of the host egg and the egg's immediate surroundings. The egg characteristics include shape, size, texture, chorion thickness, color, and chemical composition, whereas the surroundings are represented by any secretion used by host females to attach an egg to a substrate, traces left by prior host stages, and substances from the substrate itself or its interactions with the bug.

Details on such egg host units are best known for the association *Nezara viridula* (L.) (Heteroptera: Pentatomidae) – *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae). The secretion present on *N. viridula* eggs contains the host recognition kairomone used by its parasitoid *T. basalis* (Sales et al., 1980; Bin et al., 1993). In addition, parasitoid females are attracted by volatile chemicals from *N. viridula* eggs, gravid females, and males (Sales, 1979; Bin et al., 1987; Mattiacci et al., 1993; Colazza et al., 1999b). This parasitoid also shows an arrestment response to substrates contaminated by chemicals from adults of *N. viridula* (Colazza et al., 1999b). However, it is unknown whether *T. basalis* also uses cues from the nymphs. Recent data suggest parasitoid response to volatile cues induced by host oviposition on fava bean leaves (Colazza et al., 2001). All these components, therefore, should be considered as part of the host unit for *T. basalis*.

Mechanisms similar to those described for *T. basalis* were, in part, observed for *Trissolcus simoni* (Mayr) response towards *Eurydema ventrale* Kolenati (Heteroptera: Pentatomidae) (Colazza and Bin, 1988; Conti, Salerno, Bin and Vinson, unpublished data). Such information on *T. basalis* and *T. simoni* also supports previous results on host searching behavior by other Palaearctic species of *Trissolcus*

(e.g., Buleza, 1973, 1985). However, none of these papers provides a comprehensive laboratory study on the host's chemical cues that mediate host location and recognition by an egg parasitoid of pentatomid bugs.

In this paper, we show the effect of semiochemicals from the host in different stages, sexes, and/or physiological conditions, in the Nearctic association *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae) – *Murgantia histrionica* (Hahn) (Heteroptera: Pentatomidae). The parasitoid *T. brochymenae* also refers to *T. murgantiae*, which was synonymized with the former (Johnson, 1984). It is reported to be associated with at least 11 pentatomid species occurring in the New World (Salerno, 2000), including the harlequin bug, *M. histrionica* (Johnson, 1984), which mainly attacks Cruciferae and Capparidaceae in Mexico and North America and is considered a pest of cabbage and other brassicaceous crops (McPherson, 1982).

METHODS AND MATERIALS

Insects. The host, *M. histrionica*, was reared in a controlled condition chamber ($25 \pm 2^{\circ}$C, $70 \pm 10\%$ relative humidity, 14L:10D photoperiod), inside clear-plastic food containers ($300 \times 195 \times 125$ mm high) with 5-cm diam. mesh-covered holes. Separate containers were used for nymphs and adults. All stages were fed with vegetative and reproductive parts of cabbage and broccoli, *Brassica oleracea* L. Food was changed every 2–3 days, and water was provided weekly through soaked cotton wool.

The parasitoid, *T. brochymenae*, was reared in 85-ml glass tubes (30 mm diam. $\times$ 150 mm long) and kept in an incubator ($25 \pm 1^{\circ}$C, $80 \pm 5\%$ relative humidity, 15L:9D photoperiod). Adult wasps were fed with a solution of sugar (10%), honey (10%), benzoic acid (10%), yeast, and water (Safavi, 1968). About eight *M. histrionica* egg clusters ($\sim$96 eggs) were exposed to three parasitoid females for 48 hr. Females were then removed, and the eggs were stored in the same tube for incubation. After emergence, male and female parasitoids were kept together to allow mating. For experimental purposes, about 24 hr before the bioassays, 2- to 5-day-old mated females were isolated in small glass vials (10 mm diam. $\times$ 25 mm long), provided with a drop of the Safavi (1968) diet, and kept in the same incubator.

Host Location

Olfactometer Bioassays of Volatile Chemicals from Host Adults, Nymphs, and Eggs. The effect of host volatiles on parasitoid searching behavior was investigated in a Y-tube olfactometer in Plexiglas (stem 90 mm; arms 80 mm at 130° angle; internal section 15×15 mm), sandwiched between two glass sheaths (Colazza et al., 1999b). Medical-grade compressed air flowed through both arms

creating an airstream of 144 ml/min per arm. The flow was regulated by flowmeters and bubbled through a water jar to humidify the air before it passed into the olfactometer. The olfactometer was surrounded by a black fabric curtain to minimize possible cues from the room and was illuminated by two 22-W cool white fluorescent tubes located above the device. The temperature in the bioassay room was continuously maintained at ≈25°C.

For each experiment, one of the following was bioassayed in the olfactometer: mated and gravid (i.e., with enlarged and slightly bloated abdomen) *M. histrionica* females, virgin ≈2-days-old females or males, fifth instars, third instars, or 0- to 24-hr-old eggs. One adult or fifth instar, three third instars, or 60 eggs (=5 egg masses) were caged in a small brass mesh box (25 × 15 mm) and placed close to the air orifice at the end of one arm, randomly assigned, or of both arms in the case of two-choices tests. An empty box was placed in the control arm. Each bug condition was used for a set of four to five bioassays, each carried out with different parasitoid females. After each set of trials, the glass plates and brass mesh boxes were cleaned with hexane, acetone, ethanol, and distilled water, whereas the Plexiglas part of the olfactometer was cleaned with a laboratory detergent and rinsed with hot tap water (≈90°C, for 5 min) and finally with distilled water. The possible presence of bias between the two olfactometer arms was evaluated by running blank tests (i.e., with empty arms). Tests were conducted from ≈9:00 AM to 7:00 PM.

A naive female of the parasitoid was introduced into the Y-tube at the entrance of the stem. The parasitoid's walking pattern was then recorded for 10 min with a monochrome CCD videocamera (Sony SSC M370 CE) fitted with a 12.5–75 mm/F1.8 zoom lens. In order to exclude reflected light, the olfactometer was illuminated by infrared light (homogenous emission of wavelengths at 950 nm provided by 108 LEDs) from below, and the camera lens was covered with an infrared pass filter (Kodak Wratten filter 87Å). Analog video signals from the camera were digitalized by a video frame grabber, and digital data were processed by XBug, a video tracking and motion analysis system developed for the Linux operating system (Colazza et al., 1999a).

The following parameters were calculated to describe parasitoid walking patterns: residence time percentage, i.e., the percentage of total time spent in each of the olfactometer arms and average linear speed in each arm (millimeters per second).

Data were analyzed with *t* tests for paired comparisons and, prior to analysis, were subjected to angular or square-root transformation when necessary to normalize distributions (Sokal and Rohlf, 1998).

Open Arena Bioassays of Chemical Traces from Host Adults and Nymphs. Parasitoid arrestment behavior was investigated on a filter paper open arena (380 × 252 mm), where the parasitoids could move in an unconstrained field. A central circular area (58 mm diam.) in the open arena was contaminated by *M. histrionica*

in the following stages or conditions: mated gravid females, virgin ≈2-days-old females or males, fifth instars (one individual), or third instars (three individuals). Uncontaminated areas were also tested as controls. In order to contaminate the central area, the bugs were kept in place for 1 hr and forced to walk on the filter paper surface with a special device obtained from a transparent polyethylene Petri cover connected to a table watch. The axis of the watch mechanism was introduced through a hole expressly made in the middle of the cover, and only the seconds hand was kept in place, so that its movement forced the bug to move continuously and uniformly in the constrained area.

A naive female parasitoid was then placed in the middle of the central area, either host-contaminated or uncontaminated, and its walking behavior was recorded. The experiment was stopped when the female walked or flew out from the arena. Four to five bioassays were conducted for each arena set. The parasitoid's walking pattern was recorded, and digitalized data were processed as described for the olfactometer bioassays. The behavioral parameters, used to characterize parasitoid walking patterns in the host-contaminated patch, were as follows: total residence time (seconds), average linear speed (millimeters per second), average angular speed (degrees per second), and tortuosity index. The latter ranges from 0 to 1, with 0 indicating a completely linear tracking and 1 the maximum possible tortuosity (Bell, 1990; Daniele Peri and Stefano Colazza, personal communication).

Data were analyzed with one-way analysis of variance (ANOVA) and with the Tukey HSD test for multiple comparisons between the means. When necessary, heteroschedastic data were normalized with logarithmic transformation [Statistica 5.1 (Statsoft Italia, 1997); Sokal and Rohlf, 1998].

Open Arena Bioassays of Short Range Cues from Host Eggs. Possible volatile cues from host eggs, in combination with visual cues, and their effect on the final step of host searching before the encounter also were investigated in the filter paper open arena. In order to induce parasitoid arrestment and consequent searching on the substrate, the arena was contaminated with a gravid female as described in the previous section. Then, four circles (7 mm diam.) were located on the arena at the vertexes of a square (35-mm sides) centered in the contaminated area, and the sample to be tested was placed in two opposite circles (test), whereas the other two circles were left empty (control). Bioassays were carried out with host egg clusters, (12 eggs), glass dummies of the clusters, and/or egg extracts applied directly on the filter paper circles. The egg dummies were obtained from 6-mm-long portions of 50-μl microcapillary tubes, which had been previously sanded in order to prevent light reflection. Two such microcapillary portions were attached slightly offset along their main axis with a small piece of Parafilm, in order to simulate the two egg rows that constitute a normal host cluster. The chemical extracts were prepared by soaking 100, 0- to 24-hr-old host eggs in 125 μl of acetone for 1 hr, in order to obtain ∼100 μl of extract, the remaining being lost by evaporation or absorption.

Because chemical extraction is probably only partial, the amount of egg extract obtained from 24 eggs (i.e., 24 egg equivalents $\cong$24 μl) was applied to each filter paper circle. In order to prevent possible interaction between the egg extract and the host chemicals contaminating the arena, the arena in this case was made up of two filter paper layers, the upper layer being host-contaminated and with the four circles removed, the lower being treated with extracts only on two opposite circles.

A naive female of the parasitoid was released in the center of the arena, and its walking behavior was recorded as described in the previous section. The experiment was stopped when the female encountered either a test or a control circle, or when it abandoned the arena without having had any encounters. A series of 10 females and one arena set were used for each replication, and the percentage of encounters with the test or the control circles was scored.

Data on encounters with test versus control circles were analyzed with *t* tests for paired comparisons (Sokal and Rohlf, 1998). Data from the test circles were analyzed with one-way analysis of variance (ANOVA) and with the Tukey HSD test for multiple comparisons between the means [Statistica 5.1 (Statsoft Italia, 1997; Sokal and Rohlf, 1998)]. Prior to analysis, percentages were subjected to angular transformation (Sokal and Rohlf, 1998).

Host Recognition: Closed Arena Bioassays of Chemicals from Host Eggs. Host recognition behavior by *T. brochymenae* was investigated in closed arenas by exposing a simplified egg cluster of *M. histrionica* (i.e., three eggs) or a cluster dummy (i.e., three glass beads, 1.5 mm diam.) to a 2- to 5-day-old, naive, female parasitoid. To evaluate the presence of an egg recognition kairomone, chemical extracts of the eggs were obtained as previously described, with hexane instead of acetone in order to prevent extraction from the egg content. In fact, we observed that eggs treated for 1 hr with acetone were killed and collapsed after desiccation, whereas those treated with hexane for the same duration were not affected internally and hatched regularly. Therefore, it appears that hexane does not penetrate into *M. histrionica* eggs, probably because this solvent is not water soluble. Two egg equivalents ($\approx$2 μl of extract) per glass bead were applied by rolling them in a microscope slide with a central depression. In another test, to identify the source of the kairomone and on the basis of a previous investigation on the *T. basalis–N. viridula* association (Bin et al., 1993), glass beads were treated with the secretion produced by the follicular cells of *M. histrionica* ovarioles. Such secretion is used by the host to glue its eggs to each other and to the substrate. The ovaries from gravid host females were dissected, and the secretion was transferred in a drop of bidistilled water on a depression microscope slide, where beads were then rolled.

Bioassays were carried out in a multiple arena composed of a Plexiglas plate (80 $\times$ 60 $\times$ 4 mm thick) with six holes acting as single arenas (4 mm height $\times$ 13 mm diam.), sandwiched between two glass sheaths. The eggs or glass beads were placed in the center of each arena, and expressly made tunnels (3 mm

diam. × 10 mm long) on the external sides of each arena wall were used to introduce a parasitoid female. Observations were started after parasitoid introduction and were stopped after 10 min if there was no encounter, 15 min after the encounter if there was no recognition, or after recognition (glass beads) or parasitization (eggs). Only observations that led to an encounter were considered for data analysis. Parasitoid behavior was recorded with a JVC KY-M280 video camera using 12.5–75 mm/F1.8 lenses and connected to a Panasonic NV-FS100HQ video recorder. Behavioral data were collected and analyzed with The Observer Video-Pro Version 4.0 for Windows (Noldus Information Technology, 1997). Results were used to describe *T. brochymenae* recognition behavior and to compare parasitoid response to eggs, extract treated beads, follicular secretion treated beads, and untreated beads.

Frequency (number) and total duration (seconds) of behavioral steps were analyzed by one-way ANOVA and the Tukey HSD test for multiple comparisons, whereas frequencies of probing (percent of females probing the host eggs or beads) were analyzed with the Karl Pearson χ^2 test and Goodman's *post hoc* procedure for internal contrasts (Sokal and Rohlf, 1998; Marascuilo and Serlin, 1988).

RESULTS

Host Location

Volatile Chemicals from Adults, Nymphs, and Eggs. *T. brochymenae* females responded to volatile chemicals from the host, *M. histrionica*, in different stages, sexes and/or physiological conditions (Figure 1). Compared to control arms, the egg parasitoid exhibited a greater residence time percentage in the arm containing a gravid female ($t = 10.51$, $df = 14$, $P < 0.001$) or a virgin female ($t = 3.39$, $df = 15$, $P = 0.004$). When females in these different physiological conditions were compared in the same olfactometer, residence time was similar in the two arms ($t = 0.26$, $df = 19$, $P = 0.793$). The parasitoid also exhibited a higher residence time, compared to control arms, in the arm containing a virgin male ($t = 5.42$, $df = 11$, $P < 0.001$). However, when a virgin male was compared with a gravid female in the same olfactometer, parasitoid residence time was higher in the arm containing the female ($t = 2.39$, $df = 13$, $P = 0.033$). The parasitoid also responded to the arm containing a fifth instar ($t = 4.07$, $df = 14$, $P = 0.001$), third instars ($t = 2.76$, $df = 14$, $P = 0.015$), or host eggs ($t = 2.44$, $df = 13$, $P = 0.030$) (Figure 1).

Except for one case, these results were confirmed by the parasitoid's linear speed (Figure 1). This was lower, compared to the control, in the arm containing a gravid female ($t = 6.18$, $df = 13$, $P < 0.001$) or a virgin female ($t = 4.79$, $df = 15$, $P < 0.001$), whereas no differences were scored when gravid and virgin females were compared ($t = 0.36$, $df = 19$, $P = 0.725$). Linear speed also was

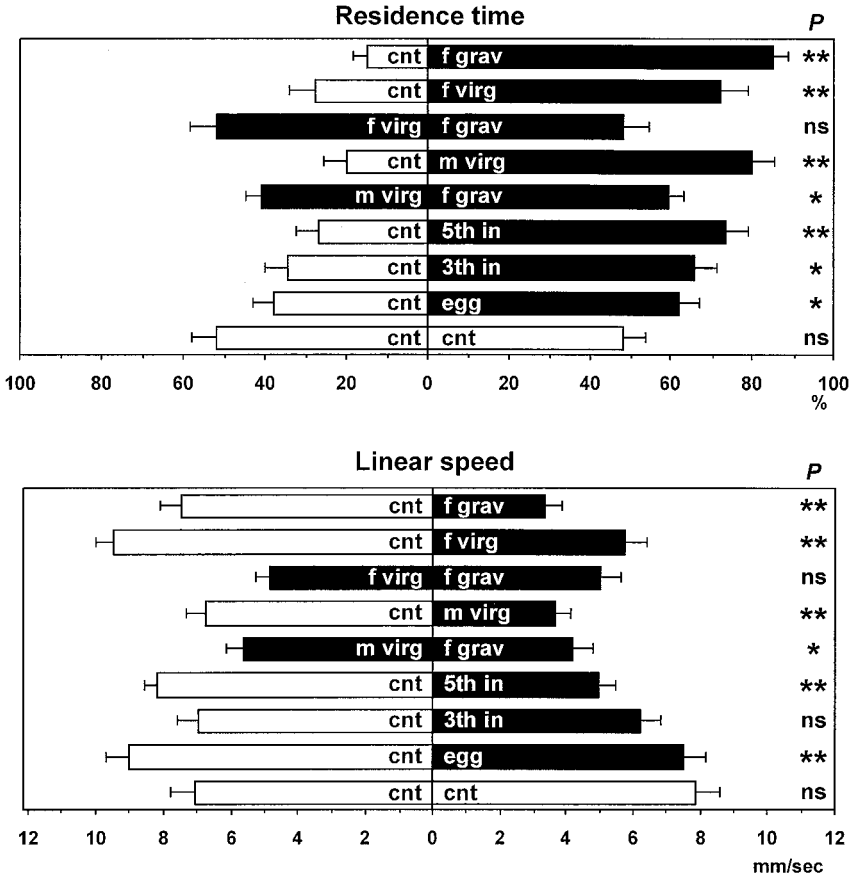

FIG. 1. Behavioral response (mean ± SE), in Y-tube olfactometer, of *Trissolcus brochymenae* to volatile chemicals from *Murgantia histrionica*. f grav: gravid females; f virg: virgin females; m virg: virgin males; 5th in: fifth instars; 3rd in: third instars; egg: eggs; cnt: control. $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant (t tests for paired comparisons).

lower, compared to the control, in the arm containing a virgin male ($t = 4.76$, $df = 11$, $P < 0.001$). When males were compared with females, linear speed was lower in the arm containing the female ($t = 2.22$, $df = 13$, $P = 0.045$). The parasitoid also responded by reducing linear speed to the arm containing a fifth instar ($t = 7.16, df = 14, P < 0.001$) or host eggs ($t = 3.39, df = 13, P = 0.005$). However, as opposed to residence time, linear speed did not change compared to the control in the arm containing third instars ($t = 1.37$, $df = 14$, $P = 0.192$) (Figure 1).

Both in terms of residence time ($t = 0.31$, $df = 14$, $P = 0.754$) and linear speed ($t = 0.98, df = 14, P = 0.345$), the blank experiment elicited no significant

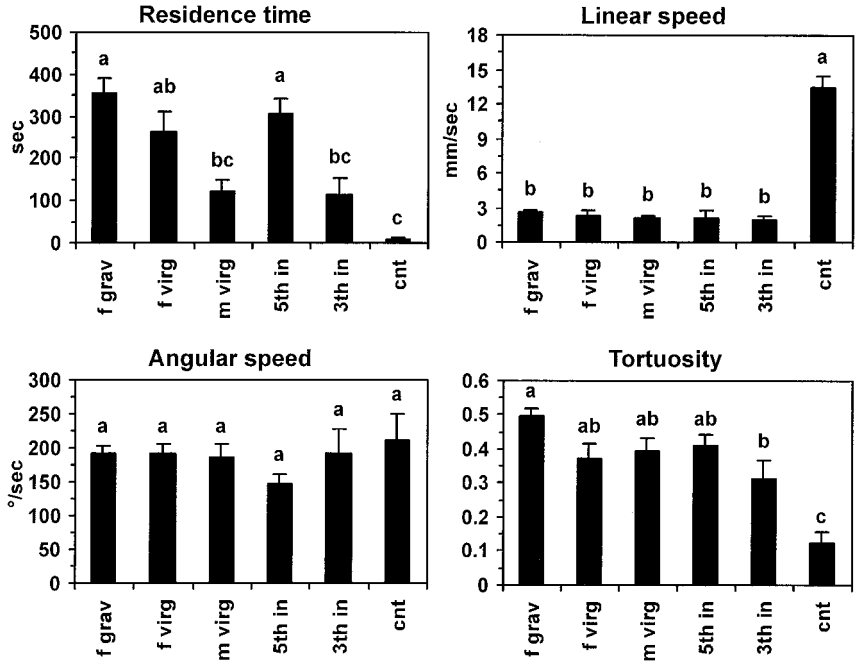

FIG. 2. Behavioral response (mean ± SE), in open arena, of *Trissolcus brochymenae* to chemical traces from *Murgantia histrionica*. Abbreviations as in Figure 1 legend. Columns with the same letter are not different at $P < 0.05$ (ANOVA, Tukey HSD).

differences between the two arms, indicating that no bias had affected the olfactometer (Figure 1).

Chemical Traces from Adults and Nymphs. Females of *T. brochymenae* also responded to chemicals left on the substrate by *M. histrionica* in the different stages, sexes, and/or physiological conditions (Figure 2). Residence time ($F = 23.35$, $df = 5, 128$, $P < 0.001$) was higher, compared to the control, in patches contaminated by females, either gravid or virgin, and by fifth instars, whereas it was not different from the control in patches contaminated by virgin males and third instars. The parasitoid walking pattern was also affected. Linear speed ($F = 53.22$, $df = 5, 128$, $P < 0.001$) was similar in all host-contaminated patches and always different from the control, whereas angular speed ($F = 0.40$, $df = 5, 128$, $P = 0.850$) did not change among the different tests and compared with the control. Tortuosity ($F = 21.36$, $df = 5, 128$, $P < 0.001$) was always higher in host-contaminated patches compared with the control. In addition, it was higher in patches contaminated by gravid females than in those contaminated by third instars, and it was intermediate in the other host-contaminated patches (Figure 2).

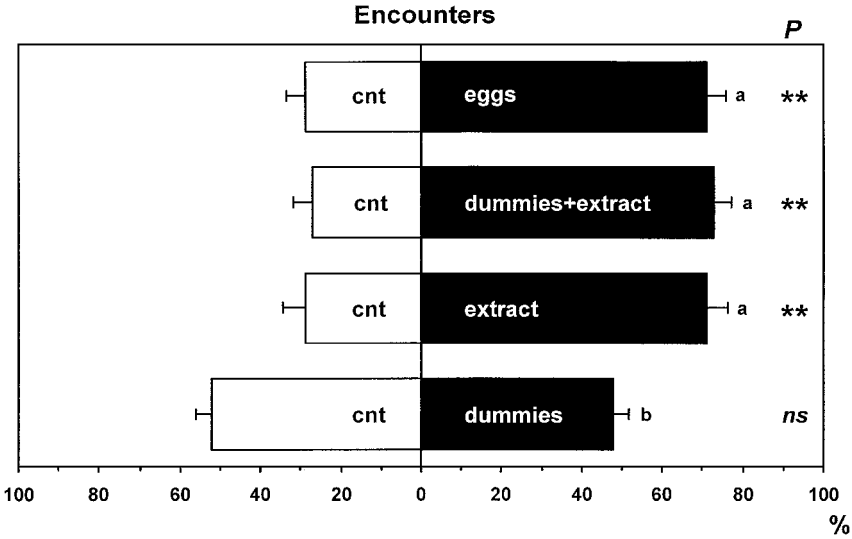

FIG. 3. Behavioral response (mean no. encounters ± SE), in open arena, of *Trissolcus brochymenae* to egg clusters of *Murgantia histrionica* (eggs), and to combinations of egg cluster dummies (dummies) and/or host egg extract (extract). $^{**}P < 0.01$; *ns* not significant (t tests for paired comparisons). Right (test) bars with the same letter are not different at $P < 0.05$ (ANOVA, Tukey HSD).

Short Range Cues from Eggs. While walking on a patch contaminated by a *M. histrionica* gravid female, *T. brochymenae* females responded to short-range cues from host egg masses (Figure 3). Parasitoids, in fact, exhibited a higher percentage of encounters with host egg clusters than with control spots ($t = 4.36$, $df = 9$, $P = 0.002$). Results were similar when glass dummies with host egg extracts ($t = 5.06$, $df = 10$, $P < 0.001$) or when egg extract only ($t = 3.84$, $df = 9$, $P = 0.004$) were bioassayed. In contrast, no parasitoid response was observed when glass dummies without extract were tested ($t = 0.51$, $df = 9$, $P = 0.619$) (Figure 3).

When parasitoid response to the tests was compared among the different treatments ($F = 6.25$, $df = 3, 37$, $P = 0.002$), percentages of encounters were similar towards host egg clusters, glass dummies with egg extract, and egg extract alone. Encounters were lower on glass dummies without extracts (Figure 3).

Host Recognition

Chemicals from Host Eggs. When a female of *T. brochymenae* encounters an egg of *M. histrionica* [frequency (N): 4.32 ± 1.93; mean ± SE], it normally starts examining the egg carefully with the antennae [frequency (N): 4.77 ± 1.99;

average duration (sec): 39.27 ± 7.87] and finally, if the host egg is recognized, it adopts a probing posture, drills the egg chorion, oviposits, and finally withdraws the ovipositor from the host egg [frequency (%): 72.7%; total duration (sec): 291.70 ± 34.96]. If oviposition has occurred, the female then marks the egg by wiping the tip of its ovipositor on it [frequency (%): 63.6%; duration (sec): 31.73 ± 11.45], and finally resumes searching on other eggs or abandons the egg cluster. During the whole process, the female may stop several times for grooming [frequency (N): 2.68 ± 0.68; duration (sec): 31.73 ± 11.45] or may remain resting [frequency (N): 0.55 ± 0.23; duration (sec): 134.02 ± 55.22].

When this behavior was compared among the different treatments (host eggs, glass beads treated with host follicular secretion, or with host egg extracts, glass beads treated with plain solvent as control), only some steps could be considered. In fact, for example, probing could not be completed with glass beads, and, therefore, the observation was interrupted at this level (Figure 4).

Frequency of encounters ($F = 1.08$, $df = 3, 113$, $P = 0.359$) did not change among the different treatments, whereas total examination duration ($F = 5.95$, $df = 3, 110$, $P < 0.001$) was higher on eggs and extract-treated glass beads compared with the control, and intermediate on secretion-treated beads. Probing frequency ($\chi^2 = 47.33$, $df = 3$, $P < 0.001$) was higher in all treatments compared to the control. In addition, it was higher on beads treated with the follicular secretion compared to those treated with the egg extract, and intermediate on eggs. Finally grooming duration was lower in all treatments compared with the control ($F = 11.95$, $df = 3, 92$, $P < 0.001$) (Figure 4).

DISCUSSION

Because host eggs are not apparent in terms of semiochemical and physical cues compared to other host stages, and because they are present in the field and/or suitable for parasitization for a short period, egg parasitoids face particular challenges in host selection (Vinson, 1998). In addition, because of their small size, egg parasitoids generally disperse passively downwind, which makes long-distance searching difficult. Consequently, the use of indirect cues from plants may be limited and more connected to arrestment behavior (Vet et al., 1995). Egg parasitoids, therefore, have evolved particular strategies that involve the use of cues from nontarget stages of the host species, which may be more detectable compared to cues from the host eggs and still relatively reliable (Vet et al., 1995; Vinson, 1998).

Such strategies were also found in host selection by *T. brochymenae*. Females of this parasitoid are attracted to volatile compounds from the host in the different stages, sexes, and/or physiological conditions, suggesting that these may act as long-distance cues used to locate the host community, and, therefore, should be

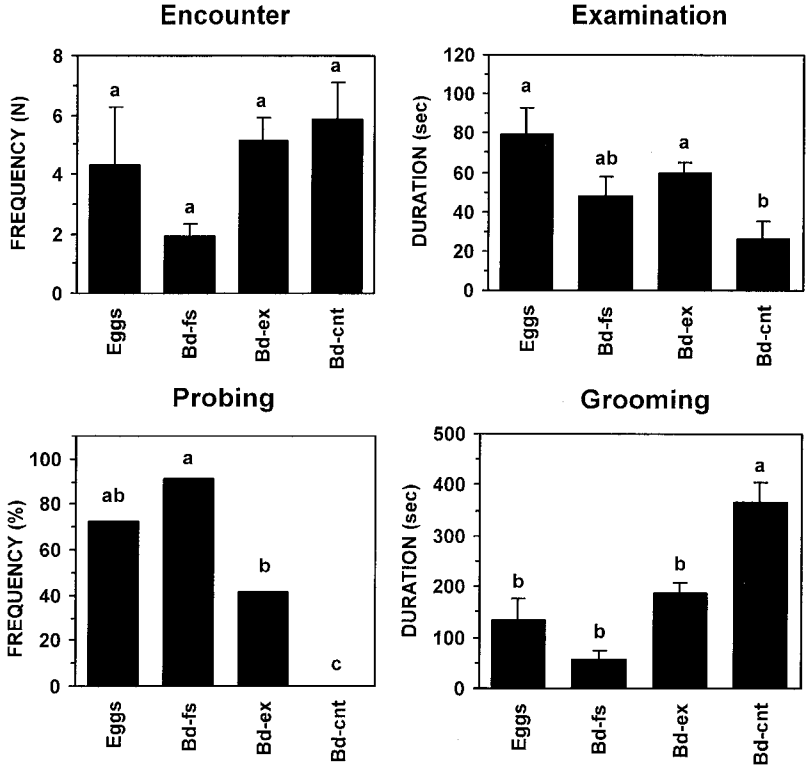

FIG. 4. Behavioral response (mean ± SE), in closed arena, of *Trissolcus brochymenae* to eggs of *Murgantia histrionica* (eggs), to glass beads treated with host follicular secretion (Bd-fs) or with chemical egg extracts (Bd-ex), or to untreated beads as control (Bd-cnt). Columns with the same letter are not different at $P < 0.05$ (Encounter, examination, and grooming: ANOVA, Tukey HSD. Probing: Karl Pearson χ^2 test, Goodman's *post hoc* procedure).

included in the group I cues defined by Vinson (1991). Such cues also elicit host searching behavior, as indicated by an increase in residence time and a decrease in linear speed.

Considering that *M. histrionica* emit different volatile chemicals that have been partly identified (Aldrich et al., 1996), these should be considered as a possible source of host kairomones for the parasitoid. Similar to *T. brochymenae*, *T. basalis* also uses cues from gravid females and from males of its host, *N. viridula*, but it is not attracted to virgin females (Colazza et al., 1999b). In addition, *T. simoni* is attracted to females and egg masses of *E. ventrale*, but not to males (Colazza and Bin, 1988). On the other hand, no information was previously available on *Trissolcus* spp. response to volatiles from nymphs of the host.

Compared to the other treatments, the *T. brochymenae* response to gravid host females seems to be particularly intense. However, when gravid and virgin females were compared in the olfactometer arms, parasitoid responses were similar. This apparent discrepancy may be explained by the possible inability of the parasitoid to discriminate between the two sources of cues when simultaneously perceiving both of them in the common olfactometer arm. This condition may be explained, for example, if the same chemicals are involved, but in different concentrations and/or proportions. Such an explanation is supported by results from the comparison between gravid females and virgin males. In fact, although both are attractive for the parasitoid, volatiles from gravid females are preferred, both in terms of residence time and linear speed. Therefore, chemical cues from host males and females probably are different and act hierarchically, since those from gravid females are a more reliable indication of the potential presence of host eggs.

While walking on the plant substrate close to the host community, the presence of patches with chemical traces left on the substrate by the host nymphs and adults elicits an arrestment behavior in *T. brochymenae*, characterized by ortho- and klinokinetic responses (i.e., reduced linear speed and increased tortuosity index) and residence time increases. This is similar to the arrestment behavior described for *T. basalis* on patches contaminated by *N. viridula* adults (Colazza et al., 1999b). In addition to adults, we also tested third and fifth instars of *M. histrionica* and showed that host gravid females elicit a higher response in *T. brochymenae* in terms of residence time and tortuosity index compared with males and/or third instars. However, fith instars also elicited a higher response. This suggests that although the parasitoid may be able to discriminate among different host conditions, the intensity of its response also may depend on unknown factors, such as chemical concentration and/or abundance, or host mobility on the substrate. These chemicals seem to have a low volatility and should be included in the group II cues, which act as searching stimulants (Vinson, 1991). However, their origin is unknown, and investigations are in progress in order to verify whether they are produced by host glands or whether they simply originate from the cuticle.

If host eggs are present on the adult-contaminated substrate, short-distance host location follows, as *T. brochymenae* orients towards the eggs, thus increasing the frequency of encounters. The parasitoid responds similarly to circles treated with host egg extracts, either with or without egg dummies, whereas it does not respond to blank dummies. This is an interesting new piece of information for this group of egg parasitoids, because it shows the presence of short-range volatile chemicals from the host eggs, i.e., probably group II cues (Vinson, 1991), affecting the last step of host location. However, because such dummies may not appropriately simulate important physical characters of the host egg cluster, whether there is a combined effect of chemical and visual cues cannot be excluded and is under investigation. In addition, recent results from the *T. basalis–N. viridula* system

indicate the presence of host oviposition-induced volatiles also involved in host location (Colazza et al., 2001).

Finally, when a host egg cluster is encountered, host recognition is elicited by chemicals present in the host secretion. Such chemicals are apparently of low volatility and should be included in the group III cues (Vinson, 1991). This behavior is similar to that described for *T. basalis* on *N. viridula* (Bin et al., 1993) and *Telenomus* species on different noctuid pests (Lepidoptera: Noctuidae) (Strand and Vinson, 1982; Nordlund et al., 1987; Conti, Salerno, and Bin, unpublished data). The long duration of host examination, common in parasitoids that attack egg clusters and that therefore, may become egg-limited, could be explained by the fact that these parasitoids optimize assessment of host suitability, thus maximizing fitness gain per host encounter (Vet et al., 1995). The effects of physical factors such as host shape, size, texture, and color have not been considered in this paper, which is focused on chemical cues. However, our results indicate that bead size affects host recognition in *T. brochymenae* (Conti, Salerno, Bin and Vinson, unpublished data).

In order to provide a complete description of the cues that characterize the egg host unit (Conti et al., 2000) for *T. brochymenae*, it will be necessary to identify the chemical and physical factors involved in host selection by this parasitoid. This would provide basic information for the definition of specificity in *T. brochymenae* (Conti, Salerno, Bin and Vinson, unpublished data) and the evaluation of its potential as an effective and reliable biological control agent (Nechols et al., 1992; van Lenteren, 1997; Follett et al., 2000).

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SPECIFIC FORAGING KAIROMONES USED BY A GENERALIST PARASITOID

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Abstract—In general, it is assumed that generalist natural enemies do not innately use specific cues for the location of their host or prey species. This hypothesis was tested using naïve females of the generalist parasitoid *Lariophagus distinguendus* Förster and two of its hosts, larvae of the lesser grain borer *Rhyzopertha dominica* (F.) and of the granary weevil *Sitophilus granarius* L., feeding in wheat grains. In a four-chamber olfactometer, female parasitoids were attracted to volatiles emanating from the feces of both host species. Chemical analysis of the volatiles from the feces of *R. dominica* revealed the presence of dominicalure 1 and 2, the species specific aggregation pheromones of *R. dominica*. The main compounds in the volatiles from feces of *S. granarius* were identified as chemicals related to mites that are associated with hosts of *L. distinguendus*. Because these mites are not specific for *S. granarius* but also co-occur with other hosts, the mite chemicals have to be considered as general cues. In bioassays, synthetic dominicalure was attractive to naïve *L. distinguendus*, explaining the attraction of feces volatiles from *R. dominica*. Synthetic mite chemicals and sitophilate, the aggregation pheromone of *S. granarius*, had no effect on naïve parasitoids. It remains to be determined which innate chemical cues from feces of *S. granarius* are used by *L. distinguendus*. In contrast to our initial hypothesis, the generalist *L. distinguendus* is innately using specific cues for foraging. Two ideas are provided to explain this result.

Key Words—*Lariophagus distinguendus*, *Rhyzopertha dominica*, *Sitophilus granarius*, lesser grain borer, granary weevil, host finding, generalist, innate specific cues.

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INTRODUCTION

The location of plants, prey, or hosts for feeding or oviposition is a major problem faced by most insects. Many insects do not search randomly but use chemical cues for orientation that have been released by their target or its environment. As demonstrated for numerous species, these cues are often learned during the first encounters (Papaj and Lewis, 1993). However, freshly emerged insects without any experience have to rely exclusively on innate cues or cues acquired during development (e.g., Corbet, 1985; Cortesero and Monge, 1994; van Emden et al., 1996).

So far, many studies on the identity of innate foraging cues exist for herbivorous insects (see review by Schoonhoven et al., 1998). For carnivorous insects, innate foraging cues were examined for specialist parasitoids (e.g., Hendry et al., 1973; Udayagiri and Jones, 1993; Geervliet et al., 1996; Colazza et al., 1997; Mattiacci et al., 1999) and for specialized predators (e.g., Budenberg and Powell, 1992; Bargen et al., 1998; Shonouda et al., 1998; Al Abassi et al., 2000). These studies demonstrated the use of specific cues, either originating from the host itself or from the hosts' food plant. In contrast, only a few studies are available on innate foraging cues used by carnivorous generalists (Steidle and van Loon, 2002). In analogy to the definition used for generalist herbivores, we regard generalist carnivores as carnivores exploiting hosts or prey from different families feeding on host plants also from different families (Bernays and Chapman, 1994). It has been hypothesized that generalist carnivores do not innately use specific cues because sensory processing of the great diversity of specific cues expected from a large number of hosts or prey species should be impossible (Vet and Dicke, 1992). In fact, there is ample evidence for physiological constraints on processing a multitude of cues (Dukas, 1998). These constraints are responsible for longer decision times in generalists as compared to specialists (e.g., Bernays and Funk, 1999). Instead of using specific cues, it is assumed that generalist natural enemies use general chemical cues present in all hosts or prey species or their respective food plants (e.g., Vet and Dicke, 1992; Godfray 1994). However, to our knowledge there are almost no studies that have explicitly examined the hypothesis that a generalist carnivore does not use specific infochemical cues but rather general cues for foraging (Steidle and van Loon, 2002; but see Steidle et al, 2001a).

To test this hypothesis, the present study was initiated with the pteromalid wasp *Lariophagus distinguendus* Förster, a larval and pupal parasitoid of at least 11 different beetle species from five families. Most hosts live endophytically in the seeds of different plants from the taxa Poaceae and Fabaceae (Steidle and Schöller, 1997) and are worldwide pests of stored products. According to the abovementioned definition, *L. distinguendus* can be considered a generalist. Host finding and host recognition of *L. distinguendus* was examined in several studies (Steidle and Schöller, 1997; Ruther and Steidle, 2000; Steidle, 2000; Steidle et al., 2001b).

Using different host species feeding in different seed species, we were able to demonstrate that naïve *L. distinguendus* females prefer volatiles from host-infested seeds over volatiles from healthy seeds (Steidle et al., 2001b). To differentiate between healthy and infested seeds, wasps are using volatile chemicals from the hosts' feces, having an arresting effect on the parasitoids (Steidle and Schöller, 1997; Steidle et al., 2001b).

The experiments were performed with *L. distinguendus* and its hosts *Rhyzopertha dominica* (F.) and *Sitophilus granarius* L. feeding on wheat grains (*Triticum aestivum* L.). First, we examined whether feces are not only arresting but also having an attracting effect on naïve females of *L. distinguendus*. Then, we identified specific and general volatile chemicals present in the feces of both hosts, and examined the attractiveness of these volatiles for *L. distinguendus*.

METHODS AND MATERIALS

Insects. Insect cultures were kept at a temperature of 25°C, 30% relative humidity, and a photoperiod of 16L:8D. To rear *R. dominica* and *S. granarius*, 30 ml of adults were placed on 200 g wheat grain (var. Batis) with about 14% moisture content in glass jars (8 cm diam., 10 cm high). Adult beetles were removed 7 days later after having laid eggs in the grains.

The *L. distinguendus* strain used was collected on *Stegobium paniceum* L. in 1995 in a flourmill in Uzwil, Switzerland. In our laboratory, it was cultured on *S. granarius* in wheat grains as host. To rear *L. distinguendus*, about 50 newly emerged parasitoids were placed into Petri dishes (9 cm diam., 1 cm high) with about 50 g grains infested by third to forth instars of *S. granarius* and kept there until their death (7–10 days). After a developing time of 19–21 days, emerged parasitoids from the next generation were collected daily from each Petri dish. To obtain naive parasitoids for bioassays, freshly emerged male and female parasitoids were collected from the infested grains within 1 hr of emergence and kept in Petri dishes on moistened filter paper in a climatic chamber without host odors under the same conditions as described above. These parasitoids never had contact with adult hosts and, therefore, have to be considered naive with respect to the cues released by adult *R. dominica* or *S. granarius*. Parasitoids were tested when they were 2 days old.

For bioassays, wheat grains infested with 43- to 57-day-old larvae of *R. dominica* and wheat grains infested with 15- to 30-day-old larvae of *S. granarius* were used. The host ages used are known to be optimal for parasitization by *L. distinguendus* (Kaschef, 1959; van den Assem, 1971).

Bioassays. The response of naïve parasitoids, i.e., parasitoids without oviposition experience, towards different odor samples was examined using a static-4-chamber olfactometer as described by Steidle and Schöller (1997). The

olfactometer was made of acrylic glass and consisted of a cylinder (4 cm high, 19 cm diam.) divided by vertical plates into four chambers. On the top of the cylinder, a walking arena (1 cm high, 19 cm diam.) was placed consisting of plastic gauze (mesh 0.5 mm) with a rim of acrylic glass (0.9 cm high) and covered with a glass plate. An odor sample was placed in a Petri dish (5.5 cm diam.) with brown filter paper (4 cm diam.) in one chamber. Volatiles were allowed to diffuse through the gauze, resulting in an odor field in the walking arena above. The remaining three chambers contained Petri dishes with brown filter paper only as controls. The olfactometer was placed in a white bucket (29 cm diam., 36 cm high) and illuminated from above, resulting in 2050 lux in the olfactometer. The following odor sources were offered:

(a) Feces (200 mg) of *R. dominica* larvae from wheat grains. Two grams of wheat grains infested by 45- to 50-day-old *R. dominica* larvae contain 204 ± 126 mg (mean $\pm$ SD; $N = 10$) feces.

(b) Feces (100 g) of *S. granarius* larvae from wheat grains. This is equivalent to the amount of feces present in about 20 grains infested by 15- to 30-day-old *S. granarius* larvae (Steidle and Schöller, 1997).

(c) A brown filter paper with 50 μl of a synthetic mixture containing dominicalure 1 (300 ng/μl) and dominicalure 2 (400 ng/μl) in dichloromethane. The amounts were chosen according to the amounts found in the headspace of *R. dominica* feces. Filter papers in control chambers contained 50 μl dichloromethane.

(d) A brown filter paper with 50 μl of a synthetic mixture containing the mite-related compounds tridecane (Aldrich, Steinheim, Germany) at 60 ng/μl, citral (mixture of neral and geranial; Roth, Karlsruhe, Germany) at 10 ng/μl, and neryl formate at 5 ng/μl in dichloromethane. Neryl formate was synthesized as described elsewhere (Ruther and Steidle, 2000). To allow a comparison with data from Ruther and Steidle (2000), the amounts were chosen according to the quantitative analysis by these authors. Filter papers in control chambers contained 50 μl hexane.

(e) A brown filter paper with 50 μl of a synthetic mixture containing sitophilate (2 ng/μl) in hexane. This results in a total dose of 100 ng on the filter paper. Filter papers in control chambers contained 50 μl hexane.

(f) A brown filter paper with 50 μl of a synthetic mixture containing sitophilate (20 ng/μl) in hexane, resulting in a dose of 1000 ng on the filter paper. Filter papers in control chambers contained 50 μl hexane.

Both concentrations of sitophilate tested were within the active range of 22.5–2500 ng that stimulates reaction by *S. granarius* females (Phillips et al., 1989). Odor samples were exchanged after every five insects. Twenty-five parasitoids were tested for each type of sample. The parasitoids were released individually in the center of the walking arena and their behavior (walking, resting) and position was recorded for 600 sec using the computer software The Observer 3.0 (Noldus,

Wageningen, The Netherlands). The number of times the parasitoids entered in the odor field directly above the odor sample was compared to the control fields and used to assess the attractiveness of the odor sample. Only parasitoids with an overall walking time of >300 sec were included in the analysis. To avoid biased results due to side preferences of the parasitoids, the position of the olfactometer was rotated clockwise after every insect. For statistical comparisons, the software package Statistica for Windows 4.5 (StatSoft Inc. 1993) was used. Because data from the olfactometer fields are dependent, the nonparametric Friedman ANOVA for dependent data was used to test for homogeneity among the four fields. In case of significant differences, the Wilcoxon-Wilcox test for multiple comparisons of dependent data (Sachs, 1992) was used to determine which sectors were different from each other.

Chemical Analysis. Headspace extracts were obtained using the closed-loop stripping (CLS) technique (Grob and Zürcher, 1976). For this purpose, volatiles from 5 g feces were trapped for 4 hr at room temperature on the charcoal layer (5 mg) of the adsorption tube. The volatiles were eluted twice with 25 ml dichloromethane each (Biomol). For quantification of the volatiles, the dichloromethane contained methyl-caprylate at a concentration of 200 ng/μl as an internal standard (IS). The extracts were analyzed using gas chromatography–mass spectrometry (GC-MS). Dry feces of *R. dominica* and *S. granarius* consisted of very fine dust that often contaminated CLS extracts. To avoid contamination of the GC-MS, we used solid-phase microextraction (SPME) for introducing the CLS extracts into the GC-MS. Five microliters of each CLS extract were added to 10 μl methanol and 100 μl H_2O in a conical vial (150 μl). A SPME fiber (Supelco, Taufkirchen, Germany) coated with polydimethylsiloxane (PDMS) at a film thickness of 100 μm was immersed in the solution to allow equilibration of volatile compounds to the fiber. After 1.5 hr, the needle was inserted into the GC injection port for 1 min for desorption of the volatiles. Analytical separations were performed on a Fisons 8060 GC. Mass spectra were obtained on a Fisons MD800 quadrupole mass spectrometer (Thermoquest, Egelsbach, Germany). Analyses were carried out using a 30-m $\times$ 0.32-mm-ID DB-5ms fused silica column, film thickness 0.25 mm (J&W/CZT, Kriftel, Germany), with helium as carrier gas (head pressure 10 kPa). The temperature program started at 40°C, temperature was held for 1 min, and raised 4°C/min to 280°C. The column effluent was ionized by electron impact ionization (EI) at 70 eV. Linear retention indices (LRI) were estimated according to van den Dool and Kratz (1963) by adding 5 μl of a hydrocarbon mixture (C_7–C_{30}; 10 ng/μl of each alkane; Aldrich) to the vial with the extract before immersing the SPME fiber. Peaks were identified by comparison of mass spectra and LRI with those of synthetic reference compounds. To quantify volatiles in the CLS extracts, peak areas of the individual compounds were compared with the peak area of the internal standard. As SPME is a method mainly for qualitative and not for quantitative analysis, the resulting quantities can be considered only as rough

estimates. Based on these results, the amount of dominicalure for testing in the bioassays was chosen.

RESULTS

When feces of *R. dominica* and *S. granarius* were offered in the olfactometer, naïve parasitoids entered the odor fields above feces significantly more often than above the control fields (Figure 1). This demonstrates that feces from both hosts release volatile chemicals that are attractive to the parasitoids.

Headspace samples of the feces were analyzed using a combined CLS/SPME technique (Figure 2). The headspace of *R. dominica* contains the hydrocarbons undecane (1; numbers refer to peaks in Figure 2), dodecane (2), tridecane (5), tetradecane (7), and (*Z*)-7-pentadecene (8) as well as two compounds identified as (*S*)-(+)-1-methylbutyl (*E*)-2-methyl-2-pentenoate (3; dominicalure 1) and (*S*)-(+)-1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate (4; dominicalure 2). The compound (6) having a mass spectrum identical to dominicalure 2 but with different LRI most likely represents an isomer of dominicalure 2. Feces from *S. granarius* also contained the hydrocarbons 1, 2, 7, 8; a much larger amount of tridecane (5); the additional hydrocarbons (*Z*, *Z*)-6,9-pentadecadiene (13), (*Z*)-6-pentadecene (14), and pentadecane (15); as well as the terpenoids neral (9), geranial (10), and neryl formate (11). Compounds 5–11, and 13–15 originate from

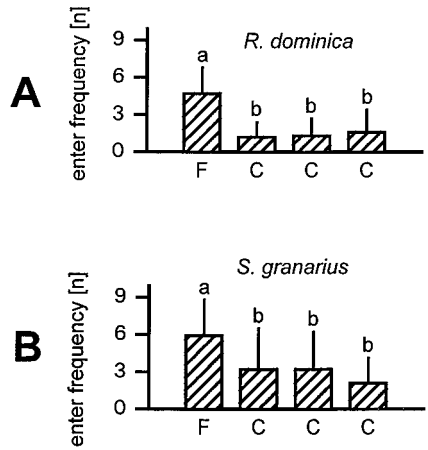

FIG. 1. Mean enter frequency (±SD) of naive females of *Lariophagus distinguendus* in odor fields of a static four-chamber-olfactometer. F: odor fields above Petri dishes with feces of *R. dominica* (A) or *S. granarius* (B); C: fields above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman's ANOVA followed by Wilcoxon-Wilcox test for multiple comparisons). $N = 25$ (A) ; $N = 23$ (B).

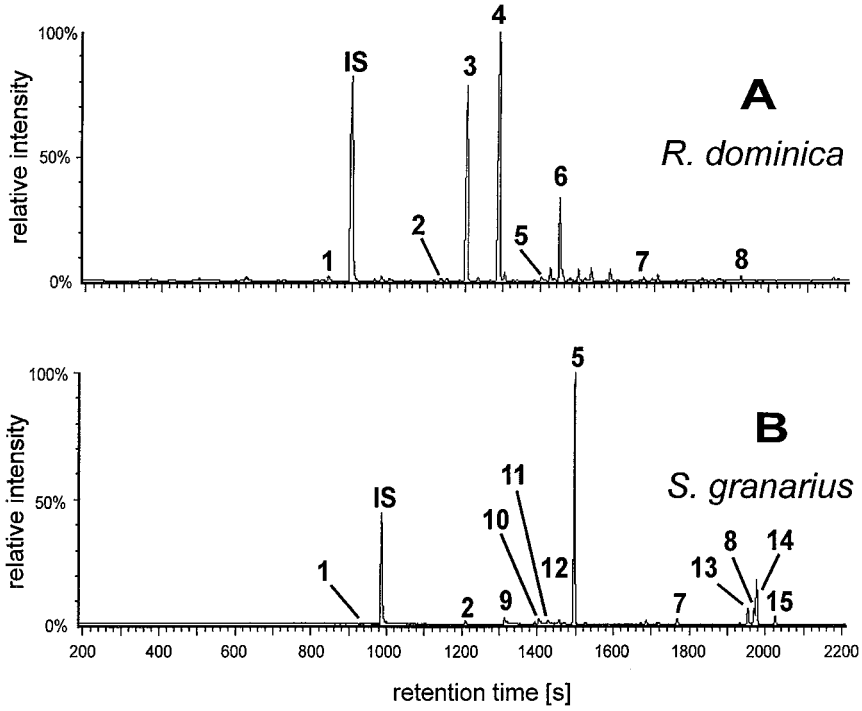

FIG. 2. Total ion current chromatograms of a CLS extract from larval feces of *R. dominica* (A) and *S. granarius* (B). Peaks with the same number were identified as being the same compounds (see text) based on linear retention indices (van den Dool and Kratz, 1963) and mass spectra. Retention times differ slightly due to different column length.

the mite *Tyrophagus putrescentiae* Schrank, which is living in our *S. granarius* cultures (Ruther and Steidle, 2000). In addition to the chemicals mentioned, feces headspace of both species contained a series of unidentified minor compounds.

In experiments using synthetic compounds (Figure 3), a mixture of dominicalure 1 and 2 was attractive to the parasitoids. A mixture of the synthetic mite chemicals 5, 9, 10, and 11 had no effect on parasitoid behavior. With sitophilate, enter frequency between fields was not different for either concentration tested using the Friedman test. At a dose of 100 ng sitophilate on the filter paper, there also was no significant attraction ($P < 0.07$, Wilcoxon-matched Pairs-test).

DISCUSSION

Fecal volatiles of the hosts *R. dominica* and *S. granarius*, both feeding on wheat grains, are attractive to *L. distinguendus* females. This result supplements

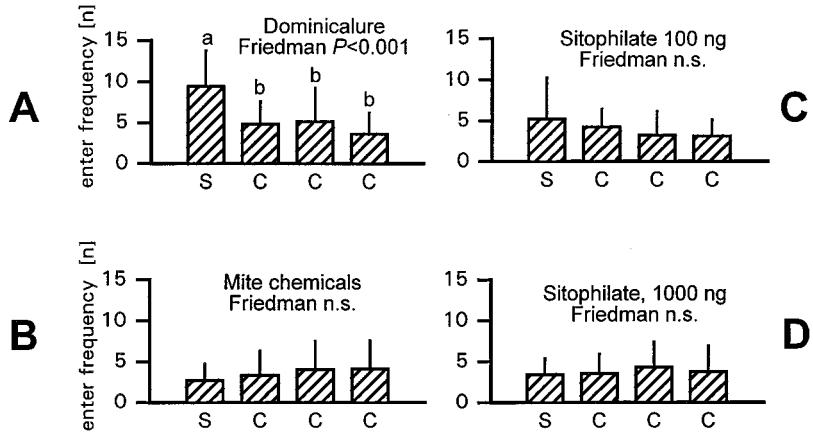

FIG. 3. Mean enter frequency (±SD; $N = 25$) of naive females of *Lariophagus distinguendus* into odor fields of a static four-chamber-olfactometer with a mixture of dominicalure 1 and 2 (A), synthetic mite chemicals (B), sitophilate (100 ng) (C), and sitophilate (1000 ng) (D). S: odor fields above Petri dishes with synthetic chemicals. C: fields above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman's ANOVA followed by Wilcoxon-Wilcox test for multiple comparisons).

earlier work revealing that fecal volatiles from these hosts have an arresting effect (Steidle and Schöller, 1997; Steidle et al., 2001). Obviously *L. distinguendus* females locate host-infested wheat grains within healthy grains by orienting towards volatiles from the host feces that cover infested grains from the outside.

To test whether *L. distinguendus* uses specific or general chemical cues for host finding, the volatile compounds identified in the headspace of feces from *R. dominica* and *S. granarius* have to be categorized as specific or general. The main compounds emanating from the feces of *R. dominica* were identified as dominicalure 1 and 2, the species-specific male-released aggregation pheromones of *R. dominica* (Williams et al., 1981). Both compounds are potential host-finding cues with high specificity. The presence of the adult pheromone in the larval feces is due to the fact that under our rearing conditions adults are never completely removed from cultures with larvae and live within the larval feces. Because *R. dominica* under natural conditions forms aggregations consisting of adults and larvae, the occurrence of dominicalure in the odor from larval feces certainly is a natural phenomenon. Potential host-finding cues specific for *S. granarius* have not been identified in this study but might be found among the hitherto unidentified trace compounds. One potential specific cue is sitophilate, the aggregation pheromone from *S. granarius* males (Phillips et al., 1989), which, like *R. dominica*, live in aggregations together with females and larvae. Fecal volatiles from both hosts contained small amounts of several alkanes, ranging from undecane

to pentadecane, some of which have been also reported as odor compounds from wheat grain (Seitz, 1995; Seitz et al., 1997). This indicates that these alkanes most likely originate from the wheat and do not represent reliable host finding cues for *L. distinguendus*. In agreement with former results (Ruther and Steidle, 2000), chemical analysis of fecal volatiles from *S. granarius* revealed the presence of terpenoids, large amounts of tridecane, and some C_{15} hydrocarbons. These compounds originate from the mite *T. putrescentiae* (Kuwahara et al., 1975, 1979; My-Yen et al., 1980) that lives associated with *S. granarius* in infested grains. Feces from mite-free *S. granarius* cultures do not contain these chemicals (Ruther, unpublished data). Being secondary pests depending on the presence of primary pests (Stein, 1986), *T. putrescentiae* and other astigmatid mites co-occur with *S. granarius* and other hosts of *L. distinguendus* like *R. dominica* in storage facilities (e.g., Sinha, 1961; Hunter et al., 1973). Therefore, the mite chemicals can be considered general host-finding cues, indicating the presence of different *L. distinguendus* hosts.

In bioassays, the attractive effect of synthetic dominicalure, as a specific cue, and also some synthetic mite compounds, representative of general cues, were tested on naïve *L. distinguendus* females. Mite chemicals had no effect and consequently were not innately used by *L. distinguendus* females for host finding. Possibly, mite pheromones are too general and not reliable enough, since astigmatid mites not only co-occur with hosts but also with nonhost species (Sinha, 1961; Sinha and Wallace, 1966; Hunter et al., 1973). Therefore, they are attractive to *L. distinguendus* females only after oviposition experience in their presence (Ruther and Steidle, 2000). From the specific cues tested, dominicalure was attractive but no significant attractiveness was found for sitophilate. Further studies to identify innate host finding cues in *S. granarius* feces are presently being carried out.

The reaction towards dominicalure must have been innate, since *L. distinguendus* females tested were reared on *S. granarius* larvae and never had contact with the pheromone releasing adult males of *R. dominica*. Thus, in contrast to the initial hypothesis, we did not find an indication for the innate use of general volatile cues but rather for the innate use of the specific dominicalure by the generalist *L. distinguendus*. This agrees with studies from at least five other generalist natural enemies, for which the innate use of specific cues has been reported before (Table 1). Altogether, these findings fail to support the initial hypothesis that generalist carnivores do not innately use specific cues (Vet and Dicke, 1992). It should be noted, however, that this does affect the idea that generalists exploit general cues. In fact, for host recognition it was found that *L. distinguendus* uses general nonvolatile compounds present in the feces of some host complexes (Steidle et al., 2001a).

With respect to *L. distinguendus*, the reaction to dominicalure could be explained by the fact that a limited use of specific cues might be possible for generalists. Assuming an average of two specific pheromone compounds for each of the

TABLE 1. EVIDENCE FOR INNATE USE OF SPECIFIC CUES BY GENERALIST CARNIVORES

Carnivore	Evidence for innate use of specific cues	Reference
Trichogramma evanescens Westwood (Trichogrammatidae)	Innately attracted by pheromone of *Mamestra brassicae* L.	Noldus et al. (1991)
Amblyseius cucumeris (Oudemans) (Phytoseiidae)	Innately attracted by pheromone of *Frankliniella occidentalis* (Pergande)	Teerling et al. (1993)
Coccinella septempunctata L. (Coccinelidae)	Specific sensory cells for the aphid alarm pheromone (*E*)-β-farnesene	Al Abassi et al. (2000)
Orius tristicolor White (Anthocoridae)	Innately attracted by specific cues from *F. occidentalis*	van Laerhoven et al. (2000)
Chrysoperla carnea Stephens (Chrysopidae)	Innately attracted to pheromone blend of *Plutella xylostella* L.	Reddy et al. (2002)

11 hosts, *L. distinguendus* would have to be able to exploit about 22 compounds. Although it is unclear how many different specific compounds can be perceived and processed, the use of 22 compounds might be physiologically possible. However, our experiments with pheromones from *S. granarius* (this study), as well as with pheromones from other *L. distinguendus* hosts (Steidle et al., 2002), did not reveal any significant attraction. Therefore, the most likely explanation to us is that *L. distinguendus* originally was a specialist on *R. dominica*, responding to the specific pheromone of this host, and became a generalist only later by expanding its host range. This might have happened after *L. distinguendus* entered the storage environment. Apart from the other species' suitability as hosts, the host range expansion could have been enabled by the agreement of old and new host complexes in host recognition cues, and in their ecological niches as seed feeders (Steidle et al., 2001a). Unfortunately, nothing is known about the origin and the natural habitat of *L. distinguendus*, which is now cosmopolitic and synantropic (Graham, 1969). Studies to identify the original hosts failed (Kaschef, 1964).

In summary, it remains unclear how the use of specific cues by the generalist *L. distinguendus* can be explained. To answer this question, more studies with cues from other hosts are necessary to determine if specific cues from only one host or from several host species are innately used. Further research with other generalist species is needed to answer the question whether the use of specific cues by generalists is a general phenomenon, thereby promoting our knowledge on the innate use of infochemicals by natural enemies in general.

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INDUCTION OF VOLATILE EMISSIONS IN MAIZE BY DIFFERENT LARVAL INSTARS OF *Spodoptera littoralis*

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Abstract—Maize plants under attack by caterpillars emit a specific blend of volatiles that is highly attractive to parasitic wasps. The release of these signals is induced by elicitors in the caterpillar regurgitant. Studies suggest that plants respond differently to different herbivore species and even to different herbivore stages, thus providing parasitoids and predators with specific signals. We tested if this is the case for different larval instars of the noctuid moth *Spodoptera littoralis* when they feed on maize plants. Cut maize plants were incubated in diluted regurgitant from second, third, or fifth instar caterpillars. There were no differences in total amount released after these treatments, but there were small differences in the release of the minor compounds phenethyl acetate and α-humulene. Regurgitant of all three instars contained the elicitor volicitin. To test the effect of actual feeding by the larvae, potted plants were infested with caterpillars of one of the three instars, and volatiles were collected the following day. The intensity of the emissions was correlated with the number of larvae feeding on a plant, and with the amount of damage inflicted, but was independent of the instar that caused the damage. We also used artificial damage to mimic the manner of feeding of each instar to test the importance of physical aspects of damages for the odor emission. The emission was highly variable, but no differences were found among the different types of damage. In olfactometer tests, *Microplitis rufiventris*, a parasitoid that can only successfully parasitize second and early third instar *S. littoralis*, did not differentiate among the odors of maize plants attacked by different instar larvae. The odor analyses as well

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as the parasitoid's responses indicate that maize odors induced by *S. littoralis* provide parasitoids with poor information on the larval developmental stage. We discuss the results in the context of variability and lack of specificity in odorous plant signals.

Key Words—larval instar, induced plant volatiles, specificity-reliability, 6-arm olfactometer, *Zea mays*, *Spodoptera littoralis*, *Microplitis rufiventris*.

INTRODUCTION

Plants subjected to feeding damage by insects respond with the release of characteristic blends of volatiles that attract parasitoids and predators (Dicke and Sabelis, 1988; Dicke et al., 1990; Turlings et al., 1990; Steinberg et al., 1992; McCall et al., 1993; Agelopoulos and Keller, 1994; Mattiacci et al., 1994; Röse et al., 1996; Du et al., 1998). These releases by attacked plants are triggered by elicitors in oral secretions of the herbivores (Dicke et al., 1993; Turlings et al., 1993a; Mattiacci et al., 1994). In the regurgitant of the caterpillar *Pieris brassicae*, the main elicitor was identified as the enzyme β-glucosidase (Mattiacci et al., 1994). Alborn et al. (1997, 2000) identified a nonprotein elicitor from the regurgitant of *Spodoptera exigua* (Lepidoptera: Noctuidae), *N*-(17-hydroxylinolenoyl)-L-glutamine, named volicitin. In maize plants, volicitin triggers a response similar to that triggered by *S. exigua* feeding. The induced odor is composed mainly of terpenoids and is highly attractive to the braconid parasitoids *Cotesia marginiventris* (Hymenoptera: Braconidae) and *Microplitis croceipes* (Hymenoptera: Braconidae) (Alborn et al., 1997; Turlings et al., 2000).

Interestingly, *M. croceipes* cannot parasitize *S. exigua* larvae, but is nevertheless highly attracted to maize odors induced by this nonhost (McCall et al., 1993; Turlings et al., 1993a). This potential limitation to the reliability of herbivore-induced plant signals has been discussed by Vet and Dicke (1992). They argue that the large quantities of the plant-provided cues make them highly detectable, but they may be poor indicators of herbivore identity. However, some recent studies suggest that plant signals can be herbivore specific. De Moraes et al. (1998) demonstrate that the parasitoid *Cardiochiles (Toxoneuron) nigriceps* (Hymenoptera: Braconidae) can distinguish odors from plants damaged by its specific host from odors induced by a nonhost. Differences in the attractiveness to the wasps were mainly ascribed to quantitative differences in a few compounds released by the plants. Guerrieri et al. (1999) showed that different aphid species elicit different emissions in bean plants and that the aphid parasitoid, *Aphidius ervi* (Hymenoptera: Braconidae: Aphidiinae), can use these to distinguish plants infested by its host, *Acyrthosiphon pisum* (Homoptera: Aphididae), from those infested by a nonhost, *Aphis fabae* (Homoptera: Aphididae). These studies suggest that herbivores can induce specific signals in plants that give information on the identity of potential hosts for parasitoids, but in some other systems this appears not to be the

case (McCall et al., 1993; Turlings et al., 1993a). Another intriguing example of specificity is that reported by Takabayashi et al. (1995) who showed that the parasitic wasp *Cotesia kariyai* (Hymenoptera: Braconidae) can differentiate between maize plants under attack by young instars and late instars of *Pseudaletia separata* (Lepidoptera: Noctuidae). In contrast, in the cabbage system, composed of cabbage plants–*Pieris brassicae* (Lepidoptera: Pieridae)–*Cotesia glomerata* (Hymenoptera: Braconidae), the parasitoid does not discriminate between plants infested by first and fifth instars (Mattiacci and Dicke, 1995). This was surprising because chemical analyses of collected cabbage odors suggested that the host instars differed in the odor they induced in the plant. The specificity of plant responses seems to differ for different systems, and the reliability of the information provided by the chemical signals varies accordingly.

In the current study, we tested if, in the tritrophic system comprising maize plants, *S. littoralis* (Boisd.) caterpillars, and the endoparasitoid *M. rufiventris* (Kok.),the induced odor differs with the developmental stage of the herbivore on the plant and whether such differences are detected and used by the wasp, which can only attack early instars. For this purpose, volatiles emitted by maize plants that were subjected to various treatments were collected and analyzed. Plants were incubated in the regurgitant of second, third, and fifth instars. They were also subjected to actual feeding damage by these three instars. Finally, artificial damage was used to mimic the damage caused by the instars. We also tested if *M. rufiventris* distinguished among the odors from plants attacked by different instars. The amount of damage was the main factor that determined the intensity of the odor emissions and their attractiveness. No major differences were found in the identities and relatives ratios of the various compounds. The results indicate that in this system odor emitted by the maize plant provides no or poor information on herbivore instar.

METHODS AND MATERIALS

Plants. Maize of the variety Delprim was used. Seeds were sown in individual plastic pots (360 ml, 10 cm diam. 7 cm high) filled with fertilized soil (Coop, Switzerland). Plants were grown in a climate chamber (type 10'US/+5 DU-PI, Weiss Umwelttechnik, Switzerland) at 23°C, 60% relative humidity, 45,000 lux, and under a 16L:8D light regime. Plants 9–10 days old were used for all experiments, at which age they carry 3–4 leaves.

Insects. S. littoralis larvae and eggs were received weekly from Syngenta (Stein, Switzerland). Batches of eggs were placed on moist filter paper in a Petri dish. Newly hatched larvae were put on maize leaves (var. Delprim), in transparent plastic boxes (13.5 × 15 × 5 cm). Larvae of second, third, and fifth instars were used. Regurgitant was collected as described by Turlings et al. (1993a).

A colony of the parasitoid *M. rufiventris* was started with cocoons provided by Dr. E. Hegazi (University of Alexandria, Alexandria, Egypt). The colony was maintained on *S. littoralis* larvae fed with artificial diet. At emergence, adults were sexed and kept in plastic cages (30 × 30 × 30 cm, Bugdorm I, MegaView Science Education Service Co, Ltd, Taiwan) under laboratory conditions (25 ± 3°C, 40% relative humidity). Insects were provided with honey, and cages were sprayed with water daily to compensate for relatively dry lab conditions.

Collection and Analysis of Induced Odor. Two systems were used to collect induced odors from plants. The first system was an all glass push–pull odor collection system modified from Turlings et al. (1991). It consists of three cylindrical Pyrex glass pieces. The first tube (14 cm) contains a glass frit, which ensures laminar airflow into the second tube. It ends with a 6-cm male ground-glass joint that is connected to a female counterpart of the second tube (29 × 7 cm). This second tube ends in a female ground glass joint (3 cm) that fits the inlet of the third tube, which tapers down and ends in a glass screw fitting. An open screw cap with a Teflon-covered rubber ferrule (6 mm ID) connects a collection trap at its upwind end on the third glass tube, while the downwind end of the filter is connected with plastic tubing to flowmeters (5-channel Adjustable Vacuum Flow Volatile Collection System, model VCS-5ASP-MAN, Analytical Research Systems, Gainesville, Florida, USA), which are connected to a vacuum pump. Humidified and purified air is pushed and pulled through the glass tubes at a rate of 600 ml/min. Collection traps were made as described by Heath and Manukian (1994). Before each collection, filters were rinsed with 500 μl of pentane, followed by 500 μl of methylene chloride.

The second system was designed to collect from growing plants (Turlings et al., 1998). The aerial part of a plant was placed in a vertical cylinder (9.5 × 54 cm), while the pot was placed outside, separated from the rest of the plant by a Teflon disk (Turlings et al., 1998). Purified and humidified air was pushed into each cylinder at a rate of 1 liter/min. For collections, air was pulled through a trap held at the base of the cylinder, at a rate of 0.8 liters/min, while the rest of the air vented through the hole in the bottom, thus preventing outside, impure air from entering. The automated part of the collection system (Analytical Research Systems) controlled the flow. The climate chamber (CMP4030, Conviron, Winnipeg, Canada) in which the collection cylinders were housed was kept at 17.5°C. Due to irradiation heat, the temperature inside the cylinders was 23 ± 3°C. During the light cycle, light intensity was about 20,000 lm/m^2.

After each collection, filters were extracted with 150 μl of methylene chloride (Lichrosolv, Merck), and 200 ng of *n*-octane and nonyl acetate (Sigma) in 10 μl methylene chloride were added to the samples as internal standards.

Analyses were done with an Hewlett Packard HP 6890 series GC equipped with an automated on-column injection system (HP G1513 A) and a flame ionization detector. A 3-μl aliquot was injected onto an apolar EC-1 capillary column

(30 m × 0.25 mm ID, 0.25-μm film thickness, Alltech Associates) preceded by a deactivated retention gap (10 m × 0.25 mm ID, Connex) and a deactivated precolumn (30 cm × 0.530 mm ID, Connex). Helium (24 cm/sec) was used as carrier gas. Following injection, the column temperature was maintained at 50°C for 3 min, increased to 230°C at 8°C/ min, and held at 230°C for 9.5 min. The detector signal was processed with HP GC Chemstation software. Compound identities had been determined in previous studies (Turlings et al., 1998; Gouinguené et al., 2001).

Effect of Regurgitant on Induction of Odor. Oral secretions of second, third, and fifth instars were tested. Three plants were used. The cut stem of each seedling was incubated in a solution of regurgitant (10% in distilled water) during one night (14–15 hr). Controls were incubated in distilled water only. Before collection, the part of the stem that had been submerged in the solution was cut off, and the fresh cut was wrapped in a piece of moist cotton to avoid desiccation. Three plants that were treated with the same regurgitant solution were placed together in a glass tube for odor collection. The experiment was replicated 13 times. Total amounts as well as the composition of the induced odor blend emitted by the plants treated with different regurgitants were compared.

Compositions of the three instars were compared to detect any variation in elicitor quality or quantity. Regurgitant samples were collected as described above. To denature enzymes and to eliminate bacterial degradation, each sample of oral secretion was diluted with an equal amount of acetonitrile immediately after collection. Samples were centrifuged at 14,000 rpm (Eppendorf Centrifuge 5415) to remove solids, and the supernatant was filtered through 0.45- and 0.22-μm sterilizing membranes (Millex-HV and Millex-GV, Millipore Bedford, Massachusetts, USA). For quantitative analyses, 5 μl of the internal standard *N*-palmitoleoyl-L-glutamine solution (1 μg/μl) in CH_3CN/H_2O (8:2 v/v) were added to each sample (50 μl) as an internal standard. Ten microliters of each sample were analyzed by HPLC with UV detection at 200 nm (constaMetric 4100 pump, SpectroMonitor 3200 detector, Spectra System AS 3500 autosampler, Thermo Separation Products, Riviera Beach, Florida, USA). A reverse-phase column (YMC-Pack ODS-AMQ, 250 × 4.6 mm ID, YMC, Kyoto, Japan) was eluted (1 ml/min) with a solvent gradient of 20–95% CH_3CN (High Purity Solvent, Burdick & Jackson, Muskegon, Mississipi, USA) containing 0.8% acetic acid (Aldrich, Milwaukee, Wisconsin, USA), in water (Milli-Q UV PLUS system, Millipore) containing 0.5% acetic acid, over 40 min, and then returned to the initial conditions at 45 min. Column temperature was maintained at 60°C. The detector response to the internal standard was used to calculate the amounts of *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin), *N*-(17-hydroxylinoleoyl)glutamine, 17-hydroxylinolenic acid, 17-hydroxylinoleic acid, *N*-linolenoyl-L-glutamine, *N*-linoleoyl glutamine, linolenic acid, and linoleic acid. These were isolated and identified earlier in beet armyworm (*S. exigua*) oral secretions (Alborn et al., 2000),

and the structures confirmed by using methods described in Mori et al. (2001).

Effect of Feeding by Second, Third, and Fifth Instars. To test if larvae of different instars induced different volatiles, we collected odor from plants attacked by different numbers of larvae. For second instars, either 1, 5, 10, 25, 50, or 70 larvae were placed on one plant. For third instars, the numbers were 1, 5, 10, 20, or 50 larvae. These numbers were chosen to obtain comparable amounts of damage. For the fifth instars, the densities were lower. Due to the size and feeding rate of the larvae, only 1, 2, 3, 4, 5, or 10 larvae were put on a plant. A cellulose bag (Celloclair, Liestal, Switzerland) was placed over each plant to prevent escape of caterpillars. Larvae were placed on the plants in the evening and were allowed to feed for 15 hr. They were removed just before an odor collection. After collection, the amount of damage on plants was estimated visually and expressed as the percentage of leaf surface removed.

Effect of Different Types of Damage. The damage caused by the different instars of *S. littoralis* is different. Young instars graze the surface of the leaf, creating "windows" and leaving most of the veins intact, while late instars remove all parts of a leaf. Intermediate instars combine both types of damage: they partly graze on the leaves and remove small parts of tissue. To determine if the different types of damage are correlated with differences in odor emissions, we mimicked the caterpillar damage. To mimic second-instar damage, the surface of the leaf was removed with a razor blade in order to leave the veins intact. In each case, 2 cm^2 were damaged per leaf. To mimic third-instar damage, the same manipulation was done, and the leaves were cut in some places. Again, 2 cm^2 were damaged per leaf. For fifth-instar damage, 10 holes were punched per leaf. Holes were 4 mm in diameter, which corresponds to a surface of 5 cm^2 per leaf, but the damaged area remaining was considerably less. For all treatments, 10 μl of caterpillar regurgitant were applied on the damaged area. Two plants were used per treatment. Plants were treated in the evening (18:00 hr), and kept in the dark until collection (09:30 hr the next morning). This was replicated nine times.

Does M. rufiventris Distinguish among Plants Fed on by Different Instars of S. littoralis? Choice tests were conducted in a six-arm olfactometer. It consists of an airtight three-part system of glass chambers interconnected by Teflon tubes. On the bottom, six glass chambers each contain a small growing plant, and purified and humidified air enters these chambers. Air passes over the plants and is pushed to the upper part of the system, where it enters the arms of the olfactometer. Half the air is pulled out at the upper part of the chamber through volatile collection traps (Heath and Manukian, 1994), which allows for trapping part of the volatiles during each bioassay. The upper part consists of a six-arm glass star, and each arm is connected with a Teflon tube to an odor source chamber from the bottom part. Thus, the air from each chamber passes through one arm to the central chamber of

the star. This central chamber is a glass cylinder that extends into the middle part of the system where the wasps are released. At the bottom, just below the release point, the remaining air is pulled out of the system. Wasps that are released in the chamber readily walk up in the direction of a light source that is placed above the olfactometer. Once they have reached this upper part they can make a "choice" for an odor by walking into one of the arms.

As odor sources, three undamaged plants were alternated with plants fed on by each instar of *S. littoralis* caterpillars in the six bottom chambers. The maximum emission of odor occurred at about 60% damage on a plant, independent of the instar. This amount of damage was correlated to a corresponding number of larvae for each instar tested. Based on this, plants were fed on by either 60 second instars, 30 third instars, or 3 fifth instars for 15 hr. Odor emissions from plants fed on by the different instars were collected for 3 hr. Four groups of six female parasitoids were tested each day. One group of six was naive females, while the other trials were conducted with six female wasps that had had an oviposition experience with plants that had been damaged by either second, third, or fifth instars overnight. During such experiences, female parasitoids learn to associate the odor they encounter with the presence of hosts and become more responsive to this odor (Lewis and Tumlinson, 1988; Turlings et al., 1993b; Vet et al., 1995). If the odors differ among the three instars, this is reflected in the responses of the different experience groups. The position of the plants remained the same on a particular day to avoid an effect of odor contamination in the arms, but the position of treated plants and control plants was changed between days of experiments. The day before each replicate, the system was thoroughly washed, rinsed with solvents, and the glass parts placed in an oven at 250°C for several hours. Experiments were conducted on 10 different days (60 wasps per experience type).

Statistical Analyses. The total amount of induced odor released was compared using one-way ANOVA. Data were ln-transformed to comply with ANOVA assumptions. The Student-Newman-Keuls test was used as the *post hoc* test for multiple comparison. Comparison of the amounts of the 20 dominant compounds was done using a multivariate ANOVA. The Dunnett T3 *post hoc* test was used for multiple comparisons.

Quadratic regressions on ln-transformed data were performed to test for the effect of the quantity of damage on the release of induced volatiles. The analysis was done for each instar separately. Confidence intervals of equation coefficients were calculated and compared. For comparison of the odor blends induced by the three instars, data were grouped in five damage classes. Multivariate analysis of variance was performed to compare the effect of instar and of the class of damage on the release of six dominant compounds. Dunnett T3 *post hoc* test was used for multiple comparison.

Chi-square analysis was performed to test for differences among proportions of wasps that choose the different odors they were offered.

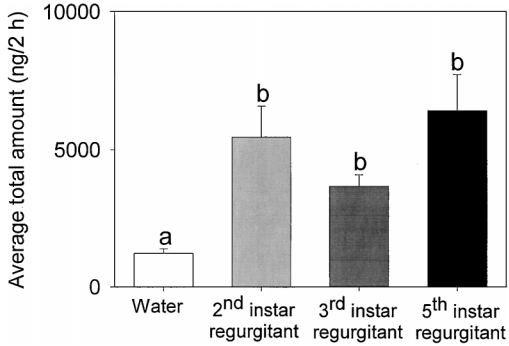

FIG. 1. Average total amount + SE of induced odor emitted by maize seedlings after incubation in a solution with regurgitant of a specific instar. Incubation in water was used as control. Different letters above each bar indicate differences among treatments according to Student-Newman-Keuls *post hoc* test after one-way ANOVA ($F = 16.138$; $P < 0.001$)

RESULTS

Effect of Regurgitant on Odor Induction. No significant differences were found in the total amount of induced volatiles emitted by maize plants incubated in regurgitant solutions from second, third, and fifth instars of *S. littoralis* (Figure 1). As expected, plants incubated only in water released significantly less compared to plants incubated in regurgitant solutions (Figure 1).

Composition of the odor blend induced by the different regurgitants was not significantly different, except for the compounds phenethyl acetate and α-humulene, which were released in higher relative proportions by plants incubated in fifth-instar regurgitant solution as compared to other treatments (Figure 2).

Analysis of the regurgitant of the three instars tested showed that all contained volicitin. For all instars, linolenic acid dominated the composition of the regurgitant. The ratios of the different compounds present varied among the three instars (Figure 3). Only one sample of second-instar regurgitant was available, so no statistical analysis was performed, but any possible difference among the three instars is not dramatic, and all instars produced volicitin.

Effect of Feeding by Second, Third, and Fifth Instars on Induced Emissions. The percentage of damage done to maize plants was correlated with the number of larvae feeding. Not surprisingly, the relationship between the quantity of damage and the number of larvae differed for the different instars (Figure 4). For each instar, the amount of volatiles released and the amount of damage inflicted closely fit a quadratic relationship (Figure 4). The maximum emission of induced odor occurred in plants from which about 60% of the surface had been removed (Figure 4). The confidence intervals for each coefficient of the three equations overlapped

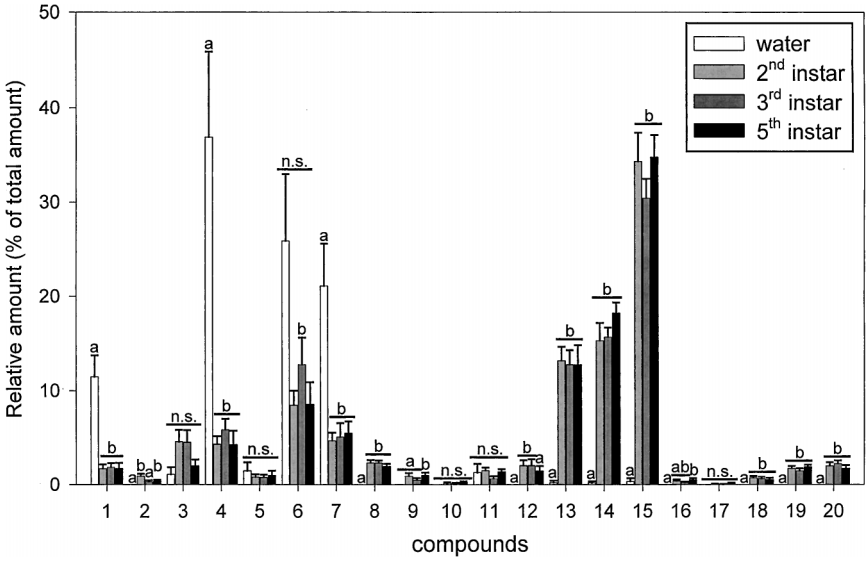

FIG. 2. Average relative amount + SE of the main compounds released by maize seedlings after incubation in a solution with regurgitant of a specific larval instar. Incubation in water was used as control. Different letters above bars indicate significant differences among treatments for the different compounds according to Student-Newman-Keuls test. Peak identities: (1) (*Z*)-3-hexenal, (2) (*E*)-2-hexenal, (3) (*Z*)-3-hexen-1-ol, (4) (*E*)-2-hexen-1-ol, (5) β-myrcene, (6) (*Z*)-3-hexen-1-yl acetate, (7) linalool, (8) (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (9) phenethyl acetate, (10) indole, (11) geranyl acetate (12) unknown, (13) β-caryophyllene, (14) (*E*)-α-bergamotene, (15) (*E*)-β-farnesene, (16) α-humulene, (17) unknown sesquiterpene, (18) β-bisabolene + (*E*, *E*)-α-farnesene, (19) β-sesquiphellandrene, (20) (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

almost completely, which means that the amount of induced volatiles released after caterpillar feeding did not differ among the three instars.

The quality of the odor blend also depended on the amount of damage done by each instar (Figure 5). There were no obvious differences among the three instars tested. Two sesquiterpenes, α-bergamotene and (*E*)-β-farnesene, were dominant in the odor blend for all instars. Figure 5 illustrates the ratios of the main compounds in the induced blend for five classes of damage and for the three instars. Multivariate analysis of variance indicated that the instar had no effect on the amount of the different induced volatiles ($F = 1.164$, $P = 0.279$), while the classes of damage had an effect ($F = 1.676$, $P = 0.004$), and the intercept between the instar and the classes was not significant ($F = 0.896$, $P = 0.755$). Among the compounds, linalool, indole, β-caryophyllene, α-bergamotene, and (*E*)-β-farnesene were released in significantly different amounts among the different

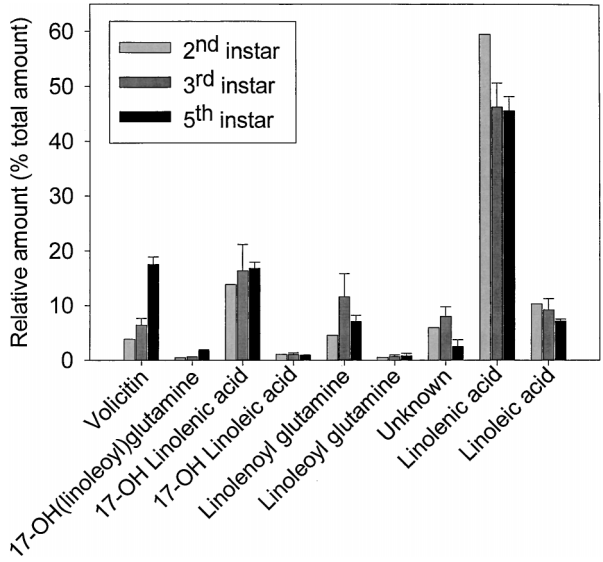

FIG. 3. Relative amount (mean + SE) of the different components present in the regurgitant of 2nd, 3rd, and 5th instars of *S. littoralis*.

classes of damage. The relative amount of linalool decreased when the amount of damage increased, except between 40 and 60% of damage where plants damaged by second instars emitted a large amount of linalool. The proportion of indole increased in the odor blend with the amount of damage. The proportion of β-caryophyllene in the induced odor blend increased slightly with the amount of damage (Figure 5). About 20% of the induced odor was composed of α-bergamotene, which represented a slightly higher proportion when 40–60 % of a plant was damaged. The same trend was observed for (E)-β-farnesene, which was the main substance in the induced blend (about 40%, Figure 5).

Effect of Different Types of Damage. Total amounts of induced volatiles for the three types of damage were not significantly different from each other, but were different from the undamaged plant (Figure 6). Multivariate analysis of variance indicated that the type of damage also had no effect on the composition of the blend (Figure 7, $F = 1.728$, $P = 0.923$).

Does M. rufiventris Distinguish among Plants Fed on by Different Instars? Female parasitoids significantly preferred the odor of plants that were fed on by larvae to undamaged plants; on average 87% of the females made a choice for the odor of damaged plants. When females had no previous experience or had experience with odors of plants fed on by third or fifth instars, no preferences were found in their choices (Figure 8A, C, D). Surprisingly, female wasps

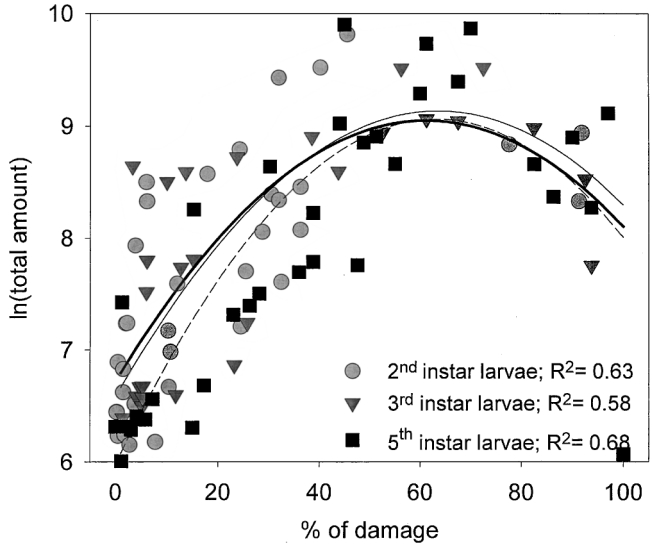

FIG. 4. Total amount of induced volatiles emitted by second, third, and fifth instars (ln-transformed data) versus percentage of damage on plants. Second instar: $F = 28.5864$, $P < 0.001$ (grey line); third instar: $F = 16.685$, $P < 0.001$ (black line); fifth instar: $F = 33.879$, $P < 0.001$ (dashed black line).

experienced with odors of plants fed on by second instars showed a slight but significant preference for the odor of plants fed on by third instars (Figure 8B). Interestingly, fewer females chose the odor of plants attacked by fifth instars, but this trend is likely due to the lower quantities of volatiles emitted by these plants (Figure 9), which showed less damage than plants eaten by second and third instar larvae.

DISCUSSION

In all cases, the three different instars induced an emission of volatiles in maize. Regurgitant of the three instars induced similar volatile blends, both in terms of quantity and quality. The only difference was a higher emission of phenethyl acetate and α-humulene (two of the minor compounds) when plants were incubated in fifth instar regurgitant (Figure 2). The composition of regurgitants of the three different instars tested showed no major differences (Figure 3), and all three instars produce the known elicitor volicitin (Alborn et al., 1997).

The total amount of volatiles released was correlated with the amount of damage done by the larvae. No differences were found among instars when comparing plants with equal amounts of damage. This was confirmed by mimicking

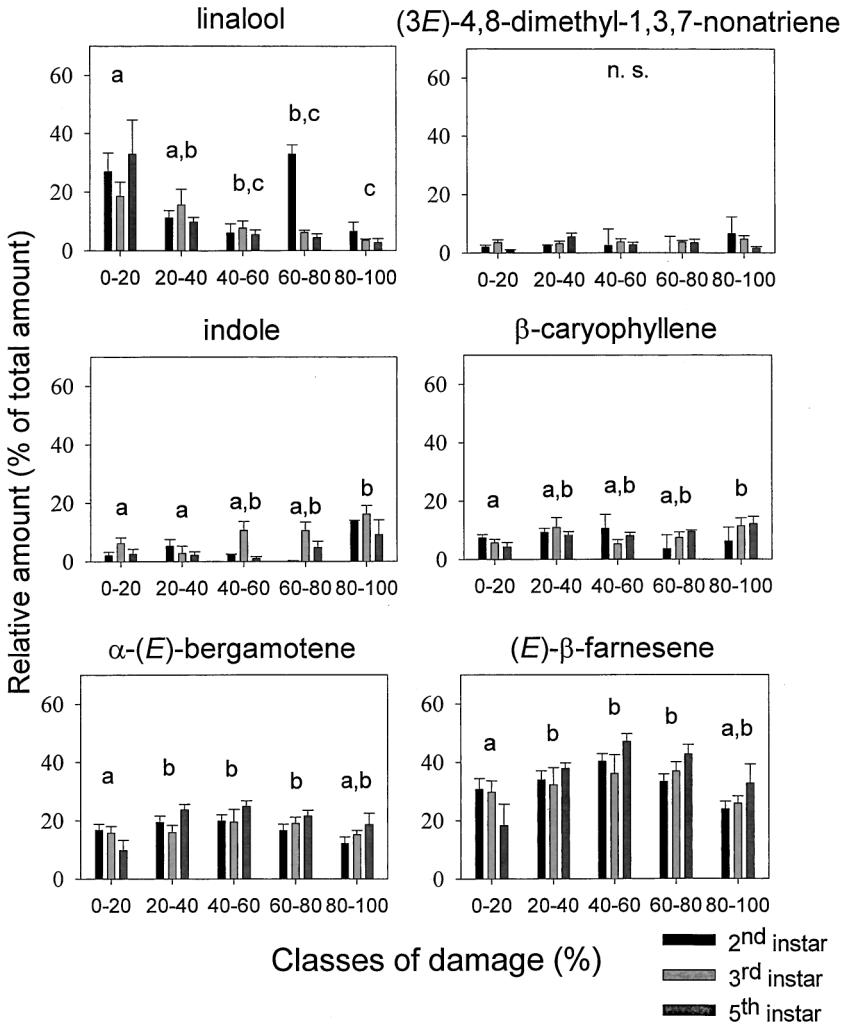

FIG. 5. Average relative amount of the six main induced volatiles (mean + SE) emitted for five classes of damage done on plants. Different letters above bars indicate significant differences between classes of damage ($\alpha = 0.05$) after Dunnett's T3 *post hoc* test.

the damage done by the different developmental stages. The type of damage did not affect the emission of induced volatiles. When larvae were feeding directly on maize plants, differences in the relative amounts of the six dominant compounds were due to the amount of damage inflicted on plants. Females of the parasitoid *M. rufiventris* did not show differences in their preference for odor induced by the

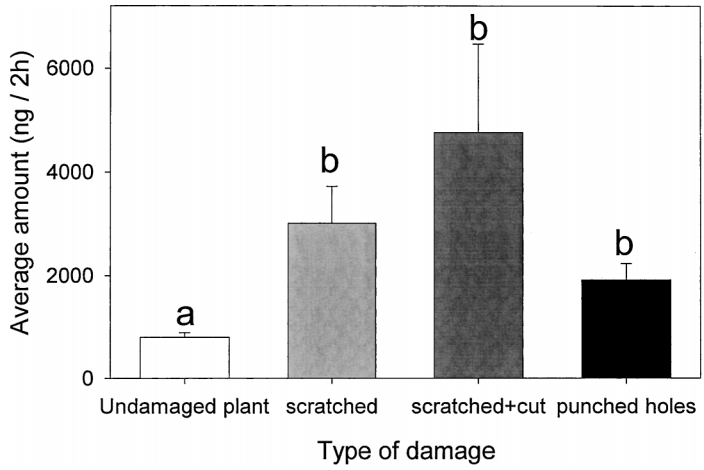

FIG. 6. Average total amount (mean + SE) of induced odor emitted by maize plants after three different types of damage. Undamaged plants served as controls. Different letters above each bar indicate significant differences after Student-Newman-Keuls *post hoc* test ($\alpha = 0.05$)

different instars. There may be some subtle differences in the odor profiles that we may not have detected because of the limitations of the analytical methods used, but the response of the wasps does not provide any evidence for differences (Figure 8).

In contrast to the results found here, Takabayashi et al. (1995) found that different developmental stages of *P. separata* differently affected the emission of volatiles in maize and that the parasitic wasp *C. kariyai* uses these differences to locate suitable young hosts. Late instars of *P. separata* do not induce the release of volatiles, while early instars do. Takabayashi et al. (1995) suggest that the plant releases different blends of induced odor that provide the parasitoid with information on stage and suitability of the herbivore. This hypothesis was not supported in a system studied by Mattiacci and Dicke (1995). The parasitoid *C. glomerata* does not discriminate among cabbage plants infested by different instars of *Pieris brassicae* even though it also can only attack young instars. Female wasps are more responsive when they have previously encountered a suitable host and they are able to learn the surrounding odor during such an encounter (Turlings et al., 1993b; Vet et al., 1995). McCall et al. (1993) showed that *Microplitis croceipes* increases its responsiveness after encountering its host, and the effectiveness of this learning process also increases with the number of experiences the wasps had previously. Turlings et al. (1993b) described associative learning in *C. marginiventris* and how this generalist is capable, after a single experience, to distinguish among

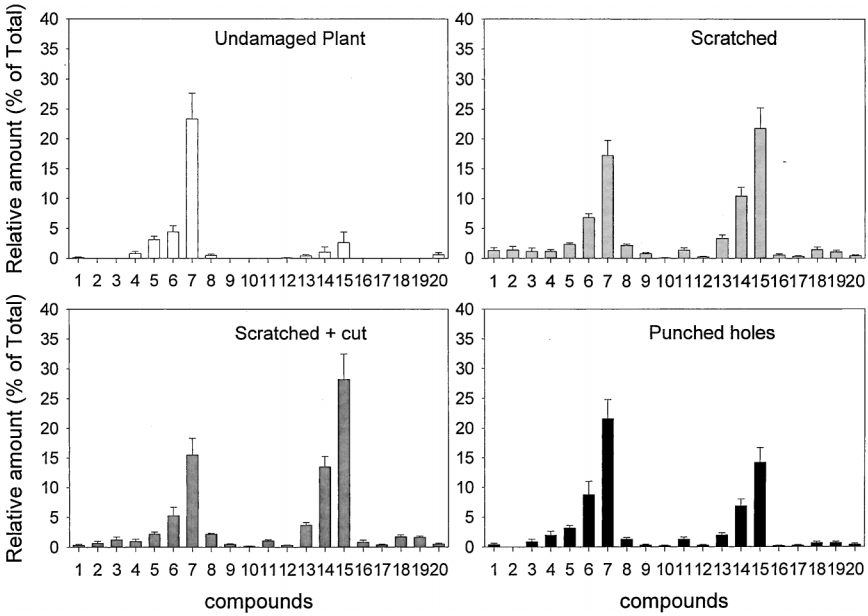

FIG. 7. Average relative amount of the different compounds of the induced odour blend (mean + SE). (1) (*Z*)-3-hexenal, (2) (*E*)-2-hexenal, (3) (*Z*)-3-hexen-1-ol, (4) (E)-2-hexen-1-ol, (5) β-myrcene, (6) (*Z*)-3-hexen-1-yl acetate, (7) linalool, (8) (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (9) phenethyl acetate, (10) indole, (11) geranyl acetate (12) unknown, (13) β-caryophyllene, (14) (*E*)-α-bergamotene, (15) (*E*)-β-farnesene, (16) α-humulene, (17) unknown sesquiterpene, (18) β-bisabolene + (*E*,*E*)-α-farnesene, (19) β-sesquiphellandrene, (20) (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

odors emitted by plants that are eaten by different host species. For *M. rufiventris*, even when female wasps had a previous experience with the odor of maize plants fed on by the different developmental stages, they did not distinguish among the induced odor of plants. The preference for plants attacked by third instars when females had an experience with the odor of maize fed on by second instars (Figure 8) also indicates that the difference among the blends is small. This can be explained by the fact that the amount of induced odor emitted in this experiment was higher for the maize plants attacked by third instars (Figure 9). The behavior of *M. rufiventris* confirmed the results obtained by analytical methods that induced maize odor does not significantly differ when different larval instars feed on the plant. Hence, the volatile signal appears limited in the specific information it provides.

The specificity of induced signal in plants has been demonstrated in several tritrophic systems. The aphid parasitoid *Aphidius ervi* can distinguish between plants fed on by its host *Acyrthosiphon pisum* and plants fed on by a nonhost

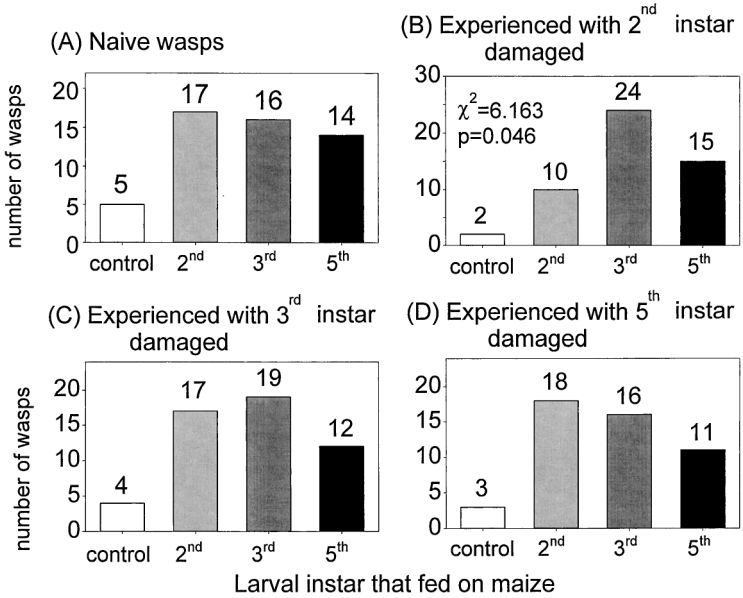

FIG. 8. The number of wasps that chose the odor of maize plants damaged by either second, third, or fifth instars, or undamaged plants. The female wasps were either naive or experienced by allowing them to oviposit in a second instar on a plant that had been damaged by either second, third, or fifth instars.

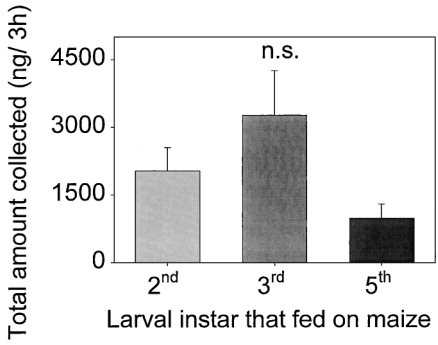

FIG. 9. Total amount of induced odor (mean + SE) released by plants fed on by second, third, and fifth instars during the 3hr of collection in the six arm olfactometer collection system. No significant differences were found among the quantity of volatiles emitted by the plants ($F = 2.793$, $P = 0.079$).

aphid. This distinction is suggested to be due to a specific compound, 6-methyl-5-hepten-2-one, of which the emission increases only when the parasitoid's host feeds on the plant (Du et al., 1998). De Moraes et al. (1998) found that the specialist parasitoid, *Cardiochiles nigriceps*, can use plant volatiles to differentiate among plants infested by its host from plants infested by a closely related non-host species. In this case, the ratios of induced volatiles in the odor blend from plants attacked by the two different noctuid hosts was somewhat different. Thus, in some systems, induced odors from plants seem to give parasitoids information on the suitability of the herbivore feeding on the plant, while in others (e.g., Mattiacci and Dicke, 1995) they do not.

Due to differences among plant species and genotypes (Gouinguené et al., 2001) as well as variation caused by abiotic factors (Gouinguené and Turlings, 2002), induced plant signals can be variable. It seems unlikely that generalist parasitoids such as *M. rufiventris* have adapted to respond to specific plant cues. Our results confirm this notion; the volatile signal induced by *S. littoralis*, a generalist herbivore, on maize does not seem to be a reliable indicator of herbivore stage. Other cues than induced plant odors might provide parasitoids with more specific information. These are likely to come from by-products of host larvae. *C. marginiventris*, for instance, is also attracted by herbivore frass and moth scale of *S. frugiperda* (Loke and Ashley, 1984). Such kairomones that come directly from the host are more reliable than plant odors, but far less detectable (Vet and Dicke, 1992). Considering the high variability in the readily available induced plants odors, even within a species (Gouinguené et al., 2001), it may be adaptive for the wasps to be flexible and exhibit specificity in their responses only after repeated experiences with rewarding odor blends.

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SALICYLIC ACID MEDIATES RESISTANCE IN THE WILLOW *Salix viminalis* AGAINST THE GALL MIDGE *Dasineura marginemtorquens*

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Abstract—Resistant willow (*Salix viminalis* L.) genotypes react with a rapid hypersensitive response (HR) when attacked by the gall midge *Dasineura marginemtorquens* Bremi. In general, infected plant genotypes or species that react hypersensitively to pathogens accumulate salicylic acid (SA) locally and systemically. Thus, pathogen-induced HRs are strongly associated with accumulation of SA. In this study, we tested the hypothesis that SA mediates resistance in *S. viminalis* against *D. marginemtorquens*. By means of HPLC, we found accumulation of SA in a resistant *S. viminalis* genotype, but not in a susceptible genotype one to two days after *D. marginemtorquens* egg hatch. To test whether this correlation between accumulation of SA and resistance is causal, we treated a normally susceptible *S. viminalis* genotype exogenously with SA and found a decreased larval survival frequency on treated shoots. Thus, the hypothesis that SA mediates resistance in the *S. viminalis/D. marginemtorquens* system was strongly supported. Interestingly, great similarity seems to exist between the biochemical signaling associated with pathogen-induced HRs and this gall-inducer induced HR.

Key Words—Induced resistance, insects, herbivory, hypersensitive response, salicylic acid.

INTRODUCTION

The existence of genotypic variation in induced resistance to plant pathogens has been known for decades. Accordingly, in plant–herbivore ecology, inducible plant resistance, resulting in reduced performance of the herbivore, is becoming a paradigm (cf. Karban and Baldwin, 1997). However, genotypic variation

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in induced plant resistance to herbivores is seldom documented (Agrawal, 2000). Furthermore, the molecular mechanisms that distinguish herbivore-resistant plant genotypes from susceptible ones are still to be sought and found in most cases. However, some studies on induction of pathogenesis-related (PR) proteins after herbivory have been successful in finding differences between resistant and susceptible plant genotypes. When infested by gall mites, resistant varieties of bittersweet nigthshade (*Solanum dulcamara* L.) react with a more rapid induction of PR proteins than susceptible varieties do (Bronner et al., 1991). Furthermore, barley and wheat cultivars that vary in resistance to the bird cherry-oat aphid (*Rhopalosiphum padi* L.) and the Russian wheat aphid (*Diuraphis noxia* Mordvilko), respectively, differ with respect to induction of PR proteins (van der Westhuizen et al., 1998; Forslund et al., 2000).

Induction of plant responses due to invading organisms is mediated through several biochemical signaling pathways. At least three pathways, mediated by jasmonic acid (JA), ethylene, and salicylic acid (SA), are known to be pathogen-induced (reviewed by Walling, 2000). However, the fact that these pathways are pathogen-induced does not exclude that they also can be induced by herbivores. Herbivorous arthropods induce one or another of these signaling pathways, depending on their mode of feeding (Karban and Baldwin, 1997; Walling, 2000). Recent data on gene expression in squash attacked by the whitefly *Bemisia argentifolii* indicate a possible novel SA/JA-independent pathway (van de Ven et al., 2000). Karban and Baldwin (1997) suggested that chewing insects primarily activate the wound-inducible JA-mediated cascade(s). This conclusion is supported by several studies (e.g., Thaler et al., 1996,1999; Thaler, 1999; Bostock et al., 2001). However, a vast array of insects inflict more subtle damage to their hosts. Phloem-feeding whiteflies and aphids, for instance, produce little injury to plant foliage and may be perceived as pathogens by the plant (Walling, 2000). Consequently, phloem-feeding insects, like pathogens, sometimes induce the SA-dependent pathway (reviewed by Walling, 2000). Thus, there seems to be no strict dichotomy between pathogen-induced and herbivore-induced pathways; but rather a more or less pronounced overlap in plant responses depending on which set of herbivore or pathogen and plant species is studied. No plant resistance manifested as reduced insect performance due to induction of the SA-dependent pathway has been reported (e.g., Inbar et al., 2001; Moran and Thompson, 2001). Even chewing insects sometimes induce accumulation of SA in their host plant. Larval feeding by the cotton bollworm (*Helicoverpa zea*) induces accumulation of foliar SA in cotton, although in this case it was concluded that SA does not mediate resistance because treatment of the cotton plants with SA does not induce resistance (Bi et al., 1997).

At the cellular level, a programmed rapid plant cell death, the hypersensitive response (HR), is a common resistance reaction to avirulent plant pathogens (Lam et al., 2001). The HR is associated with SA accumulation (reviewed by Dangl et al., 1996). McDowell and Dangl (2000) proposed a model that relates SA to the

HR, in which SA acts synergistically with reactive oxygen intermediates (ROIs) and nitric oxide (NO) to trigger HR cell death. The direct role of SA in the HR cell death remains unclear, but it seems as if SA modifies the HR cell death rather than being an elicitor of cell death (Martinez et al., 2000; Beers and McDowell, 2001; Devadas and Raina, 2002). Cell death, due to the HR, has been studied in detail for several plant–pathogen systems (reviewed by Morel and Dangl, 1997). In addition, the HR is sometimes activated by gall-inducing arthropods (Bronner et al., 1991; Fernandes and Negreiros, 2001; Ollerstam et al., 2002), but in this case, no SA induction has been reported. Although some authors have documented induced SA-dependent plant responses (Moran and Thompson, 2001) and HR-like cell collapses after aphid infestation (Belefant-Miller et al., 1994), the relation between aphid-induced HR and SA accumulation is far from clear. In fact, for some aphid–plant interactions it is concluded that SA does not mediate any defensive plant responses (Moran and Thompson, 2001).

Great genotypic variation in resistance in basket willow (*Salix viminalis* L.) against a leaf-rolling gall midge (*Dasineura marginemtorquens* Bremi) was found by Strong et al. (1993). On resistant *S. viminalis* genotypes, the great majority ($>$90%) of first-instar *D. marginemtorquens* larvae die. In contrast, on susceptible genotypes, mortality is $<$10% (Larsson and Strong, 1992).

In a previous study, we showed that resistant *S. viminalis* genotypes react hypersensitively with local collapse of up to six cell layers within 12 hr after *D. marginemtorquens* egg hatch. In contrast, no hypersensitive cell death was found in attacked, susceptible *S. viminalis* genotypes that invariably responded with gall formation (Ollerstam et al., 2002). Because SA accumulation is associated with pathogen-induced HRs (reviewed by Dangl et al., 1996), we were interested to see if this SA/HR relation existed in insect–plant systems as well. The *S. viminalis–D. marginemtorquens* system, with a distinct HR in resistant genotypes, seemed ideal for studying induction of SA in relation to insect-induced HR.

We tested two hypotheses. First, induction of SA is a feature of the resistance reaction of *S. viminalis* to attack by *D. marginemtorquens*. Second, a susceptible *S. viminalis* genotype can be made resistant by artificially increasing the SA concentration in the plant tissue. These hypotheses were formulated to answer the main question of whether SA mediates resistance in this system.

METHODS AND MATERIALS

Study Organisms. The monophagous gall midge *Dasineura marginemtorquens* induces irregular roll galls (Mani, 1964) in young leaf tissue on the willow *Salix viminalis*. Females oviposit on the leaves of the terminal buds, and larvae hatch within 3–4 days (at 20°C) and start to initiate the galls (Glynn and Larsson, 1994). On susceptible *S. viminalis* genotypes, galls are induced, and the normal

unrolling of the leaves is inhibited. Thereby, the leaf area available for photosynthesis is reduced. During *D. marginemtorquens* outbreaks, *S. viminalis* biomass production can be reduced by up to 30% (Glynn et al., 1998).

Plant material used in the present study originates from one resistant and one susceptible *S. viminalis* genotype cultivated in experimental plantations in the surroundings of Uppsala in central Sweden. It would have been advantageous to use several resistant and susceptible genotypes, but due to logistical reasons it was only possible to obtain one genotype of each category. Stems were collected from the field and cut to 10-cm pieces that were planted in 2-liter pots filled with potting soil (Hasselfors Garden Special). Thereafter, plants were cultivated in a greenhouse equipped with an air humidifier. Plants were given a daily supply of optimal nutrient solution (Wallco 51-10-43 + micronutrients, 100 mg N/liter), the technique of nutrient addition being adapted for maximal growth rate (Ingestad, 1987). The resistant genotype used was clone 1414, and the susceptible one was clone 5010. These two genotypes have been used in earlier studies (e.g., Ollerstam et al., 2002) and are well characterized as resistant (i.e., $<5\%$ larval survival) and susceptible (i.e., >90 % larval survival), respectively. The clonal numbers refer to classification within the Swedish Energy Forest Project (Sirén et al., 1987; Strong et al., 1993).

Midges were collected as pupae or third-instar larvae in galls from natural populations in *S. viminalis* plantations in the vicinity of Uppsala. Galled leaves were placed into Petri dishes, and adults were collected as they emerged and then introduced on to caged plants for controlled inoculations as previously described (Larsson et al., 1995). Females were allowed to oviposit for 24 hr on the plants used in the study. To determine the time to egg hatch, an extra plant was exposed to ovipositing females at the start of each experiment, and egg development was observed.

Genotypic Variation in Induction of Foliar SA after Larval Attack. To investigate whether resistance was correlated with local induction of SA, foliar SA was measured in one resistant and one susceptible *S. viminalis* genotype with or without *D. marginemtorquens* larvae. Resistant and susceptible plants were exposed to ovipositing females for 24 hr. The eggs hatched after 4 days, and during the following 2 days resistant and susceptible genotype leaves were sampled, in order to measure the induction of SA at 24–48 hr after egg hatch. Leaves were briefly checked for presence of larvae, and only leaves with more than 10 larvae were selected for analysis. This procedure took less than 1 min per leaf, and immediately after this the leaves were put into liquid nitrogen and stored at -80°C until extraction of SA. Two days after egg hatch, young galls were easily observed on the susceptible genotype, in contrast to the resistant genotype where no galls could be observed. The larvae were still alive on the resistant genotype at 2 days after egg hatch. Because of variation among plant individuals in number of leaves with more than 10 larvae (one to eight leaves per plant), one sample consisted

of leaves from one to three plants. However, the samples were independent because leaves from one plant individual were only used in one sample; N(attacked resistant genotype) = 6 samples, N(attacked susceptible genotype) = 6 samples, N(nonattacked resistant genotype) = 3 samples, and N(nonattacked susceptible genotype) = 3 samples. The reason for the higher number of samples from attacked (6) plants and nonattacked (3) plants was that we expected greater variation in SA level in attacked plants. Nonattacked control leaves were sampled at 2 days after egg hatch on attacked plants. From the nonattacked control plants, five leaves (in the same phenological stage as the attacked leaves) per individual plant were collected and pooled with five leaves from another plant to create one sample.

Extraction of SA. The extraction followed the procedure used by Bi et al. (1997). Frozen leaves were ground under liquid nitrogen to a fine powder with a mortar and pestle. The leaf powder was freeze-dried, and SA was extracted from 50 mg dry powder per sample for 1 min on a shaker [extraction buffer: 1.0 ml 50% (v/v) methanol per sample]. Extracts were centrifuged for 5 min at 5000 g, and the supernatants saved. Another 1.0 ml of extraction buffer was added to the remaining pellet, and the shaker/centrifuge steps were repeated two times. The three supernatants per sample were pooled and filtered through a 0.45-μm nylon filter. To each sample, 2 N HCl (1:1) was added and the extracts were hydrolyzed in a boiling water bath for 1 hr. Thus, samples were analyzed with respect to total SA, i.e., conjugated plus free SA. Each sample was then extracted three times with 1.0 ml ether, and the ether phase was dried under nitrogen and stored at -80°C.

Liquid Chromatography. Samples were dissolved in 0.2 ml 80% (v/v) methanol per sample and filtered through a 0.45-μm nylon filter. Chromatographic separation was performed on an Aquasil C-18 (Thermo Hypersil) column (50 $\times$ 2 mm, 3 μm) coupled to a system equipped with Shimadzu 10AD*VP* LC pumps, an autosampler injector (Gilson 232XL), and a column heater (Keystone Scientific). The mobile phase used was butanol–methanol–acetic acid–water (0.25:1.25:2:96.5, v/v) with 18 mM ammonium acetate. The injector temperature was 12°C and the column temperature 40°C. The flow rate was 0.2 ml/min. Under these conditions the retention time for SA was 4.2 min.

Mass Spectrometry. Samples and reference compounds were analyzed by means of a multiple reaction monitoring (MRM) mass spectrometer (Quattro Ultima, Micromass). The mass spectrometer scanned in the range m/z 137–92.5 with a dwell time of 0.5 msec. The capillary voltage was set to 2.8 kV and the cone voltage to 35 V. The source temperature was 80°C and the desolvation temperature 250°C. Cone gas flow was set to 32 l/hr, and the desolvation gas flow 300 l/hr. The collision gas pressure was 3.1×10^{-3} torr and the collision energy 16 V.

Induction of Resistance by Exogenous Application of SA. To test if SA could reduce larval survival frequency on a normally susceptible *S. viminalis* genotype, we treated a susceptible genotype with SA. To avoid direct exposure of larvae to SA, sodium salicylate was administrated to the plant tissue by placing cut 20-cm

shoots in Erlenmeyer flasks containing sodium salicylate dissolved in nutrient solution (Wallco 51-10-43 + micronutrients, 100 mg N/liter) (Ingestad, 1987). The Erlenmeyer flasks contained either pure nutrient solution ($N = 5$ shoots), 0.5 mM sodium salicylate in nutrient solution ($N = 5$ shoots), or 0.8 mM sodium salicylate in nutrient solution ($N = 5$ shoots). The shoots, while still on the plants, had been exposed to egg-laying females and were cut on the day that the larvae hatched. The experiment was interrupted 6 days after egg hatch, and living and dead larvae were counted. To reduce the risk of an exaggerated accumulation of SA in the plant tissue, shoots treated with 0.8 mM sodium salicylate were kept in 0.8 mM sodium salicylate for only 24 hr and then transferred to pure nutrient solution for the remaining 5 days.

RESULTS

Genotypic Variation in Induction of Foliar SA after Larval Attack. The two *Salix viminalis* genotypes differed with respect to foliar SA levels after larval attack. A higher SA level was detected in the resistant *S. viminalis* genotype after attack, but not in the susceptible genotype compared with nonattacked control plants (Figure 1). No differences in SA levels were found among nonattacked resistant, attacked susceptible, and nonattacked susceptible leaves ($P > 0.05$, Mann-Whitney test) (Figure 1).

Induction of Resistance by Exogenous Application of SA. The susceptible *S. viminalis* genotype became more resistant when treated with SA. *Dasineura marginemtorquens* larval survival frequency was lower on SA-treated shoots of a normally susceptible *S. viminalis* genotype than on nontreated shoots (Figure 2). We found no difference in larval survival between shoots treated with 0.8 mM sodium salicylate for 1 day or 0.5 mM sodium salicylate for 6 days (Figure 2). Unfortunately, one shoot treated with 0.8 mM sodium salicylate and one control shoot died before larval survival was estimated.

DISCUSSION

We report variation between *Salix viminalis* genotypes in SA-mediated induced resistance to the gall midge *Dasineura marginemtorquens*. The importance of SA in the resistance reaction was inferred from the SA accumulation found in attacked resistant leaves and increased larval mortality on SA-treated shoots.

Foliar SA accumulated after larval attack in a resistant *S. viminalis* genotype, but not in a susceptible one. This points to a SA-related mechanism that explains the previously observed genotypic variation in resistance (Strong et al., 1993;

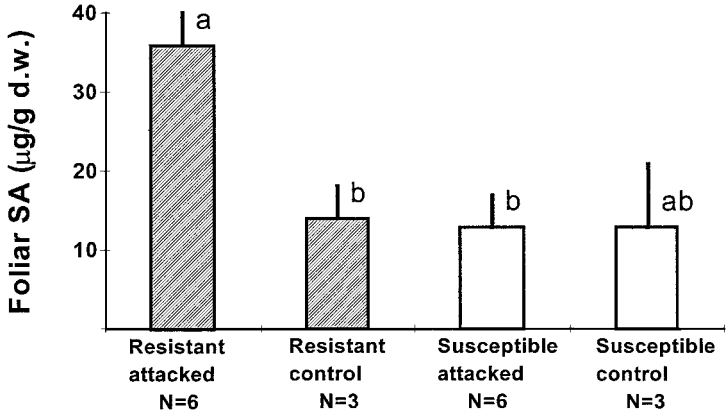

FIG. 1. Foliar SA levels in one resistant and one susceptible *Salix viminalis* genotype with or without *Dasineura marginemtorquens* larvae. A higher SA level was found in attacked resistant leaves sampled at 24–48 hr after egg hatch compared with nonattacked leaves from the same *S. viminalis* genotype ($P < 0.01$, Kruskal-Wallis test, $P = 0.01$, Mann-Whitney test, arcsine square root-transformed values). Different letters on top of bars indicate significant differences. N = number of samples. Error bars = 1 *SE*.

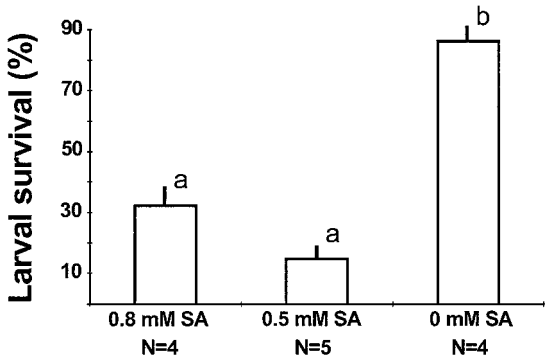

FIG. 2. Survival of *Dasineura marginemtorquens* larvae on SA-treated shoots of a susceptible *Salix viminalis* genotype. The shoots were treated either with 0.8 mM sodium salicylate for 1 day (followed by pure nutrient solution for 5 days), 0.5 mM sodium salicylate for 6 days, or 0 mM sodium salicylate (i.e., pure nutrient solution) for 6 days. Different letters on top of bars indicate significant differences ($P < 0.01$, Kruskal-Wallis test, $P = 0.01$, Mann-Whitney test, arcsine square root-transformed values). N = number of shoots. Error bars = 1 *SE*.

Ollerstam et al., 2002). The relatively large variation in SA level among samples within treatments might be accounted for by sublimation during the extraction of SA (Verberne et al., 2002). However, there is no reason to believe that sublimation of SA should be higher in some treatments than in others. Previous studies have shown that certain insects induce (Bi et al., 1997; Moran and Thompson, 2001) and respond to (Hardie et al., 1994; Glinwood and Pettersson, 2000) SA-related compounds in plants. The volatile SA ester methyl salicylate (MeSA) is a repellent for the aphid *Aphis fabae* (Hardie et al., 1994). Hence, in the case of aphids, the main resistance mechanism seems to be to reduce settling and feeding (Glinwood and Pettersson, 2000; Moran and Thompson, 2001). Because the aphid's host acceptance is affected by MeSA, it is unknown to what extent SA-signaled induction of biochemical responses within the plant tissue contributes to resistance. Similarly, egg-laying leaf miners (*Liriomyza* spp.) discriminate against tomato plants treated with the SA analog BTH [benzo(1,2,3)thiadiazole-7-carbothioic acid (S) methyl esther] (Inbar et al., 1998). Even feeding by chewing insects such as the cotton bollworm *Helicoverpa zea* induces accumulation of foliar SA. In this case, it was concluded that SA probably does not mediate any resistance (Bi et al., 1997). However, in the *D. marginemtorquens–S. viminalis* system, plant resistance is characterized by a rapid HR (Ollerstam et al., 2002), something that has been commonly associated with SA accumulation in other parasite–plant systems (reviewed by Dangl et al., 1996).

The application of exogenous SA induced resistance, manifested as reduced *D. marginemtorquens* larval survival, in a normally susceptible *S. viminalis* genotype. Thus, SA seems to be active in the resistance and not simply correlated with the true resistance reaction. In contrast, previous studies on the effects of exogenous SA or SA analogs on performance of insects have come to quite different conclusions. To test the effect of BTH-induced plant responses on two insect species, Inbar et al. (2001) treated cotton seedlings with BTH and tested the effects of BTH on host preference of the whitefly *Bemisia tabaci* and feeding efficacy of the cotton bollworm *Helicoverpa armigera*. Increased enzymatic activities were detected for peroxidase, glucanase, and chitinase in BTH-treated plants. The authors concluded that the BTH-induced responses had a negligible effect on the tested insects. In accordance, Bi et al. (1997) detected no effect on the performance of the cotton bollworm *Helicoverpa zea* when SA was exogenously applied to cotton plants. Thaler et al. (1999) showed that BTH treatment of tomato plants even increases the survivorship of the beet armyworm (*Spodoptera exigua*). Thus, earlier studies have either not detected any effect on insect preference or performance of treatment with SA or SA analogs (e.g., Bi et al., 1997; Inbar et al., 2001), or they found improved insect performance on treated plants (Thaler et al., 1999), although some aphids are repelled by SA-related compounds (Glinwood and Pettersson, 2000). However, neither of the mentioned studies has tested the effects of SA on a gall-inducing insect. It is possible that SA interacts in some way

with the galling process, explaining why the effect of SA application on insect performance is more pronounced in the *D. marginemtorquens-S. viminalis* system than in previously studied systems.

For the exogenous SA treatments, biologically relevant SA concentrations were chosen. We chose the SA concentrations based on a study on pathogen-induced accumulation of SA in cucumber (*Cucumis sativus* L.) leaf-petiole phloem (Rasmussen et al., 1991). In that study, SA concentrations of up to 0.6 mM were found in phloem exudates after infection. It is unlikely that the sodium salicylate *per se* was detrimental to the *D. marginemtorquens* larvae. An Erlenmayer flask with the highest sodium salicylate concentration (0.8 mM) contained about 38 mg sodium salicylate dissolved in 300 ml. During the 24 hr in the flask, a whole shoot consumed less than 10 ml, i.e., about 1.3 mg sodium salicylate. We do not have any data on the SA tolerance of *D. marginemtorquens* larvae because of the difficulties in culturing these larvae on artificial diets, but other insects have been shown to tolerate up to 2.5 mg SA/g food (Summers and Felton, 1994). Because the shoots weighed about 8.4 g each (8.44 g, $SE = 0.22$, $N = 10$ shoots), it could be assumed that a shoot in the 0.8 mM treatment accumulated about 0.15 mg sodium salicylate per gram of plant tissue.

In a previous study, we found an HR-like cell death in resistant *S. viminalis* genotypes at 12 hr after *D. marginemtorquens* egg hatch and accumulation of phenolic compounds, as detected by autofluorescence, in the HR-like lesions (Ollerstam et al., 2002). The timing of cell death, accumulation of autofluorescent phenolic compounds (Ollerstam et al., 2002), and accumulation of SA, all suggest a mechanistic similarity in induction and execution of pathogen-induced and gall-inducer-induced HRs. Because SA was measured at 24–48 hr after egg hatch, we cannot infer whether SA is the cause of, or a consequence of, the HR-like lesions induced by *D. marginemtorquens* attack on resistant *S. viminalis* leaves. However, SA is generally considered essential for systemic plant responses occurring subsequent to pathogen-induced HR cell death rather than being involved in the death of directly attacked cells (Gaffney et al., 1993; Martinez et al., 2000; Beers and McDowell, 2001; but see Tenhaken and Rübel, 1997). It was reasonable to measure the SA level after the time point when HR-like necrosis was first observed. The molecular mechanisms of gall induction is still to be discovered, but when plant pathogens trigger the HR, an elicitor is transferred from the pathogen to the plant (reviewed by Morel and Dangl, 1997). Similarly, induction of a gall probably involves transfer of an elicitor that triggers the reprogramming of plant cells to a functional gall. The gall elicitor might be the one that triggers the HR in incompatible interactions between a gall inducer and its host. We have previously reported that the timing of the insect-induced HR against *D. marginemtorquens* coincides with the timing of pathogen-induced HRs (Ollerstam et al., 2002). The finding of SA-mediated resistance in a *S. viminalis* genotype adds to the similarity between pathogen- and insect-induced HRs.

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ALLELOCHEMICAL EFFECTS OF EUDESMANE AND EREMOPHILANE SESQUITERPENES ON *Tribolium castaneum* LARVAE

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Abstract—Eight eudesmane and eremophilane sesquiterpenes administered to *Tribolium castaneum* larvae caused different allelochemical effects. Topical application of 3-oxo-γ-costic acid produced the greatest lengthening in the duration of the pupal stage. Morphological deformities were found, specifically when ilicic, costic, and γ-costic acids and costic aldehyde were used. Ilicic acid exhibited the major toxicity 72 hr following topical application. All compounds were significantly toxic at the end point of the experiment (60 days). Treated surface toxicity was lower than when topical assays were carried out. Responses to tessaric acid in choice bioassays had the highest attractive effect. Maximum repellency was caused by the 3-oxo-γ-costic acid. However, experimental series carried out using γ-costic acid, eremophilan-1(10),2,11(13)-trien-12-oic acid, costic aldehyde, and ilicic aldehyde showed no clear response.

Key Words—*Tribolium castaneum*, *Tessaria absinthioides*, allelochemical effects, eudesmanes, eremophilanes, sesquiterpenes, tessaric acid, ilicic acid, 3-oxo-γ-costic acid.

INTRODUCTION

Terpenoids are one of the major phytochemical classes characterized by stereochemical diversity, exceeding that of any other group of plant products (Gershenzon and Croteau, 1999). Certain sesquiterpenoids interfere with normal chemoreceptor

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function and inhibit food uptake by fifth instars of *Pieris brassicae* L. (Lepidoptera: Pieridae) (Schoonhoven and Fu-Shun, 1989). Sesquiterpene lactones, in contrast, have been shown to have deterrent, antifeedant, growth regulator, and toxic activities (Picman, 1986; Srivastara et al., 1990; Chou and Mullin, 1993).

In previous papers, we have reported on the feeding deterrent activities exhibited toward *Tenebrio molitor* (L) (Coleoptera: Tenebrionidae) larvae by some *neo*-clerodane diterpenoids isolated from natural sources, as well as on the role that hydrophobicity and stereoelectronic effects play in their antifeedant bioactivity (Enriz et al., 1994; 2000; Luco et al., 1994; Sosa et al., 1994).

Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae) is one of the most economically important insect pests, with a worldwide distribution. Several authors have studied the effects of plant secondary metabolites on it (Harmatha and Nawrot, 1984; Picman, 1986; Isman, 2000).

The present research is aimed toward discovering the allelochemical properties of known sesquiterpenes, and those obtained by chemical transformation, that might be compatible with newer integrated pest control approaches. The biological activity of compounds **1**–**8** (Figure 1) were evaluated using *T. castaneum* as an insect model.

METHODS AND MATERIALS

Chemicals. Tessaric acid (**1**), ilicic acid (**2**), and 3-oxo-γ-costic acid (**4**) (Figure 1) were isolated from aerial parts of *Tessaria absinthioides* (Hook. et Arn.) DC, as previously reported (Kurina Sanz et al., 1997). Compounds **3** and **7** were obtained from **2** by dehydration (Donadel et al., 1998). Compounds **5** was obtained from the eremophylane **1** by reduction using alane($LiAlH_4/AlCl_3$) in THF (Brown and Krishnamurthy, 1979). Derivatives **6** and **8** were prepared by reduction and dehydration of compound **2** (Guerreiro et al., 1979). Identities of all compounds were confirmed by ^{1}H NMR (200.13 MHz), ^{13}C NMR (50.23 MHz), EI-MS, and IR spectral data, as well as optical rotation.

Insects. All experiments were conducted in the laboratory using established colonies of *Tribolium castaneum* Herbst. (Coleoptera: Tenebrionidae). Larvae were reared on a mixture of flour, yeast, and starch (3:3:1) at $25 \pm 1^{\circ}$C, 65% relative humidity, and a 16L:8D photoperiod.

Bioassays

Topical Application. Fifth instars of *T. castaneum* were randomly selected. Four acetone solutions of each compound (10, 20, 40, and 80 μg/μl) were prepared.

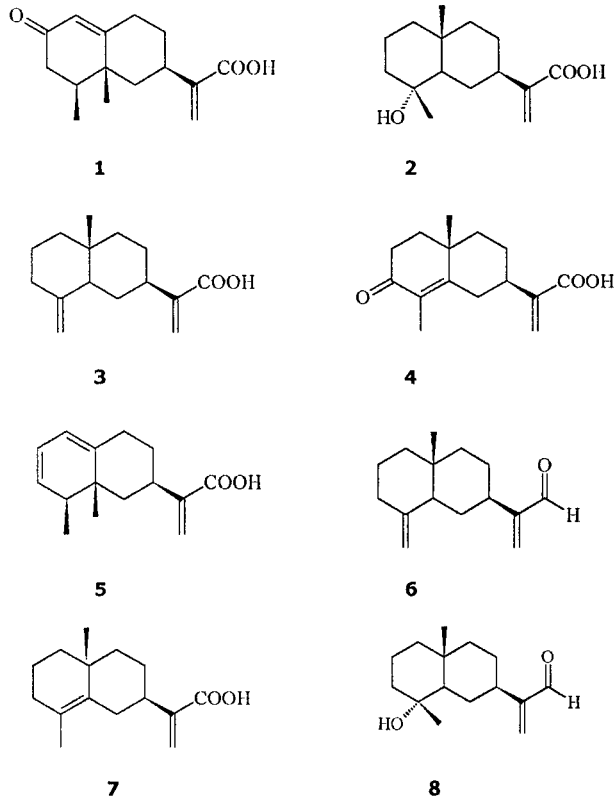

FIG. 1. Structure of compounds **1–8**.

Test solutions were topically applied to the ventral surface of the thoracic segments with a Hamilton microsyringe (1 μl/larvae). Controls were treated with the solvent alone. There were three replicates of 10 larvae for each individual compound tested. After treatments, insects were placed into plastic vials (10 cm diam. $\times$ 7 cm high) containing food and held at 25 $\pm$ 1°C with a 16L:8D photoperiod. Insects were examined daily for 60 days. The duration of the pupal stage (days) was recorded as well as the inhibition of the imaginal molt. Molt inhibition was classified according to the morphofunctional criteria of Pascual et al. (1990) using five basic categories (see description in Table 1 below). This classification represents an arbitrary categorization of a continuum from normal adults, to specimens with different inhibitory effects simply affecting ecdysis and the pre-ecdysial cuticle deposition process.

TABLE 1. RESPONSE OF *T. castaneum* LARVAE TO SESQUITERPENE COMPOUNDS IN TOPICAL BIOASSAYS[a]

Chemical (dose, μg/μl)	*T. castanueum*						
	Duration of pupal instar (days±SEM)	MI					
		0	1	2	3	4	5
Control	9(±0.1)	28	—	—	—	—	—
1 (10)	10 ± 1.0	7	—	—	2	—	2
1 (20)	13 ± 1.3	3	2	—	2	—	—
1 (40)	12 ± 0.3	5	—	—	2	—	1
1 (80)	9 ± 1.5	8	—	—	—	—	2
2 (10)	16 ± 0.2	—	4	—	2	1	—
2 (20)	16 ± 0.7**	8	—	—	1	—	—
2 (40)	16 ± 0.4**	2	3	—	2	—	—
2 (80)	14 ± 0.5*	7	2	—	2	1	2
3 (10)	16 ± 0.4**	3	3	—	1	—	—
3 (20)	13 ± 1.3	8	2	—	3	1	5
3 (40)	11 ± 0.8	8	—	—	—	1	—
3 (80)	15 ± 1.3	4	1	—	2	1	—
4 (10)	15 ± 1.2	5	—	—	—	—	2
4 (20)	9 ± 0.7	5	3	—	2	—	—
4 (40)	18 ± 0.4***	4	3	—	—	—	—
4 (80)	13 ± 0.8	8	—	1	2	3	—
5 (10)	15 ± 0.5***	17	2	1	1	2	—
5 (20)	15 ± 0.4***	19	1	—	2	1	—
5 (40)	15 ± 0.5***	18	—	2	—	—	—
5 (80)	13 ± 0.5**	20	2	—	1	1	—
6 (10)	15 ± 0.5***	20	—	—	1	1	1
6 (20)	13 ± 0.6**	18	3	2	—	1	—
6 (40)	14 ± 0.5***	24	1	—	—	—	—
6 (80)	15 ± 0.7***	15	3	2	3	—	1
7 (10)	15 ± 1.2**	6	2	—	—	3	2
7 (20)	16 ± 0.1***	8	2	—	3	—	1
7 (40)	12 ± 0.9	12	4	—	—	—	2
7 (80)	13 ± 1.1	3	2	1	1	1	—
8 (10)	12 ± 0.6	22	1	—	—	—	—
8 (20)	14 ± 0.6***	21	—	—	—	—	2
8 (40)	14 ± 0.7**	16	—	1	—	2	1
8 (80)	12 ± 0.6	13	1	1	1	—	1

Note: The values are mean (± SEM); $N = 30$. Data followed by *,**,*** are significantly different from the control ($P < 0.05$, $P < 0.01$, $P < 0.001$, by Kruskal–Wallis test; multiple comparisons Dunn's test). M I, Number of insects showing different levels of molt inhibition (Pascual et al., 1990). 0, Normal adults. 1, Adults with normal levels of sclerotization. First and second pair of wings showing certain degree of deformation. Pupal exuviae attached to the posterior part of the abdomen and to the appendages. 2, Adultoids completely enclosed in pupal exuviae, which is usually ruptured along the midline of the pronotum and in the articulation areas of the legs. Cuticle is well tanned as in normal adults. 3, Pupa with adult cuticular level. Pupal exuviae remains intact. 4, Pupa that suffered a complete inhibition of ecdysis and severe deficiencies in the process of new cuticle deposition. Appendages are partially unsegmented. 5, Pupa that suffered a complete inhibition of ecdysis and no cuticle deposition. The cephalic capsule is not normally formed and the appendages are partially unsegmented and with an abnormal position.—, no individual showing this category.

[a] Effects on the duration of pupal instar (days ± SEM) and different degrees of imaginal moult inhibition.

Insect mortality was recorded after 1, 2, 3, and 60 days (end point of the experiment) after treatment. The mortality percentage was calculated by the equation $[1 - A * B/X * D] * 100$, where A is the number of treated larvae that remain alive at the end of the experiment; B is the number of untreated larvae at the beginning of the experiment; X is the number of treated larvae at the beginning of the experiment; and D is the number of untreated larvae living at the end of the experiment (Henderson and Tilton, 1985).

Contact Toxicity. An aliquot of 10 μl of each solution with 10, 20, 40, and 80 μg/μl concentration was diluted into 1 ml of ethanol. The solution was applied to the surface of a plastic Petri dish (5.5 cm diam. $\times$ 1 cm high) and homogeneously dispersed. Controls were treated with solvent alone. The solvent was allowed to evaporate for 2 hr prior to the introduction of 10 terminal instars of *T. castaneum*. Treated Petri dishes were kept at 25 $\pm$ 1°C with a 16L:8D photoperiod. Each treatment was replicated tree times. Insect mortality was recorded at 1, 2, and 3 days, and mortality (%) was calculated using the Henderson and Tilton (1985) evaluation.

Choice Bioassay. The experiment utilized a two-choice arena based on an area preference test described by Obeng-Ofori and Reichmuth (1997). The test arena consisted of a glass Petri dish (9 cm diam. $\times$ 1.5 cm high) divided in half with adhesive tape (1 mm wide). A 10 μl dose of test solution containing 10, 20, 40, and 80 μg/μl concentration was dissolved in 0.2 ml of acetone. Each solution was applied to half of the Petri dish arena as uniformly as possible with a microsyringe. The control side (the other half of the arena) was treated with solvent alone. Petri dishes were air dried to evaporate the solvent completely. Once the Petri dish was dry, the barrier tape was removed, 10 terminal instars of *T. castaneum* were released at the center of the dish, and then the dishes were covered. Each treatment was replicated 10 times. Bioassays were conducted in complete darkness at 25 $\pm$ 1°C and 65% humidity. After 30 min, a preference index (PI) for larvae in the two-choice bioassay was calculated as $PI = (T - C/\text{Tot})*100$ (Phillips et al., 1993), where T is the number of larvae responding to the treatment; C is the number responding to the control, and Tot is the total number of insects released.

Positive PIs indicate attraction to the treatment and negative PIs indicate repellency; values could theoretically range from -100 for complete repellency, to $+100$ for complete attraction.

Data Analysis. Growth assays were analyzed by using the Kruskal-Wallis test, and Dunn's multiple comparisons test at the $P < 0.05$ level. Data from the first 72 hr (topical application assays) were analyzed using the Kruskal-Wallis test, and Dunn's multiple comparisons test at the $P < 0.05$ level. A χ^2 test was used to analyze data from the end point of the experiment. For contact toxicity assays, data were analyzed using the Kruskal-Wallis test, and Dunn's multiple comparisons test at the $P < 0.05$ level. In response index assays, numbers responding to treatments and controls were subjected to the Kruskal-Wallis test, and Dunn's

multiple comparisons test. PriProbit analysis (Sakuma, 1998) was used to determine ED_{50}, ED_{90}, LD_{50}, LD_{90}, and the corresponding 95% confidence limits.

RESULTS AND DISCUSSION

Although the sesquiterpenes studied possessed similar structures, our results indicate that *T. castaneum* responded quite differently to the related compounds. Tessaric acid (**1**) produced a certain degree of morphological abnormalities, without a significant alteration in the duration of the pupal stage (Table 1). In the topical toxicity test, no significant mortality was observed after 3 days, and the highest mortality was reached by the lower concentration after 60 days of treatment (Table 2). This suggests a toxic, but delayed, action against *T. castaneum* larvae. Similar effects were seen in *T. castaneum* adults exposed to food treated with extracts of *Crambe abyssinica* Hochst ex. R.E. Fr. (Cruciferae) (Tsao et al., 1996).

Compound **1**, which is the the most abundant sesquiterpene in *Tessaria absinthioides*, showed a significant toxicity when it was applied in the contact bioassay at 16.84 μg/cm^2 after 3 days of exposure (Table 3). It shows attractive properties with an ED_{50} (95% CL) = 18.16 μg/cm^2 (11.14–38.26) and an ED_{90}(95% CL) = 103.28 μg/cm^2 (45.04–316.62) (Figure 2), and toxic activity. Therefore, it is not surprising that this secondary metabolite acts as an attracticide. This activity could have important implications in the defense of plants against insect attack (Phillips, 1994).

Ilicic acid (**2**) increased pupal stage duration when applied at 20, 40, and 80 μg/μl dose (topical application), and interfered with larval development (Table 1). This was the only compound to cause significant mortality after 2 days at the lower dose (Table 2). This product was toxic in topical and surface contact bioassays, although the latter had more significant differences after 1, 2, and 3, days (Table 3). This phenomenon may be related to the physicochemical behavior of this compound on different surfaces. For instance, the insects on a treated surface receive a topical application (due to their own movement inside the capsule) as a chronic treatment. However, the topically treated larvae received the application only at the beginning of the assay. The highest mortality occurred at the end point of the experiment (Table 2). This confirms a moderate toxicity during the first 3 days and an acute toxicity over long periods of exposure. The highest mortality occurred in response to the lowest concentration of compound. This could result from higher doses acting as a trigger for the activation of detoxification enzymes, i.e., gluthatione-S-transferase (Wood et al., 1986). Finally, compound **2** (Figure 2) had an ED_{50}(95% CL) = 21.39 μg/cm^2 (16.21–25.32) and an ED_{90}(95% CL) = 36.12 μg/cm^2(29.01–90.32), acting as a moderate repellent against *T. castaneum* larvae.

TABLE 2. MORTALITY OF *T. castaneum* LARVAE TREATED WITH SESQUITERPENES IN A TOPICAL BIOASSAYS 1, 2, AND 3 DAYS AFTER STARTING AND AT END POINT OF EXPERIMENT

	Mortality (%)[a]															
	10 μg/μl				20 μg/μl				40μg/μl				80μg/μl			
Chemical	24 hr	48 hr	72 hr	60 days	24 hr	48 hr	72 hr	60 days	24 hr	48 hr	72 hr	60 days	24 hr	48 hr	72 hr	60 days
Control	3 ± 3.3	3 ± 3.3	3 ± 3.3	4 ± 2.4	3 ± 3.3	3 ± 3.3	3 ± 3.3	4 ± 2.4	3 ± 3.3	3 ± 3.3	3 ± 3.3	4 ± 2.4	3 ± 3.3	3 ± 3.3	3 ± 3.3	4 ± 2.4
1	3 ± 3.3	20 ± 5.8	20 ± 5.8	70 ± 15.2§	0 ± 0.0	27 ± 9.0	38 ± 9.2	63 ± 8.8§	6 ± 3.3	10 ± 5.7	13 ± 8.8	53 ± 8.8§	3 ± 3.3	10 ± 5.7	20 ± 9.8	60 ± 5.7§
2	16 ± 12.0	45 ± 12.6*	69 ± 9.6*	86 ± 6.6§	10 ± 5.7	20 ± 5.8	24 ± 3.0	66 ± 14.5§	20 ± 5.8	27 ± 9.0	38 ± 9.2*	56 ± 12.0§	10 ± 5.7	17 ± 8.9	31 ± 5.8	66 ± 3.3§
3	0 ± 0.0	10 ± 5.7	13 ± 3.1	76 ± 3.3§	3 ± 3.3	6 ± 3.3	6 ± 3.3	66 ± 3.3§	3 ± 3.3	13 ± 3.1	13 ± 3.3	53 ± 3.3§	0 ± 0.0	3 ± 3.3	13 ± 3.3	76 ± 8.8§
4	3 ± 3.3	13 ± 3.1	13 ± 3.1	66 ± 14.5§	13 ± 8.8	16 ± 12.0	34 ± 8.2	66 ± 3.3§	3 ± 3.3	6 ± 3.3	27 ± 2.5	76 ± 14.5§	3 ± 3.3	10 ± 5.7	17 ± 6.4	56 ± 3.3§
5	3 ± 3.3	3 ± 3.3	3 ± 3.3	36 ± 6.6§	0 ± 0.0	0 ± 0.0	0 ± 0.0	33 ± 8.8§	6 ± 3.3	10 ± 8.8	10 ± 8.8	43 ± 3.3§	0 ± 0.0	0 ± 0.0	0 ± 0.0	30 ± 5.7§
6	0 ± 0.0	0 ± 0.0	0 ± 0.0	33 ± 3.3§	0 ± 0.0	0 ± 0.0	0 ± 0.0	30 ± 10.0§	0 ± 0.0	0 ± 0.0	0 ± 0.0	16 ± 6.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	40 ± 5.7§
7	6 ± 6.6	6 ± 6.6	6 ± 6.6	73 ± 8.7§	3 ± 3.3	20 ± 5.8	34 ± 2.9	66 ± 12.0§	13 ± 3.1	20 ± 5.8	20 ± 5.8	46 ± 3.3§	0 ± 0.0	20 ± 5.8	41 ± 5.9*	83 ± 3.3§
8	0 ± 0.0	0 ± 0.0	0 ± 0.0	26 ± 3.3§	0 ± 0.0	0 ± 0.0	0 ± 0.0	30 ± 5.7§	0 ± 0.0	0 ± 0.0	0 ± 0.0	46 ± 8.8§	0 ± 0.0	0 ± 0.0	3 ± 3.3	53 ± 3.3§

[a] Values are mean $\pm$ SEM ($N = 30$). Mean $\pm$ SEM followed by * are significantly different from the control ($P < 0.05$, Kruskal – Wallis test; multiple comparisons Dunn's test. Mean $\pm$ SEM followed by §are significantly different from the control ($P < 0.01$, (χ^2 test).

TABLE 3. MORTALITY OF *T. castaneum* LARVAE TREATED WITH SESQUITERPENES IN A CONTACT BIOASSAYS 1, 2, AND 3 DAYS AFTER STARTING THE EXPERIMENT

	Mortality (%)[a]											
	4.21μg/cm^2			8.42μg/cm^2			16.84μg/cm^2			33.68μg/cm^2		
Chemical	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Control	0 ± 0.0	3 ± 3.3	3 ± 3.3	0 ± 0.0	3 ± 3.3	3 ± 3.3	0 ± 0.0	3 ± 3.3	3 ± 3.3	0 ± 0.0	3 ± 3.3	3 ± 3.3
1	0 ± 0.0	10 ± 0.3	10 ± 0.3	3 ± 3.3	10 ± 5.7	20 ± 0.6	0 ± 0.0	13 ± 6.6	$30 \pm 5.1^*$	13 ± 3.3	14 ± 4.0	20 ± 0.6
2	6 ± 3.3	$30 \pm 9.5^*$	$34 \pm 2.8^*$	6 ± 3.3	13 ± 6.6	$33 \pm 2.6^*$	6 ± 3.3	23 ± 5.8	$41 \pm 4.7^{**}$	$23 \pm 6.6^*$	$23 \pm 6.3^*$	30 ± 9.5
3	$16 \pm 3.3^*$	20 ± 10.0	31 ± 1.0	0 ± 0.0	6 ± 3.2	10 ± 0.3	0 ± 0.0	6 ± 3.2	13 ± 3.1	6 ± 3.3	20 ± 0.6	$51 \pm 3.1^{**}$
4	16 ± 6.6	21 ± 6.7	24 ± 4.4	0 ± 0.0	10 ± 0.3	24 ± 4.4	0 ± 0.0	6 ± 3.2	10 ± 0.3	6 ± 3.3	10 ± 0.3	23 ± 5.8
5	3 ± 3.3	3 ± 3.3	3 ± 3.3	0 ± 0.0	0 ± 0.0	0 ± 0.0	3 ± 3.3	3 ± 3.3	3 ± 3.3	16 ± 12.0	16 ± 12.0	16 ± 12.0
6	0 ± 0.0	3 ± 3.3	3 ± 3.3	3 ± 3.3	3 ± 3.3	3 ± 3.3	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
7	0 ± 0.0	10 ± 0.3	30 ± 5.1	$13 \pm 3.3^*$	$20 \pm 0.6^*$	$41 \pm 1.4^*$	6 ± 3.3	10 ± 5.7	20 ± 0.6	0 ± 0.0	10 ± 5.7	20 ± 5.4
8	0 ± 0.0	0 ± 0.0	0 ± 0.0	3 ± 3.3	3 ± 3.3	3 ± 3.3	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0

[a] Values are mean $\pm$ SEM ($N = 30$). Means $\pm$ SEM followed by * are significantly different from the control ($P < 0.05$, Kruskal – Wallis test; multiple comparisons Dunn's test).

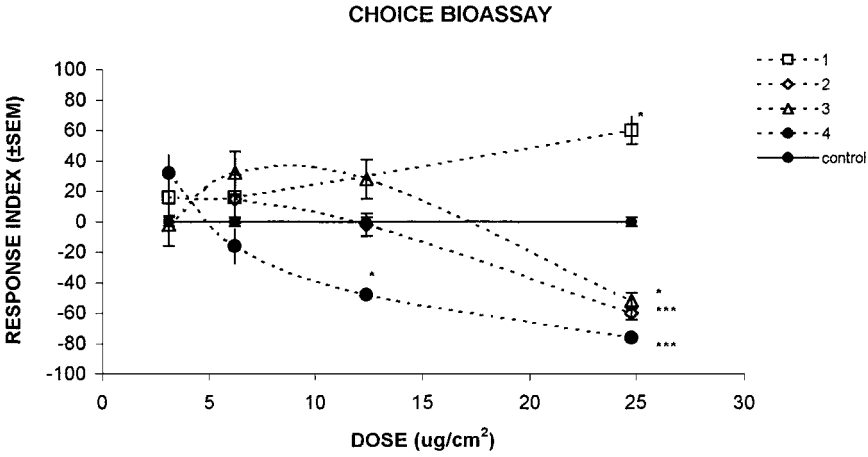

FIG. 2. Mean response index of *T. castaneum* terminal instar larvae to diferent doses of compounds in two-choice bioassays (numbers associated with particular symbols correspond to the number of the structure as seen in Figure 1. 1 = tessarie acid; 2 = ilicic acid; 3 = costic acid; 4 = 3-oxo-λ-costic acid) Response index = $(T - C/Tot) \times 100$; T is the number responding to treatment, C is the number responding to the control, and *Tot* is the total number of larvae released. There were significant differences ($P < 0.001$, Kruskal-Wallis test; multiple responding to control at two doses. Significant differences between treatment and control responses indicated by $^{*}(P < 0.05)$ $^{**}(P < 0.01)$ $^{***}(P < 0.001)$; $N = 10$ bioassays at each dose.

Costic acid (**3**) only affected the pupation period at the 10 μg/μl dose, with different kinds of deformities including severe deficiencies in development of the cephalic capsule, lack of cuticle deposition, and abnormal disposition of legs and wings (Table 1). Mortality was significant at 60 days only (Table 2). When the contact surface biotest was carried out for compound **3**, mortality was 51% after 3 days. The repellent activity, $ED_{50} = 24.65$ μg/cm^2 and $ED_{90} = 28.53$ μg/cm^2, should be considered provisional, because the confidence limits could not be calculated due to variation (Figure 2).

3-oxo-γ-costic acid (**4**) showed one of the highest repellency activities in the two-choice bioassay, with an ED_{50}(95% CL) = 13.46 μg/cm^2 (9.84–17.08) and ED_{90}(95% CL) = 39.5 μg/cm^2 (27.56–96.32) (Figure 2). According to this, this product could play an important role in plant defense. A toxic effect was not evident after 3 days of treatment, but toxic action was confirmed after 60 days of exposure (Table 2).

Both 3-oxo-γ-costic acid (**4**) and tessaric acid (**1**) have an α, β-unsaturated ketone function in the A ring of the decaline moiety, with an electrophilic-β-carbon. This electrophilic carbon could act as an acceptor in a Michael reaction with

nucleophilic functional groups such as sulfhydryl, hydroxyl, and amino groups from proteins. These kinds of electrophilic functional groups play an important role in the bioactivity of sesquiterpene lactones against many insects (Harmatha and Nawrot, 1984; Arnason et al., 1986; Picman, 1986; Chou and Mullin, 1993; Carrizo et al., 1998). Our studies indicate that compound **4** interferes with the normal development of *T. castaneum*, significantly increasing the duration of the pupal stage at 40 μg/μl dose (Table 1).

Neither eremophilan-1(10),2,11(13)-trien-12-oic acid (**5**) nor the aldehyde **6** showed activity in the two-choice bioassay. These compounds produced a significant elongation of the duration of the pupal stage at every concentration evaluated, as well as a series of morphological alterations (Table 1). This suggests that they could produce an antifeedant effect resulting in reduction in growth or death by starvation. Likewise, Carrizo et al. (1998) found a similar response to benzofuranes by *T. molitor* larvae as did Isman and Rodriguez, (1983) using *Heliothis zea* Booddie (Lepidoptera: Noctuidae) larvae treated with sesquiterpene esters. The presence of morphogenetic defects could be related to action as an insect growth regulator.

γ-Costic acid (**7**) did not show activity in the two-choice bioassay. At the end point of the topical experiment, the mortality was significantly increased at all concentrations (Table 2). Some alterations in the length of the pupal stage as well as many morphological abnormalities (Table 1) were detected for this product.

Compound **8** was the only compound to produce a dose-related effect in topical application assays. This toxic response was reached at the end point of the experiment, LD_{50} (95% CL) = 61.19 μg/μl and LD_{90} (95% CL) = 1935.68 μg/μl (confidence limits could not be calculated due to variation) (Table 2). Significant alterations in the duration of the pupal stage were produced. As mentioned above, the highly reactive β-position of the C-7 side-chain unsaturated carbonyl system could act as an alkylation agent. In the two-choice bioassay, ilicic aldehyde (**8**) exhibited an attractive activity at the lower doses. However, we were unable to detect a trend when higher doses were used.

The lack of a dose–response effect on growth and toxicity by contact biotests made it inaccurate to estimate the ED_{50}, ED_{90} or the LD_{50}, LD_{90} values, respectively.

Several reports deal with morphological alterations mediated by secondary natural products. Bowers and Thompson (1963), Thompson and Uebel (1966), and Low et al. (1968), using *Oncopeltus fasciatus* (Dallas) (Heteroptera: Lygaeidae) and *T. molitor* L. specimens treated with related sesquiterpenoids, found similar morphological abnormalities. More recently, these kind of results were studied by Pascual (1990) and Quennedey (1998) when the response of *T. molitor* was treated with azadirachtin and 20-hydroxyecdysone. These authors showed how the compounds were acting at the endocrine level, producing morphological

abnormalities. In agreement with the results obtained in our study, we postulate that some of the sesquiterpenes evaluated here could affect the endocrine system. New investigations at the microscopic, enzymatic, and cytological level are necessary in order to determine whether the abnormalities observed in the present studies are in agreement with hormonal disorders.

The deleterious properties of plant natural products could be related to an antifeedant effect and the subsequent death by starvation. Our results are consistent with those found by Alfatafta and Mullin (1992), in which eudesmanoic acid showed antifeedant action against *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae). These authors documented a reduction in the food uptake by at least 50%.

In summary, the results reported here allow us to confirm the hypothesis that the compounds studied alter development in *T. castaneum* larvae. In general, the natural products evaluated here affect larval survival, but only after a long period of exposure. Finally, it would be desirable to extend this investigation to other insect species before drawing more general conclusions.

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DIETARY INFLUENCES ON TERPENOIDS SEQUESTERED BY THE BIOLOGICAL CONTROL AGENT *Oxyops vitiosa*: EFFECT OF PLANT VOLATILES FROM DIFFERENT *Melaleuca quinquenervia* CHEMOTYPES AND LABORATORY HOST SPECIES

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Abstract—The weevil *Oxyops vitiosa* is an Australian species imported to Florida, USA, for the biological control of the invasive species *Melaleuca quinquenervia*. The larvae of this species feed on the leaves of their host and produce a shiny orange secretion that covers their integument. Previous results indicated that a major component of this secretion, viridiflorol, is sequestered from the host plant and repels a generalist predator, the red imported fire ant, *Solenopsis invicta*. When the larvae fed on a different chemotype of *M. quinquenervia*, which lacked viridiflorol but was rich in a different sesquiterpene, (*E*)-nerolidol, similar protection occurred. Solvent washes of these larvae indicated that (*E*)-nerolidol was sequestered from *M. quinquenervia* leaves and repelled *S. invicta* workers when applied to dog food baits at physiological concentrations (17.5, 35.0, and 52.5 μg/mg). Additionally, β-caryophyllene also repelled *S. invicta* workers when applied to dog food baits at concentrations that approximated those in the *O. vitiosa* larval secretions (3.5 and 35 μg/mg). When the *O. vitiosa* larvae were fed leaves from laboratory hosts (nonfield hosts), similar repellent activity was found. This activity was traced to several of the same compounds (e.g., 1,8-cineole, viridiflorol) found to be active in their field host

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M. quinquenervia. These weevil larvae are opportunistic, sequestering the primary terpenoids in their host leaves that confer antipredator activity.

Key Words—Antipredator defense, biological control of weeds, β-caryophyllene, Formicidae, *Melaleuca quinquenervia*, Myrtaceae, *Myrica cerifera*, Myricaceae *Oxyops vitiosa*, Curculionidae, plant defensive compounds, red imported fire ant, sequestration, *Solenopsis invicta*, terpenoids, (*E*)-nerolidol.

INTRODUCTION

Many phytophagous insects use chemical defenses against predators, some of which are synthesized, while others are sequestered from their host plant. Those herbivores whose defenses are host plant-derived may be closely linked behaviorally and ecologically to their host plant. By restricting feeding to only these plant species, these herbivores may have a selective advantage over other species by the protection obtained from their hosts from generalist predators. Although not all species that sequester plant defenses are monophagous (Rowell-Rahier and Pasteels, 1992), a continuum of specificity is evident from a few species that feed on a relatively wide host range, to species that are restricted to a small number of related hosts, to highly evolved host specific relationships (Jones et al., 1988). These host-specific herbivores may become dependent upon a single host or a small group of typically related plant species with similar chemistry that confers protection against generalist predators by having defensive compounds available for sequestration. Examples of this close relationship between host plant and herbivore species are numerous and summarized elsewhere (Jones et al., 1988; Bowers, 1990; Rowell-Rahier and Pasteels, 1992).

The Australian weevil *Oxyops vitiosa*, Pascoe (Coleoptera: Curculionidae) was introduced in south Florida, USA, in 1997 for the biological control of the invasive weed *Melaleuca quinquenervia* (Cav.) S. T. Blake (Myrtaceae) (Center et al., 2000). This species has since been established throughout the infested area of Florida. Larvae feed on the young leaves and have a characteristic odor that resembles that of the foliage of their host tree. The bright orange larvae are diurnally active and feed exposed during all instars on the foliage, then drop to the ground where they form a protective pupal cell in the soil (Purcell and Balciunas, 1994). The cuticle of the larvae is completely covered with a shiny oily substance that functions in defense against generalist predators (Montgomery and Wheeler, 2000; Wheeler et al., 2002). The chemical nature of this defense was found to include primarily the sesquiterpenoid viridiflorol and secondarily the monoterpenoids 1,8-cineole and α-terpineol (Wheeler et al., 2002). However, another chemotype of *M. quinquenervia* occurs in Australia (Brophy et al., 1989; Ireland et al. 2002) and Florida (Dray and Wheeler unpublished data), which lacks these compounds but contains significant concentrations of another sesquiterpenoid, (*E*)-nerolidol. The susceptibility of *O. vitiosa* larvae to generalist predators when fed this *M. quinquenervia* chemotype has yet to be determined.

Australian field collections of *Oxyops vitiosa* indicated that this species restricts its feeding to its primary host *Melaleuca quinquenervia* and a few *Melaleuca* relatives (Balciunas et al., 1994). In no-choice laboratory trials, feeding and oviposition by this species occurred on other members of the Myrtaceae, but the larvae only completed development on *M. quinquenervia* (Balciunas et al., 1994). Subsequent research with *O. vitiosa* indicated that suitable laboratory hosts included two species of Australian myrtaceous plants commonly sold commercially in south Florida (Wheeler, unpublished data). These include species of *Callistemon*, a genus whose members are closely related to *Melaleuca* (Byrnes, 1986; Craven , 1999) and include *C. citrina* (Curtis) Skeels and *C. viminalis* (Sol. Ex Gaertner.) G. Don ex Loundon. The taxonomy of these genera is still being revised, and although these were identified as *Melaleuca* spp. by Craven (unpublished data), the more traditional names are included here, as both are described as doubtful species (Craven, 1999). Additionally, *O. vitiosa* larval feeding and development occurred when neonates that had previously been fed *M. quinquenervia* leaves until the third instar were transferred to the unrelated *Myrica cerifera* L. (Myricaceae). A factor that may limit the exploitation of the Australian myrtaceous hosts and North American *M. cerifera* in the field is the availability of compounds, like those of its primary host *M. quinquenervia*, that can be sequestered and, thus, impart protection from generalist predators. The susceptibility of *O. vitiosa* larvae to generalist predators when fed these laboratory hosts has yet to be determined.

Biological control efforts may benefit by recruiting agents that sequester plant defensive compounds that impart protection from generalist predators. Sequestration of plant defenses may be more common than appreciated by biological control practitioners, as this phenomenon is best known from herbivores with narrow host ranges (Jones et al., 1988; Bowers, 1990; but see Rowell-Rahier and Pasteels, 1992). Several weed biological control agents are known to sequester plant defensive compounds, namely the pyrrolizidine alkaloids of the *Senecio jacobaea–Tyria jacobaeae* association (Aplin et al., 1968), the quinolizidine alkaloids of the Scotch broom–aphid association (Wink et al., 1982), and the bisanthraquinone hypericin of the *Hypericum–Chrysolina association* (Rees, 1969). The purpose of this study was to determine the nature of the defensive secretions produced by the larvae of *O. vitiosa* toward the generalist predator *S. invicta* when fed a different *M. quinquenervia* chemotype from that tested previously (Wheeler et al., 2002) and to determine the susceptibility of larvae to *S. invicta* when fed leaves from several laboratory hosts.

METHODS AND MATERIALS

Insects. Larvae of *O. vitiosa* were collected ($N = 20$) from a field site infested with *M. quinquenervia* in Estero, Lee County, Florida, USA. All larvae were

weighed alive prior to analysis. Solvent washes were conducted on fourth instars by dipping each larva in 1 ml of chloroform ($CHCl_3$) for 15 sec. The $CHCl_3$ larval wash was filtered through glass wool and dried over sodium sulfate (Na_2SO_4) prior to analysis by gas chromatography.

Red imported fire ants were collected at the University of Florida, Ft. Lauderdale Research and Education Center, as described previously (Montgomery and Wheeler, 2000; Wheeler et al., 2002). Colonies, consisting of brood, eggs, and workers, were established in plastic boxes (20×12 cm) and provided with deionized water in a glass tube (45×4 mm) plugged with cotton. Ants were fed kibbled dog food (Pedigree Prime) freely for 2–5 days prior to each test. The food was removed 24 hr prior to each test. Newly collected ant colonies were used for each test.

Plants. Leaves of *M. quinquenervia* were collected from Estero, Lee County, Florida. The *M. quinquenervia* plants collected at this site were most often a distinct chemotype that was rich in the sesquiterpenoid (*E*)-nerolidol (Dray et al., unpublished data). This was in contrast to that reported previously where the *M. quinquenervia* chemotype was rich in another sesquiterpenoid, viridiflorol (Wheeler et al., 2002). Leaves of *C. citrina*, *C. viminalis*, and *M. cerifera* were collected from ornamental plants in Ft. Lauderdale, Florida. Tip leaves were clipped from undamaged young trees and brought to the laboratory where they were either fed to *O. vitiosa* larvae or frozen (-10°C) for terpenoid analysis. The leaf components were extracted by a modified microwave technique (Southwell et al., 1995; Degen and Stadler, 1998; Gomez et al., 1999; Wheeler et al, 2002). Fresh leaves (75–150 mg fresh weight) were immersed in 1 ml EtOH (95%) and irradiated with microwaves (750 W) for 60 sec. An aliquot (500 μl) of the EtOH extract was mixed with deionized water (500 μl), followed by 500 μl of $CHCl_3$. The sample was vortexed for 1 min and then centrifuged for 10 min at 10,000 rpm. An aliquot (200 μl) of the $CHCl_3$ layer was removed and dried over Na_2SO_4. Internal standards were added as 10 ng/μl each of *n*-tridecane and *n*-eicosane in 10 μl $CHCl_3$ to the extract prior to analysis by gas chromatography.

Chemicals. Terpenoids were purchased from commercial sources, except where mentioned, and were of the highest purity available. The list included the primary compounds reported by Brophy et al. (1989), Ramanoelina et al. (1994), and Wheeler et al. (2002). These included (+)-α-pinene (purity = 98%), (−)-β-pinene (99%), α-terpinene (98%), (−)-limonene (92%), 1,8-cineole (99%), γ-terpinene (98%), terpinen 4-ol (97%), α-terpineol (98%), β-caryophyllene (98%), and (*E*)-nerolidol (95%). Viridiflorol (86%) was extracted from *M. quinquenervia* foliage and the compound 2,4-dihydroxy-6-methoxytoluene was synthesized by one of the authors (I.A.S.).

Gas Chromatography. Samples were analyzed with either a Hewlett-Packard model 6890 or 5890 gas chromatograph. Data collection, storage, and analysis were conducted with the ChromPerfect data system. Helium at a linear flow rate

of 37 cm/sec was used as a carrier gas. Samples were analyzed on three fused silica capillary columns [HP-5 (Hewlett Packard Company, Wilmington, Delaware, USA) DB-17MS, and a DB-WAXetr (J & W Scientific, Folsom, California, USA)] all 30 m $\times$ 0.32 mm ID, 0.25-μm thick film]. Injector temperature was 200°C and FID temperature was 250°C. The oven temperature was held at 50°C for 2 min and then increased 8°C/min to 250°C where it was held for 5 min. Compounds were quantified relative to the peak areas of the internal standard, and values were adjusted by calculation of their response factors relative to *n*-tridecane (Debbrecht, 1985).

Compound identities were confirmed by GC-MS using a Hewlett Packard 6890 instrument fitted with an HP-5MS (30.3 m $\times$ 0.25 mm, 0.25-μm film thickness) FSOT column with helium (36 cm/sec) as a carrier gas, injector port (split 1:50) at 250°C, mass selective detector (HP 5973) at 250°C (source) and 150°C (quad), with transfer line at 280°C and ion source filament voltage of 70 eV. Component identification was made on the basis of mass spectral fragmentation, retention index with *n*-paraffins, and comparison with authentic constituents and mass spectral and retention matching with commercial (NIST, Wiley, and Adams) libraries.

Bioassay of Defensive Secretions. The material washed from the *O. vitiosa* larvae or select commercial terpenoids was dissolved in 50 μl of $CHCl_3$ and applied to kibbled dog food cut into 50-mg pieces. The solvent was allowed to dry for 0.5 hr in a fume hood under ambient conditions. The treated dog food bait was stored for 12–15 hr at 4°C. Each test consisted of offering the ants a choice between a solvent ($CHCl_3$)- treated control and either a crude larval wash or a select commercial terpenoid formulated at three concentrations. Previous results (Wheeler et al., 2002) indicated that the percent loss of the terpenoids during the experiment was from 53.9% ($\pm$2.5%; viridiflorol) to 93.7% ($\pm$0.9%; α-pinene).

Five choice tests were conducted, all under ambient conditions between 9 AM to 3 PM. For all tests, ant consumption was determined by comparing the dog food mass before and after each 6-hr test. Consumption values were adjusted by measuring the mass change in similar dog food pieces that were adjacent to, but not exposed to, fire ants. Additionally, the number of ants visiting each dog food bait was counted at regular intervals. In study 1, choice tests were conducted between a $CHCl_3$-treated control and a crude extract of larvae fed leaves from *M. quinquenervia* ($N = 8$). Prior to the larval solvent wash, the chemotype [(rich in either (*E*)-nerolidol or viridiflorol)] of the plant from which the larva was collected was confirmed by GC analysis of a leaf sample. In study 2, choice tests were conducted between a $CHCl_3$-treated control and the primary terpenoid found from GC analysis of the larval washes ($N = 5$). This sesquiterpenoid, (*E*)-nerolidol, was formulated (50, 100, and 150 μg/mg) to approximate the range of concentrations (32.1–141.4 μg/mg larva) of this compound found in the crude larval washes (Table 1). In study 3, a similar choice test was conducted between a $CHCl_3$-treated control and

TABLE 1. COMPARISON OF CONCENTRATION OF 11 TERPENOID COMPONENTS FROM $CHCl_3$-EXTRACTED *Melaleuca quinquenervia* LEAVES, NEROLIDOL CHEMOTYPE ($N = 15$) OR $CHCl_3$-WASHED *Oxyops vitiosa* LARVAE ($N = 15$)

Compound	Concentration (μg/mg, mean ± SE)		F	df	P
	Leaf extract	Larval wash			
α-pinene	0.06 ± 0.01	0.07 ± 0.05	0.20	1,27	>0.5
β-pinene	0.01 ± 0.00	0.00 ± 0.00	0.30	1,27	>0.5
α-terpinene	tr	tr			
limonene	0.03 ± 0.01	0.02 ± 0.02	0.60	1,27	>0.4
1,8-cineole	0.01 ± 0.00	0.01 ± 0.01	0.90	1,27	>0.2
γ-terpinene	tr	tr			
terpinen 4-ol	tr	tr			
α-terpineol	tr	tr			
β-caryophyllene	1.71 ± 0.24	3.35 ± 1.28	1.48	1,27	>0.2
(*E*)-nerolidol	28.34 ± 3.74	70.30 ± 7.01	26.74	1,27	<0.001
viridiflorol	0.20 ± 0.04	0.76 ± 0.17	10.33	1,27	<0.001

[a] Results of ANOVA are presented for comparisons between the leaf extract and larval washes for each compound. Values were estimated by gas chromatography. tr = trace.

β-caryophyllene formulated at 0.35, 3.5, and 35.0 μg/mg, which approximated the range of this compound recovered from larval washes (0.2–29.3 μg/mg larva). In study 4, a similar choice test was conducted between a $CHCl_3$-treated control and 2,4-dihydroxy-6-methoxytoluene formulated at 0.2, 2.0, and 20.0 μg/mg, which approximated the range of this compound recovered from larval washes. In study 5, *O. vitiosa* larvae were fed leaves of one of four plant species, and the 4th instars were washed by dipping for 15 sec in $CHCl_3$. These plants included *C. citrina*, *C. viminalis*, *M. cerifera*, and *M. quinquenervia*. Neonates ($N = 10$) of *O. vitiosa* were fed leaves of all the species except *M. cerifera*, which were reared initially on *M. quinquenervia* until the 3rd instar and then switched to *M. cerifera*, on which they fed until the 4th instar. At the time of larval washes, the larvae fed *M. quinquenervia* weighed 95.3 (±3.7)mg, those fed *C. citrina* weighed 52.0 (±3.7)mg, those fed *C. viminalis* weighed 63.7 (±4.2)mg, and those fed *M. cerifera* weighed 40.1 (±2.2)mg. Choice tests were conducted between a $CHCl_3$-treated control and the crude *O. vitiosa* larval washes (1-larval equivalent) applied to a piece of dog food ($N = 5$).

Statistical Analysis. All data, terpenoid extraction yields, *S. invicta* consumption, and the number of fire ants visiting each dog food bait were analyzed with SAS software (SAS Institute, 1990). The terpenoid yields of the leaf extracts and the larval washes were compared with an ANOVA for each terpenoid analyzed. The consumption and the fire ant activity data were analyzed with a Kruskal-Wallis one-way ANOVA on ranks (Conover, 1999).

RESULTS

Chemical Analysis. Melaleuca quinquenervia *Leaves and Larvae.* The terpenoid constituents of the leaf extracts were quantitatively and qualitatively similar to the larval washes (Table 1; Figure 1). The major constituents recovered from both the leaf extracts and the larval washes included the sesquiterpenoids β-caryophyllene, and (*E*)-nerolidol and the phenol 2,4-dihydroxy-6-methoxytoluene.

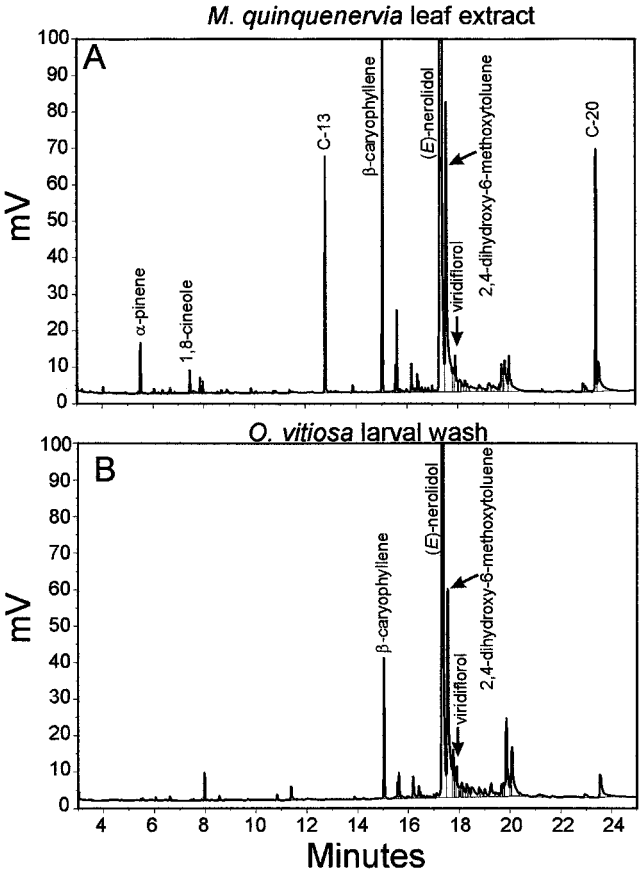


FIG. 1. Representative gas chromatographs of *Melaleuca quinquenervia* [(*E*)-nerolidol-rich chemotype] leaves (A) and washes from *Oxyops vitiosa* fourth instars fed these leaves (B) analyzed with a DB17-MS capillary column (see Methods and Materials for details on GC analyses). Leaves were digested in 95% EtOH by 60 sec microwave irradiation. Larval washes consisted of dipping fourth instar larvae in $CHCl_3$ for 15 sec. The internal standards *n*-tridecane and *n*-eicosane (10 ng/μl) were added to the leaf extract only.

The concentration of the predominant sesquiterpenoids (*E*)-nerolidol and viridiflorol increased significantly in the larval washes compared with the leaf extractions (Table 1). This suggests that as the *O. vitiosa* larvae consume *M. quinquenervia* foliage, they absorb and retain these less volatile foliar terpenoids on their cuticle. The concentration of the 2,4-dihydroxy-6-methoxytoluene was not determined in either the leaves or the larvae; however, they comprised as much as 4.6 and 9.7%, respectively, of the total ion count of the samples. The identity of numerous later-eluting constituents from leaf and larval samples was not investigated even though GC-MS data and spectral library matches (>90%) indicated fatty acids (e.g., palmitic acid), waxes, and the polyphenol 5,7-dihydroxyflavanone or its chalcone equivalent (MW 256) with two of its aromatic ring C- or O-methyl homologues (MW 270). The larval weights averaged (±SE) 56.8 (±6.3)mg, and the amount of material removed by washing each larva averaged 4.3 (±0.5)mg.

Laboratory Host Leaves and Larvae. As in the previous analysis, considerable similarity in terpenoid profiles occurred among the leaf extracts of each species and the washes of the larvae that ate those leaves (Table 2). However, unlike the previous study, none of the terpenoids increased in concentration in the larval washes compared with the leaf extracts. Several of the same terpenoids reported from *M. quinquenervia* leaves were also found in the leaves of *C. citrina*, *C. viminalis*, and *M. cerifera*. Not surprisingly, the terpenoid profiles from leaves of *C. citrina* and *C. viminalis* were similar to one another (Table 1) and to those of the other chemotype of *M. quinquenervia* dominated by viridiflorol (Wheeler et al.,

TABLE 2. COMPARISON OF CONCENTRATION OF 11 TERPENOID COMPONENTS FROM $CHCl_3$-EXTRACTED *Callistemon citrina*, *C. viminalis*, AND *Myrica cerifera* LEAVES ($N = 10$) OR $CHCl_3$-WASHED *Oxyops vitiosa* LARVAE ($N = 3$)[a]

	Concentration (μg/mg, mean ± SE)					
	C. citrina		*C. viminalis*		*M. cerifera*	
Compound	Leaf extract	Larval wash	Leaf extract	Larval wash	Leaf extract	Larval wash
α-Pinene	6.04 ± 0.33	0.86 ± 0.43	2.45 ± 0.25	1.23 ± 0.66	1.75 ± 0.31	0.06 ± 0.001
β-Pinene	0.43 ± 0.02	0.05 ± 0.01	0.16 ± 0.02	0.07 ± 0.03	0.14 ± 0.03	0.01 ± 0.002
α-Terpinene	0.01 ± 0	0.00 ± 0	0.01 ± 0	0.00 ± 0.002	0.02 ± 0	0.00 ± 0.002
Limonene	0.45 ± 0.02	0.04 ± 0.02	0.39 ± 0.04	0.06 ± 0.03	0.08 ± 0.01	0.01 ± 0.001
1,8-Cineole	2.92 ± 0.13	0.52 ± 0.27	2.59 ± 0.27	0.61 ± 0.20	0.48 ± 0.12	0.14 ± 0.09
γ-Terpinene	0.01 ± 0	0.00 ± 0.003	0.04 ± 0.01	0.01 ± 0.004	0.19 ± 0.04	0.01 ± 0.001
Terpinen 4-ol	0.03 ± 0.01	0.03 ± 0.03	0.02 ± 0.01	0.06 ± 0.004	0.06 ± 0.01	0.04 ± 0.04
α-Terpineol	0.06 ± 0.01	0.04 ± 0.02	0.29 ± 0.06	0.17 ± 0.09	0.05 ± 0.02	0.09 ± 0.05
β-Caryophyllene	0.17 ± 0.01	0.01 ± 0.01	0.14 ± 0.04	0.05 ± 0.01	0.47 ± 0.09	0.19 ± 0.07
(*E*)-Nerolidol	0.00	0.00	0.00	0.00	1.32 ± 0.27	0.23 ± 0.17
Viridiflorol	0.00	0.00	0.00	0.00	0.00 ± 0.00	5.19 ± 1.29

[a] Values estimated by gas chromatography.

2002). However, there also was considerable similarity in the terpenoid profiles of these species, especially the previously analyzed chemotype of *M. quinquenervia* (Wheeler et al., 2002) and the unrelated *M. cerifera*. The terpenoids α-pinene, β-pinene, limonene, 1,8-cineole, α-terpineol, and β-caryophyllene were present in the leaves of the test plants and the larvae that ate those leaves. Although the sesquiterpenoids (*E*)-nerolidol and viridiflorol were apparently absent from the foliage of both *C. citrina* and *C. viminalis*, (*E*)-nerolidol was present in *M. cerifera* leaves and the washes of the larvae fed these leaves (Table 2). Possibly, viridiflorol was also present in three of the 10 samples of *M. cerifera* leaves analyzed by GC alone, but this was not confirmed by GC-MS. Thus, the origin of the viridiflorol in the larval washes of larvae fed *M. cerifera* leaves is curious. However, it must be noted that the larvae do not survive from egg hatch to pupation on *M. cerifera* leaves and that they were raised during the first two instars on leaves of the chemotype of *M. quinquenervia* dominated by viridiflorol and then switched to *M. cerifera* for the remainder of their development (see *Methods and Materials*). It is possible that the viridiflorol recovered from these larvae was sequestered while feeding on *M. quinquenervia* leaves and retained until the larval wash during the fourth instar. Similarly, the phenol 2,4-dihydroxy-6-methoxytoluene, although not found from the leaves of *M. cerifera*, was recovered from the larvae that ate the leaves of this species. As with the larvae fed *M. quinquenervia*, this compound also was retained by the larvae at relatively high concentrations (8.6% of the total ion count) from their previous diet. The larval weights averaged 44.5 ($\pm$14.4)mg for larvae fed *C. citrina*, 79.9 ($\pm$5.7)mg for larvae fed *C. viminalis*, and 54.2 ($\pm$4.5)mg for larvae fed *M. cerifera*. The amount of material removed by washing each larva averaged 3.9 ($\pm$1.5)mg for larvae fed *C. citrina*, 4.4 ($\pm$0.8)mg for larvae fed *C. viminalis*, and 4.7 ($\pm$1.4)mg for larvae fed *M. cerifera*.

Bioassay of Larval Secretions

Crude Larval Washes. Fire ant consumption was greater ($\chi_1^2 = 7.1930$; $P < 0.001$) on the control dog food bait (treated with $CHCl_3$), as was fire ant visitation after 15 min ($\chi_1^2 = 12.9293$; $P < 0.001$), 3 hr ($\chi_1^2 = 8.4540$; $P < 0.001$), and 6 hr ($\chi_1^2 = 12.9293$; $P < 0.001$), compared with that treated with the *O. vitiosa* larval wash. During the 6-hr test, the fire ants consumed on average 3.7 ($\pm$0.4)mg dog food in the controls compared with 2.4 ($\pm$0.1)mg in the larval wash-treated dog food. Additionally, fire ant visits to the control dog food averaged 13.5 ($\pm$0.9)ants, 1.9 ($\pm$0.6)ants, and 8.3 ($\pm$0.9)ants, during the 15-min, 3-hr, and 6-hr observations, respectively, whereas they were never observed visiting the larval wash-treated dog food. Despite this apparent lack of fire ant visitation to the treated dog food, some mass loss occurred. This suggests that either consumption of the treated dog food occurred between observations or the adjustment made for mass changes was inaccurate (see *Methods and Materials*).

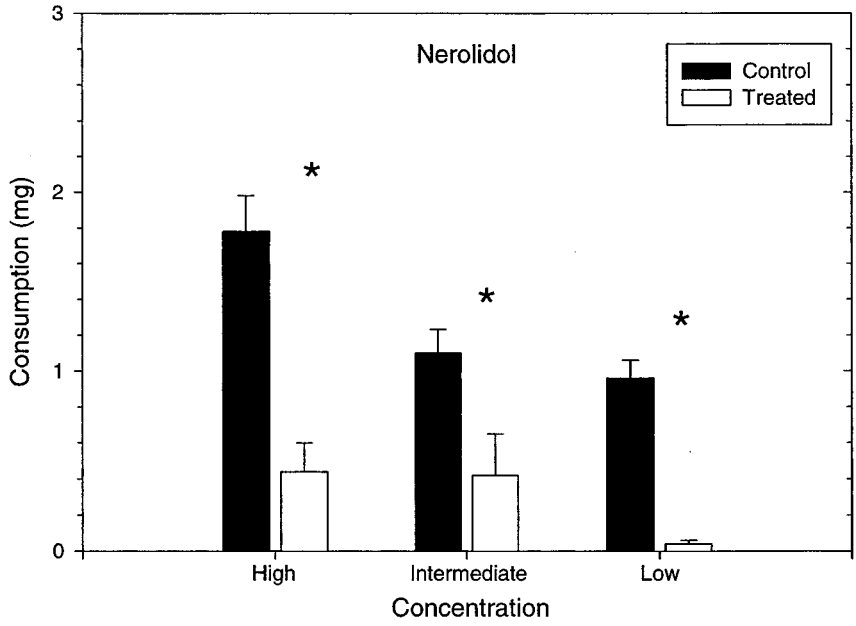
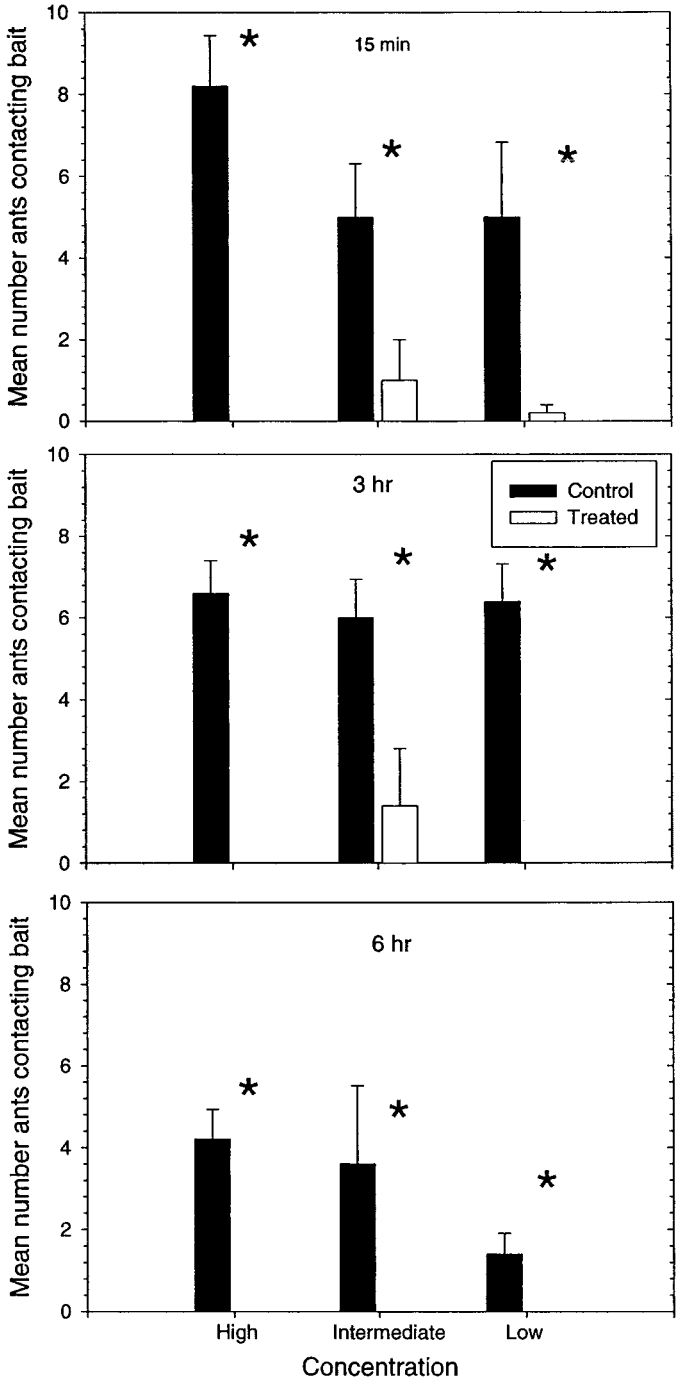


FIG. 2. Consumption mean (±SE) by *Solenopsis invicta* workers offered a choice between a dog food bait treated with $CHCl_3$ (control) and the major terpenoid (*E*)-nerolidol found from $CHCl_3$-washed *Oxyops vitiosa* larvae. The range of concentrations applied approximated that recovered from single larval washes. Asterisks indicate significant differences between the control and treated means. Consumption was significantly reduced in high ($\chi_1^2 = 6.8182$; $P < 0.001$), intermediate ($\chi_1^2 = 2.8981$; $P < 0.001$), and low ($\chi_1^2 = 7.0313$; $P < 0.001$) formulations.

(E)-Nerolidol. Fire ant consumption and visitation of dog food treated with three concentrations of the primary terpenoid (*E*)-nerolidol were decreased compared with the solvent-treated controls. In the high, intermediate, and low formulations, consumption was reduced compared with that of the control-treated dog food (Figure 2). Furthermore, fire ant visitation was reduced (Figure 3) during the 15-min observation period in the high, intermediate, and low (*E*)-nerolidol treatments compared with the solvent-treated controls. Differences were also found 3 hr after the study began in the high, intermediate, and low concentrations. Finally, fewer fire ants were observed visiting the (*E*)-nerolidol-treated dog food after 6 hr on the high, intermediate, and low concentrations.


15 min
3 hr
6 hr
Control
Treated
Mean number ants contacting bait
Concentration
High
Intermediate
Low
0
2
4
6
8
10
*

β-Caryophyllene. Fire ant visitation of dog food treated with the highest concentration of β-caryophyllene (35 μg/mg) decreased during all observations (Figure 4), and visitation of the dog food treated with the intermediate concentration (3.5 μg/mg) decreased only during the first two observation periods. The lowest concentration (0.35 μg/mg) did not reduce ant visitation during any of the observation periods. Fire ant consumption was not reduced in any of the treatments.

2,4-Dihydroxy-6-Methoxytoluene. The phenol, 2,4-dihydroxy-6-methoxytoluene, apparently had no repellent activity against fire ants, as neither worker consumption nor visitation differed between the control and the treated dog food at all concentrations.

Laboratory Hosts. When the crude washes from larvae fed the different plant species were applied to dog food, fire ant visitation generally decreased (Figure 5). However, consumption by fire ants was not significantly different between the solvent-treated controls and treated dog food bait. Fire ant visitation was reduced after 15 min in those treated with crude extracts from larvae fed each plant species. Similar results occurred after 3 hr and 6 hr.

DISCUSSION

Repellency of O. vitiosa Regardless of Host. These results demonstrate the repellent nature of the larval secretions of the *M. quinquenervia* biological control agent *O. vitiosa* fed the chemotype rich in (*E*)-nerolidol and laboratory host species toward the red imported fire ant *S. invicta*. Our results indicated repellent activity from the crude larval washes, the primary terpenoids (*E*)-nerolidol and β-caryophyllene, and crude larval washes from larvae fed leaves of *C. citrina*, *C. viminalis*, and *M. cerifera*. In all experiments except that with the phenol 2,4-dihydroxy-6-methoxytoluene, fire ant visitation to the treated baits decreased significantly compared with the solvent-treated controls. The repellency of

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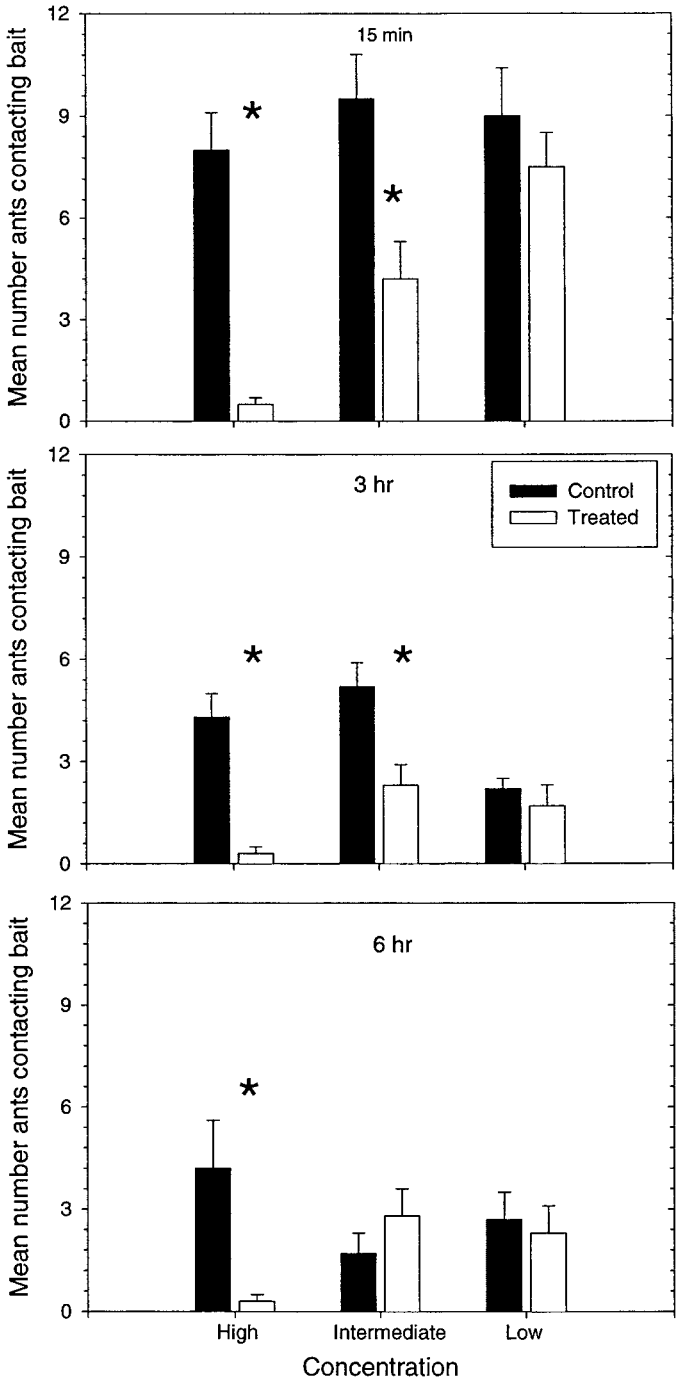
FIG. 3. Number mean (±SE) of *Solenopsis invicta* workers visiting dog food bait treated with $CHCl_3$ (control) and the major terpenoid (*E*)-nerolidol $CHCl_3$-washed from *Oxyops vitiosa* larvae. The range of concentrations applied (high: 150; intermediate: 100; and low: 50 μg/mg) approximated the range recovered from single larval washes. Asterisks indicate significant differences between the control and treated means. Fire ant visitation was significantly reduced after 15 min in the high ($\chi_1^2 = 7.7586$; $P < 0.001$), intermediate ($\chi_1^2 = 3.8118$; $P < 0.001$) and the low ($\chi_1^2 = 7.3052$; $P < 0.001$), after 3 hr in the high ($\chi_1^2 = 7.8125$; $P < 0.001$), intermediate ($\chi_1^2 = 3.8118$; $P < 0.001$), and the low ($\chi_1^2 = 7.7586$; $P < 0.001$) concentrations and after 6 hr in the high ($\chi_1^2 = 5.4482$; $P < 0.001$), intermediate ($\chi_1^2 = 5.5814$; $P < 0.001$), and the low ($\chi_1^2 = 5.5814$; $P < 0.001$) concentrations.

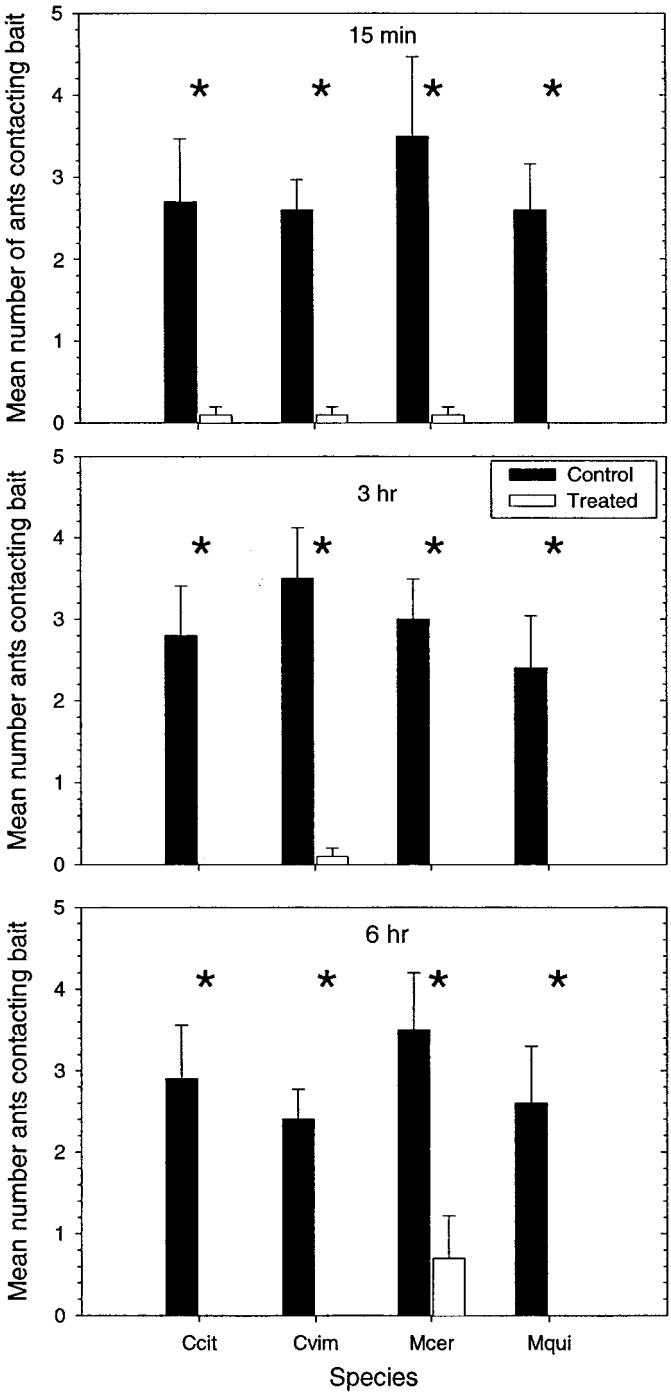

15 min
3 hr
6 hr
Control
Treated
Mean number ants contacting bait
0
3
6
9
12
*
High
Intermediate
Low
Concentration

(*E*)-nerolidol was demonstrated throughout the duration of the experiment (6 hr) at all concentrations that approximated those recovered from larval washes. Another sesquiterpenoid, β-caryophyllene, when tested at relatively high and intermediate larval concentrations was repellent for 6 and 3 hr, respectively.

The larvae fed leaves from *C. citrina* and *C. viminalis* apparently lacked the two sesquiterpenoids (*E*)-nerolidol and viridiflorol that were the most repellent compounds found here and in a previous study (Wheeler et al., 2002). However, the *O. vitiosa* larval washes of individuals fed leaves of these two laboratory host species were repellent to *S. invicta*. Possibly, in live larvae, the more volatile terpenoids found would be responsible for repelling *S. invicta*. These components may include α-pinene, limonene, and 1,8-cineole that may evaporate more quickly from dog food bioassays, whereas the less volatile components like β-caryophyllene, (*E*)-nerolidol, and viridiflorol may remain at repellent concentrations throughout the experiment. Larvae fed leaves of *C. citrina* and *C. viminalis* may constantly replenish these more volatile components and thereby maintain their repellent concentration(s). However intriguing, this hypothesis has yet to be tested. Alternatively, the unidentified flavonoids from all species may also be repellent. However, these compounds were not tested. Possibly there are more compounds yet to be investigated with potential for repellent activity.

Although terpenoids occur widely in both gymnosperms and angiosperms (Seigler, 1981), each plant species or population may have a distinct terpenoid profile (Gershenzon and Croteau, 1991). As expected from species in closely related genera (Gibbs, 1974; Cronquist, 1981; Young and Seigler, 1981; Brophy and Doran, 1996; Southwell and Lowe, 1999), considerable chemical similarity was found among the species of the Myrtaceae sampled. However, quantitative and qualitative differences were evident among species in our analyses. The importance of these terpenoids for selection and utilization by host-specific biological control agents is a topic of another study (Wheeler, unpublished data). Like the weed *M. quinquenervia*, the ornamental *C. viminalis* has at least two described chemotypes (Brophy et al., 1985, 1997). Although the results of *C. viminalis* terpenoid profiles do not closely match those of either Australian chemotype, the ornamental

FIG. 4. Number mean (±SE) of *Solenopsis invicta* workers visiting dog food bait treated with $CHCl_3$ (control) and the terpenoid β-caryophyllene $CHCl_3$-washed from *Oxyops vitiosa* larvae. The range of concentrations applied (high: 35.0; intermediate: 3.5; and low: 0.35 μg/mg) approximated the range recovered from single larval washes. Asterisks indicate significant differences between the control and treated means. Fire ant visitation was significantly reduced at the high concentrations after 15 min ($\chi_1^2 = 8.6715$; $P < 0.001$), 3 hr ($\chi_1^2 = 8.6715$; $P < 0.001$), and 6 hr ($\chi_1^2 = 7.8204$; $P < 0.001$). Visitation of the dog food treated with the intermediate concentration decreased significantly only during the first two observation periods (15 min; $\chi_1^2 = 5.0610$; $P < 0.001$; 3 hr: $\chi_1^2 = 5.4870$; $P < 0.001$).


15 min
3 hr
6 hr
Control
Treated
Mean number of ants contacting bait
Mean number ants contacting bait
Mean number ants contacting bait
0
1
2
3
4
5
*
Ccit
Cvim
Mcer
Mqui
Species

variety analyzed here was most similar to the chemotype that was lower in 1,8-cineole (24.1% of total area), most prevalent in the more tropical latitudes (Brophy et al., 1997).

The leaf extracts and the washes of larvae fed leaves of the unrelated *M. cerifera* had relatively high concentrations of β-caryophyllene and (*E*)-nerolidol. Considering that these terpenoids were present in the larval washes, it was not surprising that they were also repellent to *S. invicta*. However, it was surprising that *M. cerifera*, a North American species unrelated to *M. quinquenervia*, apparently has several terpenoids in common with *M. quinquenervia*. Possibly, the presence of these terpenoids function as behavioral cues for *O. vitiosa* to recognize and initiate oviposition and feeding by this specialist. This could explain the adult feeding and oviposition results reported in no-choice tests on this species during host range testing (Balciunas and Buckingham, unpublished data). However, *M. cerifera* is a nutritionally inadequate host plant of this herbivore species, as the neonates do not survive to the adult stage (Balciunas and Buckingham, unpublished data, Wheeler, in preparation).

Sequestration. In the secretions of larvae fed *M. quinquenervia*, the concentration (based on fresh mass) of (*E*)-nerolidol was more than twice that of the host foliage, suggesting that larvae sequester it from leaves. A similar increase was found with viridiflorol both here and in a previous study where larvae were fed a chemotype rich in this sesquiterpenoid (Wheeler et al., 2002). As in the previous study (Wheeler et al., 2002), the most active ant repellent compounds increased the most from plant to larvae. The proportion of the terpenoids retained by the larvae on the cuticle can be calculated and expressed as the conversion efficiency for each ingested terpenoid (ECI_t), analogous to the calculation of the ingested food conversion efficiency (ECD_f, of Slansky and Scriber (1985). The ECI_t values were estimated as described previously (Wheeler et al., 2002). To determine if select terpenoids were actively sequestered and deposited on the larvae, the conversion efficiency of (*E*)-nerolidol washed from the cuticle (ECI_t) can be compared with the

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FIG. 5. Number mean (±SE) of *Solenopsis invicta* workers visiting dog food bait treated with $CHCl_3$ (control) and each of the crude $CHCl_3$-washes from *Oxyops vitiosa* larvae fed the different plant species (Ccit: *Callistemon citrina*; Cvim: *Callistemon viminalis*; Mcer: *Myrica cerifera*; Mqui: *Melaleuca quinquenervia*). Asterisks indicate significant differences between the control and treated means. Fire ant visitation was reduced after 15 min (*C. citrina*: $\chi_1^2 = 10.1947$; $P < 0.001$; *C. viminalis*: $\chi_1^2 = 15.2444$; $P < 0.001$; *M. cerifera*: $\chi_1^2 = 12.4189$; $P < 0.001$; *M. quinquenervia*: $\chi_1^2 = 14.0036$; $P < 0.001$) 3 hr (*C. citrina*: $\chi_1^2 = 13.9024$; $P < 0.001$; *C. viminalis*: $\chi_1^2 = 15.4667$; $P < 0.001$; *M. cerifera*: $\chi_1^2 = 13.9529$; $P < 0.001$; *M. quinquenervia*: $\chi_1^2 = 13.9402$; $P < 0.001$) and after 6 hr (*C. citrina*: $\chi_1^2 = 11.6923$; $P < 0.001$; *C. viminalis*: $\chi_1^2 = 16.4502$; $P < 0.001$; *M. cerifera*: $\chi_1^2 = 8.0150$; $P < 0.001$; *M. quinquenervia*: $\chi_1^2 = 14.0036$; $P < 0.001$).

conversion efficiency of the food (ECI_f). The results of these calculations suggest that more than one third ($34.9 \pm 7.3\%$) the quantity consumed was retained and deposited on the larval cuticle. These results are consistent with the interpretation that (*E*)-nerolidol was actively sequestered, as this value was considerably greater than the conversion efficiency value for food ($ECI_f = 7.3\%$). However, an alternative interpretation suggests that greater concentrations of these compounds may be induced in the leaves by insect feeding. Instead of selectively accumulating these compounds, the insects feeding on these induced leaves may passively sequester these higher levels of terpenoids. This induced response, as found in other species (e.g., Turlings et al., 1990; Loughrin et al. 1995), has yet to be demonstrated for *M. quinquenervia*.

Sequestration and Biological Control. The ecological host range of this biological control agent may be influenced by the availability of foliar compounds that serve as effective repellents against generalist predators. Larvae may escape from predators and become dependent upon plant species that have the compounds that can be sequestered for antipredator defense (Jeffries and Lawton, 1984). For example, the cuticular waxes of *Manduca sexta* were influenced by different plant species or an artificial diet (Espelie and Bernays, 1989), and these differences influenced the susceptibility of the larvae to ant predation (Cornelius and Bernays, 1995). Laboratory studies with *O. vitiosa* indicated that, in addition to the preferred host *M. quinquenervia*, several *Callistemon* species were suitable laboratory hosts (Balciunas and Buckingham, unpublished data; Wheeler, unpublished data). However, in the presence of predators, field studies conducted in Australia prior to introduction of *O. vitiosa* into the United States suggested that it was a narrow specialist, collected only from *M. quinquenervia* (Balciunas et al., 1994). This observation led us to test the hypothesis that the field host range of *O. vitiosa* was restricted to *M. quinquenervia* because of specific sequestered terpenoids. Contrary to our predictions, the results indicated that *O. vitiosa* larvae were equally protected from the South American predator *S. invicta* regardless of the test plant consumed, possibly because of the similarity in terpenoid chemistry. Possibly the predominant Australian generalist predators respond differently to *O. vitiosa* larvae that feed and sequester compounds from other host species.

Predators are well known antagonists of arthropods used for biological control of weeds (Crawley, 1989; Julien and Griffiths, 1998), and predation is so common that it may be assumed that it likely influences the establishment of all agents (Goeden and Louda, 1976). In general, natural enemies, including predators, parasitoids, and diseases interfere with approximately half the biological control projects (Goeden and Louda, 1976). This *M. quinquenervia* biological control agent *O. vitiosa* is apparently well protected from one of the most common invertebrate generalist predators in the southeastern United States (Lofgren et al., 1975; Elvin et al., 1983; Kharboutli and Mack, 1991; Montgomery and Wheeler, 2000; Wheeler et al., 2002), and this ant species has been previously implicated in

reducing the efficacy of *Tyta luctuosa* (Denis & Schiffermüller) (Lepidoptera: Noctuidae), a biological control agent of field bindweed (Ciomperlik et al., 1992). However, this *M. quinquenervia* biological control agent has become well established in Florida (Center et al., 2000). Several factors have contributed to its success; among them is the integumental terpenoid defense that is flexible and adaptable to sequestering defensive compounds from different *M. quinquenervia* chemotypes.

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CYNIPID GALL-WASP COMMUNITIES CORRELATE WITH OAK CHEMISTRY

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Abstract—Host-plant association data, gathered from field surveys conducted throughout Florida and from the literature, were used to identify the specificity of cynipid gall inducers to one or more of six *Quercus* species that occur at Archbold Biological Station, Lake Placid, Florida, USA, including the red oaks *Q. laevis*, *Q. myrtifolia*, and *Q. inopina*, and the white oaks *Q. chapmanii*, *Q. geminata*, and *Q. minima*. *Quercus myrtifolia* had the highest cynipid richness and diversity (37 cynipid species, Shannon $H' = 3.61$, Simpson's $D = 0.97$), followed by *Q. chapmanii*, *Q. laevis*, *Q. inopina*, *Q. geminata*, and finally *Q. minima* (10 species, $H' = 2.30$, $D = 0.90$). All cynipid species showed strong fidelity to a particular host plant or a restricted set of host plants. An ordination of gall-wasp host associations indicated that the cynipid communities of each oak species were distinct and specific to a given oak species. Leaf samples taken from each oak species were analyzed for condensed and hydrolyzable tannins, total phenolics, lignin, cellulose and hemicellulose, nitrogen, and carbon. All of these chemical traits, with the exception of carbon, differed by oak species, and the differences were strongly correlated with the axes of the cynipid–species ordination. These results suggest that gall-wasp occurrence is influenced by oak chemistry and imply that experimental studies of cynipid gall inducers that

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examine host-plant chemistry and female oviposition choice and larval performance will yield useful insights.

Key Words—Cynipidae, Fagaceae, gall, herbivore communities, host-plant association, insect–plant interactions, ordination, plant chemistry, *Quercus*.

INTRODUCTION

Gall-inducing insects have long been known to exhibit a high degree of host-plant specificity (e.g., Felt, 1917, 1940; Mani, 1964; Abrahamson and Weis, 1987; Abrahamson et al. 1998a,b). This specificity is, in part, a consequence of the required intimate relationship that must exist between a gall inducer and its host plant if a gall-inducing insect is to stimulate the appropriate host-plant reactions to produce a gall (Shorthouse, 1982; Cornell, 1983; Raman, 1993, 1996; Abrahamson and Weis, 1997). Small differences in factors such as host-plant phenology, physiology, development, and chemistry are crucial to the success of these insects (Horner and Abrahamson, 1992, 1999; Craig et al., 1993, 1999, 2000; How et al., 1993). Gall inducers, for example, discriminate among closely related species of host plants. Studies of hybrids in *Quercus* (Aguilar and Boecklen, 1992), *Salix* (Fritz et al., 1994), and *Populus* (Floate and Whitham, 1995) have demonstrated that gall inducers can distinguish hybrid host plants from parental hosts in hybrid zones. Furthermore, cecidomyiid and tephritid gall inducers discern subspecies of the composite *Chrysothamnus nauseosus* (Floate et al., 1996).

Among gall-inducing insects, cynipid wasps (tribus Cynipini, Cynipidae: Hymenoptera) are noted for their fidelity to their host plants. Approximately 87% of all North American cynipids attack species of *Quercus* (Fagaceae), and the host range of each wasp species is generally restricted to one or a few oak species (Burks, 1979). Elsewhere we have shown that cynipid species distributions reflect similarities and relatedness among their oak hosts and mirror the phylogenetic relationships of oak species (Abrahamson et al., 1998a,b).

Plant chemistry has played an appreciable role in the host shifts and evolutionary radiation of herbivorous insects (Becerra, 1997; Camara, 1997; Berenbaum and Zangerl, 1998; Carter and Feeny, 1999). Although a number of categories of host-plant traits (e.g., morphology, phenology, plant vigor) are known to influence host choice by gall-inducing insects (e.g., Anderson et al., 1989; Eliason and Potter, 2000; Pires and Price, 2000; Yukawa, 2000), we would expect that host-plant chemistry should be among the most important factors, given the specificity of interaction between the gall inducer and its host. Unfortunately, little is known about the importance of plant chemical traits to host-plant choice by gall-inducing insects in spite of some knowledge of the chemical make-up of the galls themselves (Cornell, 1983; Abrahamson et al., 1991; Hartley, 1998). For example, in no cases are ovipositional cues from plants known for cynipids, although they have

been identified for sawfly gall inducers (Kolehmainen et al., 1994; Roininen et al., 1999).

The study reported here represents an initial exploration of the involvement of oak chemistry in host-association patterns and diversity of gall-inducing cynipid wasps. We first asked the extent of overlap and relatedness among the cynipid gall-wasp species inhabiting six species of co-occurring *Quercus*. Next, we asked how the leaf chemistries vary among these oaks and, finally, whether oak chemistry is correlated with the distributions of cynipids. Our results indicate that the host-plant associations of cynipid gall-inducing insects are highly specific, host-plant chemistries vary significantly across oak species, and host-plant chemistry is correlated with the host-plant distributions of cynipid species.

METHODS AND MATERIALS

Cynipid Host-Plant Associations. To ascertain the host-plant associations of Florida cynipid gall inducers, we sampled oaks of varying sizes in a wide variety of vegetative associations for the presence of cynipid gall inducers. Sampling of cynipid gall inducers took place from November 1994 to May 1995 and during January 1996 at least weekly in Highlands County (Archbold Biological Station, MacArthur Agro-ecology Research Center, Avon Park Air Force Range, Josephine Creek, Venus, Highland Hammock State Park, Flamingo Valley), and one or more times in Brevard County (Merritt Island National Wildlife Refuge, Coconut Point Sanctuary, Malabar Scrub Sanctuary, Enchanted Forest Sanctuary), Dade County (Deering Estate Pineland), DeSoto County (Arcadia Town Park), Levy County (Cedar Key), Hernando County (Brooksville Ridge), Jackson County (Florida Caverns State Park), Liberty County (Torreya State Park), Mantee County (Lake Mantee Recreation Area), Martin County (Jonathan Dickinson State Park), Monroe County (Everglades National Park), Okaloosa and Walton Counties (Eglin Air Force Base, Defuniak Spring, Gratin Beach Island), Orange County (Orlando vicinity), Polk County (Bok Tower), Sarasota County (Myakka River State Park), and Wakulla County (Wakulla Spring State Park). In all, 130 species of cynipid gall-inducing insects were recorded from rearings of adult wasps from collected galls or host-association reports from the literature (Weld, 1951, 1952, 1959; Burks, 1979) for *Quercus* known to occur in Florida. Plant nomenclature follows that of the *Flora of North America* (Jensen, 1997; Nixon, 1997; Nixon and Muller, 1997), while insect nomenclature follows Burks (1979). For the present study, we extracted the cynipid association data for six oak species that occur sympatrically at the Archbold Biological Station (ABS) including *Q. laevis*, *Q. myrtifolia*, *Q. inopina*, *Q. chapmanii*, *Q. geminata*, and *Q. minima*.

Study Site. Archbold Biological Station (27°11′N, 81°21′W) is located near the southern terminus of the Florida peninsula's Lake Wales Ridge, 12 km south of the town of Lake Placid. The lowest elevations commonly occupied by oaks

are in flatwoods on poorly drained to somewhat poorly drained soils, while the highest elevations inhabited by oaks are in southern ridge sandhill vegetation on excessively well-drained sands. Between these extremes, oaks occur in scrubby flatwoods and, at slightly higher elevations relative to the water table, in sand pine scrub (Abrahamson et al., 1984).

Flatwoods range from low, open savanna-like associations dominated by *Serenoa repens*, *Aristida stricta*, and scattered *Pinus elliottii* var. *densa* or *P. palustris* to dense pine stands with thick shrub understories of *S. repens*, *Ilex glabra*, *Lyonia lucida*, and *Panicum abscissum*. The evergreen, xeromorphic *Q. minima* and *Q. geminata* are frequent components of flatwoods. Scrubby flatwoods are a low shrubby association dominated by the evergreen, xeromorphic oaks *Q. inopina*, *Q. chapmanii*, *Q. geminata*, and the much more occasional *Q. myrtifolia*, as well as abundant *S. repens* and *Sabal etonia*. The pines, *P. elliottii* var. *densa* and *P. clausa*, are typically widely scattered and occur at variable densities. In contrast, sand pine scrub is characterized by a tree overstory of nearly even-aged *P. clausa*; an intermediate canopy of shrubby trees including *Carya floridana*, *Q. myrtifolia*, *Q. geminata*, *Q. chapmanii*, and occasional *Q. laevis*, and an understory of *S. repens* and *S. etonia*. Also a multilayered community, the southern ridge sandhill has an overstory of *P. elliottii* var. *densa*, an intermediate deciduous canopy of *Q. laevis* and *C. floridana*, and a shrub and understory layer of *Q. myrtifolia*, *Q. geminata*, *Q. chapmanii*, *S. repens*, *S. etonia*, *A. stricta*, and forbs. Details of these vegetative associations are available in Abrahamson et al. (1984), Abrahamson and Hartnett (1990), Myers (1990), and Menges (1999).

The climate of the study area is characterized by hot, wet summers and mild, dry winters. The highest monthly mean temperature (27.5°C) occurs in August and the lowest (16°C) in January. The rainy season normally extends from June through September, with an average of 60% of the annual precipitation (135.1 cm) falling during this 4-month period (Abrahamson et al., 1984).

Chemical Analyses of Oak Leaves. Foliar concentrations of condensed and hydrolyzable tannin, total phenolics, lignin, cellulose, hemicellulose, carbon, and nitrogen were estimated from leaf tissue of our six focal oak species; *Q. myrtifolia*, *Q. inopina*, *Q. laevis*, *Q. geminata*, *Q. chapmanii*, and *Q. minima*. Leaf samples were collected on October 9, 2001, from 10 individuals of each species at ABS from an area of approximately 0.2 ha. Samples for tannin analysis were collected by punching leaf disks, 12 per individual oak tree, directly into 70% acetone in the field. Disks were punched from 12 different leaves per tree. Companion disks were also punched from each leaf for estimates of the dry mass of samples. After the leaf disks had been removed, the same twelve leaves were then removed from each tree for the analysis of lignin, cellulose, hemicellulose, carbon, and nitrogen. Leaves were dried at 65°C for 3 days, ground to a fine powder in a Wiley mill, and stored at −80°C prior to analysis.

Carbon and nitrogen concentrations were determined on an Alpkem flow-injector analyzer and are reported as percentage dry mass. Cellulose, hemicellulose, and lignin concentrations were estimated by sequential neutral detergent/acid detergent digestion on an Ankom fiber analyzer and are also reported as percentage dry mass. We conducted three separate analyses for phenolics; condensed tannins, hydrolyzable tannins, and total phenolics. Samples for tannin analysis were extracted in 70% acetone with 1 mM ascorbic acid and evaporated under reduced pressure to provide aqueous extracts. Given concerns about the purity of tannic acid as a standard in tannin analysis (Hagerman and Butler, 1991), sample extracts were tested against tannin standards (Forkner and Hunter, 2000) prepared from pooled leaves (all six oak species combined). The standards were prepared by collecting bulk samples of fresh leaf material directly into acetone on October 9, as above. Samples were extracted in acetone and then lyophilized to provide a bulk tannin powder. Condensed tannins were estimated as proanthocyanidins using methods described in Rossiter et al. (1988). Hydrolyzable tannins were estimated using a potassium iodate technique developed by Bate-Smith (1977) and modified by Schultz and Baldwin (1982). Total phenolics were estimated using the Folin-Denis assay (Swain, 1979). Because the foliar phenolics of each species were compared against a common tannin standard, the values reported reflect relative indices of concentration, not absolute values. In addition, samples were taken in October, after the majority of cynipid oviposition and feeding have taken place. However, our goal was to explore species-specific differences in foliar chemistry rather than to associate directly the chemistry of foliage with particular samples of cynipid populations on trees. By sampling in October, we avoided the effects of phenological variation among individual trees, associated with leaf-flush, that can have a profound effect of the chemistry of sandhill oaks earlier in the season (Klaper et al., 2001).

Data Analyses. To organize the cynipid communities of the six oak species according to their similarities, we performed a Bray-Curtis ordination using PC-ORD version 4.13 for Windows (McCune and Mefford, 1999; MjM Software Design, PO Box 129, Gleneden Beach, Oregon 97388, USA) on a matrix of presence and absence data for each cynipid species on each oak. We used Bray-Curtis ordination with the Sorensen distance coefficient because of its utility with presence–absence data, retention of sensitivity in heterogeneous data sets, and less weighting of outliers. Variance-regression was used to select the end points of axes (Beals, 1984), and axes were projected with Euclidean geometry. The first three axes of the Bray-Curtis ordination were next correlated with the oak-chemistry variables contained in a second matrix. Axes were scaled proportionate to longest axis (% of maximum) with no rotation.

Although our aim was to understand the distributions of cynipid species according to potential gradients of oak chemistry, we chose to use the indirect gradient analysis of Bray-Curtis ordination because the Bray-Curtis procedure,

unlike the direct gradient analysis of canonical correspondence analysis, does not constrain the ordination by a multiple regression on the measured environmental variables (i.e., oak chemistry). Furthermore, indirect gradient analysis does not presume that all appropriate environmental variables (i.e., oak chemistry) have been measured. By secondarily relating the ordination axes to measured oak-chemistry variables, indirect gradient analysis offers an independent judgment of the significance of the parameters of the secondary matrix (McCune and Mefford, 1999).

PC-ORD was also used to calculate measures of cynipid diversity including species richness (= number of cynipid species recorded for a given oak species), Shannon's diversity index $\{= -\Sigma[p_i{}^* \ln(p_i)]$ of cynipids on each oak species$\}$, and Simpson's index of diversity for an infinite population $[= 1 - \text{sum}\ (p_i{}^* p_i)]$ (where p_i = importance probability in element i) (McCune and Mefford, 1999). The latter measure is the likelihood that two randomly chosen individuals will be different species.

The GLM procedure of SPSS version 11.0.1 (SPSS Inc., Chicago, Illinois, USA) was used to perform a multivariate ANOVA on oak chemistry variables to determine whether oak species varied. This ANOVA was followed by Student-Newman-Keuls *post-hoc* tests to determine which oak species differed. All variables except carbon/nitrogen ratio were measured as percentages and were arcsine square-root transformed to improve normality prior to performing the ANOVA. The reported means characterize the untransformed data, however, statistical results are reported for the transformed variables.

RESULTS

Relationships among Cynipid Communities. Of the 88 cynipid species recorded across the six oak species, the gall-wasp communities on the red oak *Q. myrtifolia* and the white oak *Q. chapmanii* were the richest and most diverse, while the communities on the red oaks *Q. laevis* and *Q. inopina* exhibited intermediate levels of richness and diversity, and the white oaks *Q. geminata* and *Q. minima* showed the lowest levels (Table 1). Elsewhere we have analyzed the correlation between species richness of cynipid gall inducers and biographic and host-plant architectural factors (Price, Abrahamson, Hunter, and Melika, unpublished manuscript).

The ordination of the cynipid occurrences illustrates that cynipid gall wasps have distinctive host associations with their oak hosts, and as a consequence, the cynipid community on each oak species is unique (Figure 1). For example, the distributions of cynipid communities associated with white oaks had lower values on axis 1 (low end point was *Q. minima*), while those on red oaks had higher values on axis 1 (high end point was *Q. myrtifolia*). The end points for axis 2 were *Q. geminata* and *Q. chapmanii*, both white oaks, and the end points for axis 3 were

TABLE 1. SPECIES RICHNESS, SHANNON'S DIVERSITY INDEX, AND SIMPSON'S DIVERSITY INDEX FOR AN INFINITE POPULATION

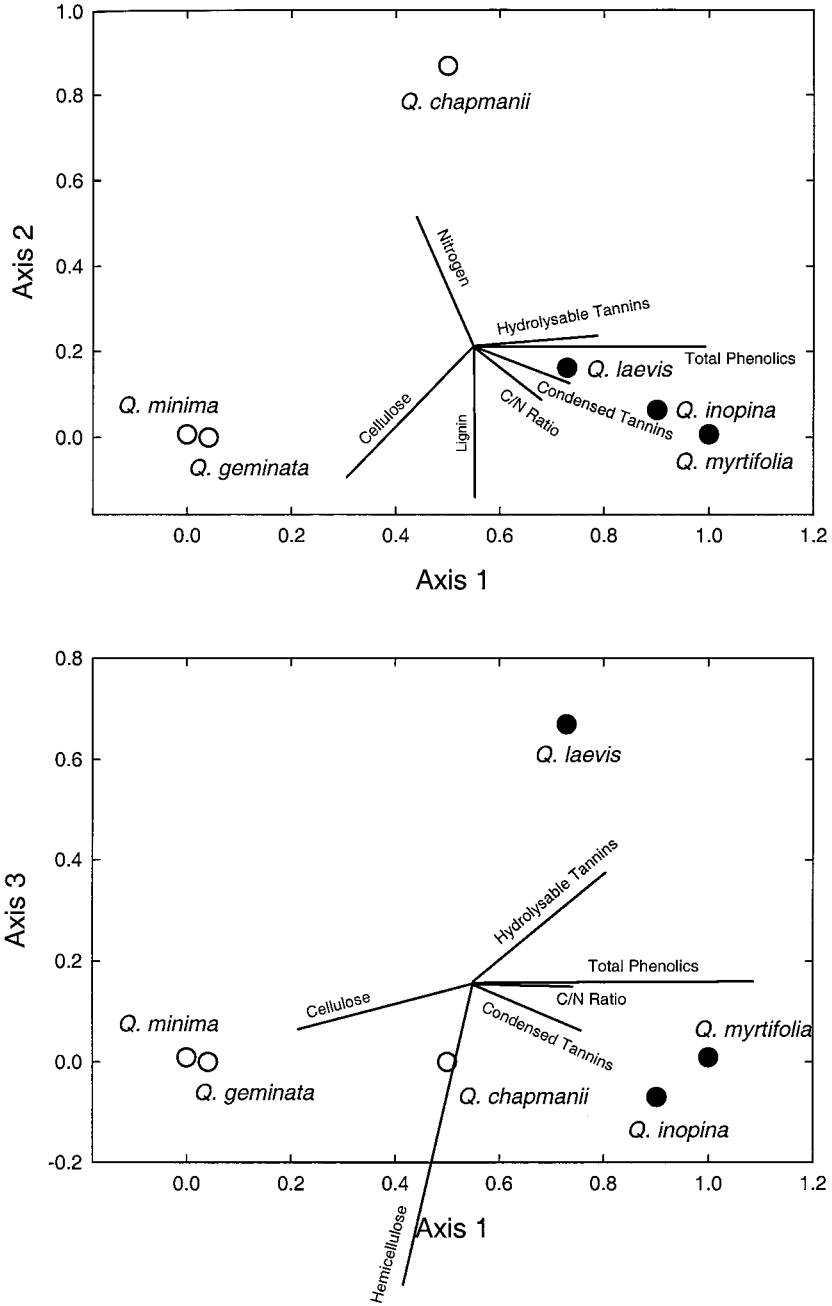
Species	Richness[a]	Shannon's diversity index[b]	Simpson diversity index[c]
Red oaks			
Q. laevis	16	2.77	0.9375
Q. myrtifolia	37	3.61	0.9730
Q. inopina	17	2.83	0.9412
White oaks			
Q. chapmanii	28	3.33	0.9643
Q. geminata	12	2.49	0.9167
Q. minima	10	2.30	0.9000

[a] Number of cynipid species recorded for a given oak species.
[b] $-\Sigma\ (p_i * \ln(p_i)$, where p_i = importance probability in element i of cynipids on each oak species.
[c] 1 − sum $(p_i * p_i)$ (McCune and Mefford, 1999). The Simpson's index is the likelihood that two randomly chosen individuals will be different species.

the red oaks *Q. inopina* and *Q. laevis*. Thus, the first axis separates red oaks and white oaks, the second axis divides white oaks, and the third axis splits red oaks. The distributions of cynipid communities on the three axes followed expectations based on the presumed phylogenies of their oak hosts (e.g., Trelease, 1924; West, 1948; Li and Hsiao, 1973, 1976; Solomon, 1983a,b). For example, the cynipid communities on the closely related *Q. geminata* and *Q. minima* were similar, as were the cynipid communities on the related *Q. myrtifolia* and *Q. inopina*.

The coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space determined an r^2 for axis 1 of 0.442, an r^2 for axis 2 of 0.269, and an r^2 for axis 3 of 0.238. The cumulative r^2 for the three axes was 0.949. These increment and cumulative r^2 values were adjusted for any lack of orthogonality of axes. Thus, axis 1 extracted 44% of the original distance matrix, axis 2 described 27%, and axis 3 explained 24% for a total of 95%.

Chemical Attributes of Oak Leaves. One-way ANOVAs with species nested within oak section showed no significant differences between red oaks and white oaks with the exception of total phenolics. Red oaks had significantly higher total phenolics levels (41.99 ± 1.98) than did the white oaks (28.17 ± 0.99; $F_{1,53} = 12.49$, $P = 0.024$). Consequently, we dropped oak section and reran a single multivariate ANOVA with chemistry measurements as dependent variables and species as a fixed factor, followed by Student-Newman-Keuls *post-hoc* tests. The multivariate results showed that oak species differed chemically (Pillai's trace = 2.678, $F_{45,240} = 6.15$, $P < 0.001$). Furthermore, the between-subjects results demonstrated that all chemical attributes differed significantly among the oak species (Figure 2), with the exception of carbon content, which is not illustrated. Foliar concentrations of condensed tannin were higher in the red oaks *Q. inopina* and


Axis 2
Axis 1
Axis 3
Q. chapmanii
Q. laevis
Q. inopina
Q. myrtifolia
Q. minima
Q. geminata
Nitrogen
Hydrolysable Tannins
Total Phenolics
Condensed Tannins
C/N Ratio
Cellulose
Lignin
Hemicellulose

Q. myrtifolia and lower in *Q. chapmanii*, *Q. geminata*, and *Q. laevis*. Hydrolyzable tannin concentrations were greater in *Q. laevis* and *Q. inopina* and occurred at lower levels in *Q. geminata*. Because of the correlation between condensed tannins and total phenolics (Pearson $r = 0.849$, $P = 0.032$), the pattern for total phenolics nearly paralleled that of condensed tannins. Lignin and cellulose concentrations varied considerably among the oak species with high levels of lignin recorded for the white oak *Q. geminata* and the red oak *Q. myrtifolia*. Cellulose was highest in *Q. geminata* and *Q. minima* and lowest in *Q. chapmanii*. Hemicellulose followed a somewhat different pattern with the highest (*Q. minima, Q. geminata, Q. inopina, Q. laevis*) and lowest levels (*Q. myrtifolia, Q. chapmanii*) in both white and red oaks. The red-oak *Q. inopina* had the lowest nitrogen content, while the white-oak *Q. chapmanii* had the highest. Given the lack of difference among oaks for carbon content, the pattern of variation in carbon/nitrogen ratios among oaks was a near inverse of that for nitrogen.

Chemical Correlates of Cynipid Wasp Ordination. In order to interpret the cynipid distributions according to differences among the oak species, we correlated the matrix of chemistry variables by oak species with the axes generated by the ordination. A number of chemical attributes were correlated with axis 1 including total phenolics ($r = 0.915$), cellulose ($r = -0.710$), hydrolyzable tannins ($r = 0.660$), condensed tannins ($r = 0.578$), and carbon/nitrogen ratio ($r = 0.504$) (Figure 1). Cynipid species on red oaks were associated with higher total phenolics and tannins, while those on *Q. minima* and *Q. geminata* were associated with higher cellulose levels.

Three oak chemistry traits correlated with axis 2 including lignin ($r = -0.762$), cellulose ($r = -0.675$), and nitrogen ($r = 0.723$). Consequently, the cynipid species on *Q. chapmanii* were associated with higher levels of nitrogen but with lower levels of lignin and cellulose compared to the cynipid species associated with the other oak hosts. Two oak chemistry variables were correlated with axis 3 including hemicellulose ($r = -0.967$) and hydrolyzable tannins ($r = 0.553$). Thus, cynipid species on *Q. laevis* were associated with higher levels of hydrolyzable tannins but lower levels of hemicellulose relative to the cynipid species on the other oaks.

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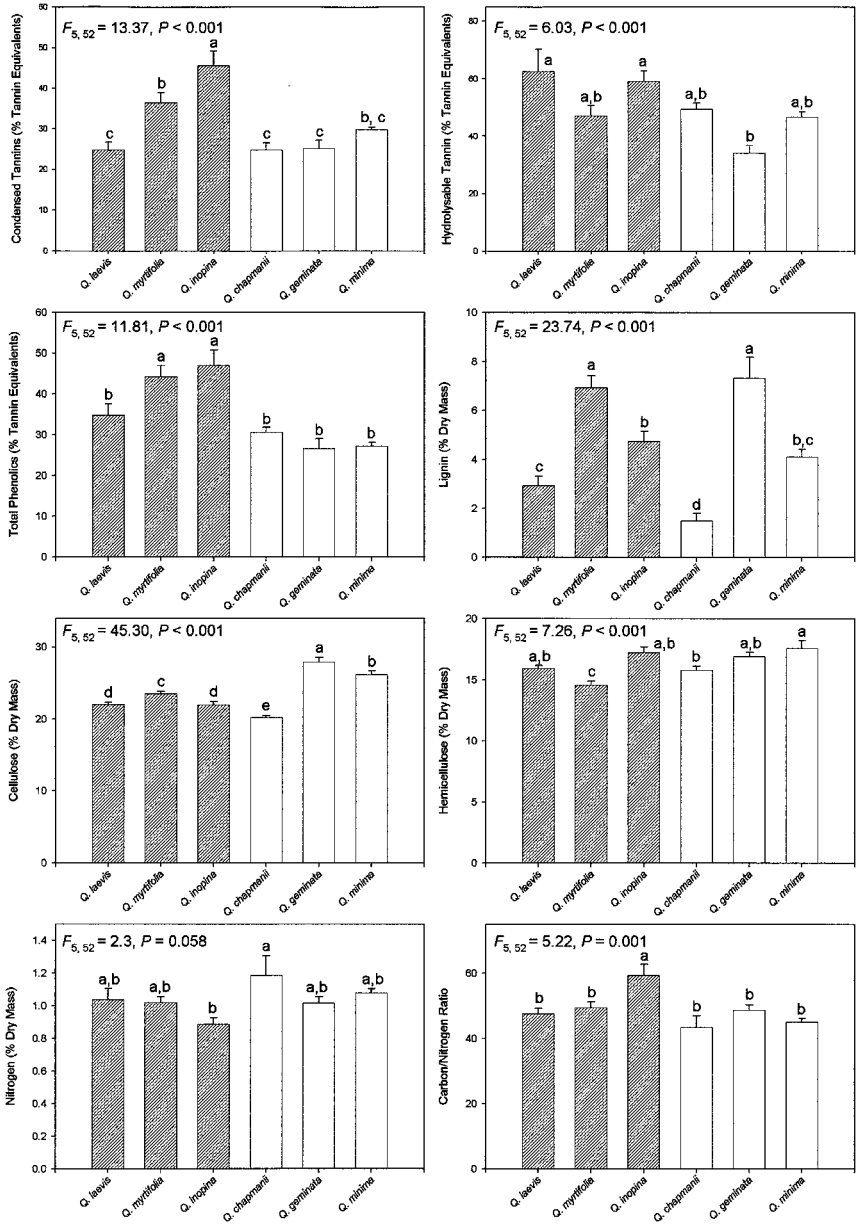
FIG. 1. Joint plots showing the ordination positions for the six cynipid wasp communities (based on a matrix of 88 species of cynipids across the six oaks; red oaks: filled circles, and white oaks: open circles) and the relationship between leaf-chemistry attributes and the ordination scores on axes 1 and 2 (top) and on axes 1 and 3 (bottom) of a Bray-Curtis ordination. Axis 1 extracted 44.2% of the original distance matrix, axis 2 described 26.9%, and axis 3 explained 23.8%, making the cumulative extraction for the first three axes 94.9%. The lines radiate from the centroid of the ordination scores and the angle and length of the lines illustrate the direction and strength of their relationships to the ordination scores.


Condensed Tannins (% Tannin Equivalents)
$F_{5,52}$ = 13.37, P < 0.001
Hydrolysable Tannin (% Tannin Equivalents)
$F_{5,52}$ = 6.03, P < 0.001
Total Phenolics (% Tannin Equivalents)
$F_{5,52}$ = 11.81, P < 0.001
Lignin (% Dry Mass)
$F_{5,52}$ = 23.74, P < 0.001
Cellulose (% Dry Mass)
$F_{5,52}$ = 45.30, P < 0.001
Hemicellulose (% Dry Mass)
$F_{5,52}$ = 7.26, P < 0.001
Nitrogen (% Dry Mass)
$F_{5,52}$ = 2.3, P = 0.058
Carbon/Nitrogen Ratio
$F_{5,52}$ = 5.22, P = 0.001
Q. laevis
Q. myrtifolia
Q. incopina
Q. chapmanii
Q. geminata
Q. minima

DISCUSSION

The most important finding of our study was the correlation of eight of the nine measured oak chemistry attributes with the axes of an ordination of the 88 cynipid species associated with six scrub oaks. Furthermore, the correlated chemical traits included both primary and secondary plant metabolites, suggesting the possibility that oak chemistry has a fundamental influence on cynipid host choice by adult females and/or on offspring performance and survival. The extent and strength of the correlations of oak chemistry variables with cynipid distributions were unexpected given that our sampling of leaves was done in the autumn, well after oviposition and gall development occurred for the majority of the cynipids sampled. Thus, the autumn samples that we examined may not completely reflect the chemistries of host-plant tissue at the time of oviposition or of larval and gall development. Furthermore, many cynipids included in our analysis attack plant organs other than leaves; instead they attack stems, flowers, and acorns. Regardless, the variation in positions on the ordination axes "explained" by oak chemistry variables was high, including 93.6% by hemicellulose on axis 3, 83.7% by total phenolics on axis 1, and 58.0% by lignin on axis 2.

Nonetheless, the correlated oak chemistry variables may not necessarily be the ones that are crucial to female oviposition choice or offspring performance. Instead, the measured chemical variables may be correlated with other unmeasured variables, which in turn are compounds crucial for host choice or larval performance. The fact that some chemical traits were correlated with one another (e.g., condensed tannins with total phenolics and with carbon/nitrogen ratio, $r = 0.849$ and 0.866, respectively) implies that other correlations with unmeasured variables may be frequent. For example, one or two functional phenolic compounds (or some specific combination of phenolic compounds) may be key stimulants for oviposition. In turn, these key compounds may be correlated with total phenolic content, as in the case for gall-inducing sawflies (Kolehmainen et al., 1994; Roininen et al., 1999). Similarly, although the levels of lignin or cellulose present

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FIG. 2. Means ± standard errors for condensed and hydrolyzable tannins, total phenolics, lignin, cellulose, hemicellulose, nitrogen, and carbon/nitrogen ratio for oak leaves from the red oaks (filled bars) *Q. laevis*, *Q. myrtifolia*, and *Q. inopina*, and the white oaks (open bars) *Q. chapmanii*, *Q. geminata*, and *Q. minima* sampled on October 9, 2001, at the Archbold Biological Station, Lake Placid, Florida. All variables except carbon/nitrogen ratio were arcsine square-root transformed prior to multivariate ANOVA. Bars with the same letter within a graph are not significantly different according to a Student-Newman-Keuls *post-hoc* test. Lignin, cellulose, hemicellulose, and nitrogen are expressed as percent dry mass. Condensed tannin, hydrolyzable tannin, and total phenolics are relative concentrations based upon comparison to a single oak tannin standard.

may be important to larval performance, adult females may use the presence of correlated volatile compound(s) in oviposition choice. In addition, the interiors of galls, which are fed upon by developing larvae in galls, typically have markedly different levels of chemical compounds from adjacent ungalled plant tissues (Abrahamson and McCrea, 1986; Abrahamson et al., 1991; Raman, 1993; Hartley, 1998; Nyman and Julkunen-Tiitto, 2000).

As our previous work has shown (Abrahamson et al., 1998a,b), the results presented here illustrate the high degree of specialization within Cynipinae species that attack species of *Quercus*. Preferences for specific oak species are strong. For example, the cynipid species that attacked white-oak species did not attack red-oak species and vice versa. This specificity of cynipid gall-inducing species may result from highly specialized responses to particular host-plant chemicals, as has been shown in other herbivorous insects (e.g., Berenbaum, 1981; Schoni et al., 1987; McGovern and Ladd, 1990; Kolehmainen et al., 1994; Berdegue and Trumble, 1996; Carter and Feeny, 1999; Roininen et al., 1999). Such specificity within cynipid gall-inducing species suggests that experimental studies of their oviposition choice and larval performance will prove profitable.

Furthermore, the numerous species of oaks [e.g., 90 species in North America north of Mexico (Nixon, 1997; Nixon and Muller, 1997)] combined with the richness of cynipid gall inducers [485 species reported for North America, north of Mexico (Burks, 1979)] and the variation among oaks in their interactions with gall-wasp species offer tremendous opportunities for studies of the role of host-plant chemistry in oviposition preference and larval performance. The responses of herbivorous insects to host-plant chemistry can be crucial to oviposition choice, larval performance, host shifts, and host-race formation, which in turn create opportunities for host-race formation, speciation, radiation, and even escalatory changes in host-plant defenses and the abilities of herbivores to utilize a given population of host plant (Zangerl and Berenbaum, 1993; Becerra, 1997; Berenbaum and Zangerl, 1998). Future studies with the cynipid–oak system should provide important insights into the processes of evolution.

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ROLE OF CUCURBITACIN C IN RESISTANCE TO SPIDER MITE (*Tetranychus urticae*) IN CUCUMBER (*Cucumis sativus* L.)

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Abstract—Cucurbitacins are bitter triterpenoid compounds that are toxic to most organisms and occur widely in wild and cultivated Cucurbitaceae. The only cucurbitacin identified in *Cucumis sativus* is cucurbitacin C. The bitter taste of cucumber has been correlated with resistance to the spider mite *Tetranychus urticae*, but a quantitative relationship has not been established. We determined the spider mite resistance and cucurbitacin C content in the dihaploid progeny derived from the F_1 generation of a cross between a bitter, spider-mite-resistant cucumber line and a bitter-free, spider-mite-susceptible line. The ratio of the number of bitter to bitter-free dihaploids conformed to the expected 1:1 ratio, based on a monogenic segregation pattern. Genetic analysis ascribed 69% of the variance of the difference in spider mite survival rate to the bitterness locus. Within the group of bitter dihaploids, cucurbitacin C content was significantly correlated with spider mite resistance. Thus, a quantitative relationship between cucurbitacin C content and spider mite resistance could be established.

Key Words—*Cucumis sativus*, *Tetranychus urticae*, cucumber, spider mite, bitterness, cucurbitacin C, resistance.

INTRODUCTION

Many authors have reported on the involvement of secondary metabolites in the interaction between plants and herbivore insects (for review see Gershenzon, 1994). Of all possible secondary metabolites, terpenoids in particular have been shown to be involved in both indirect and direct plant defense. Examples of the former

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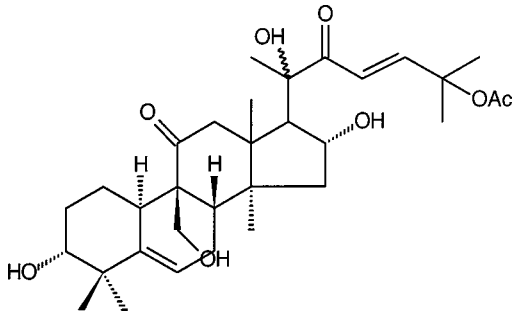

FIG. 1. Structure of cucurbitacin C.

include the herbivory-induced production of volatiles that attract predators of the herbivores, including the production of 4,8-dimethyl-1,3(*E*),7-nonatriene by cucumber and maize in response to feeding of spider mites, and *Spodoptera littoralis* (Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000). Direct defense compounds are produced by plants constitutively or upon induction to kill, repel, or weaken attacking organisms.

An important example of these direct defense compounds is the cucurbitacins. Cucurbitacins are bitter triterpenoid compounds that are toxic to most organisms, including mammals such as man, and occur widely in wild and cultivated Cucurbitaceae (Mirò, 1995). Many different forms of cucurbitacins have been identified, all with the general cucurbitane carbon skeleton: 19-(10->9β)-abeo-10α-lanost-5-ene (Figure 1) (Mirò, 1995). So far, the only form identified in *Cucumis sativus* is cucurbitacin C (Rice et al., 1981; Gorski et al., 1986), although there is some evidence that another cucurbitacin-like compound (perhaps a cucurbitacin C precursor or derivative) could be present in the nonbitter variety Eversweet (Rice et al., 1981).

In addition to being toxic repellents, cucurbitacins are also well known as phagostimulants for chrysomelid beetles (*Diabroticina, Acalyma, Ceratoma* spp.), a group of herbivores that feed on cucurbitacin-producing plants. The beetles sequester cucurbitacins that embitter their tissue, which has been suggested to protect them from predators, parasites, or pathogens (Ferguson and Metcalf, 1985; Bar-Nun and Mayer, 1989; Nishida and Fukami, 1990). Thus, in *C. sativus*, cucurbitacin C is coupled to the bitter taste and susceptibility to cucumber beetles (DaCosta and Jones, 1971a, 1971b; Rice et al., 1981; Ferguson et al., 1983; Gorski et al., 1986). For example, wilted cucumber seedlings contained twice as much cucurbitacin as nonwilted bitter seedlings and were more damaged by spotted (*Diabrotica undecimpunctata howardi* Barber) and striped (*Acalymma vittata* Fab.) cucumber beetles (Haynes and Jones, 1975).

Plant compounds that act as cues for specialist herbivores may act as deterrents for generalist herbivores, and the bitter taste of cucumber, which makes

it susceptible to the cucumber beetle, has been correlated with resistance to the polyphagous spider mite *Tetranychus urticae* Koch. This spider mite is an important pest of cucumber, and several authors have speculated about the involvement of the bitter triterpenoid cucurbitacins in resistance toward this organism (Gould, 1978; Ponti and Garretsen, 1980). After the first reports about spider-mite-resistant cucumber (Da Costa and Jones, 1971a, 1971b; Kooistra, 1971), the combination of nonbitterness and spider mite resistance in a commercial variety has been an aim of several breeding programs. The link between the presence of the bitter tasting cucurbitacins and resistance has been subject of discussion ever since (Gould, 1978; Ponti and Garretsen, 1980), but breeders have not succeeded in introducing spider mite resistance into non-bitter-tasting cultivars.

Normally, cucumber has bitter foliage and nonbitter fruits, but the fruits may become bitter as a result of stress. After discovery of one nonbitter plant in the variety Improved Long Green (Andeweg and De Bruyn, 1959), European plant breeders introduced nonbitterness into their varieties, and bitter fruit has become an undesirable trait in modern cucumber cultivars. The gene *Bi* confers bitterness to the entire plant, which can easily be determined in the cotyledons. Nonbitter plants possess the recessive allele *bi* (Pierce and Wehner, 1990). The *bi* gene prevents the fruit from becoming bitter under stress. A second recessive gene for bitter-free foliage was described by Wehner et al. (1998). This gene, designated *bi-2*, segregates independently from the first gene *bi*.

Despite suggestions that cucurbitacins are involved in spider mite resistance of cucumber, a quantitative relationship has not been established. In a preliminary study with dihaploid (completely homozygous) progenies from the F_1 generation of a cross between the nonbitter, susceptible inbred line G6 and the resistant, bitter accession 9140, we noticed an almost absolute link between spider mite resistance and bitterness as judged by tasting young cotyledons. All non-bitter-tasting dihaploid lines were highly susceptible, and the bitter-tasting dihaploid lines were shown to be resistant. In the present paper, we used this breeding material to further elucidate the role of cucurbitacins in resistance to spider mite.

METHODS AND MATERIALS

Twenty-six dihaploid lines were produced from the F_1 generation of a cross between a spider-mite-susceptible, nonbitter inbred line (G6; *bi/bi*) and a resistant, bitter-tasting dihaploid (DH103 derived from accession 9140). Haploid plants were obtained by culturing unfertilized ovaries; the plantlets were treated with colchicine to reach the regular diploid level (courtesy of G. Reuling, Nunhems Zaden, The Netherlands). When heterozygous flowers (e.g., *Bi/bi*) as in an F_1 generation are used, dihaploids will be homozyous either for the allele from the mother (e.g., *bi/bi*) or for the allele from the father (e.g., *Bi/Bi*) of that cross. Dihaploid plants are useful for genetic analysis since they are completely homozygous and

plants can be reproduced without genetic segregation. Thus, contrary to a classical segregating F_2 generation, replicated tests are possible.

Plants were grown in four successive experiments in the greenhouse from August until November 1999 with a day/night regime of 23/20°C. Daylength was extended to 16 hr with 24 W/m^2. Plants were arranged in a randomized block design with one plant of each genotype per block. Because the greenhouse could not be cooled, day temperatures occasionally reached 30°C during the first two experiments. In the first experiment, cotyledons of the 5-day-old seedlings were tasted to judge the presence of bitter compounds. In the last experiment, the cucurbitacin C content of the bitter-tasting dihaploids was determined using HPLC analysis. Cucurbitacin C was also determined from leaf disks of plants of the original resistant accession 9140, grown in four successive experiments during 1999 and 2000, to determine the effect of environmental conditions on cucurbitacin content.

For the spider mite test, four successive experiments were performed in which, respectively three, three, three, and four 4-week-old plants of each dihaploid line and parent were tested. A leaf disk (49 mm in diam.) was cut from a fully expanded leaf of each plant to be tested. Disks were placed upside down in a tray (29 × 42 cm, 48 disks/tray) lined with filter paper and moistened with a 10 ppm benzimidazole solution such that the disks nearly floated. This prevented the mites from moving from one leaf disk to another and kept the leaf disks in good condition. Trays were sealed with a transparent cover to maintain a high relative humidity. Ponti (1978) demonstrated that the genotype of the cucumber plants used for rearing the spider mites did not affect the results of the subsequent resistance tests; therefore, a nonbitter genotype was used for mass rearing. Three nonsynchronized female spider mites from the mass rearing on bitter-free cucumber plants were placed on each disk. The disks were incubated at 25°C for 24 hr. After this, mites were removed and the number of eggs produced counted. Disks were further incubated at 25°C for about 10 days until the next generation of adult mites was present and could be counted. During this period, a 16L:8 D regime was maintained. It has been shown that the genotype of the host does not affect the number of eggs produced on the disks by females from a mass rearing on nonbitter cucumber plants. In contrast, the number of individuals reaching the adult stage does depend on the genotype of the host plant. Individuals not reaching the adult stage die during the successive molting stages. The ratio of the number of individuals reaching the adult stage relative to the number of eggs (hereafter: survival rate) is used as a measure for the susceptibility of the plant (Balkema-Boomstra et al., 1999).

Cucurbitacin C content was determined on another disk of the same leaf that was used for the spider mite resistance test in the fourth experiment. HPLC analysis of cucurbitacins was carried out essentially as described by Gorski et al. (1986). Leaf tissue was frozen in liquid N_2 and ground in a mortar and pestle. The powder, resulting (0.5 g) was weighed in a glass centrifuge tube to which

2 ml of MeOH were added. The suspension was homogenized for 15 sec using an Ultraturrax homogenizer (Tamson, Zoetermeer, The Netherlands). After grinding, the homogenizer was washed with 1 ml of MeOH, and the extract centrifuged at 4°C for 20 min at 20,000 g. The supernatant was transferred to another centrifuge tube, and the pellet washed with 1 ml of MeOH and centrifuged again. The combined MeOH supernatants were diluted 1:1 with demineralized water and filtered using a Gelman 0.2 μm, 13 mm Acrodisk filter. The filtrate was (500 μl) analyzed on a Waters HPLC system equipped with Waters 510 pump, Waters 680 gradient controller, and Waters 486 detector (at 254 nm).The HPLC was equipped with a Hypersil ODS column (5 μm, 250 $\times$ 4.6 mm) using 55% MeOH in NANO pure water as the eluent at 1 ml/min and a column temperature of 25°C. The presence of cucurbitacins B, C, D, and E glucoside was checked using standards provided by Drs. P.M. Gorski and D.W. Tallamy by using both HPLC and TLC (Gorski et al., 1986). The linearity of the detector response was determined from standards. Sample preparation was carried out such that cucurbitacin concentrations were in this linear range.

Chi-square (χ^2) was calculated for the segregation ratio between the number of bitter-tasting dihaploid lines and the non-bitter-tasting dihaploid lines in the progeny of the F_1 generation (in Crow, 1967). Components of variance were determined, and regression analysis was performed by using GenStat (2001), to explain the genetic relationship between bitterness and spider mite resistance.

RESULTS

Cotyledons from 12 of 26 dihaploids, derived from the F_1 generation of the cross between the bitter-free inbred line G6 and the bitter dihaploid DH103, tasted bitter, and 14 did not. This is in agreement with the expected 1:1 segregation ratio for a trait governed by a single gene ($\chi^2 = 0.154$; $P = 0.7$). Particularly in the first experiment, the difference in spider mite survival rate between the bitter and non-bitter dihaploids was striking (mean = 11.5%, SD = 11.7 and mean = 71.0%, SD = 14.3, respectively, for the average of the groups). In later experiments, the bitter dihaploid lines were less resistant, but the level of susceptibility of the nonbitter dihaploids did not change much. Genetic analysis ascribed 69% of the variance of the difference in spider mite survival between the parents to the bitterness locus. The range and mean survival rates of spider mites on bitter and nonbitter dihaploids and parents over the four consecutive experiments are shown in Figure 2. Survival rates of the spidermites on the 12 bitter dihaploids, averaged over the four experiments, are presented in Table 1.

As reported by Gorski et al. (1986), only cucurbitacin C could be detected in cucumber leaves and cotyledons of the bitter tasting plants and not cucurbitacins

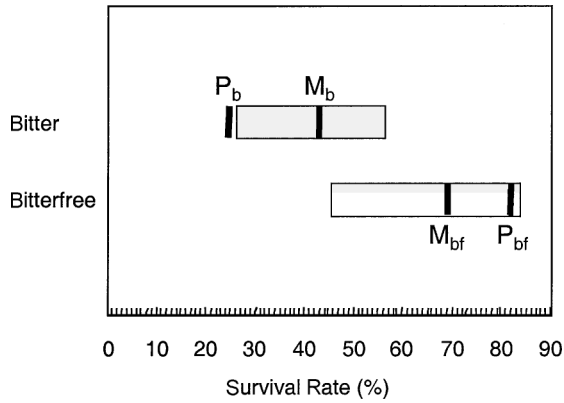

FIG. 2. Range and mean of spider mite survival rate of 12 bitter and 14 nonbitter dihaploid lines and their crossing parents/averaged over four experiments. P_b, P_{bf}: survival rate on bitter and nonbitter parent, respectively, M_b, M_{bf}: mean survival rate on bitter and nonbitter progeny, respectively: Bars indicate the variation between individual lines within the bitter and bitter-free group.

TABLE 1. MEAN CUCURBITACIN C CONTENT ($N = 3$; DETERMINED IN LAST EXPERIMENT) AND SPIDER MITE SURVIVAL RATE ($N = 13$; AVERAGED OVER FOUR EXPERIMENTS) OF 12 BITTER DIHAPLOID CUCUMBER LINES

Dihaploid code	Cucurbitacin C (μg/g fwt)	Survival rate (%)
15	49.5 a[a]	46.5 bc
26	51.3 a	36.2 ab
5	52.9 a	55.9 c
20	62.8 a	53.8 c
19	79.7 ab	51.1 c
18	88.3 ab	47.6 bc
9	98.1 abc	44.1 bc
25	149.0 bc	46.3 bc
13	169.9 c	36.5 ab
7	194.5 c	35.4 ab
23	233.5 c	33.8 ab
10	314.4 d	26.3 a
LSD	81.7	17.3

[a] Means within columns followed by the same letter are not significantly different ($P > 0.05$).

TABLE 2. AVERAGE AND TOTAL GLOBAL RADIATION AND MAXIMUM TEMPERATURE OUTSIDE THE GREENHOUSE[a] AND CUCURBITACIN C CONTENT OF 9140 PLANTS/GROWN IN FOUR SUCCESSIVE EXPERIMENTS

	Experiment			
	1	2	3	4
Sowing date	Sep 24, 1999	Mar 31, 2000	Apr 21, 2000	May 19, 2000
Harvest date	Nov 08, 1999	May 02, 2000	May 24, 2000	Jun 21, 2000
Avg. global radiation (MJ/m^2/day)	6.0	13.1	16.6	18.8
Total global radiation (MJ/m^2)	274	433	565	639
Avg. maximum temp (°C)	14.8	14.7	20.5	20.0
Plant 1 (μg cucC/g FW)	129.9	97.6	96.6	33.3
Plant 2 (μg cucC/g FW)	148.2	109.2	130.5	33.7
Plant 3 (μg cucC/g FW)	267.6	191.2	326.4	41.6
Mean (μg cucC/g FW)	181.9	132.8	184.5	36.2

[a] Calculated from weather data obtained from the meteorological station at Wageningen.

B, D, and E glucoside. Using the cucurbitacin C standard, the detector response proved to be linear ($R^2 = 0.99$) over a concentration range of at least 0.1–2.5 μg. In general, levels of cucurbitacin C in cotyledons were about 10-fold higher than in cucumber leaves.

Cucurbitacin C content, determined by using HPLC in the fourth experiment with a disk from the same leaf as used for the spider mite test, varied considerably between the different dihaploid lines and ranged from 49.5 to 314.4 μg cucurbitacin C per gram fresh weight (Table 1). A similar cucurbitacin C range was detected between plants of accession 9140, within and among four different experiments (Table 2). Although there were fairly large differences between the four experiments with accession 9140 in the amount of global radiation (measured outside the greenhouse and not taking into account the additional lighting) and the average maximum outside temperature, there seems to be no consistent correlation between these parameters and cucurbitacin C content. Possibly, the high of temperatures that occurred during a brief period in the third experiment (the temperature in the greenhouse occasionally reached 35°C) may have induced water stress in this experiment, resulting in a higher cucurbitacin content (Table 2).

There was a significant negative correlation ($r = -0.61^*$) between spider mite survival and cucurbitacin C content of the individual dihaploid lines (Figure 3A) when only data of the fourth experiment where cucurbitacin C content was determined ($N = 4$) were considered. When the data on spider mite survival of all four experiments ($N = 13$) are plotted against cucurbitacin C data of the fourth experiment, the correlation is stronger ($r = -0.81^{**}$; significant at $P = 0.01$; Figure 3B).

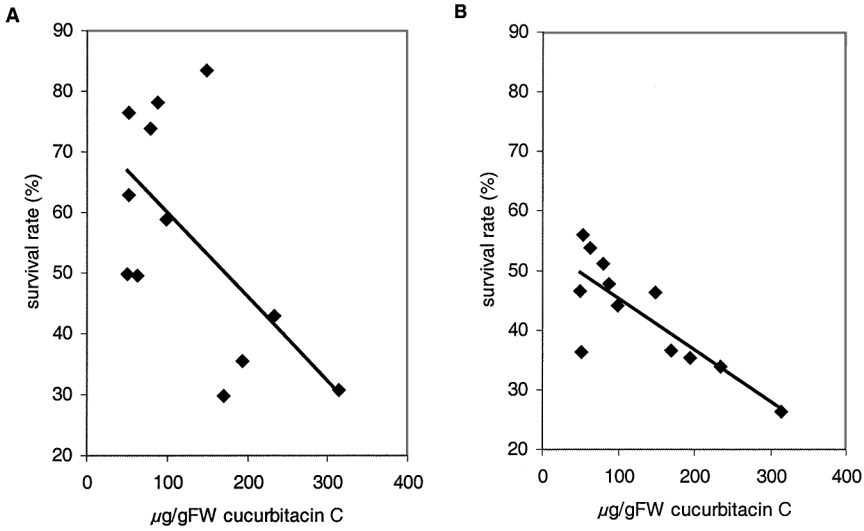

FIG. 3. Correlation between cucurbitacin C content, as determined by HPLC analysis, and spider mite survival rate. (A) Data from experiment 4 only ($y = -0.1384 + 73.735$; $R^2 = 0.38$). (B) spidermite survival rate averaged over the four experiments ($y = -0.0861\text{x} + 53.871$; $R^2 = 0.65$). See Methods and Materials for details.

DISCUSSION

Our results demonstrate that the concentration of cucurbitacin C in the leaves is an important parameter in spider mite resistance in cucumber. Susceptibility to spider mites, expressed as the percentage of adults developed from eggs, produced in 24 hr on a leaf disk, was shown to be negatively correlated to cucurbitacin C content in noninfected leaves for the dihaploid progenies of a cross between the resistant bitter dihaploid DH103 and the susceptible nonbitter, inbred line G6 (Figure 3A, B). This is in line with the results of Agrawal et al. (1999), who showed that cucurbitacin formation is induced by spider mite feeding and that this induction decreases spider mite population growth (by about 40%).

Dinan et al. (1997) demonstrated that cucurbitacins B and D, isolated from seeds of *Iberis umbellata*, act as antagonists at the ecdysteroid receptor in a *Drosophila melanogaster* cell line. Ecdysteroids are known to be involved in the molting process. Our observations on the bitter lines, indicate development of mites is hampered during the successive developmental stages from egg to adult. It is, therefore, likely that cucurbitacin C is an important factor the spidermite resistant lines of cucumber, perhaps by acting as an antagonist of a spider mite ecdysteroid receptor.

A large variation in the amount of cucurbitacin C was detected in leaves of accession 9140 plants grown in different experiments. Environmental conditions have been suggested to affect cucurbitacin content of cucumber. For example, Haynes and Jones (1975) reported that wilted seedlings contained a twofold higher cucurbitacin content compared to nonwilted seedlings. Gould (1978) also suggested the involvement of cucurbitacin in spider mite resistance and supported this by demonstrating significantly less mite survival on water-stressed bitter cucumber seedlings compared to nonstressed bitter seedlings. Water stress did not affect mite survival on nonbitter seedlings. Genetic variation in constitutive and induced cucurbitacin production may also influence cucurbitacin content in individual plants (Agrawal et al., 1999).

Differences in the level of spider mite resistance of the bitter dihaploids among the successive experiments could perhaps be explained by differences in cucurbitacin C content. Unfortunately, levels were only determined in the last experiment. Nevertheless, the first experiment showed the highest difference in resistance between bitter and nonbitter plants. Stress, because of high temperatures, is more likely to have occurred in plants durings this experiment (started in August) than in the following experiments (that were started throughout September). It is tempting to speculate that cucurbitacin C content was highest in the first experiment and lower in later experiments.

Agrawal (2000) showed that cucurbitacin D, applied to cotton cotyledons, inhibited spidermite reproduction. In similar experiments where cucurbitacin C was dissolved in 10% MeOH and applied to cucumber leaves, we were unable to demonstrate a direct relationship between the presence of cucurbitacin C and spider mite resistance. In experiments by Agrawal, spider mite survival was measured after several generations. In our experiments, spider mite fitness was determined after one generation.

In the present experiments, the linkage between plant bitterness and spidermite resistance has been confirmed, and this may be discouraging for European breeding programs. It remains to be seen whether the recently described second gene for bitter foliage *Bi-2* also causes fruits to be bitter under stress similar to *Bi*. If not, this may provide an opportunity to create a spider mite resistant genotype with leaves containing cucurbitacins in combination with nonbitter fruits.

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EFFECT OF SMOKE, CHARRED WOOD, AND NITROGENOUS COMPOUNDS ON SEED GERMINATION OF TEN SPECIES FROM WOODLAND IN CENTRAL-WESTERN SPAIN[1]

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Abstract—The effect of smoke, charred wood, and nitrogenous compounds on germination was tested on 10 species of the Cistaceae, Poaceae, Fabaceae, and Asteraceae, from fire-prone, shrubby woodlands in central-western Spain. Dry seeds were exposed to smoke, by watering with distilled water–charred wood suspensions, or $NaNO_2$, KNO_3, NH_4Cl, and NH_4NO_3. Smoke enhanced germination in 9 of 10 of the species. In species of Poaceae, germination was stimulated by 20 min of smoke exposure. In Asteraceae and Fabaceae species, 10 min of smoke exposure was the most effective treatment for enhancing germination. Three species—*Cistus ladanifer*, *Cistus crispus*, and *Cistus monspeliensis*—had a positive response to 20 min of smoke exposure; germination of *Cistus salviifolius* L. was also enhanced after 10 min. The effect of charred wood was variable, with no consistent germination pattern within the families. *Trifolium angustifolium* and *Retama sphaerocarpa* showed no stimulation of germination under most of the charred wood concentrations. Similarly, germination of *Senecio jacobea* under the charred wood treatment did not surpass that of the control. $NaNO_2$ promoted seed germination in *Dactylis glomerata* (10 mM), *Cistus ladanifer* (1, 10, and 25 mM), and *Cistus crispus* (1 and 10 mM). KNO_3 enhanced germination in *Dactylis glomerata* (1 and 25 mM), *Dittrichia viscosa* (10 and 25 mM), *C. ladanifer* (1, 10, and 25 mM), *Cistus crispus* (1 and 25 mM), and *C. salviifolius* aud *C. monspeliensis* (25 mM). NH_4Cl induced germination of *Dactylis glomerata* and *Dittrichia viscosa* (1 mM), and *Cistus* species germinated best in 25 mM of this salt. NH_4NO_3 induced germination only in

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[1] Nomenclature follows Tutin et al. (1964–1980).

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Cistus species. *Holcus lanatus* had the highest level of germination regardless of treatment.

Key Words—Smoke, charred wood, Cistaceae, Poaceae, Fabaceae, Asteraceae, pH, dormancy.

INTRODUCTION

Natural fires are a recurrent perturbations that contribute to plant community structure in Mediterranean-type ecosystems such as the Mediterranean garrigue (Trabaud, 1984), Californian chaparral (Keeley, 1991), African fynbos (Kruger and Bigalke, 1984; Pierce et al., 1995), and Australian kwongan (Dixon et al., 1995; Malcolm and Williams, 1996). Fire usually destroys the above-ground biomass, although certain species are capable of maintaining their populations from seeds or by resprouting from roots or from lignotubes that remain buried and favor subsequent plant regeneration (Matlack et al., 1993; Fernández-Santos and Gómez-Gutiérrez, 1994). In the first year after a fire, it is common to observe two peaks of vegetation recovery: one that coincides with vegetative regeneration of shrubs (i.e., resprouting), and the second with a flush of seed germination, followed by abundant seedling growth of herbaceous and subfrutescent species (Christensen and Muller, 1975; Thanos and Rundel, 1995). Moreover, some shrubby species are able to use both the sexual and vegetative methods to regenerate, an ability that has been interpreted as an evolutionary response to fire (Trabaud, 1984). Four regenerative responses can be identified: (1) obligate seeders, species that need stimulation by fire to germinate (Keeley, 1991); (2) facultative resprouters, species that are able to use either resprouting or seedlings to recover; (3) obligate resprouters, species that are constrained to the resprouting method to regenerate (Keeley, 1987); and (4) fire annuals, those species that dominate a site in the first year after a fire, but then disappear after one or two years, persisting only as dormant seeds until another fire.

The requirement for fire as a cue to the onset of germination has been interpreted as an adaptation of species living in fire-prone environments (Gónzález-Rabanal and Casal, 1995; Pugnaire and Lozano, 1997). Fire plays an active role in breaking dormancy by several direct and indirect means that include high temperatures generated by the fire (Baskin and Baskin, 1989; Gónzález-Rabanal and Casal, 1995; Ferrandis et al., 1999), presence of smoke (De Lange and Boucher, 1990; Baxter et al., 1994; Dixon et al., 1995; Keeley and Fotheringham, 1997), presence of ash (Gónzález-Rabanal and Casal, 1995; Enright et al., 1997), and release of nitrogenous compounds due to combustion of plant matter (Humphreys and Craig, 1981; Gónzález-Rabanal and Casal, 1995).

Fire plays a major role in breaking seed dormancy in many species with impermeable, hard-coated seeds such as is characteristic of many Cistaceae and

Fabaceae (Añorbe-Urmeneta et al., 1990; Thanos et al. 1992; Bradstock and Auld, 1995; Ferrandis et al., 1999). During a wildfire, high soil temperature is the main factor responsible for germination, since the thermal shock associated with fire can physically modify the seed coat of buried seeds or seeds accumulated on the upper soil surface, thus overcoming physical dormancy (Baskin and Baskin, 1989). This idea is supported by laboratory studies where Cistaceae and Fabaceae seed germination were significantly enhanced by heat treatments that simulated temperatures recorded in the upper soil layers during a fire (Añorbe-Urmeneta et al., 1990; Thanos et al., 1992; Valbuena et al., 1992; González-Rabanal and Casal, 1995; Ferrandis et al., 1999). Nevertheless, fire is also responsible for other disturbances such as smoke, changes in soil water, and chemical properties that have an ecological relevance in germination and, therefore, in plant distribution. Smoke has been interpreted as a chemical scarification method or as a "signal-mediated" stimulation, a category encompassing a number of chemical cues (Baldwin et al., 1994; Egerton-Warburton, 1998). Charred wood has a special relevance to seed germination after a fire (Keeley, 1987). However, the extent to which plant material is burned will determine the composition of chemical compounds that are released. Charred wood is a heterogeneous mixture, which includes the charred or partially burned plant residue; however, fire intensity will determine the degree of ashing, thereby dictating its final chemical profile. After fire, nitrogenous compounds are released from combustion of living and dead plant material or simply made more available within the soil (Humphreys and Craig, 1981; Franco-Vizcaíno and Sosa-Ramírez, 1997). Seed germination following fire in several California chaparral species has been related to the postfire presence of water-soluble nitrogenous compounds in soils (Thanos and Rundel, 1995) aud several Western Australia species had their germination enhanced in the presence of KNO_3 (Bell et al., 1999).

Postfire germination of species that dominate Mediterranean-type shrublands has been reported in many other papers (Portlock et al., 1990; Bell et al., 1993; González-Rabanal and Casal, 1995; Roche et al., 1998; Ferrandis et al., 1999). However, the effect of smoke, charred wood, and nitrogenous compounds on germination is not clearly understood. To evaluate the separate effect of these factors on plant germination, different treatments were performed by using 10 species of Cistaceae, Poaceae, Fabaceae, and Asteraceae from fire-prone shrubby woodlands in central-western Spain. Species were selected from among those most representative and abundant in plant communities mixed with tree cover. Their dormancy attributes and pyric responses are divergent. Cistaceae and Fabaceae species have enforced dormancy due to seed hardness, and their germination is known to be stimulated by heat and fire (Auld, 1986; Thanos et al., 1992; Valbuena et al., 1992; Pugnaire and Lozano, 1997; Ferrandis et al., 1999). Poaceae and Asteraceae species regenerate well after fire and are abundant in fire-prone shrublands in central-western Spain. Nevertheless, it is unknown whether they have innate and/or induced dormancy and fire-stimulated germination. Postfire germination response

will determine the structure of the ecosystem after a fire. Due to the lack of information regarding the latter species mentioned above, we can not predict whether these species have fire-enhanced germination or whether they regenerate as a consequence of the appearance of gaps after fire.

In this study, we examined the effect of smoke, charred wood, and nitrogenous solutions on germination in six dormant plant species and in four species where germination has not been previously investigated. The hypotheses tested are (1) that direct and indirect effects of fire, i.e., smoke, charred wood, and nitrogenous compounds enhance seed germination, and (2) that responses to fire vary among plant species.

METHODS AND MATERIALS

Plants. Cistus crispus L., *Cistus salviifolius* L., *Cistus ladanifer* L., *Cistus monspeliensis* L. , *Dittrichia viscosa* (L.) Greuter, *Senecio jacobea* L., *Holcus lanatus* L., *Dactylis glomerata* L., *Trifolium angustifolium* L., and *Retama sphaerocarpa* (L.) Boiss were used. Seeds of all these species were hand-harvested in a *Quercus rotundifolia* Lam. dehesa woodland located in the vicinity of Badajoz, central-western Spain (39°10′N, 7°00′W, 240 m above sea level). A dehesa is a seminatural, savannalike woodland, representative of the landscape in the central west of the Iberian Peninsula, which at the same time is a silvipastoral system. The dominant trees are oak (*Quercus pyrenaica* Willd.), evergreen oak (*Quercus rotundifolia* Lam.), cork oaks (*Quercus suber* L.), and ash (*Fraxinus* spp), in mono- or plurispecific distribution with a highly variable density of 2–1000 trees/ha (Gómez-Gutiérrez and Pérez-Fernández, 1996). This area has a Mediterranean-type climate with a mean annual rainfall of 450–600 mm and a temperature range of 3.7–34.5°C.

Seeds were collected at the end of the summer of 1998, coinciding with the dispersal period of these species (Table 1), and bulk stored in paper bags at room temperature. Seeds of Asteraceae and Cistaceae species were stored dry at 18 ± 1°C and 20% relative humidity.

Germination Experiments. Promotion of seed germination in wild species often requires the application of dormancy-breaking treatments. Although there is no specific germination or seed dormancy information on 5 of the 10 species studied, information is available on all the genera (ISTA, 1993; Linington et al. 1994). Fabaceae seeds were treated by placing them in boiling water followed by cooling for 8 hr (Pérez-Fernández et al., 2000).

Germination experiments were conducted in plastic Petri dishes (9 cm diam.) lined with sterile cotton disks, and moistened with either distilled water, charred wood solution, or nitrogen salt solutions, according to the trial. Experiments were carried out in an incubator equipped with cool-white fluorescent tubes under a

TABLE 1. ATTRIBUTES OF SPECIES USED IN THIS STUDY[a]

Species	Plant family	Life form	Dispersal period	Distribution	Viability (%)
Holcus lanatus	Poaceae	Perennial herb	Apr–Sep	Farmlands and dehesa woodlands	100
Dactylis glomerata	Poaceae	Annual	Mar–Jul	Grasslands and dehesa woodlands	89.5
Senecio jacobaea	Asteraceae	Annual	Jun–Feb (Apr)	Pastureland and dehesa woodlands	78.75
Dittrichia viscosa	Asteraceae	Perennial herb	Aug–Oct (Nov)	Farmlands, grasslands and dehesa woodlands	98.75
Trifolium angustifolium	Fabaceae	Annual	Mar–Jul	Pastureland and dehesa woodlands	58
Retama sphaerocarpa	Fabaceae	Shrub	Apr–Jul	Pastureland and dehesa woodlands	76.25
Cistus ladanifer	Cistaceae	Shrub	(Feb)Mar–Jun	Pastureland and dehesa woodlands	61
Cistus crispus	Cistaceae	Small Shrub	Mar–May	Pastureland and dehesa woodlands	35
Cistus monspeliensis	Cistaceae	Shrub	Mar–Jun	Pastureland and dehesa woodlands	28
Cistus salviifolius	Cistaceae	Small Shrub	Mar–Jun	Pastureland and dehesa woodlands	42

[a] Distribution and life form data from Tutin et al. (1964–1980) and Devesa (1995).

thermoperiod and photoperiod regime of 10 hr at $18 \pm 1^{\circ}$C in darkness and 14 hr at $24 \pm 1^{\circ}$C in light. These experimental conditions were chosen in an attempt to emulate the environmental thermoperiods and photoperiods that a seed would experience on the soil surface in the dry season when forest fires are most likely to occur. This season is characterized by periods of rain in sporadic storms that provide enough soil moisture to trigger seed germination.

Germination was expressed as a percentage from four replicates of 20 seeds each. Germination was defined as emergence of the radicle (Thanos and Rundel, 1995; Cochrane et al. 1999) and was recorded daily over a period of 35 days.

TABLE 2. pH OF SMOKE, CHARRED WOOD, AND NITROGENOUS SOLUTIONS USED IN GERMINATION EXPERIMENTS.

	pH				
	1 mM	10 mM	25 mM	50 mM	100mM
$NaNO_2$	6.25	6.55	6.55	6.57	6.6
KNO_3	5.13	5.76	5.83	5.86	6.01
NH_4Cl	5.1	5.65	5.76	5.83	5.8
NH_4NO_3	5.5	6.36	6.58	6.8	6.8
Charred wood solutions	7.5 (10g)	8.5 (5g)	8.5 (2.5g)		
Smoke 4.2					

Germinated seeds were removed promptly. At the end of the period, nongerminated seeds were individually dissected to check their viability (embryo and endosperm intact and not discolored) (ISTA, 1993; Cochrane et al. 1999). The final results were adjusted to account for non-viable seeds. Control treatments were performed using untreated seeds of each species (four Petri dishes per species, with 20 seeds each) that were incubated on cotton disks watered only with distilled water.

Smoke Treatment. Two smoke treatments were applied by placing seeds in a 250-ml vacuum flask into which smoke was continuously funneled for 10 or 20 min, respectively (Keeley and Fotheringham, 1997). A mixture of dry and green leaf and fine stem material from each woodland species was used as fuel (Dixon, 1995; Roche et al., 1998). Smoke pH was monitored using a pH strip contained in the vacuum glass at the same time as the seeds (Table 2). Germination was assessed as described above.

Charred Wood Treatment. The effect of charred wood on germination was studied by watering four Petri dishes, each containing 20 seeds, with 10 ml of an aqueous suspension of charred wood, following the same procedure as González-Rabanal and Casal (1995). This suspension was obtained from the combustion of leaves and branches from several representative species from the woodland. Biomass was burned to obtain the maximum degree of incineration and until there was no plant material left for further combustion. The proportion of combusted species was 200 g of each of the *Cistus* species and 33.3 g of each of the remaining six species. The charred wood was diluted with distilled water to a concentration of 10 g of charred wood per liter of water. The greater contribution of *Cistus* to the mixture coincides with its greater representation in the woodland compared with the other species. The charred wood suspension was diluted to produce two suspensions with concentrations of 5.0 and 2.5 g/liter of charred wood. Four Petri dishes containing 20 seeds each were watered with each of the charred wood treatment solutions. The pH of each solution was recorded (Table 2).

Nitrogen Salts Treatment. Nitrogenous compounds were applied as aqueous solutions of $NaNO_2$, KNO_3, NH_4Cl, and NH_4NO_3, each at concentrations of 1, 10, 25, 50, and 100 mM. Four replicates, each with 20 seeds, were prepared for each treatment. The amount of each nitrogen solution applied was 10 ml. The pH of all solutions was recorded (Table 2).

Data Analyses. Data on percentage germination were analyzed by one- or two-way analysis of variance (ANOVA) using StatView to determine the effects of smoke, charred wood, and nitrogenous compounds on seed germination. Arcsine transformations were performed on the percentage germination data prior to analysis. Where two-way ANOVA showed significant effects at 95% ($P < 0.05$), Fisher's least significant differences (Lyman, 1998) were determined using one-way ANOVA for all replicates showing some germination. Normality and homocedasticity of data were tested by means of David's and Cochran's tests, respectively.

RESULTS

Effect of Charred Wood and Smoke. Smoke had a positive effect on germination in nine of the tested species (Figure 1). The exception was *Holcus lanatus*, which had high germination (100%) in the absence of any treatment. Germination enhancement varied between 13.3% in *R. sphaerocarpa* and 39.2% in *D. glomerata*. Germination in *D. glomerata,* (Poaceae) was stimulated by 20 min of exposure to smoke. Germination of the two Asteraceae and the two Fabaceae species was significantly enhanced by 10 min of exposure to smoke, whereas germination was maximized after 20 min of exposure to smoke for the four species of *Cistus* (Figure 1).

The effect of charred wood on germination was variable (Figure 1). *Holcus lanatus* did not increase its germination with charred wood due to its inherent high level of germination. Germination in *D. glomerata,* the other member of the Poaceae, increased, but only at a concentration of 5 g/liter of charred wood. For *S. jacobea,* the presence of the maximum concentration of charred wood (10 g/liter) increased germination. *Dittrichia viscosa* showed significant differences above the control with all three charred wood treatments. Differences were not observed with *T. angustifolium, R. sphaerocarpa,* and *C. monspeliensis*. Germination of *C. ladanifer* and *C. crispus* increased at the highest concentration of charred wood (10 g/liter), while *C. salviifolius* increased its germination at the lowest concentration of charred wood (2.5 g/liter) only. The effect of smoke treatment (10 or 20 min) was similar to certain concentrations of charred wood, but there was no general pattern for the 10 species. However, neither the longest period of smoke exposure nor the charred wood treatment had any effect on the level of germination in the two legumes. The longest exposure to smoke in *C. ladanifer* and *C. crispus* was similar to that of the highest concentrations of charred wood.

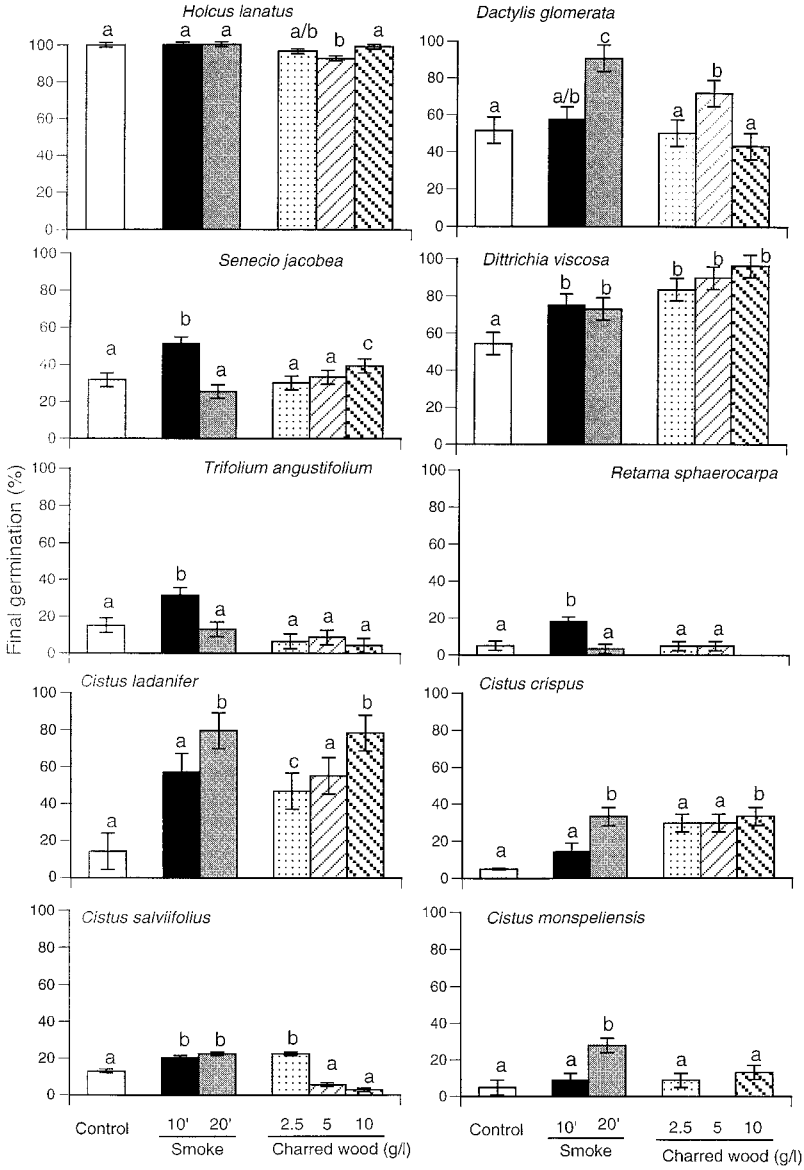

FIG. 1. Final percentages of germination of *H. lanatus*, *D. glomerata*, *S. jacobea*, *D. viscosa*, *T. angustifolium*, *R. sphaerocarpa*, *C. ladanifer*, *C. crispus*, *C. salviifolius*, and *C. monspeliensis* seeds (after 35 days incubation) treated with pulses of smoke (10 or 20 min) and three concentrations of charred wood in solution (2.5, 5 or 10 g/liter). Columns with different letters were significantly different for each species (Fisher's $\alpha = 0.05$).

Effect of Nitrogenous Compounds. Exogenous application of $NaNO_2$ at all concentrations failed to enhance significantly the germination of most species (Figure 2), although germination was promoted in *D. glomerata* at 10 mM, in *C. ladanifer* at concentrations below 25 mM, and in *C. crispus* at 1 mM.

Potassium nitrate (KNO_3) enhanced germination in *D. glomerata* at concentrations of 1 and 25 mM. In *C. ladanifer* and *C. crispus*, germination was enhanced at concentrations of 1 and 25 mM of KNO_3. Additionally, germination was enhanced in *C. ladanifer* at concentrations of 10 and 50 mM of KNO_3. The remaining concentrations inhibited germination of this species. Concentrations of 50 and 100 mM of this same nitrogenous compound inhibited germination of *C. crispus*. In *C. salviifolius*, the positive effect of the KNO_3 on germination was observed at a concentration of 25 mM. Application of NH_4Cl resulted in an increase in germination for *C. crispus* at concentrations of 10 and 25 mM and in *C. salviifolius* at concentrations of 1 and 25 mM. Ammonium chloride (NH_4Cl) also promoted germination in *C. monspeliensis* at a concentration of 25 mM. *C. ladanifer* had its germination increased at all concentrations of NH_4Cl. Finally, application of NH_4NO_3 induced germination only in the *Cistus* species and inhibited germination in the remaining species at the highest concentrations. The optimal concentrations that promoted germination were 1 and 10 mM NH_4NO_3 for *C. ladanifer* and *C. crispus*; 10 and 25 mM NH_4NO_3 for *C. salviifolius* and *C. monspeliensis.* None of the nitrogenous compounds increased germination in *T. angustifolium* or in *R. sphaerocarpa*.

DISCUSSION

Smoke, a direct effect of fire, is a germination trigger and can be interpreted as an adaptive trait because most species showed similar responses to it. Germination was not homogeneous for all the species under the same length of smoke exposure. Whereas Asteraceae and Fabaceae species showed the highest percentages of germination after 10 min of exposure, *Cistus* species required longer applications of smoke to reach similar germination percentages. Indeed, it is possible that three of the four *Cistus* species require even longer exposures to smoke to further raise their inherently low germination level; this has been demonstrated in other smoke studies (Dixon et al. 1995) in which 1 hr of exposure to smoke is needed to enhance germination. While Cistaceae species and the grass *D. glomerata* require as long as 20 min to activate their germination, the same length of smoke application is inhibitory for *S. jacobea* and the two Fabaceae species. However, it is not surprising that different species responded positively to one duration of smoke treatment and not to another. Excess exposure to smoke inhibits germination (Baldwin et al., 1994; Enright et al., 1997). Keeley and Fotheringham (1997) noted that seeds of *Emmenanthe penduliflora* tolerate only 10 min of direct exposure to smoke.

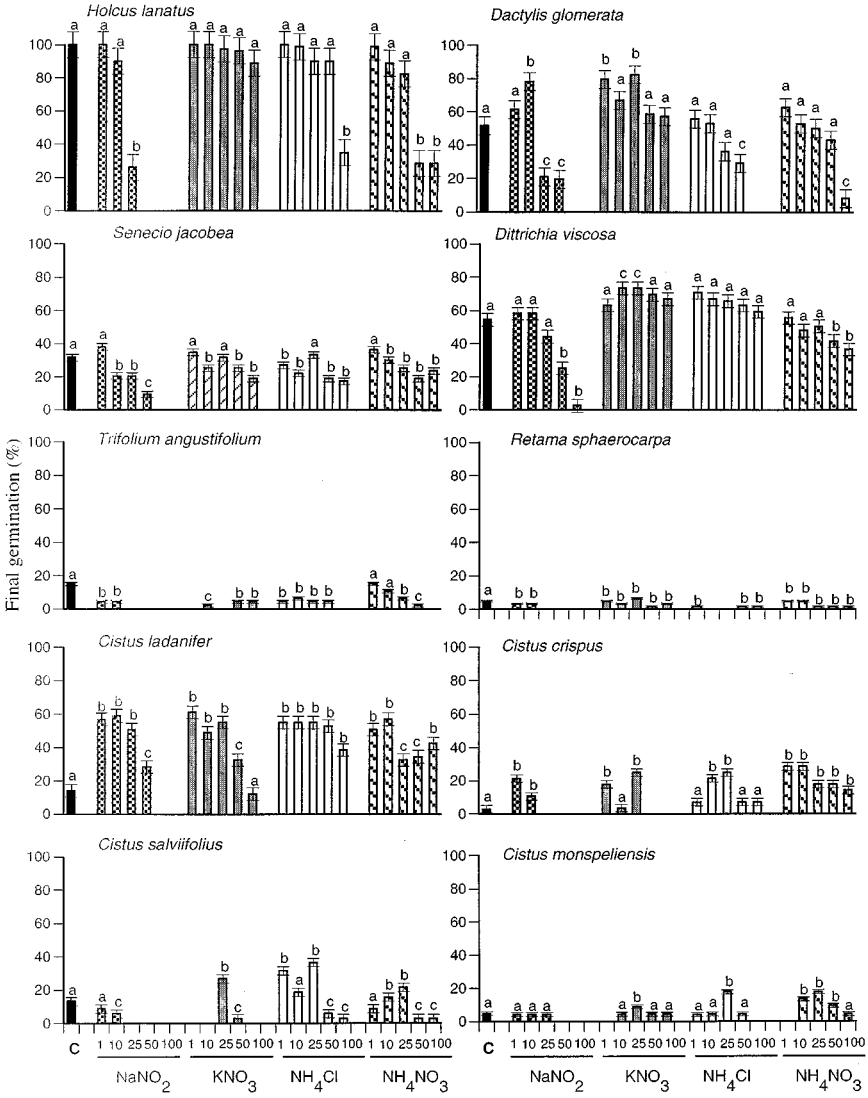

FIG. 2. Final percentages of germination of *H. lanatus*, *D. glomerata*, *S. jacobea*, *D. viscosa*, *T. angustifolium*, *R. sphaerocarpa*, *C. ladanifer*, *C. crispus*, *C. salviifolius*, and *C. monspeliensis* seeds (after 35 incubation days) treated with four nitrogenous solutions at five concentrations. Different letters indicate significant differences (Fisher's $\alpha = 0.05$).

In hard-seeded species from the Cistaceae, Fabaceae, and Rhamnaceae families (Aronne and Mazzoleni, 1989; Portlock et al., 1990; Gónzález-Rabanal and Casal, 1995; Roche et al., 1998; Read et al., 2000) and certain non-hard-seeded species, such as *Avena fatua* and *Hibertia hypericoides* (Hsiao et al., 1983; Schatral, 1996), mechanical scarification induces germination by a mechanism similar to that of natural heat-shock from fire. Since no heating of the seeds, except those from Fabaceae species, was associated with the smoke effects studied here, the presence of a chemical enhancement of seed germination is implicated. Although seeds from Fabaceae species were previously heated, the final percentage of germination was higher after 10 min of smoke exposure than in the control, indicating a positive effect of this chemical trigger on germination.

Scarification-triggered germination may be a result of wound-induced production of chemicals such as hormones and carbon dioxide (De Lange and Boucher, 1990; Craston et al., 1996; Taylor and van Staden, 1996; Voesenek and Blom, 1996; Plummer et al., 1997). Baxter et al. (1994) demonstrated that while smoke stimulated germination of a southern African grass, ethylene did not, thus eliminating it as the active component of plant-derived smoke for that species. The chemical triggers present in smoke that are responsible for enhanced germination in this study remain unknown.

The same variation in germination among species was observed after application of charred wood solutions. One can be sure that this treatment, no matter what concentration is used, does not affect germination of *C. monspeliensis* and the two legumes. Charred wood effects on germination (like those of smoke) are probably chemically mediated. However, we have not identified the chemical compounds of charred wood that enhance germination in these species. The species that require the presence of charred wood to break dormancy are also likely to be dependent on the chemical properties of the charred wood medium. A particular pH may exert a strong control on seed dormancy and the onset of germination (Sanders et al., 1981; Keeley and Fotheringham, 1998). *Retama sphaerocarpa* as well as other legumes, requires an acidic medium to break dormancy (Pérez-Fernández et al., 1996). This might explain the high germination level reached by *R. sphaerocarpa* and *T. angustifolium* treated with smoke, which is acidic (pH 4.2), and the lower germination with the charred wood, which is alkaline. However, some of the *Cistus* species that grow in naturally acidic soils (López-González, 1995) had high germination percentages in both smoke and charred wood treatments, suggesting that chemical triggers other than pH may have an effect on the dormancy of these seeds.

Compared with smoke and charred wood, nitrogenous compounds were a less potent inducing factor for the 10 species tested. In fact, they had a negative effect on germination in both *T. angustifolium* and *R. sphaerocarpa*, and this could be related to the ability of these species to colonize soils with low nitrogen concentration. Surprisingly, the germination of *C. ladanifer*, a species that typically grows in poor soils, was enhanced by all nitrogen compounds. With few exceptions, high

concentrations of nitrogenous compounds resulted in lower germination percentages than controls. The highest concentration of any of the nitrogenous solutions that resulted in a high level of germination was attained at 25 mM, whereas concentrations of 50 and 100 mM rarely produced levels of germination of biological relevance. This contrasts with other studies that have highlighted the importance of nitrogenous compounds as postfire germination stimulants (Mayer and Poljakoff-Mayber, 1989; Pons, 1989; Plummer et al., 1997; Bell et al., 1999). However, our results are in agreement with those of Bell et al. (1999) for species from Western Australia, for which nitrates enhanced germination in the range of 0–50 mM. The lowest germination rates were at concentrations close to or higher than 50 mM. Also, the International Seed Testing Association has adopted the systematic use of 20 mM KNO_3 in their germination protocols for a number of species (ISTA, 1993).

These results indicate that smoke and charred wood treatment and, to a lesser extent, exogenous application of nitrogenous compounds increase germination of dormant seeds of species from a fire-prone environment in central-western Spain. The clear trigger effect of smoke on germination indicates that at least 9 of the 10 species studied are postfire species with smoke-induced germination. More precisely, we can classify the species as follows: *Holcus lanatus*, obligate seeder with non-fire-dependent germination; *Dactylis glomerata* and *Senecio jacobea*, fire annuals; *Dittrichia viscosa*, facultative seeder; *Trifolium angustifolium*, obligate seeder with non-fire-stimulated germination; *Retama sphaerocarpa* and *Cistus ladanifer*, facultative resprouters with fire-stimulated germination; and *C. crispus*, *C. salviifolius*, and *C. monspeliensis*, facultative resprouters with fire-dependent germination.

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ODOR COMPOUND DETECTION IN MALE EUGLOSSINE BEES

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Abstract—Male euglossine bees collect fragrances from various sources, which they store and use for as yet unknown purposes. They are attracted, often specifically, to single odor compounds and blends thereof. We used gas chromatography with electroantennographic detection (GC-EAD) and electroantennography (EAG) to investigate the response to 8 odor compounds by males of two euglossine species, *Euglossa cybelia* Moure and *Eulaema polychroma* (Mocsàry). In *E. cybelia*, we recorded EAD reactions in response to 1,8-cineole, methyl benzoate, benzyl actetate, methyl salicylate, eugenbl, and methyl cinnamate. *E. polychroma* responded to the same compounds in EAG experiments, while (ls)(-)α-pinene and β-pinene failed to trigger EAD or EAG responses in the bees. Blends of two compounds triggered larger responses than single compounds in EAG experiments with E. *polychroma*, however, when α-pinene was added, reactions decreased. In the light of existing data on the bees' behavior towards these odor compounds, our work indicates that both peripheral and central nervous processes influence the attraction of euglossine bees to odors.

Key Words—Euglossine bees, GC-EAD, EAG, odor compound detection.

INTRODUCTION

Male euglossine bees are attracted by the scent of certain orchid flowers where they collect odor substances and thereby pollinate flowers (Vogel, 1966; Dodson et al., 1969). Although this behavior has attracted considerable research interest, the purpose of the odor collection behavior remains elusive (Eltz et al., 1999). Synthetics of chemicals found in orchid flowers have been used to perform tests

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that show bees are attracted selectively to certain compounds and/or specific mixtures of volatiles (Dodson et al., 1969; Williams and Dodson, 1972; Ackerman, 1989). Orchid flowers pollinated by euglossine bees may produce specific floral odor bouquets that are thought to be responsible for attraction of their pollinators (Hills et al., 1972; Williams and Whitten, 1983; summary in Roubik and Hanson, in press). The olfactory mechanisms underlying selective behavioral attraction towards odor compounds and mixtures thereof have not previously been investigated.

Odor detection by olfactory neurons may have a major influence on the behavior of euglossine bees, while compounds undetected on a receptor level may have little effect. In a blend of two or more compounds, however, undetected substances may inhibit responses of olfactory receptors to active compounds (Cromarty and Derby, 1998). On the other hand, if more than one compound in a blend is detected individually, then interactions of the signals in the central nervous system may govern behavior (Smith and Getz, 1994). Our study investigates for the first time the detection of odor compounds and mixtures thereof in two species of euglossine bees, *Euglossa cybelia* and *Eulaema polychroma*, by using gas chromatography with electroantennographic detection (GC-EAD) and electroantennography (EAG).

METHODS AND MATERIALS

Male *Euglossa cybelia* Moure and *Eulaema polychroma* (Mocsàry) were collected at 800 m elevation on 8 April, 2002 in central Panama. The bees were transferred to Zürich, Switzerland, where the electrophysiological investigations were conducted.

Gas Chromatography with Electroantennographic Detection (GC-EAD). We used 8 synthetic compounds for our recordings [(ls)(-)α-pinene, β-pinene, 1,8-cineole, methyl benzoate, benzyl acetate, methyl salicylate, eugenol, and methyl cinnamate], which are common odor constituents of euglossine-pollinated orchids (Hills et al., 1972). Many such odors are also known as general attractants for euglossine bees (Dodson et al., 1969; Ackerman, 1989). One μl of a mixture of all compounds (100 μg/ml each) was injected splitless at 50°C (1 min) into a gas chromatograph (HP 6890N) followed by opening the split valve and programming to 300°C at a rate of 10°C/min. The GC was equipped with an HP-5 column (30 m $\times$ 0.32 mm $\times$ 0.25 μm); helium was used as carrier gas. A GC effluent splitter (SGE Australia; split ratio 1:1) was used, and the outlet was placed in a purified and humidified airstream. This air was directed over a male bee's antenna prepared as follows: The tip of the excised antenna was cut off and the antenna mounted between an electrode holder using electrode gel. One electrode was grounded, the other was connected via interface box (Syntech, Hilversum) to a PC. EAD signals and flame ionization detector (FID) responses were simultaneously recorded. GC-EAD analyses of two males of *Euglossa cybelia* were obtained.

Electroantennography (EAG). One antenna of *Eulaema polychroma* was mounted on an electrode holder, and signals were recorded as described in the previous paragraph. 10 μg of a synthetic compound in 10 μl solvent, and mixtures of synthetic compounds with 10 μg each were applied on a filter paper in a pipette tip. A control filter paper was treated with 10 μl solvent only. Enough time was allowed for the solvent to evaporate. During stimulation, 2.5 ml of air were puffed over the filter paper and into an airstream directed over the antenna. Two stimulations of each test compound were applied with minimum 40 set intervals. To normalize and compensate for mechanoreceptive artifacts, EAG responses (−mV) were expressed as percent response of the control stimulant. From these values, mean responses to each compound and compound mixture were calculated.

RESULTS AND DISCUSSION

In *Euglossa cybelia*, consistent EAD reactions were recorded from all tested compounds except α- and β-pinene (Figure 1). Responses to most compounds were of similar intensity; however, the reactions to 1,8-cineole and methyl cinnamate were weaker than to other compounds. Similarly, *Eulaema polychroma* responded

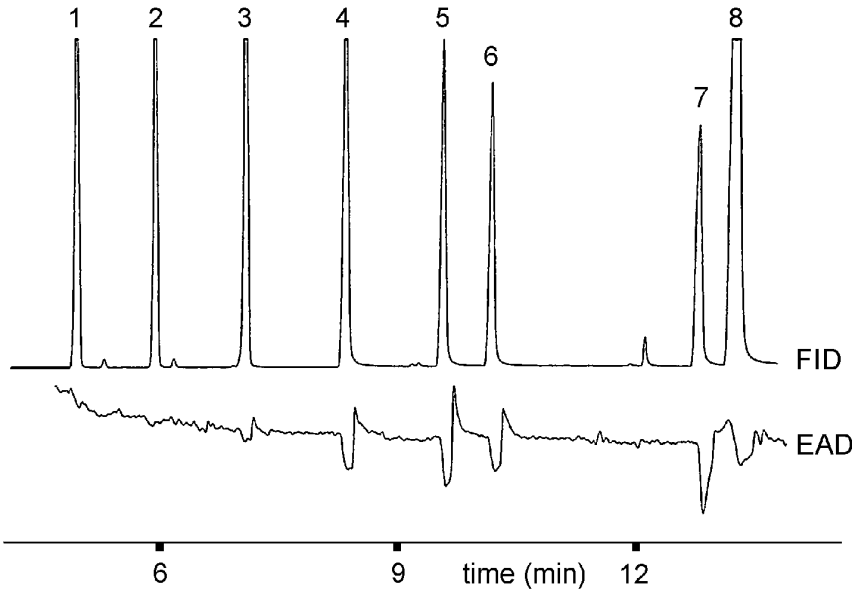

FIG. 1. Gas chromatographic analysis of a mixture of synthetic compounds with flame ionization detector (FID) and electroantennographic detection (EAD) recorded simultaneously using an antenna of a *Euglossa cybelia* male. For names of compounds see Table 1.

TABLE 1. RELATIVE EAG RESPONSES OF A *Eulaema Polychroma* MALE TO VARIOUS ODOR COMPOUNDS AND MIXTURES THEREOF (CONTROL = 100%)

Compound/mixture[a]	Mean relative EAG response (in %) ± s.e.m.
1) (1s)(-)α-pinene	85.71 ± 0.00
2) β-pinene	85.71 ± 0.00
3) 1,8-cineole	192.86 ± 21.43
4) methyl benzoate	350.00 ± 78.57
5) benzyl acetate	600.00 ± 14.29
6) methyl salicylate	571.43 ± 100.00
7) eugenol	300.00 ± 0.00
8) methyl cinnamate	200.00 ± 14.29
methyl salicylate + benzyl acetate	864.29 ± 21.43
1,8-cineole + benzyl acetate	871.43 ± 0.00
α-pinene + 1,8-cineole + benzyl acetate	664.29 ± 35.71

[a] Each compound/blend was applied two times on the antenna.

to all of the tested compounds except α- and β-pinene in the EAG experiments (Table 1). Mixtures of 1,8-cineole + benzyl acetate and benzyl acetate + methyl salicylate resulted in stronger reactions than application of single compounds. However, the addition of α-pinene to the mixture of 1,8-cineole + benzyl acetetae lead to a smaller reaction than with 1,8-cineole + benzyl acetetae alone.

It is now well established that odor compounds may attract euglossine bees selectively. Certain compounds attract certain species, and mixtures of compounds often attract the bees more selectively than single compounds (Dodson et al., 1969; Williams and Dodson, 1972; Ackerman, 1989). Ackerman (1989) showed that the compounds used in our investigation are attractive for a range of *Euglossa* and *Eulaema* species. *E. cybelia* is, however, attracted only to 1,8-cineole, benzyl acetate, and methyl salicylate, but not to eugenol, methyl benzoate, or methyl cinnamate. The chemicals α- and β-pinene were not tested by Ackerman (1989), but are generally known as poor attractants (Dodson et al., 1969). *E. polychroma* is attracted to 1,8-cineole, but not by the other compounds tested here. Because both bee species could detect all the tested compounds except α- and β-pinene, we conclude that a lack of attraction does not necessarily imply a lack of detection ability. The attractiveness of a certain chemical seems, however, to be influenced by processes in the central nervous system, which mediate behavioral patterns of the bees. Electrophysiologically active floral fragrances may in general not only attract, but also deter flower visitors.

In our EAG experiments, it was evident that the addition of α-pinene to a blend reduced the EAG response. Myrcene and α-, β-pinene were shown to reduce the attractiveness of 1,8-cineole, in field assays, by attracting fewer species and each species in smaller numbers than 1,8-cineole alone (Williams and Dodson, 1972). Our results indicate that such a reduced attractiveness may, at least in part, be due

to inhibition of receptor responses by certain compounds, which may influence the detection of molecules by the bee. Such inhibition mechanisms may include binding interactions, in which compounds mutually inhibit binding to receptor sites (Cromarty and Derby, 1998). Since α-pinene appears not to be detected as a single compound, an interaction with signals triggered by other compounds on the central nervous system level seems unlikely.

In summary, we present evidence that both detection mechanisms at the peripheral and central nervous system processes may be important for the selective attraction of euglossine bees to odor compounds and mixtures thereof. Our study has further shown that electrophysiological methods such as EAG and GC-EAD are tools that may help to better understand complex interactions of euglossine bees and floral odors.

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ANNOUNCEMENT

FIRST JOINT MEETING (http://aginfo.snu.ac.kr/isce2003) OF 20TH INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY (ISCE) AND 3RD ASIA-PACIFIC ASSOCIATION OF CHEMICAL ECOLOGISTS (APACE) GYEONGJU, SOUTH KOREA JULY 14–18, 2003

The first joint meeting between ISCE (20th) (http://www.chemecol.org) and APACE (3rd) (http://aginfo.snu.ac.kr/apace) will be held at the Hotel Concorde, Gyeongju, South Korea. The joint meeting will begin on the evening of July 14, 2003 (Monday) and end on the evening of July 18 (Friday) with the farewell banquet. The conference will be hosted by Kyung Saeng Boo (ksboo@plaza.snu.ac.kr; ksboo@hotmail.com) of Seoul National University.

There will be four symposia covering aspects of chemical ecology in semiochemical physiology and chemistry, insect-plant interactions, and practical application of semiochemicals. Both oral and poster presentations are invited from participants on all aspects of chemical ecology including allelopathy.

This will be a wonderful opportunity for chemical ecologists from the West and the East to interact. The dates are set to accommodate those attending the 15th International Plant Protection Congress (July 6–11) in Beijing. Participants are encouraged to arrive on Monday, July 14.

Further information may be obtained from the web sites above or K. S. Boo.

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CHANGE IN ACCEPTABILITY OF BARLEY PLANTS TO APHIDS AFTER EXPOSURE TO ALLELOCHEMICALS FROM COUCH-GRASS (*Elytrigia repens*)

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Abstract—The response of the bird cherry-oat aphid, *Rhopalosiphum padi*, to barley plants was investigated following exposure of the plants to root allelochemicals from the aggressive weed couch-grass, *Elytrigia* (*Agropyron*) *repens.* Plants were treated either with root exudates from living couch-grass plants or with previously identified couch-grass root compounds [5-hydroxyindole-3-acetic acid, DL-5-hydroxytryptophan, L-5-hydroxytryptophan hydrate, and 6-hydroxy-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (carboline)] either separately or in mixtures. In choice and no-choice settling tests, aphid acceptance of barley plants was significantly reduced following treatment with root exudates, and the carboline when tested alone or in combination with the other compounds. In contrast, the other compounds without the carboline were less active in reducing aphid acceptance. In a probing bioassay, individual substances were either neutral or stimulatory to aphids, indicating that the reduced settling was probably not due to direct effects on aphids, but rather due to effects on the plant. This was confirmed in olfactometer assays, in which aphids were repelled by odors from barley plants following treatment with a mixture containing all four chemicals.

Key Words—*Elytrigia repens*, *Agropyron repens*, allelopathy, aphid, herbivore, barley.

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INTRODUCTION

Plants face stress in many forms, such as extreme temperatures, drought and attack by pathogens and herbivores. However, for plants growing in a favorable environment, the most serious threat is likely to come from competition with other plants. Plants have evolved a range of strategies for dealing with competition, such as changes in production and allocation of biomass, optimized seed production and alternative propagation methods.

Although allelopathy is considered a separate phenomenon to competition (Muller, 1969), it is a factor that can influence the outcome of competitive interactions (Reigosa et al., 1999). Allelopathy is defined as "direct or indirect (harmful or beneficial) effects of a plant, on another plant through the release of compounds that escape into the environment" (Rice, 1984). Allelopathic substances may be released from the roots, or from the aerial parts, either as volatiles in the atmosphere or via decaying ground foliage with subsequent transferal through the rhizosphere (Rice, 1984).

The effects of allelopathy on the receiving plant have most often been classified in terms of reduced germination, growth and yield, or changes in various physiological parameters (Reigosa et al., 1999). However, the effects of allelopathic provocation on the next trophic level, namely the herbivores that feed on the affected plant, are only starting to be addressed. Pettersson et al. (1999), for example, showed that aphid acceptance of barley cultivars is reduced when the plants are exposed to volatiles emitted from undamaged plants of other cultivars.

Couch-grass, *Elytrigia* (*Agropyron*) *repens* L. Beauv (Poaceae), an aggressive perennial plant with mainly rhizomal propagation, is a serious weed in the Northern Hemisphere and has long been known to produce allelopathic exudates that affect other plants (Oswald, 1948; Phlak, 1967). Allelochemicals that have been identified from *E. repens* include (2,4-dihydroxy-1,4-benzoxazin-3-one) (DIBOA), ferulic acid (Schulz et al., 1994), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Friebe et al., 1995) and vanillin (Bobnick and Hagin, 1985). Other biologically active compounds isolated from *E. repens* include 5-hydroxyindole-3-acetic acid (indole acetic acid) and DL-5-hydroxytryptophan (hydroxytryptophan), both of which inhibit seedling growth in corn and bean (Hagin, 1989), L-5-hydroxytryptophan hydrate (hydroxytryptophan hydrate), and 6-hydroxy-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (carboline) the latter of which has molluscicidal activity (Hagin and Bobnick, 1991). To test the hypothesis that rhizosphere chemicals affect plant interactions at the next trophic, i.e., plant–herbivore level, the effects of tryptophan-derived chemicals on recipient plants were investigated. Barley, *Hordeum vulgare*, was used as the model recipient plant as it has been used successfully in demonstrating reduced acceptability following exposure to volatiles from neighboring barley plants (Pettersson et al., 1999). The bird cherry-oat aphid, *Rhopalosiphum padi*, was chosen as a

model phloem-feeding herbivore that is also an important pest and virus vector in northern and western Europe. Barley plants were exposed to roots from living *E. repens* using a simple step-watering system and were treated with *E. repens* chemicals both alone and in mixtures. Aphid acceptance of treated and untreated plants was assessed by means of both choice and no-choice settling tests and by measuring in an olfactometer responses to odors from treated and untreated plants. Direct aphid responses to the chemicals were also measured in a probing bioassay.

METHODS AND MATERIALS

Plants. Couch-grass, *E. repens*, was collected from Sätuna, north of Uppsala, Sweden, in 2000. Individual plants were removed from the ground, transferred to pots (8.5 cm wide × 7 cm high) with potting soil (Hasselfors Garden) and sand (approx 30% sand in the mixture), and kept in a glasshouse at 18–22°C with a 16L:8D light cycle. Plants were occasionally cut back with scissors to encourage growth and, as individual plants became larger, they were divided and transferred to new pots. Plants were watered sparingly when required.

Barley plants, *Hordeum vulgare*, used for experiments (cv. Kara), and as host plants for aphid rearing (cv. Golf), were grown in the same glasshouse as *E. repens*, but the different cultivars were separated by a distance of at least 10 m from each other and from *E. repens*. Experimental plants were grown in plastic pots (8.5 cm wide × 7 cm high) with six seeds per pot, and were at the two-leaf stage at the beginning of exposure to *E. repens* and chemicals. Cultivar Kara was selected for the experiments because it had previously been found to respond to exposure to plants of other barley cultivars, while at the same time being unresponsive to exposure to plants of the same cultivar (self-provocation) (Pettersson et al., 1999).

Insects. Bird cherry-oat aphids, *Rhopalosiphum padi*, were reared on barley in multiclonal cultures in a glasshouse at 18–22°C, with a 16L:8D light cycle. Aphids used in the experiments were all mixed-instar apterae, and were collected from cultures immediately prior to bioassays.

Treatment of Barley Plants with E. repens Root Exudates. Experiments were performed using specially designed two-chamber Perspex cages attached to a vacuum tank extractor, as described previously by Pettersson et al. (1996, 1999). In its standard configuration, when connected to the extractor, air is drawn through an opening into the front chamber, passes through a second opening into the rear chamber before being drawn from the top of this chamber into the vacuum tank, and then is vented outside the greenhouse. Both chambers are sealed at the top opening with cellophane. For this experiment, the two chambers of the exposure cage were separated and arranged with one directly on top of the other to form a two-tier cage. At the base of the upper chamber was placed a shelf made from inert polythene foam plastic (Plastazote PZ940) in which a hole was cut and a

glass funnel inserted. A Pasteur pipet (230 mm) was attached to the spout of the funnel with a short length of Teflon tube and extended down into the lower chamber through a small hole in the cellophane sealing the chamber opening. On the shelf, directly above the opening of the funnel was placed a plastic Petri dish (9 cm) into which a hole was cut matching the diameter of the funnel. One piece of filter paper was placed into the Petri dish to filter soil and particulates, and a small piece of glass wool was inserted into the funnel spout for the same reason. The funnels and glass wool were baked at 180°C prior to use, and fresh Petri dishes and filter papers were used. Into the upper chamber were introduced either a potted *E. repens*, or a pot of the same size containing only fresh potting soil as a control. A barley plant was placed into a Petri dish in the lower chamber of each two-tier cage, and the upper and lower chambers were connected separately to the same vacuum extractor to prevent transfer of air and volatiles between the chambers. Water was provided only to the pots in the upper chamber containing either *E. repens* or potting soil, which subsequently percolated through the soil, before collecting into the funnel and dripping directly via the pipet onto the soil containing the barley plants in the lower chamber. Water was supplied via an automated water drop system, with a single 8-min delivery occurring daily at 8 AM (2 hr into the photophase), which delivered a total of 98 ml to each cage. Eight barley plants were exposed for a period of 5 days in the experiment, four to *E. repens* and four to pots with potting soil. The experiment was repeated twice and was performed in a glasshouse at 18–22°C, with a 16L:8D light cycle. Aphid settling on treated and control plants was assessed by no-choice settling tests as described below.

Chemicals. DL-5-Hydroxytryptophan (hydroxytrytophan), L-5-hydroxytryptophan hydrate (hydroxytrytophan hydrate), and 5-hydroxy-3-indoleacetic acid (indoleacetic acid) were purchased from Sigma-Aldrich (Stockholm, Sweden). (3*S*)-6-Hydroxy-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (carboline) was synthesized by condensation of L-5-hydroxytryptophan (Lancaster Synthesis Ltd, Morecambe, UK) with formaldehyde (Brossi et al., 1973). NMR spectra for the carboline (Bruker 500 Avance NMR spectrometer): ^{1}H NMR (500 MHz, DMSO-d6) δ 10.70 (1H, s, H-9), 6.97 (1H, d, J 8.5, H-8), 6.64 (1H, s, H-5), 6.44 (1H, d, J 8.5, H-7), 4.14 (1H, d, J 15.5, H-1), 4.08 (1H, d, J 15.5, H-1), 3.58 (1H, dd, J 11, 4.5, H-3), 2.99 (1H, dd, J 15.5, 4.5, H-4), and 2.67 (1H, dd, J 15.5, 11, H-4); ^{13}C NMR (125 MHz, DMSO-d6) δ 171.2 (C3), 151.7 (C6), 131.5 (C8a), 128.3 (C9a), 127.7 (C4b), 112.5 (C8), 112.3 (C7), 106.4 (C4a), 102.9 (C5), 57.5 (C3), 41.3 (C1), and 23.6 (C4). Mass spectral data (VG Autospec, probe-tip analysis; EI at 70 eV, 250°C): m/z 232 (35%, M^{+}), 184 (60), 159 (100), 155 (17), 130 (13), 103 (6), 77 (8), 44 (12).

Treatment of Barley Plants with Chemicals. Barley plants were placed individually into the front chamber of exposure cages (as described above, the second chamber was empty in this case) to avoid transfer of volatiles between plants. Cages were assigned to either treatment or control, and placed in alternating positions

TABLE 1. PROTOCOLS USED TO TREAT BARLEY PLANTS WITH *E. repens* CHEMICALS AND TO TEST SETTLING RESPONSE OF *R. padi* TO TREATED PLANTS

Substance	Concentration (mg/ml)[a]	Experiment duration (days)	Treated on days[b]	Total subst./ cage (mg)	Settling test
1. Hydroxytryptophan	0.5	6	4,5	3	choice
2. Hydroxytrypt. hydr.	0.5	6	4,5	3	choice
3a. Indoleacetic acid	0.5	6	4,5	3	choice
3b. Indoleacetic acid	0.5	5	3,4	3	no-choice
4a. Carboline	0.13	5	2,3,4	1.17	no-choice
4b. Carboline	0.13	5	2,3	0.78	no-choice
5a. Mixture					
Hydroxytryptophan	0.5				
Hydroxytrypt. hydr.	0.5	6	4,5	9.36	choice
Indoleacetic acid	0.5				
5b. Mixture					
Hydroxytryptophan	2				
Hydroxytrypt. hydr.	2	6	2,3,4,5	74.3	no-choice
Indoleacetic acid	2				
Carboline	0.2				
5c. Mixture					
Hydroxytryptophan	1				
Hydroxytrypt. hydr.	1	5	2,3,4	28.8	no-choice
Indoleacetic acid	1				
Carboline	0.2				

[a] Solution in distilled water. The carboline was first dissolved in 1 ml acetone.
[b] Each treatment consisted of 3 ml solution.

along the bench. There were equal numbers of treatment and control cages (five or six). Experiments ran for 5 or 6 days, during which time plants were treated with *E. repens* root allelochemicals (or the appropriate control) according to various regimes described in Table 1. Hydroxytryptophan, hydroxytryptophan hydrate, and indoleacetic acid were dissolved in distilled water to the concentrations listed in Table 1. Concentrations in the initial experiments (1–3) were chosen based on preliminary studies (J. Pettersson, unpublished data), and in later experiments were varied to ascertain the effects of concentration on plant and insect response. Aqueous solutions (3 ml volume) were applied using disposable plastic syringes (5 ml) directly to the soil surface of the pots in which barley plants were growing. Control plants received distilled water (3 ml). Because the carboline had low solubility in water, it was dissolved in distilled acetone (1 ml) prior to the addition of distilled water. In experiments where the carboline was included, control plants received distilled water (3 ml) containing acetone at an identical level to that for the treatment solution. Treatments with chemicals were made between 14:00 and 17:00 hr. Water was delivered via an automated water drop system, with a single 2-min delivery occurring daily at 8 AM (2 hr into the photophase), which delivered a total

of 12 ml to each cage. Experiments were performed in a glasshouse at 18–22°C, with a 16L:8D light cycle.

Aphid Settling Tests. In initial experiments, aphid settling on treated and control barley plants was assessed by means of a choice test (Pettersson et al., 1999), in which the aphids were presented with a control and a treated plant simultaneously. One plant each from a control and treated cage was carefully removed from the pot, with soil still surrounding the seed and roots, and laid side by side, 5 cm apart, on a piece of white paper on a glasshouse bench. Ten mixed-instar apterous *R. padi* were released between the plants, and a net-covered PVC cylinder (10 cm high × 12 cm diam.) was placed over the leaves and aphids. The number of aphids settling (not walking) on each leaf in a pair was recorded after 2 hr. Data from choice tests were analyzed with *t*-tests for dependent samples (SAS, 1997).

In subsequent experiments, a no-choice test (Ninkovic et al., 2002) was preferred because this gave an absolute measure of aphid acceptance of the plant presented *in situ*, with a minimum amount of disturbance to the plant. The no-choice test is considered more rigorous because, in a choice test the aphids' apparent response to one plant may be accentuated by the presence of a (potentially attractive or repellent) second plant. Although in a no-choice situation all aphids may eventually be forced to accept the plant, this should occur over a longer time period than the 2 hr used in the current experiments, which is sufficient for aphids to settle and reach the phloem (Prado and Tjallingii, 1997). In the no-choice test, a 50-ml polystyrene tube was placed over the youngest, fully developed leaf. The upper end of the tube was covered with a net and the lower end with a foam plastic plug with a slit for the leaf. To minimize mechanical damage to the test plant, a stick was used to support the tube. Ten mixed-instar apterous aphids were placed into the tube and, after 2 hr, the number of aphids settled (not walking) on the leaf was recorded. Occasionally aphids were able to escape from the tubes, but the results were expressed as a proportion of the number of aphids originally introduced, in case the propensity of an aphid to escape from the tube was influenced by the experimental treatment. The number of replicates (tubes) varied among experiments, but there were always equal numbers of control and treatment plants. Typically, four plants per pot (and, therefore, per cage since each cage held a single pot) were randomly selected for the test, and each pot was considered to be a block for the statistical analysis. Data were expressed as proportions and analyzed by ANOVA (SAS, 1997).

Olfactometry. Olfactory responses of *R. padi* to barley plants provoked by *E. repens* chemicals were measured with a modified two-way airflow olfactometer. This was a standard four-arm device (Pettersson, 1970), but with two of the arms closed with silicone rubber inserts, effectively creating a two-way olfactometer consisting of two stimulus zones (arms) directly opposite each other and a central neutral zone separating them. The olfactometer was connected to the vacuum

extraction system in the glasshouse, as described for the two-chamber cages, and each separate olfactometer was connected to a cage containing a control barley plant and a cage containing a treated barley plant (details of experiments below). A single *R. padi* aptera was introduced into the olfactometer, and its position was recorded every 3 min over a 30-min period. If an aphid was inactive in the olfactometer (i.e., observed to be stationary in the same position for three consecutive observations), it was removed and the bioassay started with a fresh aphid. Experiments were replicated 20–24 times. For analysis, the number of observations in the control and treatment arms were compared using Wilcoxon matched pairs tests (SAS, 1997). Olfactometry was performed after barley plants had been treated in four different ways. In all cases, the methodology was identical to the experiments previously described above; however, olfactometry was done using plants exposed in separate experiments, not plants from the same exposures as in the aphid settling experiments.

Treatments comprised: (I) barley plants exposed to root exudates from *E. repens* as described above; (II) barley plants exposed to the carboline, with the protocol as in 4b (Table 1); (III) barley plants exposed to a mixture of the hydroxytryptophan, hydroxytryptophan hydrate, and indoleacetic acid as in 5a (Table 1); and (IV) barley plants exposed to a mixture of the hydroxytryptophan, hydroxytryptophan hydrate, indoleacetic acid and the carboline as in 5c (Table 1).

Aphid Probing Bioassay. To test the direct effects of the chemical compounds on *R. padi*, a bioassay was developed that allowed measurement of aphid probing activity on an artificial feeding substrate containing the chemicals. In preliminary tests, a solution containing sugar (commercially available sugar cubes, Danisco Sugar AB, Sweden) was found to stimulate probing by *R. padi* at a concentration of 0.4 M. This standard medium was used to make separate solutions containing the hydroxytryptophan, hydroxytryptophan hydrate, indoleacetic acid, and the carboline, each at 4 mM concentration. No attempt was made to buffer the solutions, and the pH values at 19˚C were as follows: control sugar solution, 7.1; hydroxytrytrophan, 6.8; hydroxytryptophan hydrate, 7.1; indoleacetic acid, 3.2; carboline/acetone, 7.1.

Probing bioassays were made using a specially designed Perspex sheet (17 $\times$ 17 cm), in which were cut eight separate chambers, each consisting of two circular subchambers (2.8 cm diam.), connected by a short rectangular corridor (1 cm long). One face of the Perspex sheet was sealed with fine Terrylene netting, and the opposite face was open. The sheet was laid flat on a bench, with the open face uppermost. Ten apterous *R. padi* were introduced into the central corridor of each of the eight chambers, and the open face of the chambers sealed by stretching a strip of Parafilm (Parafilm M, American National Can) over each one. An aliquot (150 μl) of test solution was dispensed onto the Parafilm membrane directly above one of the circular subchambers, and the same amount of control solution was dispensed above the opposite chamber. A circular glass microscope cover slip (1.9 cm diam.)

was gently lowered onto each drop of solution, which then formed an even layer on the Parafilm membrane.

Aphids climbed onto the underside of the Parafilm membrane and probed through it into the solutions. Every 30 min over a period of 5 hr, the numbers of aphids probing into the test and the control solutions were recorded. Because aphids tended to walk around between periods of probing, the results were expressed as the mean number of aphids observed over all of the time intervals. The mean number of aphids observed feeding on the test substance was compared with the mean number feeding on the control substance using *t*-tests (SAS, 1997). Each experiment was replicated in two separate Perspex sheets (total of 16 replicates per experiment). Indoleacetic acid and the carboline were tested in single experiments, the hydroxytryptophan and hydroxytryptophan hydrate were tested twice on different occasions with freshly made test and control solutions.

RESULTS

Aphid Settling and Olfactory Response to Barley Plants Exposed to Root Exudates from E. repens. The number of aphids settling on plants exposed to root exudates from *E. repens* was significantly lower than on unexposed plants (Table 2). In the olfactometer, there was no difference between the number of times aphids were observed in the arms containing odor from exposed or unexposed plants (Table 3).

Aphid Settling and Olfactory Response to Barley Plants Treated with E. repens *Chemical Substances*. When offered a choice between untreated plants and plants treated with the hydroxytryptophan (1, Table 1), or between untreated

TABLE 2. *R. padi* SETTLING ON BARLEY LEAF AFTER 2 HOURS IN NO-CHOICE SETTLING TESTS, AFTER EXPOSURE OF BARLEY PLANTS TO SOLUTIONS WITH *E. repens* CHEMICALS

Experimental treatment[a]	N[b]	Proportion of aphids settling[c] (mean ± SE)		ANOVA	
		Treated	Control	F	P
3b. Indoleacetic acid	5/20	0.66 ± 0.04	0.75 ± 0.03	2.8	0.11
4a. Carboline	6/24	0.62 ± 0.03	0.80 ± 0.03	21.4	<0.001
4b. Carboline	6/24	0.58 ± 0.03	0.71 ± 0.02	10.1	0.003
5b. Mixture	6/24	0.68 ± 0.03	0.74 ± 0.04	7.8	0.008
5c. Mixture	6/24	0.72 ± 0.03	0.84 ± 0.02	13.8	<0.001
E.repens root exudates	3/15	0.74 ± 0.03	0.89 ± 0.03	11.1	0.03
E.repens root exudates	3/15	0.67 ± 0.03	0.86 ± 0.03	16.2	0.005

[a] Numbers refer to experimental treatments detailed in Table 1.
[b] Number of pots/number of settling tests (a single pot with six barley plants was kept in each exposure cage, settling tests were performed on four or five plants from each pot).
[c] Ten aphids were used in each settling test.

TABLE 3. *R. padi* VISTS IN OLFACTMETER ARMS WITH ODORS OF BARLEY PLANT EXPOSED TO SOLUTIONS WITH *E. repens* CHEMICALS OR UNTREATED PLANT

Experimental treatment[a]	N[b]	Aphid visits (mean ± SE)		Wilcoxon test	
		Treated arm	Control arm	Z	P
E.repens root exudates	18	3.89 ± 0.32	3.06 ± 0.42	1.22	0.22
Mixture 5a	20	2.70 ± 0.23	3.45 ± 0.40	1.29	0.19
Carboline 4b	14	2.43 ± 0.63	4.13 ± 0.63	1.33	0.18
Mixture incl. carboline 5c	20	2.55 ± 0.40	4.95 ± 0.38	2.79	0.005

[a] Numbers refer to experimental treatments detailed in Table 1.
[b] Number aphids tested (aphids were tested individually).

plants and plants treated with the hydroxytryptophan hydrate (2, Table 1), no difference was found in the number of aphids settling on treated and untreated plants (Table 4). When offered a choice between untreated and indoleacetic acid-treated plants (3a, Table 1), fewer aphids settled on the treated plants (Table 4). However, in a no-choice test (3b, Table 1), there was no difference in the proportion of aphids settling on untreated plants and those treated with the indoleacetic acid (Table 2).

In both experiments in which barley plants were treated with the carboline (4a,b, Table 1), a lower proportion of aphids settled on carboline-treated plants than on untreated plants (Table 2).

In all experiments in which barley plants were treated with mixtures of chemicals (5a,b,c, Table 1), aphid settling on treated plants was lower than on untreated plants (5a, Table 4; 5b,c, Table 2). There were no differences between experimental blocks (cages/pots) in any of the no-choice experiments, apart from experiment 4a with the carboline (ANOVA, $F = 3.29$, $P = 0.015$).

TABLE 4. *R. padi* SETTLING ON BARLEY LEAF AFTER 2 HOURS IN SETTLING TESTS WHEN OFFERED A CHOICE OF BARLEY PLANT EXPOSED TO SOLUTIONS WITH *E. repens* CHEMICALS OR UNTREATED PLANT

Experimental treatment[a]	N[b]	Aphids setting[c] (mean ± SE)		t test	
		Treated	Control	t	P
1. Hydroxytryptophan	20	3.20 ± 0.30	3.60 ± 0.42	0.60	0.55
2. Hydroxytrypt. hydrate	20	2.95 ± 0.41	3.63 ± 0.37	1.31	0.20
3a. Indoleacetic acid	20	2.30 ± 0.34	4.80 ± 0.41	3.75	0.001
5a. Mixture	20	3.15 ± 0.35	5.70 ± 0.41	3.94	<0.001

[a] Numbers refer to experimental treatments detailed in Table 1.
[b] Number of settling choice tests (a single pot with six barley plants was kept in each exposure cage, settling tests were performed using one plant from each pot).
[c] Ten aphids were used in each settling choice test.

TABLE 5. *R. padi* OBSERVED PROBING IN CHOICE TESTS OF SUGAR SOLUTIONS WITH AND WITHOUT *E. repens* CHEMICALS

Text substance	Aphids probing[a] (mean ± SE)		*t* test	
	Treated	Control	*t*	*P*
Hydroxytryptophan I	1.93 ± 0.34	1.10 ± 0.27	2.14	0.09
Hydroxytryptophan II	0.93 ± 0.17	0.40 ± 0.18	2.15	0.04
Hydroxytrytophan hydrate I	1.20 ± 0.19	0.59 ± 0.17	2.14	0.03
Hydroxytrytophan hydrate II	1.29 ± 0.17	0.48 ± 0.11	2.18	0.001
Indoleacetic acid	0.34 ± 0.07	0.65 ± 0.16	2.29	0.11
Carboline	1.18 ± 0.35	0.96 ± 0.27	2.16	0.64

[a] Mean of observations every 30 min over 5 hr.

When plants were treated with the carboline alone (4b, Table 1), or a mixture of chemicals excluding the carboline (5a, Table1), there was no difference between the number of times aphids were observed in the arms containing odor from treated or untreated plants in the olfactometer (Table 3). However, when plants were treated with a mixture containing carboline (5c, Table 1), aphids were observed less often in the arm containing odor from a treated plant (Table 3).

Aphid Probing Bioassay. Aphids were observed to probe more often in response to solutions containing the hydroxytryptophan and hydroxytryptophan hydrate than in response to control solutions. The indoleacetic acid and carboline had no effect on aphid probing (Table 5).

DISCUSSION

Our results show that the allelopathic interaction between *E. repens* and barley can affect host plant selection by *R. padi*. Similar effects on aphid settling were found after transfer of volatiles between different barley cultivars (Pettersson et al., 1999; Ninkovic et al., 2002). *E. repens* is known to produce allelochemicals both in the laboratory and field (Oswald, 1948; Welbank, 1960; Phlak, 1967), but has traditionally been studied only in terms of its effect on the growth and performance of receiving plants. Using a recirculation system, Schulz et al. (1994) showed that *E. repens* root exudates reduced the biomass and shoot length of cress seedlings, and root extracts inhibited seed germination and root growth in a range of leguminous plants (Weston and Putnam, 1986). In the field, *E. repens* shoot and root residues reduced the yield of alfalfa (Touchette et al., 1988) and living *E. repens* reduced legume nodulation and root and shoot weights (Weston and Putnam, 1985).

The results in this study show that the effects of allelopathic provocation can reach beyond the recipient plant itself and affect insect herbivores that use

the recipient plant as a host. Root exudates of living *E. repens*, when applied directly to the soil in which barley was growing, reduced aphid acceptance of barley plants. Thus, the active compounds responsible for the effects on barley were exuded from the roots naturally and in sufficient concentrations. The known chemicals in many cases had the same effect, although they may not be fully responsible for the activity of the living plants since *E. repens* produces a wide range of allelochemicals (Hagin, 1989; Hagin and Bobnik, 1991; Schulz et al., 1994; Friebe et al., 1995).

Individually, hydroxytryptophan (experiment 1) and hydroxytryptophan hydrate (experiment 2) did not cause barley to become less acceptable to the aphid, and indoleacetic acid was inactive in the no-choice test (experiment 3b). When aphids could choose between untreated and indoleacetic acid-treated plants, there was a reduction in settling on the treated plant (experiment 3a). This may reflect the less stringent conditions imposed by the choice situation, as discussed earlier. A mixture of all three of the above chemicals was active (experiment 5a), but in this case it is not possible to conclude whether this was due to a synergistic or additive effect of combining the chemicals or due to the action of the indoleacetic acid alone. The carboline was highly active, reducing the proportion of aphids settling on treated barley plants. There was a slightly stronger effect when plants were given an extra treatment (and thus received more active substance) (experiment 4a compared with 4b), but the increase was not dramatic. Mixtures of all four chemicals were active. There was a suggestion that the reduction in aphid settling provoked by the high dosage of active substances in experiment 5b was higher. However, our interpretation is that there seems to be no great increase in activity on combining the allelopathic compounds, and, indeed, the strongest reduction in aphid settling is provoked by the carboline alone.

Olfactory information is used extensively by aphids in the host location and selection process (Pickett et al., 1992; Pettersson, 1994). Therefore, the olfactometer responses of *R. padi* to odors from provoked plants are interesting because they can potentially influence plant selection even prior to plant contact. Aphids did not discriminate between odor from unprovoked barley plants and from plants that had been exposed to root exudates from living *E. repens*. Treatment with either a mixture of the hydroxytryptophan, hydroxytryptophan hydrate, and indoleacetic acid or with carboline alone, did not alter aphid response to barley odor. However, aphids spent significantly less time in the olfactometer arm containing odor from barley treated with a mixture of all four compounds (treatment protocol as for 5c). It is not clear how the effects on aphid settling and olfactory response are related. Reduction in settling does not appear to be caused by a change in volatile profile alone since there was no change in aphid response to odors following the majority of the treatments, including exudates from living *E. repens* roots. It is possible that the mixture of chemicals that causes the change in the volatile profile of the barley was either not expressed by *E. repens*

in the experiments, or was expressed at a lower concentration than that supplied artificially.

The results suggest that barley plants underwent chemical or physiological changes as a consequence of exposure to *E. repens* allelochemicals and that these changes affected the behavior of *R. padi*. Three possible explanations can be advanced. The simplest explanation is that the *E. repens* substances are either directly toxic to the aphids or act through the phloem as systemic antifeedants. However, the aphid probing bioassay (Table 5) suggests that this is unlikely to explain the results since all the substances were either neutral or had a somewhat stimulatory effect on aphid probing. The stimulatory effects seen with the hydroxytryptophan and hydroxytryptophan hydrate may be due to the structural similarity with the amino acid tryptophan, which may be difficult for *R. padi* to obtain from plants under normal circumstances (Sandström et al., 1998). The two remaining explanations involve true allelopathy, in which the effects on insects are mediated indirectly *via* the action of chemicals in the plant. The chemicals may be phytotoxic, causing the plant to produce cues that signal reduced food quality to the aphid. It is possible, for example, that root development was affected in treated plants, although water was not limiting in the experimental regime, this is distinct, however, from the possibility that allelopathic provocation causes induced responses in the receiving plant. Karban et al. (2000) showed that wild tobacco plants respond to a volatile signal, methyl jasmonate, received from neighboring sagebrush (*Artemisia tridentata*) with increased levels of an oxidative enzyme associated with induced resistance. Treatment with methyl jasmonate caused wheat seedlings to have increased concentrations of hydroxamic acids, which made them less acceptable for *R. padi* (Slesak et al., 2001). Barley does not contain hydroxamic acids, but it does contain the indole alkaloids gramine and hordenine, which have been implicated in plant defense (Liu and Lovett, 1993) and may themselves be allelopathically active (Lovett et al., 1995). Gramine content was shown to increase in barley after aphid infestation (Velozo et al., 1999). However, the effects of hordenine on aphids have not been tested, and recent evidence suggests that gramine does not mediate barley resistance to *R. padi* (Forslund et al, 1998; Åhman et al., 2000). Thus, although there is no direct evidence for allelopathic triggering of induced effects on barley phytochemistry, the least that can be said is that barley phytochemistry is dynamic and appears to be susceptible to modification by external provocation.

This study shows that exposure of barley plants to phytotoxic compounds reduces their acceptability to *R. padi* compared to unexposed plants and that the aphid can detect this both on contacting, probing and feeding on the plant as well as by using information in the volatile profile. This lends weight to the theory that allelopathic interactions may be an overlooked mechanism contributing to the reduction of insect herbivore populations in mixed cropping (Pettersson et al., 1999). Furthermore, the results indicate that chemicals involved in allelopathy between plants may have value in new aphid pest control strategies, e.g., through crop

spraying. In preliminary field trials, in which barley was watered with solutions containing the couch-grass substances, even greater reductions in aphid settling were observed (J. Pettersson, unpublished data). Further studies are underway to provide a greater understanding of the allelopathic interactions between barley couch-grass and their effects at the next trophic level.

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FIRST ISOLATION OF NATURAL CYANAMIDE AS A POSSIBLE ALLELOCHEMICAL FROM HAIRY VETCH *Vicia villosa*

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Abstract—Cyanamide was isolated from the leaves and stems of hairy vetch (*Vicia villosa*), guided by plant growth inhibitory activity against lettuce (*Lectuca sativa*) seedlings. A large proportion of the inhibitory activity in the crude extract was explained by the presence of cyanamide, suggesting it to be a possible allelochemical in this species. The amount in a 9-day-old seedling, which had been grown without nutrients, reached approx. 40 times that of a nongerminated seed, demonstrating cyanamide biosynthesis in the seedlings. This is the first report on the isolation of a possible allelochemical from hairy vetch and also of the finding of cyanamide as a natural product.

Key Words—*Vicia*, hairy vetch, cyanamide, allelochemical, phytotoxic, cover crop.

INTRODUCTION

Hairy vetch (*Vicia villosa* Roth), a leguminous plant, is utilized as a winter cover crop in the United States (Hoffman et al., 1993) and in Japanese orchards and abandoned paddy fields (Fujii, 2001). In addition to providing a large amount of nitrogen to the soil and preventing evaporation of water and soil erosion (Teasdale, 1993), it efficiently suppresses weed growth (Teasdale et al., 1991; Hoffman et al., 1993). Weed growth inhibition by chemicals contained in this plant (allelopathy) is thought to be an important factor in weed suppression, together with competition for light, nutrients, and water (White et al., 1989; Bradow and Connick, 1990).

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Leaf extracts show much stronger phytotoxity than many other plants (White et al., 1989; Teasdale and Daughtry, 1993; Fujii, 2001). However, none of the plant growth inhibitors have been identified. Inderjit and Asakawa (2001) concluded that allelopathy does not play an important role in its suppressive ability, although this was determined based only on the total amount of phenolics as allelochemicals without identifying the individual compounds.

To examine whether allelopathy is involved in weed growth suppression, the possible allelochemicals of this crop should be identified. This paper describes the isolation of cyanamide as a possible principal allelochemical from hairy vetch and estimates its contribution to the total plant growth inhibitory activity in the crude extract. Although cyanamide is mass-produced as an industrial material (e.g., nitrogen fertilizer, herbicide, etc.) throughout the world, it has never been found as a natural product (Estermaier et al., 1992). Its natural occurrence was confirmed by detecting its accumulation in seedlings of hairy vetch cultivated under laboratory conditions. The importance of cyanamide as a toxic substance, which could cause vetch-associated diseases in cattle (Panciera et al., 1966), is also discussed.

METHODS AND MATERIALS

Plant Materials. For the isolation of plant growth inhibitors, hairy vetch (*V. villosa*; Snow Brand Seed Co., Ltd., Sapporo, Japan), which had been cultivated in our institute's experimental field (Tsukuba, Japan) from October 2000 to May 2001, was used. Neither fertilizer nor pesticide had been applied to the field for at least 5 years. For quantification of cyanamide in seedlings, seven seeds were planted in a pot (8 cm wide $\times$ 8 cm long $\times$ 5 cm high) containing vermiculite (50 g; Fukushima Vermi, Fukushima, Japan) at a depth of 5 mm. Three of the seven seeds germinated. The pot was incubated in an illuminated growth chamber (Eyelatron FLI-301NH; Eyela, Tokyo, Japan) for 9 days at 25°C with 25,000 lux. Thirty milliliters of water was added to the pot every 24 hr.

Instrumentation. Nuclear magnetic resonance (NMR) spectra for ^{1}H and ^{13}C were recorded using a JNM α-600 (Jeol, Tokyo, Tokyo) spectrometer. Infrared (IR) spectra were obtained using an FT/IR-480 plus (Jasco, Tokyo, Tokyo). A high-performance liquid chromatography unit (HPLC, 626 pump with 996 photodiode array detector; Waters, Milford, Massachusetts, USA) equipped with an ODS column (Inertsil ODS-3, 250 $\times$ 4.6 mm ID; GL-Sciences, Tokyo, Japan) was used for the isolation and quantification of cyanamide. Compounds were eluted with water at a flow rate of 1.0 ml/min with detection at UV 210 nm.

Isolation of a Plant Growth Inhibitor. Fresh leaves and stems (200 g) were cut into approx. 5-mm lengths and soaked in Me_2CO (900 ml) for 7 days at room temperature. A preliminary examination revealed that Me_2CO was more effective than MeOH or EtOAc, in extracting active components. The Me_2CO extract was

concentrated to 150 ml using a rotary vacuum evaporator. The resulting water suspension of the extract was acidified to pH 3 with 1 N HCl and extracted with EtOAc (200 ml $\times$ 3). The EtOAc layer was washed with H_2O (100 ml $\times$ 3), dried over anhydrous Na_2SO_4, and filtered. The major plant growth inhibitory activity was found in the EtOAc layer using a lettuce-seedling bioassay described below. The filtrate was concentrated to dryness, yielding a crude extract (910 mg). The crude extract was subjected to silica gel (Wakogel C-200, 100 g; Wako Pure Chemical Industries, Osaka, Japan) column chromatography and eluted successively with mixtures of *n*-hexane–EtOAc (10:0, 9:1, 8:2, 5:5, and 0:10), EtOAc–MeOH (9:1), and EtOAc–MeOH–HOAc (90:10:1), yielding an active fraction (126 mg) that eluted with 100% EtOAc. This fraction was concentrated to dryness, and the residue was subjected to silica gel (Wakogel C-200, 20 g) column chromatography and eluted successively with mixtures of *n*-hexane–$CHCl_3$–MeOH (8:12:1) and $CHCl_3$–MeOH (9:1) to give an active fraction (20 mg) that eluted with $CHCl_3$–MeOH. The active eluate was concentrated to dryness, and the residue was subjected to ODS (10 g, AQ 120-S50; YMC, Kyoto, Japan) column chromatography eluted with mixtures of MeOH-H_2O (3:7 to 10:0) to give an active fraction that eluted with 30% MeOH. The active fraction was concentrated to dryness followed by purification with preparative HPLC. The active fraction that eluted at a retention time between 3.3 and 3.4 min was collected repeatedly and concentrated to dryness, giving cyanamide as a colorless oil (7.4 mg).

Measurement of Plant Growth Inhibitory Activity. Lettuce seeds (*Lectuca sativa* L. cv. Great Lakes 366; Takii Co., Ltd., Kyoto, Japan) were allowed to germinate in the dark on wet filter paper (No. 1; Toyo Ltd., Tokyo, Japan) for 18 hr at 25°C. The five resulting germinated seeds (2-mm radicle length) were placed onto filter paper (4 cm diam., No. 1; Toyo Ltd.) containing 1 ml of test solution in a Petri dish (4 cm ID). The seedlings were grown in the dark at 25°C for 2 days, and then elongation of the hypocotyl and radicle was measured. The elongation ratio was defined as $(A/B) \times 100\%$, where A is the mean elongation length of the hypocotyl or radicle in the presence of test compound, and B is the mean elongation length of the hypocotyl or radicle in the absence of test compound (control). Each treatment was performed in triplicate ($N = 15$).

Quantification of Cyanamide in Crude Extract. Leaves and stems (50 g) were processed as mentioned above and concentrated to dryness. The residue was dissolved into 5 ml of MeOH, and 1 μl of the solution was analyzed with HPLC. A peak corresponding to cyanamide was identified by comparing the retention time (3.35 min) and UV spectrum (only end absorption) with those of an authentic sample (Aldrich, Milwaukee, Wisconsin, USA). The concentration in the extract was determined by comparing the peak area of the samples with that of an authentic sample.

Quantification of Cyanamide in Seeds and 9-Day-Old Seedlings. Seeds (7 g) were ground into a fine powder using a mortar and pestle for 15 min, and then

extracted using Me_2CO (30 ml) overnight at room temperature. The extracted Me_2CO solution was filtered, concentrated to dryness, and dissolved into MeOH (2 ml). The methanolic solution was filtered through a membrane-filter (cellulose acetate, 0.20 μm; Toyo Ltd.) and concentrated to 1 ml of MeOH. Five microliters of the solution was analyzed with HPLC. A 9-day-old plant grown in an illuminated growth chamber was divided into shoot, endosperm surrounded by a seed coat, and root parts. These three parts were cut into approx. 5-mm lengths and extracted using Me_2CO (1 ml) overnight at room temperature. Each extracted solution was then filtered through a membrane filter (polytetrafluoroethylene, 0.20 μm; Toyo Ltd.) and concentrated to dryness. The residue was dissolved into 100 μl of MeOH and an aliquot of the solution (10 μl) was analyzed by means of HPLC. Identification and quantification of cyanamide was accomplished by using the method described above. Three independent experiments were performed.

RESULTS

The crude extract of fresh leaves and stems of hairy vetch inhibited hypocotyl and radicle elongation in lettuce. The crude extract from 0.1 g fresh wt of hairy vetch per dish inhibited 50% of the hypocotyl elongation of lettuce. This growth inhibitory activity was used as an isolation guide during all the fractionation procedures. The amounts of samples used for bioassay were calculated on the basis of the fresh wt of the extract. The fraction showing the strongest activity was further fractionated chromatographically, finally producing a major plant growth inhibitor.

The IR spectrum suggested the presence of NH (3384 cm^{-1} and 1630 cm^{-1}) and C≡N (2260 cm^{-1}) groups from the absorbent bands (Figure 1). This compound showed only a broad signal at δ 5.39 ppm in the ^1H NMR spectrum (600 MHz, acetone-d_6), corresponding to a proton on the NH group based on the chemical shift value and broadened peak shape. A signal at δ 117.7 ppm, observed only in the ^{13}C NMR spectrum (150 MHz, MeOH-d_4), was assigned to a carbon on the C≡N group based on the chemical shift value. The UV spectrum consisted of end absorption only. These observations suggested that the compound was cyanamide (NH_2CN). This was confirmed by comparing all spectra with those of an authentic sample.

It was confirmed that the plants used for the isolation of a plant growth inhibitor contain 130 μg of cyanamide/g fresh wt. The crude extract and authentic cyanamide were compared with regard to their ability to inhibit the growth of lettuce hypocotyls on the basis of their cyanamide concentration (Figure 2a). At all the concentrations examined, the growth inhibition of the crude extract on the lettuce hypocotyls was explained by the presence of cyanamide in the extract. Growth inhibition of lettuce radicles, however, was not as well correlated, especially at lower

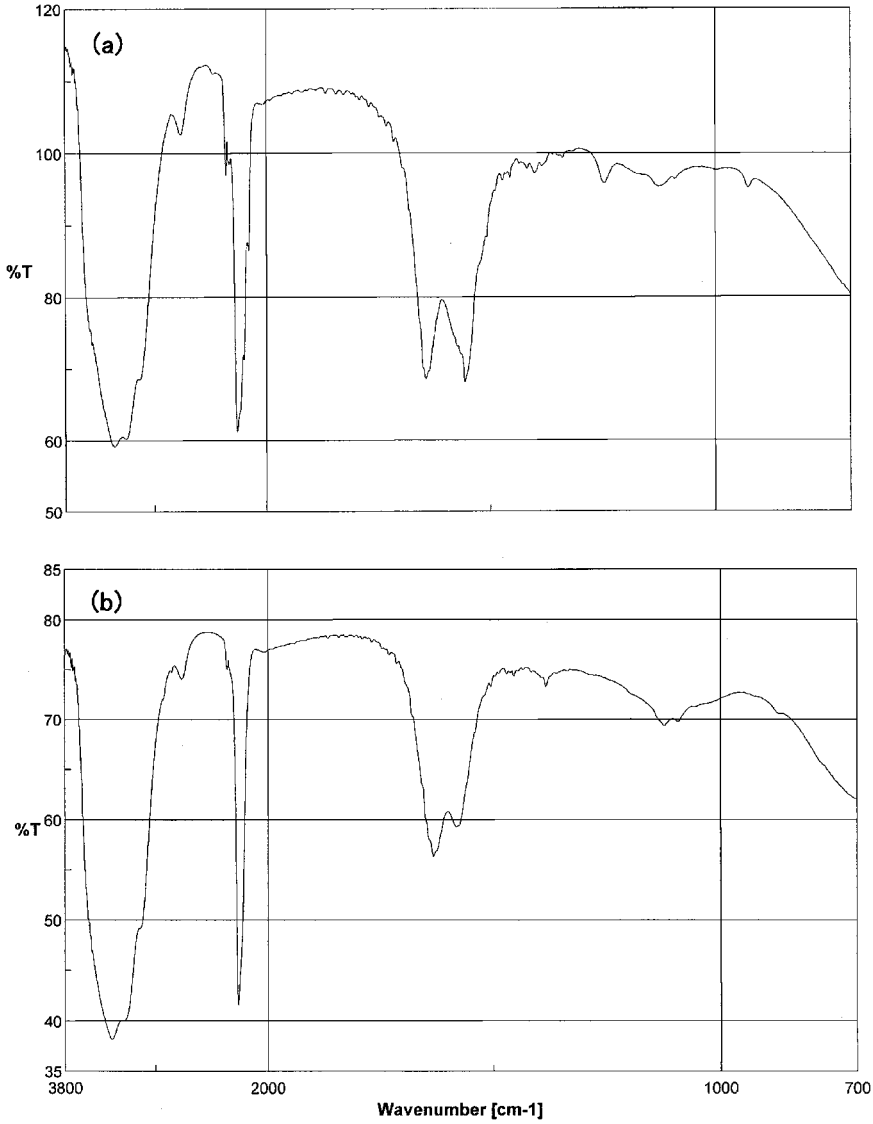

FIG. 1. The infrared spectra of (a) the plant growth inhibitor isolated from hairy vetch and (b) authentic cyanamide.

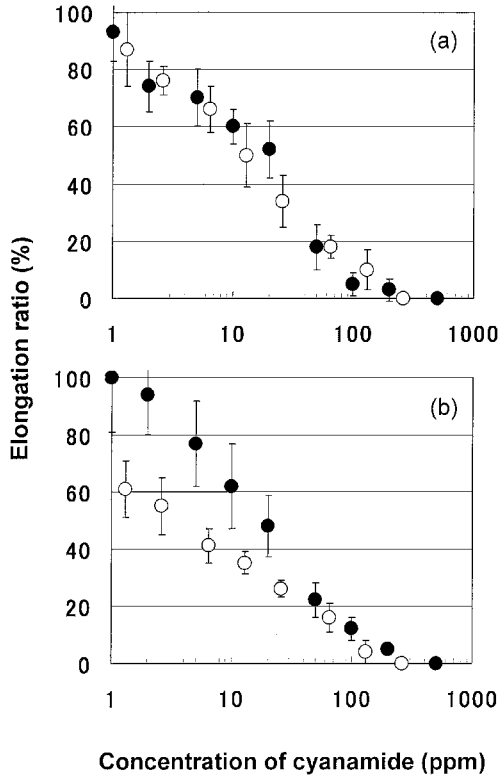

FIG. 2. Comparison of growth inhibition activity between the crude extract of hairy vetch (○) and authentic cyanamide (●) on lettuce (a) hypocotyl and (b) radicle on the basis of cyanamide concentration. Values are mean ± SD ($N = 15$).

concentrations (Figure 2b). The crude extract containing 1.3 ppm of cyanamide inhibited 40% of radicle growth, but 1.3 ppm of the authentic sample had no effect on radicle growth, and 10 ppm was required to inhibit 40% of radicle growth using authentic cyanamide alone. This suggests that the crude extract may contain radicle growth inhibitor(s) other than cyanamide. At higher concentrations, the crude extract's ability to inhibit radicle growth could be explained by the presence of cyanamide in the extract.

The amount of cyanamide in hairy vetch seeds was 5.0 ± 1.6 μg/g fresh wt (mean ± SD). Since the weight of a seed was 24.9 ± 5.3 mg ($N = 30$), the content of cyanamide in a seed amounted to 0.13 ± 0.04 μg. To clarify whether cyanamide is biosynthesized *in vivo* or not, plants were grown without nutrients in an illuminated growth chamber for 9 days, and changes in the cyanamide content in each plant were determined. Fresh weights, concentrations of cyanamide, and

TABLE 1. CONTENT OF CYANAMIDE IN 9-DAY-OLD SEEDLING OF HAIRY VETCH[a]

	Shoot	Endosperm	Root	Total
Fresh weight (mg/seedling)	78 ± 12	66 ± 4	69 ± 9	213 ± 11
Concentration of cyanamide (μg/g fresh wt)	444 ± 326	163 ± 36	89 ± 32	697 ± 358
Content of cyanamide (μg/seedling)	3.4 ± 2.4	1.1 ± 0.4	0.6 ± 0.2	5.1 ± 2.8

[a] Values are mean $\pm$ SD, $N = 3$.

contents of cyanamide in the seedlings are summarized in Table 1. Cyanamide was mainly present in the shoot but was also found in small amounts in the endosperm surrounded by the seed coat and in the root. The total amount was 5.1 ± 2.8 μg per whole seedling, indicating the presence of approx. 40 times more cyanamide than that in nongerminated seeds.

DISCUSSION

Cyanamide was identified as a major plant growth inhibitor in the leaves and stems of hairy vetch (Figure 1), accounting for the major proportion of the crude extract's inhibitory effect on the elongation of lettuce hypocotyl (Figure 2a). Other lesser-contributing phytotoxic compounds in the crude extract could also contribute to the total inhibitory activity on elongation of lettuce radicle (Figure 2b). These results indicate that cyanamide is a possible major allelochemical in hairy vetch. To demonstrate a significant role of allelopathy in the weed suppression by hairy vetch, it would be necessary to demonstrate that a sufficient concentration of cyanamide is present in the soil to inhibit the growth of other plants (Gershenzon, 1998).

The present report is the first to describe the existence of natural cyanamide. Cyanamide has been produced industrially and utilized for drugs and agrochemicals, but is not considered to occur naturally (Estermaier et al., 1992). At the early stage of this study, it was not certain whether the cyanamide isolated was from a natural source, since unexpected contamination by artificial forms might be possible in the field. To exclude this possibility, hairy vetch seedlings, that had been grown on vermiculite medium in an illuminated growth chamber and supplied with only water were analyzed for their cyanamide content. The amount in a 9-day-old seedling increased by approx. 40 times compared with that in a nongerminated seed, indicating that cyanamide is biosynthesized in hairy vetch. The possibility of contamination by artificial forms was excluded by this experiment. L-β-Cyanoalanine, known as a neurotoxic compound, has been reported to occur in seeds of common vetch, *V. sativa*, and other *Vicia* spp. (Harborne and Baxter, 1993). *Vicia* spp. apparently biosynthesize cyanides such as cyanamide and L-β-cyanoalanine.

A disease of cattle that graze in a hairy vetch pasture was first described in the United States (Panciera et al., 1966). This disease appears in cattle fed in a vetch-dominant meadow and is characterized by dermatitis, conjunctivitis, diarrhea, etc. Similar syndromes have also been reported in Australia (Peet and Cardner, 1986) and Argentina (Odriozola et al., 1990). Morbidity ranges from 1% to 30%, and mortality reaches 50% (Odriozola et al., 1990). Panciera et al. (1992) experimentally caused this vetch-associated disease in an adult cow by feeding it with hairy vetch. They also concluded that contamination by insects, molds, or other seasonally prevalent factors would not be causative, since the disease occurred in all seasons. However, no specific toxic substances causing vetch-associated disease have been identified. Cyanogenic glycosides and L-β-cyanoalanine were suspected as causal compounds (Panciera et al., 1966), but they induced quite different symptoms to those reported in the vetch-associated disease (Odriozola et al., 1990). In the present report, the presence of cyanamide in hairy vetch was demonstrated. Cyanamide is a toxic compound that is both irritating and caustic; it causes severe dermatitis on moist skin and has an LD_{50} in rats of 125 mg/kg orally (Budavari, 1989). These symptoms are identical to those seen in cattle with vetch-associated disease. In the feeding experiment (Panciera et al., 1992), a cow (approx. 432 kg) fed with hairy vetch (9 kg fresh wt/day) died after 24 days. The total amount fed was estimated to be 200 kg fresh wt, which would contain 26 g of cyanamide, if the vetch used contained the same amount as that found in this experiment (130 μg/g fresh wt). The lethal dose in this case is estimated to be 60 mg/kg, which correlates well with the value in rats. Thus, it would be possible that cyanamide is a causal compound of intoxication in cattle due to ingestion of hairy vetch. Changes in the cyanamide content of hairy vetch meadows and vetch-dominant silage and determination of the amount required to induce the characteristic symptoms of vetch-associated disease should be investigated.

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GENETIC VARIATION AND RELATIONSHIPS OF CONSTITUTIVE AND HERBIVORE-INDUCED GLUCOSINOLATES, TRYPSIN INHIBITORS, AND HERBIVORE RESISTANCE IN *Brassica rapa*

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Abstract—We examined genetic variation in inducibility and in constitutive and herbivore-induced levels of glucosinolates, trypsin inhibitors, and resistance to herbivory in families of *Brassica rapa* originating from a wild population. We also examined phenotypic and genetic correlations among absolute levels of these traits in control and induced plants. We grew seedlings of 10 half-sib families in pairs in pots, and exposed one plant per pair to folivory by *Trichoplusia ni* larvae. Two days later, we sampled all plants for total glucosinolate and trypsin inhibitor levels and examined the preference and consumption by *T. ni* larvae of previously damaged (induced) and undamaged (control) plants. There was no significant variation among sire families in the induction of glucosinolates or trypsin inhibitors by *T. ni* feeding. Total glucosinolate levels in either control or induced plants did not vary by family. In contrast, trypsin inhibitor levels in both control and induced plants varied significantly by family. *Trichoplusia ni* fed less on induced plants than on control plants in the bioassay, but neither the

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induction of resistance by prior *T. ni* feeding nor absolute levels of damage done to control and induced plants varied significantly by sire family. Temporal blocking strongly affected trypsin inhibitor levels and the response of some families in the bioassays. There were no significant phenotypic or genetic correlations of levels of glucosinolates or trypsin inhibitors with each other or with damage in either control or induced plants. Overall, these results suggest that in the *B. rapa* population that we studied, both total glucosinolate content and biological resistance to herbivory by *T. ni* was nonvariable and almost universally inducible by prior *T. ni* feeding. In contrast, control and induced levels of trypsin inhibitors varied genetically and have the capacity to respond to future selection imposed by herbivores. However, the role of these defenses in constitutive or induced resistance to *T. ni* in this species remains unclear.

Key Words—*Brassica rapa*, induction, genetic correlation, glucosinolates, herbivory, phenotypic plasticity, resistance, quantitative genetics, trypsin inhibitors, variation.

INTRODUCTION

Most plant species must defend themselves against damage by insect herbivores and other natural enemies (Simms and Rausher, 1987). In the face of such enemies, selection for increased resistance to herbivory is thought to have generated a vast taxonomic diversity in proteins and secondary metabolites with defensive function (Erlich and Raven, 1964; Rosenthal and Berenbaum, 1992). Accordingly, to examine the hypothesis that putative plant defenses are adaptive in the face of herbivore attack, evolutionary ecologists have long sought to determine the costs and benefits of the expression of plant resistance.

The production of chemical and physical defenses is assumed to be costly to fitness in plants, although the detection of costs has remained challenging (Brown, 1988; Simms, 1992; Bergelson and Purrington, 1996; Zangerl et al., 1997; Agrawal, 1998; Baldwin, 1998; Mauricio, 1998; Purrington, 2000; Cipollini et al., 2002). Allocation of limited resources to defense is thought to incur direct "physiological costs" by decreasing the amount of resources that could otherwise be allocated to fitness (Chew and Rodman, 1979; Zangerl et al., 1997; Mauricio, 1998; Cipollini, 2002). Allocation to defense may also incur indirect "ecological costs," such as reduced competitive ability or tolerance to herbivory, that are seen only when plants are growing in an ecologically relevant context (e.g., Bergelson, 1994; Stowe, 1998; van Dam and Baldwin, 1998; Cipollini, 2002). If defense is indeed costly to fitness, then plant fitness would be maximized if individuals expressed costly defenses only when warranted by the environment (i.e., when spatially and temporally variable populations of insect herbivores are present) (Karban and Baldwin, 1997). Phenotypic plasticity in the production of defenses (i.e., inducibility) may serve as a cost-saving mechanism, by restricting the production of defenses to environments in which herbivores are present (Karban and Baldwin, 1997). However, some

authors have suggested that unique benefits (rather than cost-savings) of employing inducible defenses could favor inducibility over constitutive strategies in plants (Karban et al., 1997).

Induced responses allow plants to maintain higher fitness than uninduced plants when herbivores are present in the field (Agrawal, 1998, 2000; Baldwin, 1998). Nevertheless, inducibility may not continue to evolve in response to herbivores if there is not heritable genetic variation in a population for this form of phenotypic plasticity. Although several studies have demonstrated genetic variation in constitutive levels of defenses (e.g., Kollipara et al., 1994; Mauricio, 1998; Kliebenstein et al., 2001), few studies have examined genetic variation in the induction of defenses within a plant species (Zangerl and Berenbaum, 1990; van Dam and Vrieling, 1994; Agrawal et al., 2003). The first evidence of genetic variation in induction was in a study of wild parsnip (Zangerl and Berenbaum, 1990), in which heritable genetic variation in constitutive and induced levels of furanocoumarins was detected in two populations that historically experienced different selective pressures from the parsnip webworm, *Depressaria pastivacella*.

Brassica rapa is defended from herbivores in part by glucosinolates. Glucosinolates are ubiquitous throughout the Brassicaceae (Rodman, 1991) and are known to vary quantitatively and qualitatively at the constitutive level in species such as *Arabidopsis thaliana* (Mauricio, 1998; Kliebenstein et al., 2001). Upon wounding, preformed glucosinolates are hydrolyzed by the enzyme myrosinase (thioglucosidase) to glucose and sulfate, along with various nitrile, isothiocyanate, and thiocyanate ions that are thought to be toxic or deterrent to generalist insect herbivores and some pathogens (Giamoustaris and Mithen, 1995; Hopkins et al., 1998a; Lambrix et al., 2001; Kliebenstein et al., 2002). Although constitutively produced, glucosinolates and myrosinase are also wound-inducible in many brassicaceous plants, including *B. rapa* (Broadway and Colvin, 1992; Bodnaryk, 1994; but see Agrawal, 2000), and are commonly associated with induced resistance to generalist pests (e.g., Agrawal, 1998). In contrast, glucosinolates serve as host-acceptance cues and feeding stimulants for specialist beetles and lepidopterans (e.g., Renwick and Lopez, 1999), which appear to be relatively undeterred at constitutive or wound-induced levels (Broadway and Colvin, 1992; Bodnaryk, 1994; Giamoustaris and Mithen, 1995; Kliebenstein et al., 2002; but see Agrawal, 2000).

In addition to glucosinolates, brassicaceous plants may be defended by proteinase inhibitors. Serine proteinase inhibitors are found at relatively high levels in many brassicaceous species, including *B. rapa* (Broadway, 1989) and competitively inhibit serine proteases, such as trypsin and chymotrypsin, thereby inhibiting proteolysis in animals and microorganisms (Broadway, 1995). Serine proteinase inhibitors are constitutively produced and developmentally regulated in *Brassica napus* and *Brassica oleraceae*, but are also wound-inducible (Broadway and Missurelli, 1990; Cipollini and Bergelson, 2000, 2001). Proteinase inhibitors can lower *in situ* growth of many insect herbivores (Broadway and Colvin, 1992),

are associated with induced resistance in many plants (Thaler et al., 1996; Zhao et al., 1997), and can deter herbivory and reduce insect performance when expressed in transgenic plants (Johnson et al., 1990). As for glucosinolates, some insects appear to possess effective physiological counteradaptations to the presence of host-plant proteinase inhibitors in their gut and are less affected by proteinase inhibitors than are other herbivores (Broadway, 1995; Jongsma and Bolter, 1997).

Data from a pilot study of *B. rapa* indicated that genetic variation existed among paternal half-sib families (individuals sharing the same paternal parent, but different maternal parents) in the induction of glucosinolates by larval *Trichoplusia ni* feeding but that levels were not correlated with the amount of leaf area removed by *T. ni* in a bioassay of the same plants (K. Stowe, J. Busch, and E. Simms, unpublished data). Here, we examined whether induction of trypsin inhibitors by *T. ni* feeding, in conjunction with glucosinolates, varies among families and can explain resistance to herbivory in *B. rapa*. Genetic variation in inducibility and in absolute levels of glucosinolates and trypsin inhibitors has never been examined in an integrated study. Relative roles in defense and trade-offs that could constrain their evolution also remain unclear. Specifically, this experiment was designed to address the following questions: (1) Does *B. rapa* exhibit genetic variation in the induction of glucosinolates, trypsin inhibitors, and biological resistance elicited by *T. ni* feeding? (2) Do constitutive and induced levels of glucosinolates, proteinase inhibitors, and biological resistance vary genetically in *B. rapa*? (3) Are there significant genetic or phenotypic correlations between glucosinolates, trypsin inhibitors, and biological resistance in control and induced plants?

METHODS AND MATERIALS

Study Species. Brassica rapa L. (syn. *B. campestris*: Brassicaceae; turnip rape) is a self-incompatible annual plant, native to Eurasia, that is represented by both crop cultivars and naturalized populations throughout North America. Seeds used in the current study were the second-generation offspring of plants collected from a wild population at the San Joaquin Fresh Water Reserve near the University of California, Irvine. *Trichoplusia ni* (Lepidoptera: Noctuidae; cabbage looper moth) is a generalist herbivore that uses brassicaceous plants as preferred hosts (Broadway, 1995). *T. ni* larvae have often been used in induction experiments and bioassays of resistance with brassicaceous plants, and they appear to vary in their sensitivity to different resistance factors (e.g., Broadway and Colvin, 1992; Broadway, 1995; Agrawal, 2000).

Breeding Design. We used plants from preexisting paternal half-sib families, created for an earlier experiment, as seed sires in a quantitative genetics breeding

design to produce plants for the current study. Based on data from the preliminary experiment, we identified 10 paternal half-sib families that appeared to vary in mean glucosinolate levels. We used them as sires to increase our ability to detect genetic correlations between glucosinolates, trypsin inhibitors, and biological resistance (Falconer and Mackay, 1989). During the spring of 1999, we raised an individual from each chosen half-sib family (10 total plants) in the University of Chicago greenhouse along with 45 randomly chosen individuals from the wild population in California. Following the onset of flowering, each individual from the 10 half-sib families (sires) was crossed with three randomly chosen wild plants (dams), producing 30 full-sib seed families nested within 10 paternal half-sib seed families. To control for maternal effects on seed development (Agrawal et al., 1999), five flowers on each dam were hand-pollinated in each cross with pollen collected from several flowers from each individual sire. A separate camel hair paintbrush was used to collect and transfer pollen for each cross. Seeds were collected following maturation on maternal plants in late spring.

Experimental Design. During the summer of 1999, experimental plants were grown from seed in a temperature-controlled greenhouse at the University of Chicago (20-25°C) under ambient light supplemented by sodium vapor lights. Within each of the two temporal blocks, six plants from each of the 30 full-sib families were planted, yielding 180 individuals per block and 360 individuals in the entire experiment. Several seeds from each family were initially planted in flats of Promix BX potting medium, and seedlings were watered twice daily. Four days following germination, selected plants were transplanted to 15-cm pots in the same medium to decrease the effect of root binding on the uptake of nutrients, which could interfere with the production of chemical defenses (Baldwin, 1988; Cipollini and Bergelson, 2001). At this time, two individuals from each full-sib family were paired by size and transplanted into each pot. Thus, there were three full-sib pairs of plants per half-sib family used in each temporal block of this experiment. One individual from each pair was subsequently induced (see below) while the other plant served as an undamaged control. Full-sib pairs were generated based upon similarity in size to control for any potential size effects on levels of glucosinolates or proteinase inhibitors (Broadway and Missurelli, 1990; Hopkins et al., 1998a). Paired plants did have the potential to communicate chemically through soil- or airborne factors (e.g., Siemens et al., 2002). The extent of communication among seedlings was unknown, but likely made our results conservative if it occurred. Moreover, paired plant designs are statistically powerful, and facilitated our bioassay that integrated both preference and consumption of control and induced plants.

Plants within each pot were randomly assigned control or induced status at the four-leaf stage, 14 days following transplantation. *T. ni* larvae used to induce and to bioassay resistance were raised from eggs (obtained from Entopath Inc.), on artificial cabbage looper diet (Southland Products Inc.). Approximately 15 larvae were raised in each diet cup in a growth chamber set to 22°C with a

14L:10D light cycle. To ensure that similar instars were used to induce and to bioassay the plants during each temporal block, one cohort of larvae was started to be used for the induction of plant defenses, and another was started 2 days later for use in bioassays of resistance. Larvae used to induce plants were contained on leaves in clip cages. Clip cages were constructed out of the base plates of 30-mm clear plastic Petri dishes, using a metallic hair clip glued to each cage as a hinge. Additionally, a 15-cm thin wooden dowel was glued to each cage that could be inserted into the soil at the base of each plant to suspend the cage on a leaf without weighing down and damaging the leaf. On induced plants, one 9-day-old larva was placed in a cage on the terminal end of the second true leaf and was allowed to feed. An empty cage was placed on the second true leaf of each control plant. After approximately 12 hr, during which time larvae ate $\sim$9.6 cm^2 of leaf area, all larvae and cages were removed from all plants. In the three cases where larvae did not feed during this time period, an equivalent amount of leaf area was mechanically damaged by crushing. This has been shown to induce trypsin inhibitors and glucosinolates in *Brassica* (Bodnaryk, 1994; Cipollini and Bergelson, 2000, 2001).

Three samples of leaf tissue were taken for chemical analysis from the third true leaf from all plants 48 hr after removal of cages. Two leaf disks ($\sim$1 cm diam.) were taken from either side of the midrib to quantify glucosinolate content and leaf disk dry weight. A leaf sample ($\sim$4 cm^2) was removed from the tip of the same leaf to quantify trypsin inhibitors. Two leaf samples were placed in prelabeled microfuge tubes, flash frozen in liquid nitrogen, placed in a cooler of Dry Ice, and stored in a -80°C freezer until analysis. One of the leaf disks was placed in a small, prelabeled coin envelope, placed in a drying oven, and subsequently weighed on a microbalance.

T. ni larvae raised as described were used to bioassay resistance of each pair of plants. Although all plants were wounded during the leaf sampling protocol, only previously herbivore-induced plants had had sufficient time to respond to damage with changes in leaf chemistry (Cipollini and Bergelson, 2000). One 9-day-old larva was placed on the soil between each pair of plants and allowed to choose freely and feed on the plants. To eliminate a directional bias, larvae were placed such that the long axis of the body was perpendicular to each of the two plants. Following larval placement, pots were enclosed in spun mesh polyester bags to prevent larval escape. Twelve hours later, all larvae were removed, and damage to each leaf on each control and induced plant was estimated by using a clear acetate grid. Induction of biological resistance was measured as the difference between the amount of leaf area removed from each induced plant and the amount of leaf area removed from its paired control plant (yielding a negative value if resistance was induced in the previously damaged plant). This bioassay measure integrates both insect preference for control or induced plants and the amount of damage it caused to either plant.

Chemical Analyses. Total glucosinolate content of a leaf sample can be inferred from the amount of glucose liberated from a glucosinolate extract upon hydrolysis with the enzyme, myrosinase (Broadway and Colvin, 1992; Siemens and Mitchell-Olds, 1998). We analyzed glucosinolate content according to Siemens and Mitchell-Olds (1998). Our method of sample preparation differed from that protocol only in that leaf disk samples were heated for 5 min at 62°C in 0.5m MeOH to denature endogenous myrosinase. Glucosinolate concentrations were expressed as milligrams of glucose per gram of leaf dry weight. Trypsin inhibitor content was measured in soluble protein extracts by using a radial diffusion assay through a trypsin-containing agar as in Cipollini and Bergelson (2000). Protein concentration in leaf extracts was quantified by using the Bradford assay (Bradford, 1976) with Biorad protein dye reagent and bovine serum albumin as a standard. Trypsin inhibitor concentrations were expressed as milligrams of trypsin inhibitor per gram of extract protein. For each chemical defense, leaf samples obtained from control plants were used to quantify constitutive defense levels, and leaf samples obtained from damaged plants were used to quantify induced defense levels for each full-sib pair in the experiment.

Statistical Analyses. Variation in Induction of Chemical Defense and Biological Resistance. We first tested for variation in induction (damaged levels minus control levels for each full-sib pair) of glucosinolates, trypsin inhibitors, and biological resistance using three separate ANOVAs. Each analysis was conducted using sire, block, and dam (nested within sire) as main effects. To determine whether half-sib families performed differently in the two blocks, a sire $\times$ block interaction term was also included. All statistical analyses were performed on SAS (Version 8, SAS Institute, Inc., Cary, North Carolina, USA).

Variation in Constitutive and Induced Levels of Defenses and Biological Resistance. Subsequently, we tested for variation in constitutive and induced levels of glucosinolates and trypsin inhibitors and for variation in damage to control and induced plants using six separate ANOVAs. Each analysis was conducted using sire, block, and dam (nested within sire) as main effects, and a sire $\times$ block interaction term.

Genetic Correlations among Defenses and Biological Resistance. To determine the degree to which the expression of glucosinolates, trypsin inhibitors, and the amount of damage received in the bioassay reflect the same genetic character, we calculated genetic correlations among the 10 sire half-sib family means of these traits (Falconer and Mackay, 1989). Genetic correlations were performed separately for control and induced plants. Alpha levels were Bonferroni adjusted to 0.0168 for both control and induced plants to maintain an overall alpha level of 0.05 for three tests.

Phenotypic Correlations among Defenses and Biological Resistance. To examine for the presence of nonrandom associations between putative defenses and damage, phenotypic correlations among glucosinolate levels, trypsin inhibitor

levels, and the amount of damage received in the bioassay for each individual in the experiment were performed. Phenotypic correlations were performed separately for control and induced plants. Alpha levels were Bonferroni adjusted to 0.0168 for both control and induced plants to maintain an overall alpha level of 0.05 for three tests.

RESULTS

Variation in Induction of Glucosinolates, Trypsin Inhibitors, and Biological Resistance. No model effect explained variation in the induction of glucosinolates (Table 1), or trypsin inhibitors (Table 1) by *T. ni* feeding (data not shown). No model effect affected the induction of biological resistance by *T. ni* feeding, except the sire × block interaction (Table 1, Figure 1). These results reveal a lack of variation among sires in the induction of defense chemicals and biological resistance by prior *T. ni* feeding in the *B. rapa* population that we studied, but they indicate that unknown factors associated with temporal blocking affected the induction of biological resistance in different sires.

Variation in Constitutive and Induced Levels of Glucosinolates. No model effect explained phenotypic variation in glucosinolate levels in either control plants (Table 2) or in induced plants (Table 3) (data not shown). These results indicate that there is a lack of variation among the sire families for the genes responsible for glucosinolate production, and no effect of experimental blocking.

Variation in Constitutive and Induced Levels of Trypsin Inhibitors. In contrast to total glucosinolates, some model effects explained a significant amount of the phenotypic variation in trypsin inhibitor levels. Sire and dam (nested within sire) influenced the level of trypsin inhibitors in control plants (Table 2, Figure 2A), revealing the importance of additive genetic variation and maternal effects on constitutive levels. Moreover, control plants in the first block had higher trypsin inhibitor levels than control plants in the second block, revealing the importance

TABLE 1. *F* VALUES FROM ANOVAS EXAMINING INDUCTION OF GLUCOSINOLATE, TRYPSIN INHIBITOR, AND BIOLOGICAL RESISTANCE IN *B. rapa* BY *T. ni* FEEDING[a]

Source	*df*	Glucosinolate	Trypsin inhibitor	Biological resistance
Sire	9	1.37 (0.208)	0.98 (0.460)	0.38 (0.944)
Dam (sire)	20	0.93 (0.556)	1.35 (0.161)	0.94 (0.533)
Block	1	0.01 (0.943)	0.59 (0.445)	0.11 (0.737)
Sire × block	9	0.74 (0.671)	0.67 (0.735)	**1.98 (0.046)**

[a] *P* values are shown in parentheses. Bold type indicates significance.

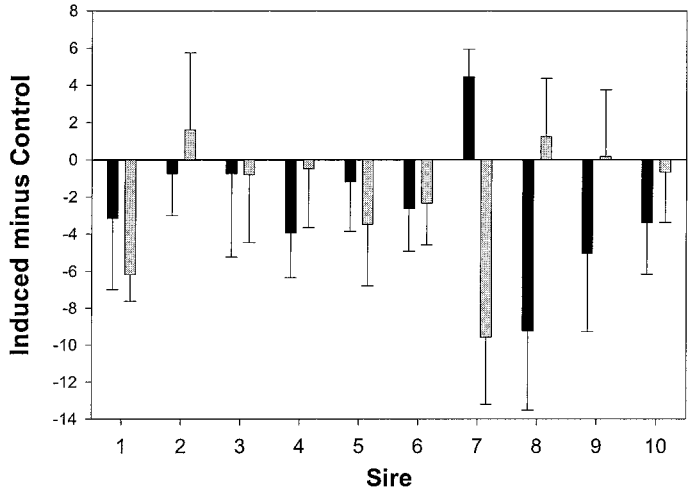

FIG. 1. Induction by *T. ni* feeding of biological resistance to *T. ni* in sire half-sib families of *B. rapa.* Black bars represent block 1 and gray bars represent block 2. Bars represent mean differences (± SE) in leaf damage by *T. ni* between paired induced and control plants.

of environmental or experimental variation among blocks. Both sire and block affected the level of trypsin inhibitors in induced plants, with trypsin inhibitor levels again being much higher in the first block than in the second block (Table 3, Figure 3A). These results indicate genetic variation in induced levels of trypsin inhibitors, as well as an effect of environmental or experimental differences between temporal blocks.

Variation in Damage on Control and Induced Plants. Only the sire × block interaction explained variation in damage to control plants in the bioassay (Table 2, Figure 2B), while only the blocking term explained variation in damage to induced plants in the bioassay (Table 3, Figure 3B). The results indicate a general lack of

TABLE 2. *F* VALUES FROM ANOVAS EXAMINING ABSOLUTE LEVELS OF GLUCOSINOLATE, TRYPSIN INHIBITOR, AND LEAF DAMAGE IN *B. rapa* PLANTS PREVIOUSLY UNDAMAGED BY *T. ni* FEEDING[a]

Source	*df*	Glucosinolate	Trypsin inhibitor	Damage
Sire	9	0.78 (0.635)	**3.80 (<0.001)**	0.59 (0.807)
Dam (sire)	20	0.97 (0.498)	**1.78 (0.029)**	0.78 (0.737)
Block	1	0.44 (0.506)	**85.0 (0.001)**	1.99 (0.161)
Sire × block	9	1.17 (0.322)	1.32 (0.231)	**2.08 (0.036)**

[a] *P* values are shown in parentheses. Bold type indicates significance.

TABLE 3. *F* VALUES FROM ANOVAS EXAMINING ABSOLUTE LEVELS OF GLUCOSINOLATE, TRYPSIN INHIBITOR, AND LEAF DAMAGE IN *B. rapa* PLANTS PREVIOUSLY DAMAGED BY *T. ni* FEEDING[a]

Source	*df*	Glucosinolate	Trypsin inhibitor	Damage
Sire	9	1.19 (0.310)	**2.12 (0.032)**	1.45 (0.173)
Dam (sire)	20	1.12 (0.339)	1.13 (0.324)	0.92 (0.562)
Block	1	0.50 (0.480)	**51.4 (<0.001)**	**7.51 (0.007)**
Sire × block	9	0.39 (0.940)	0.51 (0.867)	1.24 (0.278)

[a] *P* values are shown in parentheses. Bold type indicates significance.

genetic variation in resistance to *T. ni* in control or induced plants, although *T. ni* tended to consume less leaf tissue from induced plants than control plants overall. However, unknown factors associated with temporal blocking affected the amount of damage received by different sires in the control treatment, and the amount of damage received overall by induced plants.

Correlations among Glucosinolates, Trypsin Inhibitors, and Damage. Despite a tendency for trypsin inhibitor levels to be negatively correlated with glucosinolate levels in control plants, no genetic correlations were significant after Bonferroni adjustment of the alpha level in either the control (Table 4) or induced plants (Table 5). In turn, despite a tendency for glucosinolate levels to be negatively correlated with damage in control plants and for trypsin inhibitor levels to be negatively correlated with damage in induced plants, no phenotypic correlations were significant after Bonferroni adjustment of the alpha level in either control (Table 4) or induced plants (Tables 5).

DISCUSSION

Genetic Variation in Expression of Chemical Defenses and Biological Resistance. The detection of significant genetic variation in inducibility or in constitutive and wound-induced levels of chemical defenses in plants would indicate that inducibility and levels of these chemical defenses can respond to selection by herbivores in natural populations. The absence of sire-based genetic variation in expression of glucosinolates implies that induciblity and levels of this putative defense compound may not evolve in response to selection pressure imposed by herbivores in the *B. rapa* population that we studied. In a recent experiment employing a similar paternal half-sib design, Agrawal et al. (2003) also found no variation in constitutive levels of glucosinolates in a population of wild radish, but found significant variation in induced levels. Our inability to detect variation may be due to the genuine lack of any real genetic variation or because a small number of sire

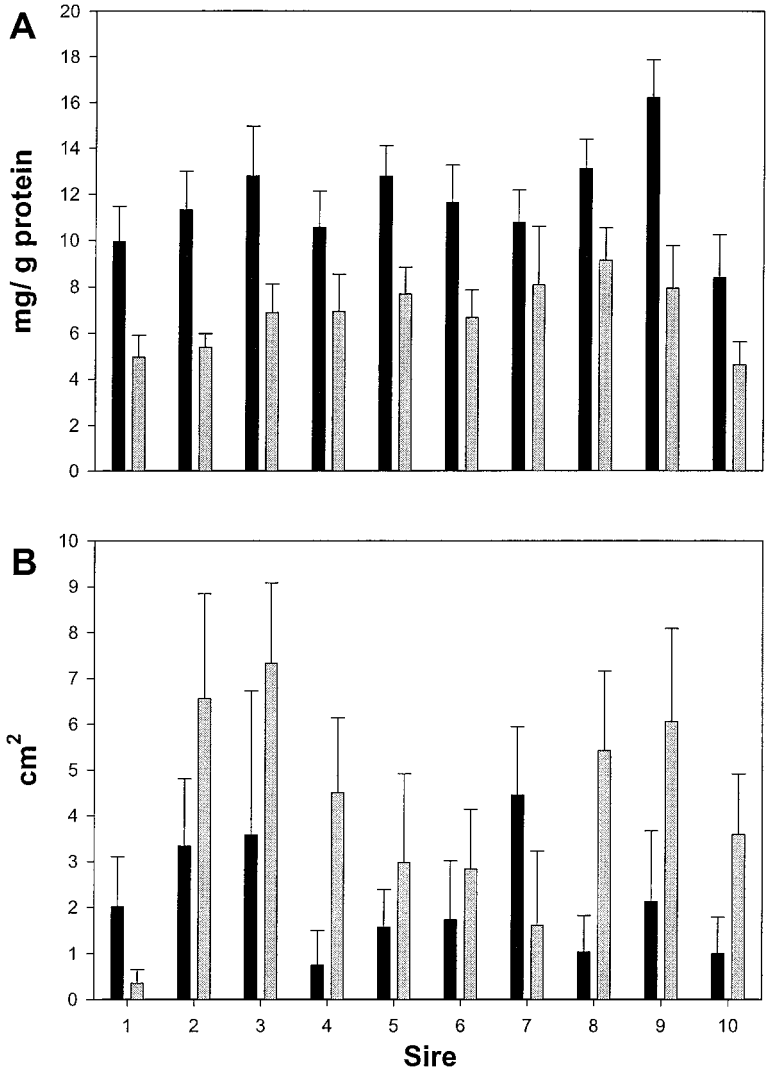

FIG. 2. Absolute levels of chemical defenses and leaf damage in sire half-sib families of *B. rapa* previously undamaged by *T. ni* feeding. Black bars represent block 1 and gray bars represent block 2. (A) Mean trypsin inhibitor levels (± SE) in control plants, (B) mean leaf damage (± SE) in control plants.

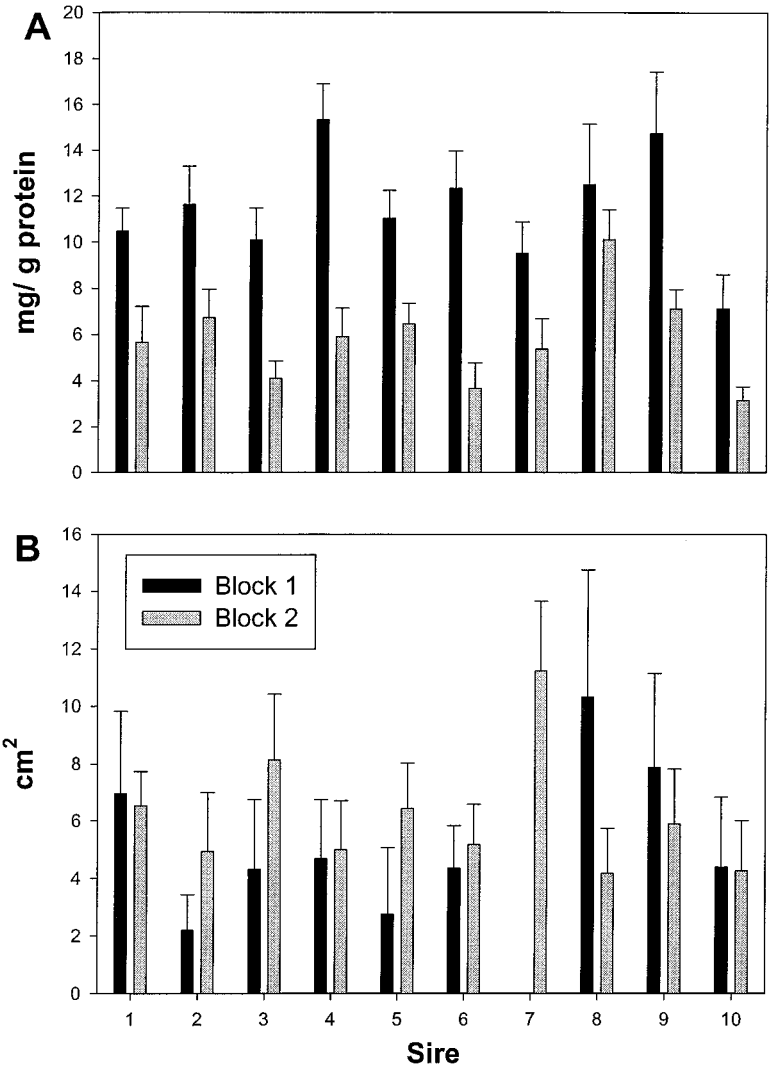

FIG. 3. Absolute levels of chemical defenses and leaf damage in sire half-sib families of *B. rapa* in previously damaged by *T. ni* feeding. Black bars represent block 1 and gray bars represent block 2. (A) Mean trypsin inhibitor levels (± SE) in induced plants, (B) mean leaf damage (± SE) in induced plants. Note that no damage was incurred in block 1 by induced plants in sire family 7.

TABLE 4. CORRELATIONS BETWEEN DEFENSE LEVELS AND DAMAGE IN *B. rapa* PLANTS PREVIOUSLY UNDAMAGED BY *T. ni* FEEDING[a]

	Glucosinolate	Trypsin inhibitor	Damage
Glucosinolate	—	−0.047 (0.546)	−0.159 (0.038)
Trypsin inhibitor	−0.316 (0.038)	—	−0.033 (0.670)
Damage	0.014 (0.971)	−0.163 (0.652)	—

[a] Values within cells are correlation coefficients (ρ) between variables. P values are shown in parentheses. Values below the diagonal are genetic correlations while those above the diagonal are phenotypic correlations.

families prevented us from detecting a genetic signal in the face of high environmental variance in the trait. Previous work has identified important influences of environmental factors on the expression of defense traits in *Brassica* (Cipollini and Bergelson, 2001). In addition, the glucosinolate protocol used in this experiment (after Siemens and Mitchell-Olds, 1998) quantifies total leaf glucosinolates, which can exist as a diverse mixture of specific types that are differentially inducible by herbivory. For example, insect herbivory can sometimes increase levels of indolyl glucosinolates in brassicaceous plants, while decreasing or having no effect on levels of aliphatic glucosinolates and total glucosinolates (e.g., Hopkins et al., 1998b; Agrawal et al., 1999). Agrawal et al. (2003) found that herbivore damage simultaneously increased indolyl, nonindolyl, and total glucosinolate levels in wild radish, albeit to different degrees. Despite the lack of genetic variation in inducibility and in absolute levels of total glucosinolates, genetic variation in the diversity or in expression levels of specific types of glucosinolate may have existed in the *B. rapa* population that we studied, but our methods did not allow us to resolve glucosinolate profiles.

Contrary to our findings on variation in total glucosinolate levels, we detected significant genetic variation in constitutive and wound-induced trypsin inhibitor levels in the population that we studied, but no variation in their inducibility. Our results indicate that both constitutive and damage-induced levels of trypsin inhibitors

TABLE 5. CORRELATIONS BETWEEN DEFENSE LEVELS AND DAMAGE IN *B. rapa* PLANTS PREVIOUSLY DAMAGED BY *T. ni* FEEDING

	Glucosinolate	Trypsin inhibitor	Damage
Glucosinolate	—	−0.139 (0.072)	−0.054 (0.483)
Trypsin inhibitor	0.007 (0.980)	—	−0.149 (0.052)
Damage	0.389 (0.266)	−0.458 (0.183)	—

[a] Values within cells are correlation coefficients (ρ) between variables. P values are shown in the parentheses. Values below the diagonal are genetic correlations while those above the diagonal are phenotypic correlations.

have the capacity to evolve in our study population. Variation in trypsin inhibitor levels has been little studied (Kollipara et al., 1994) and has never been demonstrated in any wild plant population. The degree of systemic induction apparently does not vary, however, and presumably does not have the capacity to respond to future selection. Trypsin inhibitor levels were also strongly affected by temporal blocking. Because the constitutive and wound-induced expression of trypsin inhibitors has been shown to be sensitive to environmental conditions (Cipollini and Bergelson, 2001), it is likely that unmeasured differences in growing conditions (e.g., light levels, temperatures, soil mixtures) among blocks contributed to this effect.

There was an overall lack of sire-based genetic variation in the induction of resistance to *T. ni* feeding by prior *T. ni* feeding in our study population. In a similar experiment, Agrawal et al. (2003) also found no variation in induction of resistance to *Pieris rapae* growth in several paternal half-sib families of wild radish by prior *P. rapae* feeding, and in fact, no induction of resistance at all. However, a subsequent experiment designed to maximize the detection of induced resistance did reveal significant induction and variation in selected wild radish families. While most sire half-sib families expressed induced resistance invariably in our study, the performance of some families was affected by temporal blocking. For example, sire half-sib family 7 expressed induced susceptibility in block 1, but strong induced resistance in block 2, while sire half-sib family 8 expressed induced resistance in block 1, but induced susceptibility in block 2. In turn, the absolute level of damage to control plants was affected by the sire $\times$ block interaction, and the absolute level of damage to induced plants was affected by block. The persistent effect of block on plant chemical and resistance responses could indicate variation in environmental conditions among blocks or variation in the insects used as inducers or in bioassays in each block.

Mechanisms of Resistance. A correlation between levels of a chemical defense and plant resistance would provide evidence that the chemical of interest is directly involved in conferring physiological resistance to herbivorous insects. We were unable to demonstrate a significant negative genetic or phenotypic relationship between levels of either constitutive or herbivore-induced glucosinolates and trypsin inhibitors and leaf damage in our plants. We could only show a tendency for glucosinolate levels to be negatively phenotypically correlated with damage in control plants and for trypsin inhibitors to be negatively phenotypically correlated with damage in induced plants.

Interpreting the lack of genetic or phenotypic correlation between glucosinolates or trypsin inhibitors and resistance to *T. ni* as evidence that these chemicals are not physiologically relevant to plant defense would be misguided. It is clear that plants employ a large diversity of physical and chemical defenses that have physiological effects on insect herbivores (Simms, 1992; Karban and Baldwin, 1997). Even within single classes of defense compounds, including

glucosinolates, there may be many different molecules with distinct physiological functions (Berenbaum, 1991, 1995; Lambrix et al., 2001). Consequently, plants may utilize a large number of defenses with additive or synergistic effects that help protect them from insect herbivory. On the other hand, in light of the controversy surrounding the ecological role of glucosinolates in mediating plant-insect interactions (e.g., Chew, 1988), the lack of a strong relationship between total glucosinolate levels and resistance may not be surprising for some insects. Studies have shown that high glucosinolate content or the presence of certain glucosinolates is associated with lower levels of herbivory by some generalist insects (e.g., Hopkins et al., 1998a; Lambrix et al., 2001; Kliebenstein et al., 2002). Other studies have demonstrated that high glucosinolate levels or the presence of certain glucosinolates can serve as oviposition cues and feeding stimulants for some specialist insects (e.g., Giamoustaris and Mithen, 1995; Renwick and Lopez, 1999). As a generalist that prefers to feed on brassicaceous plants, *T. ni* appears to respond idosyncratically to different resistance factors in different brassicaceous plants (Broadway and Colvin, 1992; Broadway, 1995; Agrawal, 1999; D. Cipollini and C. Slemmons, unpublished data). In several recombinant inbred lines of *Arabidopsis thaliana*, feeding by *T. ni* was deterred by high glucosinolate and myrosinase levels and by the presence of certain glucosinolates, while feeding by the specialist *Plutella xylostella* was unaffected by variation in any of these traits (Kliebenstein et al., 2002). Thus, different results (perhaps stronger correlations between defense levels and damage) could be expected from different bioassay insects (e.g., Agrawal, 1999).

Moreover, in our one-time phytocentric measure of resistance (leaf area removed by the herbivore over 12 hr), we did not consider the effects of these chemical defenses on the insect (e.g., on insect growth rates), which may have been significant, especially if given enough time. Correlations between levels of chemical defenses and insect growth rate (as in Cipollini and Redman, 1999) may prove to be more important in a short-term bioassay situation than correlations between chemical defenses and damage. However, short-term effects on insect growth must translate into long-term effects on plant fitness for such effects to be important for the evolution of plant traits. Experiments attempting to ascertain the overall defensive function of both glucosinolates and proteinase inhibitors should include examinations of their physiological effects upon insect herbivores, in addition to their effect on insect damage, which would be important in predicting the long-term effects on plant fitness. It is clear, however, that other putative resistance factors need to be considered.

Trade-Offs. The marginally significant negative genetic correlation between glucosinolate and proteinase inhibitor levels in control plants suggests that there may be negative pleiotropy or linkage disequilibrium operating on the expression of these two traits (Falconer and Mackay, 1989). Negative correlations between these two defenses in agricultural *Brassica* spp. has been anecdotally observed in

the literature (Broadway, 1990). However, this marginal relationship disappears entirely in induced plants, so its importance as a constraint on the expression and evolution of the two traits is unknown. The main goal of this experiment was to examine the evolutionary implications of variation in multichemical induced defenses on herbivore resistance. With a more thorough knowledge of the costs, benefits, and variation in these different defense compounds, future studies may elucidate the possible adaptive roles these characters play in the complex story of how plants protect themselves from insect herbivores.

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HERBIVORE-INDUCED RESPONSES IN ALFALFA (*Medicago sativa*)

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Abstract—The herbivore-induced response of alfalfa (*Medicago sativa*) was examined through assays with *Spodoptera littoralis* larvae and analyses of important secondary substances. In food preference experiments, larvae preferred young undamaged alfalfa plants over plants that had been damaged by feeding larvae 5 and 7 days earlier, while no difference in feeding preferences could be detected 1, 9, and 14 days after damage. This suggests a peak in the herbivore induced resistance of alfalfa approximately one week after initial damage. The induced resistance in young plants was also shown to be systemic, while older flowering plants failed to show increased resistance after defoliation. Larvae gained weight slower and had lower pupal mass when fed damaged alfalfa than when fed undamaged alfalfa. Levels of total saponins were increased in foliage of damaged alfalfa, and detailed analyses of specific saponin components revealed doubled concentrations of 3GlcA,28AraRhaXyl medicagenate (medicagenic acid bidesmoside) and 3GlcAGalRha soyasapogenol B (soyasaponin I). Levels of the flavonoid apigenin (as free aglycone) also were increased in herbivore damaged plants. The herbivore-induced response of alfalfa was significantly weaker than that of cotton: *S. littoralis* larvae given a choice of undamaged cotton and undamaged alfalfa preferred to feed on cotton, whereas preferences shifted towards alfalfa when plants were damaged.

Key Words—*Medicago sativa*, induced resistance, herbivore damage, bioassays, *Spodoptera littoralis*, phenology, phytochemistry, defense compounds, saponins, flavonoids.

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INTRODUCTION

Plants may lose substantial proportions of their biomass to herbivores (Coley et al., 1985). Since loss of biomass is costly, plants have evolved different ways to minimize negative effects of herbivory, e.g., by defending themselves against the damaging agent (Herms and Mattson, 1992; Karban and Baldwin, 1997). Chemical defenses can be categorized as either constitutive or inducible, depending on whether the defense is always present or if activation of the defense requires environmental stimuli (Harvell, 1990). Induced responses leading to increased resistance against herbivores have now been reported for over 100 plant species, and observed effects on associated herbivores include altered mortality, growth, fecundity, behavior, and substantial changes in herbivore populations (Karban and Baldwin, 1997).

The level and effect of an herbivore-induced response may show great temporal and spatial variation. Thus, plants may respond to injury within hours or days, with relatively short lasting effects (Edwards et al., 1992; Baldwin and Schmelz, 1996; McAuslane et al., 1997), whereas long-term induced responses can affect herbivores in subsequent seasons (Haukioja and Niemelä, 1979; Hunter, 1987; Bryant et al., 1991, see review by Karban and Baldwin, 1997). Within a damaged plant, elevated levels of defense chemicals are commonly detected also at sites far from the initial injury [systemic response (Karban and Myers, 1989; Alborn et al., 1996)], but localized responses, i.e., increased levels primarily close to the initial injury, have also been demonstrated (Croxford et al., 1989; Edwards et al., 1992).

The aim of this study was to investigate the responses of alfalfa, *Medicago sativa* L., to damage by an insect herbivore, and the associated effects on herbivore performance and behavior. Alfalfa is the world's oldest and most important livestock feeding crop (Small, 1996). Identification and quantification of alfalfa secondary compounds have been intensively analyzed (e.g., Classen et al., 1982; Hernandez et al., 1991; Massiot et al., 1991; Oleszek et al., 1992; Oleszek, 1999; Stochmal et al., 2001a,b). Several studies have also examined interactions between alfalfa constitutive chemistry and associated herbivores (reviewed by Oleszek et al., 1999). Regarding induced responses, detailed data exist on interactions between alfalfa roots and pathogens (e.g., Kapulnik et al., 1996; Buffard et al., 1996; Tang and Smith, 2001), but data on responses, to insect herbivore damage are more limited. Although insect attacks are known to alter photosynthetic rates and stem flow in alfalfa (Petersen et al., 1992; Nielsen et al., 1999), effects on alfalfa defense levels have so far not been examined.

In this study, we used bioassays and phytochemical analyses to examine the temporal and spatial distribution of herbivore induced resistance in alfalfa. We also investigated if plant phenology affected alfalfa responses to herbivore damage. For bioassays, we used the Egyptian cotton leaf worm, *Spodoptera littoralis* Boisduval, a generalist herbivore that is a major pest on cotton (*Gossypium*

hirsutum L.) and alfalfa (Brown and Dewhurst, 1975). Assays with *S. littoralis* larvae were performed as feeding preference tests, but we also reared larvae on different food sources to examine effects on development time, growth, and pupal mass. Our chemical analyses were focused on alfalfa saponins and flavonoids. We analyzed total saponin content, as well as levels of specific saponins including zanhic acid tridesmoside (3GlcGlcGlc,23Ara,28AraRhaXylApi, Za), medicagenic acid bidesmoside (3GlcA,28AraRhaXyl, medicagenate), and soyasaponin I (3GlcAGalRha, soyasapogenol B), i.e., the dominant saponins in alfalfa aerial parts (Oleszek et al., 1992). Alfalfa saponins are suggested to play an important role in defense against generalist herbivores (Nozzolillo et al., 1997; Oleszek et al., 1999) and have been shown to strongly affect performance of *S. littoralis* (Adel et al., 2001). Flavonoid analyses included total flavonoids, apigenin (as free aglycone), and 7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranozyl (1$\rightarrow$2)-*O*-β-D-glucuronopyranozyl]-4'-*O*-β-D-glucuronopyranoside apigenin (apigenin glycoside). The role of flavonoids is less well understood, but these substances are suggested to have pronounced impacts on insect behavior and physiological performance (Harborne, 1993; Simmonds, 2001). Finally, to determine the relative strength of the induced resistance in alfalfa, we made comparisons with cotton, one of the most thoroughly studied species in this respect (e.g., Karban, 1985; Alborn et al., 1996; McAuslane et al., 1997; Anderson and Alborn, 1999).

Our specific aims were:

(1) to determine, through bioassays, if alfalfa has induced resistance against insect herbivores, i.e., responds to damage by becoming less palatable;
(2) if so, to examine how this resistance varies temporally (with time after damage), spatially (distribution within the plant), and phenologically (with plant age);
(3) to determine how the induced resistance of alfalfa affects physiological performance of associated insect herbivores;
(4) to examine changes in levels of alfalfa saponins and flavonoids after insect damage; and
(5) to compare the strength of the induced resistance of alfalfa with that of cotton.

METHODS AND MATERIALS

The study was performed between October 1999 and September 2000 at the Swedish Agricultural University, Alnarp, Sweden. All experiments were conducted indoors in greenhouses and climate chambers with constant temperatures, light conditions, and relative humidity, as described below.

Plants. Alfalfa seeds (variety: Julus) were sown individually in 1.5 l plastic pots, and plants were grown in greenhouses at 25 $\pm$ 5°C and 70 $\pm$ 5% relative

humidity. From mid-October to April, additional artificial lighting was provided. In feeding preference tests examining the temporal and spatial variation of alfalfa induced resistance, as well as in larval development assays, we used nonflowering (5 to 7-weeks old) plants. To determine effects of phenology, we also performed preference tests with flowering plants (8–10 weeks old). In all insect assays, only the distal half of nonflowering alfalfa stems was used. For comparison with the induced resistance of another species, we conducted dual-species preference tests with alfalfa versus cotton. Cotton plants were grown individually under the same conditions as alfalfa and were used in experiments when they had developed five true leaves (5–7 weeks old). Only the youngest leaf (Y1) was used in preference assays, since induced resistance of cotton is activated in the young, top leaves (Alborn et al., 1996; McAuslane et al., 1997).

At 5 weeks of age, the alfalfa plants were randomly assigned to become either damaged or undamaged (control) plants. To obtain insect-damaged plants, two third-instar *S. littoralis* were placed into plastic bags enclosing the outer 15 cm of one side branch at the lower half of one of the main branches. Larvae were left to feed for approximately 24 hr, after which the bags and larvae were removed. By this time, 80–90% of the enclosed foliage had been consumed. Similarly, on cotton plants, larvae were enclosed in plastic bags on the second true leaf and left for 24 hr, after which 40–60% of the leaf was consumed. For both plant species, the damage treatment resulted in loss of approximately 10% of the total foliage. Undamaged (control) plants of both alfalfa and cotton had a branch/leaf enclosed by an empty plastic bag, thereby controlling for potential effects of the plastic bags *per se*. Damaged and control cotton plants were kept together under the same environmental conditions until used in experiments.

Insects. S. littoralis larvae were used for all food preference experiments, as well as in the larval development experiment. Larvae were obtained from a laboratory culture reared on a semisynthetic diet (Hinks and Byers, 1976) and had no previous experience with either alfalfa or cotton. Larvae were kept at 25°C, 70% relative humidity and a 16L:8D photoperiod.

Food Preference Assays. In alfalfa food preference assays, fourth instar *S. littoralis* were given a choice between an insect-damaged and a control plant. From alfalfa plants, we used the distal parts (8–10 cm) of main branches. The branches were cut off above the damaged side branch on damaged plants and on a corresponding position on the undamaged controls. To examine the temporal variation in induced resistance, damaged plants were tested against controls 1, 5, 7, 9, and 14 days after damage. These times were selected because pilot preference experiments suggested a resistance peak in alfalfa approximately 7 days after herbivore damage. The spatial distribution of the resistance within the plant was examined through tests with a main branch (from a nonflowering plant) that had not been exposed to larval feeding on any of its lower side branches versus a control branch. These experiments were conducted 1, 7, and 14 days after damage. Finally,

the effects of phenology were determined by identical tests with flowering plants, using either a damaged or an undamaged branch from a damaged or control, plant, performed 1 and 7 days after damage.

At the start of each bioassay, the two branches, one from a damaged and one from a control plant, were weighed to the closest 10^{-4} g to obtain the wet mass. The cut ends of the branches were inserted into 1-ml glass vials filled with tap water to prevent wilting. Vials were sealed with parafilm, and the branches were placed horizontally with the glass vials facing the walls at opposite ends of a transparent plastic box (24 × 18 × 7 cm). The bioassay was initiated by placing one *S. littoralis* larva into the middle of the box, which was then closed with a ventilated lid. The larva was left to feed on the alfalfa for 24 hr. If the larva molted during the night, and had not consumed any significant amount, it was left to feed for another 24 hr. At the end of the experiment, the larva was removed, the branches were visually inspected for feeding damage, put in to labeled paper bags, completely dried (70°C for a minimum of 3 days), and weighed to the nearest 10^{-4} g. The general procedure for dual species preference tests, i.e., alfalfa versus cotton, was identical to the one for alfalfa tests. We tested control alfalfa against control cotton, as well as damaged alfalfa against damaged cotton (7 days after damage). Tests were replicated 12–16 times for each experimental treatment.

To determine the amount of plant biomass consumed by larvae in food preference tests, we calculated the relationship between dry mass (DM) and wet mass (WM) for the plant material used in the experiments. For alfalfa, one additional branch, from a similar position and of approximately the same size as the experimental branch, was cut off, weighed, dried, and reweighed. After determining the plant specific DM/WM relationship, we could calculate DM of the experimental branch at beginning of the experiment, and then (by subtracting DM remaining at the end of the experiment) the biomass consumed. For cotton plants, no leaf corresponding to the Y1 (experimental) leaf could be obtained from the same plant, and therefore we calculated a general regression between DM and WM for 25 Y1 leaves identical to those used in the experiments and harvested at the same time. The relationship between DM and WM ($DM = 0.1609\ WM + 0.0015$), which turned out to be robust ($R = 0.974$), was then used to calculate cotton leaf DM at the start of the experiment and, hence, the consumed biomass. When only minute damage (<2 mm^2 eaten) was detected on a plant during the visual inspection at the end of an assay, consumed DM was arbitrarily set to 1 mg. In cases where no damage was observed, biomass consumed was set to zero.

Larval Growth and Development. To examine the effects of the induced resistance on herbivore performance, 300 *S. littoralis* eggs were hatched and larvae placed into each of 10 plastic boxes (30 larvae per box) and fed foliage either from damaged alfalfa (5 boxes) or from undamaged alfalfa (5 boxes). The damaged plants were used for feeding larvae 5–8 days after induction, since pilot assays indicated that the induced resistance of alfalfa peaked around one week

after damage. Branches fed to the larvae were changed every second day or more often if necessary. Thus, larvae had an unlimited supply of food throughout the experiment. Larval numbers in each box were randomly reduced to 15 on day 7 and to 10 on day 9. Starting on day 7, larvae were weighed every second day to obtain the larval mean mass for each box, and, at the same time, survival was recorded. This continued until the larvae pupated. During the days near pupation, the boxes were checked daily for pupating individuals to determine time from hatching to pupation. After pupation was completed, the sex and fresh mass of each pupa were recorded. During the entire rearing experiment, boxes with larvae were kept in the larval climate chamber.

Chemical Analyses. Plant material for chemical analysis was collected in parallel with the bioassays. From both control and insect-damaged alfalfa plants, the outer parts of branches were cut off, placed into plastic bags, and immediately frozen. After freeze drying, the material was ground in a Retsch mixing mill (Wuppertal, Germany), and refrozen until analysis. Eight samples from control plants and eight samples from damaged plants were collected and analyzed for saponins and flavonoids, since these alfalfa substances are suggested to have antiherbivore effects (Harborne, 1993; Oleszek et al., 1999; Simmonds, 2001).

Total saponin content was estimated through assays with the fungus *Trichoderma viride*. Saponin levels were indirectly measured as the inhibitory activity of 500 mg dried alfalfa powder on radial growth of *T. viride* (see Oleszek et al., 1990). In addition, we made detailed analyses of three dominant saponins: zanhic acid tridesmoside, medicagenic acid bidesmoside (MAB), and soyasaponin I, using HPLC. Following Nowacka and Oleszek (1994), dried alfalfa powder was repeatedly extracted in 30% MeOH, and the extract was purified by using a C_{18} Sep-Pak cartridge (Waters, Milford, Massachusetts, USA). Determination of compounds was performed on a Waters system with a DAD detector. Chromatograms were registered and integrated at 210 nm. For separation, a Eurospher 80 C_{18} column was used at 50°C. The mobile phase consisted of a linear gradient of 1% $H_3PO_4 \rightarrow$ AcN (10–90% AcN) with a flow rate of 1 ml/min. Saponin compounds were identified by comparing their retention times to those of authentic standards.

Flavonoid analyses, including total flavonoid content, free apigenin and 7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranozyl (1$\rightarrow$2)-*O*-β-D-glucuronopyranozyl]-4′-*O*-β-D-glucuronopyranozide apigenin (apigenin glycoside), followed Stochmal and Oleszek (2001). In short, each powdered alfalfa sample was refluxed for 1.5 hr in 70% MeOH, and the extract purified on a short (3 $\times$ 5 cm) LiChroprep RP-18 column that was washed with 40% MeOH. Flavonoids in the 40% MeOH solution were then separated on a Eurospher 80 C_{18} column at 50°C, using a linear gradient of 20% acetonitrile in 1% H_3PO_4 $\rightarrow$ 40% acetonitrile in 1% H_3PO_4 at flow rate of 1 ml/min. Chromatograms were registered and integrated at 350 nm. Standards of apigenin and apigenin glycoside were used for calibration curve

preparation. Total flavonoid concentration was calculated from total integration area (350 nm) using the calibration curve of apigenin glycoside.

Statistics. All statistical tests were performed using SPSS 9.0 (SPSS Inc., 1999). Paired *t*-tests were used to analyze the results from food preference assays. To avoid pseudoreplication in the rearing experiment, the mean value for all larvae/pupae in each of the 10 rearing boxes was calculated (i.e., larval mass, development time, pupal mass, mortality, and sex ratio) and used as one observation. Larval mass was then analyzed with repeated-measures ANOVA, whereas development time (from hatching to pupation), pupal fresh mass, mortality, and sex ratio were analyzed with *t*-tests. Pupal mass data were analyzed separately for the two sexes to account for any size difference. Differences in foliar chemistry between control and damaged alfalfa plants were analyzed with *t*-tests.

RESULTS

Variation in Induced Resistance of Alfalfa. Larval food preference tests revealed a temporal variation in the induced resistance of alfalfa (Figure 1). One day after damage, *S. littoralis* larvae showed no significant preference for damaged or undamaged alfalfa plants (paired *t*-test: $N = 15$, $t = 1.37$, $P > 0.10$). However, 5 and 7 days after damage, larvae fed more on undamaged (control) plants compared to damaged plants ($N = 16$, $t = 2.74$, $P = 0.016$ and $N = 16$, $t = 2.41$, $P = 0.029$, respectively). In both cases, more than two thirds of the

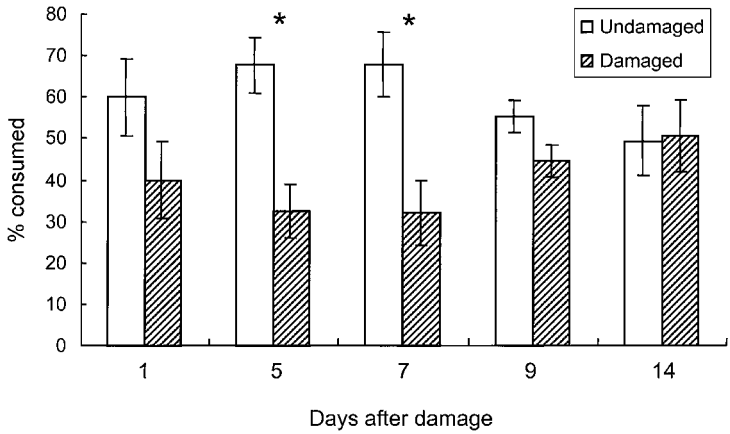

FIG. 1. Relative consumption of damaged (on same branch) and undamaged alfalfa in two-choice assays with fourth instar *S. littoralis*. Damaged alfalfa had been exposed to *S. littoralis* feeding for 24 hr either 1, 5, 7, 9, or 14 days prior to the experiment. Damage was caused to the lower part of the branch used in the assay. Vertical bars represent SE. *Significant differences between treatments at $P < 0.05$.

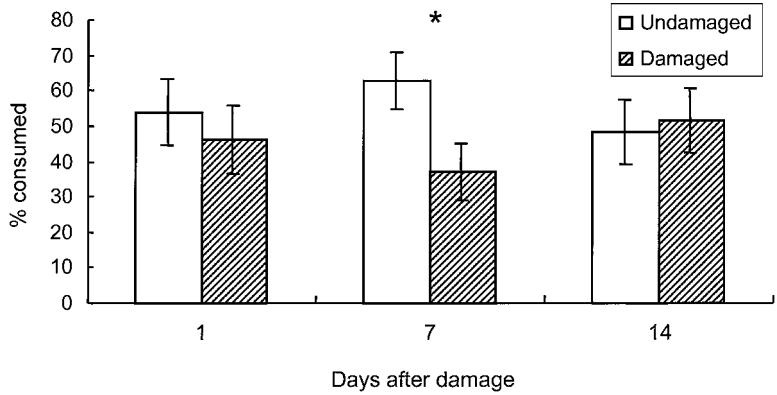

FIG. 2. Relative consumption of damaged (on other branch) and undamaged alfalfa in two-choice assays with fourth instar *S. littoralis*. Damaged alfalfa had been exposed to *S. littoralis* feeding for 24 hr either 1, 7, or 14 days prior to the experiment. Damage was caused to the lower part of another branch than the one used in the assay. Vertical bars represent SE. *Significant differences between treatments at $P < 0.05$.

biomass consumed during the tests was from control plants. This difference had diminished 9 days after damage, when larvae no longer distinguished between damaged and control plants ($N = 16$, $t = 1.41$, $P > 0.10$). Similarly, no significant preference could be detected after 14 days ($N = 15$, $t = 0.082$, $P > 0.10$).

To investigate whether herbivore injury increased resistance also in undamaged parts of the damaged plant (the spatial distribution), we tested a main branch other than the damaged one against a control branch (Figure 2). The observed pattern was largely the same as when the damaged main branch was used. Seven days after damage, larvae fed more on control compared with damaged plants, so that on average 63% of the biomass consumed was from the control plant ($N = 16$, $t = 2.243$, $P = 0.040$). No significant preference for any of the plants could be detected 1 and 14 days after damage (paired t-test: $N = 15$, $t = 0.560$, $P > 0.10$, and $N = 14$, $t = 0.213$, $P > 0.10$, respectively).

Phenological Differences. The induced resistance of flowering alfalfa plants was examined through preference assays 1 and 7 days after damage. As for nonflowering plants, there were no detectable signs of an induced resistance in flowering alfalfa 1 day after damage, i.e., the larvae showed similar preferences for damaged plants and undamaged control plants. In tests with damaged main versus control branches, larval relative consumption was 47.2% on the damaged branch (SE = 14.1; paired t-test: $N = 12$, $t = 0.184$, $P > 0.10$). Similar results were obtained when testing undamaged main branches from damaged plants against controls, i.e., with, on average, 56% consumed from damaged branches (SE = 11.8; $N = 12$, $t = 0.669$, $P > 0.10$). However, in contrast to tests with nonflowering

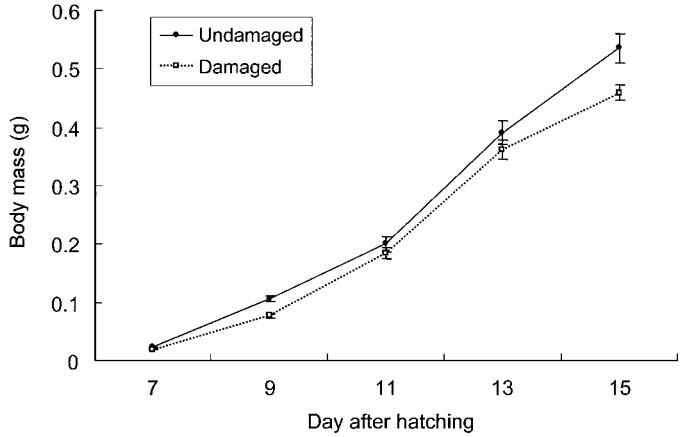

FIG. 3. Larval mass of *S. littoralis* larvae raised on undamaged alfalfa (solid line) and damaged alfalfa (dotted line). Data were obtained for days 7–15 after hatching. Vertical bars represent SE.

plants, no significant effect of previous damage on larval preference could be established 7 days after damage. Larvae consumed 49.8% from damaged main branches (SE = 8.9), and 47.3% from undamaged main branches of damaged plants (paired t-tests: damaged branch versus control, $N = 12, t = 0.132, P > 0.10$; undamaged branch versus control, $N = 12$, $t = 0.393$, $P > 0.10$).

Larval Growth and Development. During rearing from hatching to pupation, individual fresh mass was higher for larvae fed undamaged (control) alfalfa than for larvae fed alfalfa that had been damaged 5–8 days earlier (repeated-measures ANOVA: $N = 10$, $F_{(1,8)}$, $P = 0.012$). Mean larval mass was always found to be higher for control larvae, although differences compared to larvae fed damaged plants varied during the assay (Figure 3). The largest absolute difference in larval mass was found just prior to pupation (day 15), when control larvae were, on average, 14% (76 mg) heavier than larvae fed damaged alfalfa. Larval development time was similar in the two treatments. Time from hatching to completed pupation was, on average, 18.60 ($\pm$0.23) days for larvae fed undamaged plants and 19.09 ($\pm$0.40) days for larvae fed damaged plants, which was not significantly different (Figure 4a, t-test: $N = 10$, $t = 1.037$, $P > 0.10$). Pupal mass, on the other hand, showed pronounced differences between feeding treatments: masses of both female and male pupae were higher if the larvae had been fed control alfalfa compared with damaged alfalfa (Figure 4b, t-tests: $N = 10$, females: $t = 3.763$, $P < 0.01$; males: $t = 3.252$, $P < 0.05$).

The sex ratio of individuals completing pupation was similar in the two treatments, 56% of the control larvae were females, whereas the corresponding figure

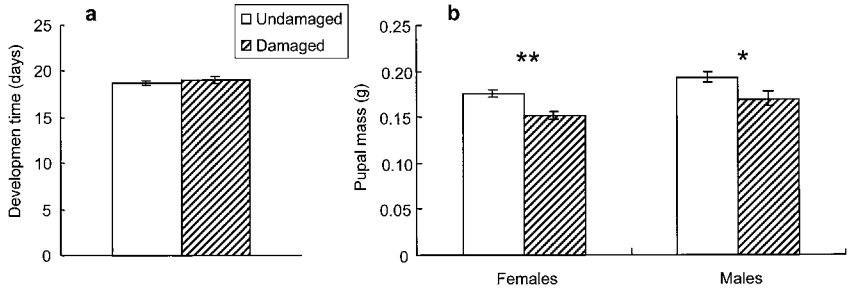

FIG. 4. Development time (a) and pupal dry mass (b) for larvae raised on undamaged and damaged alfalfa. Damaged plants had been exposed to *S. littoralis* feeding for 24 hr seven days before being fed to the larvae. Due to slight mass differences between sexes, data on pupal mass are presented separately for females and males. Vertical bars represent SE. Significant differences between treatments at $^{*}P < 0.05$ and $^{**}P < 0.01$.

for larvae fed damaged plants was 53% (t-test: $N = 10$, $t = 0.717$, $P > 0.10$). Mortality, calculated as the proportion dying during the whole larval period, was also similar in the two treatments. Of the larvae fed control plants, 18% died and among larvae fed damaged plants, 21% died during the experiment (t-test: $N = 10$, $t = 0.614$, $P > 0.10$). Most (>50%) of the total mortality occurred during the first week after hatching.

Alfalfa versus Cotton. Herbivore damage altered *S. littoralis* feeding preferences for alfalfa and cotton (Figure 5). In assays with undamaged plants, larvae preferred feeding on cotton compared with alfalfa: 64% of the total biomass

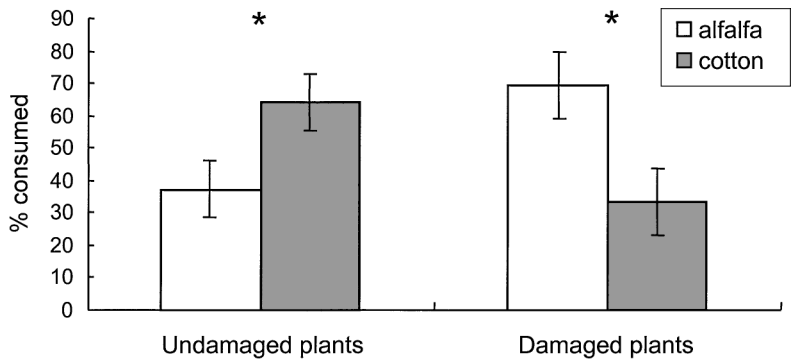

FIG. 5. Relative consumption of alfalfa and cotton in assays with either damaged or undamaged plants in two-choice assays with fourth instar *S. littoralis*. Damaged plants had been exposed to *S. littoralis* feeding for 24 hr seven days prior to the experiment. Vertical bars represent SE. *Significant differences between treatments at $P < 0.05$.

TABLE 1. FOLIAR CHEMISTRY OF UNDAMAGED AND INSECT DAMAGED ALFALFA PLANTS[a]

Substance category	Analysis	Alfalfa plants: Undamaged plants ($N = 8$)	Alfalfa plants: Damaged plants ($N = 8$)	Statistics: P	Statistics: t
Saponins	Total saponins	23.05 ± 4.59	35.30 ± 3.58	0.041	15.46
	ZAT	4.17 ± 0.66	4.97 ± 0.69	0.384	1.45
	MAB	2.21 ± 0.40	4.62 ± 0.98	0.029	22.27
	SsI	0.232 ± 0.066	0.475 ± 0.061	0.012	52.88
Flavonoids	Total flavonoids	26.00 ± 3.04	21.78 ± 3.52	0.349	1.63
	Free apigenin	0.0374 ± 0.0058	0.0673 ± 0.0137	0.049	12.71
	Apigenin glycoside	5.61 ± 1.26	3.79 ± 1.14	0.275	2.16

[a] Data are presented as percent of dry mass (mean ± 1 SE) for levels of total saponins, zanhic acid tridesmoside (ZAT), 3GlcA,28AraRhaXyl medicagenate (MAB), soyasaponin I (SsI), total flavonoids, free apigenin, and 7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranozyl (1→2)-*O*-β-D-glucuronopyranozyl]-4′-*O*-β-D-glucuronopyranozide apigenin (apigenin glycoside). Total saponin content was estimated through *T. viride* assays (% inhibition), whereas levels of the other substances were determined with HPLC (mg/g). Differences between undamaged and damaged plants were analyzed with the *t*-test.

consumed was from cotton (paired *t*-test: $N = 14$, $t = 2.719$, $P = 0.020$). However, when given a choice between damaged alfalfa and damaged cotton, larvae changed their preferences and fed more on alfalfa than on cotton (67 vs. 33%, respectively: $N = 13$, $t = 2.647$, $P = 0.021$).

Alfalfa Phytochemistry. Levels of total saponins, determined through the inhibitory effect on growth of *T. viride*, differed significantly between damaged and undamaged alfalfa (Table 1). Samples from damaged plants showed inhibitory effects that were more than 50% higher than corresponding values for undamaged plants. Separate HPLC analyses of the three dominating saponin components revealed that whereas levels of zanhic acid tridesmoside concentrations were unaffected by experimental treatments, levels of medicagenic acid bidesmoside (MAB) and soyasaponin I were significantly increased by herbivore damage, being doubled in damaged plants compared with undamaged control plants (Table 1). Flavonoid analyses revealed a substantial individual variation, but no significant treatment effects could be detected for total flavonoids and apigenin glycoside. Levels of free apigenin, on the other hand, were increased by 80% following larval damage (Table 1).

DISCUSSION

Variations in Induced Resistance of Alfalfa. This study demonstrates that alfalfa has an herbivore-induced resistance. Bioassays with *S. littoralis* larvae showed that strength of the induced resistance varied over time, building towards a peak

just before one week after herbivore damage. However, 14 days after damage, there was no indication of any induced resistance. The induced resistance of alfalfa seems to be a short-term response to herbivore damage with resistance levels increasing during the first days after damage and decreasing again after approximately one week. This type of short-term induced resistance has been observed in several other plant species. Cotton has a resistance peak around 7 days after induction (McAuslane et al., 1997), although effects can be detected after only 6 hr (Alborn et al., 1996). Similar findings have been reported for tobacco (Baldwin, 1989), soybean (Underwood, 1998), and cucumber (Agrawal et al., 1999).

Short-term responses are considered to be active responses: cues from the damaging agent activate specific defense responses in the plant, and chemicals are synthesized following wounding (Karban and Baldwin, 1997). The time it takes from initial damage to activation of the defense varies depending upon the type of defense mechanism activated (Karban and Myers, 1989). In the present study, there was a time lag after herbivore damage before any strong effect on herbivores could be observed. Although we did not establish the exact length of this time lag, we can narrow it down to be between 2 and 4 days, a time span that may be due to the time it takes for alfalfa to synthesize the defensive chemicals and produce amounts large enough to affect herbivores.

We cannot rule out that the effect of herbivore damage was stronger near the site of wounding during the first day(s). Although the difference was too small to be significant, preference, on average, was higher for control branches when these were tested against damaged branches (60%, Figure 1), than when tested against undamaged branches from damaged plants (53%, Figure 2). After 7 days, however, the induced resistance of alfalfa was systemic, i.e., could be detected also in undamaged parts of damaged plants. Systemic responses should be favored in plants that are small in relation to the expected amount of herbivory (Järemo, 1999) and are especially effective against mobile herbivores that can easily move away from the induced plant (Karban and Baldwin, 1997). A last-instar *S. littoralis* defoliates an alfalfa plant within a few days (Agrell, unpublished data) and is highly mobile. The benefits of locally induced resistance against lepidopteran larvae and other large insect herbivores would, therefore, be rather limited, since the herbivores would probably keep on feeding on another part of the damaged plant (c.f. Edwards et al., 1992). Instead, systemic induced resistance, like the one observed in this study, is likely to be most beneficial for alfalfa through its potential to deter any further feeding on the attacked plant.

In addition to temporal changes in the induced resistance of alfalfa, we also established a significant effect of plant phenology. In contrast to nonflowering plants, flowering plants showed no signs of any response to herbivore damage. Our results agree with indications from previous studies. Blodgett and Higgins (1990) reported more insect attacks on reproductive alfalfa plants than on nonreproductive ones, indicating reduced defense levels during later phenological stages. Moreover,

damage by leafhoppers (*Empoasca fabae*) has been found to affect translocation of photoassimilate in vegetative, but not reproductive alfalfa plants (Nielsen et al., 1999). One potential explanation for the lack of responses in flowering plants is that plant reproduction is associated with a physiological cost requiring resources that would otherwise support other processes in the plant (Bazzaz et al., 1987). Since maintaining a high level of resistance is assumed to be costly (Karban and Baldwin, 1997), the plant faces a trade-off between allocation of resources to reproductive tissue and defense during flowering and seed formation. Alternative explanations for reduced resistance during later phenological stages are that older plants are, in general, less nutritious and need less protection (Perevolotsky, 1994) or that the chemical compounds involved in the defense can be produced only during periods of active growth (c.f. Wolfson and Murdock, 1990; Baldwin and Schmelz, 1996). For alfalfa, an opposite explanation is also possible, i.e., that constitutive saponin levels peaking around the time of flowering (Oleszek, 2000) either mask or cause a down-regulation of herbivore induced responses. However, from the present study, it is not possible to know whether the lack of induced resistance in flowering alfalfa is due to an investment trade-off, reduced need for defense, or to phenological constraints.

The induced resistance of alfalfa not only affected feeding behavior, but also showed negative effects on herbivore physiological performance. This is important for two reasons. First, the reduced growth and pupal mass of *S. littoralis* fed damaged alfalfa demonstrates the negative fitness consequences for larvae that continue to feed on such plants, and, therefore, the evolutionary force behind the observed behavior to avoid previously attacked plants. Second, both larval growth and pupal mass are physiological parameters directly influencing demography. Thus, although the main effect of induced responses in alfalfa may be deterrence of herbivores, consequences on herbivore density and population dynamics are also possible. However, such effects are likely to occur primarily if a substantial proportion of the alfalfa population has been attacked and access to undamaged alfalfa or alternative food plants is limited.

Chemical Constituents and Strength of Induced Resistance. Chemical compounds that make up the constitutive defense are also likely to be involved in the inducible defense of a plant (Agrawal et al., 1999). The constitutive defense of alfalfa is based primarily on saponins and condensed tannins (Jones et al., 1976; Oleszek et al., 1992; Nozzolillo et al., 1997), but flavonoids also have been suggested to deter herbivores (Harborne, 1993), including *S. littoralis* (Simmonds, 2001). Indirect quantification of saponin levels using *T. viride* indicated that levels of total saponins were increased by approximately 50% in damaged plants compared with control plants, suggesting that saponins are important constituents in the induced resistance of alfalfa. Adel et al. (2000) reported that *S. littoralis* larvae fed high saponin diets exhibited reduced growth rate and pupal mass, and in the present study, we documented the same effects

for larvae fed damaged plants. Although other factors can cause these nonspecific effects, our combined results on phytochemistry and larval performance support the idea that high saponin levels increased the resistance of damaged alfalfa plants.

Regarding the specific saponins involved in the induced resistance, we established doubled levels of MAB and soyasaponin I in plants after herbivore damage. No such effect was observed for zanhic acid tridesmoside. Furthermore, we found strong indications that flavonoids are also involved in the herbivore-induced response of alfalfa. Levels of free apigenin were higher in damaged compared with control plants, whereas concentrations of total flavonoids and apigenin glycoside were unaffected by herbivore exposure. Interestingly, free apigenin is assumed to be the active part of the glycoside molecule, whereas glycosides are considered as transport forms or products of deactivation of active aglycone. Absorption and bioactivity of these compounds in insects are unknown, however. According to Simmonds (2001), there are no previous examples of plants showing an altered flavonoid profile after herbivore attack, but increases in the concentration of the isoflavone coumestrol in alfalfa herbage after infestation by bluegreen lucerne aphid (*Acyrthosiphum kondoi*) and pea aphid (*A. pisum*) have been observed (Kain and Biggs, 1980). Feeding deterrence of flavonoids and related phenolics in wheat towards the aphids *Schizaphis graminum* and *Myzus persicae* has also been reported (Dreyer and Jones, 1981).

We cannot exclude that the induced resistance of alfalfa also includes chemicals not examined in the present study. One possibility would be condensed tannins, although these substances have primarily been shown to be involved in more long-term induced resistance of woody species (e.g., Rossiter et al., 1988; Schultz and Baldwin, 1982), or to be nonresponsive to herbivore damage (Perevolotsky, 1994).

Despite significant effects on herbivore performance and levels of saponin and flavonoid constituents, herbivore damage seems to elicit a comparably limited response in alfalfa. We found that mass of both last-instar larvae and pupae were 12% lower on damaged compared with control plants. However, for several plant species, herbivore damage induces changes that cause more dramatic effects on herbivore performance. For example, mite populations were reduced by 50% on damaged compared with control cucumber (Agrawal et al., 1999). Similarly, *S. littoralis* larvae fed damaged cotton plants may show pupal masses that are 30% lower than when fed control plants (McAuslane and Alborn, 2000, Agrell, unpublished data), or even stop growing and die (Alborn et al., 1996). Chemical changes observed in alfalfa after herbivore damage are on a par with those recorded for tobacco, which showed approximately doubled levels of alkaloids in damaged compared with control plants (Baldwin, 1989). However, substantially greater changes have been observed. In cotton, concentrations of terpenoid aldehydes can increase 2–5 times after insect damage (McAuslane and Alborn,

1998; Agrell, unpublished data). The impression that induced resistance of alfalfa is relatively weak was further confirmed by the preference assays with alfalfa and cotton: with undamaged plants, larvae preferred cotton, whereas with damaged plants, this preference changed to alfalfa. The weak induced resistance in alfalfa may in part be explained by its use as a crop plant. Selection for minimal digestive problems for domestic animals has probably resulted in a general reduction in chemical defenses (Nozzolillo et al., 1997), and responses to damage may have been further reduced through selection for resource allocation to growth instead of defense after harvesting or grazing by livestock (Small, 1996). The explanation for differences in induced resistance of alfalfa and cotton may, however, partly lie in different life-history strategies evolved by these two species. Herms and Mattson (1992) discussed the plant dilemma regarding allocation of resources to growth versus defense, and suggested that this trade-off situation forces plants investing heavily in growth to have reduced defense levels, and *vice versa*. Both alfalfa and cotton are crop plants commonly grown and harvested on an annual basis, but whereas *Gossypium* (cotton) species in general have a more woody character with slower growth, *Medicago* species (including alfalfa) are typical fast-growing herbs. Possibly the growth strategy of alfalfa has been shaped more by plant–plant competition and to a lesser extent by herbivory, resulting in a comparably weak defense system.

In summary, this study demonstrates that alfalfa has short-term inducible resistance that peaks approximately one week after initial damage by herbivores. Although the response may start locally soon after initial damage, effects are systemic during the resistance peak. Foliar changes after herbivore damage include pronounced increases in levels of saponin and flavonoid substances. During flowering, alfalfa does not show any response to herbivore damage. From an agricultural and pest management perspective, detailed knowledge about herbivore-induced changes in plant chemical composition may be important. First, for a plant species primarily grown for its nutritional value, even small changes in levels of nutrients and/or secondary chemistry can have substantial effects. Second, since the competitive ability of a plant species may depend on how palatable it is in relation to other plant species (Pacala and Crawley, 1992), the relative strength of a plant's defense may influence its risk of being attacked by pest insects. Variation in induced responses to herbivory may have ecological consequences by causing shifts in the competitive balance among plant species within a plant community. Finally, caution must be taken when herbivore host plant preferences or pest damages are predicted using undamaged plants only, since the induced state can be very different, and for many plant species this might be the most common one (Karban and Myers, 1989).

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IDENTIFICATION OF HOST FRUIT VOLATILES FROM HAWTHORN (*Crataegus* spp.) ATTRACTIVE TO HAWTHORN-ORIGIN *Rhagoletis pomonella* FLIES

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Abstract—Solid-phase microextraction (SPME) and gas chromatography coupled with electroantennographic detection (GC-EAD) were used to identify volatile compounds from hawthorn fruit (*Crataegus* spp.) acting as behavioral attractants for hawthorn-infesting *Rhagoletis pomonella* flies. Consistent EAD activity was obtained for six chemicals: ethyl acetate (94.3%), 3-methylbutan-1-ol (4.0%), isoamyl acetate (1.5%), 4,8-dimethyl-1,3(*E*),7-nonatriene (0.07%), butyl hexanoate (0.01%), and dihydro-β-ionone (0.10%). In a flight-tunnel bioassay, there was a dose-related increase in the percentage of flies flying upwind to the six-component mixture. Hawthorn-origin flies also made equivalent levels of upwind flight with the synthetic blend and an adsorbent extract of volatiles collected from whole fruit, each containing the same amount of the 3-methylbutan-1-ol compound. Significantly lower levels of upwind flight occurred to a previously identified volatile blend of ester compounds that attracts *R. pomonella* flies infesting domestic apples, compared with the hawthorn volatile mix. Selected subtraction assays showed further that the four-component mixture of 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone also elicited levels of upwind flight equivalent to the six-component mix. Removal of 3-methylbutan-1-ol from the four-component blend resulted in complete loss of upwind flight behavior. Removal of dihydro-β-ionone, 4,8-dimethyl-1,3(*E*),7-nonatriene, or butyl hexanoate from the

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four-component mixture resulted in significant decreases in the mean number of upwind flights compared to the four- or six-component mixtures.

Key Words—Solid-phase microextraction, gas chromatographic electroantennographic detection, flight tunnel, *Crataegus* spp., fruit volatiles.

INTRODUCTION

The apple maggot fly, *Rhagoletis pomonella* (Walsh), occurs in North America as two races, one on native hawthorn (*Crataegus*), and one on introduced apple (Bush, 1966; Feder, 1998). Electrophoretic analysis has confirmed the taxonomic status of apple- and hawthorn-infesting populations of *R. pomonella* as partially reproductively isolated "host races," the hypothesized first stage in sympatric speciation (Feder et al., 1988, 1990, 1994, 1998; Feder and Bush, 1989; Berlocher and Feder, 2002). Behavioral studies have shown that differences in host plant preference play an important role in reproductively isolating the apple and hawthorn host races, and volatile host fruit odors have been proposed as one of several important cues in locating host plants (Prokopy et al., 1988; Feder et al., 1993, 1994). In earlier work, apple-origin flies were shown to be attracted to odors from apples (Prokopy et al., 1973) and to synthetic compounds isolated from apple fruit (Fein et al., 1982; Reissig et al., 1982; Zhang et al., 1999).

Here we present details of a protocol, using solid-phase microextraction [SPME, Supelco (see Bartelt, 1997; Agelopoulos and Pickett, 1998)], in conjunction with GC-EAD analysis that allowed for effective screening of key volatiles from hawthorn fruit to which adult flies are most sensitive. We also present evidence that hawthorn-origin flies from a well-studied field site at Grant, Michigan, USA (Feder et al., 1998), respond behaviorally to, and display a preference for, a blend of volatiles identified from hawthorn fruit that is different from the volatile blend identified from domestic apple.

METHODS AND MATERIALS

Insects. Hawthorn-origin *R. pomonella* pupae were collected from fruit at Grant, Michigan, USA. Postdiapause pupae (Feder et al., 1994) were shipped to the laboratory in Geneva, New York, USA, and kept in an environmental chamber at 23–24°C temperature, 16L:8D photoperiod, and 55–60% relative humidity. Adults were maintained on an artificial diet (Fein et al., 1982). Adult flies at 0–7 and 10–21 days old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively. The difference in age was because younger flies were more stable in the EAD apparatus, whereas flies do not respond behaviorally to volatiles until reproductive maturity at 7–10 days old.

Adult *R. pomonella* from a laboratory colony maintained on Red Delicious apples (Neilson and McAllen, 1965) also were used for some of the EAD

measurements (see GC-EAD section below). Adults were kept in an environmental chamber at 23°C temperature, 50% relative humidity, and 16L:8D photoperiod and maintained on artificial diet.

Fruit. Hawthorn fruit was collected at Grant, Michigan, and shipped to the New York laboratory. Hawthorn fruit from trees at the Experiment Station in Geneva, New York, also was analyzed for volatiles. This allowed us to compare the profiles from fruit that had been freshly picked versus shipments of fruit sent via overnight express. Fruit was collected at the two sites during August and September to include fruit that was in preripe (green fruit), ripe (red), and overripe (fruit that had fallen from the tree and might be infested) stages. Fruit from the Michigan site was held in cold storage (4°C; never more than 2 weeks) until sampled. Fruit was taken from cold storage and placed in the sampling containers (see below) at room temperature at least 1 hr before SPME sampling began. Analysis of fruit taken from cold storage continued for up to 1 week, whereas analysis of freshly picked fruit in New York began immediately after picking and continued for 1–2 days.

Adsorbent Sampling. A glass desiccator (20 liters) with a charcoal filter tube in the inlet was used for collection of volatiles from whole fruit. Volatiles from the head space were drawn (ca. 0.5 liters/min) by a vacuum pump onto an ORBO solvent desorption tube (150 mg active coconut charcoal, 20–40 mesh, 4 mm ID, 70 mm length; Supelco Inc., Bellefonte, Pennsylvania, USA). Adsorbent samplings were made for 7 days at room temperature from 3 kg of ripe fruit collected at the Grant, Michigan, USA site. The ORBO tubes were changed every day. The volatiles were eluted with 1 ml methylene chloride (Nanograde, Mallinckrodt Baker, Inc., Phillipsburg, New Jersey, USA), dried over anhydrous sodium sulfate, and then combined. The extract was kept in a freezer (−20°C) and subjected to GC and GC-MS analysis and flight tunnel bioassay.

SPME Sampling. A glass jar (500 ml) with Teflon liner screwcap (Wheaton, Milliville, New Jersey, USA) was used for a sampling container. The inner wall of the container was deactivated with 5% dimethylchlorosilane in toluene (Glass Prep; Alltech Associates, Inc., Deerfield, Illinois), and then cleaned and oven baked (ca. 250°C). Fruit (ca. 150 g) was placed in the container for SPME sampling. A carboxen–polydimethylsiloxane-coated SPME fiber (film thickness 85 μm; Supelco) was conditioned in the GC injector (280°C) for 20 min, and then passed through the small hole on the cap into the headspace of the jar. After a 10 to 20-min exposure, collected volatiles on the fiber were immediately subjected to GC-EAD analysis. For the chemical analysis of the volatiles with GC-MS, SPME samplings were made for a longer time (a few hours to overnight) if necessary.

Coupled Gas Chromatographic–Electroantennographic Detection (GC-EAD) Analysis. A Hewlett Packard 5890 series II gas chromatograph equipped with a nonpolar SPB-1 capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Supelco) or a polar EC-Wax Econo-Cap capillary column (30 m × 0.25 mm

ID, 0.25 mm film thickness; Alltech Associates, Inc., Deerfield, Illinois, USA) in the splitless mode was used for GC-EAD analysis. For SPME sample injection, a 0.75-mm-ID glass inlet liner (Supelco) was used, but for liquid sample injection, a 4-mm-ID liner was used. Nitrogen was used as the carrier gas at a head pressure of 138 kPa (flow rate, 2.0 ml/min). The time for splitless injection was 0.25 and 1.0 min for SPME samples and liquid samples, respectively. Oven temperature was set initially at 40°C for 2 min, increased at 15°C/min to 250°C, and held for 10 min. Injector and detector temperatures were set at 280°C. Septum purge flow rate was set at 3 ml/min and total flow rate at 60 ml/min.

The column effluent was combined with nitrogen makeup gas (30 ml/min) and then split 1:1 to the flame ionization detector (FID) and EAD with a TCD capillary column adapter (Agilent Technology, Wilmington, Delaware, USA), two deactivated capillary columns (nonpolar, 0.25 mm ID, Alltech Associates) of equal length as transfer lines, and a universal capillary Y connector (Alltech Associates). The TCD adapter and the Y connector were connected with a short length of deactivated wide-bore capillary column (0.53 mm ID) and a stainless reducing union (3.2 mm OD to 1.6 mm OD). The end of the column was fixed to the TCD adapter with a regular column nut so that the column was extended to the inside of the wide-bore column to facilitate easy column changes.

An extra FID port of the GC was modified and used for the EAD. The transfer line in the EAD heating block was protected and insulated with glass-lined stainless steel tube (1.6 mm OD $\times$ 0.3 mm ID, Alltech Associates), and a wider regular grade stainless steel tube. The EAD port also was insulated with glass wool and capped with a stainless steel cap. The EAD outlet was through a small hole on the top of the cap and was secured in a charcoal-filtered and humidified air stream, which was refrigerated by a modified condenser flushed with 0°C water (Zhang et al., 1999) flowing at ca. 500 ml/min over a *Rhagoletis* fly antennal preparation.

A fly head, separated at the prothorax, was mounted on the tip of a plastic pipet chip (1 ml), which was filled with *Drosophila* ringer solution (46 mmol NaCl, 182 mmol KCl, 3 mmol $CaCl_2$ and 10 mmol Tris HCl at pH 7.2), and equipped with a pure gold wire indifferent electrode. The chip was mounted with adhesive glue on a custom acrylic holder (Figure 1). A pure gold wire recording electrode was extended into the cavity (2 mm ID) in the acrylic holder, and a capillary tube (3 μl micropipet, Drumond Scientific Company, Broomall, Pennsylvania, USA) was fixed in a hole (2 mm ID) at the end of the acrylic holder with a tiny rubber stopper (made with a GC septa). The cavity and the capillary tube were filled with saline. The plastic pipet chip with a mounted fly head was slid back and forth on the acrylic holder so that the tips of both antennae were maneuvered to make electrical contact with the saline of the capillary tube. This device allowed easy mounting of variable size fly heads. The holder was placed inside a cooling condenser and was maintained at 5°C. The distance between the antennae and the EAD outlet

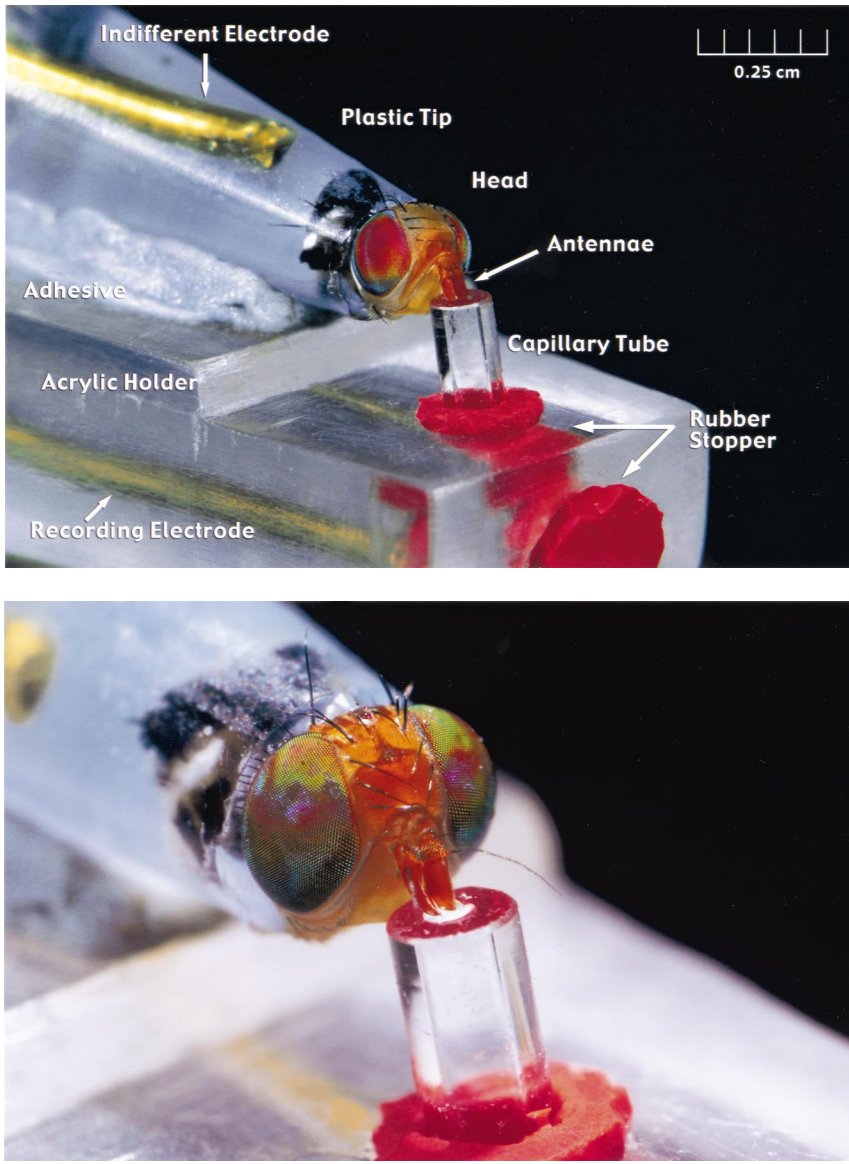


FIG. 1. (A) Acrylic holder for antennal EAD preparation; (B) close-up view.

was 1 cm. With this apparatus, excellent EAD recordings could be made for a few hours on an antennal preparation without cutting off the tip of the antenna.

The output signal from the antennae was amplified by a customized single-step high-input impedance DC amplifier. An OPA 121 precision monolithic dielectrically isolated FET operational amplifier chip (Burr-Brown, Tucson, Arizona, USA) was used at 100 $\times$ amplification. The output signal was filtered by a simple resistance/capacitance (*RC*) high-pass filter (Horowitz and Hill, 1989) with a cutoff frequency of about 0.5 Hz, experimentally determined by examining the signal-to-noise level. A metallized polyester film capacitor (3.3 μF, Matsushita Electric Industrial Co., Ltd., Tokyo, Japan) and a metal film 1/4 W resistor (100 kΩ, Yageo, Taipei, Taiwan) were used for the *RC* filter. All electrical parts were obtained through Digi-Key Corporation, Thief River Falls, Minnesota, USA (www.Digi-key.com) or RadioShack, Fort Worth, Texas, USA (www.radioshack.com). A dual-tracking variable constant voltage power supply (Akidzuki Co. Ltd, Tokyo, Japan) was used at $\pm$ 15 V and connected to the amplifier with a shielded three-conductor cable. The signal was recorded on an HP 3390 A integrator and synchronized with a GC integrator. The amplifier and the integrator were connected with a shielded coaxial cable with a BNC connector. All devices were grounded to a clean source, so that the signal could be recorded without a Faraday cage.

In preliminary experiments, no significant qualitative differences in EAD responses were found between apple-origin flies from our laboratory colony and hawthorn-origin flies from nature, or between sexes within each race in the various GC-EAD runs. Because of limited availability of hawthorn-origin flies needed for behavioral testing, key host volatiles from hawthorn fruit were first estimated by using the laboratory colony flies reared on apple, and then the results were confirmed with hawthorn-origin flies from Grant, Michigan.

Chemical Analysis. GC-MS analyses were performed on a Shimadzu GC-17A equipped with a nonpolar DB-1ms capillary column (30 m $\times$ 0.25 mm ID, 0.25 mm film thickness; J&W Scientific, Folsom, California, USA) or a polar EC-WAX Econo-Cap capillary column (30 m $\times$ 0.25 mm ID, 0.25 mm film thickness; Alltech Associates) and coupled to a Shimadzu QP-5000 quadrupole mass spectrometer running in the EI (at 70 eV) scan mode. For SPME sample injection, a 0.75-mm-ID glass inlet liner (Supelco) was used, but for liquid injection, a 3-mm-ID liner was used. Helium was used as the carrier gas at an initial head pressure of 54 kPa at constant flow rate (1.0 ml/min). The time for splitless injection was 0.3 and 1.0 min for SPME and liquid samples, respectively. Oven temperature was programmed at 40°C for 2 min, increased at 15°C/min to 250°C, and held for 10 min. The injector and interface temperatures were set at 280°C and 260°C, respectively. Volatiles were identified by mass spectral matches to library spectra as well as by retention time matches to available known standards. The EAD activity of compounds were verified by the GC-EAD analysis with authentic standards. Quantification of the relative ratio of the EAD active compounds was made from

the adsorbent collection. Compounds were quantified from their ion abundances from GC-MS analyses, or FID responses, according to the standard curves made from each authentic standard.

Chemicals. Butyl hexanoate, 3-methylbutan-1-ol, isoamyl acetate, and ethyl acetate were purchased from Aldrich, Milwaukee, Wisconsin, USA (purities $> 98\%$). Dihydro-β-ionone was purchased from Scientific Exchange, Inc., Center Ossipee, New Hampshire, USA, and purity was $>89\%$ based on GC-MS analysis. The 4,8-dimethyl-1,3(*E*),7-nonatriene compound was synthesized according to Greenwald et al. (1963), producing an 87:13 ratio of *E* and *Z* isomers, based on GC-MS analysis. The net proportion of isomers was $>96\%$.

Flight Tunnel. The response of flies to host fruit volatiles was measured in a sustained-flight tunnel (Zhang et al., 1999). Outside dimensions of the glass and metal frame tunnel were 183 cm long and 61 $\times$ 61 cm square. Flight-tunnel conditions were 23–24°C, 50–70% relative humidity, 35–40 cm/sec wind speed, and 800 lux light intensity. Light was provided by fluorescent bulbs hung from the ceiling. Adult flies were tested during the third to eleventh hours of the 16-hr photophase period (6 AM to 10 PM EST). All flight tunnel tests involved hawthorn-origin flies from the Grant, Michigan, site.

The floor of the tunnel was covered with white paper and 15 green paper circles of 10 cm diameter placed in a random pattern. The sides of the tunnel also were covered with white paper, since this was found to decrease the flies' distraction by visual stimuli in the testing environment, significantly increasing the number of flies exhibiting upwind flight in the odor plume (Cossé and Baker, 1996). Adult flies (mixed-sex, 10–21 days old) were selected from holding cages located in a separate, environmentally controlled, room, placed singly in glass vials, taken to the room housing the flight tunnel, and allowed to acclimate in the room for 15 min.

Individual flies then were transferred to a screen holding cage, which was placed inside the tunnel on a screen stand 1 m downwind of the odor source, a red rubber septum (Thomas Scientific, Swedesboro, New Jersey, USA; Cat. No. 1780J07) containing solutions of volatile extracts or synthetic compounds prepared in methylene chloride. Rubber septa were prepared 60 min prior to a test and were clipped onto the bottom of a 7.5-cm red plastic sphere (Gempler's Inc., Mt. Horeb, Wisconsin, USA), which was hung from the ceiling at the upwind end of the tunnel. Blank spheres contained rubber septa treated with methylene chloride. Fresh sources and red spheres were used for each test period. Flies were recovered after testing and used on consecutive days, but never more than four times in a weekly period. Each fly was given 1 min to respond, and a positive response was scored if the fly flew upwind in a continuous flight and landed on the red sphere containing the rubber septum source.

For comparative purposes, the dosage of the hawthorn synthetic blends and the adsorbent extract (reported below) reflects the amount of 3-methylbutan-1-ol,

TABLE 1. ACTIVE CHEMICALS FROM HAWTHORN FRUIT DETERMINED BY INTEGRATION OF GC-FID AND GC-MS TOTAL ION CURRENT USING ADSORBENT SAMPLE FROM RIPE FRUIT COLLECTED AT GRANT, MICHIGAN[a]

Chemicals	GC/MS; GC/FID (%)	FT (%)
I. Ethyl acetate	94.3	2000
II. 3-methylbutan-1-ol	4.0	100
III. Isoamyl acetate	1.5	40
IV. 4,8-dimethyl-1,3(E),7-nonatriene	0.07	2
V. Butyl hexanoate	0.01	1
VI. Dihydro-β-ionone	0.10	2

[a] FT indicates the percentage of each compound in the synthetic blend used in the flight tunnel.

with the other components added in the proportions shown in Table 1. Thus, 100 μg doses of the adsorbent extracts of head space volatile collections from whole fruit and the synthetic blend both contained approximately 100 μg of 3-methylbutan-1-ol. Hawthorn-origin flies also were tested to the synthetic blend of ester compounds identified from domestic apple (Zhang et al., 1999). The synthetic apple volatile mixture, in methylene chloride, comprised the following compounds and proportions: butyl butanoate (10%), propyl hexanoate (4%), butyl hexanoate (37%), hexyl butanoate (44%), and pentyl hexanoate (5%). The reported dose of this mixture reflects the total amount of the compounds.

Four different experiments were conducted. In the first, a 100-μg dose of the adsorbent volatile extract from hawthorn fruit was compared with a 100-μg dose of the complete six-component hawthorn fruit blend identified from SPME-EAD analysis. The 100-μg dose was selected based on analysis of the adsorbent extract showing that it contained approximately 0.5 μg/μl 3-methylbutan-1-ol, resulting in 100 μg of the compound when 200 μl of the solution was applied to the rubber septum. In the second experiment, 50- and 200-μg doses of the synthetic blend were compared. In the third experiment, the 200-μg dose of the six-component blend was compared with a 200-μg dose (total quantity) of the 5-component apple volatile blend. In the fourth experiment, the 200 μg dose of the 6-component blend was compared with mixtures in which single or multiple compounds were removed from the complete blend. In all experiments, 25 flies were tested individually each day with each treatment until there were $N = 100$ flies for experiments 1 and 2, and $N = 75$ for experiments 3 and 4. On each day when testing occurred, 10–15 males were tested to a red sphere with a solvent-treated rubber septum.

Statistical Analysis. The number of flies in each treatment that flew upwind to the source was converted to a percent value for graphical display. Treatment

comparisons within each experiment were made using χ^2 analysis, according to the JMP statistical analysis program for Macintosh ($P < 0.05$).

RESULTS

Identification of Key Host Volatiles from Hawthorn Fruit. A total of 9 fruit samples collected during August and September, 2000 (6 samples) and 2001 (3) at the Michigan (5) and New York (4) sites, were analyzed using SPME with GC-EAD. A total of 98 different antennal pairs (1–6 runs/pair; 28 female and 24 male colony apple-origin flies; 31 female and 15 male hawthorn-origin flies) were used for the analysis. Comparison of EAD samples from Michigan and New York hawthorn fruit with both Michigan hawthorn-origin flies and New York apple-origin flies allowed us to determine the key group of volatiles that consistently gave EAD responses. Figure 2 shows a GC-EAD recording from the antennae of an apple-origin female *R. pomonella* exposed to SPME samples of host hawthorn fruit. This sample was from fruit collected at the Geneva site in 2000, at a stage when the majority of the fruit had turned red and were in the ripe stage. The corresponding active compounds were identified as (I) ethyl acetate, (II) 3-methylbutan-1-ol, (III) isoamyl acetate, (IV) 4,8-dimethyl-1,3(*E*),7-nonatriene, (V) butyl hexanoate, and (VI) dihydro-β-ionone.

All of the responses in Figure 2, except for the one earlier than compound I, were consistently found in the majority of fruit samples taken from the two

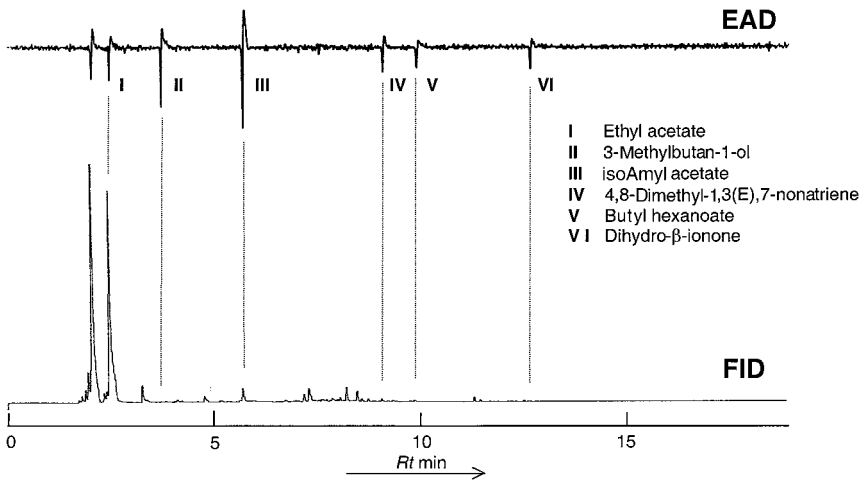


FIG. 2. Simultaneous responses of FID and EAD using the antennae of apple-origin *R. pomonella* female adult to SPME desorbed volatiles from fresh hawthorn fruit collected at the Geneva, New York, site on a nonpolar capillary column.

geographical locations during the period when fruit was red, in the ripe or overripe stage. In green fruit, EAD measurements for compounds III, V, and VI often were low or not evident, and the response to compound IV was higher than in ripe and overripe (infested) fruit. Similarly, in overripe fruit, EAD responses to compounds II, V, and VI increased compared to those from ripe fruit. Samples that were shipped from Michigan often displayed additional EAD peaks, probably due to changes in fruit quality that occurred after collection and shipment. Although these additional peaks were often present, they were not consistent among samples, as were the compounds shown in Figure 2.

A single adsorbent extract of volatiles from whole fruit, for quantitative analysis of key volatiles (GC-MS) and flight tunnel analysis, was made from ripe fruit collected at the Michigan site in September 2000. The relative ratios of the EAD active compounds in this sample, estimated with GC-FID and GC-MS, are listed in Table 1. Flight tunnel tests (see below) confirmed that the proportion of flies responding to this adsorbent collection was equivalent to a sample of the six-component synthetic blend at a comparable dose.

Behavioral Responses of Hawthorn-Origin Flies to Red Sphere with No Volatiles. Over the four experiments, a total of 90 flies were tested individually to a blank treatment, comprised of a red sphere with solvent treated septum. None of these flies initiated upwind flights. The majority presented with a red sphere and no volatiles did not leave the release cage, instead exhibiting bouts of walking and grooming. Flies that did leave the cage flew to the floor or sides of the tunnel. Thus, flies were not attracted by the visual stimulus of the red sphere from a distance of 1 m downwind.

Experiment 1. Behavioral Responses of Hawthorn-Origin Flies to Adsorbent Collection Extract and Synthetic Blend. With the 100-μg dose of extract from volatile collections from whole fruit, 42% of the flies made successful flights to the sphere, and this value was not significantly different from the proportion (44%) of upwind flights to 100 μg of the six-component synthetic blend ($\chi^2 = 0.06$, $P > 0.05$). Flies responding to the volatiles first turned and faced upwind (within 3–5 sec), walked a short distance (0.5–1.5 cm) in the upwind direction, and then initiated upwind flight. Flies that did not reach the sphere typically flew to the floor, side, or top of the tunnel, or remained in the release cage.

Experiment 2. Behavioral Response of Hawthorn-Origin Flies to Doses of the Synthetic Blend. Twenty-two percent of the flies tested with a 50-μg dose of the six-component synthetic blend flew upwind to the sphere (Figure 3). The response level increased significantly to 72% with the 200-μg source ($\chi^2 = 44.4$, $P < 0.05$). Although not tested at the same time, the data from experiment 1 show that the 42% response value for the 100-μg dose falls between those for the lower 50-μg and the higher 200-μg doses.

Experiment 3. Behavioral Response of Hawthorn-Origin Flies to Hawthorn and Apple Volatile Blends. Significantly fewer hawthorn-origin flies flew upwind

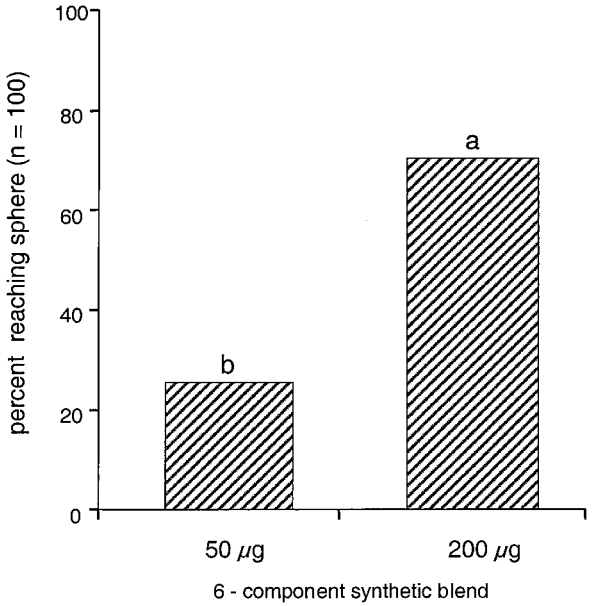


FIG. 3. Percentage of hawthorn-origin *R. pomonella* flying upwind in a sustained-flight tunnel to a red sphere + rubber septum containing 50 or 200 μg of the six-component synthetic mix of hawthorn fruit volatiles (Table 1). The doses are amounts of 3-methylbutan-1-ol applied to the septum, with the remaining compounds in the proportions given in Table 1. $N = 100$ adult flies, mixed-sex, 10–21 day old per treatment. Different letters indicate significant differences in response according to χ^2 analysis ($P < 0.05$).

and made contact with a red sphere containing a 200-μg dose of the apple volatile blend compared with a 200-μg dose of the hawthorn blend (Figure 4, 16% vs. 67%, $\chi^2 = 54$; $P < 0.05$).

Experiment 4. Behavioral Responses of Hawthorn-Origin Flies to Partial Mixtures of Synthetic Blend. There was no significant difference in the proportion of upwind flights to a four-component mixture of 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone (66%; Figure 5, treatment 2), and the response to the full six-component blend (62%, Figure 5, treatment 1; $\chi^2 = 0.06$; $P > 0.05$). Removing 3-methylbutan-1-ol from the four-component mix resulted in complete loss of upwind flight behavior, most likely because 3-methylbutan-1-ol comprised 95% of the amount applied to the rubber septum (Figure 5, treatment 3; see Table 1). Removing 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, or dihydro-β-ionone from the four-component mix (treatments 4–6, Figure 5) resulted in levels of upwind flight that were not different from each other (21, 32, and 24%, respectively; $\chi^2 = 2.36$; $P > 0.05$). However,

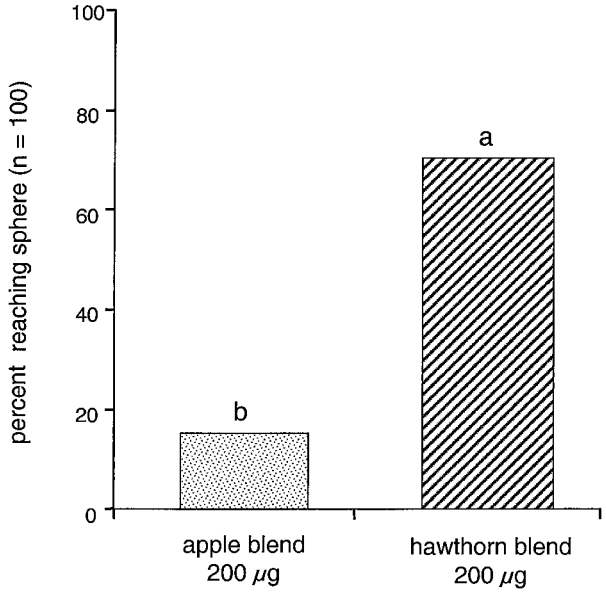
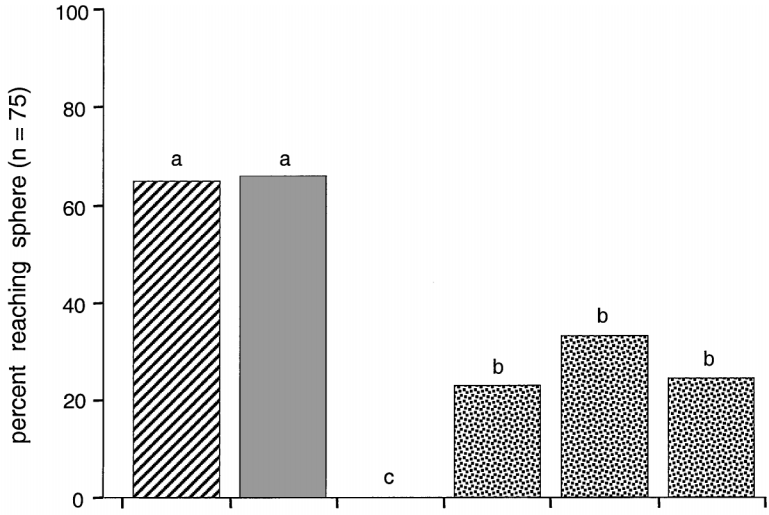


FIG. 4. Percentage of hawthorn-origin *R. pomonella* flying upwind in a sustained-flight tunnel to a red sphere + rubber septum containing the six-component synthetic blend (200 μg 3-methylbutan-1-ol with the remaining compounds in the proportions given in Table 1), compared with response of a 200-μg dose of the apple volatile blend. $N = 75$ adult flies, mixed-sex, 10–21 day old per treatment. Different letters indicate significant differences in response according to χ^2 analysis ($P < 0.05$).

the response to the four-component blend lacking butyl hexanoate (32%; treatment 5, Figure 5) was lower than the response with the six-component mix (64%; treatment 1; $\chi^2 = 15.5$; $P < 0.05$).

DISCUSSION

Identification of Key Host Volatiles from Hawthorn Fruit. We have identified a blend of volatiles from hawthorn fruit, each chemical giving consistent EAD activity from *R. pomonella* flies originating from hawthorn fruit: ethyl acetate, isoamyl acetate, 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone. Adult hawthorn-origin flies responded in a flight tunnel assay at equivalent levels to an adsorbent collection of volatiles from whole fruit and a synthetic blend of the six chemicals that contained the same amount of the 3-methylbutan-1-ol compound. However, based on results of subtraction assays, the four-component mix of 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone also provided full activity. The



treatment	1	2	3	4	5	6
Ethyl acetate	x					
3-Methylbutan-1-ol	x	x		x	x	x
isoAmy acetate	x					
Nonatriene	x	x	x		x	x
Butyl hexanoate	x	x	x	x		x
Ionone	x	x	x	x	x	

FIG. 5. Percentage of hawthorn-origin *R. pomonella* flying upwind in a sustained-flight tunnel to a red sphere + rubber septum containing the six-component synthetic blend (200 μg 3-methylbutan-1-ol with the remaining compounds in the proportions given in Table 1); a four-component blend containing 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*), 7-nonatriene, butyl hexanoate, and dihydro-β-ionone (200 μg 3-methylbutan-1-ol); or sources of the latter four-component blend with one of the components removed. $N = 75$ adult flies, mixed sex, 10–21 days old per treatment. Different letters indicate significant differences in response according to χ^2 analysis ($P < 0.05$).

results also show that hawthorn-origin flies from Grant, Michigan, responded in greater proportions to the hawthorn volatile blend than to a previously identified blend of ester compounds from domestic apple, a blend known to attract *R. pomonella* in apple orchards (Zhang et al., 1999).

The use of SPME with GC-EAD allowed very small quantities of headspace to be analyzed. Our hypothesis was that if the flies are sensitive to a specific set of volatiles, the SPME analysis at very low concentrations of chemicals should reveal those few compounds among the many volatiles being released from the fruit. The procedures we used obtained clear EAD responses from small quantities of headspace volatiles. In particular, the use of *Drosophila* ringer solution and

an acrylic holder and amplifier custom-designed for the *Rhagoletis* head/antenna complex allowed stable EAD recordings from an antennal preparation to be made for a few hours (see Bjostad, 1998), and the analysis of key volatiles from minimal headspace volumes. Furthermore, whereas there are qualitative and quantitative differences in headspace volatiles between hawthorn fruit picked fresh versus late in the season (green fruit just beginning to express a red blush versus red, maggot-infested fruit), we believe the results reflect the set of key volatiles that hawthorn-origin flies use in host selection. In almost all samples, the same group of key volatiles elicited EAD responses, and there was no difference between the behavioral response of flies to an adsorbent extract of volatiles collected from ripe fruit and the synthetic mixture.

Our flight tunnel assay was modified (after Cossé and Baker, 1996) from that in Zhang et al. (1999), resulting in individual flies making upwind oriented flights to the odor source. This is the first flight tunnel demonstration that *R. pomonella* flies can exhibit odor-modulated anemotaxis and supports the hypothesis that flies can recognize specific odor mixtures from a distance and make directed flights to a particular tree. Other studies demonstrating that adult flies can execute oriented flights to an odor source include tsetse flies, *Glossina* spp. (Gibson and Brady, 1985; Brady et al., 1989; Colvin et al., 1989), the Queensland fruit fly, *Bactrocera tryoni* (Meats and Hartland, 1999); a biting midge, *Culicoides impunctatus* (Bhasin et al., 2000); and the house fly, *Musca domestica* (Cossé and Baker, 1996).

Host Fruit Volatiles and Host Fidelity in Rhagoletis flies. Because *Rhagoletis* adults court and mate exclusively on or near the fruit of their host plants (Prokopy et al., 1971, 1972), differences in host preference behaviors translate directly into mate choice decisions and premating isolation (Prokopy et al., 1988; Feder et al., 1994; Berlocher and Feder, 2002). Both *R. pomonella* host races coexist in sympatry at the Grant, Michigan, site and have been the focus of study for 20 years (Feder, 1998). Here, we show that hawthorn-origin flies from the Grant site responded to, and displayed a preference for, a blend of volatiles from hawthorn fruit that is different from that previously characterized for domestic apple, supporting the hypothesis that discrimination of host fruit odors is an important trait in premating isolation. The fact that 16% of the hawthorn-origin flies made upwind flights to the apple blend is in agreement with allozyme analysis indicating that there is gene flow between the two sympatric host races at the Grant site (Feder, 1998). Additional behavioral tests, which include apple-origin flies from Grant, Michigan, as well as field trapping tests for both the apple and hawthorn populations, will be reported elswhere.

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SEQUESTRATION AND METABOLISM OF PROTOXIC PYRROLIZIDINE ALKALOIDS BY LARVAE OF THE LEAF BEETLE *Platyphora boucardi* AND THEIR TRANSFER VIA PUPAE INTO DEFENSIVE SECRETIONS OF ADULTS

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Abstract—Several neotropical leaf-beetles of the genus *Platyphora* ingest and specifically metabolize plant acquired pyrrolizidine alkaloids (PAs) of the lycopsamine type (e.g., rinderine or intermedine) and enrich the processed alkaloids in their exocrine defensive secretions. In contrast to the related palaearctic leaf beetles of the genus *Oreina*, which absorb and store only the non-toxic alkaloid *N*-oxides, *Platyphora* sequesters PAs exclusively as protoxic tertiary amines. In this study, the ability of *P. boucardi* larvae to accumulate PAs was investigated. Tracer studies with [^{14}C]rinderine and its *N*-oxide revealed that *P. boucardi* larvae, like adult beetles, utilize the two alkaloidal forms with the same efficiency, but accumulate the alkaloid as a tertiary amine exclusively. Ingested rinderine is rapidly epimerized to intermedine, which is localized in the hemolymph and all other tissues; it is also detected on the larval surface. Like adults, larvae are able to synthesize their own alkaloid esters (beetle PAs) from orally administered [^{14}C]retronecine and endogenous aliphatic 2-hydroxy acids. These retronecine esters show the same tissue distribution as intermedine. A long-term feeding experiment lasting for almost four months revealed that retronecine esters synthesized from [^{14}C]retronecine in the larvae are transferred from larvae via pupae

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[3]Ludger Witte died 27 September 2001 in a tragic accident. We are sadly missing a good friend and excellent scientist.

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into the exocrine glands of adult beetles. Pupae contain ca. 45% of the labeled retronecine originally ingested, metabolized, and stored by larvae; ca. 12% of larval radioactivity could be recovered from the defensive secretions of adults sampled successively over two and a half months. Almost all of this radioactivity is found in the insect-made retronecine esters that are highly enriched in the defensive secretions, i.e., more than 200-fold higher concentration compared to pupae.

Key Words—*Platyphora boucardi*, Chrysomelinae, larvae, pyrrolizidine alkaloids, alkaloid metabolism, chemical defense, alkaloid sequestration.

INTRODUCTION

The Chrysomelidae (leaf beetles), which represent one of the largest families of the Coleoptera, are known for their spectacular defense mechanisms, including mechanical, behavioral, and chemical responses (Deroe and Pasteels, 1982). Chemical defense plays an important role in the protection of both larvae and adults in many leaf beetle species (Pasteels et al., 1982). Prominent examples are members of the subfamily Chrysomelinae. In aposematic adults, the defensive substances are released from exocrine glands located in the elytra and pronotum (Pasteels et al., 1988a, 1994). Although most chrysomelines are specialist herbivores, which often feed on plants containing deterrent and toxic secondary compounds, chemical defense in adults appears to be generally autogenous, although host-derived defense has also been reported. Sequestration of plant secondary compounds for beetle defense has been demonstrated for a few species from two taxonomically related chrysomeline genera, the palearctic genus *Oreina* (Pasteels et al., 1988b) and more recently the neotropical genus *Platyphora* (Pasteels et al., 2001). In both cases, the beetles sequester pyrrolizidine alkaloids (PAs) from their host plants. *Oreina* and *Platyphora* show many parallels in the evolution of their chemical defenses. *Oreina* species release cardenolides from their exocrine glands, autogenously produced from cholesterol, which must be derived from ubiquitous phytosterols (Pasteels et al., 1988b), while *Platyphora* species release pentacyclic saponins, probably synthesized from plant-derived amyrins, found widely in leaf cuticular waxes (Plasman et al., 2000a,b).

In the two genera, it is assumed that PA acquisition evolved in species that already possessed the ability to synthesize and store cardenolides and triterpene saponins, respectively (Hsiao and Pasteels, 1999; Termonia et al., 2002). In *Oreina*, sequestration evolved in species that feed on *Senecio* and *Adenostyles* (Asteraceae tribe Senecioneae) (Pasteels et al., 1988b, 1996; Hartmann et al., 1997), while in *Platyphora*, sequestration evolved in species adapted to *Tournefortia* (Boraginaceae), *Prestonia* (Apocynaceae), or *Koanophyllon* (Asteraceae tribe Eupatorieae) (Pasteels et al., 2001). The last named three families represent the only taxa known to contain PAs of the lycopsamine type. In contrast, species of the Asteraceae tribe Senecioneae contain macrocyclic PAs of the senecionine type

(Hartmann and Witte, 1995). With one exception, all PA sequestering leaf-beetle species continue to produce their ancestral defenses. The only exception is *Oreina cacaliae*, which abandoned the ability to synthesize cardenolides (Pasteels et al., 1992, 1995).

PAs may exist in two molecular forms: as protoxic tertiary amines or as nontoxic *N*-oxides. Most alkaloid sequestering insects avoid accumulating PAs as tertiary amines (for review see Hartmann, 1999; Hartmann and Ober, 2000). Despite their close taxonomic relationship, *Oreina* and *Platyphora* leaf beetles have developed completely different strategies to maintain and store plant acquired PAs. *Oreina* beetles absorb PAs from ingested plant food exclusively as *N*-oxides. The *N*-oxides are accumulated in the hemolymph and body tissue. The body-stored PA *N*-oxides constitute a reservoir that is continuously utilized to refill the defensive glands (Pasteels et al., 1992) and maintain high alkaloid concentrations (i.e., up to 0.3 mol/liter) in the defensive secretions (Ehmke et al., 1991; Rowell-Rahier et al., 1991; Hartmann et al., 1997). Any PA that is passively absorbed as the tertiary amine (e.g., senecionine or seneciphylline), will be efficiently detoxified in the beetles by glucosylation (Hartmann et al., 1999). In contrast, *Platyphora* beetles absorb PAs exclusively as tertiary amines. However, they avoid PA accumulation in the hemolymph by efficient alkaloid transfer into their defensive secretions, where they may be concentrated up to 0.038 M/liter (Hartmann et al., 2001).

In contrast to adults, *Oreina* and *Platyphora* larvae do not possess defensive glands. However, lack of such glands does not exclude chemical defense. Larvae of Oreina species, which as adults accumulate cardenolides in their defensive secretions, are also able to produce cardenolides and store them in the hemolymph and body tissue (Eggenberger and Rowell-Rahier, 1993; Dobler and Rowell-Rahier, 1994). In the same manner, larvae of PA sequestering *Oreina* species accumulate PAs and store them mainly in the hemolymph, but also in the solid tissues (Dobler and Rowell-Rahier, 1994; Ehmke et al., 1999). Like adults, *Oreina* larvae sequester PAs only as *N*-oxides and accumulate them with the same efficiency and specificity as adult beetles store the PA *N*-oxides in their body compartment.

Here, we report on the ability of *Platyphora boucardi* larvae to take up, metabolize, and maintain PAs as tertiary amines. Feeding experiments with ^{14}C-labeled and deuterated alkaloids provide evidence that larvae possess all the biochemical abilities to specifically metabolize PAs that have recently been established for adults (Hartmann et al., 2001). Unlike adults, larvae are able to store these alkaloids in the body as tertiary amines and keep them throughout all stages of metamorphosis.

METHODS AND MATERIALS

Insects and Host Plants. Platyphora boucardi and its host plant *Prestonia portobellensis* (Apocynaceae) were collected in Cerro Campana Panama. The

beetles were reared for several generations in Brussels at 25°C and a 12L:12D regime. *P. portobellensis* was grown in a greenhouse.

Radioactively Labeled and Deuterated Alkaloids. [^{14}C]Rinderine and its *N*-oxide, [^{14}C]retronecine, and double labeled [^{14}C][^{2}H]rinderine were prepared biosynthetically using root cultures of PA synthesizing plant species as described previously (Hartmann, 1994; Hartmann et al., 2001).

Tracer Feeding Experiments. P. boucardi larvae were fed individually in Petri dishes with the respective tracer as indicated. The tracer was painted as a methanolic solution on the upper surface of a fresh leaf disk (10 mm diam) of *Prestonia portobellensis*. The leaf disc was folded to hold the tracer between the folded halves. In this way, smearing of the tracer on the filter paper covering the Petri dish and any surface contamination of the larvae was prevented. Larvae were allowed to feed for 24–48 hr. Individuals that had finished their labeled meal (usually between 24 and 48 hr) were transferred to fresh, untreated host-plant leaves and allowed to feed until termination of the experiment. Then, larvae were either dissected (see below) or preserved directly after weighing.

Dissection of Larvae and Sampling of Surface Radioactivity. Larvae were killed by freezing and dissected within 30 min after removal from the freezer. The body was divided into four tissues: (1) hemolymph, containing as much body fluid as could be recovered with a capillary tube; (2) the dorsal integument (i.e., tergites, pro-, meso-, and metanotum); (3) the ventral integument (i.e., sternites); and (4) the remainder, containing viscera (gut, Malpighian tubules, and fat body) plus head and thorax, but without pro-, meso-, and metanotum. The separated tissues were weighed, preserved in methanol, and kept until analysis. Radioactivity from the larval surface was collected as follows: on the surface of the defrosting larvae, small droplets of condensed water appeared. These droplets were collected by rubbing the larvae with small pieces of filter paper, which were subsequently preserved in methanol. Control experiments with living larvae confirmed the validity of the method. Sampling the surface radioactivity of living larvae was done as follows. The dorsal surface of the larva was thoroughly wiped with a Q-tip moistened with water, followed by wiping with a Q-tip moistened with methanol; the cotton of the Q-tips containing any surface radioactivity was collected separately and preserved in methanol. Then, the larva was killed by freezing and treated for collection of surface radioactivity as described above.

Feeding of [^{14}C][^{2}H]Rinderine. The feeding experiment with larvae, as well as the subsequent calculation of the degree of deuteration in labeled metabolites, was performed in exactly the same manner as described for experiments with *P. boucardi* beetles (Hartmann et al., 2001).

Analytical Procedures. Alkaloid extraction and separation of the crude extracts by thin-layer chromatography (TLC) with radiodetection was performed as described elsewhere (Hartmann et al., 1999, 2001). TLC separation was achieved on silica gel 60 (Merck) using the solvent system ethyl acetate–isopropanol–

NH_4OH (25%) (45:35:20, by volume). Gas chromatography (GC) and GC-mass spectrometry (GC-MS) of deuterated alkaloids was performed as reported previously (Witte et al., 1993; Hartmann et al., 2001).

RESULTS

Uptake and Metabolism of [^{14}C]Rinderine. Larvae of *P. boucardi* were fed with [^{14}C]rinderine and analyzed 3 and 9 days after tracer uptake and transfered to untreated host-plant leaves. For analysis, individuals were dissected as described under Methods and Materials. The total amount of radioactivity in the various tissues and the respective concentrations were calculated on a fresh weight basis (Figure 1). Radioactivity was detected in all tissues and even on the larval surface. The total amount of radioactivity sequestered by the larvae decreased from 12.9% after 3 days to 4% after 9 days. Based on the relative concentrations, there is an almost equal distribution of radioactivity between hemolymph and the analyzed tissues after nine days.

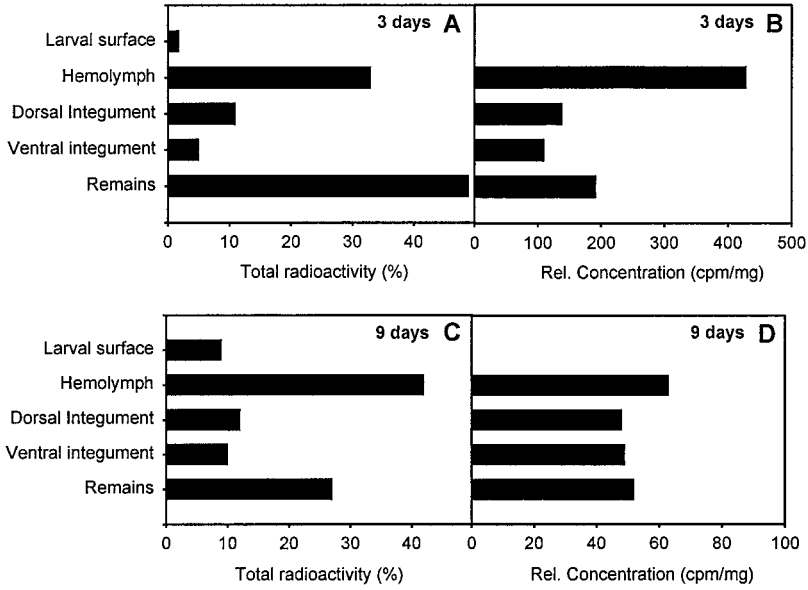

FIG. 1. Uptake and tissue distribution of radioactivity in larvae of *P. boucardi* that previously had been fed on [^{14}C]rinderine (1.5×10^5 cpm/larva). Larvae received their labeled diet for 1 day and were killed and dissected 3 (A, B) and 9 days (C, D) after transfer onto untreated host-plant leaves. Recovery of total radioactivity was 12.9% after 3 days and 4.0% after 9 days. $N = 3$ in each series.

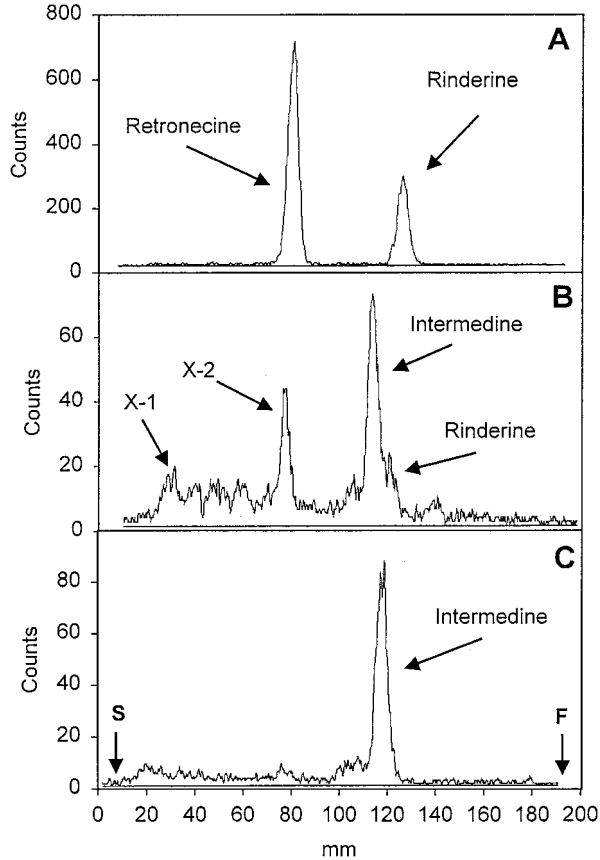

FIG. 2. Metabolism of [^{14}C]rinderine fed to larvae of *P. boucardi*. TLC separation of crude hemolymph extracts. (A) reference compounds; (B) hemolymph collected 3 days after transfer of larvae from the radioactively labeled diet onto untreated host-plant leaves; (C) the same but hemolymph collected after 9 days. See Figure 1 for details.

The extracts were separated by TLC (Figure 2) and the relative abundances of the major metabolites were evaluated (Table 1). Larvae efficiently metabolize the ingested [^{14}C]rinderine. The major proportion of the radioactivity was present as unidentified polar metabolites (Table 1, X-1 and X-2) or intermedine, the C-7 epimer of rinderine. After nine days, the tissues contained almost exlusively intermedine, while the other metabolites were no longer detectable (Figure 2B,C; Table 1). A comparison of the patterns observed after 3 and 9 days suggests that the polar and other metabolites were eliminated by the larvae, while the intermedine was retained. This is in accord with the low recovery of total radioactivity after

TABLE 1. TISSUE DISTRIBUTION OF RADIOACTIVELY LABELED METABOLITES IN LARVAE OF *Platyphora boucardi* 3 AND 9 DAYS AFTER FEEDING ON [^{14}C]RINDERINE[a]

	Relative abundance of radioactivity (%)							
	3 Days					9 Days		
Tissue	X-1	X-2	Int	Rin	Oth	X-2	Int	Oth
Larval surface	tr	47	10	10	33	60	21	19
Hemolymph	tr	31	57	12	nd	tr	>95	nd
Dorsal integument	54	17	29	tr	29	tr	>95	nd
Ventral integument	43	15	10	6	13	tr	>95	nd
Remainder	57	23	20	tr	tr	tr	>95	nd

[a] Int = intermedine; Rin = rinderine; X-1 = unidentified polar metabolite (R_f = 30–40); X-2 = unidentified metabolite (R_f = 70); Oth = others, unidentified metabolites (R_f > intermedine); tr = trace, nd = not detectable. See Figure 1 for further details.

9 days (4%) as compared to 3 days (12.9%). The amount of radioactively labeled intermedine in larval extracts after 3 days accounts for approximately one third of the total radioactivity recovered (Table 1). This fits well with the proportion of residual radioactivity recovered after day nine.

The amounts of radioactively labeled polar metabolites detected by TLC (Figure 2) were too small to allow unequivocal chemical identification. However, the identity of X-2 with retronecine, which migrates with the same R_f, could be excluded by additional HPLC analysis.

The radioactivity detected in the aqueous droplets that formed on the surface of larvae killed by freezing indicates the presence of extracellularly localized alkaloid metabolites. A control experiment in which the dry surface of living larvae was wiped successively with water-and methanol-moistened cotton wool confirmed that radioactivity is genuinely present on the larval surface. The composition of the surface sample differs from tissue extracts, particularly in larvae analyzed after 13 days (Table 1). The aqueous surface fluid contains the polar metabolite X-2 as the major constituent, while hemolymph and tissues contain this metabolite only in trace amounts.

Uptake and Metabolism of [^{14}C]Rinderine N-Oxide. Adults of *P. boucardi* sequester and metabolize [^{14}C]rinderine *N*-oxide with the same efficiency as rinderine itself and display the same metabolite pattern. Alkaloid *N*-oxide was never detected in beetle extracts, indicating an effective *N*-oxide reduction in the gut (Hartmann et al., 2001). In a similarly designed experiment, labeled rinderine *N*-oxide was fed to larvae. The results (data not shown) corresponded exactly to those obtained with rinderine and displayed the same metabolite patterns as documented in Figure 2. Alkaloid *N*-oxide could not be detected in larval extracts. Some 20% of the total radioactivity offered was recovered from the larvae after 7 days.

TABLE 2. GC-MS ANLYSIS OF DEUTERATED ALKALOIDS FROM PREPURIFIED EXTRACTS OF *P. boucardi* LARVAE PREVIOUSLY FED [^{14}C][^{2}H]RINDERINE

Alkaloid in larval extracts[a]	M/z[M$^+$]	RI	Relative abundance (%)	Deuterium incorporated (%)[b]
Rinderine	299	2155	17	106
Intermedine	299	2133	83	101
Lycopsamine	299	2145	nd	

[a] After ingestion of the tracer, larvae were kept on untreated host-plant leaves for 7 days before extraction.
[b] Relative intensity of the isotope signal of the [^{14}C][^{2}H]rinderine fed as tracer (i.e., 26.3%) was set to 100%; nd = not detectable; $N = 5$

Conversion of [^{14}C][^{2}H]Rinderine into [^{14}C][^{2}H]Intermedine. To confirm the epimerization of rinderine, a tracer experiment with double-labeled [^{14}C][^{2}H] rinderine was performed. Five larvae received a total of 325 μg [^{14}C][^{2}H] rinderine for 48 hr. Then, larvae were transferred to untreated host-plant leaves and were killed and extracted after another 7 days. The alkaloid extracts were prepurified and analyzed by GC-MS as described previously (Hartmann et al., 2001). Analysis revealed the presence of both deuterated rinderine and intermedine (Table 2). Intermedine showed the same relative abundance of deuterium as rinderine applied as tracer, indicating its entire origin was from the fed tracer. Besides rinderine and intermedine, neither lycopsamine, the 3' epimer of intermedine, nor any other metabolite could be detected by GC-MS.

Uptake and Metabolism of [^{14}C]Retronecine. Adults of *P. boucardi* are capable of esterifying plant derived retronecine with α-hydroxy analogs of aliphatic amino acids such as alanine and valine (Hartmann et al., 2001). In an experiment designed in the same way as the rinderine feeding experiment, [^{14}C]retronecine was fed to larvae. The distribution of total radioactivity recovered from various larval tissues and its relative concentration in the tissues after 12 hr and 4 days is illustrated in Figure 3. The total amount of radioactivity recovered from larvae was 21.9% and 24.4%, respectively. Moreover, no noticeable decrease in the radioactivity stored in the bodies was observed between the two dates of analysis. Twelve hours after tracer feeding, TLC separation of the tissue extracts revealed the presence of largely unchanged retronecine in most tissues (Table 3; Figure 4A). After 4 days, the typical pattern of retronecine esters (beetle alkaloids) became visible (Figure 4B)[(see Hartmann et al. (2001) and Figure 8) below]. Substantial amounts of retronecine esters were found in all analyzed tissues; in the hemolymph their concentration exceeded 50% of the total radioactivity (Table 3). Besides retronecine esters and unaltered retronecine, small quantities of polar metabolites (X-1) were detectable. As in the rinderine experiment, a substantial portion of

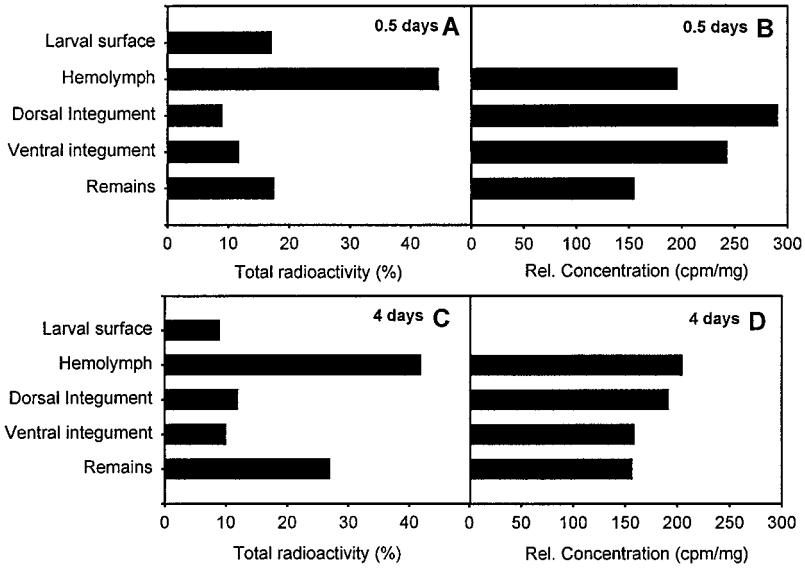

FIG. 3. Uptake and tissue distribution of radioactivity in larvae of *P. boucardi* that previously had been fed on [^{14}C]retronecine (1.2×10^5 cpm/larva). Larvae received their labeled diet for 1 day and were killed and dissected 12 hr (A, B) and 4 days (C, D) after transfer onto untreated host-plant leaves. Recovery of total radioactivity was 21.9% after 12 hr and 24.1% after 4 days (C, D). $N = 3$ in each series.

absorbed radioactivity (ca. 9% after 4 days) was recovered from the larval surface. The composition of this extracellular radioactivity reflects the metabolite pattern found in the tissues, but appears to contain a lower proportion of the retronecine esters.

TABLE 3. TISSUE DISTRIBUTION OF RADIOACTIVELY LABELED METABOLITES IN LARVAE OF *Platyphora boucardi* 12 HOURS AND 4 DAYS AFTER FEEDING ON [^{14}C]RETRONECINE

	12 Hours			4 Days		
Tissue	X-1	Retronecine	Retronecine esters	X-1	Retronecine	Retronecine esters
Larval surface	nd	100	Nd	27	48	24
Hemolymph	nd	>95	Tr	15	30	55
Dorsal integument	<7	>90	Nd	10	46	44
Ventral integument	tr	>95	Tr	20	46	34
Remainder	20–30[a]	<35	10–20[a]	21	47	32

[a] Poor separation, assignment not possible; X-1 = unidentified polar metabolites (R_f < retronecine); tr = trace; nd = not detectable. See Figure 3 for further details.

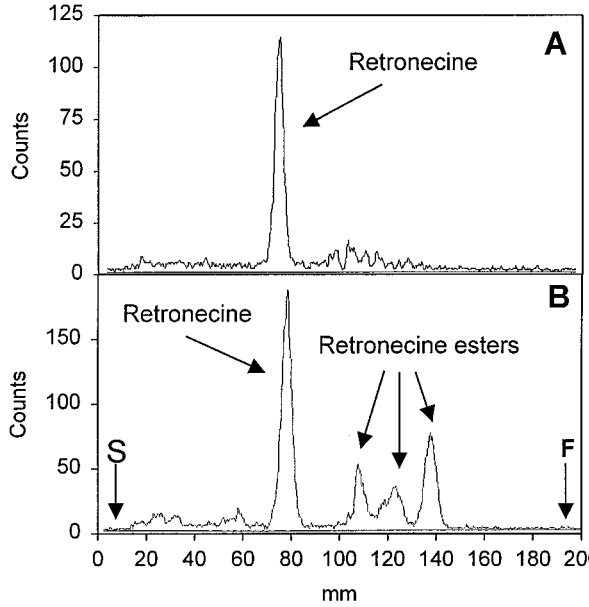

FIG. 4. Metabolism of [^{14}C]retronecine fed to larvae of *P. boucardi*. TLC separation of crude hemolymph extracts. (A) hemolymph was collected 12 hr after transfer of larvae from the radioactively labeled diet onto untreated host-plant leaves; (B) the same, but hemolymph was collected after 4 days. See Figure 3 for details.

Transfer of Retronecine Esters from Larvae to Adults. The preceding results demonstrate that larvae of *P. boucardi* are not only able to store, but also to metabolize, plant-acquired PAs in the same manner as adult beetles (Hartmann et al., 2001). To verify whether alkaloids stored by larvae are retained during the further stages of metamorphosis, larvae were fed with radioactively labeled retronecine and individuals were analyzed during all stages of metamorphosis over a period of almost four months. The results are illustrated in Figures 5 and 6. Four days after tracer feeding an average of 18.2% of the radioactivity offered with the diet was recovered from two larvae. This number was chosen as a reference value (100%) for all subsequent data (Figure 5). Pupae still carried 43% of the larval radioactivity and the defensive secretions of the adults collected five times successively over a period of two and a half months altogether still contained 12% of the original larval load. TLC analysis of the extracts from larvae and pupae (Figure 6) gave results similar to those already described for the retronecine feeding experiment (Figure 4, Table 2). Besides unaltered retronecine, polar metabolites (X-1), and newly formed retronecine esters were detected. Great individual variation was found to exist between the relative proportions of these

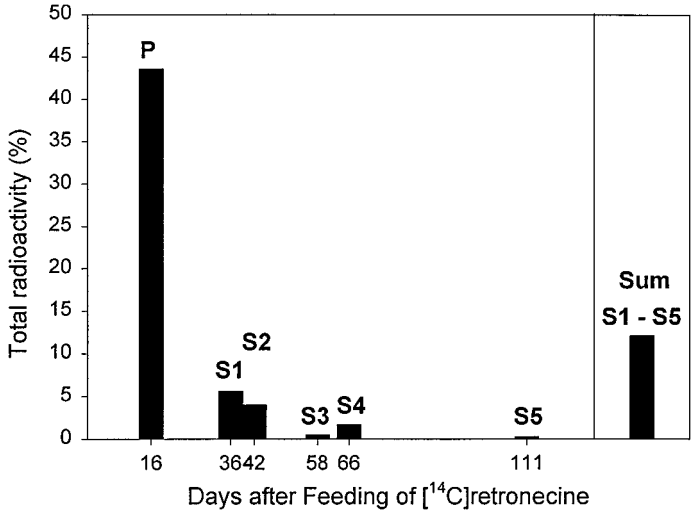

FIG. 5. Transfer of [^{14}C]retronecine and its metabolites from larvae via pupae into the exocrine defensive secretions of *P. boucardi* adults. Ten larvae were fed with 5×10^5 cpm [^{14}C]retronecine and transferred onto untreated host plant leaves after termination of the feeding-pulse. Two larvae were killed and analyzed for total radioactivity 2 days later, four pupae (P) 16 days later; and four beetles hatched from pupae and were kept on their host plant; secretions (S1 to S5) were collected at time intervals as indicated. Total radioactivity refers to radioactivity recovered from larvae (100%).

three compounds. Particularly the percentage of retronecine esters varied among individuals between, 11 and 62% (data not shown). In adult beetles, localization of radioactivity is restricted to defensive glands. TLC analysis revealed that retronecine esters accounted for 80–90% of the total radioactivity found in the secretions (Figure 7). The composition of retronecine esters did not vary significantly in the secretions sampled successively over two and a half months. Retronecine esters in the secretions were always accompanied by a polar metabolite (abundance 10 to 15%), that was also present in the secretions of adult beetles that had received labeled retronecine (Hartmann et al., 2001). Compared to retronecine esters identified by GC-MS from the secretions of adults that previously had received deuterated retronecine (Hartmann et al., 2001), compounds 2, 3, and 4 (Figure 7) display identical TLC mobility as O^7-and O^9-(2'-hydroxyisovaleryl)-retronecine, and O^7-lactyl-O^9-(2'hydroxyisovaleryl)-retronecine respectively (Figure 8).

The result of a more detailed quantitative analysis of successively collected secretions is summarized in Table 4. The data show that the labeled retronecine esters are retained in the glands over a long period. Even after two and a half months of the beetle's life and five successive samplings, 20% of the total amount and almost 15% of the original concentration is detectable in the secretion.

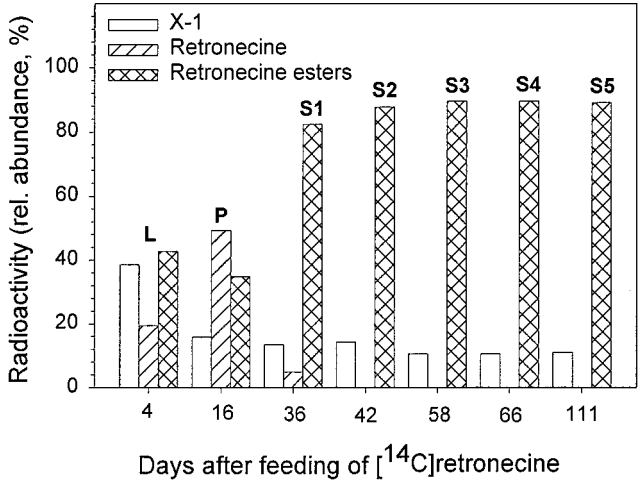

FIG. 6. Transfer of [^{14}C]retronecine and its metabolites from larvae via pupae into the exocrine defensive secretions of *P. boucardi* adults. Metabolite composition of extracts from larvae (L), pupae (P) and five successively collected secretions (S1 to S5) of adult beetles. X-1, polar metabolite. See Figure 5 for further details.

Although the total amount of alkaloids recovered from secretions of adult beetles is <15% (Figure 5), they are highly concentrated compared to tissue concentrations in larvae or pupae (Table 5). Assuming an equal tissue distribution of the alkaloids in larvae, as demonstrated by the results shown in Figure 3D, the alkaloid concentration in the first secretions (Figure 5, S1) is 65-fold higher than in larvae (accumulation factor 0.015). Compared to pupae, the concentration is 200-fold higher (accumulation factor 0.005).

DISCUSSION

Adults of *P. boucardi* (Hartmann et al., 2001) and, as described here, larvae, sequester and metabolize PAs in the same manner: (1) both accumulate, with the same efficiency, PAs only as tertiary amines, when feeding either on labeled [^{14}C]rinderine *N*-oxide or [^{14}C]rinderine; (2) both epimerize rinderine to intermedine (Figure 8A); and (3) both are capable of esterifying labeled retronecine to insect-specific retronecine esters (Figure 8B). Thus, larvae and adults of *P. boucardi* express the same specific biochemical mechanisms to process plant-acquired PAs. This is surprising, since only adult beetles have exocrine defense glands that function as an efficient sink to store ingested host-plant alkaloids, leaving the hemolymph and remaining body almost devoid of alkaloids (Hartmann

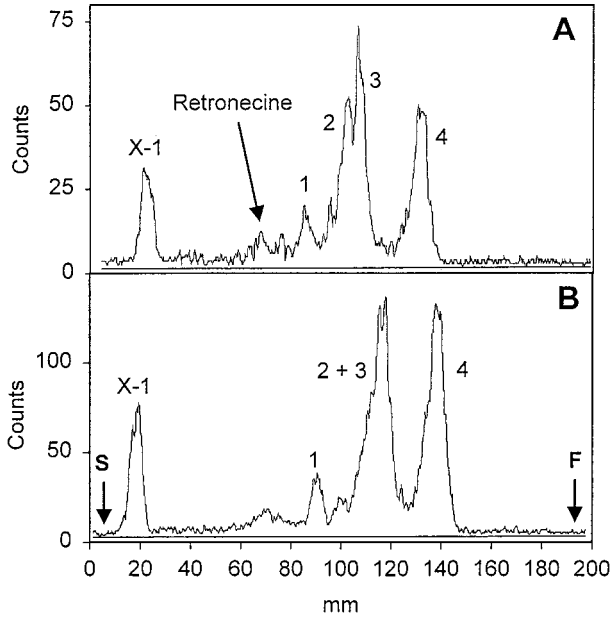

FIG. 7. Transfer of [^{14}C]retronecine and its metabolites from larvae via pupae into the exocrine defensive secretions of *P. boucardi* adults. TLC separation of crude secretion: (A) sampled after 36 days (S 1); (B) sampled after 66 days (S4). Peaks marked 1–4 represent retronecine esters (beetle alkaloids). Peaks 2 and 3 correspond to O^7-and O^9-(2'-hydroxyisovaleryl)-retronecine and peak 4 to O^7-lactyl-O^9-(2'hydroxyisovaleryl)-retronecine according to their R_f values. See Figure 8 for chemical structures.

et al., 2001; Pasteels et al., 2001). In contrast, larvae, which are devoid of specific storage compartments for PAs, apparently store ingested PAs as tertiary amines and their metabolites nonspecifically in the hemolymph and body. As tertiary amines, PAs are potentially toxic to vertebrates and insects (Mattocks, 1986; Frei et al., 1992; Cheeke, 1994). Almost all insects so far studied, prevent the accumulation of detrimental concentrations of PAs as tertiary amines in their hemolymph and body. They do this either by enzymatic *N*-oxidation, as in larvae of arctiid moths (Lindigkeit et al., 1997), ithomiine butterflies (Brückmann et al., 2000), and the grasshopper *Zonocerus variegatus* (Lindigkeit et al., 1997), or by direct uptake of the alkaloid *N*-oxides from their host plants as in *Oreina* leaf beetles (Ehmke et al., 1991). *Oreina* appears to suppress spontaneous or microbial reduction of the PA *N*-oxides in the gut. In addition, host-plant PAs absorbed as tertiary amines from the gut are detoxified by glycosylation (Hartmann et al., 1999). Besides *Platyphora*, the only known example for sequestration of PAs as tertiary amines is the tritrophic interaction involving *Senecio*, aphids, and

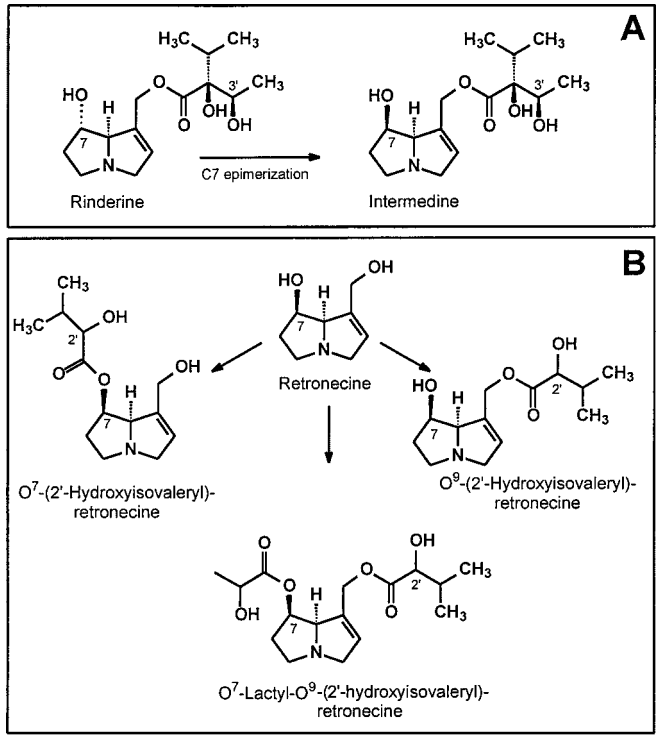

FIG. 8. Transformations catalyzed by larvae of *P. boucardi*. (A) O^7 epimerization of renderine to intermedine; (B) esterification of retronecine with 2-hydroxy analogs of alanine and valine derived from insect metabolism.

ladybirds (*Coccinella septempunctata*). Ladybirds, which infest colonies of the specialist aphid *Aphis jacobaeae*, accumulate PAs, which the aphids had previously sequestered from the phloem-sap of various *Senecio species*. Although the PAs are present as *N*-oxides in the phloem sap of *Senecio* (Hartmann et al., 1989), both aphids and ladybirds store the ingested PAs mainly as tertiary amines (Witte et al., 1990).

The identical metabolism of PAs in *P. boucardi* larvae and adults indicates the same specific biochemical adaptation to PAs in the two metamorphotic stages. From the experiment with deuterated rinderine (Table 5), it can be calculated that larvae accumulated approximately 30 μg (0.1 μmol) per gram body weight. In comparison, *Oreina cacaliae* larvae accumulate alkaloid *N*-oxides from their host plants in 50- to 200-fold higher concentrations (Dobler and Rowell-Rahier, 1994; Ehmke et al., 1999). In wild-collected larvae of another PA-sequestering *Platyphora* species, i.e., *P. bella*, feeding on *Tournefortia* (Boraginaceae), no PAs could

TABLE 4. RADIOACTIVITY IN SUCCESSIVELY COLLECTED DEFENSIVE SECRETIONS OF *Platyphora bourcardi* ADULTS THAT RECEIVED [^{14}C]RETRONECINE AS LARVAE

Days following the tracer pulse ($N = 4$)	Total radioactivity per individual		Concentration of radioactivity	
	cpm ($\times 10^3$)	%	cpm ($\times 10^3$)/μl	%
36	5.1	100	24.3	100
42	3.6	71	16.5	68
58	1.8	35	6.9	28
66	1.4	28	3.1	13
111	1.0	20	3.5	14

be detected (Pasteels et al., 2001), possibly due to their very low concentration in the larval body. Further studies are needed to evaluate the capacity of *Platyphora* larvae to ingest and store PAs as tertiary amines.

In this context, the detection of PAs and PA metabolites on the larval surface is remarkable. There is evidence that absorbed and metabolized PAs are translocated to the cuticular surface. Artifacts by exogenous contamination as well as by the sampling technique can be excluded. However, the results of several feeding experiments with labeled PAs showed great variation in the proportion of ingested radioactivity recovered from the larval surface, ranging from zero to more than 30%. It is not yet known whether the alkaloids are localized on the surface or in the cuticle near the surface, but the compounds are water soluble and easy to remove. The surface of *P. boucardi* larvae is entirely dry, and there is no evidence for surface secretions as in other species, e.g., *P. opima* or *P. decorata*, the larvae of which are covered by a viscous fluid. Preliminary TLC analyses of these larval surface-secretions suggest the presence of pentacyclic saponins similar to

TABLE 5. TRANSFER OF [^{14}C]RETRONECINE ESTERS FROM LARVAE VIA PUPAE INTO ADULT BEETLES OF *P. boucardi* AND ENRICHMENT IN EXOCRINE DEFENSIVE SECRETIONS

Parameter[a]	Larva ($N = 2$)	Pupa ($N = 4$)	Secretions (S1) ($N = 4$)
Body weight or volume of secretions (mg or μl)	127	143	0.213
Sequestered radioactivity (total cpm/individual)	9.1×10^4	4.0×10^4	5.1×10^3
Sequestered radioactivity (total cpm/g or/ml)	7.2×10^5	2.8×10^5	2.4×10^7
Relative abundance of retronecine esters (%)	43	35	82
Retronecine esters (total cpm/g or/ml)	3.1×10^5	9.8×10^4	2.0×10^7
Accumulation factor	0.015	0.005	1

[a] Data are taken from the experiments illustrated in Figures 5 and 6. Labeled retronecine esters were synthesized by the insects from [^{14}C]retronecine fed to larvae. Data were obtained from larvae, pupae, and defensive secretions analyzed 4, 16, and 36 days following the tracer feeding pulse with [^{14}C]retronecine, corresponding to L, P, and S1, respectively, in Figure 5.

those present in the exocrine defensive secretions of adults (Pasteels, unpublished results). The phenomenon needs to be studied in more detail to understand the secretion mechanism and its potential function in larval defense.

Although it is still open whether *Platyphora* larvae under natural conditions are protected by plant acquired PAs and their metabolites, the long-term experiment demonstrates that there is a specific and efficient alkaloid transfer from larvae via pupae into the defensive secretions of adult beetles. The retronecine esters synthesized from ingested retronecine are not only transferred, but greatly enriched in the adult defensive secretions (i.e., the alkaloid concentrations are ca. 200-fold higher than in pupae). *P. boucardi* is the first leaf beetle for which a transfer of PAs from the larval to the adult stage has been directly demonstrated. Enrichment of the alkaloids of larval origin in adult defensive secretions again indicates the presence of a highly efficient "membrane pump" responsible for this process (Hartmann et al., 2001). Detailed studies are needed to clarify the extent to which larvae may participate in the loading of the adult's defensive glands with plant acquired PAs and their metabolites.

One intriguing question remains unanswered. How do *P. boucardi* larvae and pupae tolerate PAs in the state of their protoxic tertiary amines? We do not know whether larvae and pupae are biochemically adapted to avoid bioactivation of the alkaloids, i.e., convert them into alkylating pyrrolic intermediates (Mattocks, 1986; Winter and Segall, 1989). In the case of retronecine, which itself should be nontoxic (Frei et al., 1992; Lindigkeit et al., 1997), larvae of *P. boucardi* convert it into the potentially toxic retronecine esters and transfer these insect-made PAs via pupae into the defensive secretions of adults. Again, one can only speculate whether *P. boucardi* larvae are biochemically adapted to these esters. It interesting to note that the same specific metabolic reactions observed in adults and larvae of *P. boucardi*, i.e., the stereochemical inversion at C-7 (Figure 8) and the esterification of retronecine with aliphatic branched-chain 2-hydroxy acid (Figure 8) have been observed in PA-sequestering species of taxonomically unrelated Lepidoptera: (1) during the formation of the male pheromone (7*R*)-hydroxydanaidal, larvae of the PA-sequestering arctiid moth *Creatonotos* convert PAs with the 7*S*-configured necine base (heliotridine) into PAs containing the C-7 epimer (retronecine) (Bell et al., 1984; Wink et al., 1988; Schulz et al., 1993); (2) ithomiine butterflies epimerize ingested stereoisomeric PAs of the lycopsamine type at C-7 (again 7*S* to 7*R*) and C-3' (Trigo et al., 1994); (3) larvae of several arctiids esterify retronecine with aliphatic branched-chain 2-hydroxy acids producing insect PAs such as creatonotine and callimorphine that are closely related to the retronecine esters synthesized by *P. boucardi* (Ehmke et al., 1990; Hartmann et al., 1990; Schulz et al., 1993).

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EFFECT OF CONSPECIFIC AND HETEROSPECIFIC FECES ON FORAGING AND OVIPOSITION OF TWO PREDATORY LADYBIRDS: ROLE OF FECAL CUES IN PREDATOR AVOIDANCE

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Abstract—Growing evidence suggests a flow of chemical information from higher to lower trophic levels that affects foraging and oviposition of 'prey' in response to potential risks from predators. This was investigated in two species of ladybird predators of aphids, *Harmonia axyridis* and *Propylea japonica. H. axyridis* is known to be the stronger intraguild predator and *P. japonica* to be the more frequent intraguild prey in interactions of these two species. These ladybirds share aphid prey on mugworts, hibiscus, and Italian ryegrasses in fields of northern Japan but largely avoid each other on the same plant. Fecal cues of these ladybird predators were found to contribute in their assessment of predation risk from conspecific and heterospecific competitors in common habitats. Gravid females of *H. axyridis* reduced rates of feeding and oviposition when exposed to feces of conspecifics, but not when exposed to feces of *P. japonica.* In contrast, gravid females of *P. japonica* reduced feeding and oviposition when exposed to feces of both *H. axyridis* and its own species. Females of both ladybird species exhibited similar behavior in response to water extracts of feces. For *P. japonica*, the influence of heterospecific feces was greater than that of conspecific feces. Our results demonstrate that feces of ladybirds contain odors that have the potential to deter the feeding and oviposition activities of conspecific as well as heterospecific ladybirds. Such deterrence allows these insects to avoid predation risk. Differences in responses of the two predators are discussed.

Key Words—Foraging behavior, ladybird predators, aphid prey, predator avoidance, feces, chemical cues.

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INTRODUCTION

Ladybird predators foraging in aphid patches exploit the rising phase of aphid populations for oviposition in order to maximize fitness (Kindlmann and Dixon, 1993; Dixon, 1997; Agarwala and Bardhanroy, 1999). Aphid patches attract several species of predators. Because of the temporary nature of aphid colonies in space and time (Dixon, 1998) and depletion of the prey population caused by predation, ladybird predators often face competition for limited food and risk of intraguild predation (Agarwala and Dixon, 1992; Rosenheim et al., 1995; Hironori and Katsuhiro, 1997). Eggs and young larvae are particularly vulnerable to cannibalism (Agarwala, 1991; Lucas et al., 1997) and interspecific predation (Agarwala et al., 1998; Obrycki et al., 1998a,b; Phoofolo and Obrycki, 1998; Yasuda and Ohnuma, 1999). How do foraging ladybirds decide which patches to attack and which to avoid? Species that can assess predation risk and avoid predator-rich patches would increase their fitness for survival and reproduction (Krebs and Davies, 1987; Polis et al., 1989; Grostal and Dicke, 2000; Dicke, 2000). The role of female ladybirds is assumed to be important in assessing risk as they determine the site of oviposition (Gutierrez et al., 1984; Dixon, 2000).

Oviposition-deterring semiochemicals have been reported in a number of aphidophagous predators such as chrysopids (Růžička, 1994, 1996, 1997a, 1998), coccinellids (Růžička, 1997b; Doumbia et al., 1998; Hemptinne et al., 1998, 2001; Hemptinne and Dixon, 2000; Yasuda et al., 2000), and cecidomyiid (Růžička and Havelka, 1998). The effects of these semiochemicals are associated with either larval tracks of conspecifics (Yasuda et al., 2000; Hemptinne et al., 2001), heterospecifics (Růžička, 1997b), or both (Růžička, 2001), or possibly feces (Doumbia et al., 1998). Semiochemicals present in the larval tracks of ladybird beetles contain certain alkanes or their mixtures that deter conspecific females of *Coccinellia septempunctata* L., *Adalia bipunctata* (L.) (Hemptinne et al., 2001), and *Harmonia axyridis* Pallas (Yasuda et al., 2000) from laying eggs in aphid colonies already attacked by larvae. Another study demonstrated that females of different coccinellid species vary in their oviposition-deterrence response to larval tracks of different predator species, thereby suggesting that larval tracks might contain different semiochemicals or different mixtures (Růžička, 2001). These semiochemicals, however, do not affect the time spent by female beetles in searching, as evident by the similar frequency of their fecal spots between clear substrate and those contaminated with larval tracks (Růžička, 2001).

According to Dicke and Grostal (2001), of all chemical information gathered by animals, cues about predation risk are of special significance because predation risk usually has important and immediate consequences on fitness. Feces from herbivorous insects are known to either deter predators (Whitman et al., 1990; Olmstead, 1994; Müller and Hilker, 1999) or to be exploited by predators and parasitoids (Grewal et al., 1993). Feces from herbivorous insects are also known to deter

conspecifics from oviposition (Renwick and Radke, 1980; Prokopy et al., 1984; Hilker, 1985; Hilker and Klein, 1989; Anderson et al., 1993). Insect feces contain chemicals derived from the insect's food, and some of these may be volatiles that may be recognized from a distance (Aegolopoulos et al., 1995). Feces of carnivorous insects are produced in small quantity and may contain protein metabolites that could be effective oviposition deterrents for species that serve as prey of these predators (Nolte et al., 1994; Kats and Dill, 1998; Grostal and Dicke, 2000). In northern Japan (Tsuruoka, Yamagata Prefecture), *Harmonia axyridis* Pallas and *Propylea japonica* (Thunberg) are the primary ladybird predators of aphids on mugworts, *Artemisia vulgaris* var. *indica*, hibiscus, *Hibiscus syriacus*, and Italian ryegrass, *Lolium multiforum*, but the two predators mostly avoid co-occurring in the same patch. Thus, these ladybirds feed on the same prey aphid species yet tend to be spatially separated. This could be adaptive as the co-occurrence of differently sized larvae often leads to interspecific predation (Agarwala and Dixon, 1992; Rosenheim et al., 1995; Lucas et al., 1997), and, in the laboratory study, eggs and larvae of *P. japonica* are preyed upon by *H. axyridis* (Agarwala, unpublished). *H. axyridis* is a large and polyphagous predator with larvae that can complete development on aphids and on many nonaphid insect prey, whereas *P. japonica* is a smaller and more specialized predator of aphids and some coccids (Hodek and Honek, 1996; Dixon, 2000). We hypothesized that the smaller ladybird species might be able to assess predation risk in its environment and avoid interaction with the larger species. We, therefore, investigated the effects of fecal cues of the two species of ladybird predators on conspecific and heterospecific females. Because of their poor dispersal ability, larval ladybirds likely stay and complete their development within a patch, and, therefore, their feces likely accumulate on plants in the patch. Feces may also accumulate from ovipositing females when they stay in a patch for a certain length of time. This accumulating feces might contain substances that can affect aggregation and oviposition of conspecifics and heterospecifics in the competitive environment of patchy resources.

Oviposition-deterring properties of feces in aphidophagous ladybirds have not been studied previously. Therefore, we first ascertained that feces of the two species accumulate on different plant species where they often forage for aphids. We also determined the rate of fecal production by larvae and adults and the length of time that feces remain on plants. We then exposed adult female ladybirds to larval and adult feces and their water extracts to determine effects on prey consumption, oviposition, and behavior.

METHODS AND MATERIALS

Incidence of Ladybirds and Their Fecal Deposits in Fields. To ascertain the incidence of the two species and their feces in fields, aphid-infested leaves of three

hosts plants ($N = 100$ per species)—mugworts, hibiscus, and Italian ryegrass—were sampled in an area of about 1.5 km^2 around the premises of the Yamagata University at Tsuruoka, Japan. Leaves containing aphids, larvae, and/or adults of *H. axyridis* and *P. japonica* and their fecal deposits were collected individually in 9-cm-diam. Petri dishes and brought to the laboratory. Feces from these leaves were collected separately and the wet weight recorded within 3 hr of collection. It was possible to identify feces of the two ladybird species in the field because of the shape and size. *H. axyridis* excreted feces as pellets that were longer and thicker in comparison to the smaller pellets excreted by *P. japonica*. The numbers of aphids of each species and the active stages of ladybird predators present in the samples ($N = 100$ leaves per plant species) were also recorded.

Laboratory Protocol. Fourth instars and adult females of *P. japonica* and *H. axyridis* were used in experiments. Each stock culture was kept in 9-cm-diam. Petri dishes with fresh bean leaves, damp paper tissue, and a piece of corrugated filter paper and fed with pea aphids, *Acyrthosiphon pisum* (Harris). Insects were kept at $22 \pm 1^{\circ}$C and a photoperiod of 16L:8D. Two-day-old fourth instars or 18- to 20-day-old adult females were starved for 24 hr in order to induce similar levels of hunger. Feces of larvae or adults of the two species were distinguished as 1, 5, or 10 days old after their deposition and obtained from the stock culture. Ladybirds often deposited feces as pellets on bean leaves or tissue and filter papers in Petri dishes. One-day-old feces were easily collected with the tip of a fine paintbrush, but 5- and 10 day old feces were dry and often fixed to surfaces. These were removed by applying a little pressure with the tip of a needle or a pair of fine forceps at the base of fecal deposits.

Experiments were done either in Petri dishes (5 or 9 cm diam.) or on potted plants. Dishes were lined with filter paper before use. Individual 16-day-old bean plants, *Vicia faba*, about 15 cm in height, bearing 6–7 leaves and grown in 9-cm-diam. pots, were used. A 9-cm-diam. filter paper was placed on the soil around the base of each bean plant. A grease band around the base of the stem, near the soil, of each plant prevented larvae or adults from leaving. A ventilated plastic cylinder was placed over each plant to isolate it from adjacent ones. Live adult pea aphids were offered to larvae and adult ladybirds, 40 per larva or adult female to *H. axyridis* and 20 per larva or adult female to *P. japonica*, on leaves of the bean plant, both in Petri dishes and on potted plants (mean fresh weight $\pm$ SE of adult pea aphid $= 2.70 \pm 0.08$ mg; $N = 24$). In a separate study, these numbers of aphids were determined to meet the optimal daily requirements for larvae and adults of the two ladybird species (data unpublished). Any ladybirds or aphids that dropped off the potted plants were placed back on during observations at periodic intervals.

Rates of Deposit of Feces by Larvae and Adult Ladybirds on Optimal Diets. Fourth instars and adult females of the two species were kept individually in

9-cm-diam. Petri dishes or released at 1 cm above the grease bands of potted plants ($N = 10$). These were offered pea aphids as described. The fecal pellets that were deposited in 24 hr were separately collected from each dish and potted plant and weighed on a microbalance. The number of aphids eaten by individual larvae and adults during the same period in each treatment was recorded.

Length of Time Feces Remain on Plants. To determine how long feces remained on plants after being deposited by beetles, 30 larvae of each species were individually released at the bases of infested potted plants. After 24 hr, larvae were removed from all plants. Fecal pellets were collected separately from 10 plants each with feces of *H. axyridis* and *P. japonica*. The remaining plants were left undisturbed and examined for fecal remains after 5 and 10 days. On each occasion, fecal pellets were collected from 10 plants each for each species. All feces were weighed on the day of collection within 3 hr.

Effects of Feces in Combination with Prey and Host Plant on Behavior of Female Ladybirds. Ten gravid females of both *P. japonica* and *H. axyridis* were kept individually with 1-, 5-, or 10-day-old feces of larvae or adults of their own or the other species. Each female of both species was kept with a bean leaf infested with an optimal number of pea aphids and evenly contaminated with 1 mg of 1-, 5-, or 10-day-old feces. In fields, nearly the same amount of feces per leaf was found on the three plant species (Table 1 below). The numbers of aphids eaten and eggs laid by the females were recorded after 24 hr.

Influence of Feces on Behavior of Female Ladybirds. To evaluate whether feces of conspecifics or heterospecifics influence encounter rates of female ladybirds and their reactions to feces, two experiments were carried out. Individual *H. axyridis* and *P. japonica* females were kept with 1 mg of 1-day-old feces of larvae or adults of their own or the other species in 5-cm-diam. Petri dishes. Each experiment, one on *H. axyridis* females and the other on *P. japonica* females, consisted of four treatments. In each treatment, 10 females were exposed to one kind of feces only. Thus, eight treatments were set up. Feces were placed on a piece of 1-cm-diam. filter paper and kept in the center of the Petri dish. The numbers of encounters made and reaction to feces (avoidance, i.e., backing off or moving away after approaching feces, no reaction) of their own versus the other species for both larvae and adults, or feeding by a female beetle in 5 min were analyzed with χ^2 test.

Effectiveness of Fecal Cues in Water-Extract on Oviposition by Female Ladybirds. To examine whether chemical cues from the feces of larvae and adults were effective in influencing the oviposition by female beetles, water extracts of feces were used. To control for the effect of water, a control of sterile distilled water was used. One-day-old feces of larvae and adults of the two species (10 mg each) were homogenized in 1 ml of sterile distilled water in a clean dry glass tube. The mixture was allowed to precipitate for 1 hr before the supernatant was collected. In each replicate, 200 μl of this supernatant was transferred by a micropipet to the

center of a 5-cm-diam. Whatman filter paper and placed in the center of a 9-cm-diam. Petri dish. In a trial study, supernatant prepared from this ratio (10 mg/ml) was found to be oviposition deterrent in both ladybird species. Ten *P. japonica* and 10 *H. axyridis* females were kept individually with water extracts of feces of larvae or adults of their own or the other species and provided 20 (*P. japonica*) or 40 (*H. axyridis*) adult pea aphids. Ten females of each species were kept individually with the control extract. Thus, for each ladybird species, 50 females were used in five replicates. The number of eggs laid by individual females was recorded after 24 hr. Bean leaves were not used for providing aphids in order to exclude the possible influence of host plant volatiles.

Statistical Analyses. Results of paired treatments (e.g., for production of feces by larvae or adult females in Petri dishes and on potted plants) were analyzed by using the Student's t test. Results of multiple comparisons, including amount of fecal deposits onto three plant species in fields, length of time feces remained on plants, and effects of larval and adult feces or their water extracts on prey consumption or oviposition of conspecific and heterospecific female ladybirds were analyzed by one-way or two-way analyses of variance (ANOVA). Significant differences between means of different treatments were established with the Scheffé multiple range test. Results of behavioral responses by female ladybirds to conspecific and heterospecific feces and proportions of plants visited by only one or both ladybird species in fields were analyzed by a χ^2 test. The level of significance for all statistical tests was $P = 0.05$.

RESULTS

Incidence of Ladybirds and Fecal Deposits in Fields. In fields, 37–53% of the aphid-infested leaves of the three host plant species had deposits of ladybird feces. The ratio of ladybird predators to aphids per leaf of a host plant species was higher for mugwort, and its leaves were contaminated with greater amounts of feces in comparison to aphid-infested leaves of hibiscus and Italian ryegrasses (Table 1; total feces: $F_{2,297} = 6.73$, $P = 0.001$; feces per leaf contaminated: $F_{2,129} = 6.49$, $P = 0.002$). For the three host plant species, the proportion of plants with only *P. japonica* or only *H. axyridis* was either equal to (hibiscus) or significantly higher than (mugworts and Italian ryegrass) those with both species (Figure 1).

Rates of Deposit of Feces by Larvae and Adult Ladybirds on Optimal Diets. In both species, fourth instars produced more feces per aphid consumed than did adult females (Figure 2; *H. axyridis*: potted plants: $t_{18} = 3.49$, $P < 0.001$, Petri dishes: $t_{18} = 2.88$, $P < 0.01$; *P. japonica*: potted plants: $t_{18} = 3.86$, $P < 0.001$, Petri dishes: $t_{18} = 2.13$, $P < 0.01$). The number of aphids consumed by a fourth instar or an adult female after 24 hr was nearly the same on potted plants and in Petri dishes (Table 2; *H. axyridis*: larvae: $t_{18} = 0.66$, $P > 0.05$; adults: $t_{18} = 0.70$, $P > 0.05$; *P. japonica*: larvae: $t_{18} = 0.91$, $P > 0.05$; adults: $t_{18} = 2.52$, $P > 0.05$). Larvae

TABLE 1. RATIO OF LADYBIRD LARVAE AND ADULTS TO APHIDS AND AMOUNT OF FECES COLLECTED FROM APHID-INFESTED LEAVES OF THREE HOST PLANT SPECIES IN FIELDS (100 LEAVES)

Host plants and aphids[a]	Ratio of ladybirds to aphids	Leaves with ladybird (total)	Feces collected per leaf (mean ± SE, mg)[b]	
			Feces (%)	Contaminated
Mugworts (*Macrosiphoniella yomogifoliae* Shinji)	0.03	53	63.75 ± 0.64(a)	1.18 ± 0.04(a)
Hibiscus (*Aphis gossypii* Glover)	0.021	41	43.60 ± 0.55(b)	1.06 ± 0.04(a)
Italian ryegrasses (*Sitobion avenae* Takahashi)	0.008	37	35.30 ± 0.50(b)	0.95 ± 0.05(b)

[a] Aphid species in parenthesis.
[b] Dissimilar letters in parenthesis following means in a column indicate significant difference at $P < 0.05$; Scheffé's multiple range test.

of the two ladybird species deposited similar amounts of feces in Petri dishes and on potted plants, but adult females deposited more feces in dishes than on plants (Table 2; *H. axyridis*: larvae: $t_{18} = 0.48$, $P > 0.05$; adults: $t_{18} = 4.49$, $P = 0.001$; *P. japonica*: larvae: $t_{18} = 1.49$, $P > 0.05$; adults: $t_{18} = 5.94$, $P = 0.001$). These results suggest that some feces deposited on plants by adult females (but not larvae) drop to the soil or the lower parts of the plants.

Length of Time Feces Remain on Plants. The wet weight of the feces deposited by beetles in a 24-hr period declined in the days thereafter (amount of fecal material remaining for *H. axyridis*: after 1 day = 1.66 ± 0.08 mg, after 5 days = 0.71 ± 0.05 mg, after 10 days = 0.23 ± 0.03; *P. japonica*: after 1 day = 0.72 ± 0.03 mg, after 5 days = 0.33 ± 0.03 mg, after 10 days = 0.13 ± 0.02 mg). The reduction in the wet weight of feces remaining was statistically significant for both species (*H. axyridis*: $F_{2,27} = 247.08$, $P < 0.001$; *P. japonica*: $F_{2,27} = 104.70$, $P < 0.001$) and might be attributed to two factors: (1) the drying of feces [*H. axyridis*: dry weight of feces (mg) = 0.90 × fresh weight of feces (mg), $N = 25$; *P. japonica*: dry weight (mg) = 0.86 × fresh weight (mg), $N = 25$], and (2) dislodgement as aphids and predators walked over the material (during the period ladybird larvae remain on plants) that caused some feces to fall to the soil or the lower parts of the plants.

Effects of Feces in Combination with Prey and Host Plant on Behavior of Female Ladybirds. Females modified their foraging and oviposition in response to fecal cues of larval and adult stages. *Propylea japonica* females consumed significantly fewer aphids and laid fewer eggs in the presence of 1- or 5-day-old

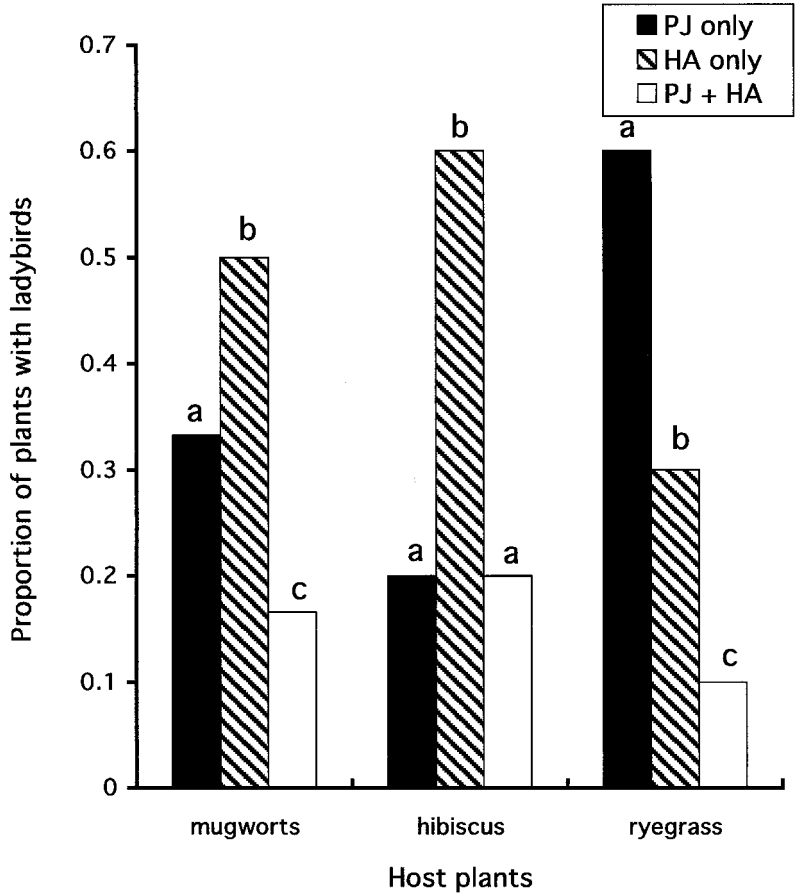

FIG. 1. Proportion of feces-contaminated plants of the three plant species ($N = 20$ plants per species) that harbored larvae and/or adults of only *P. japonica* (PJ), only *H. axyridis* (HA), or both predators. Bars of a host plant with different letters indicate significant differences at $P < 0.05$ (χ^2 test).

H. axyridis feces of both larval and adult stages than in the presence of feces of larvae and adults of their own species (Table 3; Figure 3a,b). However, prey consumption and oviposition by *P. japonica* females in the presence of 10-day-old feces did not differ depending on whether the feces were from conspecifics or from *H. axyridis*.

Harmonia axyridis females consumed signficantly fewer aphids and laid fewer eggs in the presence of 1-day-old feces of their own species than they did in the presence of *P. japonica* feces. This was true for feces of both larvae and adult

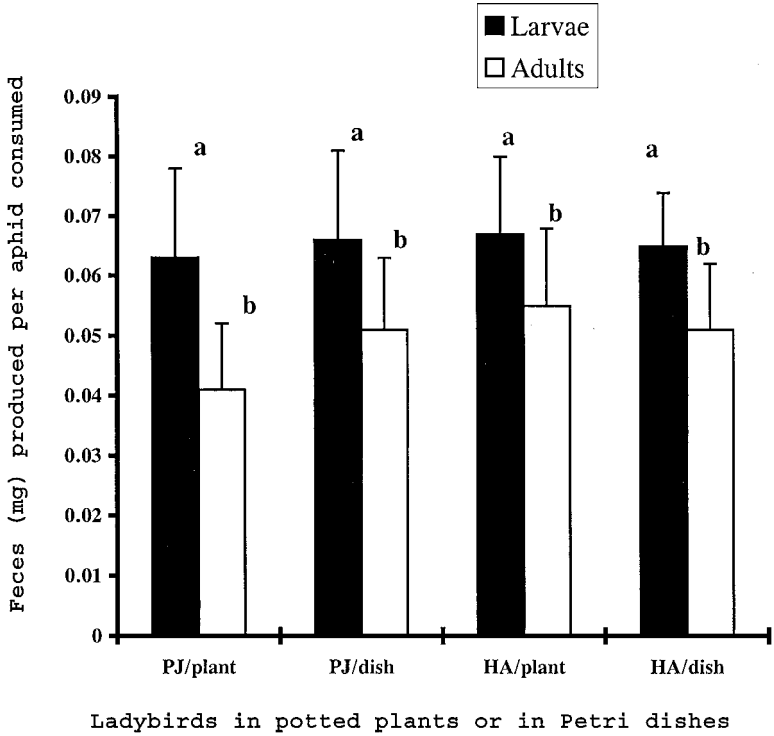

FIG. 2. Wet weight (mean ± SD) of feces produced per aphid consumed by a fourth instar or an adult female of *P. japonica* (PJ), or *H. axyridis* (HA) in 24 hr when kept on an optimal diet of the pea aphid either in 9-cm-diam. Petri dishes or on potted plants ($N = 10$). Bars of a treatment with different letters indicate significant difference at $P < 0.05$ (Student's *t* test).

beetles (Table 4; Figure 4a,b). Prey consumption and oviposition by *H. axyridis* females did not differ in the presence of 5- or 10-day-old feces of their own species versus those of *P. japonia*.

Influence of Feces on Behavior of Adult Ladybirds. Ladybird females did not differ in their encounter rate with feces of their own versus the other species for either larval or adult feces (*P. japonica* female: larval feces: $\chi^2 = 0.82$, $P > 0.05$; adult feces: $\chi^2 = 0.03$, $P > 0.05$; *H. axyridis* female: larval feces: $\chi^2 = 0.22$, $P > 0.05$; adult feces: $\chi^2 = 0.00$, $P > 0.05$). However, the nature of responses to the feces differed. In *P. japonica*, females avoided feces of both larval and adult *H. axyridis* upon contact more frequently than they did feces of conspecifics (larval feces: $\chi^2 = 36.38$, $P < 0.001$; adult feces: $\chi^2 = 41.78$, $P < 0.001$; Figure 5a). In contrast, *H. axyridis* females avoided conspecific feces more frequently than

TABLE 2. PEA APHIDS EATEN AND FECES COLLECTED AFTER 24 HOURS FROM FOURTH INSTARS OR ADULT FEMALES OF *H. axyridis* OR *P. japonica* KEPT IN PETRI DISHES OR ON POTTED PLANTS ($N = 10$)[a]

Treatment	Aphids eaten (N)		Feces collected (mg)	
	Potted plants	Petri dishes	Potted plants	Petri dishes
H. axyridis				
Larvae	27.10 ± 1.06(a)	28.20 ± 1.53(a)	1.68 ± 0.14(a)	1.79 ± 0.16(a)
Adults	32.10 ± 1.59(a)	33.90 ± 1.57(a)	1.29 ± 0.13(a)	1.58 ± 0.13(b)
P. japonica				
Larvae	13.60 ± 0.89(a)	12.50 ± 0.75(a)	0.73 ± 0.02(a)	0.83 ± 0.05(a)
Adults	14.20 ± 2.39(a)	15.80 ± 0.73(a)	0.58 ± 0.05(a)	0.84 ± 0.05(b)

[a] Dissimilar letters in parenthesis following means in a row for each experiment indicate significant difference at $P < 0.05$, Student's t test.

P. japonica faces (larval feces: $\chi^2 = 21.51$, $P < 0.001$; adult feces: $\chi^2 = 21.64$, $P < 0.001$; Figure 5b).

The contrast was also apparent in the number of encounters to which there were no reactions. In *P. japonica*, females failed to react to conspecific feces upon encounter more frequently than they did to *H. axyridis* (Figure 5a; larval feces: $\chi^2 = 28.74$, $P < 0.001$; adult feces: $\chi^2 = 35.62$, $P < 0.001$). In *H. axyridis*, however, females failed to react to feces of *P. japonica* more frequently than they did to feces of conspecifics (Figure 5b; larval feces: $\chi^2 = 23.70$, $P < 0.001$; adult feces: $\chi^2 = 23.96$, $P < 0.001$). Few encounters of females of either species resulted in feeding on conspecific feces.

Effectiveness of Fecal Cues in Water Extracts on Oviposition by Female Ladybirds. Females of *P. japonica* laid significantly fewer eggs in treatments with water extracts of ladybird feces than in controls (Table 5). Among treatments, numbers of eggs laid were higher in the presence of water extracts of conspecific feces than in the presence of water extracts of *H. axyridis* feces ($F_{4,45} = 30.02$, $P < 0.001$). Females of *H. axyridis* laid more eggs both in the control and in treatments with water extracts of *P. japonica* feces than females in treatments with water extract of conspecific feces (Table 5; $F_{5,45} = 16.01$, $P < 0.001$).

DISCUSSION

Previous studies showed the adult ladybird beetles response to oviposition-deterring semiochemicals in larval tracks of conspecifics and heterospecifics (Hemptinne and Dixon, 2000; Yasuda et al., 2000; Růžička, 2001; Hemptinne et al., 2001) and the deleterious effects on the development of larger species by eating semiochemical-coated eggs of smaller ladybird species (Agarwala and Dixon, 1992; Agarwala et al., 1998). The results of this study have advanced the existing

TABLE 3. TWO-WAY ANOVA FOR MEAN NUMBER OF PEA APHIDS CONSUMED AND EGGS LAID BY *P. japonica* FEMALES PROVIDED WITH 1-, 5-, OR 10-DAY-OLD FECES FROM LARVAE OR ADULTS OF THEIR OWN SPECIES OR *H. axyridis*

		Feces of larvae or adults of conspecific and heterospecific ladybirds used					
		1 day old		5 days old		10 days old	
Source	*df*	*F*	*P*	*F*	*P*	*F*	*P*
Aphids consumed, main effects:							
Between ladybird species	1	28.90	<0.001	45.59	<0.001	0.13	0.724
Between ladybird stages	1	5.39	0.027	0.06	0.800	0.28	0.597
Interaction species × stages	1	0.004	0.947	1.62	0.132	0.008	0.930
Error	36						
Eggs laid, main effects:							
Between ladybird species	1	23.41	<0.001	78.78	<0.001	0.06	0.809
Between stages	1	1.64	0.132	1.97	0.123	3.59	0.072
Interaction species × stages	1	0.08	0.778	0.45	0.506	0.002	0.962
Error	36						

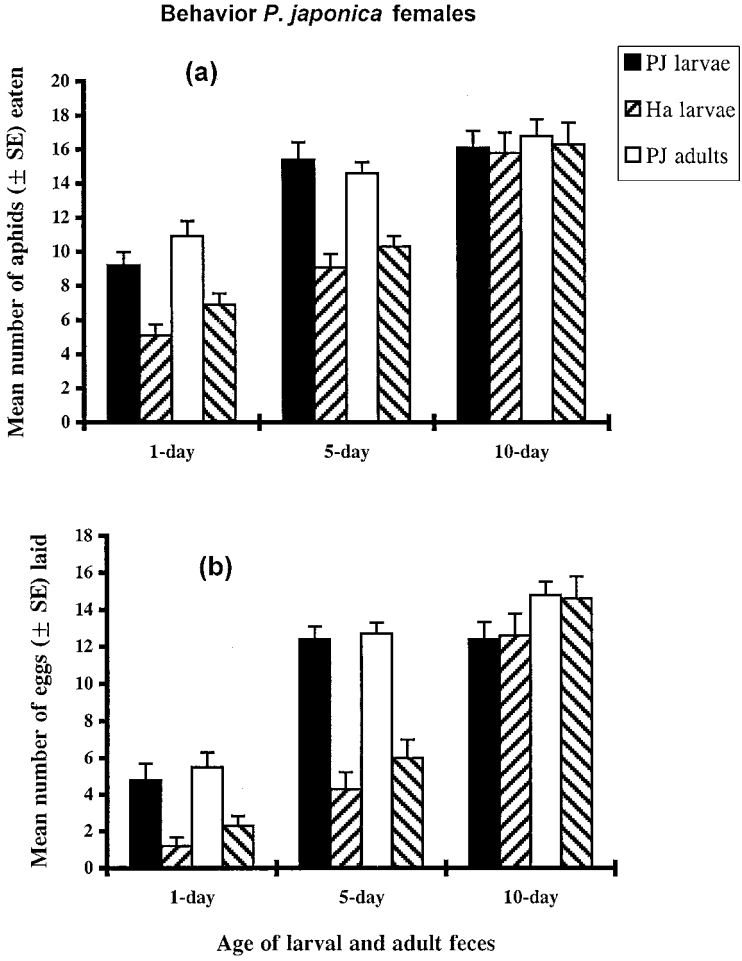

FIG. 3. Numbers (mean ± SE) of aphids consumed (a) and eggs laid (b) by a *P. japonica* (PJ) female when maintained on an optimal diet of pea aphids (20 aphids/female) while exposed to 1 mg of 1-, 5-, or 10-day-old feces of larvae or adults of conspecifics or *H. axyridis* (HA) ($N = 10$).

knowledge by identifying a new source of semiochemicals in ladybird feces and the effects of them on prey consumption and oviposition of female beetles. In this study, larvae and adults of *H. axyridis* and *P. japonica* foraged in the same habitats, but largely occupied mutually exclusive spaces. Feces accumulate on plants as the predators remain in patches of high prey density. Fourth instars produced more feces per prey consumed than did adult females, and larval feces accumulated in

TABLE 4. TWO-WAY ANALYSIS OF VARIANCE FOR MEAN NUMBER OF PEA APHIDS CONSUMED AND EGGS LAID BY *H. axyridis* FEMALES PROVIDED WITH 1-, 5-, OR 10-DAY-OLD FECES OF LARVAE OR ADULTS OF THEIR OWN SPECIES OR *P. japonica*

		Feces of larvae or adults of conspecific and heterospecific ladybird used					
		1 day old		5 days old		10 days old	
Source	*df*	*F*	*P*	*F*	*P*	*F*	*P*
Aphids consumed, main effects:							
Between ladybird species	1	161.05	<0.001	0.08	0.779	0.00	1.00
Between ladybird stages	1	6.61	0.014	0.02	0.898	1.49	0.131
Interaction species × stages	1	0.73	0.397	1.86	0.127	0.98	0.327
Error	36						
Eggs laid, main effects:							
Between ladybird species	1	24.51	<0.001	0.04	0.837	0.17	0.686
Between ladybird stages	1	1.31	0.122	0.05	0.820	0.49	0.489
Interaction species × stages	1	0.138	0.712	0.31	0.582	0.00	1.00
Error	36						

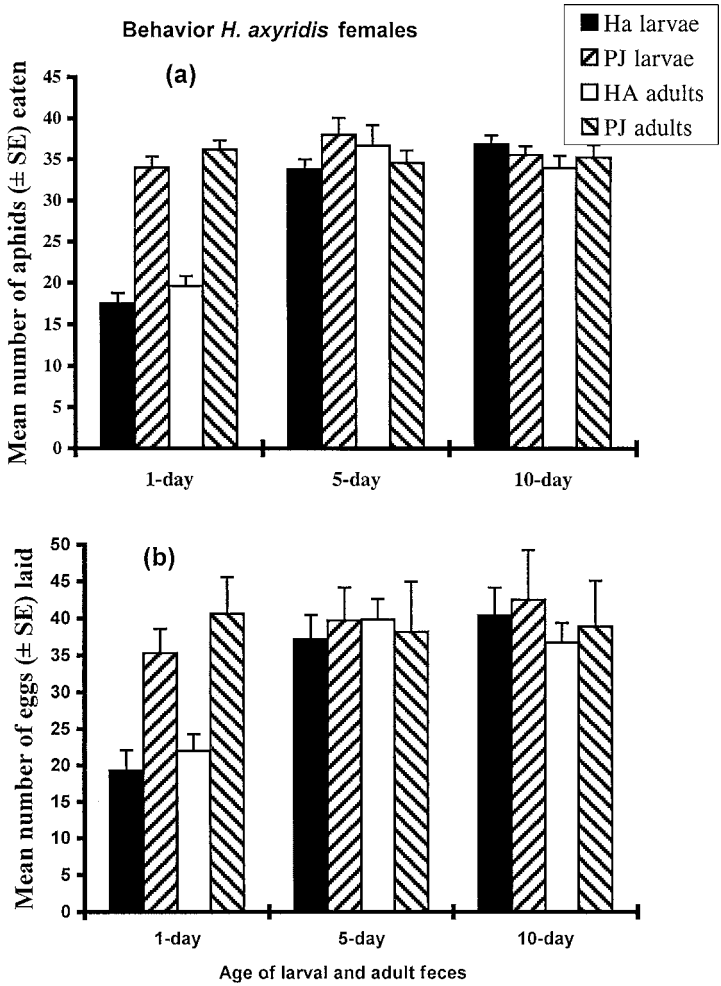

FIG. 4. Numbers (mean ± SE) of aphids consumed (a) and eggs laid (b) by a *H. axyridis* (HA) female when maintained on an optimal diet of pea aphids (40 aphids/female) while exposed to 1 mg of 1-, 5-, or 10-day-old feces of larvae or adults of conspecifics or *P. japonica* (PJ) ($N = 10$).

larger quantity on plants. These differences can be attributed to three factors: first, larvae are flat-bodied, adults are domed-shaped second, larvae stay longer than adults in a patch (Dixon, 2000); and finally, fourth instars have poorer food conversion efficiency than do adult females (Hodek and Honek, 1996). Results showed that fecal deposits on plants shrank in size with time, which may be

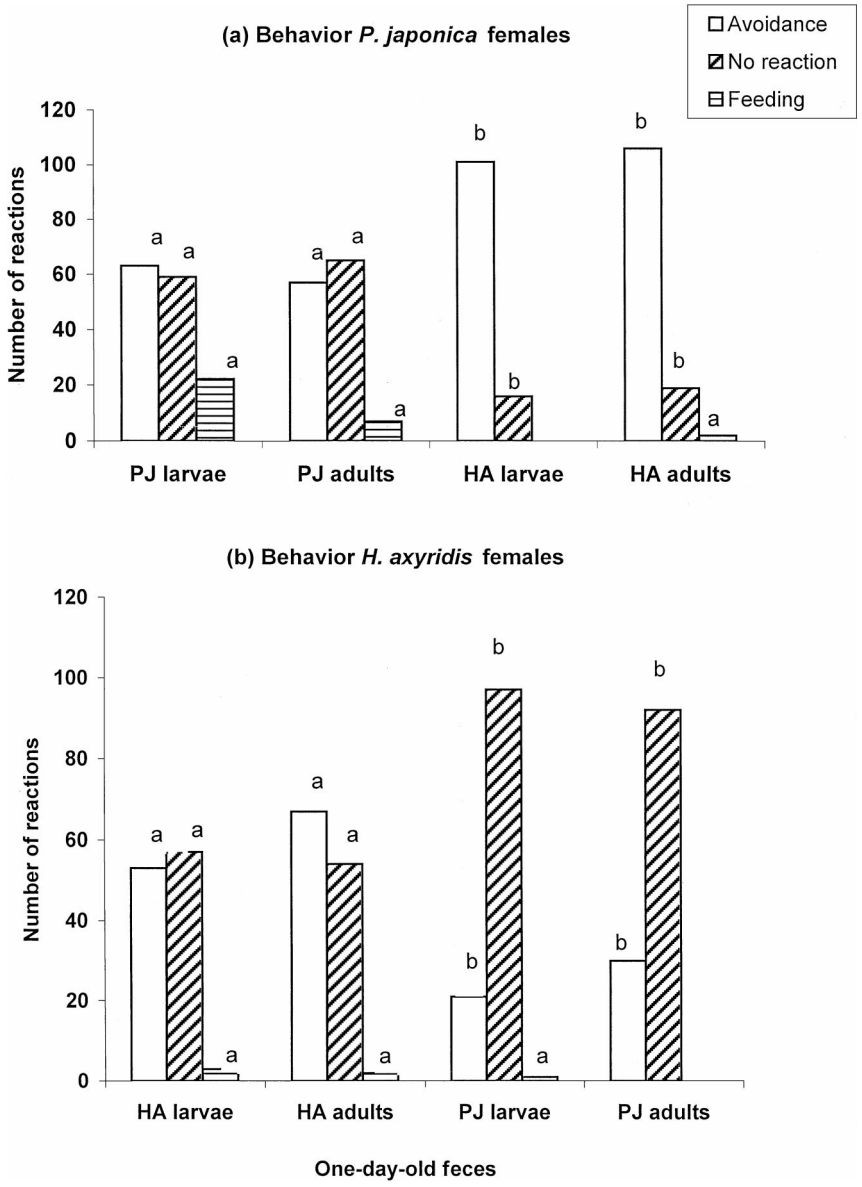

FIG. 5. Number of instances in which avoidance, no reaction, or feeding on fecal pellets occurred when a *P. japonica* (PJ) female (a) or *H. axyridis* (HA) female (b) encountered 1-day-old feces of larvae or adults of conspecifics and heterospecifics during 5 min of observation in 5-cm-diameter Petri dishes ($N = 10$). Similar bars with different letters in each graph indicate significant differences at $P < 0.05$ (χ^2 test).

TABLE 5. EFFECT OF WATER EXTRACTS OF LARVAL AND ADULT FECES OF CONSPECIFICS AND HETEROSPECIFICS ON OVIPOSITION BY *P. janponica* AND *H. axyridis* ($N = 10$)[a]

Treatment	Eggs laid after 24 hr by female (MEAN ± SE)	
	P. japonica	*H. axyridis*
Water extract		
P. japonica feces		
Adults	7.80 ± 0.95(a)	40.50 ± 2.58(a)
Larvae	7.30 ± 0.53(a)	37.50 ± 2.31(a)
H. axyridis feces		
Adults	3.70 ± 0.81(b)	23.30 ± 2.57(b)
Larvae	3.10 ± 0.67(b)	21.80 ± 1.73(b)
Water only (control)	14.80 ± 1.14(c)	38.90 ± 2.00(a)

[a] Dissimilar letters in parenthesis following means in a column indicate significant difference at $P < 0.05$; Scheffé's multiple range test.

attributed to the effects of drying and shaking of plants by foraging animals, with the consequence that some feces fall to the surrounding ground or lower parts of plants. This implies that recently produced and still wet and sticky feces of predators were most likely to be effective sources of signals to other predators. Similarly, wet feces of *Pieris* butterflies were found to contain more volatiles than dry feces and to be more helpful to parasitoids in host-searching (Aegolopoulos et al., 1995).

Our results further demonstrate that fecal cues of the larger species *H. axyridis* caused conspecific as well as *P. japonica* females to reduce their rates of feeding and oviposition. In contrast, fecal cues of the smaller species, *P. japonica*, affected rates of feeding and oviposition of conspecifc females but not *H. axyridis* females. Grostal and Dicke (1999, 2000) and Dicke and Grostal (2001) provided several examples from both invertebrates and vertebrates (including insects and mites) where potential prey avoid predation by reducing feeding and breeding upon perceiving kairomones contained in the feces of intraguild predators. Reduced feeding by ladybirds in the present study may be attributed to reduced foraging caused by avoidance of patches containing feces of predators.

Durations of the effectiveness of fecal cues differed between the two species. In the case of *H. axyridis*, 1-day and 5-day-old feces were avoided by *P. japonica* females but only 1-day-old feces were avoided by conspecific females. In *P. japonica*, only 1-day-old feces were avoided by conspecific females. The differences in avoidance responses may be attributed to the relative risk of predation. *H. axyridis* is a more generalist predator with higher voracity (more than twice that of *P. japonica*; Agarwala, unpublished), and its eggs and larvae are avoided by larvae and adults of *P. japonica* (Agarwala, unpublished). In contrast, *P. japonica*

is a more specialist predator, and its eggs and larvae are not known to be toxic. By avoiding both conspecific and heterospecific fecal cues, *P. japonica* females seem to be seeking nearly enemy-free spaces that would enhance their fitness for foraging and oviposition. Lack of a avoidance response in *H. axyridis* females to feces of *P. japonica* suggests that larger ladybirds avoid conspecific interactions but not interspecific ones in which they are most likely to prevail as intraguild predators. A classical study of interactions involving the ladybird species of this study and *Coccinella septempunctata bruckii* in the presence of extraguild prey (aphids) (Sato, unpublished), as cited by Dixon (2000), suggests that larvae of *H. axyridis* survive better when kept with larvae of *P. japonica* and *C. septempunctata bruckii* than when kept with aphids alone. In marked contrast, larvae of *P. japonica* survive less well when kept with larvae of *H. axyridis* or *C. septempunctata bruckii* than with aphids alone. These results support the hypotheses that *H. axyridis* is a superior predator, that its larvae prevail in interspecific competition, and that its interactions with other species may often result in those species leaving the patch or plant rather than remaining to risk becoming intraguild prey.

H. axyridis is a polyphagous predator (Hodek and Honek, 1996) and is often the last ladybird to arrive and attack aphids in a patch because of its requirement for high prey density (Takahashi, 1989; Agarwala and Yasuda, 2001). Given the temporary nature of short-lived aphid colonies, growing larvae of *H. axyridis* may often face food scarcity. This could lead to intense intraspecific competition. Avoiding oviposition in sites already occupied by conspecifics, as suggested by our findings, could, therefore, be advantageous. Further study is now called for to identify the chemicals in feces that affect foraging and oviposition beahvior of *H. axyridis* and *P. japonica*.

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IDENTIFICATION OF MALE-SPECIFIC CHIRAL COMPOUND FROM THE SUGARCANE WEEVIL *Sphenophorus levis*

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Abstract—Comparative gas chromatographic analyses of airborne volatiles produced by males and females of the sugarcane weevil *Sphenophorus levis*, showed one male-specific compound. Gas chromatography–mass spectrometry data indicated an aliphatic alcohol that was identified as 2-methyl-4-octanol. Both optical isomers were synthesized in five steps by employing commercially available (*R*)- and (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol as starting material. Enantiomeric resolution by gas chromatography with a chiral column demonstrated that the natural alcohol possessed the *S* configuration. Preliminary indoor observations suggested that the alcohol elicited aggregation behavior among adults. The same compound has been previously described as an aggregation pheromone in several other curculionid species.

Key Words—Sugarcane weevil, *Sphenophorus levis*, aggregation pheromone, 2-methyl-4-octanol, 3-methyl-4-octanol, *Metamasius hemipterus*.

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INTRODUCTION

Sphenophorus levis (Coleoptera: Curculionidae) is a weevil found in northern Argentina, Paraguay, and Brazil and develops mainly on sugarcane (Vanin, 1988). This species was described as a pest in 1977 and was classified as a new species in 1978 (Precetti, and Arrigoni, 1990). In Brazil it has been detected in sugarcane only in the state of São Paulo, with an endemic incidence in a southwestern region of the state (Vanin, 1988). The use of synthetic insecticides for control of this insect has been unsuccessful due to its behavior at the larval stage. During this time, similar to other species of this family, *S. levis* tunnels in sugarcane stalks, destroying tissues and weakening the plant. (Cerda et al., 1999). The increasing economic and agricultural importance of this pest in Brazil led us to investigate the feasibility of using its pheromone system for pest management.

Male-produced aggregation pheromones have been described in several curculionidae belonging to the same subfamily (Rhynchophorinae) as *S. levis* (Rochat et al., 1991; Hallett et al., 1993; Oehlschlager et al., 1995; Giblin-Davis et al., 2000). A similar species also found in Brazil, *Metamasius hemipterus*, produces six male-specific compounds (Ramirez-Lucas et al., 1996a; Perez et al., 1997) but only two of them seem to be essential as attractants (Ramirez-Lucas et al., 1996b; Perez et al., 1997). *M. hemipterus* and *S. levis* belong to the same taxonomic class; however, huge morphological and behavioral differences have been found between them.

Here we are compiling the first results of our studies on the chemical ecology of *S. levis*, reporting the identification, synthesis, and absolute configuration of a male-specific chiral compound that we believe to be the aggregation pheromone of this species.

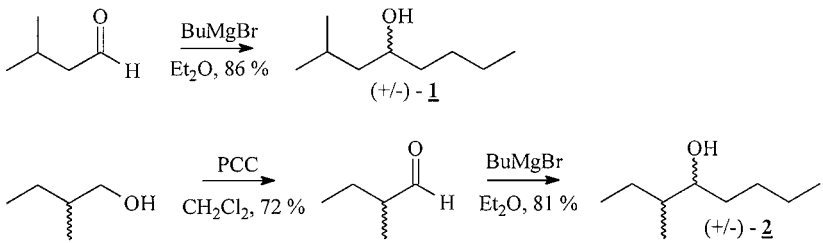
METHODS AND MATERIALS

Collection of Insect Volatiles. Adult weevils of mixed age, sex, and mating status were collected in a sugarcane plantation at the Centro de Tecnologia da Copersucar, Piracicaba, in December 1999 and January 2000. They were provided with sugarcane stalks and transported to Curitiba. Weevils were separated by sex and kept in the laboratory at 25°C under a 14 L:10 D photoperiod. Groups of 60 males and females were placed in different all-glass aeration chambers (Zarbin et al., 1998a), and the volatiles were trapped on Super Q (Alltech, Deerfield, Illinois, USA) columns for 6 days. A humidified charcoal-filtered airstream (1 liter/min) was maintained through the aeration apparatus. The columns were washed with distilled hexane, and the extracts were concentrated to 10 insect equivalents (10 IE/μL) under an argon stream in a clean conical bottom vial. (Zarbin, 2001).

General Analytical Procedures. Gas chromatographic analyses were performed on a Varian 3800 GC equipped with FID, eletronic pressure control, and operated in splitless mode. The following capillary columns were used: VA-5 (30 m $\times$ 0.25 mm $\times$ 0.25 μm), oven temperature 50°C for 3 min, programmed at 5°C/min to 150°C, held for 1 min, then rising 10°C/min to 270°C and held at this temperature for additional 10 min; and VA-Wax (30 m $\times$ 0.25 mm $\times$ 0.25 μm), oven temperature 50°C for 3 min, increased to 220°C at a rate of 7°C/min, and held for 10 min. Mass spectra were recorded on a Varian Saturn 2000 GC-MS-MS ion trap detector using the same type VA-5 capillary column under the same conditions as described above. The equipment was operated in the electron impact (EI) ionization mode (70 eV) and in the chemical ionization (CI) mode (CH_3CN). Chiral separations were obtained on a cyclodextrin-based capillary column [heptakis (2,6-di-*O*-methyl-3-*O*-phenyl)-β-CD, 20% in OV-1701, w/w, 25 m, 0.25 mm ID] using helium as carrier gas (38 psi) at 60°C. The IR spectra refer to films and were measured on a Bomem M-102 spectrometer. The ^{1}HNMR spectra were recorded with TMS as an internal standard at 400 MHz on a Bruker ARX 400 spectrometer. The ^{13}CNMR spectra were recorded with TMS as an internal standard at 100 MHz on a Bruker ARX 400. Optical rotations were measured on a Bellinghan + Stanley Ltd model D polarimeter.

Purification of Male-Specific Compound. Crude male aeration extracts were subjected to purification on silica gel columns eluted with hexane–ether mixtures of increasing polarities (100:0, 90:10, 80:20, 70:30, 50:50, 20:80, 0:100). The male-specific compound was recovered along with a small amount of other impurities in the hexane–ether 70:30 fraction. This sample was again carefully fractionated by eluting with hexane:ether mixtures (90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40). In the hexane–ether 75:25 fraction, the compound was isolated in good purity to be submitted to chiral analyses.

Synthesis. All reagents and solvents used in the syntheses were of highest commercially available standard. Chromatographic purifications were carried out on silica gel 60, Merck, 230–400 mesh. Racemic 2-methyl-4-octanol **(1)** (Scheme 1) was prepared by reaction of butyl magnesium bromide with



SCHEME 1. Synthesis of (±)-2-methyl-4-octanol **(1)** and (±)-3-methyl-4-octanol **(2)**.

isovaleraldehyde in ether solution at 0°C. Standard work up yielded **(1)** in 86% (Perez et al., 1997). Racemic 3-methyl-4-octanol **(2)** and 5-nonanol were synthesized using the same conditions (as described above, employing 2-mehtylbutanal [prepared by oxidation of 2-mehyl-1-butanol (Aldrich) with PCC in dichloromethane, 72% yield (Zarbin et al., 1998b)] and butanal, respectively [81% yield for **(2)** and 74% yield for 5-nonanol].

*(*4R*)- and (*4S*)-2,2-Dimethyl-4-Toluene-4-Sulfonyloxymethyl 1,3-Dioxolane **(4)***. Tosyl chloride (2.07 g, 10.88 mmol) was added in small portions (1 hr) to a solution of alcohol **(3)** (1.20 g, 9.08 mmol; *R* or *S*), triethylamine (1.38 g, 13,61 mmol, 1.90 ml), and DMAP (112 mg, 0.09 mmol) in dry dichloromethane (20 ml), with magnetic stirring at 0°C. After 5 hr, ether was added, and the solution was washed with aqueous HCl (10%), saturated aqueous $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was chromatographed over silica gel (hexane–ethyl acetate, 5:1), and the product **(4)** was obtained in 88% yield (2.2 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.30 (s, 3H); 1.35 (s, 3H); 2.45 (s, 3H); 3.76 (dd, $J = 5.1$, $J = 10.7$ Hz, 1H); 3.94–4.09 (m, 3H); 4.26 (dq, $J = 5.1$, $J = 6.1$ Hz, 1H); 7.35 (d, $J = 6.1$ Hz, 2H); 7.80 (d, $J = 6.1$ Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.66; 25.14; 26.64; 66.19; 69.60; 72.92; 110.07; 128.00; 130.01; 132.66; 145.00. {(4*S*)-**(4)**, $[\alpha]_D^{25} = +4.3$ (*c* 1.0, EtOH); (4*R*)-**(4)**, $[\alpha]_D^{25} = -4.6$ (*c* 1.2, EtOH)}.

*(*4R*)- and (*4S*)-4-Butyl-2,2-Dimethyl-1,3-Dioxolane **(5)***. Propylmagnesium bromide (5 eq. in THF) and $CuBr \cdot Me_2S$ (1.4 mmol, 286 mg) were added to a solution of **4** (2.0 g, 6.70 mmol) in dry THF (70 ml) at −40°C under an argon atmosphere. The temperature was gradually raised to room temperature and the reaction was stirred for 24 hr. Subsequently, 10 ml of saturated NH_4Cl was added, and the organic layer was diluted with 60 ml of ether. The extract was washed with water (5 ml), saturated $NaHCO_3$, and saturated NaCl. The layers were separated, and the organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was subjected to column chromatography (hexane–ethyl ether, 3:1) on silica gel to yield 0.61 g (55.6 %) of **(5)**. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.91 (t, $J = 7.0$ Hz, 3H); 1.14–1.59 (m, 5H); 1.37 (s, 3H); 1.42 (s, 3H); 1.60–1.71 (m, 1H); 3.50 (t, $J = 7.2$ Hz, 1H); 4.01–4.11 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$)δ: 14.04; 22.75; 25.77; 26.96; 27.94; 33.30; 69.55; 76.17; 108.56. {(4*S*)-**(5)**, $[\alpha]_D^{25} = +20.1$ (*c* 1.45, $CHCl_3$); (4*R*)-**(5)**, $[\alpha]_D^{25} = -20.5$ (*c* 1.32, $CHCl_3$)}.

*(*2R*)- and (*2S*)-2-Hexanediol **(6)***. To a mixture of methanol (5 ml) and 2 N HCl (5 ml) was added dropwise compound **5** (0.54 g; 3.44 mmol) in methanol (1 ml). The solution was stirred for 2 hr at room temperature, and then a saturated aqueous solution of K_2CO_3 was slowly added at 0°C. Ether (50 ml) was added, and the organic layer was washed with saturated NaCl solution, separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residual oil was chromatographed on a silica gel column (hexane; ethyl ether, 1:1) to afford 0.4 g of **(6)** (99 %). IR (ν_{max}, film cm^{-1}): 3338; 2932; 2861; 1466; 1062. ^{1}H NMR (400 MHz,

$CDCl_3$) δ: 0.91 (t, $J = 7.1$ Hz, 3H); 1.25–1.50 (m, 6H); 2.85 (bs, 2H); 3.42 (dd, $J = 7.7$, $J = 11.2$ Hz, 1H); 3.62-3.73 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.01; 22.75; 27.79; 32.80; 66.76; 72.40. GC-MS (70 eV) m/z (%): 117 (M-1, 2); 101 (14); 83 (100); 69 (77); 55 (8). {(2*S*)-**(6)**, $[\alpha]_D^{25} = +2.0$ (*c* 3.38, $CHCl_3$); (2*R*)-**(6)**, $[\alpha]_D^{25} = -2.17$ (*c* 9.5, $CHCl_3$)}.

*(*2R*)- and (*2S*)-2-Butyloxirane **(7)***. The diol **(6)** (0.39 g, 3.30 mmol) was added to a suspension of KOH (1.10 g, 19.50 mmol) in dry ethyl ether (10 ml) at 0°C, followed by slow addition of an ethereal solution (5 ml) of tosyl chloride (0.65 g, 3.4 mmol). The mixture was stirred for 2 hr at room temperature. After addition of hot water (5 ml), the mixture was extracted with ether (3 × 10 ml), the organic layer was separated, dried over $MgSO_4$, and the solvent was carefully removed by distillation. The residue (0.12 g, 37%) was employed in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.92 (t, $J = 7.3$ Hz, 3H); 1.32–1.58 (m, 6H); 2.46 (dd, $J = 2.8$, $J = 5.1$ Hz; 1H); 2.74 (dd, $J = 4.0$, $J = 5.1$ Hz, 1H); 2.90 (ddt, $J = 2.8$, $J = 4.0$, $J = 5.6$ Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.99; 22.56; 28.12; 32.21; 47.11; 52.39. {(2*S*)-**(7)**, $[\alpha]_D^{25} = -9.3$ (*c* 1.20, $CHCl_3$), [Lit: $[\alpha]_D^{20} = -10.1$ (*c* 1.53, $CHCl_3$); Francke et al., 1995); (2*R*)-**(7)**, $[\alpha]_D^{25} = +9.0$ (*c* 10.5, $CHCl_3$)}.

*(*4R*)- and (*4S*)-2-Methyl-4-Octanol **(1)***. To a stirred solution of **7** (0.27 g, 2.7 mmol) in dry THF (5 ml) kept below −40°C, was added CuI (0.52 g, 2.7 mmol) followed by dropwise addition of PrMgBr (2M solution in ether, 13.5 ml, 27 mmol). After the addition was completed, the mixture turned dark. The mixture was allowed to warm to room temperature, and stirring was continued for 1 hr. The reaction was quenched by addition of saturated NH_4Cl (10 ml). The aqueous layer was extracted with hexane–ethyl ether, 1:1 (3 × 20 ml), and the organic layer was dried over $MgSO_4$ and concentrated carefully *in vacuo*. The product was purified by flash chromatography on silica gel using a mixture of hexane–ethyl ether, 5:1, yielding compound **1** as a colorless oil (0.32 g, 82 %). IR (ν_{max}, film cm^{-1}): 3346; 2956; 2928; 2870; 1466; 1026. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (d, $J = 6.6$ Hz, 3H); 0.91 (t, $J = 7.0$ Hz, 3H); 0.92 (d, $J = 6.6$ Hz; 3H); 1.19–1.48 (m, 8H); 1.70 (bs, 1H); 1.71–1.81 (m, 1H); 3.64–3.72 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.50; 22.46; 23.17; 23.91; 25.03; 28.22; 38.19; 47.22; 70.40. {(4*S*)-**(1)**, $[\alpha]_D^{25} = +9.17$ (*c* 2.3, $CHCl_3$), 92% *ee* by chiral GC analyses, $R_t = 7.81$ min; (4*R*)-**(1)**, $[\alpha]_D^{25} = -9.21$ (*c* 1.5, $CHCl_3$), 93% *ee* by chiral GC analyses, $R_t = 7.35$ min}.

RESULTS AND DISCUSSION

Gas chromatographic (GC-FID) analysis of the airborne volatiles produced by males and females of the sugarcane weevil *S. levis* showed the presence of one male-specific compound (Figure 1). The base peak observed on GC-EI mass

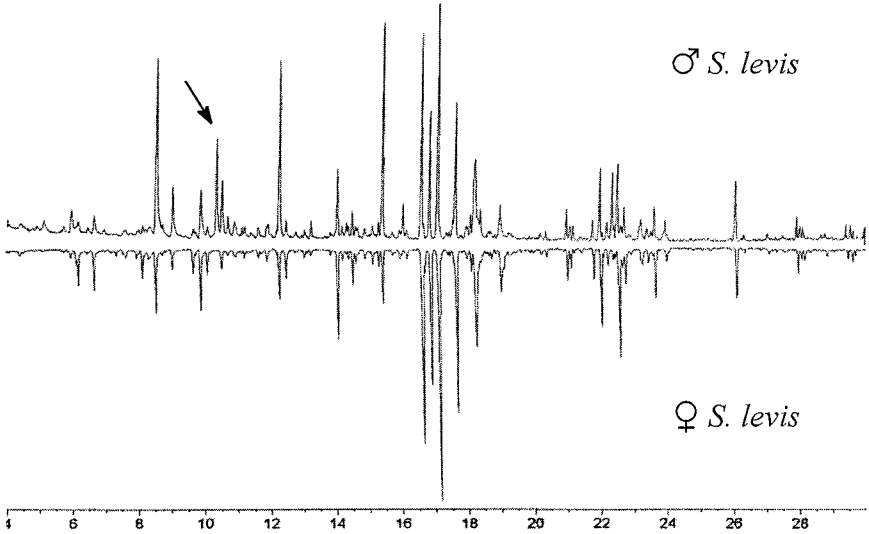


FIG. 1. GC profiles of the extracts of airborne volatiles collected from male and female sugarcane weevils *S. levis*. The male-specific compound is marked by an arrow.

spectra (MS) at m/z 69 along with the intense peak at m/z 87 strongly suggested the existence of a hydroxyl group attached to a five-carbon fragment. The m/z 126 was deduced to be due to a M−18 peak, resulting from loss of water, indicating a possible molecular ion (M^+) at m/z 144 (Figure 2). However, even employing chemical ionization with CH_3CN, the molecular ion of the compound could not be detected. The hypothesis that the target molecule was a linear and symmetrical structure such as 5-nonanol was ruled out, since the MS of the compound gave an ion at m/z 111, typical M−33 of methyl branched alcohol that results from loss of CH_3 and H_2O (Silverstein et al., 1991). Moreover, the retention time (R_t) of

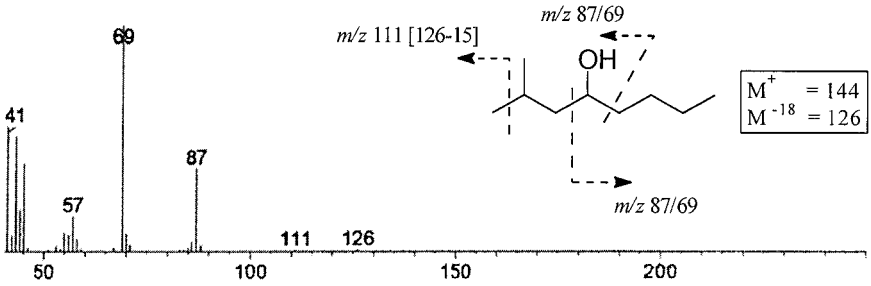
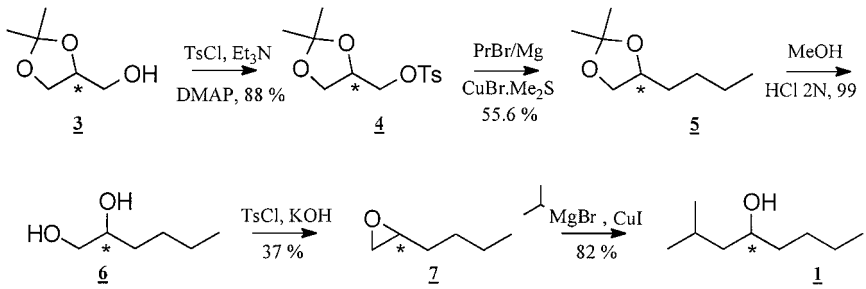


FIG. 2. Mass spectra of the male-specific compound of *S. levis*. Inset: major fragmentation patterns of the molecule.

a synthetic sample of 5-nonanol was higher than that of the natural product. At this point, the data suggested only two possible chemical structures for this compound; 2-methyl-4-octanol **(1)** or 3-methyl-4-octanol **(2)**. In order to determine unambiguously the correct structure of this male-specific compound, both alcohols were synthesized as racemates and used as authentic references (Scheme 1). The mass spectrometric characteristics of both standards were identical to those of the natural product, but only the 2-methyl-4-octanol **(1)** exhibited identical retention times on two different GC columns (VA-5 and VA-Wax). Thus, the chemical structure of the male-specific compound was deduced as 2-methyl-4-octanol.

While the carbon skeleton was determined, nothing was known about the configuration of the stereogenic center. One way to investigate the stereochemistry of chiral volatiles is to obtain enantiomeric resolution on a chiral column. The assignments of the peaks can be carried out with samples of known stereochemistry, and the absolute configuration of the natural product is determined by comparison of its retention time under the same conditions (Leal et al., 1996).

In order to determine the absolute configuration of naturally occurring **1**, we developed a short enantioselective synthesis for both enantiomers, as described in Scheme 2. Our route utilized commercially available (*R*)- and (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol **(3)** as a starting material, which were transformed into the tosylates **(4)** in 88% yield (Perkins et al., 1992). Compounds **(5)** were obtained by coupling tosylates **(4)** with *n*-propyl magnesium bromide, catalyzed by $CuBr \cdot Me_2S$, in 55.6% yield (Zarbin et al., 2000). Hydrolysis of ketals **(5)** under acidic conditions furnished the diols **(6)** in 99% yield. The highly volatile epoxides **(7)** were obtained in "one pot" from diols **(6)** in 37% isolated yield, by reaction with tosyl chloride and powdered KOH (Francke et al., 1995; Jurczak et al., 1986; Kuwahara et al., 1997). Finally, the epoxides **(7)** were reacted with 1-methylethyl magnesium bromide catalyzed by CuI, affording the target compounds **(1)** in 82% yield (Takenaka et al., 1996; Baraldi et al., 2002).



SCHEME 2. Enantioselective synthesis of (*R*)- and (*S*)-2-methyl-4-octanol **(1)**.

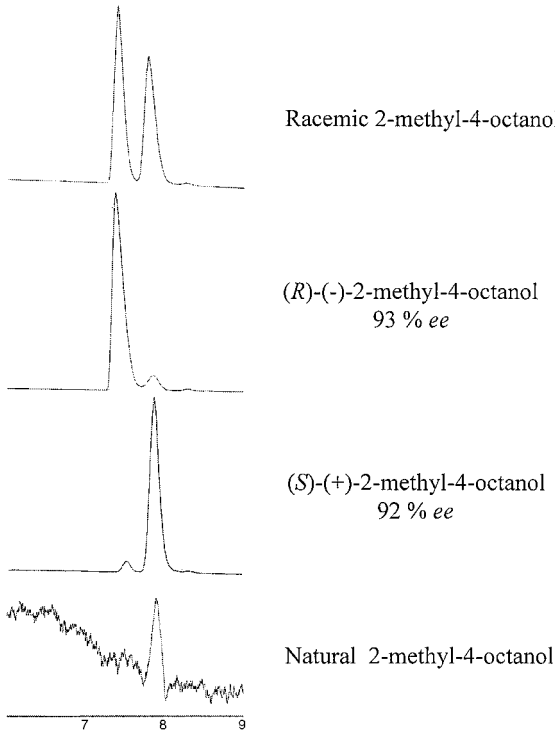


FIG. 3. Resolution of enantiomers of 2-methyl-4-octanol **(1)** on a β-CD column. The peaks correspond to7.35 and 7.81 min.

Racemic **(1)** was resolved almost to baseline on a β-CD capillary column, showing two peaks with retention times of 7.35 and 7.81 min (Figure 3). Synthetic (*R*)-**(1)** corresponded to the earlier eluting peak, while the (*S*)-**(1)** isomer gave a peak coeluting with the later one. The purified natural product had the same retention time as the second peak. Therefore, the male-specific compound produced by the sugarcane weevil *S. levis* was fully characterized as (*S*)-(+)-2-methyl-4-octanol.

At present, the biological role of the compound identified in this work is unknown or, at least, uncertain. However, preliminary laboratory observations employing rubber septa as releaser suggest that the racemic compound elicits aggregation behavior among both males and females of this species. As already mentioned, the same compound has been identified as a male-produced aggregation pheromone in several other curculionidae species (Perez et al., 1997; Ramirez-Lucas et al., 1996a,b; Giblin-Davis et al., 2000). A detailed study concerning the biological activity of racemic, as well as (*R*)- and

(*S*)-2-methyl-4-octanol, including the electrophysiological measurements, is now in progress.

In summary, we have identified a male-specific chiral compound (possibly an aggregation pheromonce) produced by the weevil *S. levis*, an important sugarcane pest in South America.

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SEX PHEROMONE OF HORSE-CHESTNUT LEAFMINER *Cameraria ohridella* AND ITS USE IN A PHEROMONE-BASED MONITORING SYSTEM

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Abstract—Gas chromatography combined with electroantennographic detection (GC-EAD), electroantennography (EAG), and wind-tunnel and field experiments were used to reinvestigate the composition of *Cameraria ohridella* (Lepidoptera, Gracillariidae, Lithocolletinae) sex pheromone. The GC-EAD experiments showed one EAD-active area corresponding to the major pheromone component, (8*E*,10*Z*)-tetradeca-8,10-dienal. The EAG experiments proved that (9*E*)-tetracedecenal and stereoisomers of (8*E*,10*Z*)-tetradeca-8,10-dienal exhibited significant electrophysiological activity and could, therefore, be considered as possible minor pheromone components. However, wind-tunnel and field experiments demonstrated that none of these compounds affect the efficacy of the main pheromone component. A monitoring system based on (8*E*,10*Z*)-tetradeca-8,10-dienal was developed and used to study the flight activity of *C. ohridella.*

Key Words—Pheromone, (8*E*,10*Z*)-tetradeca-8,10-dienal, *Cameraria ohridella*, Lepidoptera, horse-chestnut leafminer, antennal sensitivity, field experiments, monitoring.

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INTRODUCTION

Cameraria ohridella Deschka et Dimić (Lepidoptera, Gracillariidae, Lithocolletinae), a species recently found and described in Macedonia (Simova-Tošić and Filov, 1985; Deschka and Dimić, 1986), has spread almost everywhere in central Europe and is moving towards the west (Puchberger, 1990; Holzschuh and Krehan, 1992; Butin and Führer, 1994; Szabóky, 1997; Tomiczek, 1997; Krehan, 1997; Tomiczek and Krehan, 1998; Šefrová and Laštùvka, 2001). This tiny leafminer selectively infests horse-chestnut trees (*Aesculus hippocastanum*, Hippocastanaceae).

Females of *C. ohridella* produce a sex pheromone to attract males for mating (Svatoš et al., 1999a). The major sex pheromone component was recently identified as (8*E*,10*Z*)-tetradeca-8,10-dienal [8*E*10*Z*-14:Al] (Svatoš et al., 1999b). In this study, we reinvestigated the pheromone with particular attention to EAG active compounds that could be considered as minor pheromone components. Using a novel pheromone-based monitoring system, we describe the pattern of *C. ohridella* flight activity.

METHODS AND MATERIALS

Insects. Horse-chestnut leaves infested with overwintering *C. ohridella* pupae were field-collected in autumn. Collected leaves were kept outdoors. After the first freezing period, leaves were placed in nylon cages in the laboratory, and adults were allowed to emerge (temperature 20–23°C, 50% relative humidity, 16L:8D photoperiod regime). Emerged adults were kept individually in 5-ml glass vials to prevent mating. Pheromone glands of 1- to 2-day-old virgin calling females were excised and extracted for 1 hr with hexane (5 μl/gland). The EAGs were recorded from antennae of 1- to 2-day-old males kept at 5°C. In behavioral experiments, 1- to 2-day-old males kept at 20–23°C, 16L:8D photoperiod and 50% relative humidity were flown in a wind tunnel. Field experiments were performed during the 2000–2001 seasons.

Scanning Electron Microscopy. For scanning electron microscopy (SEM), male antennae and female abdominal tips were dehydrated for 15 min each in an ascending ethanol series starting at 70% and in acetone, mounted on stubs, sputter-coated with gold (300 Å), and viewed in a scanning electron microscope (Jeol 6300, Jeol Ltd., Akishima, Japan).

EAG Recordings. The whole animal preparation (Svatoš et al., 1999a,b), Ag–AgCl electrodes, CyberAmp 320 amplifier (Axon Instruments) equipped with AI402 probe (Axon Instruments), PC with A/D conventer board (Metrabyte DAS-16), and Stand-Alone Enhanced Data Acquisition System (Version 3.0, RUN Technologies) were used for EAG recordings.

EAG PROFILES

The saturated and monounsaturated C_{12} and C_{14} alcohols, acetates, and aldehydes (12:Ac, 12:OH, 12:Al, 14:Ac, 14:OH, 14:Al; purity 99%) were dissolved in hexane. One microgram of the respective compound was loaded on a filter paper disk (1 cm diam.), hexane was evaporated, and the loaded disk was placed into a Pasteur pipet, sealed with Parafilm, and stored in a freezer until use. To achieve stimulation, a solenoid-activated odor delivery system was used to divert a continuous clean airstream (charcoal filtered and humidified, velocity 1 liter/min) through the loaded Pasteur pipet (stimulus duration: 0.5 sec) and then over the antenna of the fixed moth. Maximum negative deflections of EAG potentials were recorded. Successive stimuli were separated by 5-min intervals to allow the antennae to recover from the previous stimulation. The antennal sensitivity and its possible change during the experiment were checked by standard stimulation [1 μg of (10*Z*)-tetradec-10-en-1-yl acetate]. Stimuli were applied to the antenna randomly to prevent systematic errors caused by previous stimulations. Data from six preparations with approximately constant responses to standard stimulation within the experimental session were evaluated: EAG mean values were calculated for each compound. Means were plotted against tested chemical identities to create EAG profiles.

EAG Dose–Response Experiments. The test compounds were diluted in decadic steps in hexane. Pasteur pipets were loaded with aliquots of 10 μl (the amount of substance in the pipets ranged between 0.1 and 1 μg). The test substances were presented to the antenna at all dose levels, starting with the lowest ones. The stimuli were presented with an interval of 2 min. Where reasonable, the EAG data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$).

GC-EAD Experiments. In GC-EAD experiments, intact antennal preparations served as biological detectors for the HP 5890A gas chromatograph. Three columns with different polarities: DB-Wax, DB-5, and DB-1 (30 m length, 0.25 ID, film thickness 0.25 μm; J & W Scientific) were used. The inlet and FID detector temperatures were set at 200°C and 260°C, respectively. Samples were injected splitless. Helium was used as a carrier gas. The temperature program used for the analyses: 70°C for 2 min, 4°C/min to 140°C, 5°C/min to 220°C, 20°C/min to 280°C, and then 5 min at this temperature. The column was split at the end by a four-arm splitter (Graphpack 3D/2). The first arm of the splitter was linked to the original column, the second arm was connected to the FID detector by means of 0.5 m of a deactivated silica column (0.33 mm id), and the third arm (0.5 m of deactivated silica column) led through a transfer line (heated up to 180°C) to the outside of the GC (Effluent Conditioning Assembly, Syntech, Hilversum, The Netherlands). The third arm ended in a mixing chamber, from which a continual airstream (1 liter/min) was blowing onto the antennal preparation via a PTFE-coated stainless steel flow

tube. Makeup gas (20 ml/min, N_2) was introduced through the fourth arm of the splitter to prevent condensation.

Chemicals. The pheromone, 8*E*10*Z*-14:Al, and its geometric isomers were synthesized in our laboratory (Hoskovec et al., 2000). Synthetic pheromone standards were purchased from Pherobank, IPO-DLO, Wageningen, The Netherlands.

Wind-Tunnel Experiments. The flight tunnel consisted of a rectangular tube (clear Plexiglas) 186 cm long. Its upwind and downwind ends were closed with a mesh screen (2-mm openings). Charcoal filtered and humidified air was pushed through the tunnel by means of four ventilators creating an airspeed of 0.25 m/sec. The flight tunnel was diffusely illuminated from above with a light intensity of about 1000 lux. The stimuli were applied on a filter paper disk (1 cm diam.) positioned in a holder fixed in the center of the upwind mesh. The plume parameters and orientation were checked periodically using $TiCl_4$ smoke. The experiments were performed during the first 2 hr of the photophase. Prior to an experiment, males were placed individually into clean glass tubes (10 cm long, 4 cm diam.), closed by nylon cloth. These release cages were kept in an illuminated quiet place until use (at least 15 min). The stimulus source was set in place, and the release cage was then opened and placed into a holder positioned in the central part of the flight compartment near the bottom. In this position, the male inside the cage was still outside the odor plume and usually did not respond. Then, the holder was moved up into the odor plume, and the male's behavior was observed during the following 5 min. Males that did not respond (no antennal movement, walking, wing fluttering, or taking off) were considered nonresponding. The behavior of responding males was classified into four categories: (1) activation (walking, antennal movements, and wing fanning), (2) take off, (3) oriented flight, and (4) source localization and landing. Each male was tested only once. Twenty-five males were flown in five replicates to each treatment. An extract of three pheromone glands served as a standard stimulus and was presented at the beginning of every experiment. When 4 of 5 males responded to the standard stimulus, other treatments were tested. After each experimental session, the glass tubes and holders were washed with light petroleum and oven-dried at 200°C. The data were analyzed by χ^2 test ($\alpha = 0.05$).

Field Experiments. All field experiments were performed in Prague, Czech Republic. In the majority, delta traps (Biolatrap Delta 155; ZD Chelčice, Czech Republic) containing sticky paper inserts (200 × 80 mm), covered with Lonamelt PS 3199/05 glue (Vetox, Prague, Czech Republic) were used. Traps were placed within the canopy of horse-chestnut trees at 1–2 m above the ground. Filter paper, PE foil, and rubber septa (Thomas Scientific, Swedesboro, New Jersey, USA; Cat. No.1780-J07) were used as pheromone dispensers. Filter paper and PE foil dispensers were replaced daily, and rubber dispensers were replaced every 14 days. Traps were rotated daily to eliminate any position effect (Latin-square design) (Perry et al., 1980). Sticky inserts were replaced when necessary. The data were

analyzed by two-way (position and treatment) ANOVA ($\alpha = 0.05$) in all cases, except for monitoring experiments.

RESULTS

Scanning Electron Microscopy. Scanning electron microscopy (SEM) of female abdominal tips exposed during calling behavior revealed that the sex pheromone gland is associated with the intersegmental membrane between the eighth and ninth segments and the papillae anales. The gland is located dorsally in the intersegmental membrane (Figure 1A).

Olfactory sensilla were found over the whole annular surface of long flagellate antennae interspaced between regularly arranged scale rows (Figures 1B,C). SEM of male and female antennae showed that male antennae possess male-specific olfactory sensilla trichodea, presumably specialized for pheromone perception.

GC-EAD and EAG Experiments. The GC-EAD analyses of pheromone gland extracts consistently showed only one EAD response (Figure 2A). The respective

FIG. 1. (A) Scanning electron micrographs (SEM) of exposed pheromone gland and papilla annales; (B) scanning electron micrograph (SEM) of two annuli of flagellate male antenna covered by scales and olfactory sensillae; (C) SEM of male-specific sensilla trichodea.

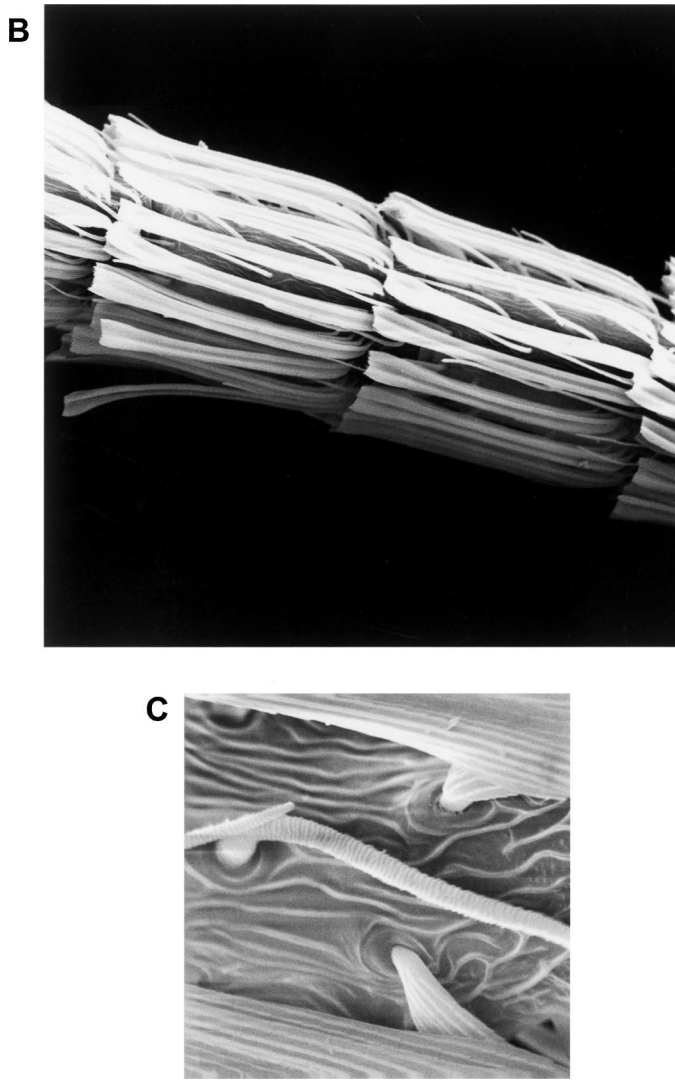

FIG. 1. Continued.

compound eliciting the EAG response was not FID- or MS-detectable, even though highly concentrated extracts were analyzed (>100 FE). The Kovats' indices of the EAD active peak obtained on three different GC columns matched with the retention times of 8*E*,10*Z*-14:Al (Table 1). No GC-EAD evidence about the eventual minor pheromone component(s) was obtained. The GC-EAD recordings with

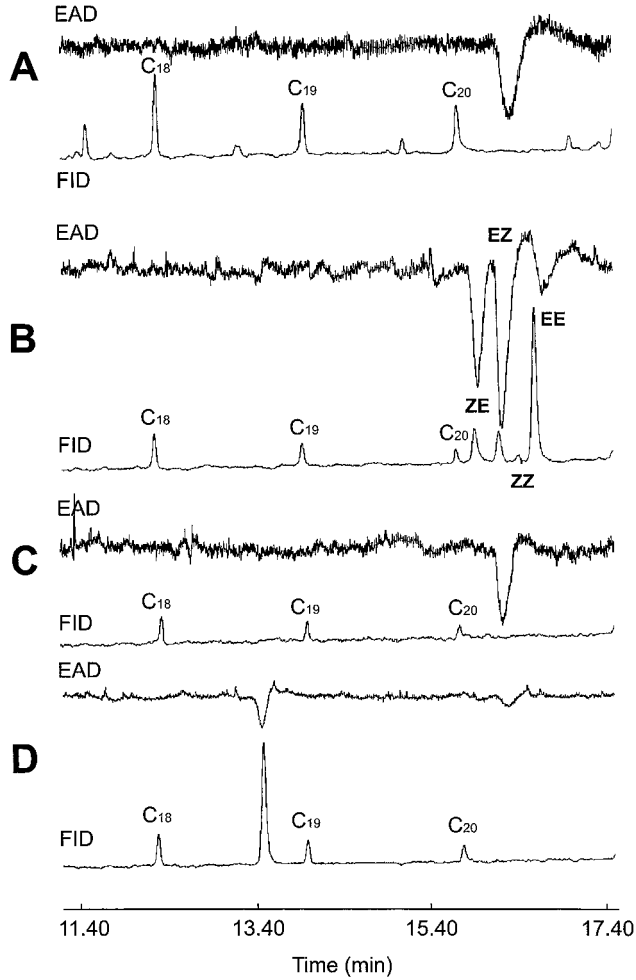

FIG. 2. Sections of GC-EAD/FID traces: (A) pheromone gland extract (ca. 10 FE); (B) 1 μg of a solution of the four synthetic stereoisomers of 8, 10–14:Al; (C) 1 ng of 8*E*10*Z*-14:Al; (D) 1 μg of 9*E*-14:Ald. A mixture of C_9–C_{22} hydrocarbons was coinjected with tested samples to help peak identification.

synthetic standards revealed also the EAD activity for other stereoisomers of 8,10–14:Al (Figure 2B). The EAG activity of the *EZ* stereoisomer (Figure 2C) was followed by *ZZ*, *EE*, and *ZE*, respectively. As can be seen in Figure 3, the *EZ* dose–response curve exhibited a typical sigmoidal shape with a threshold dose of 0.1 ng/pipet (one-way ANOVA, Dunett's multiple comparison test, $\alpha = 0.05$) and

TABLE 1. KOVATS' INDICES[a] OF EAD PEAK IN *C. ohridella* FEMALE EXTRACTS AND SOME OTHER SYNTHETIC COMPOUNDS

GC phase[b]	Female extract[c] (ca. 3 FE)	Compounds			
		12:Ac	14:Ald	14:OH	8*E*10*Z*-14:Al[c]
DB-1[d]	1623.3				1623.9
DB-5[d]	1674.4	1605.9	1610.8	1675.4	1674.3
DB-WAX[e]	2031.2				2031.8

[a] Based on saturated hydrocarbons.
[b] J & W Scientific, 30 m × 0.25 mm, film thickness 0.25 μm.
[c] For EAD trace.
[d] 170°C.
[e] 140–240°C at 5°C/min.

saturation above 100 ng/pipet. The *ZZ* curve was less sigmoidal, reached lower maximum EAG values, and was shifted by one order of magnitude to higher doses than the *EZ* isomer (threshold dose 1 ng). The *EE* and *ZE* curves were much flatter. The *EE* isomer exhibited the highest threshold dose (10 ng) and lowest saturation amplitude.

EAG Profile Spectra. The EAG profile spectra showed a pattern compatible with 8*E*,10*Z*-14:Al as the main pheromone component and provided no clues about the prospective minor ones. Among linear saturated C_{12} and C_{14} standards,

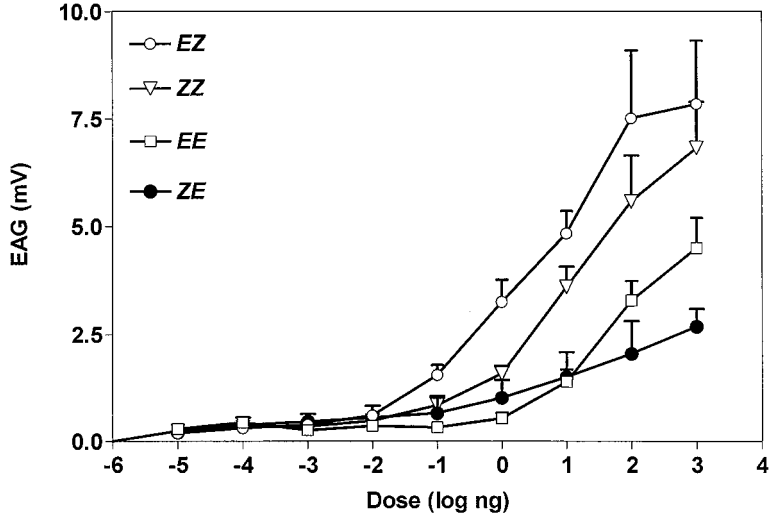

FIG. 3. The dose-response curves of the four stereoisomers of 8, 10–14:Al. Each value represents mean from six antennal preparations and standard error of mean.

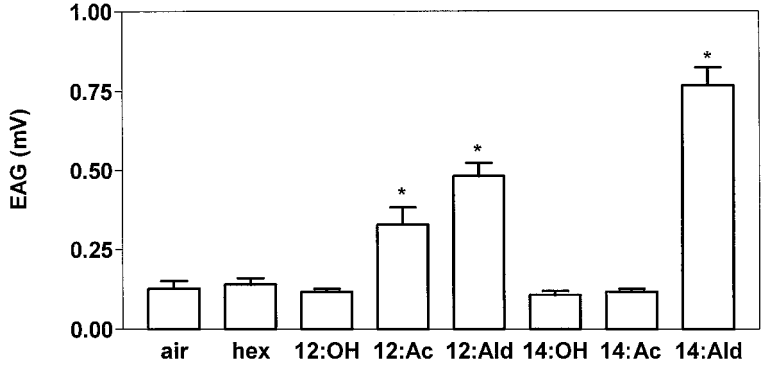

FIG. 4. Male EAG responses to 1 μg of saturated C_{12} and C_{14} alcohols, acetates, and aldehydes (mean + standard error of mean, $N = 6$). Asterisks represent significantly higher EAG response than to hexane (one-way ANOVA, $\alpha = 0.05$; hex = hexane).

aldehydes elicited higher EAG responses than alcohols or the corresponding acetates. Tetradecanal (14:Al) elicited higher EAG responses than dodecanal (12:Al) and dodecyl and tetradecyl acetates (12:Ac, 14:Ac) and alcohols (12:OH, 14:OH). 12:Ac gave higher responses than 14:Ac (Figure 4). In the monounsaturated C_{14} series, EAG activity increased gradually when the double bond was moved from position 2 to position 7, remained high between positions 8 and 10, and decreased again when the double bond was moved to the penultimate position (Figure 5). Maximum EAG activity was observed with the double bond in position 9. The *E*-isomer, 9–14:Al (9*E*-14:Al) was more active than the corresponding *Z* isomer (9*Z*-14:Al)

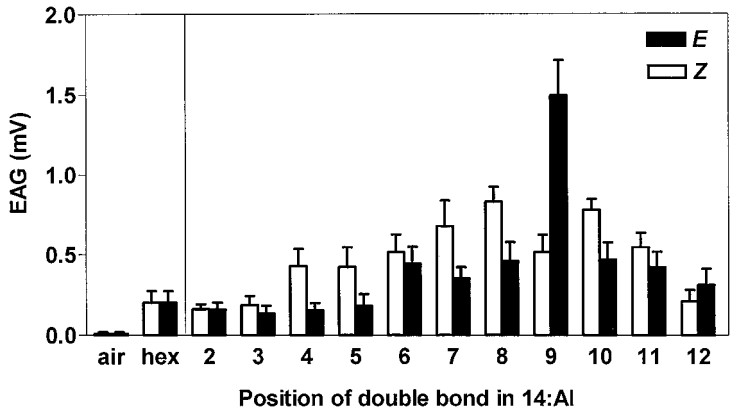

FIG. 5. Male EAG responses to tetradecenals with different double bond positions (mean + standard error of mean, $N = 6$; hex = hexane).

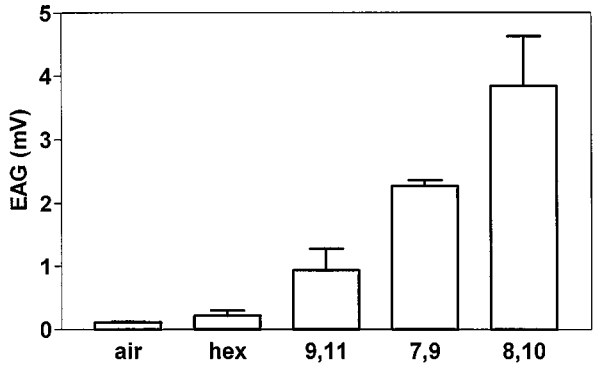

FIG. 6. Male EAG responses to three thermodynamic mixtures of tetradecadienals with dienic system in positions 9,11; 7,9, and 8,10 (mean + standard error of mean, $N = 6$; hex = hexane).

and in GC-EAD experiments elicited significant EAD activity (Figure 2D). From conjugated tetradecadienals with carbon–carbon double bonds in positions 7,9, 8,10, and 9,11, male antennae were the most sensitive to 8,10-14:Al (Figure 6).

Wind Tunnel Experiments. In wind-tunnel experiments, 8*E*10*Z*-14:Al alone was attractive to *C. ohridella* males in extremely low doses. Males responded readily to 0.1 pg loaded onto a filter paper disk. 8*E*10*Z*-14:Al (1 pg) elicited a male behavioral response comparable to that evoked by an extract of 3 female equivalents (FE) (Figure 7).

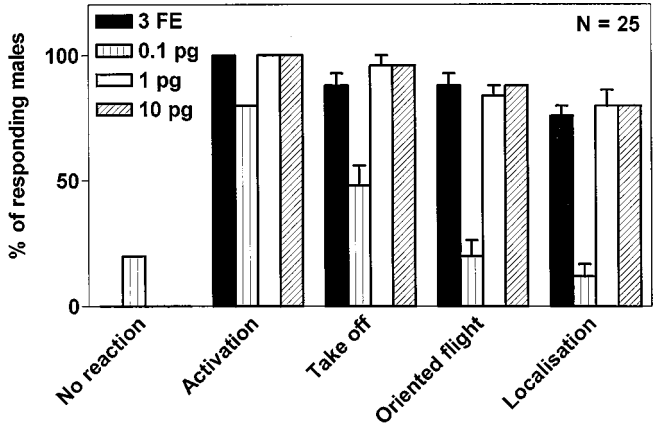

FIG. 7. Male behavioral responses to pheromone gland extract (3 FE; black bar) and to 0.1, 1 and 10 pg of synthetic 8*E*10*Z*-14:Al (see legend). X-axis: the respective behavioral category. Y-axis: percentage of males displaying the behavior.

TABLE 2. ATTRACTIVENESS OF MIXTURES OF 8*E*10*Z*-14:Al AND 9*E*-14:Al TO *Cameraria ohridella* MALES IN FIELD EXPERIMENTS[a]

	Pheromone–9*E*-14:Al ratio		
	1:0	1:1	5:1
Catches/trap (mean ± SEM)	386.6 ± 58.8	371.7 ± 29.2	389.6 ± 30.5

[a] Delta traps, dispenser = 1 cm^2 of PE–Aluminum foil, sticky inserts replaced daily, Latin-square design, 2 replicates. Loadings: 1:0 ratio = 5 ng of 8*E*10*Z*-14:Al; 1:1 ratio = 5 ng of 8*E*10*Z*-14:Al and 5 ng of 9*E*-14:Al; 5:1 ratio = 5 ng of 8*E*10*Z*-14:Al and 1 ng of 9*E*-14:Al (mean catches per trap and day ± standard error of mean, 2 replicates). Different treatments were not statistically different (two-way ANOVA, $\alpha = 0.05$).

Neither 9*E*-14:Al nor stereoisomers of 8*E*10*Z*-14:Al attracted males when presented alone in wind-tunnel or field experiments (data not shown). When they were presented in 1:1 or 10:1 mixtures with the pheromone (10 pg *EZ*), they did not significantly affect the effectiveness of the main pheromone (Tables 2 and 3), suggesting that they do not take part in the sexual communication system of *C. ohridella.*

TABLE 3. BEHAVIORAL RESPONSES OF *C. ohridella* MALES TO 1:1 AND 10:1 BLENDS OF 10 pg OF 8*E*10*Z*-14:Al AND 10 OR 1 pg OF RESPECTIVE ISOMERS IN WIND-TUNNEL EXPERIMENTS

	Responding males (%, mean ± SEM)			
	EE	*ZE*	*ZZ*	Standard stimulus
EZ–isomer = 1:1				
No reaction	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Activation	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Take off	88 ± 4.89	96 ± 4.00	88 ± 4.89	96 ± 4.00
Oriented flight	84 ± 4.00	88 ± 4.89	72 ± 4.89	72 ± 4.89
Localization	80 ± 0	80 ± 6.33	68 ± 4.86	72 ± 4.89
EZ–isomer = 10:1				
No reaction	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Activation	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Take off	92 ± 4.89	96 ± 4.00	96 ± 4.00	100 ± 0
Oriented flight	84 ± 4.00	80 ± 6.32	92 ± 8.00	92 ± 4.89
Localization	80 ± 0	76 ± 4.0	76 ± 7.48	80 ± 6.32

[a] One or 10 pg of the respective isomer was added to 10 pg of 8*E*10*Z*-14:Al and compared with a standard stimulus (10 pg of 8*E*10*Z*-14:Al). Mean percentages of responding males by the respective behavior + standard error of mean ($N = 25$). No significant differences between various treatments and the standard stimulus was observed (χ^2 test; $\alpha = 0.05$).

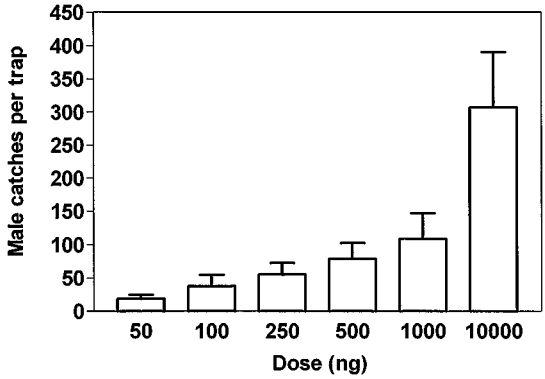

FIG. 8. The effect of pheromone dose on trap effectiveness (delta trap, pheromone was loaded on red rubber septa). The number of caught males was counted after 3 hr of trap exposure (10:00–13:00 hr; mean + standard error of mean, $N = 12$).

Monitoring System. Traps baited with 8*E*10*Z*-14:Al, were remarkably attractive. Their efficacy depended on the pheromone dose loaded onto the dispenser. Figure 8 shows the effect of pheromone doses ranging from 50 ng to 10 μg of 8*E*10*Z*-14:Al per dispenser on trap catches (tested in August 2001 in Matěj, Prague 6). In some traps, moths were attracted by a dose as small as 10 ng. Males of *C. ohridella* were active mainly during the period from 06:00 to 18:00 hr with a peak between 10:00 and 12:00 hr (tested on August 8, 2001, in Čimice; Figure 9).

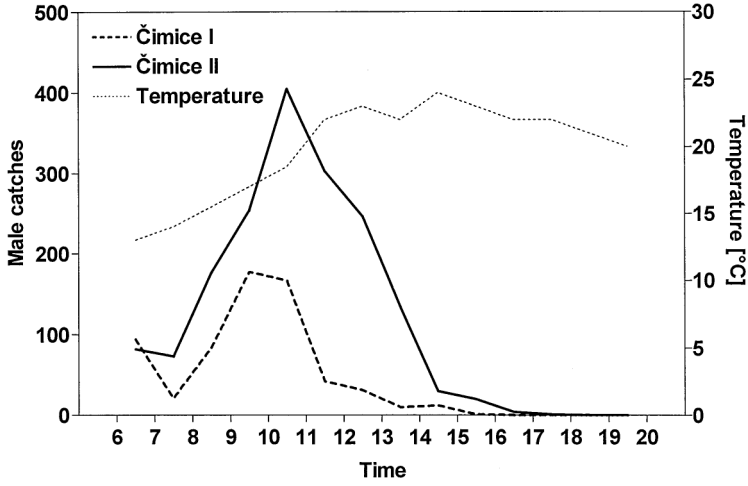

FIG. 9. The circadian activity of *C. ohridella* monitored by delta traps baited with 100 ng of 8*E*10*Z*-14:Al.

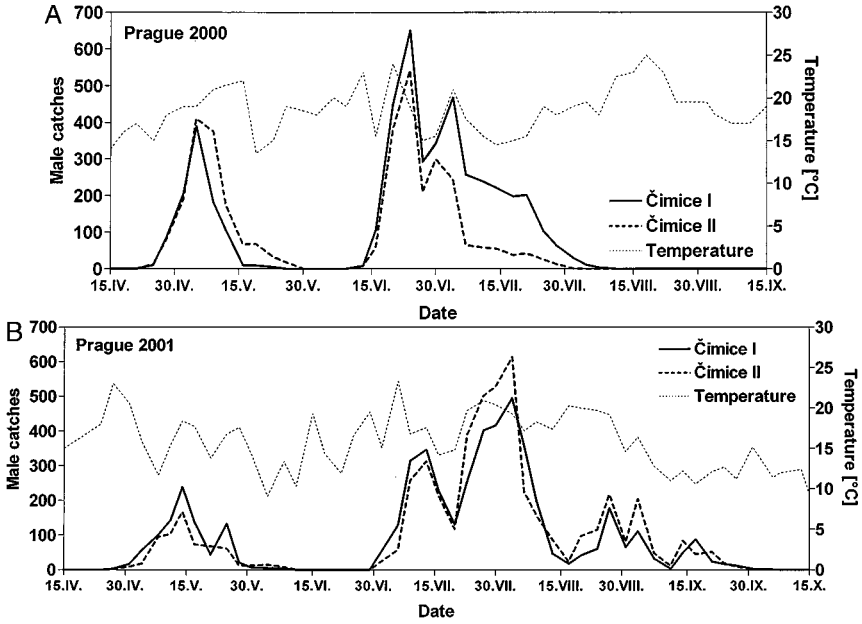

FIG. 10. Flight activity of *C. ohridella* monitored by delta traps baited with 100 ng of 8*E*10*Z*-14:Al loaded on red rubber septa. Sticky inserts were exposed two times per week for 24 hr**,** pheromone dispensers were replaced every 14 days. (A) year 2000 flight season; (B) year 2001 flight season.

The pattern of flight activity of *C. ohridella* estimated by pheromone trap catches was influenced by climatic conditions during the respective season. Figure 10 shows the flight activity of *C. ohridella* and temperature recorded at two localities (Čimice I and II; Prague 8) during the 2000 and 2001 seasons. During the 2000 season (Figure 10A), the flight activity exhibited two distinct peaks (May 5 and June 24) separated by a period without any catches (2–3 weeks). The second decline exhibited a smaller secondary peak on July 4. Considering the time of egg, larval, and pupal development, the two main peaks likely correspond to two generations. The whole season was relatively warm. The situation was slightly different the following year (Figure 10B). In 2000, the first moths appeared at the end of April, and trap catches of the first generation reached two maxima on May 14 and 25. Individuals of the second generation appeared at the beginning of July, created two peaks on July 13 and August 3 and reached almost zero around August 15. At the end of August, and in September and October, several smaller activity peaks occurred. The relative increase of the second generation over the first was more pronounced than in 2000. The third generation was detected during the period of late August and September. The 2001 season was cooler than that of 2000.

DISCUSSION

SEM. The compact gland structure found in *C. ohridella* located dorsally in the intersegmental membrane between the 8th and 9th abdominal segments is quite common among Lepidoptera. Families where it is found include the Ageriidae, Olethreutidae, Tortricidae, Noctuidae, Pyralidae, Gelechiidae, and others (Percy-Cunningham and MacDonald, 1987). The observed antennal dimorphism between sexes also is widespread among Lepidoptera. The male-specific sensilla trichodea of *C. ohridella* presumably contain pheromone receptor cells, since male but not female antennae respond to the pheromone (data not shown).

Pheromone. Our data confirmed that 8*E*10*Z*-14:Al is the main pheromone component of *C. ohridella.* (Svatoš, 1999b; Francke et al., 2003). None of the tested compounds synergized or inhibited the attractiveness of 8*E*10*Z*-14:Al, although they were EAG active. However, the EAG itself does not necessarily indicate that a compound is part of a pheromonal blend, since compounds chemically similar to a pheromone could elicit significant EAG responses (Roelofs, 1977, 1984; Löfstedt and Löfquist, 1983). The prospect that *C. ohridella* employs a one-component pheromone system fits with the pattern of EAG response profiles reflecting male antennal specificity. On the other hand, minor pheromone components are usually present in considerably lower amounts than the major one(s), and male antennae are less sensitive to them due to fewer olfactory receptor neurons devoted to their detection (Roelofs, 1977, 1984; Löfstedt and Löfquist, 1983). Therefore, there still is a theoretical possibility that *C. ohridella* minor pheromonal component(s) exist but are present in amounts not detectable by available analytical instrumentation. Many pheromones originally identified as a single component proved to be multicomponent blends when reinvestigated with better analytical equipment.

The great majority of lepidopteran sex pheromones are multi-, rather than single-component blends (Arn,), although one-component pheromones also exist (i.e., in *Lymantria dispar*) (Bierl et al., 1970; Cardé et al., 1977). Among the eight pheromones so far investigated in the family Gracillariidae (subfamily Gracillarinae), two are based on a single component. In *Acrocercops* sp. and in *Gracillaria elongella,* 8*E*10*E*-14:Al and 10*Z*12*E*-16:Al, respectively, were reported as single attractants for conspecific females (Ando et al., 1987; Priesner, 1985).

Pheromone-Based Monitoring System. The high efficacy of the main compound allowed the successful employment in pheromone-based monitoring systems. From available trap systems (Cardé and Elkinton, 1984), the delta sticky traps were selected because of low cost and operational simplicity. However, their suitability is limited, since the sticky surface might become saturated or loose its stickiness upon aging. Delta traps used in our experiments retain a maximum of approximately 600–700 moths per sticky insert. Thus, in a monitoring system, when the population density is high, saturation could disorder the relationships between trap catch and population density unless sticky inserts are changed frequently or

traps are baited with very low dosages of the attractant. The pheromone dose affects the trap efficiency considerably. Generally, a high rate of pheromone emission produces an increase in trap catches (Cardé and Elkinton, 1984), although trap attractiveness may be weak at high release rates. The effect of reduced responsiveness is especially pronounced in *G. molesta*, a species that exhibits attraction and mating responses to a comparatively narrow range of pheromone concentrations (Cardé et al., 1975; Baker and Cardé, 1979; Baker and Roelofs, 1981). Similar trends presumably exist in other species, but are not pronounced in *C. ohridella*, where trap catches increased over 4 orders of magnitude and inhibitory effects were only observed at high doses (over 10 μg/dispenser). The increase of trap catches with high pheromone loadings might be attributed to increased active space of the trap. Higher doses likely attract males from longer distances. Data on the estimation of trap active space for *C. ohridella* are not available. Šefrová and Laštůvka (2001), who studied *C. ohridella* dispersal in Europe for 10 years and estimated the invasion rate to be 60–90 km annually, considered air current transport as the most important factor for the dispersal of this species (Šefrová and Laštůvka, 2001). The invasion rate, however, tells nothing about how far a male can actively fly while searching for a mate, since the dispersal behavior of pheromone-responsive and nonresponsive insects might differ and males can disperse much farther than females (Cardé, 1979). High release rates, thus, may lure males from a considerable distance, thereby causing migration of populations from outside the investigated area. Therefore, such trap catches may not accurately reflect the respective population density and infestation level.

Rubber septa are the most common dispensers for controlled pheromone release currently used in monitoring traps (Daterman, 1982; Vrkoč et al., 1988). However, they do not release pheromones at a constant rate over time or at different temperatures or wind speeds (Zeoli et al., 1982; Cardé and Elkinton, 1984; Vrkoč et al., 1988; Horák et al., 1989; Streinz et al., 1993; Svatoš, unpublished data). The evaporation rate of pheromones from the red rubber septa used in our study decreased over 50% within 14 days of exposure (data not shown). Therefore, periodic replacement was necessary to ensure trap efficacy. Replacement was also required because pheromones with conjugated double bonds are generally less stable and undergo photooxidation and isomerization (Ideses et al., 1982; Brown and McDonough, 1986). Isomeric purity is critical for attraction in some species (Vrkoč et al., 1988; Horák et al., 1989). Apparently this is not the case with *C. ohridella*, where addition of the *ZE*, *ZZ* and *EE* isomers of 8*E*10*Z*-14:Al does not significantly affect the attractiveness for males of the *EZ* isomer.

The circadian rhythm observed in male flight activity corresponds closely to the circadian rhythm of calling behavior in females (Svatoš et al. 1999a). The dynamic of pheromone trap catches confirmed two to three generations of *C. ohridella* in Prague. The data show that the abundance of *C. ohridella* does not increase dramatically during the season as was originally expected (Deschka,

1993, 1995). The second generation was the most abundant, while the third generation, when present, was much smaller and did not reach the level of the first one. Paradoxically, the number of generations was lower in the warmer year, 2000, than in the following, colder 2001. One factor that could be responsible for this effect might be food availability. A significant increase in larval mortality was reported when mines on a leaf started to coalesce (Heitland and Freise, 2001). In 2000, the infestation level was much higher, and the second generation might have experienced considerable food shortage during development (due to mine coalescence), resulting in a higher ratio of pupae entering diapause and, thus, elimination of a significant third generation.

The number of *C. ohridella* generations found by pheromone trapping differs from the data of Skuhravý (1998), who reported four to five overlapping generations from the Czech Republic. However, the existence of so many generations per year is unlikely considering the time required for egg, larval, and pupal development (Heitland et al., 2000; Grabenweger and Grill, 2000; Laštůvka and Šefrová, unpublished observations). On the other hand, our phenological data agree with those reported from other areas in Europe (Deschka and Dimić, 1986; Pschorn-Walcher, 1994; Hellrigl, 1999; De Prins and Puplesiene, 2000).

In summary 8*E*10*Z*-14:Al was confirmed as the main pheromone component in *Cameraria ohridella*. No minor components were found. The scope and limitations of pheromone sticky traps were discussed. Up to three generations were detected in the Czech Republic by means of trapping of males.

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PRESENCE OF LONG-LASTING PERIPHERAL ADAPTATION IN OBLIQUE-BANDED LEAFROLLER, *Choristoneura rosaceana* AND ABSENCE OF SUCH ADAPTATION IN REDBANDED LEAFROLLER, *Argyrotaenia velutinana*

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Abstract—Pre-exposure of male oblique-banded leafrollers, *Choristoneura rosaceana* (Harris), to the main component of their pheromone blend and traces of its geometric isomer (*Z*11–14:Ac and *E*11–14:Ac, respectively) at 36 ± 12 ng/ml air for durations of 15 and 60 min in sealed Teflon chambers with continuous air exchange significantly reduced peripheral sensory responses to these compounds as measured by electroantennograms (EAGs). The EAG responses of *C. rosaceana* to all tested dosages of pheromonal stimuli and blank controls were lowered by 55–58% and made a linear recovery to 70–100% of the pre-exposure amplitude within 12.5 min at a rate of 3–4 %/min. Exposures of 5 min were insufficient to maximally adapt *C. rosaceana*; however, exposures of 15 and 60 min reduced sensory responsiveness to the same minimum. In contrast, EAG responses of redbanded leafroller, *Argyrotaenia velutinana* (Walker), after identical pheromone exposure for 5 and 60 min yielded no long-lasting peripheral sensory adaptation as measured by EAGs, even though this species shares the same main pheromone components with *C. rosaceana*. We postulate that the long-lasting peripheral adaptation observed for *C. rosaceana* is a mechanism that impedes central nervous system habituation in this species. In contrast, *A. velutinana* may be more susceptible to central nervous system habituation because it lacks the capacity for minutes-long adaptation. We propose that long-lasting adaptation may be a mechanism explaining some of the variation in efficacy of pheromone-based mating disruption across taxa.

Key Words—Long-lasting adaptation, adaptation chamber, electroantennogram, NO/cGMP signal transduction pathway.

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INTRODUCTION

Pheromone-based mating disruption for economically important lepidopteran pests has been successfully demonstrated and promises to make important contributions to biorational pest control (Cardé et al., 1975; Deland et al., 1994; Cardé and Minks, 1995). However, in certain cases, utility of pheromone mating disruption may be limited by such factors as high population densities of moths (Schmitz et al., 1995a; Weissling and Knight, 1996; Suckling and Angerelli, 1996; Knight and Turner, 1999) that increases competition between calling females and pheromone dispensers; migration of mated females into treated areas; variable canopy structure; wind direction, which affects pheromone plume structure (Cardé and Minks, 1995) and retention; and the specific tuning of the pheromone blends employed (Pfeiffer et al., 1993; Knight et al., 1998; Knight and Turner, 1999; Evenden et al., 1999a). A challenge for pest managers is to determine which pests are most conducive to management by pheromones as opposed to other strategies.

The most popular hypotheses concerning mechanisms for disruption of pheromone-based communication are: (1) sensory adaptation at the peripheral level affecting olfactory receptors, (2) habituation affecting processing of and normal responsiveness to olfactory information reaching the central nervous system, (3) camouflage of female-produced plumes, and (4) false-trail-following of synthetic pheromone plumes by male moths (Rothchild, 1981; Bartell, 1982; Cardé, 1990).

The effects of short and prolonged exposures of moths to their species-specific synthetic pheromones and geometric isomers have been the targets of various investigations (Bartell and Roelofs, 1973; Bartell and Lawrence, 1976a–c; Linn and Roelofs, 1981; Sanders, 1985). These and other studies established that prolonged exposure to pheromone decreased such male behavioral responses as wing fanning and rapid walking (Bartell and Roelofs, 1973), recapture rates in mark–release studies, and orientation in wind tunnels (Rumbo and Vickers, 1997; Daly and Figueredo, 2000). For *Trichoplusia ni* (Hübner), these effects occurred with no corresponding decrease in responses of olfactory receptor neurons as measured by EAGs (Kuenen and Baker, 1981). In a later study, male *Cydia* (=*Grapholita*) *molesta* (Busck) exhibited days-long habituation after exposure to its pheromone (Figueredo and Baker, 1992). Such studies provide evidence that habituation of the central nervous system can be an important mechanism for observed decreases in males' responsiveness to female pheromones under conditions of mating disruption. In contrast, other studies suggest that adaptation and habituation, although inducible, had no influence on the effectiveness of mating disruption in the field (Novak and Roelofs, 1985; Schmitz et al., 1995a,b, 1997). The main explanations proposed for mating disruption of these species are competition between females and pheromone dispensers as well as camouflage of female pheromone signals.

The recent review by Zufall and Leinders-Zufall (2000) has formally defined three distinct types of olfactory adaptation, using vocabulary likely to be adopted for animals generally. The categories are characterized by differing temporal dynamics. The two short-lived variants have onset times on the order of 100 msec and 4 sec and corresponding recovery times of 10 sec and 1.5 min, respectively. The third type of adaptation is termed "long-lasting"; onset occurs after repetitive exposure for 25 sec and subsequent recovery intervals are about 6 min. As shown by Zufall and Leinders-Zufall (2000), these three types of adaptation are further distinguished by separate molecular mechanisms. For example, in salamander (*Ambystoma tigrinum*) olfactory receptor neurons, the long-lasting adaptive effect depends on the carbon monoxide (CO)/cGMP second messenger system, and can be uncoupled from excitation and completely eliminated by inhibitors of CO synthesis (Zufall and Leinders-Zufall, 1997). Likewise, elevated concentrations of cGMP were observed in moth antennae [*Heliothis virescens* (F.)], after application of high doses of pheromone (Boekhoff et al., 1993). Furthermore, Boekhoff et al. (1993) established that the IP_3 pathway mediates the primary transduction of pheromone signaling in antennal neurons, whereas activation of the cGMP-cascade is a secondary effect thought to be involved in adaptation and tuning of receptor neuron sensitivity.

Few studies have characterized sensory adaptation with the intention of distinguishing long-lasting versus short-lived variants of peripheral adaptation. Kuenen and Baker (1981) documented a short-lived form of pheromonal adaptation in *T. ni* using EAG; full receptor cell recovery occurred within 1 min of exposure.Schmitz et al. (1997) characterized longer-lasting sensory adaptation in *Lobesia botrana* (Denis and Schiffermüller), from which EAG responses returned to 70% of their pretreatment amplitude after 5 min.

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris), and the redbanded leafroller, *Argyrotaenia velutinana* (Walker), share the major components of their pheromone blends: *Z*11–14:Ac and *E*11–14:Ac in a 98:2 ratio for *C. rosaceana* and 93:7 ratio for *A. velutinana* (Roelofs and Arn, 1968; Roelofs and Tette, 1970; Cardé and Roelofs, 1977; Hill and Roelofs, 1979). *A. velutinana* is reported to be easily disrupted, in some cases using only the main pheromone component, *Z*11–14:Ac (Novak et al., 1978; Reissig et al., 1978; Novak and Roelofs, 1985; Miller et al., unpublished data). In contrast, *C. rosaceana* is often described as difficult to disrupt in the field as measured by lowered captures of males by synthetically baited traps, fruit and foliar damage, and mating reductions of tethered females (Novak et al., 1978; Reissig et al., 1978; Roelofs and Novak, 1981; Deland et al., 1994; Agnello et al., 1996; Lawson et al., 1996; Miller et al., unpublished data) and possibly requiring the full natural blend of pheromone components. However, populations of *C. rosaceana* from Western Canada and Washington state, USA, which are characterized by a slightly different blend of pheromone components compared with those from central and Eastern North America (Vakenti et al.,

1988; Thomson et al., 1991), have shown some potential for successful mating disruption in small-plot trials (Knight et al., 1998; Evenden et al., 1999b,c).

We seek to understand the mechanisms underlying the differences in susceptibility to mating disruption in these two sympatric tortricids. In this first paper of a series, we characterize the differences in the capacity for long-lasting peripheral adaptation and disadaptation in *C. rosaceana* and *A. velutinana* using EAG measurements performed on moths before and after exposure to pheromone for various time intervals.

METHODS AND MATERIALS

Insect Colonies. C. rosaceana were drawn from a 4-year-old laboratory colony originally collected as first and second generation pupae from apple orchards in Southwestern Michigan. *A. velutinana* came from a long-established laboratory colony maintained at Geneva, New York, USA, by W. Roelofs. Both species were reared at 24°C on a pinto bean diet (Shorey and Hale, 1965) under a 16L : 8D photoperiod. Male pupae of each species were segregated in 1-liter plastic cages containing a 5% sucrose solution in plastic cups with dental cotton wick protruding from their lids.

Electroantennograms. Our EAG system consisted of a data acquisition interface board (type IDAC-02) and universal single-ended probe (Type PRS-1) from Syntech (Hilversum, The Netherlands). The recording and indifferent electrodes consisted of silver-coated wire in glass micropipets (10 μl microhematocrit capillary tubes) containing 0.5 M KCl. Micropipets were prepared by a Flaming/Brown micropipet puller (model P-97, Sutter Instrument Co.). The pipets were pulled at 308°C under time and velocity settings of 150 and 80, respectively. EAG data were recorded, stored, printed, and quantified using a Gateway 2000 (P-75) computer equipped with an interface card and software (PC-EAG version 2.4) from Syntech. The interface card contained a software-controlled amplifier, and an A/D conversion circuit; it operated with 12-bit resolution.

Male insects of both species were 2–4 days old when used for electroantennograms. EAGs were measured as the maximum amplitude of depolarization elicited by the applied stimulus. EAGs were conducted on live-insect preparations (Figure 1). Insects were restrained on a wax-filled, 3.5-cm-diam. Petri dish by placing clay (10 × 3 mm) over their thorax and abdomen. The terminal two segments of the antenna destined for recording were removed with fine scissors and the recording electrode was placed over the severed end. The reference electrode was inserted into the neck (Figure 1).

Stimulus Delivery. This apparatus consisted of a glass Y-tube (each arm 2 cm in length, the base 1 cm long, 0.5 cm diam.) positioned approximately 5 mm from the antenna (Figure 1). Carbon-filtered and humidified air (50 ml/min) was delivered

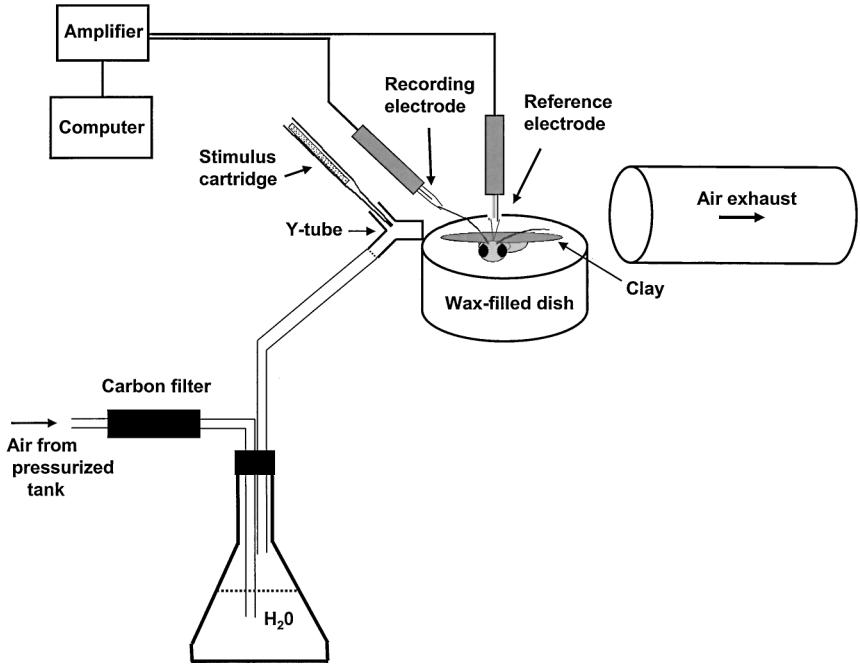

FIG. 1. Design of electroantennogram recording and stimulus delivery apparatus. Insect and electrodes are enlarged.

continuously into one arm of the Y-tube via Tygon tubing, while pheromone stimuli were delivered through the second arm of the Y-tube. *Z*11–14:Ac (lot # 10010) was obtained from Shin Etsu (Tokyo, Japan) and purity was determined with GLC (96.1% *Z*11–14:Ac and 3.9% *E*11–14:Ac). Various concentrations of this mixture of pheromone in hexane (20 μl total solution) were pipetted onto 1.4- $\times$ 0.5-cm strips of Whatman No. 1 filter paper. After 5 min in a fume hood for solvent evaporation, treated strips were inserted into disposable glass Pasteur pipets, sealed with Parafilm, and allowed to equilibrate for 24 hr prior to use. A given stimulus pipet (cartridge) was inserted into the Y-tube such that its tip was positioned at the junction of the Y-tube and 1.5 cm from antennal preparations (Figure 1). Stimulus puffs (1 ml) were generated through the cartridges with a clean hand-held 20-ml glass syringe connected to the pipets with a 1-cm piece of Tygon tubing. The time interval to expel 1 ml of stimulus odor or clean air from the syringe was quantified with video-cinematography and slow-motion playback. The 1-ml puff of air was expelled from the syringe in 120 ± 0.02 (SD) msec ($N = 20$).

Adaptation Experiments. Prior to adaptation experiments, dose–response curves were obtained for both moth species. Pheromone dosages were delivered

to individual moths (N10) either in ascending or descending order. Four puffs of each dosage spaced 10 sec apart were administered to yield duplicate depolarization amplitudes at each dosage. Appropriate dosages were chosen for further experimentation and the ascending order of stimulus–dose application was employed in all later studies.

To test for inducement of adaptation, males of both species were placed in adaptation chambers consisting of 1-liter Teflon transfer containers (Jensen, Coral Springs, Florida, USA) equipped with two 0.64-cm ports in their lids (Figure 2). Glass inlets and outlets were affixed to the lids, allowing for carbon-filtered air (30 ml/min) to pass through the chambers. Chambers were divided with wire mesh (Figure 2), such that insects were confined in the upper halves while a rubber septum impregnated with 5 mg (solvent-free) of the same pheromone blend used in EAG cartridges was placed in the lower half (Figure 2). This arrangement was designed to reduce variation in pheromone exposure relative to that possible in a static container, where moths might touch the dispenser.

All chambers were allowed to equilibrate for 15 min prior to insertion of insects. To assay the onset of peripheral adaptation, EAGs were performed on both species ($N \geq 18$) after confinement for 5, 15, or 60 min. Sham treatments ($N \geq 18$) were administered in separate, pheromone-free chambers. EAGs were performed on all insects prior to confinement, exactly 1 min after confinement, and then 12.5 or 60 min postconfinement.

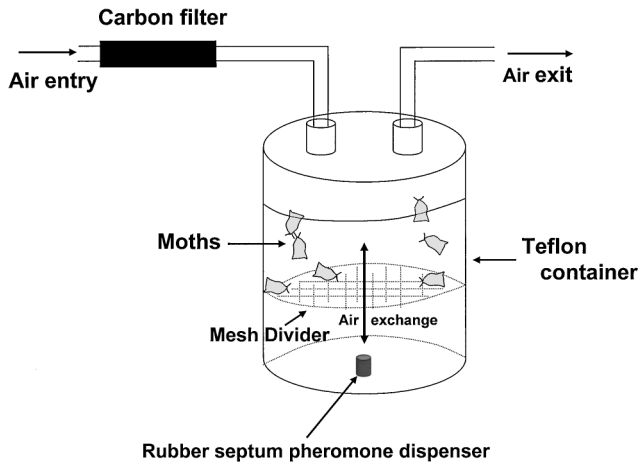

FIG. 2. Adaptation chamber: 1-liter Teflon container with glass inlet and outlet. Wire mesh divider prevents insects from contacting pheromone dispenser but allows exchange of air at throughput of 30 ml/min.

Disadaptation Study. Because minutes-long adaptation did not occur in *A. velutinana* (See Results below), only *C. rosaceana* was used for characterization of disadaptation. EAGs were performed on groups of six male *C. rosaceana* prior to confinement in adaptation chambers for 15 min of continuous pheromone exposure. After exposure, entire groups were removed from adaptation chambers and placed into clean-air containers. Individuals were assayed 1, 2.5, 5, 7.5, 10, 12.5, and 30 min thereafter. A total of 7 groups of six individuals was assayed in this manner.

Pheromone Concentration in Adaptation Chamber. Rubber septa impregnated with 5 mg of pheromone were placed into adaptation chambers and allowed to equilibrate for 15 min with air flowing through at a rate of 30 ml/min, as previously described. After equilibration, the exhaust port was replaced with a port sealed with a clean rubber septum. Immediately following port replacement, 15 ml of air was withdrawn from adaptation chambers through the septum-sealed port into a 20-ml glass syringe fitted with a 22-gauge needle. In rapid succession, 5 ml of hexane wash containing an internal standard of saturated 14:Ac at 6.4 ng/μl was drawn into the syringe. The solution within the syringe was carefully shaken for 30 sec, then expelled into a gas chromatography vial. This entire procedure was repeated 5 times with separate pheromone-impregnated septa. Prior to analysis, samples were concentrated under nitrogen by a factor of 50. Samples of 1 μl were analyzed by gas chromatography (HP-6890, Hewlett-Packard Co.) with flame ionization detection (FID) to measure the concentration of pheromone present in adaptation chambers. The GC was fitted with a DBWAXETR polar column (model 122-7332, J&W Scientific, Folsom, California, USA) 30 m long and 250 μm internal diam. The initial GC temperature was held at 100°C for 3 min and the program ran at a rate of 10°C/min from 100 to 250°C and was held for 3 min; the carrier gas was He. We calculated nanograms of pheromone present in the 15 ml of adaptation chamber air by multiplying the ratio of peak areas of the target compound (*Z*11–14:Ac) over the standard (saturated 14:Ac) by: (1) nanograms of internal standard present in 1 μl of concentrated hexane wash, and (2) 100 to account for GLC analysis of only 1% of the total concentrated sample.

The dosage of pheromone in adaptation chambers relative to pheromone cartridges was also compared to that of EAG cartridges. Adaptation chambers containing rubber septa impregnated with 5 mg of pheromone and equilibrated for 15 min were used as stimulus cartridges in EAG-assays of both *C. rosaceana* and *A. velutinana;* $N = 10$ for both species. EAGs were also carried out on individuals of both species ($N = 10$) using control chambers lacking pheromone. Chambers were modified by replacing the glass exhaust tube with a 5-cm Teflon tube. The in-current port was disconnected from the continuous airflow 1 min prior to assay and connected to a 20-ml glass syringe via a 25 cm piece of Tygon tubing. The Teflon exhaust tube was positioned ca. 1 cm from antennal preparations and 1-ml puffs were delivered through the chambers.

Statistical Analysis. Data were subjected to analysis of variance (ANOVA) and differences in pairs of means over time and between treatments were separated using Tukey's multiple comparisons test (SAS Institute, 1989). The relationship between percent recovery from adaptation and time interval after exposure to pheromone was analyzed using linear regression. All values are ± SEM unless otherwise designated.

RESULTS

EAG Dose–Response Curves. Profiles for both species are presented in Figure 3 as unnormalized millivolt responses in both ascending and descending orders of stimulus-dosage applied. Under both regimes, responses of the two species overlapped for pheromone dosages 0–2.0 μg (Figure 3). Beginning at 20 μg, the dosage–response curves diverged; *A. velutinana* consistently produced higher EAG amplitudes at the high dosages than did *C. rosaceana.* Under the ascending regime, the mean responses reached a plateau of ca. 6 and 5 mV for *A. velutinana* and *C. rosaceana*, respectively, at the 200-μg dose. The mean EAG amplitudes were significantly ($P < 0.05$) different at the 200-μg and 2-mg dosages. Quite linear dosage–response curves were observed when dosages were applied in descending order, and the divergence in EAG amplitude between species was significantly ($P < 0.05$) different at the 200-μg, 2-mg, and 20-mg dosages. The maximum mean millivolt response for a given species was consistent irrespective of the order for dosage presentation (Figure 3).

Pheromone Exposure Studies. EAG amplitudes of male *C. rosaceana* prior to and 1 min after 5 min of confinement in adaptation chambers were not different

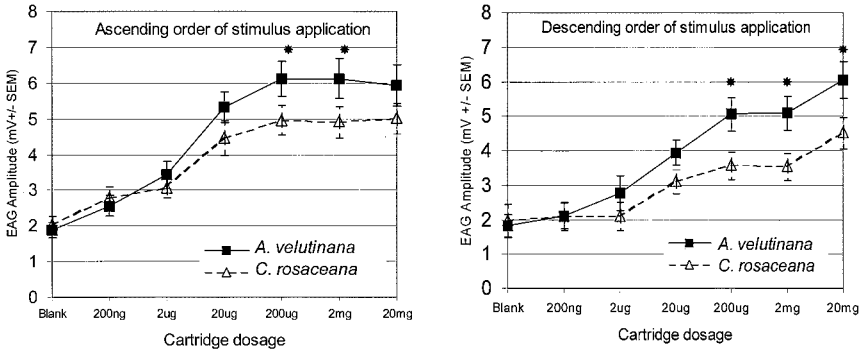

FIG. 3. Dose–response relationships for *Choristoneura rosaceana* and *Argyrotaenia velutinana* live-insect antennal prepartions in both ascending and descending orders of stimulus application. *Significant differences between pairs of means ($P < 0.05$).

(Figures 4A and 5A). However, EAG responses to the 200-μg and 2-mg dosages were ($P < 0.05$) lower in *C. rosaceana* assayed 1 min after 15 min of confinement compared with their pre-exposure responses (Figures 4B and 5B). The mean EAG responses of these individuals returned to their pre-exposure amplitudes within 30 min after exposure (Figures 4B and 5B). *C. rosaceana* exposed for 60 min in adaptation chambers produced lower mean EAG amplitudes 1 min after confinement compared with their pre-exposure responses to the 2-μg, 200-μg, and 2-mg doses (Figures 4C and 5C) ($P < 0.05$). After a 60-min post-exposure interval in clean air, the mean EAG responses of *C. rosaceana* exposed for 60 min were not different from their pre-exposure amplitudes for all dosages except 2 mg (Figures 4C and 5C).

EAG responses of *A. velutinana* prior to exposure in adaptation chambers and after exposure for 5- and 60-min durations were not different at any of the stimulus dosages applied (Figures 4E, F, and 5D).

Disadaptation Study. C. rosaceana EAG amplitudes after 15 min of exposure were lower compared with pre-exposure amplitudes for the 200-μg and 2-mg doses at 1, 2.5, and 5 min after exposure (Figures 4D) ($P < 0.05$). At 7.5 min after exposure, the mean EAG amplitudes were no longer different from pre-exposure amplitudes and responses returned to amplitudes nearly equal to pre-exposure responses within 12.5 min after exposure (Figure 4D). The mean EAG amplitudes elicited by the blank and 2-μg doses also decreased after 15 min of exposure and followed a similar trend of increase after exposure; however, the mean differences were not significant in simple pair-wise comparisons. A significant ($P < 0.05$) linear relationship was found in the percent recoveries of the mean EAG responses elicited by each dosage including the blank when regressed over time after 15 min of exposure in adaptation chambers (Figure 6). Percent recovery was calculated as the ratio of the mean EAG amplitude at the various time intervals at which individuals were assayed after exposure over the mean pre-exposure amplitude. The process of recovery was decidedly linear; recovery rate was a constant 3–4%/min and was nearly complete within 12.5 min (Figure 5).

Pheromone Concentration in Adaptation Chamber. The retention times for the 14:Ac (internal standard) and *Z*11–14:Ac were 13.7 ± 0.012 and 14.3 ± 0.008 min, respectively. The trace amount of *E*11–14:Ac present in the pheromone used to impregnate the rubber septa was undetected by FID in the air samples taken from adaptation chambers. The mean peak areas in thousands for the 14:Ac standard and *Z*11–14:Ac were 454 ± 28 and 8 ± 3, respectively. The calculated concentration of *Z*11–14:Ac present in our adaptation chambers was 36 ± 12 ng/ml of air.

For *C. rosaceana*, adaptation chambers containing pheromone elicited higher EAG responses (2.5 ± 0.24 mV) compared with depolarizations elicited by control chambers lacking pheromone (1.1 ± 0.13 mV) ($P < 0.05$). The mean amplitude elicited by the adaptation chamber closely corresponded to the mean response

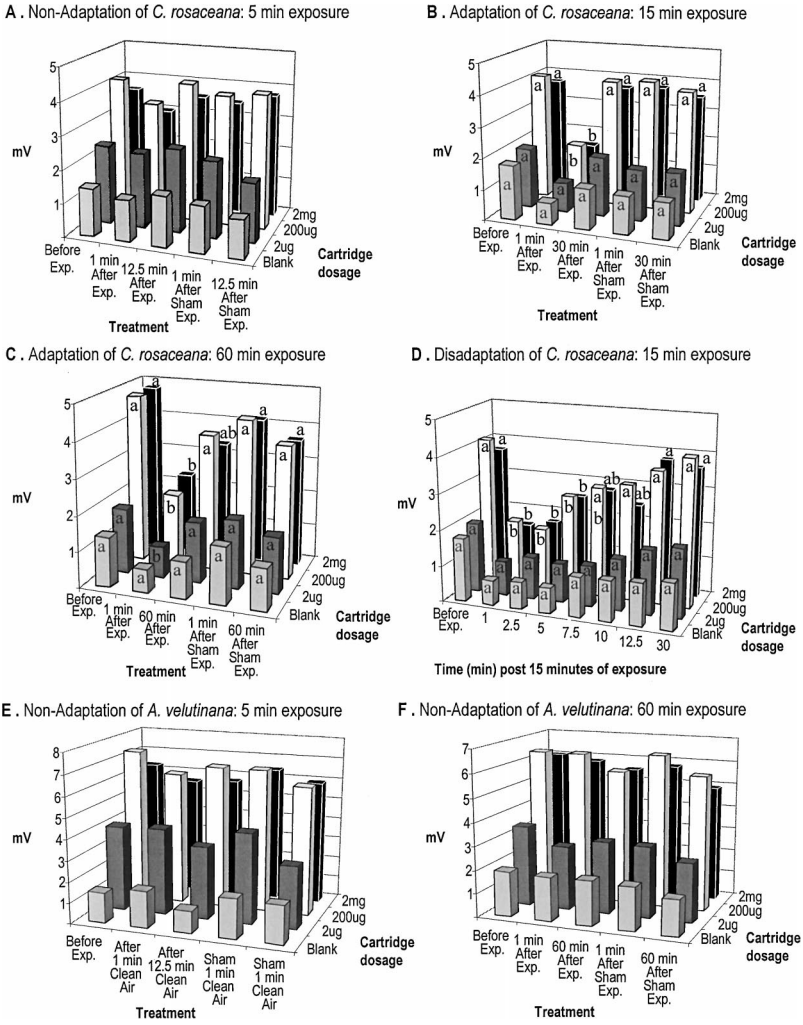

FIG. 4. (A) Effect of 5 min of confinement of *Choristoneura rosaceana* in adaptation chambers. There were no significant differences between treatment means for responses within or across dosages. (B) Effect of 15 min of confinement of *C. rosaceana* in adaptation chambers. Bars with different letters indicate significant differences between treatment means within a given dosage. (C) Effect of 60 min of confinement of *C. rosaceana* in adaptation chambers. Bars with different letters indicate significant differences between treatment means within a given dosage. (D) Disadaptation of *Choristoneura rosaceana* after 15 min of confinement in adaptation chambers. Bars with different letters indicate significant differences between treatment means within a given dosage. (E and F) Effect of 5 and 60 min, respectively of confinement of *Argyrotaenia velutinana* in adaptation chambers. There were no significant differences between treatment means for responses within a given dosage.

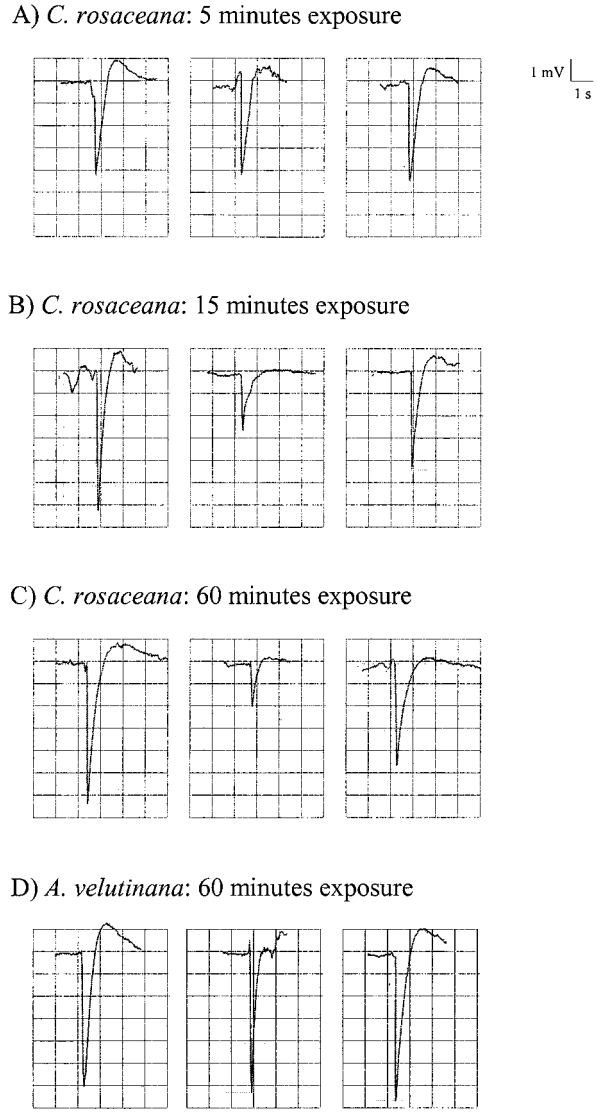

FIG. 5. Representative EAG tracings of *Choristoneura rosaceana* and *Argyrotaenia velutinana* responding to 1-ml puffs from pheromone cartridges loaded with 200 μg of pheromone. Tracings on the left within each row are responses of *C. rosaceana* or *A. velutinana* prior to exposure in adaptation chambers. Center tracings within each row are responses of the same individuals after various exposure intervals within adaptation chambers. Tracings on the right within each row were measured after 30- to 60-min intervals within pheromone-free air after exposure in adaptation chambers. Each horizontal tick mark represents 1 sec and each vertical tick mark represents 1 mV of depolarization.

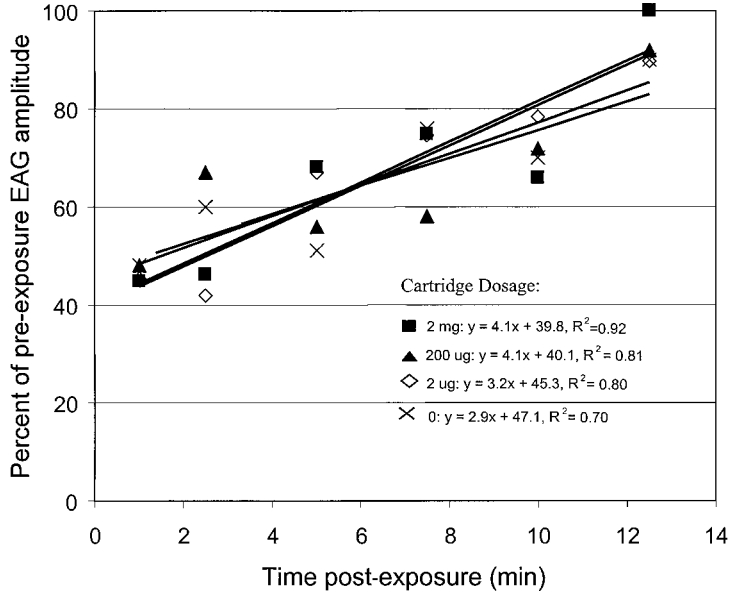

FIG. 6. Linear regressions of percent recovery of EAG responses of *Choristoneura rosaceana* after 15 min of confinement in adaptation chambers over time. Percent recovery was calculated as the ratio of the mean EAG amplitude at the various time intervals at which individuals were assayed post-exposure over the mean pre-exposure amplitude. All regressions were significant at $P < 0.01$.

generated by our stimulus cartridges at the 200-ng dosage (Figure 3). For *A. velutinana*, a response of 4.1 ± 0.4 mV was generated by the adaptation chamber with pheromone, which was also higher than the responses elicited by control chambers lacking pheromone (1.0 ± 0.13 mV) ($P < 0.05$). For this species, the adaptation chambers induced a response that closely corresponded to responses generated by stimulus cartridges charged with the 2-μg dosage (Figure 3).

DISCUSSION

Dynamics of Long-Lasting Adaptation in C. rosaceana. Pre-exposure of male *C. rosaceana* to the main components of its pheromone blend (*Z*11- and *E*11–14:Ac) decreased EAG responses to the pheromone for up to 10 min after exposure (Figure 4D). Assuming that an EAG consists of depolarization of receptor potentials summed across the antennal olfactory neurons (Roelofs, 1984), the observed decrease in response by *C. rosaceana* can be attributed to decreased sensitivity of pheromone receptor neurons. The EAG response of pre-exposed *C. rosaceana* to our stimuli was reduced by 55–58% for all cartridge dosages tested including

the blank (control). Responses returned in a linear fashion to 70–100% of the pre-exposure response within 12.5 min (Figure 6). These results establish that the process of recovery from adaptation took place at a constant rate. At our exposure dosage of 36 ± 12 ng/ml, exposures of only 5 min were not sufficient to induce adaptation in *C. rosaceana*; however, exposures of 15 and 60 min reduced sensory responsiveness to the same degree (60%). Given that exposure duration of 60 min did not increase adaptation, it seems that a plateau was reached at or even before 15 min. Currently, we have no physiological explanation for why long-lasting adaptation in *C. rosaceana* peaks at 60%. Perhaps the titer of some inhibitory signaling agent (see below) is set so as not to turn off signaling transduction completely. Alternatively, we have not ruled out the possibility that certain populations of receptors adapt completely while others do not. Given the adaptive significance of male sensitivity to female pheromone, it would seem disadvantageous for male *C. rosaceana* to become completely anosmic to its pheromone. Characterization of the dose–response relationship and threshold for long-lasting adaptation in *C. rosaceana* will be treated further in a subsequent publication.

Similarities of C. rosaceana Adaptation Response to Those of Other Moth Species. By performing recordings from single antennal neurons, Baker et al. (1989) showed that male *Agrotis segetum* (Schiffermüller) olfactory receptor neurons adapted when they were exposed to high pheromone concentrations known to cause in-flight arrestment of progress toward the source. Using the same technique, they also showed that antennal neurons from *H. virescens* failed to adapt regardless of concentration. Baker et al. (1989) proposed that, given the low emission rate of *Z*11–16:Ald from the rubber septa employed in their study, it was unlikely that *H. virescens* neurons were challenged to the same degree as *A. segetum* had been by the more volatile pheromone of that species. Alternatively, we suggest that *A. segetum* and *H. virescens* may differ in their susceptibility to peripheral sensory adaptation, as we have observed with *C. rosaceana* and *A. velutinana*. Specifically, certain species, such as *C. rosaceana* and *A. segetum*, may exhibit greater capacity for sensory adaptation at the peripheral level than others such as *A. velutinana* and possibly *H. virescens.* Other electrophysiological studies have also demonstrated differential degrees of peripheral adaptation among moth species (Kuenen and Baker, 1981; Schmitz et al., 1997).

Molecular Basis of Long-Lasting Adaptation. Adaptation of moth olfactory receptor neurons has long been recognized to occur following intense pheromonal stimulation, be it constant or pulsed (e.g., Baker et al., 1989; Figuerdo and Baker, 1992); however, delineation of the particular types of adaptation as recently defined by Zufall and Leinders-Zufall (2000) has just begun. Both invertebrate and vertebrate animal models reveal that both rapid and slower, longer-lasting forms of odor adaptation exist (Getchell, 1986; Marion-Poll and Tobin, 1992). The molecular basis and temporal dynamics of some of these differences has recently been described (Zufall and Leinders-Zufall, 2000). The rapid forms of adaptation result

from Ca^{2+}-dependent cyclic nucleotide-gated (CNG) ion channel modulation and Ca^{2+}/calmodulin kinase II-dependent phosphorylation, respectively. In contrast, long-lasting adaptation is mediated by the carbon monoxide (CO)/cGMP second messenger system. Thus, odor adaptation is a complex phenomenon mediated by diverse molecular processes; these may differ within and among taxa depending on the nature of the signal-transduction cascades mediating olfaction in those species. Boekhoff et al. (1993) showed that in moth antennae cGMP formation is a consecutive reaction to pheromone-induced elevation in IP_3 concentration. Consistent with this interpretation, exogenously applied cGMP abolished the phasic but not the tonic component of the pheromone-stimulated IP_3 signal in *H. virescens* [Figure 5B of Boekhoff et al., (1993)]. Sustained pheromonal stimulation is also known to induce cGMP signals in *Antheraea polyphemus* and *Bombyx mori* (Ziegelberger et al., 1990), and pheromone-activated cation channels sensitive to cGMP have been found in insect olfactory cilia (Zufall and Hatt, 1991). Furthermore, an IP_3–mediated increase in intracellular Ca^{2+} could be the direct stimulus for shifts in Ca^{2+}/calmodulin interactions or, alternatively, could activate NO synthase, leading to the generation of NO and subsequently activating cytoplasmic guanylyl cyclase (Steinlen et al., 1990; Boekhoff et al., 1993). Thus, as proposed by Zufall and Leinders-Zufall (2000), there is good reason to believe that insects and vertebrates share parallel mechanisms yielding long-lasting adaptation. The NO/cGMP pathway has been implicated in odor processing in vertebrates and invertebrates (Breer et al., 1992; Boekhoff et al., 1993) and should be considered along with the (CO)/cGMP second-messenger system as possible mechanisms leading to the long-lasting form of adaptation such as that observed in *C. rosaceana* in the present study. Pharmacological inhibitors of CO and NO formation (Zufall and Leinders-Zufall, 1997) will be useful in testing this hypothesis.

Adaptation to Blank Air Puffs. Throughout this study, blank (negative control) puffs of clean air elicited EAG responses of 1–2 mV from both tortricids when applied from either clean disposable Pasteur pipets or clean adaptation chambers (Figure 3), and adaptation resulted in reduced responses to both pheromonal and clean air (control) stimulus puffs (Figure 4). We took utmost care to assure that there was no pheromone contamination in controls. Pioneer studies on *B. mori* showed that EAG depolarizations occur in response to blank (control) puffs of clean pheromone-free air (Schneider 1962, Figures 2 and 3) and more contemporary studies using *A. velutinana* also showed EAG responses to 1-ml puffs of clean air (Baker and Roelofs 1976, Figure 3); however, these responses were smaller than in the present study. Mayer et al. (1984) also obtained pronounced EAG responses to their blank control stimulus puffs and proposed that stimuli such as water vapor, extraneous room contaminants, or delivery-line plasticizers may have been responsible for eliciting these responses. If the blank responses in our study were due to such extraneous contaminants, then adaptation to pheromone may

have resulted in cross-adaptation to other unknown chemical or physical stimuli. In addition, the 1-ml puffs of air momentarily trembled the filiform antennae of both species, and such movement of the antennae may have added to the apparent EAG depolarization.

Proposed Impacts of Long-Lasting Adaptation on Susceptibility to Pheromone Disruption. Exposure of *A. velutinana* to the components of its pheromone blend results in distortion and inhibition of the normal sexual response (Bartell and Roelofs, 1973). However, our results indicated that long-term exposure of *A. velutinana* to the main component of its pheromone blend and the geometric isomer had no effect on peripheral sensitivity even 1 min after exposure. Our results with *A. velutinana* are similar to those of Kuenen and Baker (1981), who showed that adaptation of receptor response in *T. ni* was also short-lived and returned to control levels 1 min after cessation of pheromone stimulation. In both cases, sensory processes at the peripheral level appear not to have declined after pheromone exposure, whereas central nervous system habituation appears to be the longer lasting and fundamental cause of sexual response-inhibition. Long-term habituation has also been shown to occur with *C. molesta* in wind-tunnel trials (Figueredo and Baker, 1992) and in field experiments (Rumbo and Vickers, 1997). Similarly, wind-tunnel and field experiments using *H. virescens* implicated central nervous system habituation, lasting as long as 96 h, as the major mechanism for modulating male moth response to female pheromone and as the underlying means for pheromone-based mating disruption (Daly and Figueredo, 2000).

Bartell and Lawrence (1977) suggested that male moth exposures to pulsed pheromonal stimuli would result in greater reductions of sexual response compared with constant stimulation, because peripheral adaptation would be circumvented so as to allow for greater central habituation. Kuenen and Baker (1981) obtained data supporting this hypothesis for *T. ni* by showing that pulsed rather than constant pre-exposure resulted in greater disorientation. They also demonstrated decreased EAG amplitudes with concurrent exposure, indicating that receptor adaptation was taking place. They concluded that receptor adaptation might have been an impediment for central nervous system habituation. Therefore, we postulate that the long-lasting peripheral adaptation documented in this study for *C. rosaceana* could be a mechanism precluding central nervous system habituation in this species and reducing susceptibility to pheromone-based mating disruption, as observed in field studies (Novak et al., 1978; Reissig et al., 1978; Roelofs and Novak, 1981; Deland et al., 1994; Agnello et al., 1996; Lawson et al., 1996; Gut and Miller, unpublished data). In contrast, *A. velutinana*, which is easily disoriented in lab and field studies (Bartell and Roelofs, 1973; Cardé et al., 1975; Novak et al., 1978; Reissig et al., 1978; Novak and Roelofs, 1985; Gut and Miller, unpublished data), may be more susceptible to central nervous system habituation, as it appears to lack the capacity for long-lasting peripheral adaptation. In addition, it is possible that the onset of long-lasting adaptation in *C. rosaceana* may result in a decrease in perception of

the active space of synthetic pheromone point sources and, therefore, a decreased inclination for false-trail-following. The opposite effect would be expected for *A. velutinana*, resulting in sustained false-trail-following or pheromone-induced excitation and arrestment precluding movement into zones of pheromone free air.

The concentration of pheromone per milliliter of air in our adaptation chambers was judged high, as it was within the range of pheromone present per abdominal tip extract of female *A. velutinana* (Roelofs et al., 1975; Miller and Roelofs, 1980). Future studies must be extended into the field to document whether similar adaptation takes place at pheromone concentrations realized within a pheromone-treated crop. Finally, studies employing intracellular recordings from central nervous system processing centers in the olfactory lobe (Anton and Gadenne, 1999; Gadenne et al., 2001), obtained from pheromone-exposed *C. rosaceana* and *A. velutinana*, may illuminate whether adaptation actually precludes habituation.

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DOES PHEROMONE-BASED AGGREGATION OF CODLING MOTH LARVAE HELP PROCURE FUTURE MATES?

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Abstract—In field and laboratory bioassay experiments, we show that larvae of the codling moth, *Cydia pomonella*, cocoon in aggregations. This aggregation behavior of fifth-instar larvae prior to pupation and arrestment of eclosed adult males by mature female pupae seems to allow mating as soon as an adult female ecloses. This synchronous timing is realized because foraging fifth-instar are attracted by cocoon-spinning larvae and prepupae, but not by pupae, and because male pupae develop faster than female pupae. Eclosed males are arrested by sex pheromone that disseminates from female pupae even before adult females eclose. Communication in *C. pomonella* within and among developmental stages (larva–larva and pupa–adult, respectively) may be a strategy to procure mates. If so, our data add to current knowledge that attraction of mates in insects relies on communication among adults, or pupae and adults.

Key Words—*Cydia pomonella*, larvae, pupae, aggregation behavior, aggregation pheromone, sex pheromone, mate attraction, delayed mating, mating strategy, protandry, fitness.

INTRODUCTION

Rapid acquisition of mates in short-lived adult moths may have positive fitness consequences on females or males. In gypsy moths, *Lymantria dispar*, delayed mating of females reduced the number of eggs deposited and egg viability. Moreover, with increasing age, females were less likely to mate or to receive a full

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complement of sperm than females exposed to males within the first few days after eclosion (Proshold, 1996). In beet armyworms, *Spodoptera exigua*, delayed mating by females decreased fecundity and fertility, and delayed mating by males decreased the number of spermatophores they transferred to females (Rogers and Marti, 1996). In polygamous codling moths, *Cydia pomonella*, mean fecundity, fertility, and net reproductive rate of females mated 4 days after eclosion were significantly lower than for females mated on the day of eclosion or 2 days later (Vickers, 1997). Male *C. pomonella* are able to produce up to five spermatophores over the course of their adult life (Howell et al., 1978; Howell, 1988). This leads to scramble competition among males, defined by early search for and swift location of mates (Anderson and Iwasa, 1996).

For these moth species, there may be strong selection for mechanisms that ensure the presence of potential mates at the time of eclosion or for mechanisms that facilitate rapid location of mates following eclosion. Such mechanisms would facilitate rapid mating, thereby optimizing fitness for one or both mates. One such mechanism is the attraction of mate-seeking males by mature female pupae, as reported in some butterflies (Dienert et al., 1994). An alternative mechanism may involve larval aggregation behavior prior to pupation. Adults eclosing from such aggregations would immediately be near potential mates.

Aggregations of cocooning *C. pomonella* larvae reared in a laboratory and in open-fluted corrugated cardboard bands wrapped around trunks of apple trees, *Prunus malus*, provided evidence for aggregation behavior in *C. pomonella* larvae. Here we demonstrate experimentally that *C. pomonella* larvae cocoon in aggregations, and we present experimental data strongly suggesting that these larval aggregations are part of an intricate reproductive strategy to procure mates and enhance fitness.

METHODS AND MATERIALS

Experimental Insects. Larvae were shipped from the Sterile Insect Release Program in Osoyoos, British Columbia, Canada. Final instars were removed from the diet and kept in individual Petri dishes (5.5 cm diam.) for 6 hr at 22°C under a photoregime of 16L:8D. The sex of all larvae bioassayed or employed as test stimuli was determined following pupation. Female and male pupae were distinguished by the presence of three and four dark bands, respectively, on the tip of the wing pad (Petterson, 1965).

Acquisition of Larvae- and Pupae-Derived Volatiles. To obtain larvae-derived volatiles, 40 fifth instars were placed in a horizontal Pyrex glass chamber (30 × 1.2 cm). An empty chamber served as a control. Water aspirators were used to draw humidified, charcoal-filtered air at a rate of 80 ml/min through each chamber and through a glass column (6 × 30 mm) filled with Porapak Q (50–80 mesh, Waters Associates, Inc., Milford, Massachusetts, USA). After 48 hr, volatiles were

desorbed with 2.5 ml of redistilled pentane plus ether (95:5). Larvae cocooning inside the glass chamber were rinsed with 30 ml of pentane plus ether (95:5). Extracts were concentrated under a fine stream of nitrogen.

To obtain pupae-derived volatiles, 40 newly formed female or male pupae were placed separately in Pyrex glass tubes (30×0.7 cm) maintained at 23°C and an airflow of 40 ml/min. During days 0–2, 3–5, 6–8, 9–10, 11, and 12, volatiles were captured with Porapak Q and desorbed as above. In addition, 12-day-old male and female pupae were rinsed with pentane plus ether (95:5).

Experiment 1: Evidence for Aggregation by Larvae in the Field. On August, 21, 2001, the trunks of 163 apple trees in orchards of the Tree Fruit Research and Extension Center of Washington State University (Wenatchee) were wrapped with an open-fluted, corrugated cardboard band (10 cm wide; flute width: $\sim$ 8 mm), 20–50 cm above ground. On October 21, 2001, all bands were collected and frozen (-10°C). For each band, the position of all cocoons was measured with callipers to the nearest 0.1 mm. The null hypothesis tested was that cocoons generally ($>95\%$) do not occur in aggregations.

Experiment 2: Evidence for Aggregation by Larvae in the Laboratory. Ten fifth-instar larvae were placed in the center of each of 10 plastic Petri dishes (14 cm diam.). After 24 hr, cocooning larvae were recorded as solitary, aggregates of 2–5, and aggregates of >5.

Experiment 3: Aggregation to Arrest Males. Each replicate consisted of six treatments (Table 1). In pairs of pupae, the pupa mentioned first was one day older and typically eclosed earlier, allowing us to record the adult's response to the presence of a conspecific pupa. Eclosion, arrestment, and departure of male and female adults were recorded from each jar (Figure 1A) at 12-hr intervals. Because the experiment tested the hypothesis that the presence of a mature female pupa arrests an closed adult male, replicates with paired pupae were terminated when both adults had eclosed. In treatments with a single male or female pupa, the replicate was terminated when the eclosed male or female had departed.

Experiments 4–6: Time of Aggregation. In olfactometers (Figure 1B) (Pierce et al., 1981), potential pupation sites (Table 1) were randomly assigned to one of two vials. For each replicate, a fifth instar was released in the center of the olfactometer and its pupation site recorded. All experiments were conducted in an environmental chamber at 22°C and a photoregime of 16L:8D.

Experiment 7: Timing of Pupal Development. Each of 60 fifth instars was placed in a Petri dish (5 cm diam.) at 23°C to record duration of the pupation period.

Experiments 8 and 9: Pheromone-Based Larval Aggregation. The same protocol was employed as in experiments 4–6, except that perforated plastic inserts in vials (Figure 1C) prevented physical contact of foraging larvae with test stimuli, which consisted of cocooning larvae (experiment 8) or collected volatiles of cocooning larvae (experiment 9) (Table 1). In experiment 9, aliquots

TABLE 1. SUMMARY OF EXPERIMENTS CONDUCTED WITH *C. pomonella*

Exp.	Title	Design or stimuli	Replicates (N)
1	Aggregation by larvae in the field	Open-fluted, corrugated cardboard band on tree trunk	163
2	Aggregation by larvae in the laboratory	10 larvae in Petri dish	10
3	Stimulus-dependent arrestment of eclosed adults	♂ + ♂ pupae	12
		♂ + ♀ pupae	12
		♂ pupa	12
		♀ + ♀ pupae	12
		♀ + ♂ pupae	12
		♀ pupa	12
		Test stimuli in choice experiments[a]	
4	Stimulus-dependent attraction of larva	Cardboard + cocooning larva Cardboard	33
5	Stimulus-dependent attraction of larva	Cardboard + prepupa in cocoon Cardboard	45
6	Stimulus-dependent attraction of larva	Cardboard + pupa in cocoon Cardboard	28
7	Duration of pupation period	1 fifth-instar in Petri dish	60
		Test stimuli in choice Experiments[b]	
8	Stimulus-dependent attraction of larva	Cardboard + cocooning larva Cardboard	28
9[c]	Stimulus-dependent attraction of larva	Cotton wick + volatiles from cocooning larvae Cardboard	26
10[d]	GC-EAD analyses of pupa-derived volatiles	Volatiles from ♀ or ♂ pupae	4

[a] Test stimuli could be contacted by the foraging larva.
[b] Perforated plastic inserts in vials (Figure 1C) prevented the foraging larva from contacting test stimuli.
[c] Aliquots tested were equivalent to a solvent rinse from 40 cocoon-spinning larvae plus 19 volatile hour equivalents of cocooning larvae [collected with Porapak Q and desorbed with pentane–ether (95:5)]; each treatment and control stimulus received 25 μl of solvent.
[d] Aliquots analyzed by GC-EAD were equivalent to solvent rinses from 18 female pupae, and volatiles from 35 male or female pupae collected with Porapak Q for 1 hr and desorbed with pentane–ether (95:5).

of both Porapak Q volatile extract and solvent rinses of cocooning larvae were released from cotton wicks to test their attractiveness *versus* that of a solvent control.

Experiment 10: GC-EAD Analyses of Pupae-Derived Volatiles. Aliquots of Porapak Q trapped volatile extracts from, and solvent rinse extracts of, 14-day-old male and female pupae were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), employing a

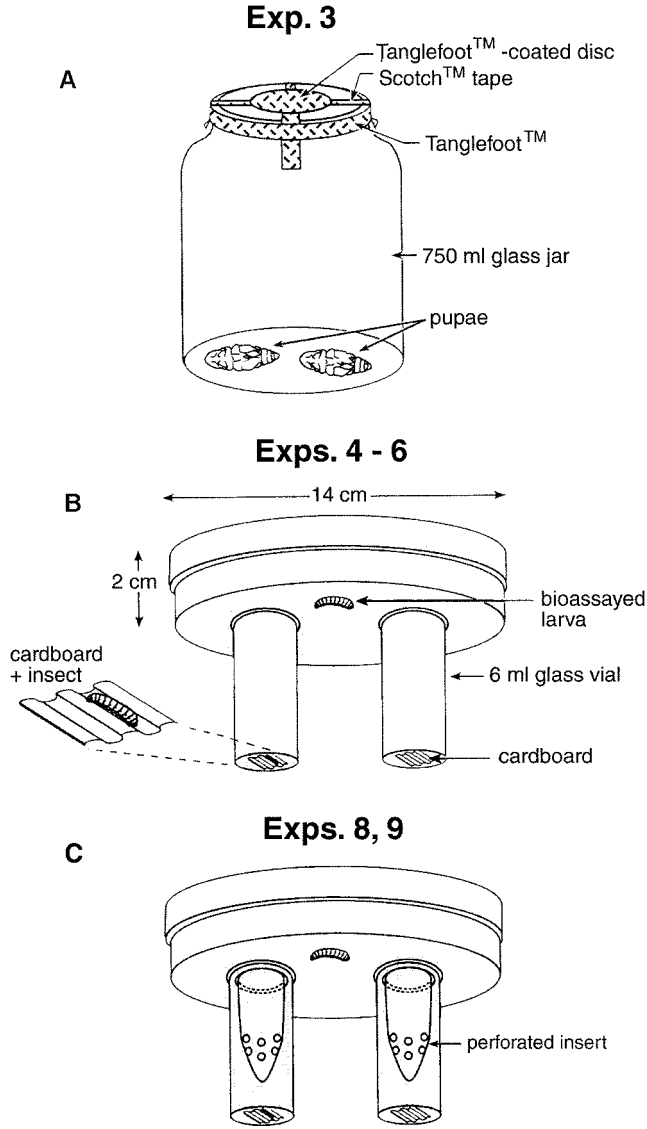

FIG. 1. Bioassay devices. (A) Glass jar modified such that eclosed *Cydia pomonella* adults could depart but not reenter. (B) Two-choice olfactometer (adapted from Pierce et al., 1981). (C) Two-choice olfactometer with perforated plastic inserts in glass vials that prevented the bioassayed *C. pomonella* larva from contracting test stimuli.

Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-23, or DB-210 (J&W Scientific, Folsom, California, USA) or SP-1000 (Supelco, Bellefonte, Pennsylvania, USA). For GC-EAD recordings, an antenna was gently removed from a male's head and suspended between glass capillary electrodes filled with insect Ringer's solution (Staddon and Everton, 1980). Identical retention times of antennal responses to synthetic and natural (E,E)-8,10-dodecadien-1-0 on each of four GC columns (see above) provided strong evidence that (E,E)-8,10-dodecadien-1-0 was produced in the late stage of female, but not male, pupae.

Statistical Analyses. The null hypothesis that *C. pomonella* cocoons do not occur in aggregations in open-fluted cardboard bands wrapped around tree trunks (experiment 1) was tested by the U^2 statistic (Watson, 1961) developed for discrete (here flute-based) data around a circle (Choulakian et al., 1994). Aggregation behavior of larvae in experiment 2, and number of larvae responding to stimuli in olfactometer bioassay experiments 4–6, 8, and 9 were analyzed with the χ^2 goodness-of-fit test using Yates' correction for continuity or Fisher's exact test to determine whether observed frequencies deviated significantly from expected frequencies (Zar, 1996). Arrestment times of newly eclosed adult moths in experiment 3 were analyzed by nonparametric analysis of variance followed by comparison of means [Bonferoni (Dunn) *t*-test]. The results for male and female pupation times in experiment 7 were analyzed with the paired-sample *t* test. In all experiments $\alpha = 0.05$.

RESULTS AND DISCUSSION

Our results show that: (1) *C. pomonella* larvae aggregate prior to pupation (2) larvae select pupation sites based on the presence and developmental stage of conspecifics (3) larval aggregations help arrest future mates, and (4) pheromones mediate both aggregation of larvae and arrestment of eclosed adults.

Aggregations of larval cocoons (Figure 2) were found in many cardboard bands and were confirmed to be statistically significant for 64 ($P \leq 0.05$) or 88 ($P \leq 0.1$) of 163 bands (Figure 3A). The null hypothesis that cocoons generally (>95%) do not occur in aggregations was rejected based on these results. Aggregations of larval cocoons were also confirmed in a laboratory experiment (Figure 3B). The adaptive significance of these aggregations of male and female larvae could be to arrest eclosing adult males and to procure future mates for females. Males that eclosed from pairs of a female plus male pupa were arrested significantly longer than males that eclosed from single or paired male pupae (Figure 3C).

Timing is of the essence in the procurement of future mates by larvae. Attraction of foraging fifth instars by cocoon-spinning larvae and prepupae, but not by pupae (Figure 4A), ensures eclosion of adults within the same relative time period.

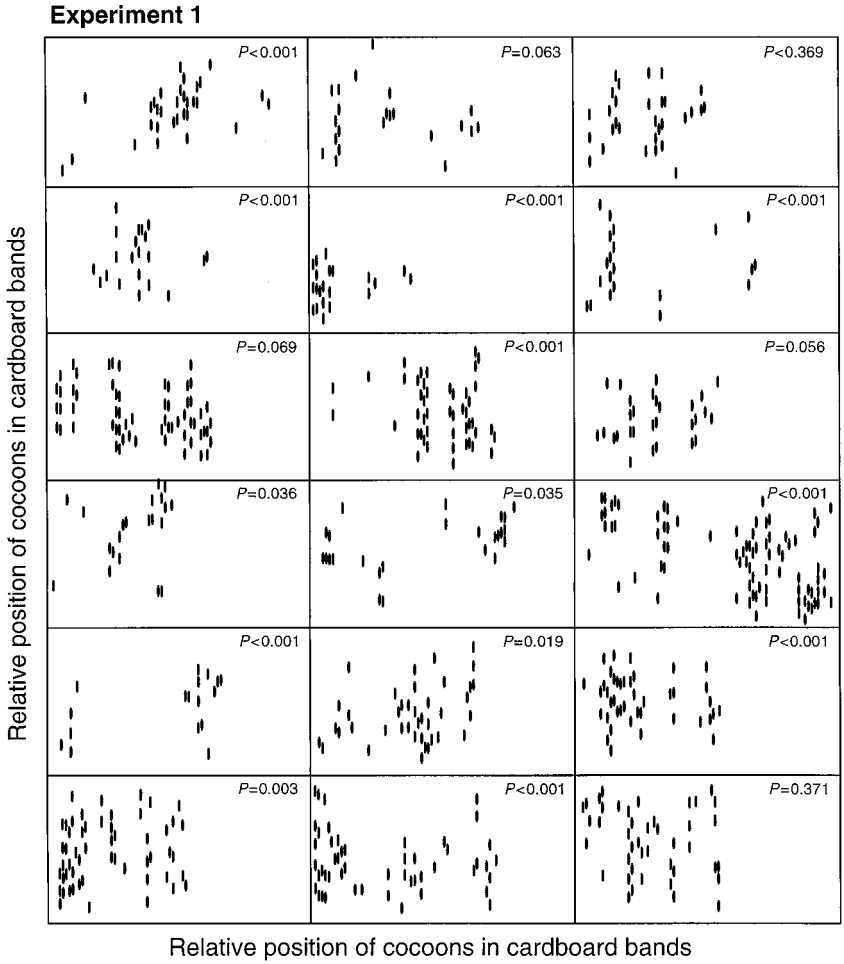

FIG. 2. Graphical presentations of relative positions of *Cydia pomonella* cocoons in 18 (of 163) open-fluted cardboard bands (10 cm wide) placed on trunks of apple trees, *Prunus malus*, in orchards of the Tree Fruit Research and Extension Centre of Washington State University, Wenatchee, August 21– October 21, 2001. In each band, $P < 0.05$ indicates aggregation of larval cocoons. Note: apparent evidence for aggregation of larval cocoons could not be confirmed statistically for each band.

Adult males eclose before females (Dolstad, 1985). A male larva, for example, being attracted to a female prepupa will pass through the pupal stage faster than the female (Figure 4B), thereby making up for the advanced development of the female prepupa. As a result, the male ecloses just in time to await eclosion of

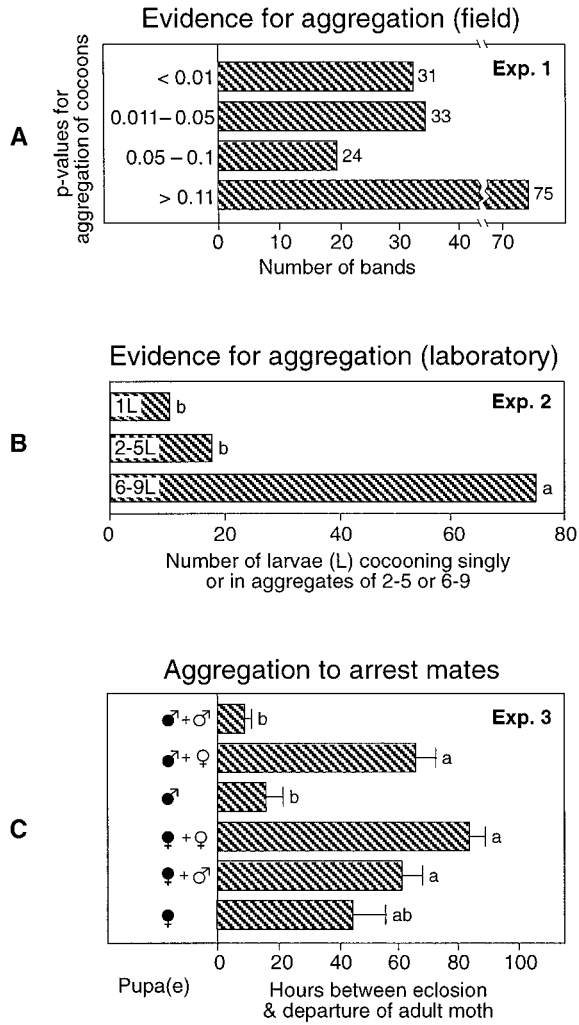

FIG. 3. (A) Number of open-fluted cardboard bands with respective levels of significance for aggregation of *Cydia pomonella* larval cocoons. (B) Number of larvae in 10 groups of 10 larvae cocooning singly or in aggregates (2–5 or >5). (C) Stimulus-dependent arrestment of eclosed adults. Solid and open symbols represent insects of identical age. In treatments of paired insects, those represented by solid symbols were one day older. Each replicate was terminated when both adults in pairs of pupae had eclosed or the adult from a single pupa had departed. Arrestment periods reported in this study may be longer than those occurring in nature, because presence or departure of the adults was recorded only every 12 hr. In each of experiments 1–3, bars with different letters are significantly different; $\alpha = 0.05$.

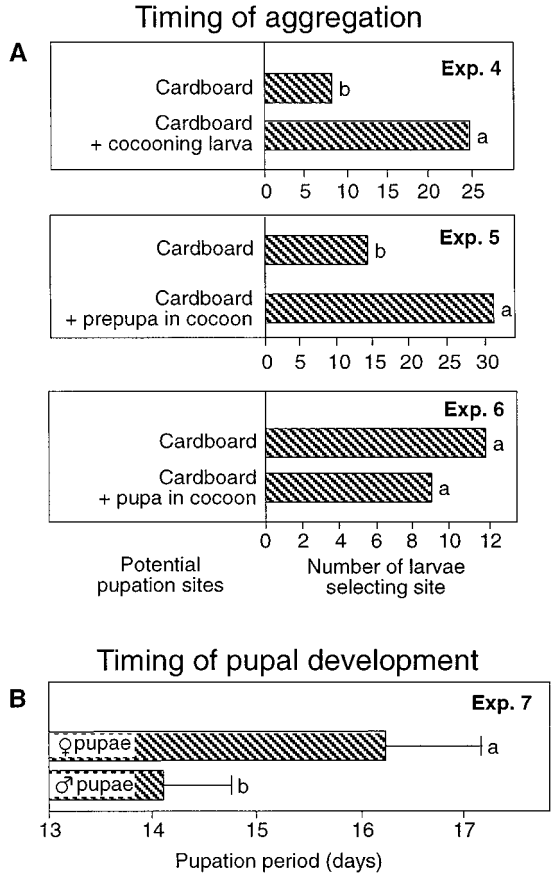

FIG. 4. (A) Selection of potential pupation sites by fifth-instar *Cydia pomonella* in olfactometer (Figure 1B) bioassay experiments in response to accessible test stimuli. (B) Average pupation periods of male and female pupae at 23°C. In each experiment, bars with different letters are significantly different; $\alpha = 0.05$.

the female. Even if the male's eclosion preceded that of a female by >24 hr, the arrestment signal from the female pupa (Figures 3C and 5B) should prevent the adult male from departing. The female's tactic of remaining at the site of eclosion for a significant time (Figure 3C) should increase her chance of securing a mate. While she is calling for a foraging male, a male from the pupal aggregate may eclose and mate with her.

Airborne pheromones mediate both aggregation of larvae (Figures 2 and 3A,B) and arrestment of adult males by female pupae (Figure 3C). These conclusions arise from the findings that: (1) foraging fifth instars were attracted to

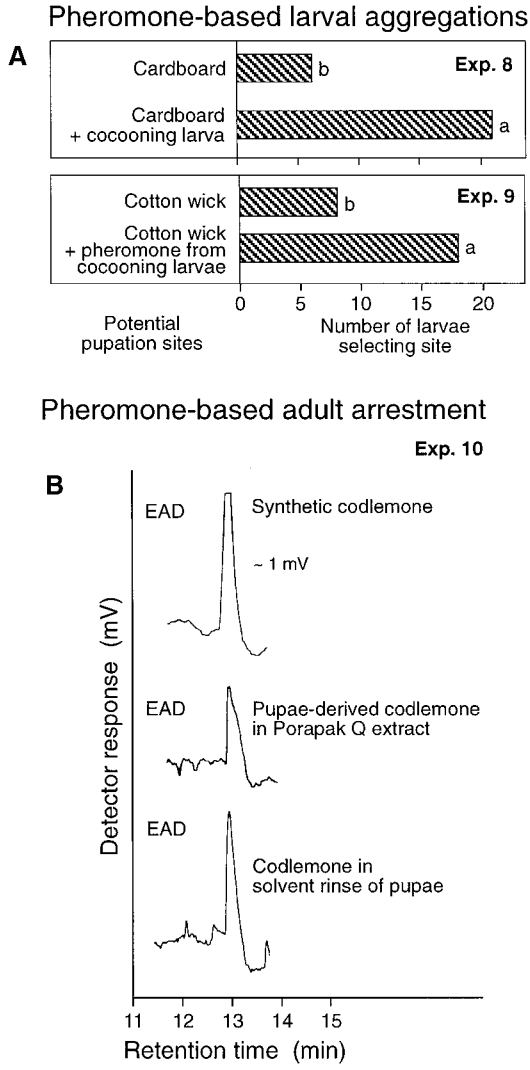

FIG. 5. (A) Selection of potential pupation sites by fifth-instar *Cydia pomonella* in olfactometer (Figure 1C) bioassay experiments in response to test stimuli. Perforated plastic inserts prevented contact with test stimuli. (B) Electroantennographic detector (EAD: male *C. pomonella* antenna) responses to 10 pg of synthetic pheromone (*E*,*E*)-8,10-dodecadienol (top), 35 volatile hour equivalents of female pupae (middle), and to solvent rinses from 18 female pupae (bottom). Chromatography: Hewlett Packard 5890A gas chromatograph equipped with a DB-23 coated column (30 m × 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector temperature: 280°C; temperature program: 1 min at 100°C, 10°C/min to 200°C.

cocoon-spinning larvae or their volatile extracts even when physical contact with the volatile source was prevented (Figure 5A), and (2) male antennae in GC-EAD analyses of volatile extracts from mature pupae detected codlemone, (*E*,*E*)-8,10-dodecadienol (Figure 5B), the known female-produced sex pheromone (Roelofs et al., 1971). Codlemone was not detectable in extract analyses of early pupal stages.

Our findings represent evidence of a fail-safe mechanism of mate procurement. Should attraction of male larvae by female larvae or prepupae (Figures 4A and 5A) and arrestment of eclosed adult males by female pupae (Figure 3C) fail, eclosed females can still resort to calling behavior to attract foraging males. Procurement of future mates by larvae would be advantageous to both sexes. Females that mated just after eclosion laid more eggs and produced more females than those that mated several days later (Vickers, 1997). Males, in turn, pass on their genes even before they commence foraging, which is adaptive should they perish during foraging or fail to locate a calling female. Males may also secure mating with several females, select the most profitable female(s), and/or gain sperm precedence (Bonduriansky, 2001).

Communication in *C. pomonella* within and among developmental stages, larva–larva (Figures 4A and 5A) and pupa–adult (Figures 3C, and 5B), may be a strategy to procure mates. If so, our data add to current knowledge that attraction of mates in insects relies on communication among adults, or pupae and adults (Phelan, 1997). They may indicate that communication in *C. pomonella*, and possibly other insects, is more intricate than previously realized.

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DOSE AND ENANTIOSPECIFIC RESPONSES OF WHITE PINE CONE BEETLES, *Conophthorus coniperda*, TO α-PINENE IN AN EASTERN WHITE PINE SEED ORCHARD

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Abstract—The white pine cone beetle, *Conophthorus coniperda*, exhibited dose and enantiospecific responses to α-pinene in stands of mature eastern white pine, *Pinus strobus*, in a seed orchard near Murphy, North Carolina, USA. (−)-α-Pinene significantly increased catches of cone beetles to traps baited with (±)-*trans*-pityol. (+)-α-Pinene did not increase catches of beetles to pityol-baited traps and interrupted the response of beetles to traps baited with (±)-*trans*-pityol and (−)-α-pinene. Maximal attraction of cone beetles to pityol-baited traps was obtained with lures releasing (−)-α-pinene at a rate of 103 mg/day at 23°C. Lures releasing (−)-α-pinene at rates lower or higher than 103 mg/day resulted in reduced catches to traps baited with (±)-*trans*-pityol. The sex ratio in all catches was heavily male biased. Attraction of the clerid predator, *Thanasimus dubius*, to traps baited with (±)-*trans*-pityol increased significantly with the presence of α-pinene, irrespective of enantiomeric composition. Maximal attraction of *T. dubius* to pityol-baited traps occurred with devices releasing (−)-α-pinene at the highest rate tested, 579 mg/d at 23°C, a sub optimal rate for cone beetles.

Key Words—Scolytidae, *Conophthorus coniperda*, kairomones, enantiospecificity, α-pinene, chirality, Cleridae, *Thanasimus dubius*.

INTRODUCTION

The white pine cone beetle, *Conophthorus coniperda* (Schwarz) (Coleoptera: Scolytidae), breeds in the cones of eastern white pine, *Pinus strobus* L. (Godwin and ODell, 1965; Ebel et al., 1980). Adult beetles emerge from aborted cones in the

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spring and fly to cones in the upper crowns of white pines. Initially, female beetles attack the base of second-year cones, girdling the stem, and tunneling along the cone axis. Eggs are laid along an egg gallery within a cone. Larvae hatch and feed indiscriminately within the cone, consuming most of the seeds. At times, beetles may also attack first-year conelets and new shoots (Godwin and ODell, 1965).

Eastern white pine is a dominant and valuable tree species throughout northeastern North America (Farrar, 1995). Cone damage by beetles can have a significant impact on natural regeneration of eastern white pine following forest fires (Godwin and ODell, 1965) and on seed production in white pine seed orchards, with losses of 60–100% at times (Graber, 1964; Odera, 1968; Morgan and Mailu, 1976). Prescribed fire can be used to control populations within seed orchards, consuming beetles while beetles are still in aborted cones on the forest floor (Wade et al., 1989).

Semiochemicals may provide a valuable tool in mitigating the impacts of cone beetles through their use in monitoring and mass-trapping programs, as well as in mating disruption or push–pull strategies (Rappaport et al., 2000). Males are attracted to infested cones (de Groot et al., 1991) by the sex pheromone, (+)-*trans*-pityol (2*R*,5*S*)-2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran], produced by females (Birgersson et al., 1995). Attractants for females have not been identified. (±)-α-Pinene synergizes the attraction of males to pityol-baited traps (de Groot and DeBarr, 1998; de Groot et al., 1998; Rappaport et al., 2000).

α-Pinene is a common monoterpene in eastern white pine with an enantiomeric composition dominated by the (−) enantiomer (Mirov, 1961). Hobson et al. (1993) demonstrated enantiospecificity in attraction of the red turpentine beetle, *Dendroctonus valens* LeConte (Scolytidae), to α-pinene in stands of ponderosa pine, *Pinus ponderosa* P. Laws. ex C. Laws. Cone beetles may show similar enantiospecific responses to host kairomones. All previous work with cone beetles dealt with racemic α-pinene only (de Groot and DeBarr, 1998; de Groot et al., 1998; Rappaport et al., 2000). In addition, the issue of release rates has not been examined and should be investigated. Miller and Borden (2000) demonstrated dose-dependent relationships with host monoterpenes for three species of bark beetles. Our objective was to determine the effect of dose and enantiomeric composition of α-pinene on the response of male cone beetles to the pheromone, (±)-*trans*-pityol. Specifically, we hypothesized that males would show enantiospecificity to (−)-α-pinene and that trap catches would increase with release rate of α-pinene.

METHODS AND MATERIALS

Chemicals and Release Devices. Phero Tech Inc. (Delta, British Columbia) supplied polyethylene bubblecap lures containing (±)-*trans*-pityol (hereafter referred to as simply pityol) (chemical purity >98%), released at about 0.14 mg/day

at 23°C. Previous studies have demonstrated that (−)-*trans*-pityol has no effect on the response of cone beetles to (+)-*trans*-pityol (Birgersson et al., 1995). Sigma-Aldrich Co. (Milwaukee, Wisconsin, USA) supplied (−)-, (+)- and (±)-α-pinene and (−)-β-pinene (chemical purities >98%). The enantiomeric composition of (−)-α-pinene and (−)-β-pinene was 98% (−), whereas the enantiomeric composition of (+)-α-pinene was 95% (+). Pinenes were released from the following types of sealed low-density polyethylene devices: (1–2) 250- and 400-μl centrifuge tubes (Fisher Scientific, Pittsburgh, Pennsylvania); (3) 8-ml Nalgene wide-mouth screw-cap bottles (Cole-Parmer Instrument Co., Vernon Hills, Illinois, USA); (4) 15-ml Boston round screw-cap bottles (O. Berk Co., Union, New Jersey, USA); and (5) 15-ml cylinder screw-cap bottles (PheroTech Inc.). The release rates (at 23°C in still air) from these devices were the same for all pinenes at about 5, 10, 47, 107, and 193 mg/day, respectively.

Experimental Design. Four experiments were conducted at the Beech Creek Seed Orchard (USDA Forest Service, Nantahala National Forest) near Murphy, North Carolina, USA in 1999–2001. In each experiment, Japanese beetle traps (Trécé Inc., Salinas, California, USA) were baited with pityol and suspended by rope and pulley in the crowns of mature eastern white pines (15–20 m height), adjacent to cones, with only one trap per tree. The distance between trees varied from 15 to 30 m. Beetles were collected in 500-ml plastic Mason jars, filled with approximately 200 ml of plumber's antifreeze (pink propylene glycol solution) as a killing and preservation agent. Trap catches were collected at 3-week intervals with the glycol solution replaced on each occasion. Sexes of captured beetles were determined by examination of the 7th and 8th abdominal tergites (Kinzer and Ridgill, 1972).

Experiments 1–3 tested the effects of enantiomeric composition of α-pinene on the attraction of cone beetles to pityol-baited traps. In each experiment, 40 traps were set in 10 replicate blocks of 4 traps/block. The trapping periods for experiments 1–3 were March 11–May 15, 1999, March 14–May 10, 2000, and March 28–May 30, 2001, respectively. In experiment 1, the following treatments were randomly assigned to pityol-baited traps within each block: (1) control; (2) (±)-α-pinene [50:50 mix of (−)- and (+)-α-pinene); (3) (−)-α-pinene; and (4) (±)-α-pinene + β-pinene. Each pinene was released separately by using release device 5. β-Pinene is a common monoterpene in eastern white pine (Mirov, 1961) and may affect attraction of cone beetles to α-pinene.

Experiment 2 was designed to separate the effect of enantiomeric composition from that of dose (release rate). The following treatments were randomly assigned to pityol-baited traps within each block: (1) (±)-α-pinene (about 193 mg/day at 23°C); (2) (−)-α-pinene (about 193 mg/day at 23°C); (3) (±)-α-pinene (about 384 mg/day at 23°C); and (4) (−)-α-pinene (about 384 mg/day at 23°C). The two release rates of α-pinene were obtained by using either one or two of release device 5. The release rate of (−)-α-pinene is the same in treatment 2 as in treatment 3,

whereas the release rate of α-pinene (irrespective of enantiomeric composition) is the same in treatment 1 as in treatment 2, and the same in treatment 3 as in treatment 4.

In experiment 3, the following treatments were randomly assigned to pityol-baited traps within each block: (1) control; (2) (−)-α-pinene; (3) (+)-α-pinene; and (4) (−)-α-pinene and (+)-α-pinene. Each pinene was released from a separate release device 5 with one device used in treatments 2 and 3, and two devices used in treatment 4.

Experiment 4 determined the dose-dependent effect of (–)-α-pinene on the attraction of beetles to baited traps. Forty-two traps were set in seven replicate blocks of 6 traps/block for the period March 28–May 30, 2001. The following treatments, differing only in the release rate of (−)-α-pinene (at 23°C), were assigned randomly to pityol-baited traps within each block: (1) about 5 mg/day; (2) about 20 mg/day; (3) about 47 mg/day; (4) about 107 mg/day; (5) about 193 mg/day; and (6) about 579 mg/day. Release rates 1, 3, 4, and 5 were obtained with one of device 1, 3, 4, and 5, respectively. Release rate 2 was obtained with two of device 2, whereas release rate 6 was obtained with three of device 5. Release rates from devices are not expected to be constant under field conditions. These data for rates at 23°C serve primarily as an index series (or set of reference values) that we expect lie within the range of rates experienced under field conditions.

GC-MS Analysis of Cone Volatiles. Second-year eastern white pine cones were collected in the spring of 2001 at the USDA Forest Service Beech Creek Seed Orchard near Murphy, North Carolina. Ten cones from each of three randomly selected trees were collected in early March and 10 cones from each of five randomly selected trees in late May different from those selected in March. Each set of 10 cones was sealed in a separate Mylar bag and stored at −10°C.

Each set of cones was aerated in a sealed glass collection chamber (10 liter) (Analytical Research Systems Inc., Gainesville, Florida, USA) with a flow of charcoal-filtered air introduced to the bottom of the chamber at approximately 1 liter/min. Volatiles were collected on 72-mm Super-Q columns (5 mm OD) (Analytical Research Systems Inc.). The columns were rinsed with pentane (1 ml), and the extract was analyzed on an Hewlett-Packard G1800C coupled gas chromatograph and mass spectrometer (GC-MS) with electron ionization detection and a chiral fused-silica capillary column (J&W Cyclodex-B, 30 m × 0.250 mm, 0.25 μm film thickness; J&W Scientific, Folsom, California, USA). Following a splitless injection, the temperature was held constant at 50°C for 2 min, then increased to 100°C at a rate of 8°C/min, held constant at 100°C for 2 min, then increased to 210°C at a rate of 12°C/min. Inlet and detector temperatures were held at 190°C. Helium was used as the carrier gas at a flow rate of 0.9 ml/min. All peaks were identified by comparing mass spectra, recorded at 70 eV and intervals of 1.0 sec, and retention times to the mass spectra and retention times of analytical standards.

Statistical Analyses. The data were analyzed with the Systat statistical package version 9.01 for Windows (SPSS Inc, Chicago, Illinois, USA). Trap catch data were transformed by $\ln(y + 1)$ or $\ln(y)$, as required, to remove heteroscedasticity. Sex ratio data were transformed by arcsine(y). Data from experiments 1–3 were subjected to either one- or two-way ANOVA, whereas data from experiment 4 were regressed on release rates, transformed by $\ln(x)$. Differences in mean catches among treatments in experiments 1–3 were determined by Fisher's least significant difference (LSD) multiple-comparison test when $P = 0.05$. Differences in sex ratios were determined by 1-way ANOVA or t tests.

RESULTS

Trap catches in experiment 1 were affected by treatments ($F_{3,16} = 5.074$, $P = 0.012$). Catches of cone beetles were highest in traps with (−)-α-pinene and pityol (Figure 1). Although male beetles dominated all catches, there was a difference in sex ratio in catches among the treatments ($F_{3,14} = 4.201$, $P = 0.026$). The proportion (mean ± SE) of males caught in traps baited with pityol, (±)-α-pinene, and β- pinene was 0.905 ± 0.017, significantly lower than the proportion of males caught in the remaining treatments (0.973 ± 0.012) (LSD multiple comparison test, $P < 0.05$).

In experiment 2, catches of cone beetles were affected by the enantiomeric composition of α-pinene ($F_{1,36} = 22.119$, $P < 0.001$). Catches were greater in traps baited with pityol and (−)-α-pinene than in those baited with pityol and

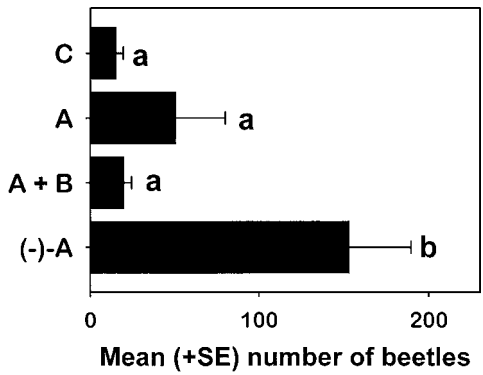

FIG. 1. Comparison of mean (+SE) total catches of *C. coniperda* in pityol-baited traps with (±)-α-pinene (A), (±)-α-pinene and (−)-β-pinene (A + B), and (−)-α-pinene [(−)-A] to pityol-baited control traps (C) from March 11 to May 15, 1999, in experiment 1 ($n = 10$). Means followed by different letters are significantly different at $P = 0.05$ (LSD multiple comparison test).

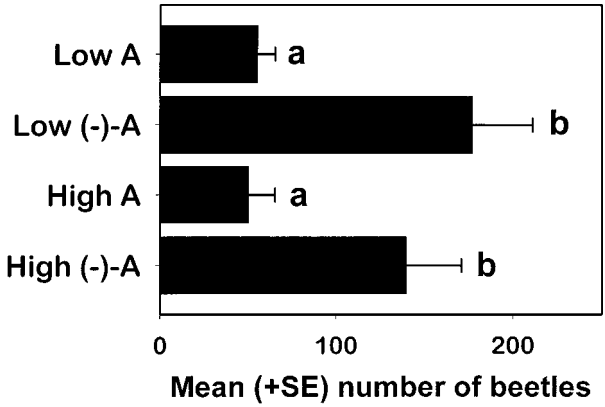

FIG. 2. Effects of (−)-α-pinene [(−)-A] and (±)-α-pinene (A), released at two different rates (low and high), on mean (+SE) total catches of *C. coniperda* in pityol-baited traps from March 14 to May 10, 2000, in experiment 2 ($N = 10$). Means followed by different letters are significantly different at $P = 0.05$ (LSD multiple comparison test).

(±)-α-pinene (Figure 2). Trap catches were unaffected by dose ($F_{1,36} = 0.545$, $P = 0.465$) or the interaction between dose and enantiomeric composition ($F_{1,36} = 0.014$, $P = 0.908$). There was no effect of treatment on sex ratios of beetles in trap catches ($F_{3,35}$, $P = 0.396$). The proportion of males in trap catches was 0.860 ± 0.013.

Catches of beetles to pityol-baited traps in experiment 3 were increased by (−)-α-pinene ($F_{1,39} = 46.998$, $P < 0.001$) but not by (+)-α-pinene alone ($F_{1,39} = 2.454$, $P = 0.125$) (Figure 3). (+)-α-Pinene interrupted the response of beetles to traps baited with (−)-α-pinene and pityol. There was no difference in sex ratio in catches for traps baited with pityol and (−)-α-pinene and those baited with pityol, (−)- and (+)-α-pinene ($t_{16} = 0.199$, $P = 0.844$). Data on sex ratio for the remaining treatments were not obtained due to low catches.

In experiment 4, catches of beetles to traps baited with pityol and (−)-α-pinene increased as the release rate of (−)-α-pinene increased up to the rate associated with device 4 (107 mg/day at 23°C) and then decreased with higher rates (Figure 4). Catches in traps baited with (−)-α-pinene released at 107 mg/day at 23°C were higher than those in traps baited with (−)-α-pinene released at the highest and lowest rates (LSD multiple comparison test, $P = 0.05$). There was no difference in catches of traps with the highest and lowest release rates of (−)-α-pinene (LSD multiple comparison test, $P = 0.05$). There was an effect of treatments on the sex ratio of beetles in trap catches ($F_{5,36} = 2.487$, $P = 0.049$). The proportion (mean ± SE) of males caught in traps baited with pityol and (−)-α-pinene released at the highest rate was 0.986 ± 0.007, significantly higher than the proportion of males

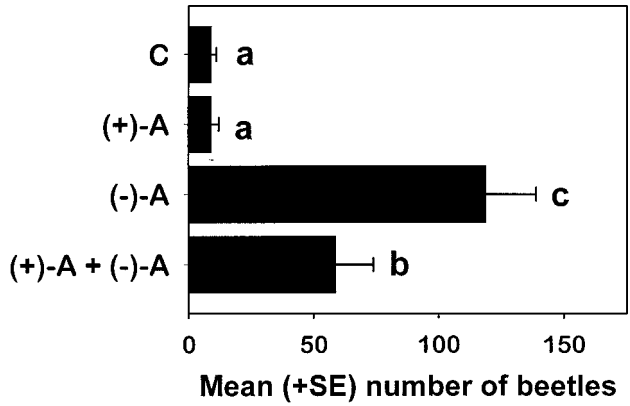

FIG. 3. Comparison of mean (+SE) total catches of *C. coniperda* in pityol-baited traps with (−)-α-pinene [(−)-A], (+)-α-pinene [(+)-A], and (+)-α-pinene and (−)-α-pinene [(+)-A + (−)-A], to pityol-baited control traps (C) from March 28 to May 30, 2001, in experiment 3 ($N = 10$). Means followed by different letters are significantly different at $P = 0.05$ (LSD multiple comparison test).

caught in the remaining treatments (0.942 ± 0.008) (LSD multiple comparison test, $P < 0.05$).

The dominant monoterpenes in cone volatiles were α-pinene, β-pinene, limonene, and myrcene (Figure 5). Myrcene seemed to be more prevalent in

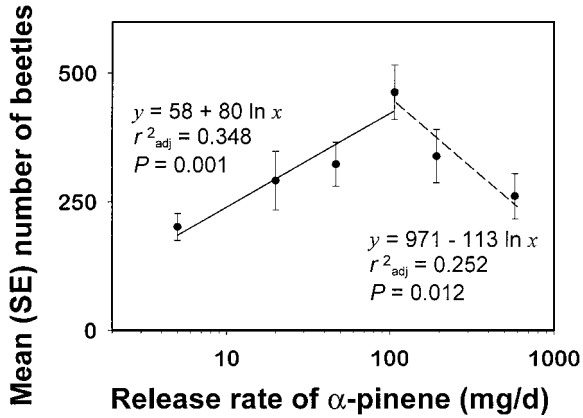

FIG. 4. Dose-dependent attraction and interruption of *C. coniperda* by (−)-α-pinene to traps baited with pityol from March 28 to May 30, 2001, in experiment 4 ($N = 7$). Slopes of regression lines are significantly different from zero (t test, $P < 0.05$). Release rates were determined in still air at 23°C.

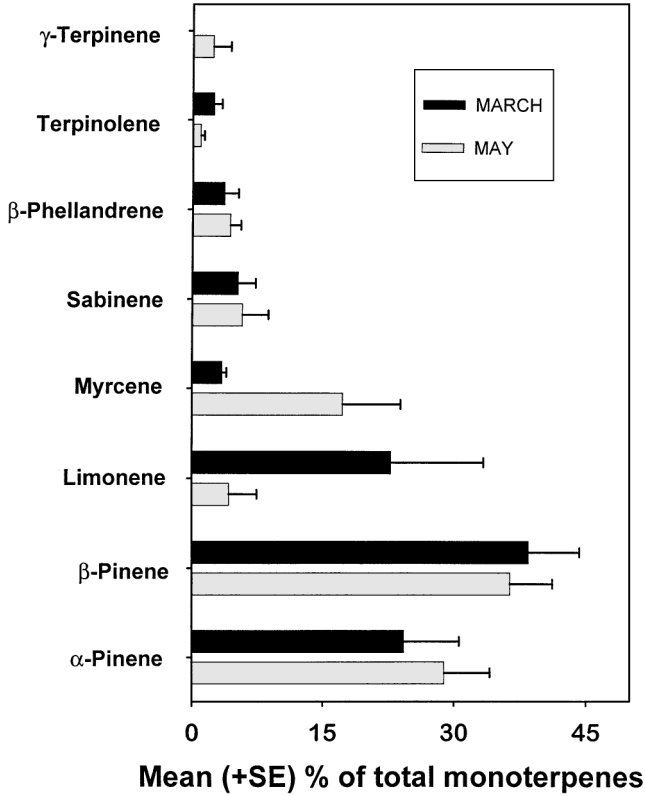

FIG. 5. Composition of volatile monoterpenes from eastern white pine cones collected in March ($N = 3$) and May 2001 ($N = 5$) from the Beech Creek Seed Orchard in Nantahala National Forest, North Carolina.

volatiles emitted from cones collected in May than in March, whereas limonene seemed to be prevalent in volatiles emitted from cones collected in March than in May. The enantiomeric composition of α-pinene in volatiles from cones collected in March was (mean $\pm$SE) $80.9 \pm 3.0\%$ $(-)$, significantly lower than the enantiomeric composition of $93.2 \pm 1.7\%$ $(-)$ emitted by cones collected in May ($t_6 = 3.863$, $P = 0.008$).

The bark beetle predator, *Thanasimus dubius* (F.) (Coleoptera: Cleridae) was affected by treatments in three of the four experiments. In experiment 1, all traps baited with pityol and α-pinene caught twice as many clerid beetles as those baited with pityol alone ($F_{5,24} = 7.256$, $P < 0.001$) (Figure 6). The same result was obtained in experiment 3, where catches in traps baited with pityol alone were lower than those baited with pityol and either $(+)$- or $(-)$-α-pinene ($F_{3,39} =$

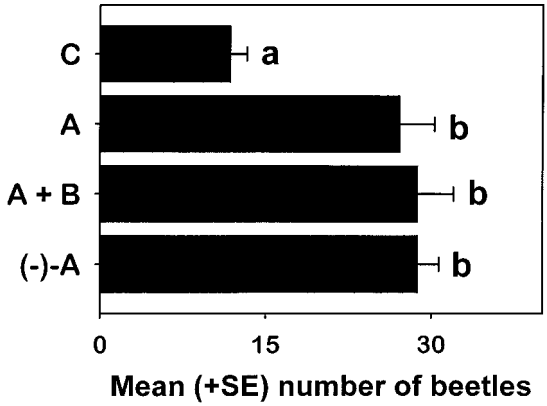

FIG. 6. Comparison of mean (+SE) total catches of the clerid, *T. dubius*, in pityol-baited traps with (±)-α-pinene (A), (±)-α-pinene and (−)-β-pinene (A + B), and (−)-α-pinene [(−)-A] to pityol-baited control traps (C) from March 11 to May 15, 1999, in experiment 1 ($N = 10$). Means followed by different letters are significantly different at $P = 0.05$ (LSD multiple comparison test).

6.639, $P = 0.001$) (Figure 7). There was no effect of treatments on catches of *T. dubius* in experiment 2, where all treatments contained pityol and α-pinene ($F_{3,35} = 0.766$, $P = 0.521$). The catch of *T. dubius* in experiment 2 was 24.3 ± 2.3 (mean ±SE). In experiment 4, the responses of *T. dubius* were affected by the release rate of (−)-α-pinene ($F_{5,36} = 3.473$, $P = 0.012$). Traps baited with pityol and (−)-α-pinene, released at the highest rate, caught significantly more clerid beetles than any other treatment (Figure 8). There was no difference among the other treatments (LSD multiple comparison test, $P = 0.05$).

DISCUSSION

Male white pine cone beetles exhibited enantiospecific responses to α-pinene. Attraction of beetles to pityol was increased by (−)-α-pinene (Figures 1–3). (+)-α-Pinene did not increase attraction of beetles to pityol (Figure 3). In fact, (+)-α-pinene interrupted the attraction of beetles to traps baited with pityol and (−)-α-pinene (Figures 2 and 3). Our results are consistent with the enantiomeric composition of α-pinene emitted by cones in the same orchard, which was primarily (−)-α-pinene. (±)-α-Pinene [50: 50 mix of (−)- and (+)] increased attraction of beetles to pityol albeit less than (−)-α-pinene alone (Figure 3). This is consistent with previous trials that demonstrated a synergistic effect of (±)-α-pinene on the attraction of white pine cone beetles to pityol in stands of eastern white pine (de Groot et al., 1998; Rappaport et al., 2000).

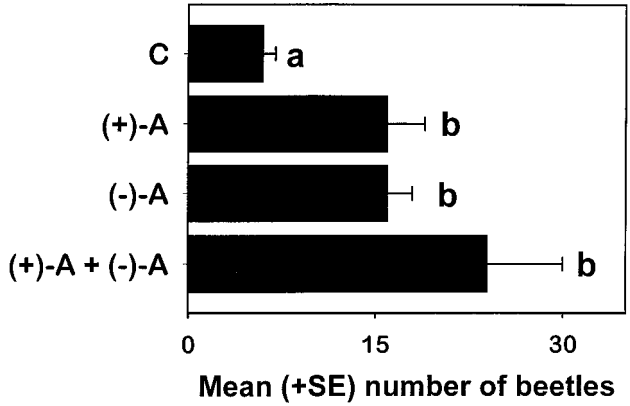

FIG. 7. Comparison of mean (+SE) total catches of the clerid, *T. dubius*, in pityol-baited traps with (−)-α-pinene [(−)-A], (+)-α-pinene [(+)-A], and (+)-α-pinene and (−)-α-pinene [(+)-A + (−)-A], to pityol-baited control traps (C) from March 28 to May 30, 2001, in experiment 3 ($N = 10$). Means followed by different letters are significantly different at $P = 0.05$ (LSD multiple comparison test).

Enantiospecific behavioral responses to α-pinene occur with a western species of cone beetles as well. In western North America, attraction of the cone beetle, *C. ponderosae* Hopkins, to pityol-baited traps was strongly increased by (−)-α-pinene in a coastal stand of western white pine, *P. monticola* Dougl. ex D. Don

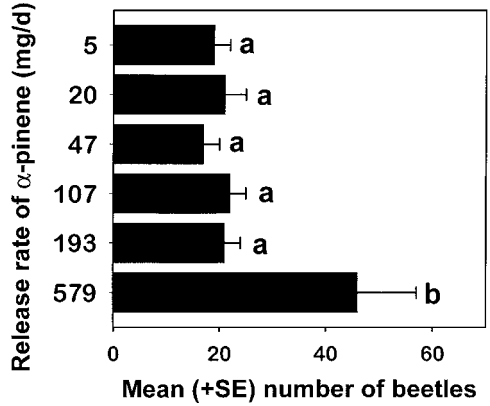

FIG. 8. Effect of (−)-α-pinene, released at six different rates (at 23°C), on mean (+SE) total catches of the clerid, *T. dubius*, in pityol-baited traps from March 14 to May 10, 2000, in experiment 4 ($N = 10$). Means followed by different letters are significantly different at $P = 0.05$ (LSD multiple comparison test).

(Miller et al., 2000) and (±)- and (−)-α-pinene in stands of western white pine in Idaho (Rappaport et al., 2000, 2002). However, Rappaport et al. (2002) found that in Idaho, traps baited with pityol and (+)-α-pinene caught more cone beetles than those baited with pityol and (−)-α-pinene, even though the mean enantiomeric composition of α-pinene emitted by cones of western white pine in the study was 96% (−). Neither (±)-α-pinene nor (+)-α-pinene was tested in the coastal stand of western white pine (Miller et al., 2000).

In contrast, de Groot et al. (2002) found that attraction of male red pine cone beetles, *Conophthorus resinosae* Hopkins, to traps baited with pityol in plantations of red pine, *Pinus resinosa* Ait., in eastern North America was interrupted by α-pinene, irrespective of enantiomeric composition. These results are surprising as de Groot et al. (2002) found that α-pinene was the predominant volatile collected from cones of red pine, accounting for >40% of the total volatiles on average. They found that attraction of red pine cone beetles was interrupted more by α-pinene with enantiomeric compositions of 12% (−) to 69% (−) than with α-pinene with an enantiomeric composition of 86% (−), the mean enantiomeric composition of α-pinene in volatiles from red pine cones.

Variation in responses among species is not surprising nor is geographic variation within a species, as numerous examples are known within the family Scolytidae (Borden, 1982; Byers, 1989). However, we should expect some correlation between odors emitted by a host and the responses of beetles to those hosts that they infest, or at least they should not interrupt attraction.

One confounding issue in previous trials may have been the release rate characteristics of devices used for α-pinene. de Groot et al. (2002) used devices that released α-pinene at 150–450 mg/day at 24°C. Rappaport et al. (2002) used devices that released α-pinene at 100–330 mg/day. In our study, we found that maximal attraction of cone beetles to pityol occurred with a lure releasing α-pinene at a rate of 107 mg/day at 23°C (Figure 4). Traps with devices releasing α-pinene at rates higher or lower than 107 mg/day (at 23°C) resulted in a reduction in beetle catches relative to those with lures releasing α-pinene at 107 mg/day. These results may be due to a mixed-functional response to (−)-α-pinene, requiring a very specific dose for maximal attraction. An alternate explanation may be that at high release rates, the interruptive effect of the antipode, (+)-α-pinene, may play a significant role. In our study, the enantiomeric composition of α-pinene was 98% (−). Release rates of 100–400 mg/day (at 23°C), therefore, would result in release rates of (+)-α-pinene of 2–8 mg/day (at 23°C), which may be above threshold levels.

Another possible explanation for these various results may relate to host preferences (Rappaport et al., 2002; de Groot et al., 2002). The cone beetle, *C. ponderosae*, attacks cones of 12 species of western pines (Wood, 1982). The predominant enantiomer of α-pinene in the resin of western white pine is (−)-α-pinene in the Pacific Northwest, whereas (+)-α-pinene is more abundant than (−)-α-pinene

in other species such as limber pine (*P. flexilis* James) and ponderosa pine (*P. ponderosa* P. Laws. ex C. Laws.) (Mirov, 1961). In some species such as lodgepole pine (*P. contorta* var. *latifolia* Engelm.), whitebark pine (*P. albicaulis* Engelm.), and ponderosa pine, the quantity of α-pinene in the resin may be low or nonexistent (Mirov, 1961). The preference for (+)-α-pinene may be a consequence of past competition rather than current host preferences (Rappaport, 2002).

In eastern North America, the cones of jack pine, *P. banksiana* Lamb., a species sympatric with red pine, are not attacked by *C. resinosae* (= *C. banksianae* McPherson), although the twigs are attacked (Mattson, 1989; de Groot and Fleming, 1994). de Groot et al. (2002) found that the emission of α-pinene from cones of jack pine was high, accounting for 41% of the total volatiles on average. However, the mean enantiomeric composition of α-pinene was 66% (−) in jack pine as opposed to a mean of 86% (−) in eastern white pine (de Groot et al., 2002). The resin of the exotic, yet abundant, Scots pine (*P. sylvestris* L.) contains primarily (+)-α-pinene (Mirov, 1961). The predominant enantiomer of α-pinene in southeastern pine species, in or near the range of white pine cone beetles, such as shortleaf pine (*P. echinata* Mill.), loblolly pine (*P. taeda* L.), and longleaf pine (*P. palustris* Mill.) is (+)-α-pinene, although in others such as Virginia pine (*P. virginia* Mill.) and pitch pine (*P. rigida* Mill.) it is (−)-α-pinene (Mirov, 1961).

Host selection may be based on monoterpene blends rather than merely the presence of α-pinene and its enantiomeric composition. In our study, we found that β-pinene was as abundant as α-pinene (Figure 4), yet we did not find any effect of β-pinene on catches of beetles to traps baited with pityol and (±)-α-pinene) (Figure 1). Limonene was initially quite abundant in March, but seemed to decrease by May (Figure 4). In contrast, the abundance of myrcene seemed to have increased over the same time period. de Groot et al. (1998) tested β-pinene, limonene, and myrcene in traps baited with pityol and α-pinene for attraction to *C. coniperda*. However, limonene and myrcene were never tested alone. Interactions between monoterpenes on the responses of cone beetles in previous studies may have masked behaviors to individual compounds. Furthermore, we should not be surprised to find variation in host volatile preferences based on local abundance of host species given the variation of hosts in some species such as *C. ponderosae*. Similarly, responses of red pine cone beetles may depend on the presence of other monoterpenes, not the dominant α-pinene.

We need to examine responses of beetles to host odors and blends for possible attractants as well as indicators of susceptibility, tolerance, and resistance of cones to attacks by cone beetles. Henson (1960, 1961) found that both male and female white pine cone beetles formed aggregations when confined in open arenas in the laboratory. Morgan and Mailu (1976) reported aggregations of up to 12 beetles/site during early feeding attacks on twigs and cones. Currently, only attractants for males have been identified, namely the combination of (+)-*trans*-pityol and

(−)-α-pinene (Birgersson et al., 1995; de Groot et al., 1998). As the attacking sex, females seem to be able to find suitable hosts prior to the appearance of males. There is no evidence that females are attracted by volatiles produced by other females such as aggregation pheromones. In laboratory assays, de Groot et al. (1991) found that both male and female white pine cone beetles were attracted to host compounds. Some trees in seed orchards of eastern white pine seem to be preferred by white pine cone beetles, whereas others are left untouched (personal observations, D. R. Miller and G. L. DeBarr). Therefore, it seems plausible that, if females use chemical attractants at all, host odors may play a significant role in host location by female cone beetles.

Predators may play a role in the semiochemical ecology of cone beetles as well. Attraction of the clerid predator, *T. dubius*, to (±)-*trans*-pityol is increased by the kairomone, α-pinene, irrespective of enantiomeric composition (Figures 6 and 7). Previously, de Groot and DeBarr (2000) found that *T. dubius* was attracted to the combination of pityol and α-pinene, but they did not test the binary combination against pityol alone. Unlike the cone beetles (Figure 4), *T. dubius* showed a strong preference for traps baited with (±)-*trans*-pityol and devices releasing α-pinene at the highest rate (Figure 8). Our observations on *T. dubius* may relate to a general predator strategy and not a specific one for cone beetles. Other potential prey, such as the twig beetles *Pityophthorus cariniceps* LeConte and *P. puberulus* (LeConte) (Scolytidae), are also attracted to the combination of pityol and α-pinene (de Groot and DeBarr, 2000). High release rates of monoterpenes are often associated with attacks by the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Scolytidae), and associated bark beetles, on southern pines (Thatcher et al., 1980). The predator, *T. dubius*, is a common and abundant predator of species in the southern pine bark beetle guild (Thatcher et al., 1980).

The phenomenon of enantiospecificity is not new for the bark beetle family. Numerous species exhibit enantiospecificity to pheromones, particularly with respect to the pheromones ipsenol and ipsdienol (Borden, 1982; Byers, 1989). However, our results and those of Rappaport et al. (2002) and de Groot et al. (2002) with cone beetles are only the second demonstration of enantiospecificity to host compounds by bark beetles. Previously, Hobson et al. (1993) found that attraction of *D. valens* to (+)-α-pinene was interrupted by (−)-α-pinene. Our results with *C. coniperda* showing dose-dependent attraction to (−)-α-pinene over a low range of release rates and dose-dependent interruption over a higher range are unique. Previous results with host compounds and bark beetles, such as these of Miller and Borden (2000), have typically demonstrated one or the other, never both.

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DISCOVERY OF 3-METHYL-2-BUTEN-1-YL ACETATE, A NEW ALARM COMPONENT IN THE STING APPARATUS OF AFRICANIZED HONEYBEES

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Abstract—We analyzed the alarm pheromone components from five colonies of Africanized honeybees and three colonies of European honeybees collected in Mexico. Analyses revealed a novel alarm pheromone component that was only present in appreciable quantities in the Africanized bee samples. Analysis of the mass spectrum and subsequent synthesis confirmed that this compound is 3-methyl-2-buten-1-yl acetate (3M2BA), an unsaturated derivative of IPA. In Africanized honeybees, sampling from stings of guards showed that 3M2BA was present at levels of 0–38% the amount of isoamyl acetate (IPA). Behavioral assays from three colonies each of Africanized and European bees showed that 3M2BA recruited worker bees from hives of both Africanized bees and European bees at least as efficiently as isopentyl acetate IPA, a compound widely reported to have the highest activity for releasing alarm and stinging behavior in honeybees. However, a mixture of of 3M2BA and IPA (1:2) recruited bees more efficiently than either of the compounds alone. None of the compounds differed in their efficacy for inducing bees to pursue the observers.

Key Words—*Apis mellifera*, honeybee, stinging, alarm, pheromone, social insect, defensive behavior.

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INTRODUCTION

Many compounds have been identified that appear to communicate alarm among nestmates in the social hymenoptera (reviewed by Blum, 1969; Free, 1987). In the honeybee (*Apis mellifera* L.), extracts from the sting apparatus function as attractants and can cause bees to rush out of the colony ready to defend the nest. Alarm pheromones, along with other cues (primarily visual and tactile), are sufficient to release stinging behavior (Free, 1961; Ghent and Gary, 1962).

The sting apparatus of the bee contains a complex blend of hydrocarbons (Blum et al., 1978; Free, 1987; Blum and Fales, 1988). Most honeybee alarm pheromone components appear to be produced by the Koshewnikov gland of the sting apparatus (Grandperrin and Cassier, 1983) and are volatilized on the setaceous membrane (Lensky et al., 1994). Of the more than 40 compounds that have been identified from the sting apparatus, about 30 are fairly volatile, oxygenated compounds that could be involved in signaling alarm (Blum and Fales, 1988). Alarm behavior has been defined in more than one way. Some researchers have defined it as agitated movement and buzzing of bees on cage assays. Others have defined alarm based on bioassays of recruitment, by counting how many bees come out of the hive entrance in response to alarm pheromone. At least 15 compounds from the sting apparatus can cause agitation in young, caged bees (Collins and Blum, 1982, 1983). Isopentyl acetate (IPA) was reported to be the alarm pheromone component that was principally responsible for releasing stinging behavior (Boch et al., 1962). Although IPA can account for most of the recruitment and stinging behavior elicited by a sting, IPA alone is not as effective in this regard as is the intact sting apparatus. The reactions of *Apis* species to sting extracts from other species do not correlate well with the IPA content of those extracts, apparently because different components are behaviorally important in different species (Koeniger et al., 1979). *cis*-11-Eicosenol also influences honeybee pursuit and stinging of targets. Its lower volatility suggests that this component aids in targeting stings (Pickett et al., 1982).

Variation in levels of defensive behavior in races or strains of bees could be influenced by differences in either production of or sensitivity to alarm pheromones. Collins (1979) demonstrated increased sensitivity to alarm pheromone in a defensive European honeybee (EHB) inbred line compared with a gentle line. Africanized honey bees (AHB), which are well known for their highly defensive behavior, respond more rapidly and in greater numbers than do gentle EHB after application of IPA to the entrance of colonies (Collins et al. 1982, 1987, 1988), and this response has a heritable component (Collins, 1979; Rinderer et al., 1983; Moritz et al., 1987). It also has been suggested, however, that different levels of IPA (Boch and Rothenbuhler, 1974; Kerr et al., 1974; Crewe, 1976) or other alarm pheromone components could account for higher defensive behavior. Collins et al. (1989) found higher levels in AHB for 9 of the 12 alarm pheromone components

analyzed in a survey involving bees from two geographic sources. When the researchers controlled for age and geographic location, EHB had higher levels for two of the nine components that varied and had twice as much IPA. Hunt et al. (1999) mapped the genomic locations of nine putative quantitative trait loci (QTL) that influenced the production of four alarm pheromone components, including IPA. The colonies used in that study were derived from an AHB/EHB F_1 queen. In general, QTL that influenced alarm pheromone levels were not the same QTL that influenced colony stinging responses mapped in the same population of colonies (Hunt et al., 1998). They also found in this particular cross that QTL alleles conferring higher levels of alarm pheromone components were more often inherited from the gentle European parent (six cases) than from the Africanized parent (three cases) and were often correlated with body size. However, at least one GC peak, representing an unidentified compound, correlated with whole-colony stinging behavior and individual guarding behavior (unpublished data). We collected new samples of stings from several unrelated sources of Africanized bees and European bees to survey for novel alarm pheromone components. Here, we report a new alarm pheromone component and its influence on behavior.

METHODS AND MATERIALS

Sampling for Alarm Pheromone. From each colony, we collected six guard bees with tweezers. The sting of each was removed with another tweezer, and the stings from all six bees were combined in a sealed vial containing 2 ml of methylene chloride and 4.86 μg/ml of *n*-tetradecane as an internal standard. All workers sampled were from naturally mated queens. Since queens mate with up to 17 males (Adams et al., 1977), open-mated queens provide a diverse genotypic sample from an area. All of the bees in this study were kept at the Center for Beekeeping Development, located near Villa Guerrero, Mexico (19°N, 99°W). Two European queens were obtained that were naturally mated in Canada and were part of a breeding program involving Buckfast bees (originating from introductions from Buckfast Abbey, UK). One European queen was obtained from a California queen breeder (Glenn Apiaries, Fallbrook, California, USA). Five presumably unrelated queens of African origin were obtained from several areas within 50 km of the center from feral swarms or commercial colonies that had not been requeened for at least two years. AHB sources were tested by morphometrics (Sylvester and Rinderer, 1987) and behavioral tests (Guzmán-Novoa et al., 1999) to ensure they had African characteristics, and also for the mitochondrial DNA type of *A. m. scutellata* (Nielsen et al., 2000).

Chemical Analyses. The gas chromatography–mass spectrometry (GC-MS) analyses were performed by using both an Hewlett-Packard Engine mass spectrometer (Hewlett-Packard, Co./Agilent Technologies, Wilmington, Delaware, USA)

and a Finnigan GCQ (ThermoFinnigan, San Jose, California, USA). In both instruments, 1-μl samples were injected onto a 15-m DB-1 capillary column (J&W Associates), with the injector temperature set at 240°C. The initial column temperature was set at 50°C (40°C for GCQ) and held for 1 min prior to being temperature programmed to 250°C (240°C for GCQ) at 10°C (12°C for GCQ)/min. Samples were ionized by electron impact (EI) and isobutane chemical ionization (CI).

The 3-methyl-2-buten-1-yl acetate (3M2BA) was synthesized starting from 3-methyl-2-buten-1-ol (cat. no. 16,235-3; Aldrich Chemical Co., Milwaukee, Wisconsin, USA). To a mixture of 3-methyl-2-buten-1-ol (0.86 g, 10 mmol) and pyridine (0.79 g, 10 mmol) at 0°C was added acetyl chloride (0.82 g, 10 mol) dropwise. The reaction was highly exothermic. The mixture was stirred at 0°C for 1 hr, followed by addition of water (15 ml) and extraction with diethyl ether (3 $\times$ 15 ml). The organic extracts were combined, washed thoroughly with water, and dried ($MgSO_4$). The dried ether solution was again passed through a short silica gel column to remove any polar side-products. Removal of ether in air, followed by bottle-to-bottle distillation gave the desired 3-methyl-2-buten-1-yl acetate (MW 128). (Anal. GC-MS, >99% pure. EI: *m/z* 128, <2%; *m/z* 85, 7%; *m/z* 71, 16%; *m/z* 69, 38%; *m/z* 68, 69%; *m/z* 67, 70%; *m/z* 53, 21%; *m/z* 43 100%; CI: $(M + H)^+$, *m/z* 129, 100%.)

Behavioral Assays. Field assays similar to the method of Collins and Kubasek (1982) were employed. A small piece of tissue paper of standard size on the end of a stick was wetted with the pheromone component, either IPA, 3M2BA, or a 1:2 mixture of 3M2BA and IPA. The stick served as a handle to present the pheromone with minimal disturbance to the colony. The test substance was diluted 1:20 in mineral oil (Sigma M-3516, St Louis, Missouri, USA) to control the rate of evaporation. The equivalent of 2.5 μl of alarm pheromone component or mixture was used in each test (50 μl of diluted pheromone). Individual stings contain about 1–5 μg of IPA (Boch and Shearer, 1966). Immediately after wetting the tissue paper, the paper on the stick was placed directly in the center of a colony entrance. A photograph was taken immediately before application of pheromone to the colony, and again 20 sec after pheromone application. The difference in number of bees on the outside of the colony after application of the pheromone component was used as a measure of the effectiveness of the compound in alerting the bees. Therefore, "alarm" in this paper is defined as recruitment at the colony entrance. Each colony was tested three times for each of the three test substances, allowing at least 4 hr between tests. In addition, the observers walked 25 m from the colony immediately after the test, and a count was made of the bees that pursued for that distance. Differences in recruitment between treatments were analyzed with Fisher's protected LSD to look at overall effects of the chemicals on behavior. The European and Africanized bees were analyzed separately because of the large differences in response observed for the two types.

RESULTS AND DISCUSSION

AHB react with behaviors that resemble EHB in response to application of IPA and synthetic alarm pheromone mixtures. In comparison to EHB, however, the AHB colony response is characterized by greater numbers of bees rushing out of the hive that continue to be agitated for longer periods of time (Collins et al., 1987). Figure 1 shows a representative GC-MS trace. The largest peak (L) corresponds to eicosenol (MW 296). A peak in this region, peak D, was identified as a solvent contaminant, chlorocyclohexanol. Peak C, however, eluting after isopentyl acetate (peak B), was a component found in most samples of AHB. The new component was tentatively identified as 3-methyl-2-butenyl acetate (3M2BA) based on the mass spectrum. This compound was synthesized (see Methods and Materials) from 3-methyl-2-buten-1-ol. The mass spectrum and GC retention time of the synthesized 3M2BA were identical to that of the unknown. 3M2BA was present primarily in AHB (Table 1) and was detected in each of the three samples from four of the five AHB colonies. The amount of 3M2BA in AHB samples ranged from 0 to 38% of IPA levels, with an average of 21%. Only one sample from one EHB colony had detectable 3M2BA in it. It is possible that this European colony had a few Africanized bees that had "drifted" into the colony and had become accepted as nest-mates. Robbing behavior in which bees try to steal honey from other colonies is often observed late in the year at the time of the honey harvest (which was prior to the time of sampling), and some drifting of bees among colonies can occur at this time.

The novel alarm pheromone component, 3M2BA, is probably synthesized from IPA. Because of the structural similarity of 3M2BA and IPA, behavioral assays were conducted with either synthetic 3M2BA, IPA, or a 1:2 mixture of 3M2BA and IPA. This ratio is similar to that found in some of the AHB samples. There was an effect on recruitment attributed to the test chemical and the type of bee (EHB versus AHB) but no significant type $\times$ chemical interaction (Table 2). Results show that AHB were more responsive, but both types of bees reacted with the same pattern of responses to the test compounds. Overall, there was higher recruitment in response to the mix than to either IPA (Fisher's LSD; $P < 0.001$) or to 3M2BA alone ($P < 0.05$) but no difference between IPA and 3M2BA ($P = 0.18$). Thus, 3M2BA recruited bees as efficiently as IPA, but the mixture of the two was more effective (Figure 2). Analyzing AHB and EHB separately showed that for both bee types the mixture was significantly more effective than IPA for eliciting a response ($P = 0.017$ and $P = 0.009$, respectively). None of the test substances differed in their ability to elicit pursuit of the observers for a distance of 25 m (results not shown). Africanized bees were more likely to pursue. Overall, an average of 25.5 AHB pursued, compared to only 2.3 EHB.

This is the first time that an alarm pheromone component has been found in honeybee stings that is as potent in behavioral assays as IPA for eliciting an alarm response. The alarm response was defined as the number of bees recruited from the

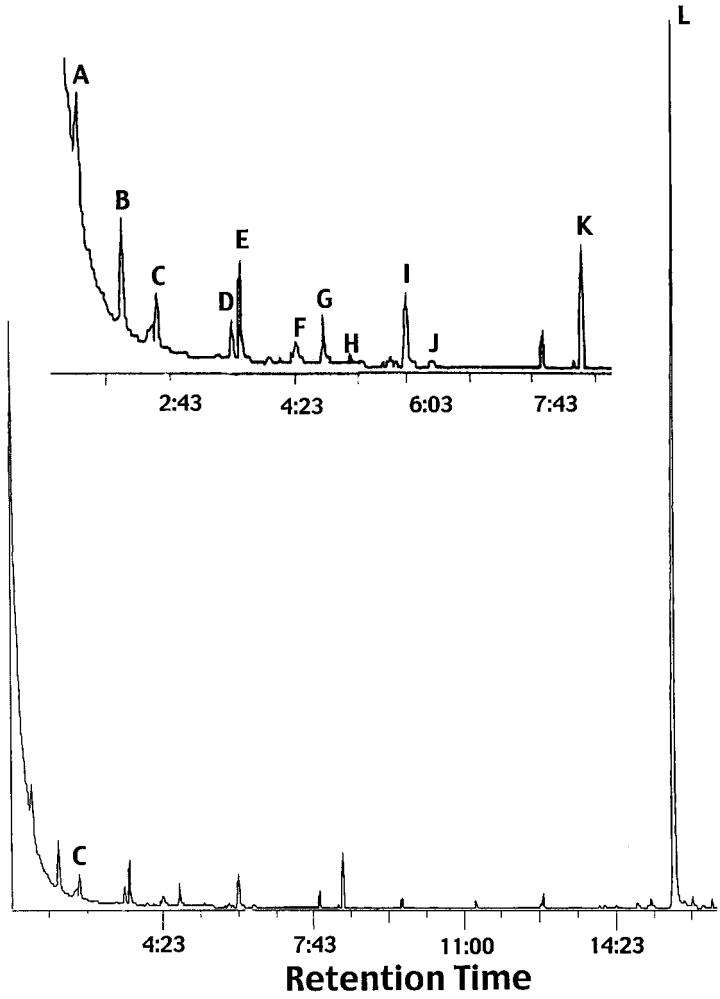

FIG. 1. Gas chromatographic trace for one representative sample of six stingers from worker bees. The peaks represent the following compounds: A, butyl acetate; B, isopentyl acetate; C, 3-methyl-2buten-1yl acetate; D, chlorocyclohexanol; E, hexyl acetate; F, octanol; G, 2-nonanol; H, benzyl acetate; I, octyl acetate; J, nonyl acetate; K, tetradecane internal standard; L, eicosenol.

hive, but the recruits were quick to sting the clothes of the researchers in response to either of the pheromone components or the mix. The unusual complexity of the pheromone blend of honeybees suggests that interactions between compounds may be important or that different components have different functions. The quality and

TABLE 1. AMOUNTS OF TWO ALARM PHEROMONE COMPONENTS IN SAMPLES OF STINGERS FROM GUARD BEES OF 8 COLONIES[a]

Colony[b]	IPA[c]	μg/sting	3M2BA[d]	μg/sting
E1	1.50 ± 0.45	2.4	0.06 ± 0.10	0.1
E2	1.86 ± 0.74	3.0	0	0
E3	0.81 ± 0.41	1.3	0	0
A1	2.17 ± 0.56	3.5	0.18 ± 0.05	0.3
A2	3.81 ± 0.99	6.2	0	0
A3	0.79 ± 0.17	1.3	0.30 ± 0.18	0.5
A4	1.75 ± 0.44	2.8	0.34 ± 0.12	0.6
A5	2.23 ± 0.17	3.6	0.29 ± 0.15	0.5

[a] Three samples of six stingers each were collected from guard bees and stored in methylene chloride. Values are the means and standard deviations of the areas of peaks from the GC referenced to an internal standard of *n*-tetradecane.
[b] The prefix "E" refers to European colonies; the prefix "A" refers to an Africanized honey bee colony.
[c] IPA = isopentyl acetate; first column contains amount relative to internal standard and second contains average micrograms per sting.
[d] 3M2BA = 3-methyl-2-buten-1-yl acetate; first column contains amount relative to internal standard and second contains average micrograms per sting.

quantity of aromatic components increases as bees reach guarding and foraging ages (Boch and Shearer, 1966; Crewe and Hastings, 1976). A number of these components have been tested individually to determine their effects on behavior. At least 10 have been reported to alert bees at the hive entrance, and nine have been reported as contributing in some way to stinging behavior (Free, 1987). Most of the stinging response elicited by a fresh intact sting in European honeybees has been attributed to IPA alone (Boch et al., 1962). However, 3M2BA was as effective as IPA for recruiting bees and was present in stings of AHB at levels up to 38% that of IPA.

TABLE 2. ANALYSIS OF VARIANCE FOR NUMBER OF BEES RECRUITED BY THREE SUBSTANCES[a]

	df	Mean square	*F*	*P*
Compound	2	12476.167	7.094	0.002
Bee type	1	93333.796	53.069	<0.001
Compound type	2	230.574	0.131	0.877
Residual	48	1758.741		

[a] The three substances tested were 3-methyl-2-butenyl acetate (3M2BA), isopentyl acetate (IPA), and a mixture of 1 part 3M2BA to 2 parts IPA. The two bee types were European honeybees and Africanized honeybees.

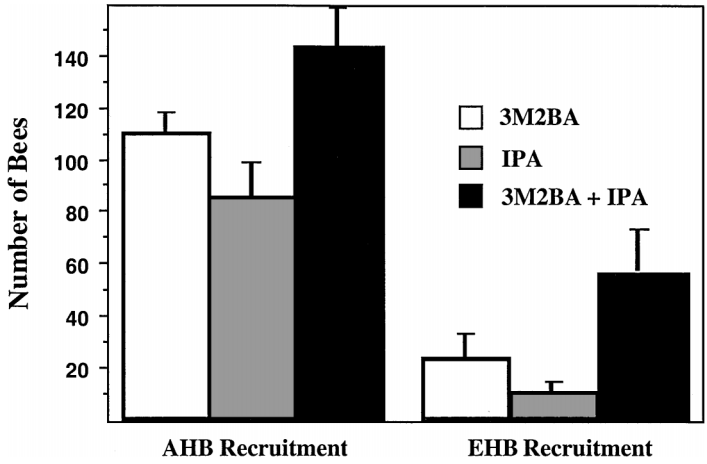

FIG. 2. Average number of bees recruited per colony by two alarm pheromone components and a 1:2 mixture of them. Bars represent the average number of bees (with standard errors) recruited in 20 sec for three tests.

The diversity of pheromone components in bees leads us to believe that natural selection acts to generate this complexity. The compound described in this study may only be present in some populations of AHB and may be spreading to others. In addition to the synergistic effects that we observed, specific components or mixtures of pheromones might aid in reinforcement of guarding behavior or nest and nestmate recognition. Bees often engage in behavior that is identical to that of a guard bee when exposed to a sting apparatus or alarm pheromone (Ghent and Gary, 1962). The level of isopentyl acetate reaches its peak in bees of guarding age (about 5 μg) (Boch and Shearer, 1966). Bees will partially extrude the sting when alarmed or while guarding. This may reinforce their own guarding behavior or recruit other bees to guard or to sting. Reinforcement of guarding behavior may cause bees to persist in guarding for a few extra days before becoming foragers, and increase the pool of bees near the entrance that are the front line in colony defense. AHB guards have been shown to be more persistent than EHB and respond to the presence of other AHB with even greater persistence in guarding (Hunt et al., unpublished data).

Complex blends of alarm pheromone components might aid bees to recognize their own colony entrance more effectively and/or discourage robbers from entering (nonnestmate bees that seek to steal honey from a colony). Components such as IPA from the sting apparatus and 2-heptanone from the mandibular glands have been shown to repel foragers (Shearer and Boch, 1965; Simpson, 1966; Free et al., 1985; Reith et al., 1986). Foragers are also repelled by scents released from disturbed bees. The repellent effect of a chamber that was preconditioned with a forager bee

was greatest just after the period of abundant nectar flow when robbing behavior is more likely (Balderrama et al., 1996). However, in those experiments, bees were repelled more by head squashes than by sting squashes, suggesting a role of mandibular gland compounds such as 2-heptanone in repelling workers.

Pheromone components are often used in more than one modality and in ways that are context specific. It would not be unusual for compounds that are produced in the quantities found in the honeybee sting apparatus to be used for multiple roles (Blum, 1996). The addition of other components may prolong the effect of pheromone for inducing alarm. Some current hypotheses to test are that additional roles for alarm pheromones include: synergistic effects in recruitment, reinforcing guarding, persistence, and perhaps nest recognition.

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MATING BEHAVIOUR AND EVIDENCE FOR A FEMALE RELEASED COURTSHIP PHEROMONE IN THE SIGNAL CRAYFISH *Pacifastacus leniusculus*

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Abstract—The mating behaviour of the signal crayfish, *Pacifastacus leniusculus* (Dana), has been categorised into seven distinct stages: orientation; contact; seizure; turning; mounting; spermatophore deposition; and dismounting. These categories were used in the development of a bioassay to test whether mature females release a sex pheromone during the breeding season to which males respond. Water conditioned by 1) mature females (MF water), 2) immature females (IF water) and 3) freshwater (C water), were injected through standard aquarium air-stones into tanks containing mature males. The behaviour of the male was recorded on video for 15 minutes before and after injection of each test water. The time that the males spent exhibiting the three behavioural categories of 'quiescent,' 'motile' and 'handling' of the air-stone, were recorded. 'Handling' of the air-stone included the mating behaviours of seizure, mounting, and spermatophore deposition onto the surface of the air-stone. Males exposed to MF water exhibited significantly increased levels of motile activity and handling behaviour, than males exposed to C water or IF water. These results demonstrate for the first time the presence of a sex pheromone, released during the breeding season by mature females, that stimulates courtship and mating behaviour in male *P. leniusculus*.

Key Words—crayfish, *Pacifastacus leniusculus*, sex pheromone, behavioural bioassay, courtship, mating.

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INTRODUCTION

Since the 1960s, the list of crustacean species that have been shown to make use of sex pheromone(s) has grown considerably (Hughes and Matthiesson, 1962; Ryan, 1966; Hazlett, 1975; McLeese, 1970; Dunham, 1979; Gleeson, 1980; Bauchau, 1986; Dunham, 1978, 1988; Cowen and Atema, 1990; Asai *et al.*, 2000; Hardege *et al.*, 2002). However, despite this, only the pheromone of the hair crab, *Erimacrus isenbeckii* has been fully characterised (Asai *et al.*, 2000).

It is viewed that the relatively slow progress in the identification of aquatic crustacean pheromones has been due to the lack of reliable behavioural bioassays (Dunham, 1978, 1988; Bentley and Watson, 2000). The two principal problems in the development of bioassays are the requirement to remove or reduce stimuli other than the pheromone being studied, and the interpretation of the induced behavioural response. Progress has been made to eliminate external stimuli, for example by spatial separation of the releaser and the receiver of the stimulus, and the use of darkened aquaria (Bamber and Naylor, 1996), but interpretations of many behavioural responses, even with reduced external influences, still remain ambiguous. Tests for sex pheromones based on courtship behaviour, however, make for reliable bioassays, as there are few alternative interpretations of such a reaction to a potential partner (Dunham, 1978).

To use the copulatory behaviour of a species in a pheromone bioassay, the behaviour itself has to be well understood and documented. Several studies have reported on the copulatory behaviour of a number of crayfish species (Mason, 1970; Ingle and Thomas, 1974; Villanelli and Gherardi, 1998), but none has documented the mating behaviour of *Pacifastacus leniusculus*.

Pheromone-induced sex recognition in conspecific crayfish has been documented in a number of species based on aggressive or submissive responses to conditioned water (Ameyaw-Akumfi and Hazlett, 1975; Tierney and Dunham, 1982, 1984; Hazlett, 1985; Bechler *et al.*, 1988; Villanelli and Gherardi, 1998). Although there have been a number of contradictory studies, Bechler (1995), in his review, assigned these to flawed bioassay techniques. Overall he concluded that despite the lack of absolute evidence for the use of such pheromones, the evidence supports the contention that pheromones are involved during some aspect of sexual behaviour or mate choice in crayfish. The use of sex pheromones by *Pacifastacus leniusculus*, however, has never been documented.

We report on the first description of the courtship and copulatory behaviour of *Pacifastacus leniusculus*, and through the development of an unambiguous, quick and reliable bioassay, we demonstrate that mature females produce a pheromone that induces courtship behaviour in mature males during breeding season.

METHODS AND MATERIALS

Culture Collection and Maintenance. Specimens of *Pacifastacus leniusculus* were collected from the River Thames, Oxford, England (grid reference OS164: 504 065) on the 17–18 September 2001. In the laboratory, animals were maintained at ambient temperature (15°C) and photoperiod. Animals were separated by sex in communal re-circulating systems, and fed daily with potato, supplemented weekly with mackerel or tinned ham. Animals were only used once in any of the experiments.

Assessment of Sexual Maturity. Sexual maturity was distinguished by the presence of well-developed glair glands in females, and whitened gonopods in males (Thomas and Ingle, 1971). Only animals with a carapace length >30 mm in females and >35 mm in males were considered mature and consequently used in the experiments.

Mating Behaviour Analysis. Mating behaviour was recorded using an overhead mounted Sharp Digital Viewcam (video camera) between October and mid-November of 2001. Twenty successful pairings in glass tanks ($18 \times 32 \times 20$ cm) containing 4L of constantly aerated freshwater (15°C) were used. A mature female was placed into the tank and left to acclimatise for 30 minutes; the male was then placed at the opposite end of the tank. The courtship behaviour was recorded, analysed and categorized into seven stages as follows (times were recorded from the point that the male was placed into the tank):

1. *Orientation.* The male orientated and moved towards the female. The female tended to remain stationary until the male approached.
2. *Contact.* Initial contact was aggressive in 13 out of the 20 observed pairings. The animals met with chelae raised, but the female soon became submissive. The larger the male (relative to the female) the more rapidly the female became submissive. Mating contact differed from non-mating contact, because the female would not attempt to swim away when grasped by the male, which is characteristic of the subordinate in non-mating contact of both male-male and male-female interactions. In the other seven there was no aggressive interaction, and the male exhibited stage 3 behaviour directly.
3. *Seizure.* Once the female had been pacified, the male grasped the female by her chelipeds, antennae or rostrum. Eleven males used both chelipeds to grasp the female, the others used only one, although they would often change the grip on the female to gain a more suitable hold. The female remained stationary throughout this period, while the male climbed onto her back.
4. *Turning.* Once on top of the female, the male began to turn the female over onto her back often adjusting his grip on her. In all cases the male used

his pereiopods, and his already established hold(s), to role the female over whilst she was held beneath him. In 18 cases the male ended up with his ventral surface parallel with the underside of the female's abdomen, in the other 2 cases the ventral surfaces were already parallel.

5. *Mounting.* The male would then move along the female's body until their ventral surfaces were opposed. During this whole period the male still had a firm hold of the female with his chelipeds, and with his other pereiopods around her cephalothorax.
6. *Spermatophore Deposition.* The male then proceeded to deposit spermatophores onto the female's ventral surface, while maintaining his hold on her. This deposition behaviour was characterised by arching and depression, in quick succession, of the male's abdomen at an average rate of 1 full cycle every 10 seconds (1Hz). The male would sometimes rest during this process for up to 30 seconds between bouts.
7. *Dismounting.* After deposition, half the females struggled and turned over, effectively throwing the males off, whilst the males moved off the other females. The female then moved away from the male. On 12 occasions the male tried to remount the female. During this stage the female constantly tried to move away, this often involved aggressive interaction. On 9 occasions the male ended up 'guarding' the female, holding her close to him with his chelipeds and not allowing her to move, despite her efforts to move away. This tended to be the case only with the larger males (relative to the female).

Conditioning of Test Water for Bioassay. Water was 'conditioned' during the breeding season by placing four females in a tank containing 2L of constantly aerated freshwater (15°C) over a 24-hour period. Water was 'conditioned' using mature females, and immature females. The control was freshwater that had been aerated over a 24-hour period with no crayfish present.

Bioassay Experimental Design. Individual mature males were placed in opaque, visually isolated tanks (12 × 18 × 15 cm) each containing 2 L of freshwater at 15°C (not aerated during recording periods). Males were left to acclimatise for 1 hour before the start of the experiment. A blue cylindrical aquarium air-stone 2 cm in length was placed at one end of the tank furthest away from the test animal 30 minutes into the acclimatisation period. A syringe containing 20 ml of the female conditioned water was attached to the air stone via 15 cm of 5 mm-diameter silicone tubing. The males' behaviour was then recorded for 15 minutes before the introduction of the treatment, and for another 15 minutes afterwards using a Sharp digital Viewcam (video camera) mounted above the test tank. Conditioned water was introduced to the test arena at a rate of approximately 5 ml/sec. Thirty-two males were exposed to water conditioned by mature females (MF water). Thirty-two males were exposed to 'blank' control (C water); and 16 males were exposed to

the water conditioned by immature females (IF water). The experiments were run during the breeding season (October to mid-November) of 2001, between 10.00 h and 15.00 h (the period of the day when the animals were least active).

Data Collection and Analysis of Bioassay. For the bioassay, three behavioural categories were recorded based on the observed mating behaviour: 'quiescent,' the animals remained motionless, 'motile,' the animal was moving around the tank, and 'handling,' where the animal was making contact with, seizing, and mounting the air-stone, the animals would show all of these behaviours to be classified as handling. Contact, seizing, turning and mounting were not recorded as separate behaviours as it was difficult to distinguish them as distinct categories without the interaction between a male and female, and spermatophore deposition happened rarely so was only noted. The time that the animals spent in each behavioural category, for before and after the addition of the treatment, was recorded and totalled. The data were ranked and analysed using a general linear model with a pair-wise comparison, at the 95% significance level (Conover and Iman, 1981).

RESULTS

Mating Behaviour. Figure 1 shows the mean start and finish times (minutes) with 95% C. I. of each of the seven categories of the mating behaviour ($N = 20$). All times shown were taken from the introduction of the male into the test arena ($T = 0$). The whole mating process took on average 32 minutes to complete,

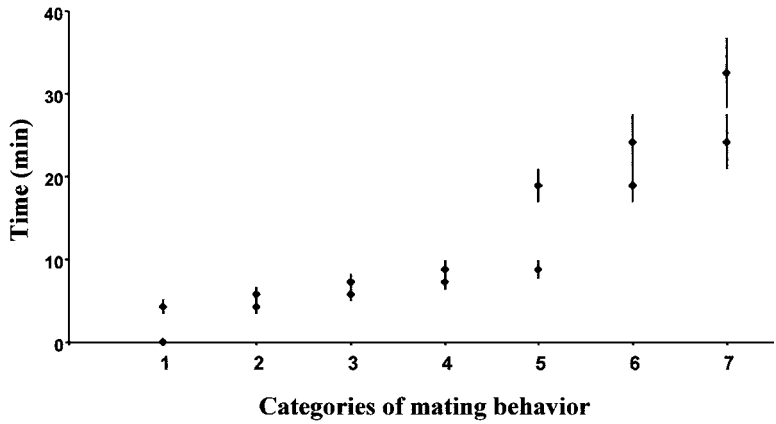

FIG. 1. Mean start and finish times (minutes) with 95% C.I. for each category of the mating process in *Pacifastacus leniusculus*: 1) orientation, 2) contact, 3) seizure, 4) turning, 5) mounting, 6) spermatophore deposition, and 7) dismounting ($N = 20$). All times were taken from the introduction of the male into the test arena ($T = 0$).

though varied between 36 and 28 minutes. Orientation towards the female took between 1–5 minutes (mean: 4 min 20 sec). Contact, seizure and turning all happened in comparative quick succession than the other stages, all taking between 1–2 minutes to complete. The mounting of the female was the longest stage, taking between 9–20 minutes, with a mean time of 10 minutes. Spermatophore deposition took on average 5 minutes 20 seconds, but did take as long as 12 minutes. Abdominal depressions took place in short bursts of up to 40 seconds before resting during spermatophore deposition. Although, the mean time for dismounting was 8.5 minutes, individual times varied between a couple of seconds and 15 minutes, depending on how much the female struggled or if the male dismounted before the female began to struggle.

Bioassays. Figure 2 shows the average time that the male spent exhibiting each behaviour before (white), and after (grey) the addition of C water treatment ($N = 32$). The data showed that there was no significant difference in the time spent quiescent ($T = -0.34$, P 0.9860), motile ($T = 0.832$, P 0.8999), or handling ($T = 1.011$, P 1.0000) for before and after the treatment. This demonstrates that the physical process of injection had no effect on the animal's behaviour.

Figure 3 shows the average time that the male spent exhibiting each behaviour before (white), and after (grey) the addition of MF water during the breeding season ($N = 32$). There was a significant decrease in the time spent quiescent after the addition of female conditioned water ($T = -6.700$, P 0.0001). There was also a slight, but insignificant ($T = 1.396$, P 0.1055), increase in the time spent motile after addition of MF water. Although there was some handling of the air-stone before the addition of the conditioned water, the handling time increased significantly ($T = 5.893$, P 0.0001) after the addition of the MF water. The males did not always go through the 'typical' mating sequence described above, often missing stages out. The main behaviour observed during the 'handling' category was seizure and mounting and although the males were, at times, clearly trying to turn the air-stone this was impossible for them to achieve. In addition to the copulatory behaviour observed in the experiments, in two cases males deposited spermatophores onto the surface of the air-stone.

Figure 4 shows the average time that the male spent exhibiting each behaviour before (white), and after (grey) the addition of IF water conditioned during breeding season ($N = 16$). There were no significant differences in the time spent quiescent ($T = -0.49$, P 0.9875), motile ($T = 0.085$, P 0.9999), or handling ($T = -0.3513$, P 0.9775) for before and after introduction of the conditioned water.

DISCUSSION

The mating behaviour described here, for *Pacifastacus leniusculus* is similar to that of other crayfish species studied. In this study, seven distinct stages of the

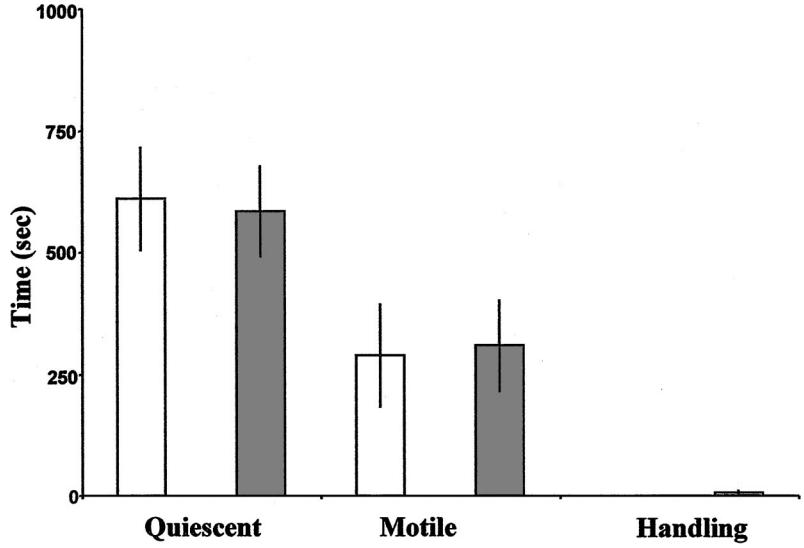

FIG. 2. Mean time (secs) that male *Pacifastacus leniusculus* spent quiescent, motile or handling before (white) and after (grey) the addition of non-conditioned water (C water) ($N = 32$), with 95% C.I.

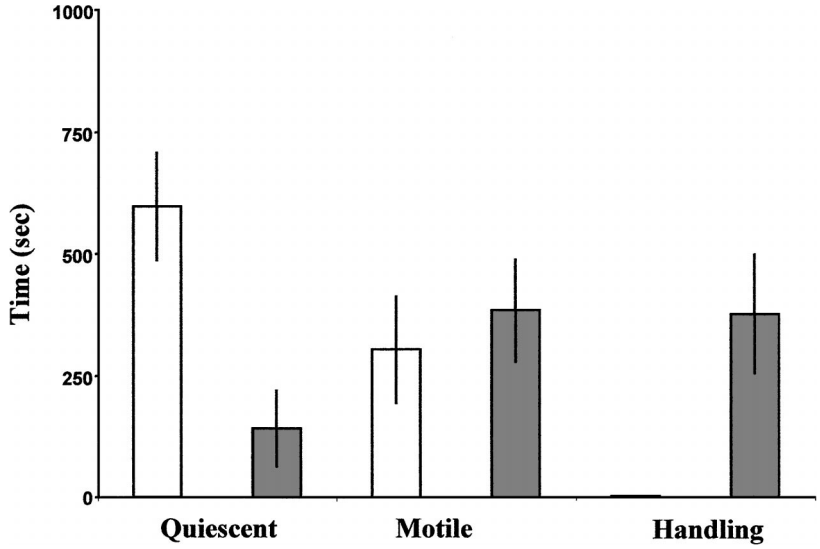

FIG. 3. Mean time (secs) that male *Pacifastacus leniusculus* spent quiescent, motile or handling before (white) and after (grey) the addition of water conditioned by mature females (MF water) ($N = 32$), with 95% C.I.

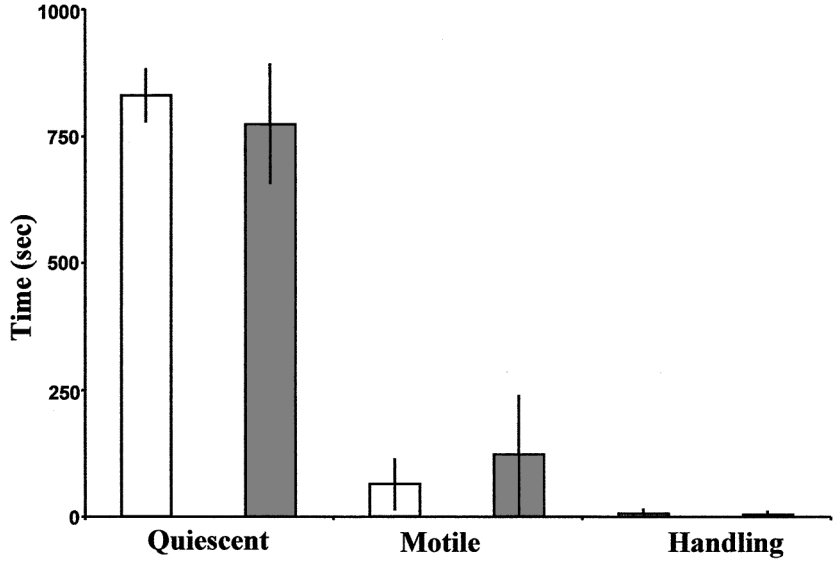

FIG. 4. Mean time (secs) that male *Pacifastacus leniusculus* spent quiescent, motile or handling before (white) and after (grey) the addition of water conditioned by immature females (IF water) ($N = 16$), with 95% C.I.

mating process have been identified, compared to 10 stages for *P. trowbridgii*, (Mason, 1970), seven for *Austropotamobius pallipes* by Ingle and Thomas, (1974) and three stages by Villanelli and Gherardi (1998) also for *A. pallipes*. Both Mason (1970), and Ingle and Thomas (1974) described the locking of the gonopods. Mason (1970) also went onto describe the unlocking and recession of the gonopods after spermatophore transfer. Neither of these behaviours was described in this study (or Villanelli and Gherardi (1998)) as recordings were made from overhead therefore not allowing a clear view of the gonopods. However, for spermatophore deposition to take place the gonopods have to be locked, and subsequently recessed, it is therefore likely that *P. leniusculus* also performs these processes. Mason's description of the actions of the gonopods accounts for three stages and post-copulatory grooming for another not described in this paper. Mason also distinguished between the release of the female and dismounting of the female, while Ingle and Thomas only mention the release of the female, and this study only mentions dismounting. Villanelli and Gherardi have not mentioned either of these stages.

The orientation and movement towards the female by the male, and post-copulatory guarding of the female by the male described in this study have not been recorded in other studies. Mason (1970) did observe post-copulatory grooming of the abdominal sterna by the female although this was not observed in

this report. The duration of the whole mating process also differs among species of crayfish; Ingle and Thomas recorded a time of between 12–15 minutes and Villanelli and Gherardi of 5 minutes for *A. pallipes*; Mason recorded a duration of 8–20 minutes for *P. trowbridgii*, and this study a duration of 28–36 minutes with a mean time of 36 minutes for *P. leniusculus*. Despite the differences, the main stages of contact, turning, mounting, and spermatophore deposition are present in all other descriptions of the mating behaviour in crayfish. The mating behaviour recorded was the basis for the bioassay, such distinctive behaviours allows for the distinction between mating behaviour and other possible reactions that the male could have to female conditioned water e.g. feeding or aggressive behaviour.

It can be concluded from the data (presented in figure 3.) that a pheromone is released by mature female *Pacifastacus leniusculus* during the breeding season that stimulates courtship and mating behaviour in males. This is evident from the increase in the amount of time that the animal spent handling the air-stone after the addition of MF water. As quiescence and motility are non-independent it can be assumed that the significant decrease in time spent quiescent, and the slight increase in motility after the introduction of the female-conditioned water can be equated to an increase in activity due to the addition of the MF water; this may be movement towards the air-stone, or at the very least increased activity due to searching for the source of the attractant, this is supported by the significant increase in the time spent handling the air-stone. The lack of significant differences when mature males were tested with IF (figure 4.) suggests that immature females do not release the pheromone during breeding season, and that the behaviour of the males observed with the introduction of MF water is of a sexual nature.

A number of studies have reported evidence for sex recognition and mate attractants in conspecifics of *Procambarus clarkii, P. zonangularis, Austropotamobius pallipes* and *Orconectes spp.* and sympatric *Orconectes spp.* (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985; Tierney and Dunham, 1982, 1984; Bechler *et al.*, 1988; Corotto *et al.*, 1999). None, however, has reported on evidence for a courtship and mating pheromone, which this study does. The use of a strong, unambiguous response such as the effective 'mating' with an air-stone clearly demonstrates the presence of a pheromone that induces courtship and mating behaviour in *Pacifastacus leniusculus* that is only released by mature females during the breeding season. Purification of the pheromone is in progress using the bioassay described in this report.

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CHEMICAL ATTRACTANTS IN HORSESHOE CRAB, *Limulus polyphemus*, EGGS: THE POTENTIAL FOR AN ARTIFICIAL BAIT

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Abstract—Horseshoe crabs, *Limulus polyphemus*, are the preferred bait in the eel and conch fisheries along the east coast of the United States. However, recent management measures have restricted the availability of horseshoe crabs to commercial fisheries, creating the need for sustainable, alternative bait sources. In this study, we examined the chemistry underlying the predator–prey attraction to determine if specific, isolable attractant metabolites from the horseshoe crab could be identified and characterized for incorporation into an artifical bait. Initial assays with the mud snail, *Ilyanassa obsoleta*, suggested that the chemoattractants were concentrated in *L. polyphemus* eggs. Chemical analyses and biological assays of the egg extract indicated the primary cue was a heat-stable, proteinaceous compound (>10 kDa). A carbohydrate-rich fraction of low molecular mass (<10 kDa) also enhanced mud snail chemotaxis. Analysis of egg digests with SDS-PAGE confirmed the presence of glycoproteins or carbohydrate-binding proteins in the horseshoe crab egg extract. Because the attractant appears to be a complex protein or glycoprotein, conventional chemical synthesis is unlikely. However, the tools of modern biotechnology offer the potential to produce this attractant in a system independent of the horseshoe crab. Such an attractant could be incorporated into an artificial bait, providing an ecologically sound alternative for commercial eel and whelk fisheries.

Key Words—Horseshoe crab, *Limulus polyphemus*, artificial bait, chemical attractant, chemoreception.

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INTRODUCTION

The Atlantic horseshoe crab (*Limulus polyphemus*) ranges from Maine, USA to the Yucatan peninsula, with the highest population density centered in the Delaware Bay (Shuster, 1982; Botton and Ropes, 1987; Smith et al., 2002; Walls et al., 2002). Horseshoe crabs are the preferred bait in the mid-Atlantic eel and conch fisheries (Bianchini et al., 1981; Reshetiloff, 1996; ASMFC, 1998, 1999; Manion et al., 2000). Landings along the US east coast have increased more than four-fold over the last decade as fishermen have moved from more heavily regulated fisheries into these fisheries, which initially had little or no regulation (ASMFC, 2000).

Current concern about the horseshoe crab population is driven by its multiple and often competing uses (Berkson and Shuster, 1999; Walls et al., 2002). In addition to their value as bait in the eel and conch fisheries, horseshoe crabs are vital to the biomedical industry, where they are the source of a clotting agent that detects the presence of endotoxins pathogenic to humans (Pearson and Weary, 1980; Manion et al., 2000; Walls and Berkson, 2000). Spawning horseshoe crabs also have an important link with migrating shorebirds. En route to arctic breeding grounds, migratory shorebirds stop over in Delaware Bay, feeding extensively on the lipid-rich horseshoe crab eggs (Clark et al., 1993; Botton et al., 1994; Clark, 1998; Berkson and Shuster, 1999; Tsipoura and Burger, 1999).

To assure that the horseshoe crab population can meet the basic needs of its multiple users, there has been pressure to manage the species more intensively (Berkson and Shuster, 1999). These management strategies have restricted the availability of horseshoe crabs for bait. In an effort to identify a suitable alternative for fishermen, we sought to isolate and characterize the specific chemical attractant from the horseshoe crab. Upon incorporation into an artificial bait, such an attractant could potentially provide a sustainable alternative to the natural horseshoe crab bait currently in use.

Chemical signals that elicit feeding behavior are often simple metabolites that are ubiquitous components of tissues (Atema, 1980a,b; Carr, 1982, 1988; Hara, 1982, 1992, 1993, 1994; Little, 1983; Atema et al., 1988; Rittschof, 1990; Marui and Caprio, 1992; Jones, 1992; Carr et al., 1996; Derby et al., 1996; Zimmer et al., 1999; Lamb, 2001), although examples of high-molecular-weight stimulants are known (Gurin and Carr, 1971; Heeb, 1973; Zimmer-Faust et al., 1984; Rittschof et al., 1984; Rittschof, 1990). Some aquatic animals respond to only a narrow range of the chemical spectrum available to them, being attracted by a specific quality of a chemical signal [i.e., a unique blend of common metabolites such as DFAAs, organic acids, amine bases, and nucleotides (e.g., Derby et al., 1989; Fine-Levy et al., 1989; Zimmer et al., 1999, Rahman et al., 2000) or the presence of a novel compound (Rittschof et al., 1984; Carr, 1988)]. We followed a bioassay-guided approach using the mud snail, *Ilyanassa obsoleta*, to isolate the specific attractant

from gravid female horseshoe crabs. Here, we report on the chemical nature of the attractant.

METHODS AND MATERIALS

Bioassay Organism. The mud snail, *Ilyanassa obsoleta*, was used for the bioassay-guided isolation of the attractant from the horseshoe crab. Chemoreception has been well documented for mud snails (Carr, 1967a,b; Gurin and Carr, 1971; Curtis, 1985; Zimmer et al., 1999; Rahman et al., 2000). These snails are natural scavengers, feeding on a mixed diet consisting of benthic diatoms, bacteria, detritus, and animal tissue. They locate their food primarily via olfaction (Zimmer et al., 1999; Rahman et al., 2000) and, like many other animals (e.g., eels, conch, catfish), are attracted to dead horseshoe crabs. Several hundred *Ilyanassa obsoleta* were collected at low tide, as needed, from the mudflats at Cape Henlopen, Delaware (38.8°N, 75.1°W). They were separated into several 5-liter tanks containing filtered seawater from Delaware Bay. The snails were maintained on a diet of frozen mullet and deprived of food for at least 24 hr prior to their use in bioassays.

Y-Choice Assays. Bioassays were conducted in a Y-choice chamber constructed of PVC pipe (diameter = 21.5 cm, arm length = 70 cm). The Y-tube design enhanced diffusion of waterborne chemical stimulants, simulating distance chemoreception in the aquatic environment. This design enabled us to determine snail preferences between paired treatments, which would help to localize the horseshoe crab attractant. Assay solutions were introduced via a peristaltic pump at a rate of 8 ml/min. Preliminary data suggested that this rate was sufficient to maintain an adequate water depth (2–3 cm) in the Y-tube. Using dyes, we established that there were two distinct streams, one traveling through the center of each arm, and no evidence of mixing at the junction of the arms. The Y-choice chamber was rinsed thoroughly with filtered seawater after each assay, and treatments were alternated between arms to prevent interference from chemical cues in snail mucous trails. Filtered seawater was used for all bioassays.

From the experimental population, random sets of 10 mud snails were chosen for each assay. None were returned to in the experimental population following use in a series of assays. Control assays (extract versus extract, control versus control) were conducted to assess whether snail trail-following behavior would confound experimental results. Treatments of egg extract were tested eight times with 10 naïve snails per test. Threshold and gel filtration treatments for egg extract were tested five times with 10 naïve snails per test. A bootstrap power analysis (Power and Precision v. 1.0 Los Angeles, California, USA) was used to determine the number of replicates per treatment.

To characterize the chemoattractant from *L. polyphemus* eggs, clutches of fertilized eggs were collected 1 hr after high tide from Cape Henlopen, Delaware, in June 1996 and 1997 and from Bowers Beach, Delaware, in May and June 1998–

2000. Fertilized eggs were stored at −80°C prior to extraction. Horseshoe crab egg extracts were prepared by extracting 1 vol of crushed eggs in 2 vols of 0.05 M Tris HCl buffer (pH 7.5) overnight, in the dark, at 4°C. After centrifugation to remove sand particles and cellular debris, the supernatant (crude extract) was decanted and immediately assayed for activity against a seawater control. Threshold limits of detection for *I. obsoleta* were determined.

Molecular Size Determination. The molecular size of the chemical cue in *L. polyphemus* eggs was estimated using gel filtration chromatography. Initially, molecular size standards ranging from 6.5 to 66 kDa were eluted on a Sephacryl S200HR column (30 cm × 1.5 cm diam.) with 0.05 M Tris KCl buffer (pH 7.5). After calibration of the column, absorbance (280 nm) of the eluate from 0.9 ml of the water-soluble egg extract was measured spectrophometrically. To determine the approximate molecular size of the chemoattractant in *L. polyphemus* eggs, elution volumes were fit to a standard curve of proteins. Fractions of the eluate from the water-soluble egg extract were collected and assayed for bioactivity in the Y-choice chamber. The protein content of each gel filtration fraction was examined with SDS-PAGE on a 15% Tris HCl Ready Gel (Bio-Rad). Proteins were visualized with Coomassie blue staining.

Ultra- and centrifugal filtration techniques were also employed to estimate the molecular size of the chemical cue in *Limulus polyphemus* eggs. The crude egg extract was filtered through a series of ultrafiltration membranes with molecular size cutoffs of 100, 30, 10, and 5 kDa in a pressurized stirred-cell system. Filtrates were assayed for biological activity in the Y-choice chamber. The crude extract was also filtered through YM-100 (100 kDa) and YM-10 (10 kDa) Centricon centrifugal filtration units overnight at 4°C. After collecting the filtrate, the retentate was resuspended in the initial volume of 0.05 M Tris HCl (pH 7.5) and washed by centrifugation overnight at 4°C. The resulting wash fraction was collected, and the retentate was resuspended in the initial volume of buffer. The Bradford method was used to determine the protein content of the crude extract, and the retentate, wash, and filtrate fractions. The biological activity of each fraction was assessed in the Y-choice chamber. Threshold detection limits for *Ilyanassa obsoleta* were determined by assaying snail response to several dilutions of each of the YM-10 fractions and the crude extract.

Ammonium Sulfate Precipitation. Ammonium sulfate precipitation was used to determine if the attractants in *L. polyphemus* eggs contained a protein component. Proteins typically precipitate out of solution between 40 and 60% saturation with $(NH_4)_2SO_4$. The boiled crude egg extract was fractionated at 0–30%, 30–50%, 50–80%, and 80–100% ammonium sulfate. Each fraction was centrifuged (7000g) for 5 min at 4°C. Pelleted precipitates were resuspended in 1 ml aliquots of 0.05 M Tris HCl buffer (pH 7.5). The activity of each precipitated fraction was assessed in Y-choice bioassays with *I. obsoleta*. Protein content of each fraction was determined with the Bradford method.

Ionic Charge. Ion-exchange chromatography on a carboxymethyl cellulose (CM 52) cation exchange column and a diethylaminoethyl cellulose (DEAE 52) anion exchange column was used to ascertain the charge of the chemical cue in *L. polyphemus* eggs. After applying 30 ml of egg extract to each column, columns were washed by gravity pressure twice with the Tris HCl buffer (pH 7.5), and then with increasing concentrations of NaCl (0.2, 0.4, 1.0 M) in the Tris HCl buffer. For each of the five fractions collected, a tenth of the volume was assayed for activity in the Y-choice chamber.

Finally, the active fractions eluted from the gel permeation and ion exchange columns and the active $(NH_4)_2SO_4$ cuts were concentrated with Centricon YM-10 ultracentrifugation devices. The purity of the concentrated fractions was established by SDS-PAGE on a 15% Tris HCl Ready Gel (Bio-Rad). Polyacrylamide gels were stained with Coomassie blue.

Thermal Stability. To examine the properties of the chemoattractant in *L. polyphemus* eggs, the crude egg extract was subjected to rigorous temperature treatments. Temperature treatments consisted of freezing the crude egg extract at 0°C or −80°C for 24 hr, heating at 90°C for 60 min, or autoclaving at 120°C for 20 min. Activity of the treated egg extract was subsequently assayed with *I. obsoleta*.

Enzymatic Digestion. Susceptibility of the chemoattractant to enzymatic digestion was determined by pepsin digestion of the crude egg extract. The egg extract was adjusted to pH 2 with HCl. Two, 5, 10, and 20 mg of pepsin were added to 10-ml aliquots of the extract, and the solutions were incubated with mild agitation for 2 hr at 25°C. After readjusting the pH to 7.5, the enzyme-digested extract was assayed for activity in the Y-choice chamber. Digestion was confirmed with SDS-PAGE on a 12.5% Tris-HCl Ready Gel (Bio-Rad). Polyacrylamide gels were stained with Coomassie blue.

Carbohydrate Analysis. The crude egg extract was precipitated with trichloracetic acid (TCA) (Bollag et al., 1996). The supernatant and pelleted proteins were assayed individually against controls in the Y-choice chamber. TCA precipitation products were analyzed for protein content using the Bradford method. Carbohydrate content was also determined using the phenol-sulfuric acid reaction with L-fucose as a standard (Dubois et al., 1956).

To elucidate the structure of the chemoattractant, the crude egg extract was digested with a carbohydrase mix consisting of α-amylase and β-glucosidase (1 μg/ml each) and a protease, either trypsin or chymotrypsin. Enzymes were allowed to react with the crude egg extract for 2 hr at 37°C, pH 7.5. Enzymatic digests were analyzed with SDS-PAGE on a 15% Tris HCl Ready Gel (Bio-Rad). The polyacrylamide gels were then silver stained (Pharmacia Biotech Silver Stain Kit) to detect dissociation of carbohydrate-protein complexes. Enzymatic digests were also precipitated with TCA. Supernatants from pre- and postdigestion products were assayed for carbohydrates as described above.

Statistical Treatment of Data. Data analysis for Y-choice assays was performed using Monte Carlo simulation (see Manly, 1991). This nonparametric statistic compares observed data to the results of simulations in which snails randomly respond to two choices. Initially, a Y-choice assay based on eight trials with 10 snails per trial was simulated. In each trial, it was assumed that snails moved independently of each other. Ten thousand iterations were performed, and the mean and median distributions were plotted. Based on these distributions, an average (across trials) of >6.4 or <3.6 snails choosing a side of the Y-choice chamber occurred in fewer than one of 100 observations. Therefore, if the average number of observed snails responding per assay was <3.6 or >6.4, the null hypothesis (H_o: $\mu = 0.5$) was rejected, and snail response was determined to be significantly different from random ($P < 0.05$). A Y-choice assay for five trials with 10 snails per trial was also simulated. In this case, $P < 0.05$ if an average of >6.5 or <3.2 snails chose one side of the Y-choice chamber.

The effects of enzymatic digestion on carbohydrate concentration in *L. polyphemus* egg extract were compared with one-way ANOVA. Differences in free sugar concentration among protease and carbohydrase treatments were analyzed *a posteriori* with the sum of squares simultaneous test procedure (SS-STP) (Sokal and Rohlf, 1996).

RESULTS

Ilyanassa obsoleta preferred *L. polyphemus* egg extract to a seawater control in Y-choice assays ($P < 0.05$) (Figure 1). Below a threshold of 3 ml/liter sea water, *Ilyanassa* response was not statistically different from random choice. In control experiments pairing seawater versus seawater and egg extract versus egg extract, we determined that snails were not preferentially attracted to either side of the Y-choice chamber throughout the duration of the experiment ($P < 0.05$). Thus, we established that the effect of the horseshoe crab attractant superceded the trail-following behavior of the mud snail.

The chemoattractant retained biological activity across a range of temperatures (-80°C to 120°C). Freezing the egg extract at 0°C or -80°C did not significantly degrade the activity of the chemoattractant. *Ilyanassa* preferred previously frozen egg extract to the seawater control ($P < 0.05$) (Table 1). When snail response to crude egg extract was compared to frozen extract, no difference was observed. Similarly, heat did not degrade the chemical cue. *I. obsoleta* preferred boiled (90°C) or autoclaved (120°C) extract to the seawater control ($P < 0.05$) (Figure 2). Response of *Ilyanassa* to heat-denatured extracts was not significantly different from crude egg extract. These data indicate that the chemoattractant demonstrates unusual tolerance to heat and cold extremes.

The active metabolite in *L. polyphemus* eggs was not characterized by a strong ionic charge. In CM52 (cation exchange) fractions, *Ilyanassa* responded positively

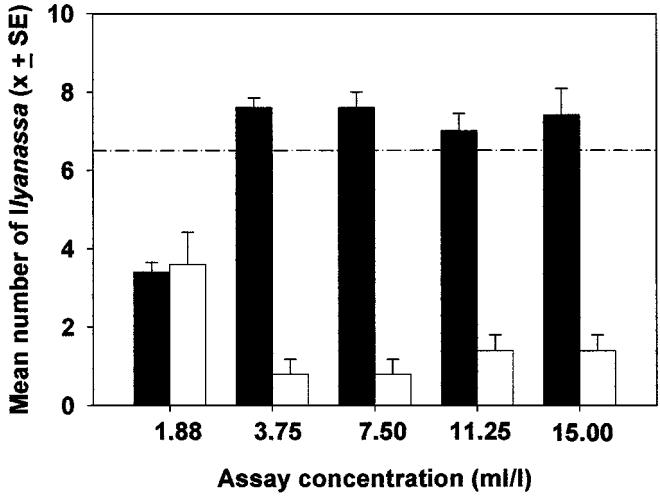

FIG. 1. Determination of threshold dilution for *Limulus polyphemus* egg extract. Black bars represent the mean number of *Ilyanassa obsoleta* attracted to egg extract. White bars represent the mean number of *Ilyanassa obsoleta* attracted to a seawater control. Means for each Y-choice assay are derived from five trials with 10 snails per trial. Error bars represent the standard error of the mean. The reference line indicates the limit for statistical significance determined by Monte Carlo simulation ($P < 0.05$). Assay concentration refers to milliliters of egg extract per liter of seawater.

to the first three washes eluted with neutral buffer (50 mM Tris) or a solution of 0.2 M NaCl in 50 mM Tris (Table 2). In DEAE (anion exchange) fractions, activity was observed in the second and third washes eluted with neutral buffer (50 mM Tris) or a solution of 0.2 M NaCl in 50 mM Tris (Table 2). Because the chemical cue in the egg extract did not bind tightly to either ion exchange column, it appeared to be weakly charged overall. As a result, ion exchange chromatography was an ineffective technique for partitioning the chemical cue in *L. polyphemus* egg extract.

TABLE 1. *Ilyanassa obsoleta* RESPONSE TO COLD-TREATED *Limulus polyphemus* EGG EXTRACT[a]

Temperature (°C)	*Ilyanassa response*	
	Egg extract	Seawater
25	8.87 ± 0.35^b	0.62 ± 0.26
0	7.25 ± 0.73^b	2.25 ± 0.73
−80	8.75 ± 0.31^b	0.25 ± 0.61

[a] Means ± SE are derived from 8 trials with 10 snails per trial.
[b] Means are significantly different from random choice ($P < 0.05$).

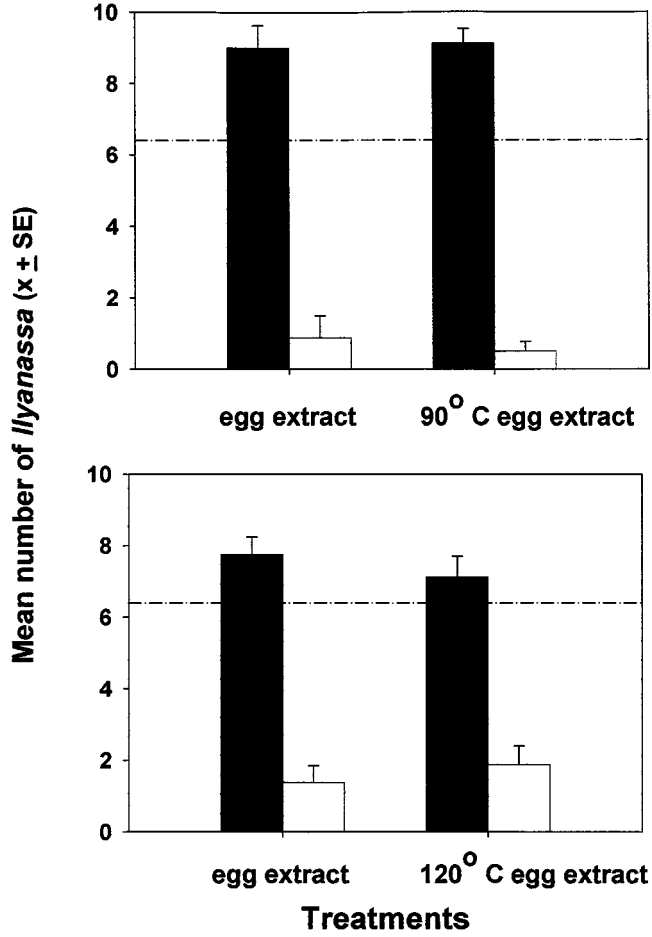

FIG. 2. *Ilyanassa obsoleta* response to heat-denatured *Limulus polyphemus* egg extract. Black bars represent the mean number of snails attracted to heat-denatured egg extract. White bars indicate the mean number of snails attracted to a seawater control. Means for each Y-choice assay are derived from eight trials with 10 snails per trial. Error bars represent the standard error of the mean. The reference line indicates the limit for statistical significance determined by Monte Carlo simulation ($P < 0.05$).

The molecular size of the chemoattractant was estimated through a combination of filtration and SDS-PAGE techniques. Gel filtration of the egg extract identified a major peak with an absorbance maximum at 280 nm. Y-choice bioassays of eluted fractions suggested a range of molecular sizes for the chemical cue in *L. polyphemus* eggs (Figure 3). Fractions 3–6 elicited a significant response from *I.*

TABLE 2. *Ilyanassa obsoleta* RESPONSE TO ION EXCHANGE CHROMATOGRAPHY FRACTIONS OF *Limulus polyphemus* EGG EXTRACT[a]

	CM52[b]		DEAE[c]	
Fraction	Egg extract	Seawater	Egg extract	Seawater
1	7.37 ± 0.46[d]	2.00 ± 0.53	5.37 ± 0.70	3.37 ± 0.73
2	8.25 ± 0.45[d]	1.12 ± 0.29	7.37 ± 0.42[d]	1.37 ± 0.62
3	8.25 ± 0.70[d]	0.75 ± 0.37	6.75 ± 0.45[d]	3.12 ± 0.48
4	4.00 ± 0.87	2.50 ± 0.42	6.37 ± 0.88	2.50 ± 0.91
5	3.62 ± 0.42	2.75 ± 0.62	5.12 ± 0.89	2.50 ± 0.63

[a] Means ± SE are derived from 8 trials with 10 snails per trial.
[b] CM52: carboxymethyl cellulose cation exchange chromatography column.
[c] DEAE: diethylaminoethyl cellulose anion exchange chromatography column.
[d] Means are significantly different from random choice ($P < 0.05$).

obsoleta ($P < 0.05$). The standard curve for the Sephacryl HR200 gel filtration column was prepared with four standards ranging from 6.5 to 66 kDa, and the corresponding molecular size of attractants in the active fractions ranged from 2 to 38 kDa. SDS-PAGE indicated that fraction 3 contained two proteins between 21 and 31 kDa. Despite *Ilyanassa's* attraction to fractions 4–6 in Y-choice assays, proteins were not visible in these fractions following Coomassie staining of

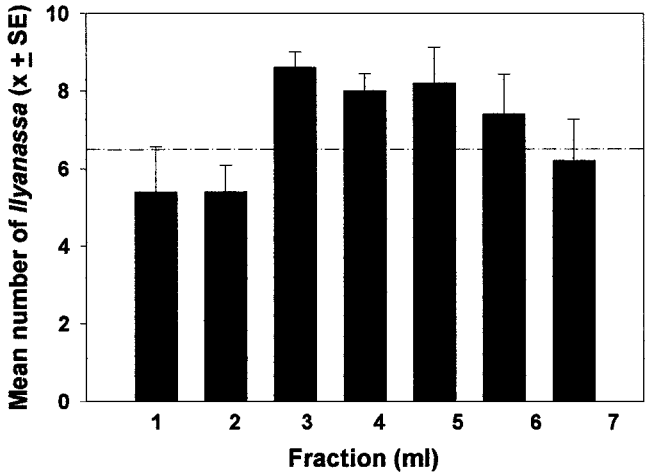

FIG. 3. *Ilyanassa obsoleta* response to gel filtration fractions. Bars represent the mean number of snails attracted to the eluate fraction when assayed against a seawater control. Means for each Y-choice assay are derived from five trials with 10 snails/trial. Error bars represent standard error of the mean. The reference line indicates the limit for statistical significance determined by Mark Carlo Simulation ($P < 0.05$).

SDS-PAGE gels. These data suggested that multiple compounds stimulated *I. obsoleta* response to the crude egg extract.

Ultrafiltration of the crude egg extract was not an effective technique for molecular size fractionation. *I. obsoleta* were significantly attracted to each fraction prepared by ultrafiltration ($P < 0.05$). Again, these data suggested that the crude egg extract contained multiple chemoattractants across a wide range of molecular sizes.

Results from centrifugal filtration with YM-10 Centricon units mirrored the ultrafiltration results. At a concentration of 3 ml/liter, each of the fractions [the retentate (>10 kDa), the filtrate (<10 kDa), and the wash (<10 kDa)] evoked a significant response from *Ilyanassa* (Figure 4). *Ilyanassa* preferred the wash

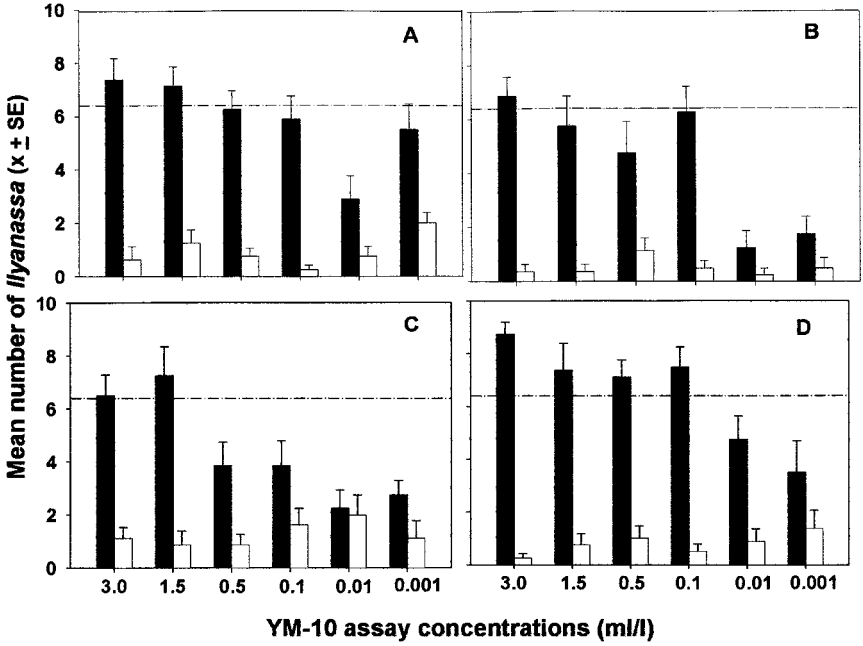

FIG. 4. *Ilyanassa obsoleta* response to dilute YM-10 fractions of *Limulus polyphemus* egg extract. Black bars represent the mean number of snails attracted to YM-10 fractions of egg extract. White bars indicate the mean number of snails attracted to a seawater control. Means for each Y-choice assay are derived from eight trials with 10 snails per trial. Error bars represent the standard error of the mean. The reference line indicates the limit for statistical significance determined by Monte Carlo simulation ($P < 0.05$). Treatment (milliliters) refers to the concentration of YM-10 fraction per liter seawater in Y-choice bioassays. A = YM-10 retentate; B = YM-10 wash; C = YM-10 filtrate; D = crude *Limulus polyphemus* egg extract.

and filtrate fractions slightly less than the retentate or crude extract, but the difference was less than 10%. Activity of the YM-10 fractions was concentration dependent; dilution decreased *Ilyanassa* response to the chemical cues. At a concentration of 0.5 ml extract/liter seawater, all fractions lost biological activity. However, *Ilyanassa* response to the crude extract and retentate fraction was >30% higher than either the wash or filtrate fractions at this concentration. *Ilyanassa* response appeared to be stimulated by two cues in *Limulus polyphemus* eggs: a large molecular size compound (>10 kDa) effective at low concentrations, and a small molecular size compound (<10 kDa) effective near the threshold concentration.

Protein content in YM-10 centrifugal filtration fractions corresponded to biological activity. Protein content was highest in the retentate (2.6 mg/ml) and was equivalent to the crude egg extract (2.7 mg/ml). Both fractions were highly active in Y-choice assays, and retained activity after dilution (Figure 4). In contrast, protein concentrations were negligible in the wash and filtrate fractions (<0.05 mg/ml). After dilution, the filtrate, low in protein content, stimulated chemotaxis less effectively (Figure 4).

L. polyphemus egg extract was fractionated with ammonium sulfate to determine whether the attractant precipitated in the typical range for proteins, 40–60% $(NH_4)_2SO_4$ saturation. In Y-choice bioassays, *I. obsoleta* responded significantly to both the 30–50% and 50–80% $(NH_4)_2SO_4$ fractions (Figure 5). However, the 0–30% and 80–100% fractions did not induce a response statistically different from random choice. The data suggested that the chemical cue in *L. polyphemus* eggs precipitated within the typical range for proteins.

Protein assays and SDS-PAGE corroborated these results. Nearly 70% of the total egg protein precipitated between 30 and 80% $(NH_4)_2SO_4$ saturation. Although some protein was present in the 0–30% fraction, little protein was detected in the 80–100% fraction (<0.32 mg/ml). The low concentration of protein in these fractions may explain the *I. obsoleta* response in Y-choice bioassays. In the inactive fractions, we observed few proteins in SDS-PAGE analysis. Although several larger proteins were present in the 0–30% fraction, few proteins were observed in the estimated range for the chemical cue. In the 80–100% fraction, proteins were barely visible in Coomassie-stained SDS–PAGE gels. Ammonium sulfate precipitation coupled with a protein assay and SDS-PAGE suggested that the chemical cue in *Limulus polyphemus* eggs is comprised of proteins.

Enzymatic digestion of the egg extract with a protease also indicated that the chemoattractant contains protein. Digestion with pepsin decreased the biological activity of the chemoattractant in Y-choice assays. In fact, treatment of the crude egg extract with as little as 2 mg of pepsin decreased *Ilyanassa* response to a level that was not significantly different from random (Figure 6). However, enzymatic digestion of the extract with 20 mg of pepsin had no effect on *I. obsoleta* response. *Ilyanassa* significantly preferred this extract to a seawater and pepsin control. Pepsin itself was not a factor in snail response; avoidance was not observed in

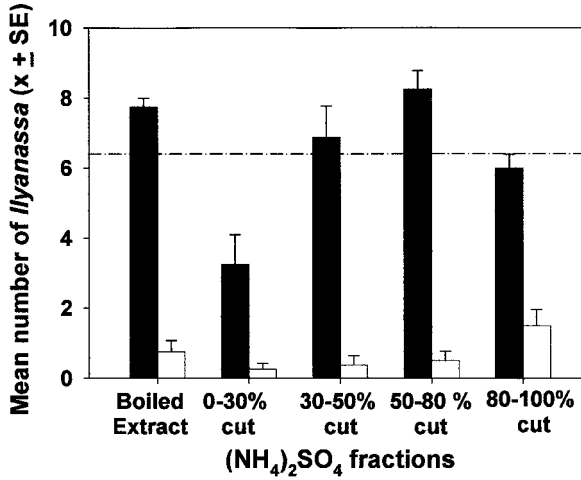

FIG. 5. *Ilyanassa obsoleta* response to $(NH_4)_2SO_4$ fractions of *Limulus polyphemus* egg extract. Black bars represent the mean number of snails attracted to $(NH_4)_2SO_4$ fractions of boiled egg extract. White bars indicate the mean number of snails attracted to a seawater control. Means for each Y-choice assay are derived from eight trials with 10 snails per trial. Error bars represent the standard error of the mean. The reference line indicates the limit for statistical significance determined by Monte Carlo simulation ($P < 0.05$).

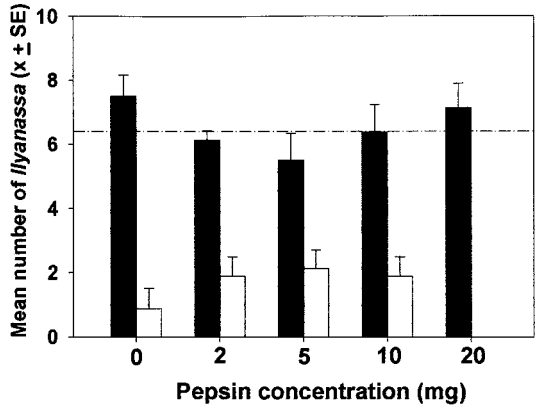

FIG. 6. *Ilyanassa obsoleta* response to pepsin digests of *Limulus polyphemus* egg extract. Black bars represent the mean number of snails attracted to pepsin digests of egg extract. White bars indicate the mean number of snails attracted to a pepsin and seawater control. Means for each Y-choice assay are derived from eight trials with 10 snails per trial. Error bars represent the standard error of the mean. The reference line indicates the limit for statistical significance determined by Monte Carlo simulation ($P < 0.05$).

an assay pairing 20 mg of pepsin against a seawater control (data not shown). SDS-PAGE analysis of postdigestion products indicated that smaller molecular size proteins characteristic of the chemical cue (<38 kDa) were barely visible after digestion with 2 or 5 mg of pepsin. However, after digestion with 10 or 20 mg of pepsin, the densities of smaller molecular size proteins that were visible with SDS-PAGE approached the densities observed in the crude egg extract.

The protein hypothesis was further investigated using TCA precipitation of the crude egg extract. After precipitation, the soluble and insoluble fractions of the crude egg extract were assayed for activity in the Y-choice chamber (Figure 7). The TCA-insoluble fraction elicited a response from *Ilyanassa obsoleta* that was different from random choice ($P < 0.05$). In contrast, biological activity was not detected in the supernatant. Proteins from the egg extract were primarily concentrated in the TCA insoluble fraction; the precipitate contained 0.2 mg/ml of protein whereas the supernatant contained 0.03 mg/ml. In comparison, carbohydrates were predominant in the TCA-soluble fraction; the supernatant contained 1.04 mg of carbohydrate whereas the precipitate contained 0.15 mg. Again, fractions containing protein compounds were more attractive to *Ilyanassa obsoleta*.

Protease and carbohydrase digests were visualized with SDS-PAGE to detect mobility shifts indicative of glycosidic bond cleavage. Comparison of enzymatic

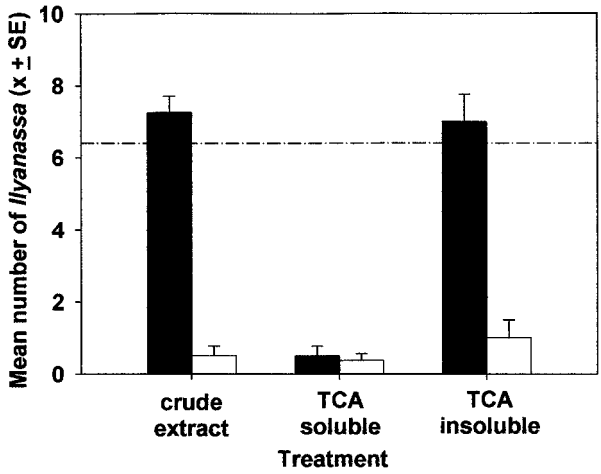

FIG. 7. *Ilyanassa obsoleta* response to TCA fractions of *Limulus polyphemus* egg extract. Black bars represent the mean number of snails attracted to the TCA fractions of egg extract. White bars indicate the mean number of snails attracted to seawater controls. Means for each Y-choice assay are derived from eight trials with 10 snails per trial. Error bars represent the standard error of the mean. The reference line indicates the limit for statistical significance determined by Monte Carlo simulation ($P < 0.05$).

digests with untreated crude egg extract confirmed the presence of glycosylated proteins. When *L. polyphemus* egg extract was digested only with trypsin or chymotrypsin, differences in protein migration were not observed with SDS-PAGE. However, when the egg extract was digested with α-amylase, β-glucosidase, and trypsin, mobility shifts were apparent. In the trypsin carbohydrase digestion, three proteins appeared, and a fourth stained more heavily between 55.4 and 36.5 kDa. These proteins were absent from the trypsin digest. The mobility shift in the trypsin and carbohydrase digest confirmed the presence of glycosylated proteins in *Limulus polyphemus* eggs.

Carbohydrates in the TCA soluble fractions of the digestion reactions were assayed to verify the SDS-PAGE results. As expected, sugar concentrations did not differ significantly after digestion with either chymotrypsin or trypsin, but they did increase after digestion with a mixture of protease and carbohydrases. Combined protease and carbohydrase digestion of *L. polyphemus* egg extract had an effect on mean carbohydrate concentration (ANOVA, $P < 0.05$) (Figure 8). Detection of glycoproteins was dependent on protease specificity. The trypsin and carbohydrase treatment increased the concentration of free carbohydrates in the egg extract by about 7 mg ($P < 0.05$); however, carbohydrate content of the chymotrypsin and carbohydrase digest did not change.

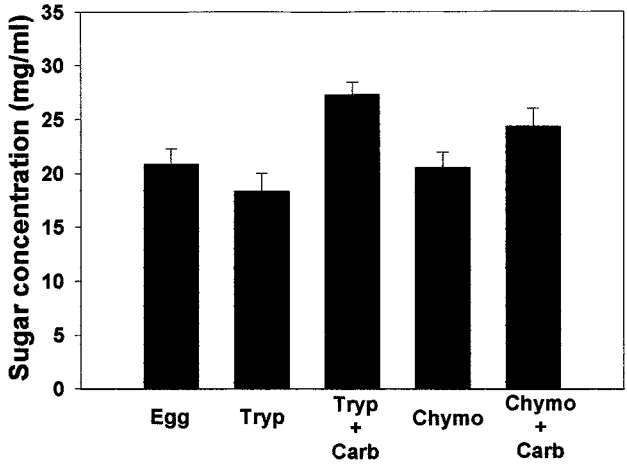

FIG. 8. Effect of protease and carbohydrase digestion on total sugar concentration in *Limulus polyphemus* egg extract. Data are mean $\pm$ SE ($N = 5$) for all treatments. Data are extrapolated from a standard curve for L-fucose (480 nm). Undigested egg extract (Egg); trypsin digest (Tryp); trypsin and carbohydrase digest (Tryp + Carb); chymotrypsin digest (Chymo); chymotrypsin and carbohydrase digest (Chymo + Carb).

DISCUSSION

Fisheries management plans along the US east coast have restricted the availability of horseshoe crab bait to eel and conch fishermen. In our study, we determined that there is a specific chemical attractant in the horseshoe crab that could potentially be incorporated into an artificial bait (see also Ferrari, 2001). Such a bait would provide a sustainable alternative to the natural horseshoe crab bait currently used by fishermen. Using a laboratory-based bioassay-guided approach, we determined that the chemoattractant, which stimulated foraging behavior in mud snails and eels (data not shown), was concentrated in *Limulus polyphemus* eggs. The primary chemical cue appears to be a heat-stable, proteinaceous compound, >10 kDa in molecular mass. Proteins have been shown to attract crustaceans, mollusks, and echinoderms to bait or live prey (Rittschof et al., 1984; Carr, 1988), and in a survey of marine invertebrates and fishes, Carr et al. (1974) found that proteins and peptides were the predominant olfactory stimulants for *Nassarius obsoletus* (= *Ilyanassa obsoleta*) in seven of the eight species assayed. Our results differ from recent studies identifying low-molecular-weight metabolites, such as amino acids (Carr et al., 1996; Coman et al., 1996; Sola and Tongiorgi, 1998) and sugars (Sears et al., 1991; Harada et al., 1994), as primary olfactory cues in the marine environment.

Centrifugal filtration, TCA and ammonium sulfate precipitation, and pepsin digestion experiments confirmed that the chemoattractant contained proteins, however, the active metabolite from *Limulus polyphemus* eggs demonstrated unusual heat tolerance. Although proteins typically lose activity after exposure to high temperatures, heat-stable proteins have been documented in marine invertebrates. Some examples include hemocyanins in arthropods and mollusks (68–91°C) (Georgieva et al., 1998), cadmium-binding proteins in *Callinectes sapidus* (90°C) (Wiedow et al., 1982), and metallothioneins in *Asterias rubens* (90°C) (Temara et al., 1997). In addition, heat-stable proteins have been identified as olfactory cues for marine mollusks and echinoderms. Gurin and Carr (1971) found that a heat-denatured proteinaceous cue from *Crassostrea virginica* extract stimulated a feeding response in *Nassarius obsoletus*. Collins (1975) identified a heat-stable macromolecule from the coral *Fungia fungites* (L.) as a feeding attractant for *Acanthaster planci* (L.). Rittschof et al. (1984) discovered the oyster drill, *Urosalpinx cinerea,* responded to a thermostable protein in living barnacles. Based on these observations, there is ample precedent for a heat-stable protein attractant in *Limulus polyphemus* eggs.

The structural conformation of the active metabolite may also be a factor in the anomalous results from heat-denaturation bioassays. A specific peptide in the protein complex rather than the entire complex may stimulate chemosensory cells. If heat denaturation cleaves peptides or protein subunits from the chemoattractant, these fragments may provide the recognition sequence for chemotaxis in *Ilyanassa*.

Results from the pepsin digestion experiments support this argument. When the extract was digested with high concentrations of pepsin (10–20 mg), *Ilyanassa* response increased significantly. Small molecular size cleavage products in the egg extract appeared to trigger the positive response, because the number and density of protein fragments and peptides increased proportionally with pepsin concentration. Rittschof (1990) postulated that interspecies communication, such as *Urosalpinx cinerea* attraction to live prey, evolved from serine protease-generated peptides naturally occurring in the body odor of marine organisms. The heightened response of *Ilyanassa obsoleta* to egg extract digested with high concentrations of pepsin supports the hypothesis of a peptide signal embedded in the proteinaceous metabolite.

Data from centrifugal and ultrafiltration techniques suggested the chemoattractant might not be a single metabolite. Chemotaxis may be enhanced by the interaction of multiple cues in horseshoe crab egg extract. Synergistic olfactory cues are well documented in marine organisms. Carr (1967b) observed that while lactate and glycine were the predominant stimuli for feeding behavior in *Nassarius obsoletus*, a mixture of 12 naturally occurring compounds in shrimp (*Penaeus duorarum*) extract enhanced chemoreception. Similarly, lobsters (*Homarus gammarus*) were not attracted to individual components of squid (*Loligo vulgaris)* extract, but resumed foraging behavior when individual stimulants were recombined (Mackie and Shelton, 1972). In *Limulus* egg extract, activity could not be attributed solely to high ($>$10 kDa) or low ($<$10 kDa) molecular size fractions; carbohydrates and proteins proved to be effective olfactory cues for *I. obsoleta*. In addition, individual filtrates were never as attractive as the crude egg extract, implying that carbohydrates and proteins must both be present to initiate the food search behavior. Therefore, a glycoprotein or carbohydrate–protein complex may function as the chemoattractant in horseshoe crab bait, with synergism between the carbohydrates and proteinaceous metabolite necessary to elicit the strong feeding response observed in Y-choice assays. A glycosylated waterborne peptide pheromone has been characterized from *Aplysia* (Painter et al., 1998). Like the horseshoe crab attractant, the *Aplysia* peptide is tissue specific and appears to be present in different isoforms.

Concentrated in the cortex of *Limulus* eggs, glycoproteins are instrumental in the formation of the embryonic egg envelope (Bennett, 1979). Several carbohydrates and glycoproteins have also been found to regulate osmotic pressure in the perivitelline fluid of *Tachypleus tridentatus* (Sugita, 1988). In our study, glycoproteins were identified in *Limulus* egg extract through SDS-PAGE of trypsin and carbohydrase digests, but surprisingly, were not detected in chymotrypsin and carbohydrase digests. This may be a function of protease specificity; trypsin targets lysine and arginine, whereas chymotrypsin generally cleaves the carboxylic side of aromatic side chains (Robyt and White, 1987).

Although eel and conch fishermen have experimented with alternatives to horseshoe crab bait, such as blue mussels (*Mytilus edulis*), blue crabs (*Callinectes*

sapidus), and surf clams (*Spisula solidissima*), horseshoe crabs remain the most effective natural bait across the Delaware Bay region (Manion et al., 2000). The success of an artificial bait will depend on its ability to attract the target species through distance chemoreception. Proteinaceous metabolites from *Limulus polyphemus* eggs are effective stimulants of distance chemoreception in *Ilyanassa obsoleta*. Preliminary laboratory assays have also confirmed the ability of these active metabolites to attract one of the target commercial fish species, the American eel, *Anguilla rostrata* (data not shown). Thus, an artificial bait impregnated with metabolites from *L. polyphemus* eggs could provide a feasible alternative for commercial eel and conch fisheries. Because the attractant is a protein, conventional chemical synthesis is unlikely, however, the tools of modern biotechnology offer the potential for cloning and expressing the protein in a suitable biological system. Accomplishing this would ensure a sustainable supply of the attractant that is independent of the horseshoe crab.

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BEHAVIORAL RESPONSES OF SEXUALLY ACTIVE MUD SNAILS: KARIOMONES AND PHEROMONES

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Abstract—We postulated that kariomones and pheromones function to organize egg capsule deposition and breeding aggregations of mud snails *(Ilyanassa obsoleta)*. Sexually active male and female mud snails were exposed to water-born odors from juvenile snails, nonaggregated female snails, oysters, egg capsule depositing snails, sexually active males, sexually active females, and copulating snails and tested for a follow response using a ring assay. Neither sex responded to juvenile odor or to odor of sexually inactive females. Snails turned and followed water born odors from oysters. when tested with the odors of sexually active males, sexually active females, and copulating pairs, we found sex-specific differences in responses. Sexually active females followed the odor of sexually active males and ignored sexually active female odor. Sexually active males followed the odor of sexually active females and ignored sexually active male odor. Both sexes followed the odor of copulating pairs and egg capsule depositing snails. Snail activities in breeding aggregations are organized by at least three pheromones and one kariomone, and reflect the multifunctionaLity of the aggregations.

Key Words—Gastropods, breeding aggregations, kariomones, pheromones.

INTRODUCTION

Mud snails, *Ilyanassa obsoleta* (= *Nassarius obsoletus* = *Nassa obsoleta*) live on soft bottom sediments in intertidal areas along the east coast of Canada and the United States from the Bay of Fundy to Florida. Many mud snail behaviors including feeding, prey detection, predator avoidance, breeding, and egg capsule deposition are chemically mediated (for review, see Rittschof et al., 2002). The

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goal of the present study was to expand and clarify the role of water born chemicals in establishing mud snail breeding aggregations.

Rittschof et al. (2002) reported that reproductive mud snails aggregate in response to odors from conspecific and heterospecific egg capsules and living bivalves. Aggregations are composed of males and inseminated females. They also reported tests of odors from mud snails that did not demonstrate existence of pheromones. However, they suggested that their tests were flawed because the gender and reproductive condition of the odor generating and responding snails were not determined.

We re-examined the attractiveness of snail odors. We tested sexually active male and female snails for attraction to odors from sexually active male and female snails, from egg laying and copulating snails, as well as odors from non-aggregated female snails, juvenile snails, and oysters.

METHODS AND MATERIALS

Collection of Material. Snails were collected in March and early April 2002, near the time of low tide, 24 hours prior to use in assays. Collection sites were Carrot Island embayment (centered at 34° 42.625 N, 76° 40.230 W) and at NOAA beach (34° 43.100 N, 76° 40.267 W), Pivers Island, Beaufort, North Carolina. We found juvenile (3–5 mm shell height) and non-aggregated adult (18–25 mm shell height) snails in the intertidal zone. Egg capsule depositing snails were collected while depositing capsules on oysters. Copulating snails (17–25 mm shell height) were collected from aggregations around submerged oysters. Only copulating pairs of snails were counted as sexually active. The snail receiving the penis was designated the female. Groups of each type were sorted into buckets and exposed to the ambient light cycle. Each bucket had its own isolating inflow of single pass running seawater. Oysters were collected from an area without mud snails near the Duke University Marine Laboratory docks (34° 43.034 N, 76° 40 242 W).

Ring Assay. Responses of sexually active male and female snails were tested using ring assays (Duval et al., 1994; Rahman et al., 2000; Rittschof et al., 2002). At least 30 mud snails were tested individually to each test solution. Each snail was placed in the center of a dry finger bowl and surrounded by a ring of odor. The ring was drawn with a Q-tip® soaked with 0.5 ml of odorant solution. Each snail's response upon contact with the ring was recorded. Two different responses were observed. First, if a snail followed the ring for at least 1 cm, a follow response was recorded. Second, if the snail moved directly across the ring without stopping, a neutral response was recorded. Avoidance or alarm responses (Rahman et al., 2000) in which snails retract or turn around upon contact with the ring were not observed in our tests.

The control odor was filtered and aged (FSW) seawater. Ambient seawater was filtered to remove particles $>1\ \mu$m and aged at room temperature with aeration

for at least one week prior to use. Test solutions of odors from juvenile mud snails, non-aggregated adult mud snails, sexually active males, sexually active females, copulating pairs, egg capsule depositing snails, and oysters were prepared by placing the living, intact odorant source of known total weight (shell plus tissue) in a known volume of seawater and incubation for a known time. Incubation was for 1–10 min. All solutions were tested at a concentration of 50 g weight/min/l of seawater (Rittschof et al., 2002).

Snails used in trials were frozen and removed from their shells to verify their gender. Males were identified by their orange gonads. Females were identified by their cream colored gonads and capsule glands (Smith, 1980). Preplanned comparisons were conducted on frequency data by calculating the Z statistic for comparison of proportions of male or female snails that showed a follow response to filtered seawater and a test solution (Walpole, 1974).

RESULTS

Mud Snail Collections. Juvenile mud snails were found touching in 10 to >100 cm diam. aggregations slightly higher in the intertidal zone than adults. Non-aggregated mud snails were scattered away from oysters on soft substrate with about 5 cm between individuals. Dissection showed all non-aggregating snails were sexually inactive females without capsule glands. Copulating snails were found in aggregations ranging from 100 to >1000 near oysters and whelk egg capsule strings. These snails were considered sexually active and separated by sex.

Responses of Sexually Active Snails to Odor. Sexually active male and female snails (30–60 individuals of each sex) were exposed to odors from juvenile snails, non aggregated snails, oysters, egg capsule depositing males, females, and copulating snails in ring assays (Figure 1). Neither sex responded to juvenile odor (female, $Z = 1.4$, $P > 0.05$; male, $Z = 0.42$, $P > 0.05$) or to the odor of non-aggregating females (female, $Z = 0.89 P > 0.05$; male, $Z = 0.83 P > 0.05$). In contrast, oyster odor evoked following in 50% of females and 42% of males (females, $Z = 3.6$, $P < .001$; males, $Z = 3.78$, $P < .001$). Similarly, 62% of females and 40% of males followed the odors of egg capsule depositing snails ($Z = 10.4$, $P < .001$) ($Z = 2.8$, $P < .01$).

When we tested sexually active males and females with the odors of sexually active females, sexually active males, and copulating pairs, we found sex differences in responses Sexually active males followed the odor of sexually active females (77%, $Z = 28.7$, $P < .001$) and ignored sexually active male odor ($Z = 0.83$, $P > 0.05$). Sexually active females followed the odor of sexually active males (64%, $Z = 8.9$, $P < 0.001$) and ignored sexually active female odor ($Z = 0.43$, $P > 0.05$). Both sexes (68% of females and 69% of males) followed the odor of copulating snails (females $Z > 10.9$, $P < 0.001$; males $Z > 11.6$, $P < 0.001$).

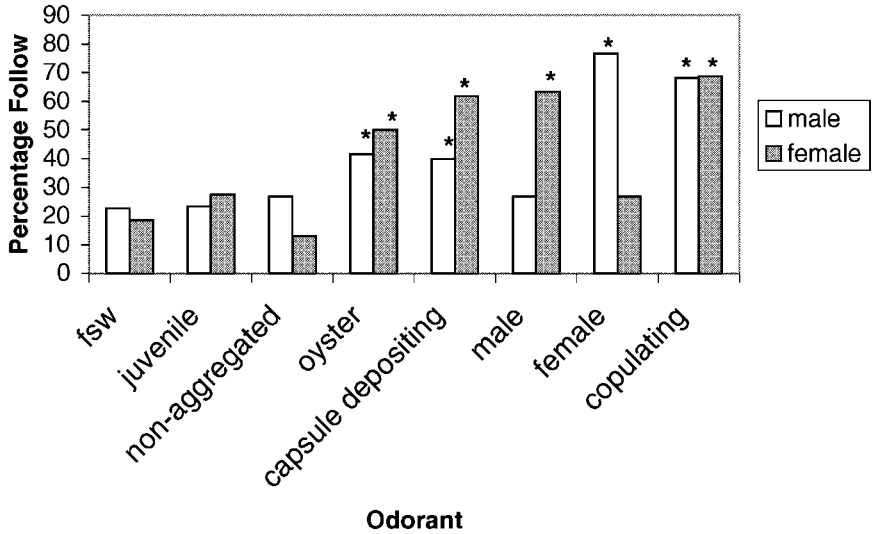

FIG. 1. Responses of sexually active male and female mud snails to water-tan odors. At least 30 snails of each sex were tested individually in ring assays for each odor source. Asterisk (*) indicates significant ($P < 0.05$ or lower, see text) follow response when compared to response to filtered seawater (FSW). Odors were standardized to 50 grams of wet weight of intact odor source soaked for 1 min in 1 titer of FSW.

DISCUSSION

Mud snail breeding aggregations are initiated in the field by odors from living bivalves and from snail egg capsules (Rittschof et al., 2002). This study supports the hypothesis that mud snails produce pheromones and suggests there are at least three sources of pheromones and one source of kariomones. Pheromones are produced by sexually active females and males, copulating pairs, and capsule depositing females. Pheromones from sexually active snails evoke sex-specific responses. These cues contain information about the sender's sex as well as reproductive status. Pheromones from capsule depositing females and copulating snails attract both sexes. Because they attract both sexes, pheromones from capsule depositing females are different compounds or blends from pheromones of sexually active females. Thus, there are at least three discrete sources of water-born pheromones that induce snail aggregations. Water-born karimones originating from bivalves attract both sexes. Our previous study (Rittschof et al., 2002) found that egg capsules also produce a kariomone that induces the following response. Only water born odors were tested in this study. It seems likely, as is the case in a copepod, *Tigriopus japonicus* (Ting et al., 2000) that contact chemorecption associated with snail trails and snail surfaces will also play a role in organizing mud snail reproductive behaviors.

Rittschof et al. (2002) postulated the similarity between mud snail aggregation, egg laying and breeding patterns and those observed in *Aplysia* spp. (Painter, 1992; Painter et al., 1991). At the behavioral level these similarities are striking. Future studies will determine if the similarity extends to the chemical level.

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MALE-SPECIFIC EAD ACTIVE COMPOUNDS PRODUCED BY FEMALE EUROPEAN CHAFER *Rhizotrogus majalis* (RAZOUMOWSKY)

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Abstract—Chemical investigation of a fmale balloon-like organ of the European chafer, *Rhizotrogus majalis* (Razoumowsky), with GC-EAD has resulted in the identification of female-specific compounds, (*R*)-3- hydroxybutan-2-one, (2*R*,3*R*)-2,3-butanediol, and *meso*-2,3-butanediol that are specifically EAD-active with male antennae. No behavioral role for any of the EAD active compounds could be discerned with this species.

Key Words—Coleoptera, Scarabaeidae, Melolonthinae, European cha-fer, *Rhizotrogus majalis*, GC-EAD, acetoin, (*R*)-3-hydroxybutan-2-one, (2*R*, 3*R*)-2,3-butanediol, *meso*-2,3-butanediol.

INTRODUCTION

The European chafer, *Rhizotrogus majalis* (Razoumowsky) was accidentally introduced into the U.S. from Europe sometime in the 1930's. It was first detected in Newark, New York in 1940, and since then has become an important pest of

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lawn and turfgrass in the northeastern U.S. and Canada (Vittum et al., 19992). Females of this species have a balloon-like organ that is produced by evagination of the vaginal tract (Vittum et al., 1999). Such abdominal sacs are observed in some other species of scarab beetle and are exposed during females' calling behavior (Leal, 1998). In a few melolonthine species, female sex pheromones have been identified from the extracts of these abdominal organs (Leal, 1998).

Although the sex pheromone of the European chafer has been investigated in the past, the existence of the pheromone was not verified (Roelofs et al., 1967). We present here the results of a chemical investigation of the female balloon-like organ using coupled gas chromatography-electroantennographic detection (GC-EAD) technique.

METHODS AND MATERIALS

Adults and last instar larvae of the European chafer were collected in Geneva NY. A total of 15 male antennae and 30 female gland extracts were used for the qualitative analysis of EAD active compounds. Larvae were individually housed in 1 oz. soil-filled plastic cups at 25°C on a 16L:8D photoperiod to obtain virgin females. Five virgin females were used for the quantitative analysis of the active compounds. Excised balloon-like organs of females were soaked in methylene chloride, and the extracts were subjected to GC-EAD and GC-MS analyses.

GC-EAD instrumentation used in this study was the same as described previously (Nojima et al., 2003) except for using au acrylic antenna holder designed for a beetle's antenna (Leal et al., 1992). A nonpolar SPB-1 capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Supelco Inc.) was used for qualitative GC-EAD analysis. The oven temperature was kept at 35°C for 5 mm and programmed to rise 15°C/min to 250°C and held for 5 min. Nitrogen was the carrier gas, with the flow rate at 2 ml/mm. The injector and detector temperatures were set at 250°C. A chiral BETA DEX 120 capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Supelco Inc.) was used for both qualitative and quantitative analyses. The oven temperature was kept at 40°C for 5 min and programmed to rise 4°C/min to 130°C then 10°C/min to 220°C and held for 20 mm. Nitrogen was the carrier gas, with the flow rate at 2 ml/mm The injector temperature was set at 100°C for quantitative or 250°C for qualitative analyses, while the detector temperatures were set at 250°C for both analyses. GC-MS was performed on a Shimadzu QP 5050A in El mode using a non-polar DB-1 ms column (30 m × 0.25 mm ID, 0.25 mm film thickness, J&W) and the same conditions as those of the qualitative GC-EAD analysis with SPB-1, but with helium as the carrier gas at 1 ml/mm. BAD active components were identified by mass spectral matches to library spectra followed by comparisons of retention times with those of authentic samples.

(*R*)-3-Hydroxybutan-2-one (>95% GC; e.e. >92%) was prepared according to Crout et al. (1983) (±)-3-Hydroxybutan-2-one (crystalline dimer at room

temperature), (2*R*,3*R*)-2,3-butanediol (97% GC; e.e. 99%), *meso*-2,3-butanediol (97% GC), 1-hexanol (98%), and (±)-linalool (97%) were purchased from Aldrich, Milwaukee, Wisconsin.

Preliminary field trap tests were performed using synthetic chemicals in Geneva, NY and Rascafria, Spain where native *Rhizotrogus* species occur, so that the behavioral responses to the chemicals could be compared between the introduced species in the United States and the native species in Europe. The blend, a 100: 1 :0.1 mixture of (*R*)-3-hydroxybutan-2-one, *meso*-2,3-butanediol, and *(2R,3R)-2,3*-butanediol, and each chemical at 1 mgrnl water solution were tested.

RESULTS AND DISCUSSION

Four electrophysiological antennal responses were observed in the GC-EAD analysis of the balloon-like organ extracts (Figure 1). The corresponding EAD active components were identified as (*R*)-3-hydroxybutan-2-one (peak 1, 350 ng/organ), (*S*)-3-hydroxybutan-2-one (peak 2, probably a thermally produced artifact, see below), (2*R*,3*R*)-2,3-butanediol (peak 3, 1 ng/organ >) and *meso*-2,3-butanediol (peak 4, 3.5 ng/organ). These components were not detected from the extracts of male genitalia.

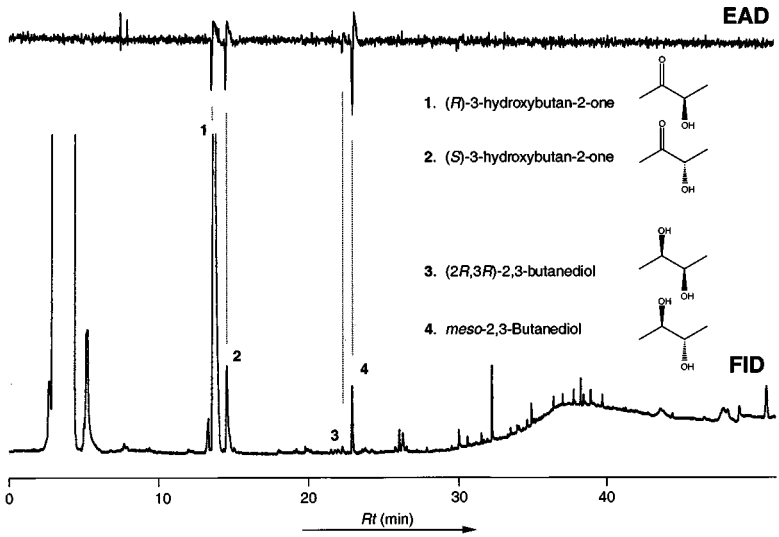

FIG. 1. Simultaneous responses of FID and EAD using the antennae of male European chafer to the extracts of female balloon-like organ on a chiral capillary column.

Authentic active components, (±)-3-hydroxybutan-2-one, *meso*-2,3-butanediol, and general stimuli (1-hexanol and linalool) were tested with the antennae of both sexes at 0.5, 5, and 25 ng of each by means of GC-EAD. Male antennae ($N = 17$) strongly responded to authentic chemicals, (±)-3-hydroxybutan-2-one, *meso*-2,3- butanediol, at similar concentrations to those in the gland extracts, and to general stimuli (1-hexanol and linalool), whereas female antennae ($N = 5$) responded slightly only to 1-hexanol, and exhibited little activity to the others at 25 ng each. Thus, (*R*)-3-hydroxybutan-2-one and *meso*-2,3-butanediol were found to be specificallyproduced by females and to elicit responses only from male antennae.

During the chiral GC analysis of enantiomeric purity of synthetic (*R*)-3-hydroxybutan-2-one, it was observed that a higher GC injection temperature resulted in an increase in the other euantiomer. Furthermore, (*S*)-3-hydroxybutan-2-one in the extracts decreased to only a trace amount at a low GC injection temperature (at 100°C). This suggested that (*S*)-3-hydroxybutan-2-one was an artifact of tbermal isomerization of (*R*)-3-hydroxybutan-2-one during GC analysis.

In the preliminary field trap tests, a total of 11 maIes were captured at the Geneva site where chafers were observed flying around the site during the test (June 17th through 30th), whereas no male was captured at the Rascafria site. No *Rhizofrogus* chafers were seen flying at the Rascafria site in June 2002 due to the unusual weather. There were no significant differences in the number of captured males between control traps and baited traps at the Geneva site ($P > 0.05$, χ^2 test), and total number of captured males was low for the numerous males seen flying during the flight period. Atso, males were not observed to respond behaviorally to the lures in the field. Males could have been captured in the traps accidentally.

Adults of the European chafer typically swarm around trees during their flight period at dusk, with a synchronicity of emergence and attraction for a silhouetted object (Vittum et al., 1999). This swarming flight would allow beetles to mate without the need for a long-distance sex pheromone as pointed out by Roelofs et al. (1967). In their study, evidence for the use of a pheromone communication system by this species was not found. Our present field studies with the female-produced chemicals also support a conclusion that the European chafer does not rely on a long-distant sex attractant to locate mates. However, it is curious to find a relatively large amount of female-specific and male antennal-specific EAD active compounds in the female reproductive organ without evidence for their involvement in mating activity. Another possibility is that the compounds are important in close-range courtship. Further investigation as well as extensive and systematic field trap experiments are needed to clarify the function of these chemicals.

Interestingly, both enantiomers of 3-hydroxybutan-2-one and three stereoisomers of 2,3-butanediol have been recently identified from summer chafer *Amphimallon solstitiale* (Melolonthinae) that occurs in Europe, and (*R*)-3-hydroxybutan-2-one showed strong attractancy to the males in the field-trap test (Tolasch et al.,

2002) Moreover, these compounds have been reported as components of the aggregation pheromone of a Rhinoceros beetle *Scupanes australis* (Scarabaeidae) (Rochat et al., 2000). Although fewer sex pheromones of Scarabaeidae beetles have been identified compared with Lepidopteran, it is suggested that these small volatile compounds may be relatively common pheromonal components in some other species of scarab beetles.

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There will be a special issue of the *Journal of Chemical Ecology* devoted entirely to papers dealing with "Molecular Chemical Ecology." Publication is planned for late fall/early winter 2003. The issue will consist of invited mini-reviews and contributed papers. Those wishing to submit a paper for consideration for this special should do so by June 1, 2003.

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ANNOUNCEMENT

The 3rd International Symposium on Insect Pheromones (ISIP3) will take place May 25–29, 2003, at Bäckaskog Castle in southern Sweden. Invited speakers will talk in sessions dealing with most aspects of insect pheromone communication. Posters will be accepted from those participants not invited as speakers. Additional information can be found at the Web site: http://www-isip.slu.se.

Participants must *register* for the meeting by *March 15, 2003*. The number of rooms at the meeting venue is limited, and there are various options (http://www-isip.slu.se/accommodation.html). The Registration and Accommodation form is at http://www-isip.sluse/accform.html.

Students are invited to register. Student status must be confirmed by an accompanying letter from the supervisor. Student travel support can be applied for on the Web site.

Abstracts should be submitted before *April 1, 2000*. Use the format at the Web site.

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ROLE OF TRAIL PHEROMONE IN FORAGING AND PROCESSIONARY BEHAVIOR OF PINE PROCESSIONARY CATERPILLARS *Thaumetopoea pityocampa*

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Abstract—Although caterpillars of *Thaumetopoea pityocampa* may mark their pathways with silk, this study shows that the material is essential to neither the elicitation nor maintenance of trail-following or processionary behavior. Trail following is dependent upon a pheromone the caterpillars deposit by brushing the ventral surfaces of the tips of their abdomens against the substrate. Earlier instars are strongly bound to their trail system; in the laboratory, caterpillars followed circular trails continuously for as long as 12 hr before breaking away from them. The trail pheromone is long-lived and soluble in nonpolar solvents, but its volatilization or degradation allows the caterpillars to distinguish new from aged trails. In contrast to trail following, processionary behavior, the head-to-tail, single-file movement of the caterpillars is dependent on neither silk nor the trail pheromone. Stimuli associated with setae found on the tip of the abdomen of the precedent caterpillar serve to hold processions together, and such stimuli take priority over those associated with either the trail pheromone or silk. Although the caterpillars discern trail strength and choose stronger over weaker trails, the trail marking system of the processionary caterpillar appears less sophisticated than those of other, previously studied species of social caterpillars, and colonies are relatively inefficient in abandoning exhausted feeding sites in favor of new food finds. In laboratory studies, females were more likely to lead processions than males, and leaders, regardless of gender, expended more energy in locomotion than followers.

Key Words—*Thaumetopoea pityocampa*, pine processionary caterpillar, thigmotaxis, social caterpillar, trail pheromone, caterpillar, foraging, Thaumetopoeidae.

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INTRODUCTION

Thaumetopoea pityocampa (Lepidoptera: Thaumetopoeidae), the pine processionary caterpillar, is a social species whose larvae form silken nests in the branches of host trees in warmer regions of southern Europe, the Near East, and North Africa. Colonies develop from egg masses containing approximately 70–300 eggs (Dajoz, 2000). The caterpillars at first forage nomadically among the branches of pines, but in the third-instar build a permanent nest (Halperin, 1990) and become central place foragers, mounting nightly forays from their nests to feeding sites on the branches of host trees. During these forays, the caterpillars move over silken pathways laid down on the branches of the host tree. When fully grown, they leave the host tree in search of pupation sites, forming long, over-the-ground, head-to-tail processions. These spectacular processions, which may consist of a few to as many as 300 caterpillars (Edwards, 1910), were first investigated by Réaumur (1736) and later by Fabre (1898). In a now famous essay, Fabre (1916) reported that during these processions the caterpillars secrete and follow silk strands and that such strands are essential to the maintenance of the assemblages. Although other investigators have reported that tactile stimuli are involved in the formation of processions (Brindley, 1910; Edwards, 1910; Demolin, 1962, 1971; Mallmann, 1962; Schmidt et al., 1990), it is still widely held that the strands that issue from the caterpillars' spinnerets play an essential role in trail following behavior (Schmidt et al., 1990; Dajoz, 2000), and there have been no definitive investigations to indicate otherwise. Studies of nonprocessionary social caterpillars have shown, however, that silk plays little or no role in their trail-following behavior but is dependent upon a trail pheromone secreted from the ventral surface of the tip of the abdomen (Fitzgerald, 1995; Fitzgerald and Underwood, 1998a,b; Ruf et al., 2001). An extra-silk trail marker was also implicated in a recent study of the processionary behavior of the neotropical saturniid, *Hylesia lineata* (Fitzgerald and Pescador-Rubio, 2002). These studies suggested that the trail-following and processionary behavior of *T. pityocampa* might also involve a trail pheromone and prompted the investigation reported here. The study sought to determine whether caterpillars produce an extra-silk trail pheromone and, if so, to ascertain its importance relative to tactile and silk-associated stimuli to the trail following and processionary behavior of the insect.

METHODS AND MATERIALS

Field Study Site and Source of Laboratory Colonies. Observations were made of processions of caterpillars foraging in trees and moving over the ground in search of pupation sites during February 2001, in Baix Penedès County, Catalonia (Spain). Egg masses were collected from Scot's pine (*Pinus sylvestris*) in the Spanish

Pyrennes in August 2001. Egg masses were brought back to the laboratory, the larvae allowed to eclose, and the colonies established on branches of Scot's pine, replaced as needed. Approximately 25 colonies of caterpillars were maintained in the laboratory during the course of the study. During the third through fifth stadia, caterpillars have urticating hairs. Contact with the setae or airborne fragments of the setae may cause dermatitis and an allergic response may develop with repeated exposure (Ducombs et al., 1981; Vega et al., 1999). An air filtration system was used in the isolation facility to clear the air of setal fragments, and latex gloves were worn when it was necessary to handle the larger caterpillars. Despite these precautions, I experienced contact dermatitis in varying degrees of severity on a daily basis during the latter part of the study period.

Persistence of the Trail. A study was conducted to determine whether processions of caterpillars mark their pathways with a persistent trail marker. Approximately 30 second-instar caterpillars were placed at the center of a large sheet of paper in the laboratory and allowed to form a procession. The pathway was marked by placing pencil dots at 1-cm intervals behind the last caterpillar in line. When the procession had traveled approximately 80 cm, it was removed and two new caterpillars were placed one at a time on different sections of the marked pathway to determine if they could detect and follow the marked sections. A response was considered positive if a caterpillar precisely followed the marked pathway for a distance of at least 5 cm. The procedure was repeated after the pathway had aged for an additional 4, 8, and 22 hr, each time using different sections of the marked pathways. The study was replicated three times with caterpillars from different colonies. Observations made prior to this test indicated that the pencil markings had no effect on the caterpillars' behavior.

Role of Silk in Trail Marking. Observations of laboratory colonies showed that all instars may secrete strands of silk as they move over the substrate. Several studies were conducted to determine if this silk is essential for trail marking and the formation of processions. For the first study, third-instar caterpillars were allowed to form a procession on a paper sheet as described above. After the procession had traveled approximately 80 cm, caterpillars were removed and their pathway, which had been marked with pencil dots, was inspected under a microscope, and the deposited silk was removed by lifting it from the substrate. Five caterpillars were then placed on different sections of the pathway, one at a time, and observed to determine if they would follow it even though it was no longer marked with silk.

To further assess the role of silk in the formation of processions, the tips of the spinnerets of 30 fourth instars were cauterized (Fitzgerald, 1993) to prevent them from spinning silk. Caterpillars were observed under a microscope as they moved about in Petri plates to be certain that they were unable to deposit silk. They were then placed at the center of a paper sheet as described above and observed to determine if they would form a procession and if so whether they left behind

a persistent trail. The sheet was also again inspected under a microscope to be certain that no silk was deposited by these caterpillars.

A third study was conducted to determine if the caterpillars were able to detect a pathway marked with silk but otherwise untouched by the bodies of the insects. Silk was wound directly from the spinnerets of caterpillars onto paper strips, 4 mm wide × 2.5 cm long, using the procedure of Fitzgerald (1993). These strips were then paired with blank strips of similar dimensions to form the alternate arms of Y mazes. The stems of the mazes were made from blank strips. To conduct a test, a caterpillar was placed at the base of the stem of a maze, allowed to move to the juncture of the arms of the maze, and its choice of arm recorded. The study was replicated 20 times with different caterpillars.

Source of Extra-Silk Trail Marker. The possibility that *T. pityocampa* caterpillars secrete an extra-silk trail marker from the ventral surfaces of the posterior tips of their abdomens was investigated by observing the response of second instars to surface residue collected from the ventral surface of the last abdominal segment. Response of caterpillars to residue collected from the ventral surface of the mid-abdomen was also observed, since one study (Costa et al., 2002) reported that the trail marker of the social caterpillar *Arsenura armida* was found in this region as well as at the tip of the abdomen. Material from these two body sites was collected on separate X mazes prepared by folding 6-cm-square paper cards along both diagonals (Fitzgerald and Underwood, 1998a) to create four ridges. The length of one of the four ridges of the X was stroked two times against one of the designated body regions of a fourth instar to obtain surface residue. The card was unfolded and flattened and a second instar placed at the center of the X, presenting it with four slightly elevated ridges (arms) along which it might travel, only one of which bore surface residue. The responses of 20 caterpillars from four different colonies to mazes bearing residue collected from each of the two body sites were observed. Residues used to prepare each of the mazes were collected from different caterpillars. Response to an arm was considered positive if a caterpillar traveled its entire length, turned at the end of the arm and retraced its path. Residues were collected from different caterpillars for each replicate of the test, and each maze was used only once.

To assess the potential importance of an extra-silk trail marker to processionary behavior, small pieces of paper card were cemented with Super Glue to the ventral surfaces of the last abdominal segments of each of 10 fourth-instar caterpillars, preventing the segments from contacting the substrate. Caterpillars were then placed together at the center of a sheet of paper to determine if they could form a procession and if so whether they left behind a persistent trail that marked their pathway. A second study was conducted by sealing the ventral surfaces of the last abdominal segments of 15 fourth instars with a thin coating of Super Glue. The caterpillars were then allowed to walk up a vertically suspended strip of chromatography paper, 45 cm long × 2.5 mm wide. The treatment had no apparent

affect on locomotive ability. Caterpillars were removed when they reached the top, and the strip was cut into sections 1.5 cm long. A second group of 10 intact caterpillars (control group) obtained from the same colony was allowed to walk up another strip, and the strip was cut into 1.5-cm-long sections. These short sections were used to form the alternate arms of Y mazes. The stems of the mazes were formed from strips walked on by the treated group or the control group, and alternated for each test. To determine if caterpillars would distinguish between strips, a second instar was placed at the base of the stem of a maze, allowed to move to the choice point, and its choice of arm recorded. A response was considered positive if a caterpillar walked the entire length of the selected arm. The responses of 20 first and second instars were recorded. The arms of the mazes were used only once, and the position of the treatment and control sections alternated to preclude a positional bias.

Response of Caterpillars to Artificial Trails Prepared from Silk Only or Trail Pheromone Only. A study was conducted to determine how caterpillars would respond when allowed to choose between trails consisting only of silk or only of trail pheromone prepared as described below. Silk pulled directly from the spinnerets of caterpillars was wound onto paper strips as described above. Strips were cut into sections 1 cm long, and a section was used to form one arm of a Y maze. The other arm was formed from a strip of identical length to which 20 μl of a stock acetone–pheromone solution was applied. The stem of the maze consisted of a blank strip 0.5 cm long. To conduct a test, a first or second instar was placed on the stem of the maze and allowed to choose between the two arms. Response to an arm was considered positive if the caterpillar walked the entire length of an arm ($N = 20$). Each maze was used only once, and arms were alternated for each successive test to preclude a potential positional bias.

To obtain an extract of the extra-silk trail marker, 10 fourth instars were killed by freezing, then soaked for 1 hr in 5 ml of either acetone or hexanes. The solvents were poured off into separate vials and allowed to evaporate completely. One milliliter of solvent was added to each of the vials to form stock solutions. To determine if extracts would elicit trail following, 20 μl of the stock solution was concentrated on a strip of card 1 mm wide $\times$ 2.5 cm long (Figure 1). A first instar was placed at one end of the strip and observed. A response was considered positive if the caterpillar followed the artificial trail from one end to the other. Control trails were prepared from similar quantities of the solvents alone. Each test was replicated 10 times with different caterpillars. Trails were used only once. The response of first instars to 5β-cholestan-3-one, a chemical shown to elicit trail following from other species of social caterpillars (Fitzgerald and Costa, 1999; Fitzgerald and Underwood, 1998a; Ruf et al., 2001), was also investigated. The pheromone was diluted with hexanes to produce concentrations of 1×10^{-9} g/mm of trail and 1×10^{-6} g/mm of trail. The response of three caterpillars to artificial trails prepared with each of these dilutions was tested using the procedures described above.

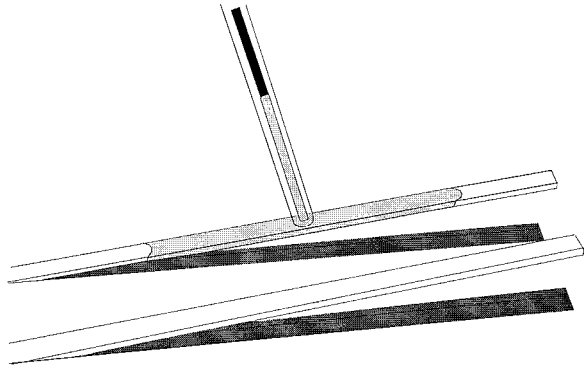

FIG. 1. Procedure for concentrating trail pheromone in a narrow line. The pheromone solution or solvent control was applied with a micropipet to a raised strip cut from a paper card to prevent the chemical from bleeding into the adjacent areas of the card. After the solvent evaporated, the strip was pressed into the groove, flush with the surrounding card.

Ability of Caterpillars to Discern Trail Strength. Different groups of second instars were allowed to walk up separate vertically suspended strips of chromatography paper as described above. Groups consisted of either 10, 20, or 30 caterpillars. Long strips were cut into sections 2 cm long and used to form Y mazes. The stem of a maze was made from the strip walked on by 30 caterpillars, and the arms from strips walked on by either 10 or 20 caterpillars. The study simulated the condition where a procession of 30 caterpillars fragmented into subgroups of 10 or 20 and sought to determine if stragglers would select the pathways of the two groups with equal frequency. Procedures for conducting the tests and criteria for a positive response were the same as those for Y-maze tests ($N = 20$).

Ability of Caterpillars to Discern Trail Age. A study was conducted to determine if caterpillars could distinguish between new and aged trails. Forty first instars were allowed to walk up a strip of suspended chromatography paper as described above. This long strip was laid out on a table and divided into 40 sections each 1 cm long. Odd-numbered sections were assigned to one group and those with even numbers to another. One group of strips was placed in a covered Petri plate and immediately stored in a freezer at -75°C to retard aging or volatilization of the marker. The other was held in a covered Petri plate at room temperature ($21 \pm 3^{\circ}$C). Twenty-two hours later the strips from the two groups were removed from their storage sites and used to form the alternate arms of Y mazes as described above. The test was replicated 20 times with second instars.

Response of Caterpillars to Tactile Stimuli. Demolin (1962, 1971) and Mallmann (1962) conducted studies that showed that caterpillars of *T. pityocampa* exhibit thigmotaxis to tactile stimuli applied to their heads and can be made to follow models made from dead caterpillars and other objects. A set of preliminary

studies was conducted to verify these observations, then a series of tests was carried out to determine if tactile stimuli were species-specific and competitive with the stimuli associated with established trails. Preliminary studies were conducted by placing third instars on sheets of paper and attempting to lure them into following a model. Models were prepared by gluing the tips of the abdomens of recently killed caterpillars of *T. pityocampa* or *Malacosoma americanum* (Lasiocampidae) to a thin dowel. The responses of 10 third-instar processionary caterpillars to each of the models, to the dowel alone, and to the fine hairs of a brush were observed. A response was considered positive if a caterpillar followed the model for a distance of at least 5 cm. In all cases, models were held above the substrate so that there was no possibility of a chemical residue from the model being deposited on the substrate.

Following these preliminary observations, attempts were made to lure caterpillars from well-established circular trails (see below) with a model. A third instar was placed on a circular trail and allowed to follow it for a distance of 10 cm or more, then a model made from the tip of the abdomen of a processionary caterpillar as described above was placed in front of the caterpillar and moved off the trail onto previously unmarked substrate. A response was considered positive if the caterpillar left the established trail and followed the model onto previously unmarked substrate for a distance of at least 5 cm. The test was replicated with 10 different third instars and five different circular trails.

Manipulation of Field Colonies. Field observations were made of fully grown caterpillars that were moving over the ground in search of pupation sites to determine the extent to which caterpillars in these final processions relied on the trail pheromone to stay together. Twelve naturally formed processions of approximately 30–65 caterpillars moving over stone terraces were manipulated by abruptly removing three caterpillars from near the centers of the processions. Observations were then made to determine if the processionaries had deposited a trail pheromone that would enable the new leaders of the isolated rear sections of the processions to locate and rejoin the forward contingents.

Tenacity of Established Trails. Ten groups of 14–58 (mean $= 34 \pm 4.0$) second instars were placed under the lids of separate 9-cm-diam. Petri plates situated at the centers of sheets of paper and allowed to form trails on the paper. Constrained by the side walls of the plates, the groups of caterpillars formed circular trails along the edges of the plates, which they were allowed to walk over for at least 30 min with the plate in place. The plates were removed, and the circling groups of liberated caterpillars were videotaped. The tapes were subsequently analyzed to determine how long the liberated caterpillars remained on the circular trails and the means by which the caterpillars eventually broke away from them. This same study was repeated using three groups of fully grown fifth instars (mean $= 17 \pm 1.5$ caterpillars per group). Due to their larger size, caterpillars were allowed to establish circular trails under glass dishes 19 cm in diameter.

Polyethism. To determine if males and females lead processions with equal frequency, groups of fifth instars consisting of five males and five females were placed at the centers of sheets of paper and allowed to form processions *ad libitum*. In each case, it was noted whether the caterpillar at the head of the procession was a male or a female. Females are readily distinguished from males by their larger size (Demolin, 1971). Caterpillars used were obtained from six laboratory colonies and 134 instances of spontaneous assembly were recorded.

Videotapes of 11 processions formed by 10 colonies of third instars as they advanced over unmarked substrate in the laboratory were analyzed to obtain a measure of the relative amount of energy expended by leaders and followers. The mean duration of episodes of locomotion used in the analysis was 55.9 ± 5.7 sec. At approximate 1-sec intervals, the positions of the bodies of the leader and the caterpillar immediately behind it were traced directly from the videoscreen onto plastic sheets. The tracings were analyzed with SigmaScan software to determine the head velocity of the caterpillars during each episode.

Efficiency with which Caterpillars Abandon Exhausted Feeding Sites. To determine the efficiency with which *T. pityocampa* caterpillars abandoned exhausted feeding sites, colonies were allowed to establish their nests on wooden stands having dowel bridges to food plants (Fitzgerald and Underwood, 1998a,b). To conduct a test, caterpillars were allowed to move to food for two consecutive nights over a new dowel bridge consisting of a proximal stem section and a distal arm. The arm was attached to the end of the stem at an angle of 15°. The food supply consisted of a branch of Scot's pine sufficiently large to provide caterpillars with needles in excess of their needs. At the start of the third night, a second arm was joined to the end of the stem to form a Y, the arms of which were separated by an angle of 30°. The branch at the end of the first arm was manually defoliated but left in place, and a new, fully foliated branch was placed at the end of the new arm. Thus, the study sought to determine how long it would take the colony to abandon the arm of the Y that led to the old, exhausted feeding site in favor of the arm leading to the new food supply. Infrared activity monitors (Fitzgerald and Underwood, 2000) were used to monitor the activity on both arms of the bridge during each of four successive nights. Monitors were placed at the distal ends of the arms, approximately 2 cm from the point where the arms abutted the host branches. The monitors were connected to event loggers (Onset Computer Corp.), which recorded the time when a caterpillar triggered a monitor. A reset delay of 5 sec was programmed into the loggers to minimize the probability that a single passing caterpillar would register its movement more than once. Data were off-loaded with BoxCar Pro software (Onset Computer Corp.) The study was replicated seven times for the first three nights and five times on the fourth night using six different colonies.

Statistics. Statistical analyses as detailed below are based on procedures outlined by Sokal and Rolf (1969) and on ProStat and SigmaStat statistical software.

RESULTS

Persistence of the Trail. Processions of caterpillars moving over paper sheets in the laboratory deposited persistent trails. In three replicates of the procedure, all 18 caterpillars placed singly on different sections of the trails 4, 8, or 22 hr after the trails were initially deposited, followed the precise pathways of the processions. The results are consistent with other studies that show that social caterpillars produce long-lived trail systems (Fitzgerald and Costa, 1999).

Role of Silk in Trail Marking. Caterpillars placed singly on separate sections of a trail previously marked by a procession of caterpillars from which all silk was removed followed the precise pathway of the procession, indicating that the persistence of the trail is not dependant on the physical presence of silk. Since it was possible that some chemical residue of silk remained even through the silk was physically removed, a second study was conducted in which the tips of the spinnerets of caterpillars were sealed, preventing them from secreting silk. Caterpillars so treated formed a procession indistinguishable from that of intact caterpillars and left behind a persistent trail that was followed precisely by all caterpillars placed on a separate section of the silkless pathway. A third study indicated that although silk is not needed to mark trails or to maintain processions, caterpillars are able to distinguish between pathways overlain with silk and silkless pathways. In Y-maze tests, all caterpillars selected the arms of mazes marked with silk collected directly from the spinnerets of caterpillars, rather than the control arms ($\chi^2 = 20$, $P < 0.001$) but showed considerable hesitation when moving over the arms, turning back frequently. It was not determined if the caterpillars' response to silk was due to the increased purchase afforded by the material or to a chemical component of the strand.

Source of Extra-Silk Trail Marker. The results of the above studies indicated that the caterpillars secreted an extra-silk marker as they moved over the substrate, accounting for the persistence of their trails. In X-maze tests, all caterpillars followed arms of mazes that bore surface residue collected from the ventral surface of the tip of the last abdominal segment ($\chi^2 = 60$, $P < 0.001$). All caterpillars also followed arms prepared from surface residue of the ventral surface of the mid-abdominal region. These results suggest that the caterpillars mark the substrate with a chemical that is a general component of the cuticle, as recently shown for the saturniid caterpillar *Arsenura armida* (Costa et al., 2002). The result differs from that of studies of the lasiocampid caterpillars *Malacosoma* (Fitzgerald, 1995) and *Gloveria* (Fitzgerald and Underwood, 1998b), the pierid *Eucheira socialis* (Fitzgerald and Underwood, 1998a), and the saturniid *Hylesia lineata* (Fitzgerald and Pescador-Rubio, 2002), all of which mark trails with a chemical found exclusively at the tip of the abdomen. It remains to be investigated, however, whether in *T. pityocampa*, the marker is produced at a discrete site and contaminates other areas of the body that may come in contact with the substrate. Furthermore, it

is likely that most of the deposition of the marker occurs when the caterpillars brush the tips of their abdomens against the substrate. When the venters of the tips of the abdomens of caterpillars were covered with small pieces of paper preventing them from contacting the substrate, they were still able to form processions. Third instars placed singly on different sections of the pathway taken by the procession, however, showed no response to the pathway, indicating that the venter is the prime site of the deposition of the trail pheromone. Y-maze tests also showed that the ventral surface of the tip of the abdomen is the primary source of the trail marker. When allowed to choose between arms of mazes walked on by 15 caterpillars that had the venters of the last abdominal segment sealed and those walked on by 10 intact caterpillars, 17 of 20 caterpillars selected that arm previously walked on by the intact caterpillars ($\chi^2 = 9.8$, $P < 0.01$) despite their fewer numbers. Both trail types were heavily marked with silk, providing additional evidence that silk plays a subordinate role to the trail pheromone in eliciting trial following. Videorecordings indicate that the caterpillars can vary the extent to which they mark with pheromone by pressing the tips of the abdomens more or less tightly to the substrate.

All caterpillars also followed artificial trails laid out in narrow lines on paper cards prepared from either acetone or hexane extracts of whole caterpillars, but showed no response to solvent control trails. The caterpillars showed no response to 5β-cholestan-3-one at any of the tested dilutions.

Response of Caterpillars to Artificial Trails Prepared from Silk Only or Trail Pheromone Only. To further test the conclusion that the extra-silk trail pheromone takes priority over silk in eliciting trail following, first or second instars were allowed to choose between the arms of Y mazes covered with either silk wound directly from the spinnerets of fourth instars or marked with the acetone–pheromone extract. In all replicates, the caterpillars chose the arm marked with the pheromone extract ($\chi^2 = 20$, $P < 0.001$).

Ability of Caterpillars to Discern Trail Strength. When allowed to choose between the arms of Y mazes previously walked on by either 10 or 20 caterpillars, all caterpillars selected the arms of the mazes walked on by the group of 20 caterpillars ($\chi^2 = 20$, $P < 0.001$). In 15 of the 20 replicates, caterpillars turned back at least once at the junctures of the stems and arms of the mazes before eventually selecting the stronger of the two arms. This hesitancy is consistent with the tendency of the caterpillar to move from weaker to stronger trails since the stem of the maze was previously walked on by a group of 30 caterpillars.

Ability of Caterpillars to Discern Trail Age. Although the results reported above indicate that the trails of the pine processionary caterpillar are long-lived, Y-maze tests indicate that the trail pheromone volatilizes or deteriorates with age. In these tests, 18 of 20 caterpillars selected arms of mazes held for 22 hr at -75°C, beginning immediately after their deposition, over trails maintained at room temperature for the same period of time ($\chi^2 = 12.8$, $P < 0.01$). That the

arms maintained at room temperature were still active was shown by the result of another Y-maze test comparing the response of caterpillars to arms held at room temperature to blank (control) arms. In nine of 10 replicates, they selected the strips aged at room temperature over the blank controls ($\chi^2 = 6.4$, $P < 0.05$). The foraging arena of established colonies of the processionary caterpillar consists of a network of variously aged trails, and the ability of the caterpillars to distinguish among them on the basis of age facilitates their nightly back and forth movements between their nest and feeding sites.

Response of Caterpillars to Tactile Stimuli. The results of the studies showing that caterpillars unable to spin silk or to mark the substrate with the trail pheromone are still able to form processions suggested that tactile cues alone may be adequate to allow caterpillars to form and maintain processions. Single third instars placed on clean paper sheets in the laboratory were readily enticed to follow models prepared by gluing the tip of the abdomen to a stick. In all replicates, caterpillars followed models prepared from the tips of previously frozen pine processionary caterpillars and eastern tent caterpillars. The response to the tent caterpillar indicates that tactile stimuli associated with the setae that issue from the tip of the abdomen rather than species-specific, chemotactile stimuli are likely to be involved. Caterpillars could not be enticed to follow the stick alone, and response to the hairs of a small brush was spotty and incomplete. In contrast, the thigomotactic response to the tip of the abdomen is best described as "magnetic." The caterpillars showed a nearly instant response to the models and followed them in contact with little or no hesitation.

Caterpillars placed individually on circular trails followed them readily, circling continuously. In all replicates, it was possible to lure circling caterpillars from the trails and onto unmarked substrate with a model prepared from the tip of a fourth-instar pine processionary caterpillar. Caterpillars showed no hesitation in following the model onto the unmarked substrate and responded nearly instantly to it. The results indicate that as caterpillars move together over trails or in long head-to-tail processions over the ground, thigomotactic cues take priority over trail-associated stimuli.

Manipulation of Field Colonies. Three of 12 processions manipulated under field conditions collapsed and disintegrated, but in the other nine instances both sections of the processions resumed moving after a brief pause. In none of these nine instances were the new leaders of the second sections of the processions able to relocate the ends of the first sections, and the two halves struck off in different directions. This occurred despite the fact that the new leaders of the rear contingent stopped abruptly, and excitedly swung the anterior portions of their bodies though wide arcs in apparent attempts to locate the caterpillars they had been following or trails that might have been left behind. These observations indicate that the caterpillars either do not deposit a trail marker when moving over the ground in search of pupation sites or that deposition is too spotty and inadequate to serve as a guide for the procession.

Tenacity of Established Trail. The partially grown caterpillars of *T. pityocampa* are strongly influenced by the trail marker as evidenced by their response to circular trails. In 10 replicates of the test, cohorts of third instars followed their circular trails for 143.2 ± 67.9 min after being liberated from the confines of the arenas used to establish the trails. One cohort circled continuously for nearly 12 hr, during which the caterpillars were estimated to have traveled a total distance of 142 m. In contrast, cohorts of near mature, fifth instars stayed on the trails for only 2.3 ± 1.1 min before breaking away. The difference between the performance of third and fifth instars was highly significant (Mann-Whitney rank sum test, $P < 0.01$) This result corroborates the observations noted above that full grown caterpillars moving over the ground in search of pupation sites may rely little if at all on the trail pheromone.

Although tactile stimuli associated with the tips of the abdomens clearly played a role in the maintenance of the circular processions of the younger caterpillars, their ranks were often not completely closed, requiring tactilely isolated caterpillars to reference trail-based stimuli to maintain their place in line. Caterpillars showed a clear reluctance to leave the trails, and individuals typically turned back after moving only short distances beyond them. Processions also sometimes collapsed centripetally, only to be "trapped" again by their circular trails when they resumed their activity. Caterpillars eventually broke away from the circular trials when leaders tentatively ventured off the trails and were pushed from behind by siblings, preventing them from turning back.

Examination of the videotapes also indicated that escape from the circular pathways was facilitated by the deposition of fresh trail pheromone by the caterpillars as they moved off the established trail onto previously unmarked substrate. This was evidenced by the fact that in eight of the 10 replicates, the newly formed, divergent pathway taken by the escaping cohort was readily differentiated from the circular pathway by small groups of caterpillars or singletons that lagged behind after the main body of the procession had departed. When these still circling remnants encountered the juncture of the circular trail and the pathway taken by the departed cohort, they followed the new pathway without hesitation. That the caterpillars could differentiate these two pathways suggests that after some period of circling they stopped depositing pheromone and that degradation or volatilization of the pheromone or a component of the pheromone from the circular trail allowed them to distinguish it from the newly marked, breakaway trail.

Polyethism. When allowed to form processions spontaneously in the laboratory, groups of caterpillars comprised of equal numbers of males and females had females at the head of the procession 88 times and males 46 times ($\chi^2 = 13.16$, $P < 0.001$). Caterpillars in the vanguard, regardless of gender, were significantly more active than caterpillars that followed behind. Leaders constantly swung the anterior sections of their bodies through broad arcs as they advanced over previously unmarked substrate, while those that followed kept their heads in contact with

FIG. 2. Positions of the bodies of *T. pityocampa* caterpillars recorded at 1 sec intervals as the procession advanced over the substrate. (Above) The caterpillar at the head of the procession, (Below) the caterpillar second in line.

the tip of the abdomen of the precedent caterpillar with little lateral deviation of the anterior ends of their bodies (Figure 2). The head velocities of leaders (0.43 ± 0.047 cm/sec) differed significantly from those of followers (0.24 ± 0.38 cm/sec) (ANOVA, $F = 9.21$, $P < 0.01$) reflecting the extensive pendulations of the anterior of the bodies of the leaders compared to those of non-leaders. Thus, followers enjoy an economy of movement at the expense of the leader.

Efficiency with which Caterpillars Abandon Exhausted Feeding Sites. During the first foray after the food supply was switched from the old to the new site, the caterpillars in all but one replicate located and fed at the new site (Table 1), although the total number of visits to the old site was significantly greater than the number to the new site. During the second foray following the switch, three colonies favored the new site, three favored the old, and one showed no preference; there was no significant difference in the pooled results. During the third foray, four of seven colonies favored the new site, and during the fourth foray, the caterpillars in all five replicates showed a preference for the new site; the pooled results in both cases were significant.

Compared to other species of social caterpillars tested under identical conditions, colonies of the pine processionary caterpillar are relatively inefficient in abandoning exhausted feeding sites in favor of new food finds. As shown in

TABLE 1. NUMBER OF EVENTS RECORDED ON BRIDGE SECTIONS LEADING TO OLD FEEDING SITE AND NEW FEEDING SITE AFTER FOOD WAS SWITCHED FROM OLD TO NEW SITE

		Events															
		Initial foray after switch				Second foray after switch				Third foray after switch				Fourth foray after switch			
Colony	Replicate	Old	New	G^a	P	Old	New	G	P	Old	New	G	P	Old	New	G	P
1	1	216	184	2.56	NS	145	221	15.89	**	69	214	77.94	**	87	179	32.48	**
2	1	146	108	5.70	*[b]	198	149	6.94	**	293	74	139.81	**				
3	1	146	214	12.92	**	0	154	213.49	**	3	158	193.35	**	0	175	242.60	**
	2	356	0	493.52	**	283	37	214.42	**	258	0	357.66	**				
4	1	204	323	27.10	**	115	314	95.94	**	114	339	116.87	**	163	288	35.11	**
5	1	306	181	32.44	**	340	217	27.38	**	205	397	62.32	**	315	403	10.82	**
6	1	270	298	1.38	ns	260	290	1.64	ns	272	315	3.15	ns	0	302	418.66	**
Total		1644	1308	575.63	**	1341	1382	575.72	**	1214	1497	951.13	**	565	1347	739.67	**
Heterogeneity				537.31	**			575.10	**			921.53	**			410.26	**
Pooled				38.32	**			0.62	ns			29.60	**			329.41	**

[a] Replicated G test statistics.
[b] $^*P < 0.05$; $^{**}P < 0.01$.

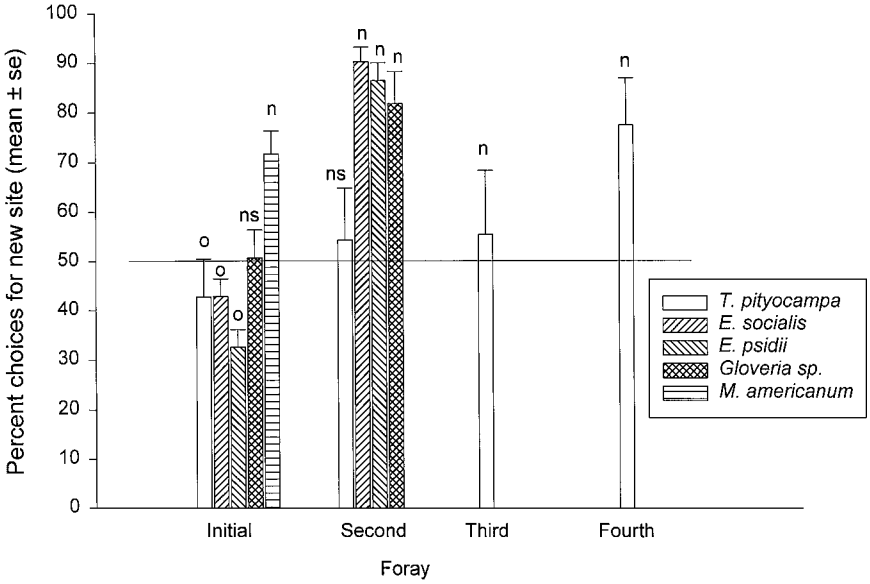

FIG. 3. Percent of caterpillars of five species selecting the arm of a Y maze leading to a new feeding site during the first to fourth forays after food was switched from the original site to the new site. o = significant preference for the original site, n = significant preference for the new site, ns = no significant preference for either site (χ^2 analyses). Data for *T. pityocampa* from this study, for other species from Fitzgerald and Underwood (1998a,b).

Figure 3, with the exception of *M. americanum*, none of the species showed a preference for the new site during the first foray, but in the second, all but *T. pityocampa* favored the new site. The performance of *T. pityocampa* was also marginal during the third foray and did not approach the performance of the other species until the fourth foray. Of the species shown in Table 1, only *M. americanum* uses its trail pheromone to recruit to food, and the others recruit to trails, facilitating the abandonment of exhausted feeding sites in favor of new food finds (Fitzgerald and Underwood, 1998b). The extent to which *T. pityocampa* marks with its trail pheromone as it moves to and from food is unknown, and the tendency of the caterpillars to follow each other, even in the absence of a trail marker, may account for the lower overall efficiency with which colonies shift from one feeding site to another.

DISCUSSION

Field observations made in association with this study show that during their nightly forays, caterpillars of *T. pityocampa* travel in small processions or as

singletons as they move over the branches of the host tree in search of needles. During these forays, they lay down strands of silk, and the upper surfaces of repeatedly used pathways eventually become thickly covered with the material. Although silk is the conspicuous component of the pathway, the data presented here provide the first evidence that a chemical trail marker secreted from the tip of the abdomen rather than silk is primarily responsible for eliciting trail following. The ability of the caterpillars to mark trails with a pheromone and to distinguish new from aged trails and stronger from weaker trails enables them to move to communal feeding sites independently and at their own pace. It also enables them to find their way back to the nest after feeding. The results of this study are consistent with studies of other social caterpillars that forage from a central place, and indicate that the primary function of the silk component of the trail is to enhance steadfastness. This function of silk may be particularly important to the pine processionary caterpillar in midwinter. Caterpillars leave the nest in search of food on the coldest of nights and may become immobilized on the branches of the host when temperatures drop to freezing or below, forcing them to depend on the mechanical entanglement of silk with their crochets to keep from falling from the tree.

In contrast to the essential role that the trail pheromone plays in allowing caterpillars to move efficiently to and from the nest, the data indicate that when prepupal caterpillars advance en masse over the ground in search of pupation sites they may rely solely on tactile or chemotactile stimulation associated with the tips of the abdomens of precedent individuals to maintain their place in a procession. Neither silk nor a trail pheromone is required. While the tendency of individuals on a trail to move forward when brushed from behind by a sibling may be a universal response of social caterpillars, no other species has yet been reported to be influenced by such stimuli to the extent that the tactile stimulus completely supplants the need for a trail pheromone to hold the traveling company together. In *T. pityocampa*, the ability of the caterpillars to rely solely on thigmotactic stimuli when moving in processions over the ground may have evolved to keep colonies together when they rapidly traverse terrain too texturally complex to mark continuously with a trail pheromone. Although the absence of a persistent trail would appear to make it difficult for caterpillars to stay together, observations of both field and laboratory processions during this study as well as previous observations by Demolin (1971) indicate that a unique behavioral mechanism may function to keep processions from fragmenting. It was observed that if the last caterpillars in processions were held back, the penultimate caterpillars sensed the loss of contact from behind and immediately stopped moving, causing them, in turn, to lose contact with the caterpillars they were following. In this manner, the loss of contact proceeded rapidly from the back to the front and caused processions to come to a complete halt. The procession began to move again when the last caterpillar in line moved forward and brushed against the tip of the abdomen of

the penultimate caterpillar causing the contacted caterpillar to move forward and tactilely stimulate the caterpillar next in line. The behavior rippled from back to front and eventually reached the leader who then began to move again. Thus, the tendency of a caterpillar to stop immediately when the tip of its abdomen is no longer stimulated by the caterpillar that has been following it increases the likelihood that the processionaries will wait for caterpillars that are momentarily detained. It is not known how rigidly the caterpillars adhere to this protocol, and in any case it is unlikely to be as reliable a mechanism as a trail pheromone in enabling stragglers to reestablish contact with their procession. Indeed, Brindley (1910) noted the ". . . great tendency of processions (of *T. pityocampa*) to break up into daughter populations," and Mills (1951) observed processions of *Orchogaster* caterpillars fragment into as many as 23 subprocessions, although nothing is known of the sensory basis for procession formation in the latter species. Moreover, because the prepupae of the pine processionary caterpillars fail to leave behind a cue that would enable them to retrace their way, these processions are necessarily unidirectional. Previous reports stating that processionaries may return to the nest after extensive over-the-ground forays (Fabre, 1916) would appear to be true only of younger colonies whose search for food off the tree is accompanied by the deposition of an unbroken pheromone trail.

Analysis of videorecordings shows that leaders of over-the-ground processions expend more energy in locomotion than other caterpillars in the processions. The leader is also likely to be at increased risk of succumbing to small predators that lie in wait, such as spiders, since the leader would likely be the first to move within striking distance of the predator and it is unprotected from frontal attack. Despite the costs, assuming the leader's role is not likely a form of altruism. Judging from their hesitant locomotion (Figure 2), caterpillars in the vanguard appear to be reluctant leaders, goaded on by the caterpillars that line up behind them. Nonetheless, laboratory studies show that females lead processions more often than males and, pending confirming field studies, indicate that the colonies may have a gender-based division of labor (polyethism) with females predisposed to expend more energy and to expose themselves to more risk than males. Polyethism was first reported in social caterpillars by Wellington (1957), who argued that colonies of tent caterpillars consisted of an adaptive mixture of siblings with different activity levels leading some to be primarily responsible for constructing trails and others for adding silk to the tent. Subsequent studies (Edgerly and Fitzgerald, 1982; Papaj and Rausher, 1983), however, failed to substantiate these earlier studies and cast doubt on the occurrence of polyethism in tent caterpillars. More recently, Weinstein and Maelzer (1997) reported a weak polyethism in which certain caterpillars in colonies of the sawfly *Perga dorsalia* were more likely than others to assume the leadership role in processions, and Underwood and Shapiro (1999) reported a gender-based division of labor in colonies of *Eucheria socialis* in which the male caterpillars were more likely to spin silk than the females. In these species, and in

T. pityocampa, polyethism is a statistical phenomenon bearing little resemblance to the genetically encoded and sharply defined division of labor found among the eusocial insects, and it is unclear whether the behavior has any adaptive significance for colonies of social caterpillars.

The significance of over-the-ground processions is unknown. It has been suggested that they constitute aposematic displays affording the highly urticating caterpillars (Lamy et al., 1986; Vega et al., 1999) more protection than they might enjoy were they to travel independently. It may be, however, that the most significant function of the procession is not to protect the traveling caterpillars but to bring them to a common burial site where they pupate. Edwards (1910) and Schmidt et al. (1990) observed that "circulating masses" of caterpillars at pupation sites may wear away a depression in soft, sandy soils while collectively attacking the sand with their mandibles and gradually bury themselves. Since the toxin in a shed urticating hair remains active after it is shed (Ducombs et al., 1981), the mass of hairs lost by the caterpillars and mixed with the substrate as they excavate en masse would likely form a more substantial defensive barrier than an individual alone could produce. If predators are deterred by lost urticating hairs or hairs attached to the outside of an unearthed cocoon, then siblings buried nearby are likely to be spared. By pupating en masse at the burial site, an individual's chance of being the caterpillar that the predator samples is reduced in proportion to the number of siblings buried nearby, favoring caterpillars that pupate near others. It also remains an unstudied possibly that the process of digging out by the moth months later may be facilitated if others nearby are simultaneously excavating tunnels to the surface and, in the process, loosening the soil or forming common pathways.

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ROLE OF CONTACT PHEROMONES IN MATE RECOGNITION IN *Xylotrechus colonus*

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Abstract—Adult male and female rustic borers, *Xylotrechus colonus* F. (Coleoptera: Cerambycidae), aggregate on cut logs and fallen trees that are the hosts of their larvae. Our studies show that male *X. colonus* actively search for females, and only respond to them after contacting them with their antennae. Stripping cuticular hydrocarbons from females with solvent rendered them unattractive to males, suggesting that males did not recognize females by mechanoreception alone. Reapplying solvent extract to washed females restored their attractiveness to males, confirming the role of cuticular hydrocarbons in mate recognition. Female cuticular hydrocarbon extracts contain *n*-pentacosane, 9-methylpentacosane, and 3-methylpentacosane, components that were either absent or present in very small amounts on males. We demonstrate that the contact pheromone is a blend of these three cuticular hydrocarbons.

Key Words—Cerambycidae, longhorned beetle, mating behavior, cuticular hydrocarbon, *n*-pentacosane, 9-methylpentacosane, 3-methylpentacosane, methyl-branched alkane.

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INTRODUCTION

Among the Coleoptera, sex pheromones have been identified for few species other than bark beetles and weevils (Mayer and McLaughlin, 1991). It has long been thought that longhorned beetles, Cerambycidae, rely on pheromones for mate location (Linsley, 1964). However, there is convincing evidence of long-range pheromones for only a few species in this family (Hanks, 1999). Hanks (1999) suggested that reproductive strategies and behavior of longhorned beetles appear to be associated with the condition of the larval host plant. Sex pheromones that operate over short or long distances are produced by either males or females of some species whose larvae feed in healthy, marginally weakened, or dead host plants (Hanks, 1999). For species that attack severely stressed or moribund trees, however, the sexes apparently are brought together by their mutual attraction to the larval host. Once on the host, males of these "stressed host species" actively search for mates, using their antennae to recognize females by contact chemoreception (Hanks, 1999).

Cerambycid species that use sex pheromones that operate over a distance include three species of the genus *Xylotrechus* in which the males produce the pheromone: Asian species *X. pyrrhoderus* (Bates) and *X. chinensis* Chevrolat, and the African *X. quadripes* Chevrolat (Iwabuchi, 1982, 1988; Sakai et al., 1984; Iwabuchi et al., 1987; Kuwahara et al., 1987; Hall et al., 1998). The North American species *Xylotrechus colonus* F., however, shows behaviors consistent with the stressed host strategy of Hanks (1999): both sexes are attracted by volatile compounds of larval hosts, and there is no evidence of long-range pheromones (Ginzel, 1999). Adult *X. colonus* are crepuscular and the larvae develop in recently killed and stressed deciduous trees of many species, primarily hickory (Yanega, 1996). The objectives of our study were to: (1) characterize the mating behavior of *X. colonus*, (2) identify the types of cues involved in mate recognition, and (3) identify and bioassay specific cuticular hydrocarbons that serve as contact pheromones.

METHODS AND MATERIALS

Field Site and Source of Specimens. Field studies were conducted at Allerton Park, a University of Illinois Natural Area in Piatt County, Illinois, USA. We studied mating behavior of *X. colonus* on two hickory trees, *Carya glabra* (Mill) Sweet, that had been felled on May 1 and July 9, 1998. The same trees, and others felled later, were the source of beetles for all of our laboratory studies. Beetles were collected directly into glass vials without handling to avoid contaminating their cuticular hydrocarbons. In the laboratory, we individually housed beetles in cylindrical cages (9 cm diam. $\times$ 12 cm tall) of aluminum window screen with 9-cm glass Petri dishes covering top and bottom. Caged beetles were provided

10% sucrose solution in an 8-ml feeder vial into which was inserted a 4-cm long cotton dental wick (Patterson Dental, South Edina, Minnesota, USA). Feeders were replaced at least every three days. Beetles used in mating trials and bioassays had been isolated in these cages for at least 24 hr and were only used in bioassays as long as they were active and vigorous.

Mating Behavior. To characterize the mating behavior of *X. colonus* and identify cues used in mate location, we observed beetles in the field as well as in laboratory arenas. In the field, we observed mating behavior of beetles on the felled hickory trees on warm evenings during May through August 1998. We observed $\sim$100 interactions among beetles of which 20 were videotaped (Panasonic AG-465 Proline S-VHS video camera). To study mating behavior in the laboratory during the same time period, we paired females and males in glass Petri dishes (9 cm diam. $\times$ 2 cm tall) lined with filter paper (No. 1, Whatman, Maidstone, England). Dishes were cleaned with acetone between trials and allowed to air dry. Twenty pairs of beetles in these arenas were videotaped in late afternoons under ambient light from exterior windows. The beetles became active when overhead lights were turned off.

We studied videotapes of beetles from field and laboratory studies to determine whether one sex showed directed movements towards the other sex from a distance, evidence that mate location might be mediated by either volatile pheromones or vision. We predicted (based on Hanks, 1999) that males would respond to females only after contacting them with their antennae, suggesting the use of contact pheromones. We also studied videotapes to characterize the copulatory behavior of *X. colonus*.

Role of Contact Chemoreception. We confirmed that male *X. colonus* used contact pheromones to recognize females via bioassay. Individual females were freeze killed (-4°C for 20 min), allowed to warm to room temperature, and presented to individual males in Petri dish arenas to confirm that males would recognize dead females and attempt to mate, demonstrating that recognition cues were intact. Cuticular hydrocarbons of females were removed by immersing them in two 1-ml aliquots of hexane for 2 min each; aliquots were combined and concentrated to 1 ml under nitrogen. Dead-washed females were then presented individually to the same males to confirm that males no longer responded (did not stop to inspect females with their antennae or otherwise orient to them), demonstrating that chemical cues had been removed and mating was not elicited by mechanoreception alone. To prove that the extract contained the pheromone, we pipetted 0.1 female equivalent (FE) of extract (0.1 ml) on each original dead-washed female, entirely coating the body with the solution, allowed it to evaporate, and presented the female again to males to confirm that the recognition cue was restored. If males did not respond, we incrementally added 0.1 FE (0.1 ml of extract) to each dead-washed female, to a maximum of 1 FE, and recorded the FE level at which males responded.

In these bioassays, conducted in late afternoons between July 10 and 22, 1998, under laboratory conditions, each of 10 dead females was presented to three different males ($N = 30$ males). We observed and videotaped the behavior of individual males toward females (dead, dead-washed, or dead-washed extract-treated) in a Petri dish arena. After a male contacts a living female with his antennae, a clear progression of behaviors leads to copulation: (1) the male stops walking, (2) aligns his body with the female, (3) mounts her, and (4) bends abdomen to connect claspers. We recorded behaviors of each male for 5 min after initial antennal contact with the female, and a trial was scored as a "response" if the male displayed at least the first behavior (stopping), and we also recorded subsequent behaviors. A trial was recorded as "no response" if the male showed none of these behaviors, but rather continued to walk after first contacting the dead female. Responses of males to dead-washed extract-treated females were compared to their response to dead-washed females using a χ^2 goodness of fit test (Sokal and Rohlf, 1995).

Identification of Female Contact Pheromone. To identify the compounds that served as contact pheromones (see Results), we extracted 10 females and males by individually immersing them in hexane as described above. Extracts were initially analyzed by electron ionization-mass spectrometry with a Hewlett-Packard 5973 mass spectrometer interfaced to a HP 6890 gas chromatograph, using a HP-5MS (cross-linked 5% phenylmethyl siloxane) capillary column (30 m $\times$ 0.25 mm, 0.25 μm film thickness) in splitless mode with helium as the carrier gas (6.1 psi head pressure, linear velocity $\sim$36 cm/sec). Oven temperature was ramped from 200 to 240°C at 5°C/min, then from 240 to 300°C at 2°C/min. Injector temperature was 250°C, and the transfer line temperature was 280°C.

We examined chromatograms for consistent female-specific peaks that might serve as contact pheromones. These compounds were tentatively identified by interpretation of their mass spectra by the methods of Blomquist et al. (1987) and Nelson and Blomquist (1995) using a Varian 3400 capillary gas chromatograph interfaced with a Finnigan 4023 mass spectrometer and INCOS data system, using a 30 m $\times$ 0.32 mm DB-5 column with helium as the carrier gas (8 psi head pressure, linear velocity $\sim$30 cm/sec). Column temperature was ramped from 200 to 280°C at a rate of 3°C/min.

The identities of three female-specific compounds (see Results) were confirmed by comparing retention times and mass spectra of insect-produced compounds with those of standards. An $\sim$100% pure standard of *n*-pentacosane (*n*-C_{25}) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 9-Methylpentacosane (9-MeC_{25}) was synthesized by G. Pomonis (approximate purity 85%) using the methods of Pomonis et al. (1989). 3-Methylpentacosane (3-MeC_{25}) was obtained from the cuticle of American cockroaches, *Periplaneta americana* (L.), by the methods of Dwyer et al. (1981). Approximately 100 late-instar and adult cockroaches were extracted with hexane, the solvent was removed

under nitrogen, and the hydrocarbons were isolated by elution of the concentrated extract through a column of Florosil with hexane. The saturated hydrocarbons were separated from alkenes by elution with hexane through a BioSil A column impregnated with 10% (w/w) silver nitrate. Straight-chain hydrocarbons were removed from the resulting eluate by inclusion in 5Å molecular sieves as described in O'Conner et al. (1962) leaving 3-MeC_{25} (approximate purity: 97%).

Testing Activity of Isolated Compounds. We prepared hexane solutions of the hydrocarbon standards in concentrations that approximated the original extracts of female *X. colonus* ($\sim 2.5 \times 10^{-3}$ mg/μl, see Results). We tested the activity of standards with the following bioassay, testing the three female-specific compounds (*n*-C_{25}, 9-MeC_{25}, and 3-MeC_{25}) separately, in solutions containing pairwise combinations, and with all three compounds combined into one solution (combinations were 1 FE of each standard in 1 ml of hexane, approximating ratios of compounds in the original extract; see Results). Freeze-killed females were individually presented to single males in a Petri dish arena to confirm that males would attempt to mate. For each test, we presented each of at least five dead females to two or three different males ($N = 16$ to 24 males). Females were then washed in hexane to remove cuticular compounds (as described above). Dead-washed females were again presented to males; a lack of response confirmed that contact pheromones had been eliminated. Finally, we pipetted 1 ml of synthetic standards, containing 1 FE of individual standard or 1 FE of each component in a blend, onto dead females, allowed solvent to evaporate, and they were again offered individually to the same males. We rated the response of males as already described and videotaped each step of the bioassays to confirm responses. The proportion of males responding to dead-washed, hydrocarbon-treated females was compared to their proportional response to dead-washed females using the χ^2 test (Sokal and Rohlf, 1995). Due to delays in obtaining standards, these bioassays were conducted from 1999 to 2001.

RESULTS

Mating Behavior. In the field, adult male and female *X. colonus* appeared on hickory logs just before sunset. They became active as light levels decreased, with males running up and down the log surface with their antennae spread, apparently searching for females. Females remained stationary, or walked slowly, probing the bark surface with their ovipositors.

In both field and laboratory studies, every male responded to a female only after contacting her with his antennae ($N = 20$ for both experiments); no male directly approached a female in a manner that would suggest they were cued by vision or volatile attractants that operated over a distance. Once a male touched a female with his antennae, he immediately mounted her, grasping her

pronotum or elytra with his forelegs, and apparently biting or palpating her tergum. All of the females were receptive to mating. The male then bent his abdomen to connect with the female's genitalia, and extracted the ovipositor by extending his hind legs, raising the abdomen, and pulling back. During copulation, females generally were immobile, or walked slowly for short distances. Average copulation time in the laboratory was 21.1 ± 13 sec ($N = 10$). After withdrawing his aedeagus, the male remained astride the female, grasping her dorsum with his forelegs. He remained in this position as the female walked, a behavior consistent with mate guarding (Thornhill and Alcock, 1983). On larval hosts, females probed the bark with their ovipositors. Pairs mated repeatedly.

Role of Contact Chemoreception. All 30 *X. colonus* males attempted to mate with freshly killed females ($N = 10$) in a Petri dish arena but did not respond to the same females after they had been washed in hexane, suggesting that a contact pheromone had been removed by the solvent and that recognition was not cued solely by mechanoreception. However, 23 of 30 males attempted to mate with dead-washed extract-treated females ($\chi^2 = 25.8$, $P < 0.001$), and each showed the full progression of mating behaviors: stopped walking, aligned his body with the female, mounted, and bent abdomen to connect genitalia. Mating behavior was elicited by 0.1 FE of extract in 57% of these beetles, and 0.2 FE in the remaining 43%. After attempting to mate for a few seconds, males abandoned the dead-washed extract-treated females. These findings are further evidence that chemical cues play an essential role in mate recognition.

Identification of Female Contact Pheromone. Gas chromatograms of cuticular extracts of female and male *X. colonus* showed consistent differences (Figure 1, Table 1). Peak number 2 (Figure 1, Table 1), which was absent in males, was identified as 9-methylpentacosane based on its retention time of about 27.3 equivalent chain lengths (ECL) and diagnostic ions at m/z 140 and m/z 252/253, which arise from cleavage on either side of the methyl branching group (Figure 2, top). A small amount of 11-methylpentacosane was also present in this peak as indicated by ions at m/z 168/169 and m/z 224/225. Peak number 4 (Figure 1, Table 1), also absent in males, was identified as 3-methylpentacosane based on its ECL of about 27.7 and diagnostic ions of M-29 at m/z 337 and M-57 at m/z 309 (Figure 2, bottom). Spectra for other hydrocarbon components were interpreted in a similar manner (Table 1). Males shared many hydrocarbons with females, but differed primarily in having greater amounts of the 11-, 13-, 15-MeC_{25} (peak 16, Figure 1, Table 1). We focused on the three early-eluting peaks that were present in females and in small amounts or absent in males, *n*-C_{25}, 9-MeC_{25}, and 3-MeC_{25}, assuming males used female-specific compounds to distinguish between the sexes. We omitted 11-MeC_{25} because it was present only in trace amounts in female extracts. Comparison of peak areas with an internal standard showed that the concentration of each of the three compounds was $\sim 2.5 \times 10^{-3}$ mg/μl. Identification of these

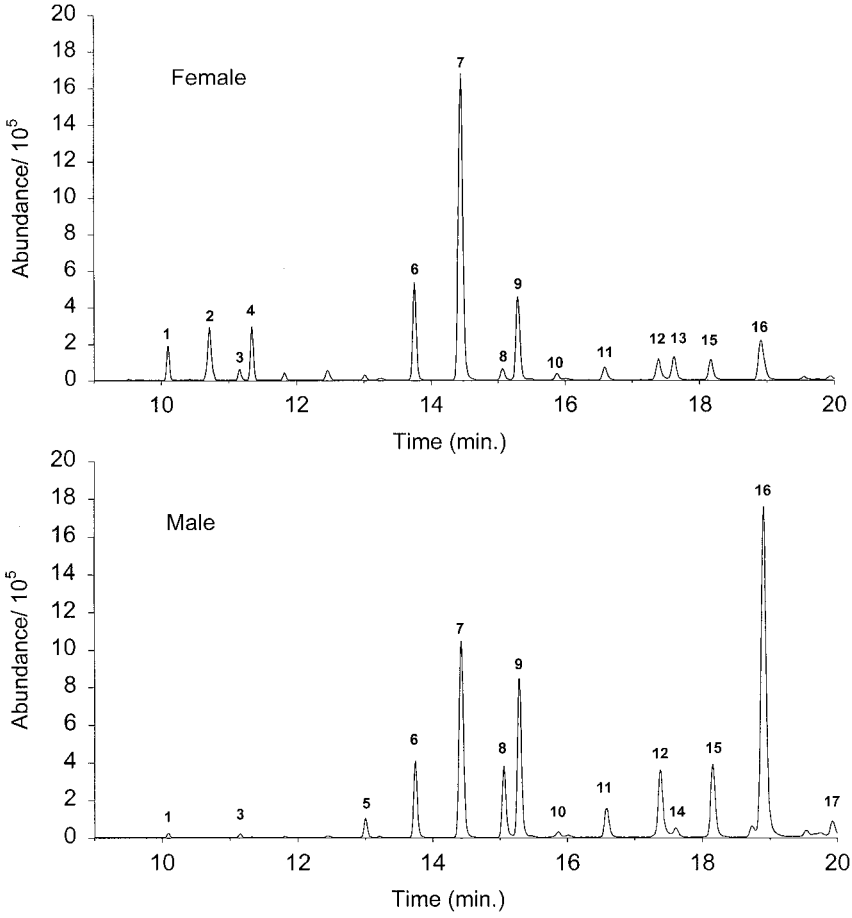

FIG. 1. Representative gas chromatograms of hexane extracts of a *Xylotrechus colonus* female (top) and male (bottom).

compounds was confirmed by comparing retention times and mass spectra with standards.

Testing Activity of Isolated Compounds. In bioassays, males showed a weak response only to *n*-C_{25} when pure compounds were added individually to dead-washed hydrocarbon-treated females (Table 2). Pairwise combinations of 1 FE of each compound did not elicit a response by males (Table 2). However, males responded to dead-washed hydrocarbon-treated females that received the blend containing 1 FE of all three components in 1 ml of hexane, showing the full sequence of mating behaviors (Table 2).

TABLE 1. CUTICULAR HYDROCARBONS OF FEMALE AND MALE *Xylotrechus colonus*[a]

Peak	Hydrocarbon	Female	Male	Diagnostic ions
1	*n*-C_{25}	+	+	352 (M^+)
2	9-MeC_{25}	+	−	140, 252/253, 366 (M^+)
2	11-MeC_{25} (trace)	+	−	168/169, 224/225
3	2-MeC_{25}	+	+	323, 351, 366 (M^+)
4	3-MeC_{25}	+	−	309, 337, 366 (M^+)
5	3-MeC_{26}	−	+	337, 365, 380 (M^+)
6	*n*-C_{27}	+	+	380 (M^+)
7	11,13-MeC_{27}	+	+	168/169, 196/197, 224/225, 252/253 (M^+)
8	2-MeC_{27}	+	+	351, 279, 394 (M^+)
9	3-MeC_{27}	+	+	337, 365, 394 (M^+)
10	*n*-C_{28}	+	+	394 (M^+)
11	13-MeC_{28}	+	+	196/197, 238/239
11	12, 11-MeC_{28} (trace)	+	+	168/169, 182/183, 252/253, 266/267
12	C_{29}:1	+	+	406 (M^+)
13	C_{29}:1	+	−	406 (M^+)
14	3-MeC_{28}	−	+	351, 379, 408 (M^+)
15	*n*-C_{29}	+	+	408 (M^+)
16	11, 13, 15-MeC_{29}	+	+	168/169, 196/197, 224/225, 252/253
17	C_{31}:1	−	+	434 (M^+)

[a] Peak numbers correspond with those in Figure 1; "+" indicates compound is present and "−" indicates it is absent. 11-MeC_{25} and 12, 11-MeC_{28} coeluted in trace amounts with other compounds. Peaks 12 and 13 represent isomers of the same alkene.

DISCUSSION

Adult *X. colonus* of both sexes are attracted by volatiles emanating from cut hickory logs in olfactometer bioassays (Ginzel, 1999), suggesting that mate location is mediated by plant volatiles, rather than long-range sex pheromones. This reliance on plant volatiles for mate location has also been observed in other cerambycid species whose larvae require stressed or dying hosts, including *Hoplocerambyx spinicornis* (New.) (Beeson and Bhatia, 1939), *Monochamus alternatus* Hope (Ikeda et al., 1980; Okamoto, 1984), and *Phoracantha semipunctata* F. (Hanks et al., 1996, 1998). Males of these species use their antennae to locate females on the larval host, and the elongate and filamentous structure of their antennae appear well suited to this purpose (Hanks et al., 1996; Hanks, 1999).

Male *X. colonus* searched for females by running on the bark surface with their antennae outstretched and only responded to a female after contacting her with their antennae. Our bioassays demonstrated that the contact pheromone is comprised of three compounds: *n*-pentacosane, 9-methylpentacosane, and 3-methylpentacosane. Males only responded to the complete blend of these compounds, and the blend elicited the same mating behaviors that males exhibited in response to crude extract, confirming that no other compounds were necessary for

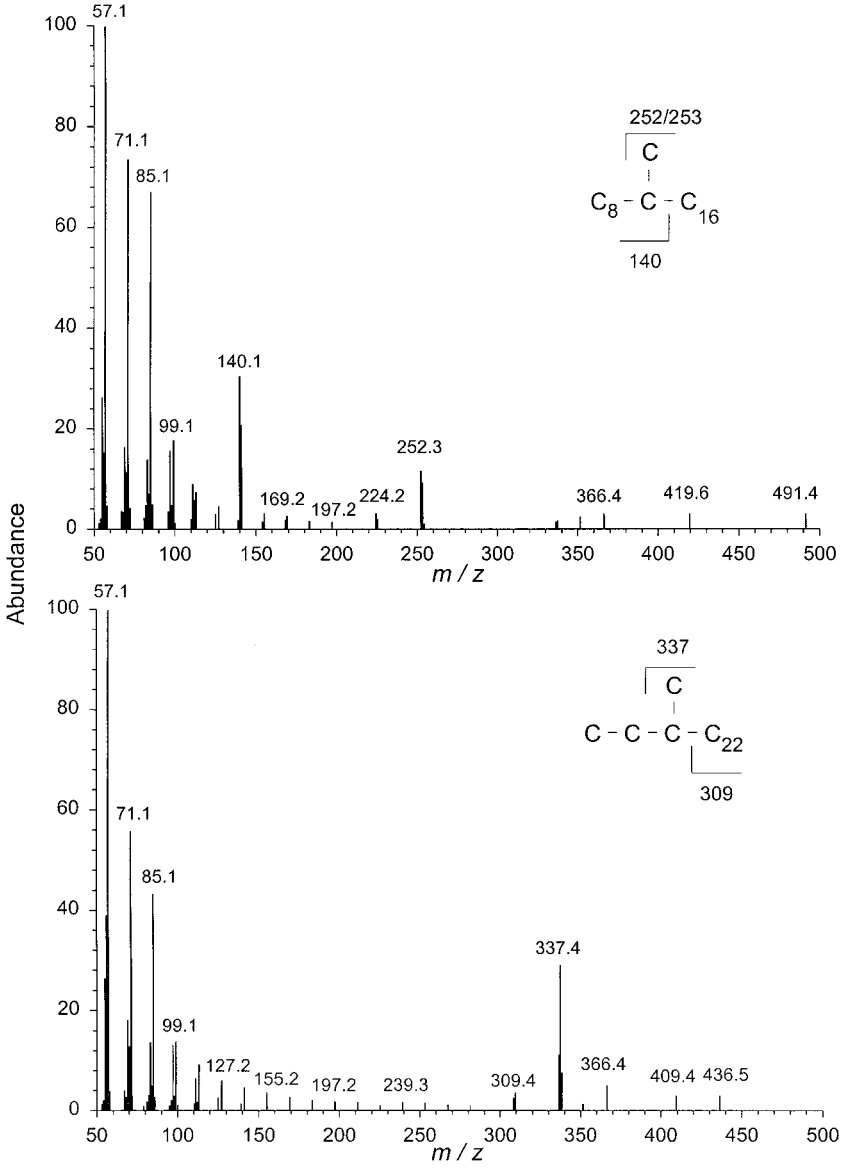

FIG. 2. EI mass spectra of 9-methylpentacosane (top) and 3-methylpentacosane (bottom), showing origins of major fragments.

TABLE 2. RESPONSE OF MALE *Xylotrechus colonus* TO SOLVENT-WASHED DEAD FEMALES (CONTROL) AND THE SAME FEMALES TREATED WITH STANDARDS OF SUSPECTED CONTACT PHEROMONES

Compound[a]	Males (N)	Response to washed females[b] (%)	Response to compound(s) (%)	No response to compound(s) (%)	(χ^2 statistic (P)[c]	Behavior of responding males
nC$_{25}$	24	0	21	79	5.6 (<0.05)	stopped walking
9-MeC$_{25}$	24	0	8.3	91.7	2.1 (>0.1)	stopped walking
3-MeC$_{25}$	21	0	4.8	95.2	1.0 (>0.1)	stopped walking
nC$_{25}$ + 9-MeC$_{25}$	19	0	11	89	2.1 (>0.1)	stopped walking
nC$_{25}$ + 3-MeC$_{25}$	18	0	11	89	2.1 (>0.1)	stopped walking
9-MeC$_{25}$ + 3-MeC$_{25}$	16	0	0	100	0 (1)	none
nC$_{25}$ + 9-MeC$_{25}$ + 3-MeC$_{25}$	24	0	71	29	26.3 ($P < 0.001$)	stopped walking, aligned body with female, mounted, bent abdomen

[a] Single compounds were tested at a dose of 1 FE/ 1 ml hexane and mixtures were tested at a concentration of 1 FE of each component in 1 ml hexane.
[b] See Methods and Materials for definition of "response" and "no response."
[c] Percentages of males responding to compounds were compared to their response to solvent-washed females using the χ^2 test.

mate recognition. The lack of response of males to dead-washed females rules out the exclusive use of mechanoreception in mate recognition, but it may work in concert with chemoreception.

Such hydrocarbon components of the lipid layer on the cuticle are involved in mate recognition in many insect species (Howard and Blomquist, 1982; Nelson, 1993), and may be saturated or unsaturated, terminally branched monomethyl alkanes, or internally branched monomethyl-, dimethyl-, and trimethylalkanes (Lockey, 1980), and tetramethylalkanes (Nelson et al. 1988). Straight chain alkanes, particularly those of odd carbon number, are ubiquitous among insects (Lockey, 1980), including *n*-pentacosane, *n*-heptacosane, and *n*-nonacosane, which were present on the cuticle of male and female *X. colonus*. The structural variability of these hydrocarbons allows for the diversity of pheromones necessary for species specificity, and their chemical stability and low volatility make them suitable as contact pheromones (Dani et al., 2001).

Although the insect cuticle often contains a complex mixture of hydrocarbons, only a few may comprise the contact pheromone of a species (Howard, 1993). Active compounds are sometimes present in both sexes, but may be more abundant on the cuticle of one sex. For example, (*Z*)-14-nonacosene, (*Z*)-13-nonacosene, and (*Z*)-13-heptacosene are present on both sexes of the fly *Musca autumnalis* De Geer, but more abundant on females for which they serve as the sex pheromone (Uebel et al., 1975). Similarly, both sexes of *X. colonus* have *n*-pentacosane, but it was far more abundant on females than on males. In general, hydrocarbon chain length was associated with beetle sex to some degree: female *X. colonus* had greater amounts of the C_{25} to C_{27} methylalkanes, while males had greater amounts of the C_{27} to C_{29} methylalkanes (Figure 1, Table 1). In fact, males had longer chain analogs of two of the female-specific compounds that comprised the contact pheromone: *n*-C_{25} and 3-MeC_{25} (Table 1). Sex-based differences in alkyl chain length also occur in the tsetse fly, but females have greater amounts of longer chain methylalkanes than do males (Nelson and Carlson, 1986).

The role of contact pheromones in cerambycids apparently was first suggested by Heintz (1925), who reported that males of some flower-visiting species responded to females only after contacting them with their antennae and that antennectomized males could not find mates. Males of other cerambycid species show behaviors that suggest they use contact pheromones. For example, male *M. alternatus* attempt to copulate with glass rods treated with a solvent extract of females (Kim et al., 1992). Male *Anoplophora malasiaca* (Thomson) show a similar response to female extract on glass rods, and cuticular hydrocarbons apparently are constituents of the contact pheromones (Fukaya et al, 2000). Male *Psacothea hilaris* (Pasc.) respond to gelatin capsules treated with extract of the female cuticle that contains the primary component (*Z*)-21-methyl-8-pentatriacontene (Fukaya et al., 1996, 1997). The structure of that pheromone is similar to the three compounds that male *X. colonus* responded to in our study.

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CONTACT CHEMOSENSORY CUES IN EGG BUNDLES ELICIT MALE–MALE AGONISTIC CONFLICTS IN THE SQUID *Loligo pealeii*

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Abstract—Male *Loligo pealeii* engage in frequent agonistic bouts to gain access to female mates while aggregated at communal egg beds. Male squids are attracted to eggs in the field and in the laboratory. It was recently demonstrated that visual detection followed by physical contact with egg capsules elicited male–male aggression. We tested specific physical and chemical features of the egg capsules that may cause this strong behavioral reaction. Male squids were presented with either natural or artificial egg stimuli and scored for four selected behaviors (egg touch, egg blowing, forward-lunge grab, and fin-beating), the last two of which are highly aggressive behaviors. First, squids were presented with natural eggs versus eggs sealed in agarose-coated tubes (ESACT), which eliminated both tactile and chemical stimuli. Second, males were presented with natural eggs versus eggs sealed in agarose coated tubes containing C_{18} Sep-Pak-purified extracts (TCPE) from squid egg capsules, which provided chemical cues from natural eggs without the physical stimulus of the egg capsules. Third, natural eggs versus heat-denatured eggs were tested to determine whether the active factor in natural eggs is heat-labile. Squids responded aggressively when contacting natural eggs and TCPE, whereas squids did not respond after touching ESACT or denatured eggs. These results suggest that aggressive behavior is elicited by a heat-labile factor that is embedded within squid egg capsules. This chemosensory cue appears to be a contact pheromone that stimulates the

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agonistic interactions that characterize the mating behavior of migratory squids on inshore spawning grounds.

Key Words—*Loligo pealeii*, squid, chemosensory, pheromone, agonistic behavior, mating behavior, aggression.

INTRODUCTION

Agonistic interactions are costly because of their high-energy expenditure and increased risk of predation (Huntingford and Turner, 1987). Therefore, animals will only engage in such behaviors when the costs are outweighed by some benefit, such as mate acquisition (Archer, 1988). Males of many taxa engage in agonistic encounters to gain access to females [e.g., arthropods (Berrill and Arsenault, 1984); avians, (Hirschenhauser et al., 2000; Johnsen et al., 2001) and lepidopterans, (Kemp and Wiklund, 2001)]. Male loliginid squids engage in such agonistic conflicts when competing for access to females at spawning sites (Hanlon and Messenger, 1996).

Loligo pealeii is a highly mobile species that occurs along the eastern coast of the Americas from Nova Scotia to the Gulf of Venezuela (Summers, 1983). Squids spend the winter months along the edge of the continental shelf in canyons where temperature fluctuations are minimal, and migrate inshore to warmer waters in the spring (Summers, 1983; Black et al., 1987). Inshore, mating is known to occur in aggregations where squids lay their eggs communally, often forming masses of egg capsules (Hanlon, 1998). There is a skewed operational sex ratio at the egg beds (Hanlon, 1998) (2.6 males to 1 female, unpublished data), and males must compete for valuable consortships with females. In this context, agonistic conflicts may influence the reproductive success of individual males.

Cephalopods are useful for studying agonistic behavior because they use distinct, conspicuous body patterning signals for communication during escalating agonistic encounters (Hanlon and Messenger, 1996). In addition, their polygamous mating systems create ample opportunities for agonistic bouts. However, there have been few studies of male–male agonistic behavior in cephalopods (Adamo and Hanlon, 1996; DiMarco and Hanlon, 1997; King et al., in press). DiMarco and Hanlon (1997) found that the presence of a female increased the level of aggression in agonistic conflicts in male *Loligo plei*. Recent work with *L. pealeii* has shown that males engage in frequent agonistic bouts and that egg capsules elicit such aggressive behavior even in the absence of females (King et al., in press).

Male squids are attracted to egg capsules both in the field and laboratory (Arnold, 1962; Hanlon, 1996; King et al., in press). Squids swim up to the eggs with their arms in a cone shape. Male squids touch the egg capsules with their arms, and often manipulate them by spreading their arms over a large number of

egg capsules and agitate the eggs with rapid movements. Males will also often "blow on the eggs" by forcing water through their funnel over the eggs, causing them to move gently in the water (unpublished data). King et al. (in press) found that this egg-manipulating behavior elicits male–male aggression in the laboratory. In a series of experiments designed to determine the sensory stimulus necessary to elicit this behavior (i.e., visual, tactile, or chemosensory), they found that vision was important for attraction, but that squids needed to touch the egg capsules to become aggressive. Their results indicated that tactile and/or chemosensory cues may be responsible for the induction of aggressive behavior.

In this paper, we attempt to determine specifically which sensory cue(s) in egg capsules elicits male–male aggression. We designed a series of experiments to isolate various sensory aspects to ascertain whether chemosensory and/or tactile input elicit aggressive behavior in male squids.

In the marine mollusk *Aplysia*, several peptide pheromones that induce mating behaviors have been characterized from egg cordons (Painter et al., 1998, 1999). This prompted us to consider the possibility that similar factors in or on egg capsules may be responsible for the male–male aggression in *L. pealeii*. Therefore, we tested whether contact with C_{18} Sep-Pak-purified extracts from whole-egg capsules would stimulate male–male aggressive behavior in squids.

METHODS AND MATERIALS

Animal Collection and Care. Squids were caught from Vineyard Sound (Falmouth, Massachusetts, USA), with trawls or jigs, during the months of April through September 2001. Trawled individuals were maintained in oval tanks (360 cm long × 240 cm wide × 90 cm deep) with flow-through natural seawater (NSW) at 12°C (salinity ranged from 30 to 32 ppt). Jigged individuals were maintained in a round tank (366 cm diam. × 91 cm deep) with flow-through NSW at 16–18°C. The tanks were exposed to the ambient light cycle (ca. 14 L: 10 D). Individuals were fed 2–4 fish per day in the evening. Healthy animals, with little or no skin damage, were selected for experiments and placed in holding tanks 12 hr prior to behavioral trials.

Experimental Design for Behavioral Trials. Two behaviors—fin-beating and forward-lunge grab (Figure 1)—were used to assess the level of aggression because they were conspicuous, easy to score, and reliable between observers. Both are highly aggressive behaviors; therefore, we were measuring the strongest level of aggression. Fin-beating occurred when two males swam parallel to each other and maneuvered to beat one another's fins (Figure 1A). Fin-beating was generally preceded by some postural and behavioral patterns that we did not score here. The forward-lunge grab was a fast, forward movement in which one male lunged toward another male with arms flared (Figure 1B); in rare cases, this was followed

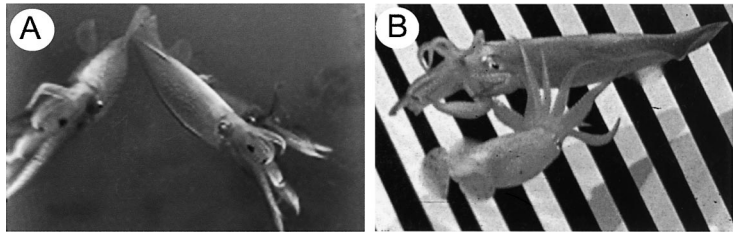

FIG. 1. (A) Two squids engaged in fin-beating. This is an aggressive behavior in which male squids swim parallel to each other and beat one another's fins. (B) A typical forward-lunge grab, the highest level of aggressive behavior. The male in front is lunging with arms flared toward the male in the back. The black and white stripes in the background are painted on the walls of the tank to discourage squids from swimming into the side. Video clips of these highly aggressive behaviors can been viewed at *http://www.mbl.edu/services/MRC/hanlon.html*.

by biting or grappling. Two other behaviors—egg blowing and egg touch—were used to determine the squid's level of interest in the eggs. Egg blowing occurred when squids swam up to an egg bundle and jetted water into it; egg touch occurred when the arms contacted the eggs.

Behavioral trials were conducted in round tanks (3 m diam. $\times$ 0.5 m deep) with aerated, flow-through NSW. Temperature was maintained at 16–18°C because previous observations indicated that temperatures above 22°C were associated with a decrease in squid responsiveness to egg capsules (King et al., in press).

Pairs of male squids were selected whose mantle lengths differed by fewer than 2 cm (population range: 12.0–30.0 cm). Individual squids were isolated in tanks for at least 12 hr prior to the trial, one in the trial tank (the resident squid) and another in a smaller holding tank (132 cm long $\times$ 76 cm wide $\times$ 43 cm deep; intruder) due to space constraints. Trials were conducted with the following protocol (Figure 2A). To begin the trial, the intruder squid and an egg stimulus were added to the resident squid's tank. Data collection began when one of the squids touched the egg stimulus, and the four behaviors were scored for a period of 20 min. Observers were visible to the animals but care was taken to minimize movement. After 20 min, the intruder squid was removed from the trial tank and placed back into the small holding tank; the stimulus was also removed. Squids remained in their individual holding tanks for 4 hr before the second half of the trial began. The second half of the trial was conducted as described above but with a different stimulus (e.g., if natural eggs were used as a stimulus in the first part of the trial, eggs sealed in agarose-coated tubes were used in the second part of the trial). Treatment order was randomized to minimize effects of the time of day since there may be a circadian rhythm to these agonistic behaviors.

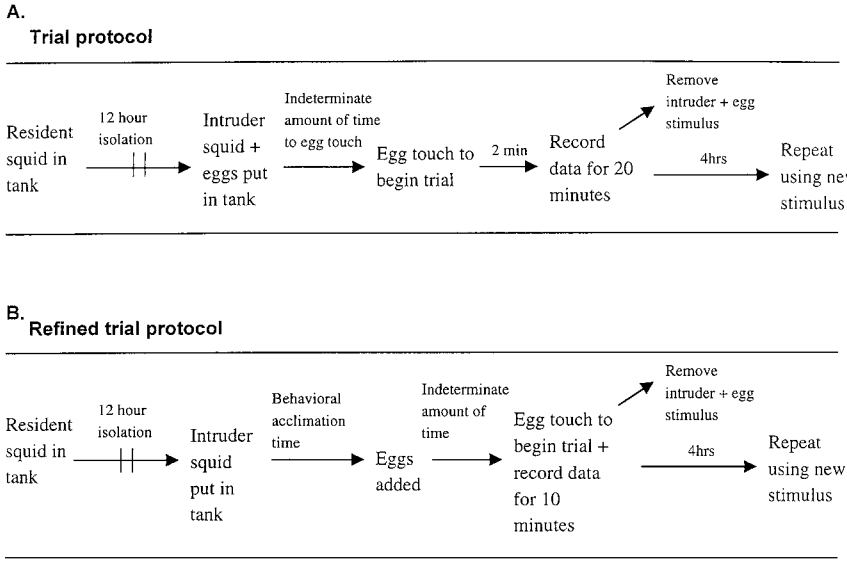

FIG. 2. Protocol A was used in May. The protocol was changed to the one shown in B because of what was learned with the previous protocol; it was used June–September.

The trial protocol was refined (Figure 2B) based on results at the beginning of the study; most trials were conducted using the refined protocol ($N = 50$ of 74). Two differences were noteworthy. First, the egg stimulus and the intruder squid were not added simultaneously. The intruder was added prior to the egg stimulus, and squids were monitored until their behavior reached baseline (i.e., no overt aggression). An egg stimulus was then added to the tank. Second, data were recorded for only 10 min after the first egg touch. To correct for differences in observation time in the two protocols, the data for egg touch, egg blowing, forward-lunge grab, and fin-beating collected from the original trial protocol were divided by two. We found that most of the agonistic behaviors occurred in the first 10 min of data collection; therefore, this should be a conservative estimate.

C_{18} Sep-Pak Purification of Egg Capsule Extracts. Squids were induced to mate and lay eggs by placing day-old eggs into a tank in which they had been isolated for 12 hr. Freshly-laid eggs were collected from several females, frozen on dry ice, and stored at –80°C until extraction. Samples from each egg were individually extracted at 4°C in 20 ml of 0.1% heptafluorobutyric acid (HFBA) using a Brinkmann Polytron homogenizer, and sonicated. Each extract was centrifuged at 48,000*g* for 20 min at 4°C, and the supernatants were purified on separate C_{18} Sep-Pak cartridges (Waters Associates, Milford, Massachusetts, USA); Sep-Paks were pretreated with 3 ml of 100% acetonitrile (CH_3CN) containing 0.1% HFBA

and rinsed with 20 ml of 0.1% HFBA. The bound sample was eluted with 5 ml of 50% CH_3CN containing 0.1% HFBA, divided into 1 ml aliquots, and lyophilized in siliconized 1.5 ml microfuge tubes.

The eluted sample was resuspended in 1.0 ml of Tris-buffered (20 mM, pH 8.0) artificial seawater. Resuspended samples were added to the agarose slurry after it cooled to 39°C so that heat-sensitive factors would not be denatured. Each test tube was coated with agarose containing the extracts from 1/6 of a squid egg capsule, meaning that each artificial egg bundle contained extracts from one egg capsule.

Statistical Analyses. All treatments were compared with natural egg treatments using a Wilcoxon's signed rank test (Sokal and Rohlf, 1995). We anticipated substantial variation in squid responsiveness; therefore, we designed the experiments to use paired analyses. In the beginning of the study, we did not have enough space to hold many animals. Thus, we were restricted to two treatments per animal. This experimental setup did not facilitate direct comparisons between experiments; differences can only be inferred.

RESULTS

Squid Responsiveness. There was considerable individual variation in squid responsiveness to the presence of natural egg capsules. Some individuals did not attempt to touch the eggs (4.0%), while others touched but did not exhibit aggressive behavior (16.2%). Data (Figure 3) were only analyzed from trials in which squids touched the natural egg stimulus. Fin-beating and egg blowing did not occur with enough frequency to compare between treatments.

Natural Eggs versus ESACT. Do male squids exhibit as many agonistic behaviors when exposed to eggs sealed in agarose-coated tubes (ESACT), which have no scent, as when exposed to natural eggs that have a scent ($N = 23$)? This experiment was designed to remove all chemosensory aspects of the egg capsules while still allowing the squids to have the visual stimulus of an egg bundle. A natural egg bundle consisting of 16–20 egg capsules was used as a behavioral control. Egg capsules were attached to an airstone to weight them to the bottom of the tank. To create an artificial egg bundle, 16 egg capsules were placed in 5 ml test tubes that were sealed with rubber stoppers or corks. The "egg bundle" was tied together with fishing line, and a weight was added to allow the tubes to easily rest on the bottom of the tank. The outsides of six of the test tubes were coated with 5% low-melting-point agarose (Sigma) to replicate the number of coated capsules that were embedded with C_{18} Sep-Pak-purified extracts (below).

Squids responded to the visual stimulus of ESACT by approaching the tubes and touching them. Some individuals repeatedly touched the tubes, while others touched only once. This behavior paralleled the response to natural egg

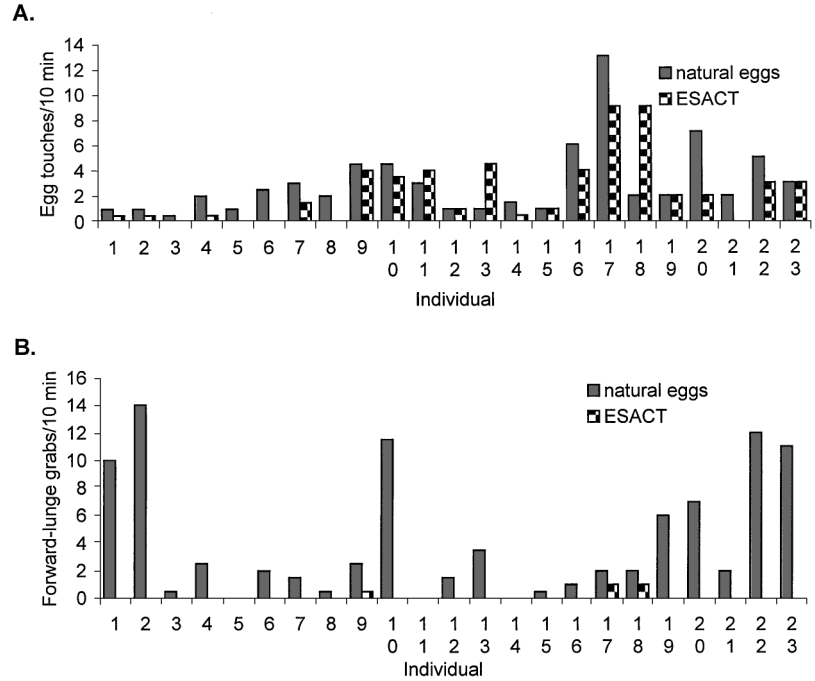

FIG. 3. Chemosensory and/or tactile cues from eggs increase the number of agonistic behaviors exhibited by individual male *Loligo*. The number of egg touches (A) was not significantly different in response to natural eggs and ESACT ($N = 23$, $P = 0.03$). The number of forward-lunge grabs (B) per 10-min period was significantly higher in individual males when exposed to natural eggs than when exposed to eggs in sealed agarose-coated tubes (ESACT; $N = 23$, $P < 0.001$).

bundles—instances of egg touch were similar between treatments (Figure 3A; Wilcoxon's sign rank test $T = 41.5$, $P = 0.03$). There was a dramatic difference in the agonistic response to natural eggs versus ESACT; squids responded much more aggressively (greater number of forward-lunge grabs) after touching natural eggs (Figure 3B; Wilcoxon's signed rank test $T = 0.00$, $P < 0.001$).

Natural Eggs versus TCPE. Do male squids exhibit as many agonistic behaviors when exposed to eggs sealed in agarose-coated tubes containing C_{18} Sep-Pak-purified extracts from squid egg capsules (TCPE), which possess the scent but not the mechanosensory qualities of natural eggs, as when exposed to natural eggs ($N = 35$)? This experiment was designed to determine if these purified extracts would elicit an agonistic response comparable to natural eggs. An artificial egg bundle was embedded with the purified extract from natural egg capsules. A natural egg bundle was used as a behavioral control as above.

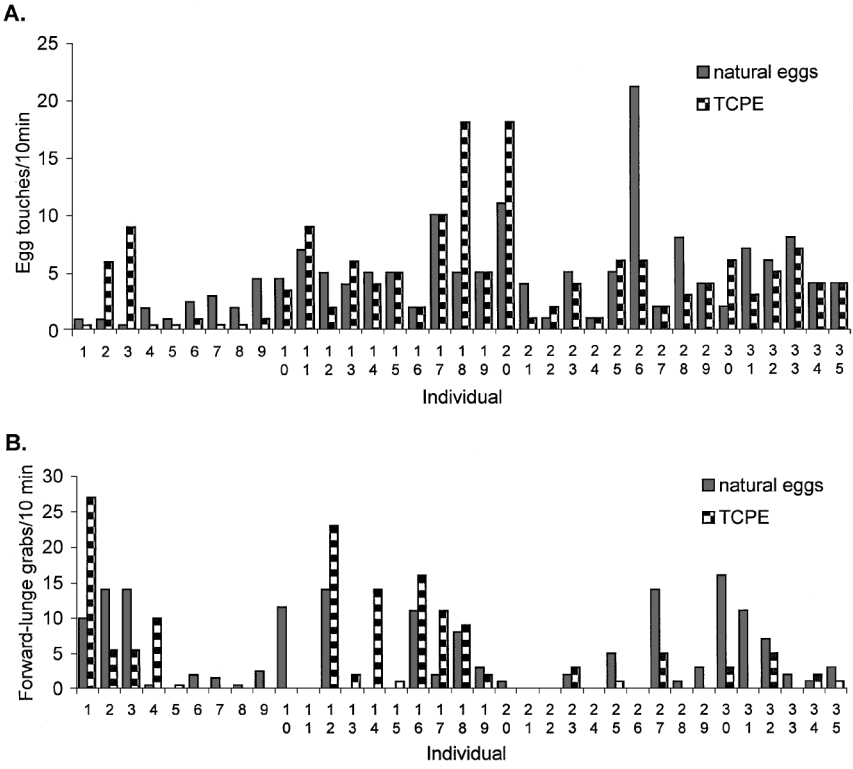

FIG. 4. Individual male *Loligo* exhibit a similar number of agonistic behaviors following physical contact with either eggs or with a C_{18} Sep-Pak-purified extract of eggs. The number of egg touches (A) was not significantly different in response to natural eggs and TCPE ($N = 35$, $P = 0.55$). The number of forward-lunge grabs (B) per 10-min period did not differ significantly in individual males that have come in physical contact with either natural eggs or TCPE ($N = 35$, $P = 0.35$).

Squids responded to the TCPE by approaching the artificial egg bundle and touching the outside of the tubes. In this experiment, they touched the TCPE with the same frequency as they touched natural eggs (Figure 4A; $T = 152.0$, $P = 0.55$). Touching TCPE also elicited an aggressive response (forward-lunge grabs) similar to responses to natural eggs (Figure 4B; $T = 187.5$, $P = 0.35$).

Natural Eggs versus Denatured Eggs. Could the chemical component be a peptide or a protein, as indicated by a loss of activity following heat-denaturation ($N = 8$) or treatment with trypsin ($N = 8$)? This experiment was designed to determine whether activity could be destroyed by heat or by treatment with trypsin. A natural egg bundle was used as a control. An egg bundle containing 16–20 egg

capsules was treated in one of two ways: (1) Egg capsules were heated to 70–100°C for 30–45 min (egg capsules remained intact after this treatment); or (2) egg capsules were incubated in a seawater–trypsin solution (100 μg/ml) at 37°C for 2 hr.

Squids responded to eggs that had been either heat-denatured or trypsin-treated by approaching the eggs and manipulating them with their arms as they would with natural eggs. The frequency of egg touching was the same among heat-denatured, trypsin-treated, and natural eggs (Wilcoxon's signed rank test, Figure 5A; heat $T = 17.5$, $P = 0.94$; Figure 6A; trypsin $T = 6.50$, $P = 0.20$). Heat-denaturing the eggs effectively eliminated or reduced the agonistic response that was present with natural eggs (Figure 5B; Wilcoxon's signed rank test

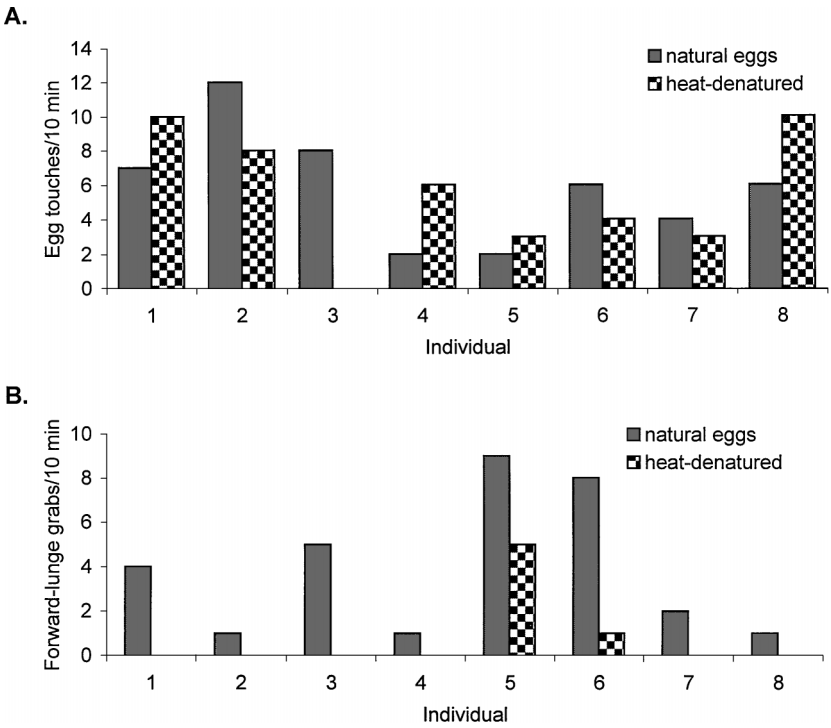

FIG. 5. The chemical that makes animals exhibit more agonistic behaviors is heat-labile. Approximately the same number of egg touches occur (A) per 10-min period when male *Loligo* make contact with either heat-denatured eggs or natural eggs ($N = 8$, $P = 0.94$). When male *Loligo* come into contact with heat-denatured eggs, they exhibit significantly fewer forward-lunge grabs (B) per 10-min period than when they contact natural eggs ($N = 8$, $P = 0.01$).

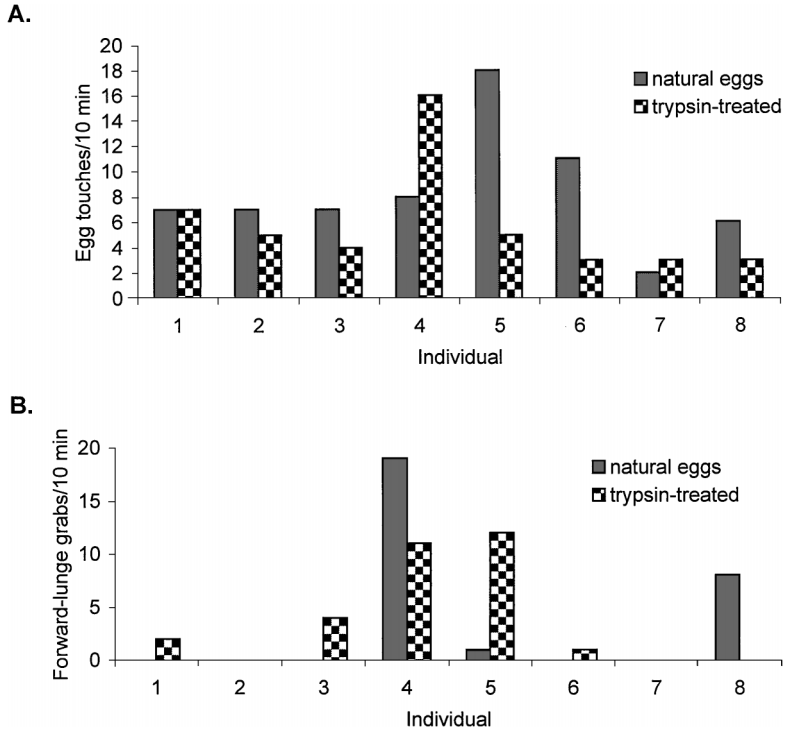

FIG. 6. Enzymatic digestion of eggs with trypsin does not reduce the frequency of agonistic behavior of male squid relative to natural eggs. Approximately the same number of egg touches occur (A) per 10-min period when male *Loligo* make contact with either trypsin-treated eggs or natural eggs ($N = 8$, $P = 0.24$). When male *Loligo* come into contact with trypsin-treated eggs, they exhibit approximately the same number of forward-lunge grabs (B) per 10-min period as they do when they contact natural eggs ($N = 8$, $P = 0.75$).

$T = 0.0$, $P = 0.01$); however, trypsin-treated eggs still elicited an aggressive response (forward-lunge grabs) similar to natural eggs (Figure 6B; Wilcoxon's signed rank test $T = 9.0$, $P = 0.75$).

DISCUSSION

Our data support the hypothesis that contact chemoreception is responsible for increased agonistic behavior. First, we showed that squids are visually stimulated to touch an artificial egg bundle but that contacting the artificial bundle does not elicit an aggressive response. Next, we showed that contact with an artificial egg bundle containing C_{18} Sep-Pak-purified extracts from squid egg capsules

elicits male–male agonistic interactions comparable to natural eggs. Finally, we demonstrated that heat denaturation of natural egg capsules greatly reduces the aggressive response. Thus, we conclude that squid egg capsules contain a heat-labile factor that functions as a contact pheromone, which in turn induces male–male aggressive behavior.

The presence of either natural eggs, ESACT, TCPE, heat-denatured, or trypsin-treated eggs stimulated squids to swim up to the egg capsules and touch them, indicating that squids were attracted visually to all treatment types. However, only treatments with intact chemosensory stimuli caused squids to become aggressive after touching the eggs or artificial egg bundles, thus revealing that chemosensory stimuli are responsible for the increased levels of agonistic behavior.

Squid Responsiveness. In contrast to the results of King et al. (in press), the squids in our experiments were not always responsive to egg capsules; i.e., they were not always attracted to them visually and did not always respond aggressively when they did make contact with the egg capsules. We are uncertain of the reasons for this. It is possible that differences resulted from unmeasured environmental effects that differed between two seasons (1999 vs. 2001) when the experiments were conducted. Nonetheless, most squids in our experiments (80%) that responded to natural eggs (by swimming up to them and manipulating them in the characteristic manner) displayed aggressive behavior.

Visual Stimuli. The experiment testing natural eggs versus ESACT illustrated the importance of visual and chemosensory stimuli in squid responsiveness to egg capsules. Squids were attracted to ESACT; however, this stimulus alone was not sufficient to cause an escalation of aggressive behavior similar to natural eggs. As expected, vision played an important role. Since placing eggs in sealed tubes eliminated any chemosensory stimuli from the artificial egg bundle, other sensory stimuli must be responsible for inducing the aggressive behavior that resulted from touching the eggs.

Chemical Stimuli. The experiment testing natural eggs versus TCPE demonstrated the importance of chemosensory stimuli to elicit aggressive interactions among males. This experiment removed chemical stimuli from the eggs (placing them in sealed tubes) but artificially restored their chemical properties by applying C_{18} Sep-Pak-purified extracts from eggs to the outside of the tubes. Squids responded similarly to natural eggs and TCPE, indicating that the agonistic response was elicited by chemical cues. However, this design did not remove the tactile stimulus entirely because squids still "touched" eggs, although they were unable to manipulate the TCPE in the same manner as natural eggs, and the TCPE did not have the same mechanosensory qualities as natural eggs.

The experiment testing real eggs versus heat-denatured or trypsin-treated eggs revealed that removing the chemosensory stimulus eliminates the aggressive response. Squids responded to the visual stimulus of heat-denatured eggs by approaching and touching them as they would natural eggs. Touching and

manipulating heat-denatured eggs, however, failed to elicit an aggressive response. This clearly indicated that it was a chemical stimulus—not physical—that was responsible for the increase in male–male aggression and that it was a heat-labile factor. We further tested this hypothesis in a subset of trials. We tested the ability of trypsin to destroy the activity responsible for increased aggression. Interestingly, the trypsin-treated eggs failed to eliminate an agonistic response, and squids responded as they would to natural eggs. This result may be due to the specificity of trypsin, which cleaves peptide bonds at arginine and lysine residues (Walsh, 1970) and may have left the active site(s) intact. It is possible that the pheromone(s) does not have these residues or does not have them in a position accessible to the enzyme.

Chemical Stimuli and Mating Behavior. Chemosensory stimuli operate in several taxa to elicit different types of mating behaviors. Several species of copepods use both water-borne and contact chemical cues for mate recognition (Lonsdale et al., 1998). In a study with a design similar to ours, the mating response of the rotifer *Asplanchna sieboldi* was induced by stimulation with agarose spheres impregnated with extracts from females (Joanidopoulos and Marwan, 1999). Recent work has illustrated the presence of a water-borne pheromone, attractin, in the marine mollusk *Aplysia*. It is secreted into the egg cordons when eggs are being prepared for deposition and then elutes into the surrounding seawater. It attracts *Aplysia* to the egg cordons and induces them to mate as males when they arrive (Painter et al., 1998, 1999). There are also contact pheromones on the eggs, which cause other animals to lay eggs (Begnoche et al., 1996). Boal (1997) suggested that female cuttlefish (*Sepia officinalis*) use chemosensory cues to select the most recently mated males as their own mates. Chemosensory cues may be important in *L. pealeii*. The presence of eggs may indicate proximity of receptive females (King et al., in press). The reproductive benefits of mating with and guarding a female would outweigh the cost of male–male agonistic interactions at this time. Chemical cues embedded in egg capsules may be responsible for allowing male–male agonistic interactions to be restricted to a time when receptive females are present. DiMarco and Hanlon (1997) found that the presence of mature females induced fighting behavior in *L. plei* males. They did not test the effect of egg bundles on fighting behavior; however, it is possible that this cue may be present in other loliginids.

We have noticed that the presence of eggs not only elicits male–male agonistic interactions but also stimulates mating behavior between males and females (unpublished observations). These observations suggest that the factor in egg capsules that is responsible for increased male aggression either has multiple effects or that several pheromonal cues are present in squid egg capsules.

This chemical stimulus (or another one embedded in egg capsules) may also act to attract squids to the egg beds, as in *Aplysia*. If the "agonistic factor" acts as an attractant to the egg beds, then it must be a water-borne pheromone rather

than a contact pheromone. More work is necessary to determine whether the agonistic factor is solely a contact pheromone or whether it could also be waterborne.

Potential Sites of Chemoreception. Squids have eight arms and two tentacles, and average adult male *L. pealeii* have an average of 63 suckers on each arm. The site responsible for contact chemoreception may be the chemoreceptors in the suckers or in the buccal mass, since squids, octopuses, and cuttlefish are known to have chemoreceptors in the lips and on the suckers (Emery, 1975; Hanlon and Messenger, 1996). When male squids manipulate egg capsules, they place their heads directly into the egg mass and manipulate the capsules with their arms. Manipulating the eggs with their arms would allow chemoreceptors on the suckers to come into contact with pheromone(s) present on the surface of the egg capsules. Placing their heads into the egg mass may cause receptors in the buccal mass to come into contact with the egg capsules, and hence the pheromone(s) embedded in them. Squids also often blow on the eggs by forcing water over them through their funnel. This may cause pheromone(s) embedded in the egg capsules to be released into the water and come into contact with chemoreceptors in the suckers and/or in the buccal area.

An alternative site for chemoreception may be the olfactory organ located beneath the eye. One report indicates that *Loligo opalescens* can detect water-borne chemicals via this organ (Gilly and Lucero, 1992). Woodhams and Messenger (1974) have suggested that octopus olfactory organs may be sensitive to sexual pheromones from conspecifics.

It is obvious that males would benefit from limiting costly agonistic interactions to a time when receptive females are present. To the best of our knowledge, using chemosensory cues from egg capsules as an indirect signal to elicit agonistic interactions is unique to this cephalopod mating system. Further investigation is necessary to determine the precise mechanism by which this chemosensory cue operates.

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MALE BUGS MODULATE PHEROMONE EMISSION IN RESPONSE TO VIBRATORY SIGNALS FROM CONSPECIFICS

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Abstract—Male *Nezara viridula* emit a volatile sex pheromone that acts as a long-range attractant to females. Both sexes communicate through vibrations once they are on the same plant. Males respond to the female calling song by emitting a male courtship song, and they orient to the female calling song on a plant. Simultaneity between vibratory and chemical communication during the last steps of mate finding suggests that pheromone emission might be modulated by signals from conspecifics. Male volatile emissions were collected with fibers for solid-phase microextraction, while male bugs were stimulated with natural and artificial signals. Percentages of males releasing pheromone and collected amounts of pheromone increased when males were stimulated with female calling song. Pheromone emission was stable in males stimulated with male rivalry songs, and it decreased in males stimulated with a 100-Hz artificial signal. The ability of male bugs to modulate their pheromone emission may reduce metabolic costs, reduce parasitism, and offer a better synchronization of sexual activity.

Key Words—Heteroptera, *Nezara viridula*, southern green stink bug, vibratory communication, sex pheromone, pheromone emission, mating behavior.

INTRODUCTION

Communication is a key component of animal reproduction, and it uses multiple sensory channels, among which olfaction is of major importance. Many insect species use volatile pheromones during sexual interactions, and research has been devoted to understanding chemical, ethological, and physiological aspects of pheromone communication. Because of the molecular nature of the signal, it is generally admitted that chemical communication offers relatively slow kinetics,

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compared to other sensory channels. In a number of species, an emitter releases a sex pheromone that attracts conspecifics from a distance. In these species, the emission of the long-range attractant takes place at the earlier steps of the communication. Environmental cues and physiological state affect emission (McNeil, 1991), but this seems independent from the presence of the receiver. Thus, the females of many moth species exhibit diel periodicity of their sex pheromone production and emission behavior. The pheromonotropic factors determining the biosynthesis of the moth pheromones have received considerable attention, showing that the biosynthetic activity of the pheromone gland is controlled by a pheromone biosynthesis activating neuropeptide (PBAN), whose release depends on photoperiod (Raina, 1997). The main issue in this neuroendocrine control is the synchronization of signal emission by females and receptivity to pheromone in male moths.

The adaptative value of a synchronization between the rhythms of sexual activity in both sexes suggests that some other insect species might have achieved a direct control of pheromone emission by signals from potential receivers. Such modulation could occur when, following long-range attraction, multimodal exchanges of signals take place between sexes. Stink bugs (Heteroptera, Pentatomidae) produce sex pheromones (Aldrich, 1988; McBrien and Millar, 1999), but in spite of the fact that sex pheromones of a number of bugs have been identified, nothing is known of the factors that regulate their emission. These bugs also communicate with substrate-borne vibrations (Čokl et al., 1978, 2001). Thus, two main sensory modalities contribute to mate finding and recognition in the southern green stink bug, *Nezara viridula*. Males emit a sex pheromone that attracts female bugs (Pavis and Malosse, 1986; Borges et al., 1987; Brézot et al., 1994), whereas males and females produce several types of vibrations (Čokl et al., 1978, 2000; Harris et al., 1982). It has been postulated that the sex pheromone works as a long range attractant, while substrate-borne vibrations serve as close-range communication once the bugs are on the same plant (Borges et al., 1987). Males orient to female calling songs (FCS) on plants (Ota and Čokl, 1991; Čokl et al., 1999) and respond to female calling by emitting male calling and courtship songs (Miklas et al., 2001). These songs cause females to continue singing (Kon et al., 1988). Females usually do not sing spontaneously, but start emitting FCS when challenged with filter papers loaded with male odors (Miklas, 2002).

Simultaneity between vibratory and chemical communication during the last steps of mate finding suggests that pheromone emission might be modulated by signals from conspecifics. Contrary to many other insect species, an overt pheromone-emission behavior has not been observed in *N. viridula*. According to its function as a primary sex attractant, one could expect that pheromone emission by the southern green stink bug depends only on the physiological state of males and environmental factors. Sex pheromone emission starts at maturity, about 8–10 days after the adult molts. Pheromone release continues over several days, but mating decreases pheromone emission (Brézot, 1994). Following the observation that

increasing the numbers of males in the laboratory glassware used for headspace collection decreases mean production, it was suggested that pheromone emission is density dependent (Brézot, 1994). The amount of pheromone collected from mature, virgin, and single males is extremely variable among males and from day to day for the same individual (Miklas et al., 2001). However, no evidence for a diel rhythm has been found. This quantitative variation suggests that male *N. viridula* modulate their pheromone emission. We hypothesized that conspecific vibrational songs that are transmitted through the plant could influence sex pheromone emission by males. To test this hypothesis we monitored the amounts of pheromone released by male bugs stimulated with prerecorded vibratory natural songs. This pheromone blend comprises three main components: (*Z*)-α-bisabolene (1-methyl-4-(1,5-dimethyl-(*Z*)-1,4-hexadienyl)-cyclohexyl and its *trans* and *cis* epoxides (*trans* and *cis* isomers of (*Z*)-2-(3′R,4′S)(−)-2-(3′,4′-epoxy-4′-methylcyclohexyl)-6-methylhepta-2,5-diene) (Baker et al., 1987), which were pooled to quantify pheromone production. Male and female songs from two geographically isolated populations were used. To determine the specificity of the vibrations, male bugs were also challenged with an artificial signal.

METHODS AND MATERIALS

Insects. We used sexually mature, virgin males from two populations of *N. viridula* maintained in the laboratory. These populations came from individuals caught in southern France or in Guadeloupe. Males were isolated from females at adult molting and kept in groups of 8–12 in cages (30 × 30 × 30 cm) in a separate room at 22–26°C, 70–80% relative humidity, and 16L:8D daily cycle. They were reared on a diet of bean plants (*Phaseolus vulgaris* L.) and sunflower seeds (*Helianthus annus* L.).

Chemical Collection. To collect pheromone, sexually mature, virgin males were introduced singly with a bean leaf into an 18-ml glass vial with lateral tubes for input and output of the airflow. Air was purified on charcoal, and its flow was adjusted at 25.2 liters/hr. To collect volatiles from males, a fiber for solid-phase microextraction (SPME, blue fiber 65 μm, PDM-DVB, Supelco, Bellefonte, Pennsylvania, USA) was introduced into the output of the vial. Effluvia were adsorbed during 20 min. Fiber contents were analyzed in a Varian 3400 chromatograph on a nonpolar column (25 m × 0.32 mm ID; 0.5-μm film, BPX5) with helium as carrier gas. The temperature program was 100°C for 0.1 min to 300°C at 10°C/min, followed by 10 min at 300°C. Injector and FID temperatures were set at 260°C and 240°C, respectively.

Preliminary experiments were performed to check the reliability of the method. A square sheet of aluminum (3.24 cm^2) loaded with 10 μg of a synthetic blend of the *trans* and *cis* epoxides of (*Z*)-α-bisabolene [a gift from Pr. K. Mori, Tokyo University (Tomioka and Mori, 1992)] was introduced into the set

up. Volatile collection lasted for 20 min. Then, the aluminum sheet was removed from the glass vial, and a clean SPME fiber was used to collect volatiles from the empty vial. Direct injections of bisabolene epoxides into the chromatograph were done for calibration. In these conditions, the amounts of epoxides of bisabolene collected during the first collection, in presence of the aluminum sheet, averaged 220 ± 111 ng ($N = 10$). This value is in agreement with results from other experiments, indicating the low volatility of bisabolene epoxides at ambient temperature. During the second collection (source of bisabolene epoxides removed), we recovered less than 1% of the amounts of epoxides collected during the first collection phase. We concluded that there was no accumulation of pheromone in the set up and that the amount of pheromone collected during a period was emitted during this period. Furthermore, all the SPME fibers were controlled at regular intervals, with the same experimental procedure, to detect variation of the affinity of the fiber for epoxides. When the affinity declined by 30%, the fiber was discarded.

Vibratory Stimulation. The acoustic courtship of sexually mature and virgin adults from the France or Guadeloupe populations, maintained in pairs on the membrane of a loudspeaker (model WS 13 BF, 8 Ω impedance, 144 mm diam., 40–6000 Hz bandpass), was recorded and digitized on a computer (6000 Hz, 16 bit) after amplification by a Revox A-77. The record of the male rivalry song was obtained by putting two males and a female on the membrane of the loudspeaker. We synthesized four vibration programs from these records and one program with an artificial signal with the software Sound Forge 4.0. The first program contained pulses of the female calling song (FCS) recorded from 10 French female bugs (Figure 1). The second program was composed of FCS pulses recorded from 7 Guadeloupe females. The third program was composed of pulses of the male rivalry song (MRS) recorded from 4 French male bugs (Figure 1). The fourth program was composed of MRS pulses recorded from 4 Guadeloupe males. Lastly, an artificial song was synthesized with 1-min-long pulses of a pure 100-Hz signal, separated by intervals of 2000 msec of silence (Figure 1).

The amplitude of each stimulation program was normalized with an average RMS power at -13dB and peak level at -3 dB. The stimulation programs were stored on the computer and played back in a loop during 20 min through the sound card to a loudspeaker (type 45605 BSA, 8 Ω impedance, 22 mm diam., and 40–22,000 Hz bandpass), the membrane of which was brought into contact with the wall of the glass vial to transmit vibrations to the insect.

Experimental Procedure. One male was introduced into the glass vial with a leaf from a bean plant. The volatile emissions were sampled with an SPME fiber during three periods of 20 min. Fibers were immediately analyzed by gas chromatography. The quantities of the three main components of the pheromone blend produced by males of *N. viridula,* the (Z)-α-bisalonene and its *trans* and *cis* epoxides, were pooled, and they are further designated as the pheromone. The glass vial was changed for each male. After use, vials were cleaned by soaking

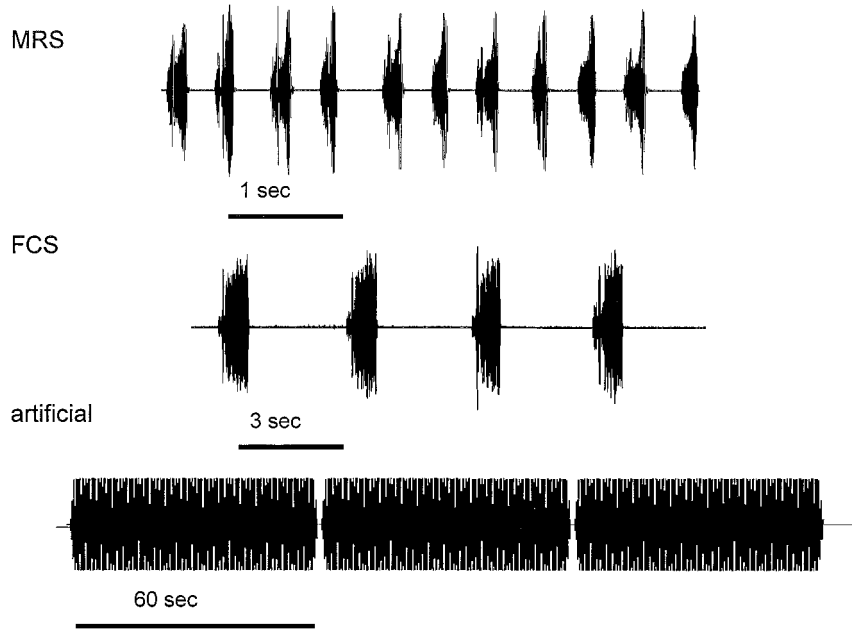

FIG. 1. Samples of recordings of the female calling song (FCS) and male rivalry song (MRS) of French bugs and of the 100 Hz artificial signal used to stimulate males.

overnight in a 5% solution of Decon 90 (Prolabo, Fontenay-sous-Bois, France), rinsed with water and acetone, and dried.

When appropriate, a prerecorded vibratory stimulus was given during the second period, through the wall of the vial. A total of 123 male bugs were stimulated, either with FCS from their own population, FCS from the other population, MRS from their own population, or the artificial signal. Special attention was paid to equalize stimulation (vibration) pressure level across all song types presented to the insects. Before each experiment, the level of the stimulation through the glass vial was measured and adjusted to obtain a record with an average RMS power of -30 dB and a peak level of -4.5 dB.

Statistics. To study the effect of each type of vibratory stimulus, we compared by paired analysis the amounts released by males during the three periods and the number of males emitting detectable amounts of pheromone. Wilcoxon's T test for paired samples was used to analyze variations in the amount of pheromone released by each male between the first period (before stimulation) and the second period (during stimulation), between the second and the third (after stimulation) periods, or between the first and the third periods. MacNemar's test (Sokal and Rohlf, 1969) for paired data was used to compare the percentage of males emitting

detectable amounts of pheromone during the three periods of the experiment with $P < 0.05$ as the significance level. The effect of the stimulation on the relative composition of pheromone blend through the three periods of experiment was analyzed by a Kruskal-Wallis test. The ratios of the *trans* and *cis* epoxides of the (*Z*)-α-bisabolene in the two populations were compared using a Mann-Whitney test.

RESULTS

The volatile emissions from a total of 149 male bugs belonging to two populations were collected. During the first phase of 20 min following the introduction of the male into the collection vial, and without any vibratory stimulus (prestimulation), 66 males (44.3%) did not produce detectable amounts of pheromone (<0.5 ng; Figure 2). The collected amount of pheromone averaged 57.9 ± 157.1 ng. This high standard deviation reflects the fact that pheromone amounts covered a very large range from male to male of between 0 and 948 ng. Thus, even in the absence of characterized stimulation from conspecifics during the collection period, 19.5% of males released more than 100 ng.

The percentages of males emitting pheromone during the prestimulus period in the different experiments (Table 1) were compared with control (no vibratory stimulation) using a χ^2 test. We considered that a male emitted when more than 0.5 ng of pheromone was recovered from the SPME fiber. There were no statistical differences among the different experiments (FCS of the same population, FCS of the other population, MRS, artificial signal) and the control. The average amounts of pheromone collected during the prestimulation phase ranged from 25.1 ng in the

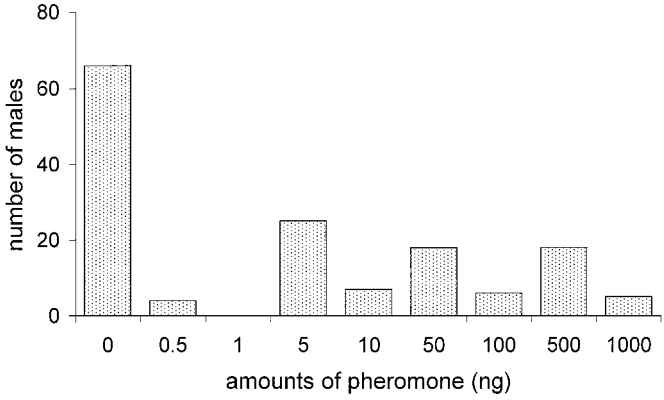

FIG. 2. Distribution of male *Nezara viridula* according to the amounts of pheromone collected with SPME fibers during the prestimulation period of 20 min.

TABLE 1. PHEROMONE EMISSION DURING PRESTIMULUS PERIOD IN DIFFERENT EXPERIMENTAL BATCHES OF MALE *N. viridula*

	Control ($N = 26$)	FCS-pop ($N = 43$)	FCS-alien ($N = 28$)	MRS ($N = 28$)	Artif ($N = 24$)
Emitting males (%)	42.3	60.5	53.6	53.6	54.2
P (χ^2)		0.137	0.405	0.405	0.399
Amount of pheromone (mean $\pm$ SD)	69.0 ± 187.0	25.1 ± 59.5	100.2 ± 228.2	67.5 ± 181.7	43.8 ± 98.4
P (Mean-Whintey)		0.469	0.526	0.709	0.831

FCS-pop to 100.2 ng, in the FCS-alien (Table 1). However, there was no statistical differences among experiments (Mann-Whitney, $P > 0.44$).

In turn, there were differences among experiments in the changes in the number of males releasing pheromone and in the pheromone release rate. In the absence of vibratory stimulus (control), the percentage of releaser males was stable during the three collection periods (Figure 3; MacNemar's test, NS). The number of males

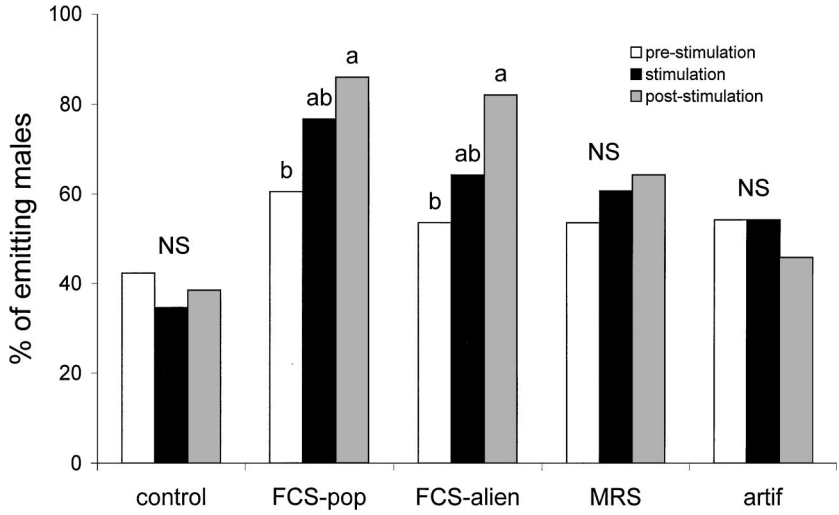

FIG. 3. Percentages of male *N. viridula* emitting detectable amounts of pheromone during the prestimulation (white bars), stimulation (black bars), and poststimulation (gray bars) periods. Males were stimulated for 20 min (simulation period) with a prerecorded female calling song of their own population (FCS-pop), with the female calling song of the other population (FCS-alien), with the male rivalry song (MRS), or with an artificial signal of 100 Hz frequency (artif). Control males were not stimulated. Different letters above bars indicate significant differences between collection periods within the same stimulus with $P < 0.05$ (MacNemar's test).

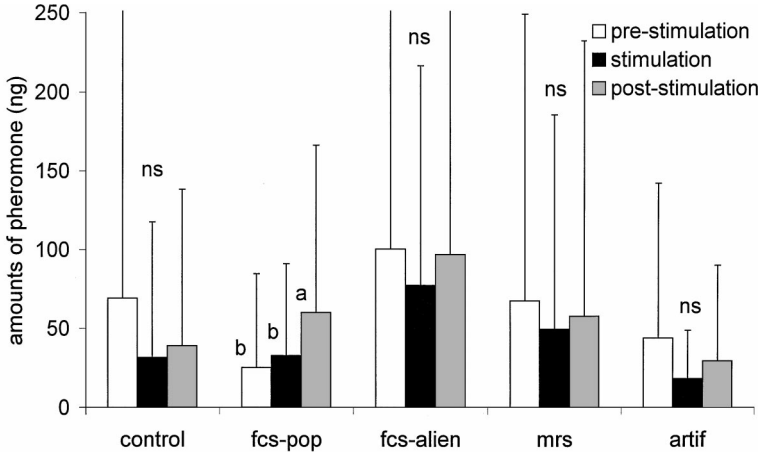

FIG. 4. Quantitative changes in pheromone emission by male *N. viridula* in response to vibratory stimuli. Error bars are standard deviations. Males were stimulated during 20 min (simulation period) with a prerecorded female calling song of their own population (FCS-pop), with the female calling song of the other population (FCS-alien), with the male rivalry song (MRS), or with an artificial signal of 100 Hz frequency (artif). Control males were not stimulated. Different letters above bars indicate significant differences between collection periods within the same stimulus with $P < 0.05$ (Wilcoxon's T test for paired samples).

releasing pheromone was stable in control experiments (Figure 3). The amounts of pheromone recovered during the first 20 min ("pre-stimulus") from control males were variable; 852 ng were collected from one individual, whereas the other control males released between 0 and 333 ng/20 min. The amounts of pheromone collected during the following 20-min period ("stimulation") decreased (Figure 4) and was stable during the third period. However, the differences between the prestimulus and the stimulus or poststimulus period were not statistically significant (Wilcoxon's T test for paired samples).

When prerecorded female calling songs of their own population (FCS-pop) were played back during the second period of stimulation, the percentage of males ($N = 43$) releasing measurable amounts of pheromone increased progressively from 60.5% during the prestimulation period to 86.0% during the poststimulation period (Figure 3; MacNemar's test, $P < 0.01$). The average amounts of pheromone collected from the males were significantly higher during the poststimulation period, compared to the prestimulation (Wilcoxon's T test for paired samples, $P < 0.001$) and the stimulation (Wilcoxon's T test for paired samples, $P < 0.01$) periods (Figure 4).

The comparison of the change of pheromone emission from unstimulated and FCS stimulated male *N. viridula* suggested that vibratory signals activated

pheromone emission by increasing the number of releasing males and the total amounts they emitted. Further experiments were necessary to ensure that this effect was dependent on the nature of the signal.

First, considering the variability in the vibratory repertoire among geographically isolated populations (Ryan, 1996; Čokl et al., 2000), cross-stimulation experiments between France and Guadeloupe stocks were performed. When female calling songs of the alien population (FCS-alien) were played back during the stimulation period, the percentage of males ($N = 28$) releasing measurable amounts of pheromone increased significantly from 53.6% during the prestimulation period, to 78.2% during the stimulation period, and to 82.1% during the poststimulation period (Figure 3; MacNemar's test, $P < 0.05$). The amounts of pheromone released by males stimulated with FCS-alien were large during the prestimulation period (Figure 4) and remained stable during the stimulation and the post-stimulation periods (Wilcoxon's T test for paired samples, NS).

Considering that the ratio of the *cis* and *trans* isomers of the (*Z*)-α-bisabolene is variable among individuals, and between strains (Aldrich et al., 1987; Ryan et al., 1995; Miklas et al., 2000), we investigated whether this ratio was affected by vibrations. The blend component ratio was different between the French (*trans* ratio of 0.65 ± 0.27) and the Guadeloupe (*trans* ratio of 0.82 ± 0.25) populations ($P = 0.004$, Mann-Whitney), but did not vary through the three periods of collection, either when males from both populations were stimulated with female calling songs of their own population or when they were stimulated with FCS of the other population (Kruskal-Wallis test, NS).

To investigate further the specificity of the effect of vibratory signals on pheromone release by males, we looked for the effects of songs of the same species, but with another communication function. We used the male rivalry song (MRS), which is produced when two males compete for a mate (Čokl et al., 1978). The percentage of males ($N = 28$) emitting detectable amounts of pheromone was stable over all three periods (Figure 3; MacNemar's test, NS). The mean amounts of pheromone collected from males during the stimulation and poststimulation periods were also stable (Figure 4; Wilcoxon's T test for paired samples, NS). The relative composition of the pheromone varied when males were stimulated with MRS (Kruskal-Wallis test, $P < 0.05$). The ratio of the *trans* epoxide was higher during the stimulation and the poststimulation periods. However, this shift towards higher ratios of the *trans* epoxide could be due to an underestimation of the minor components of the blend at total amounts close to the sensitivity threshold of the method of collection.

Finally, we investigated the effect of an artificial signal with 100 Hz frequency on the production of pheromone by males. The percentages of males ($N = 24$) releasing detectable amounts of pheromone were stable throughout the experiment (Figure 3; MacNemar's test, NS). The amounts of pheromone recovered showed a decrease between the prestimulation and the stimulation periods, but the

difference was not statistically significant (Wilcoxon's T test for paired samples, $P = 0.06$).

DISCUSSION

Quantification of pheromone release in this large sample of individuals indicates that male *N. viridula* emit the sex pheromone in the absence of a potential mate and reveals that released amounts are variable. The fact that male bugs release the sex pheromone in the absence of a potential mate is expected from a calling signal and a long-range attractant. The variability of emission from male to male has already been reported (Miklas et al., 2000), but is unexpected for such a signal. Low pheromone emitters have a strong disadvantage over high emitters in attracting a mate. In our experiments, male bugs were taken from rearing cages housing about 10 males, right before the experiments. The decrease in the amounts of pheromone collected from control males during the second and third periods suggests that the absence of an external signal might cause a decrease in pheromone release. Alternatively, pheromone emission might be cyclic, alternating high and low release rates.

The number of releasers increased when males were challenged with a female calling song of their own or of the other population. Accordingly, during the poststimulus period, the pheromone release increased by 2.5-fold over its prestimulus level. However, no significant increase in the amounts of pheromone was observed when males received an FCS song of the other population. This suggests that the FCS of the other population is less efficient in stimulating pheromone emission. Discrimination by males between female songs of different populations has already been reported. French males emit pulses of male courtship songs at higher rates in response to female calling songs of their own population, compared to FCS from Italian or Slovenian bugs (Miklas et al., 2001).

One other signal belonging to the repertoire of *N. viridula*, the male rivalry song, was able to maintain the level of male pheromone emission, whereas the artificial signal was not. Indeed, the emission profile of males submitted to that artificial signal was similar to that of unstimulated, control males.

All together these results provide evidence for a modulation of pheromone emission by vibratory signals from conspecifics. Female-specific songs were more efficient in enhancing male pheromone emission. Mechanisms of sex pheromone production and emission by Heteroptera are not accurately known. However, three main steps could be involved in the modulation of pheromone release: pheromone biosynthesis within specialized cells, excretion rate towards extracellular compartments, and volatilization in the surrounding atmosphere. In moths, the production of pheromone is regulated by direct neuroendocrine control of the biosynthetic activity of the pheromone gland (Raina, 1997), and emission is modulated by exhibiting a specific calling behavior that increases volatilization

(Baker and Cardé, 1979). Despite extensive ethological studies (Borges et al., 1987; Kon et al., 1988, 1993), no specialized calling behavior has been observed up to now in *N. viridula*. In males stimulated with the FCS, the increase in pheromone release was progressive and more important during the poststimulus period. This suggests that detection of conspecific vibrations affects an early step in the production of the chemical signal, showing slow kinetics such as biosynthesis of the pheromone or its excretion. Male production of songs was not monitored during our experiments, but it is known that *N. viridula* males respond readily to FCS by emitting male courtship songs and alternate their songs with that of females (Čokl et al., 1978). Dissections and analyses of different parts of the male body have shown that the sex pheromone is emitted from the abdominal sternites (Pavis and Malosse, 1986; Borges, 1995). Thus, vibrations of the abdomen during the process of song production by males in response to the female calling song might also have facilitated the emission of pheromone.

The modulation of the emission of chemical signals in insects by environmental or physiological factors is well documented in the literature (see a review in McNeil, 1991). Experimental demonstration of the influence of signals from conspecifics on the release of volatile long-range attractants has been limited up to now to the Scolytidae (Rudinsky et al., 1976) and the greater wax moth, *Galleria mellonella* (L.) (Spangler, 1987). In *Dendroctonus* bark beetles, acoustic signals produced by one or the other sex evoke the emission of an antiaggregation pheromone by the other sex. The interaction of chemical and acoustic signals contributes to regulate the colonization of the host plant. Male greater wax moths release a pheromone that attracts females, and they increase their rate of pheromone release in response to artificial vibrations within a range of frequencies that include that of vibrations produced by a wing-fanning female (Spangler, 1987). In *G. mellonella* and in *N. viridula*, the modulation of the pheromone release is directly related to the mate recognition system. It might offer several advantages to the male bugs. First, it limits the energetic or physiological cost of production by adjusting pheromone amounts to communication context. This metabolic cost is supposed to be high in insect species with long reproductive periods and multiple matings (McLain and Marsh, 1990) such as *N. viridula*. Second, it may improve the efficiency of olfactory communication by offering better synchronization and specificity between partners, considering the absence of external synchronization cues, such as photoperiod, and circadian rhythm of the sexual behavior. Lastly, it may reduce the risk of parasitism of males, which are more exposed than females to the attacks of *Trichopoda pennipes* (F.), a dipteran parasitoid attracted to the sex pheromone of *N. viridula* (Mitchell and Mau, 1971; Harris and Todd, 1980).

Due to the absence of an overt behavioral response of the female other than locomotion, the function of the male pheromone has been regarded up to now as that of a long-range attractant. Thus, one expects that the male would decrease its emission once it detects the presence of a female in its vicinity. The positive

modulation of pheromone emission by FCS suggests that the male pheromone also plays a role in further steps of the courtship behavior. Females have been shown to walk and fly in response to synthetic and natural male pheromone (Brézot et al., 1994), but the orientation behavior of male to female calling songs (Čokl et al., 1999) suggests that the achievement of mate finding involves an active search for females. An increase in pheromone emission offers to the male the possibility of recruiting more females or of increasing their receptivity and offers to the females selection of males based on the level of their production of sex pheromone. Both processes would give an advantage to largest producers. In natural populations of *N. viridula*, larger males mate more frequently than small ones (McLain, 1980), but selection cues have not been identified. Pheromone production, if proven to be positively correlated with body size, could provide the sensory cues for female choice. The involvement of the male pheromone in sexual selection and close-range courtship behavior would explain both the large variability observed in pheromone titer among males and why they modulate their emission.

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CHARACTERISTIC ODOR OF *Osmoderma eremita* IDENTIFIED AS A MALE-RELEASED PHEROMONE

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Abstract—*Osmoderma eremita* (Scopoli) is an endangered scarab beetle living in hollow trees. It has mainly been known for its characteristic odor, typically described as a fruity, peachlike or plumlike aroma. The odor emanating from a single beetle can sometimes be perceived from a distance of several meters. In this paper, we show that the characteristic odor from *O. eremita* is caused by the compound (*R*)-(+)-γ-decalactone, released in large quantities mainly or exclusively by male beetles. Antennae from male and female beetles responded in a similar way to (*R*)-(+)-γ-decalactone in electroantennographic recordings. Field trapping experiments showed that (*R*)-(+)-γ-decalactone is a pheromone attracting female beetles. Lactones similar to (*R*)-(+)-γ-decalactone are frequently used as female-released sex pheromones by phytophagous scarabs. This is, however, the first evidence of a lactone used as a male-produced pheromone in scarab beetles. We propose that the strong signal from males is a sexually selected trait used to compete for females and matings. The signal could work within trees but also act as a guide to tree hollows, which are an essential resource for *O. eremita*. Males may, thus, attract females dispersing from their natal tree by advertising a suitable habitat. This signal could also be exploited by other males searching for tree hollows or for females, which would explain the catch of several males in our traps.

Key Words—Pheromone identification, (*R*)-(+)-γ-decalactone, scarab beetle, conservation, sexual selection, saproxylic, habitat colonization, chirality, electroantennography, gas chromatography.

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INTRODUCTION

Osmoderma eremita (Scopoli) is a saproxylic scarab beetle that lives as a larva and adult in the hollows of old trees. In Sweden, its primary habitat is hollow oak (*Quercus robur*) and beech (*Fagus sylvatica*) (Ranius and Nilsson, 1997), but in other parts of Europe it has also been found in a large number of other deciduous and yew trees (*Taxus baccata*) forming hollows (Luce, 1996). The destruction and fragmentation of old-growth forests and later intensive cutting of old hollow trees in the agricultural landscape (Eliasson and Nilsson, 1999) has rendered *O. eremita* an endangered species all over Europe. In Sweden, it is considered vulnerable (VU) on the national redlist (Gärdenfors, 2000). It also seems to be a good indicator for species richness of saproxylic beetles in hollow trees (Ranius, 2002), and due to its value as a symbol of the diverse fauna of old hollow trees, this beetle has been chosen as a species with especially high priority for conservation efforts within the European Union (Anonymous, 1992).

Since 1995 the population biology and dispersal patterns of *O. eremita* have been investigated in one of the Swedish areas comprising relatively high population densities of the species as well as in a fragmented area nearby (Ranius and Nilsson, 1997; Ranius, 2000, 2001, 2002; Ranius and Hedin, 2001; Hedin and Ranius, 2002). Results from these studies indicate a metapopulation structure with semi-isolated populations occupying individual trees. A low proportion of individuals leave their natal trees and disperse to other trees in the vicinity, which leads to colonization of new trees or to recolonization of trees where the local population has gone extinct (Ranius, 2001; Ranius and Hedin, 2001). One aspect of the dispersal process that has received no attention is the question of how adult beetles locate suitable habitats. This is likely to have a profound influence on dispersal and colonization patterns.

Perhaps the most distinctive and well-known feature of *O. eremita* is its characteristic odor, which is somewhat fruity and peachlike or plumlike. It is reminiscent of the smell of so-called "Russia leather" or "jucht leather" (from the Russian name), an old Eastern European type of leather tanned in plant oils (Nationalencyclopedin, 1989; Oxford English Dictionary, 1989), which is likely the reason behind the scientific name of the genus (*Osmoderma* in Greek means "smelling skin"). The odor emanating from a single beetle is sometimes so strong that it can be perceived by the human nose at a distance of several meters (personal observations). The function of this strong odor has been unknown, however. We hypothesized that it could serve as an intraspecific long-distance odor signal, which could constitute an important part of our understanding of the mechanisms behind habitat colonization in *O. eremita*. An identified pheromone could also serve as a complement to already existing systems for monitoring of *O. eremita* populations by field trapping. This paper presents the chemical identification of the characteristic odor of *O. eremita* and the confirmation of its role as a pheromone.

METHODS AND MATERIALS

Collection of animals and field trapping experiments were conducted in the Bjärka-Säby area south of Linköping, Sweden. This area is dominated by old oak trees housing one of the largest populations of *O. eremita* in Europe (Ranius, 2001). Pitfall traps were placed in hollow oaks and checked every one or two days for the presence of adults. We sexed every beetle that we encountered, and sniffed it to check for odors detectable by the human nose. For chemical analyses, volatiles were collected by headspace sampling from live beetles. Some beetles were brought to the Department of Ecology at Lund University and sampled for up to 24 hr in a stationary closed-loop collecting apparatus made from glass containers and a pump with a capacity of 0.7 liters/min. (Parker Hannifin Corp. Sharon, Massachusetts, USA). Some beetles were collected at the field site and sampled indoors for 3–8 hr in a portable closed-loop collecting apparatus made from 0.5 liter polyacetate frying bags and a portable air pump with a capacity of 2.5 liters/min. (KNF Neuberger AB, Stockholm, Sweden), before being released again. *Osmoderma eremita* is a diurnal species (J. Hedin, T. Ranius, A. Malmqvist, unpublished data), and all these collections were performed during the natural photophase. Volatiles were trapped on 1.5-mg charcoal filters (Brechbühler AG, Schlieren, Switzerland) placed in the outlet of the collection chamber. The charcoal filters were extracted with 20–160 μl of acetone, sometimes containing anethol as an internal standard for quantification. Some samples were concentrated under nitrogen before analysis.

Initial analysis of the extracts was performed by splitless injection on two HP 5890 Plus gas chromatographs with flame ionization detectors (FIDs) and fitted with a polar HP-Innowax (30 m $\times$ 0.25 mm ID) or nonpolar HP-1 (30 m $\times$ 0.25 mm ID) capillary column. The carrier gas was hydrogen and the injector temperature was set at 250°C. Initial oven temperature was 40°C for 2 min, and then the temperature raised to 230°C at a rate of 10°C/min. One of the GCs was equipped for GC electrophysiological recordings with a two-way split installed at the end of the column leading half of the effluent to the FID and the other half to an outlet from the GC (Stensmyr et al., 2001). Instead of an insect antenna, we had five different human test persons sniffing the outlet of the GC to check the retention times of any compounds smelling like hermit beetle odor (Al Abassi et al., 1998). Identification and quantification of compounds in the extracts was performed by coupled GC-mass spectrometry on an HP 5890 gas chromatograph (HP Innowax column 30 m $\times$ 0.25 mm diam. splitless injection at 250°C, carrier gas helium, temperature program as above) coupled to an HP 5972 mass spectrometer. Mass spectra were compared with reference spectra from a NIST/EPA/NIH 75 K electronic mass spectral database. Enantiomeric separation of γ-lactones was carried out by using a 30 m $\times$ 0.25 mm ID fused silica column coated with a 1:1 mixture of OV-1701 and *6-tert*-butyldimethylsilyl-2,3-dimethyl-β-cyclodextrin operated at 60°C for 3 min, then programmed to 180°C at a rate of 3°C/min. Synthetic

standards like anethol and γ-lactones were purchased from Sigma-Aldrich and had purities of 98–99%. Synthetic (*R*)-(+)-γ-decalactone had an overall purity of ca. 99% and an enantiomeric purity of 97% (checked by chiral GC).

Electroantennograms (EAGs) were recorded from excised *O. eremita* antennae placed on top of a supporting piece of dental wax. Examination of general antennal morphology and handling of antennal specimens were performed under an Olympus × 100 prep microscope. The three lamellae comprising the antennal club and housing the olfactory sensilla were held apart by cactus needles inserted into the wax. Electrical signals were recorded through glass capillary electrodes filled with Beadle-Ephrussi ringer (Bjostad, 1998) and connected via silver wires to an electrophysiological recording unit with combined joystick micromanipulators and amplifier (Syntech INR-02, Hilversum, The Netherlands). The ground electrode was slipped over the base of the antenna, while the recording electrode was pressed to one of the antennal lamellae. Although the antennal preparation usually remained viable for at least an hour, all measurements were completed within 10 min after excision. The signal was stored on a computer through a Syntech digital interface and analyzed with the Syntech EAG software v. 1.0. The preparation of stimuli and the method for odor stimulation of the antenna has been described before (Stensmyr et al., 2001). Odor stimuli were delivered into an airstream flushing over the antenna, from Pasteur pipets containing filter paper loaded with different amounts of synthetic (*R*)-(+)-γ-decalactone. The response strength to an odor stimulus was calculated as the amplitude of the EAG response, after subtraction of the blank response.

For field trapping experiments, conducted in 2001, we used 10 replicates of Lindgren funnel traps (Phero Tech Inc., Delta, British Columbia, Canada), each containing a treatment trap and a control trap spaced at least 4 m apart. The replicates were placed in the vicinity (within 2–20 m) of trees known to contain substantial populations of *O. eremita* (Ranius, 2001) in several different separate stands of oaks in the area. Each treatment trap contained an odor dispenser made from a 2-ml glass vial with a piece of cotton dental roll inserted as a wick from the opening to the bottom of the vial. Odor dispensers were initially loaded with 400 μl of neat (*R*)-(+)-γ-decalactone and supplemented with another 200 μl after 20 days. Laboratory experiments had shown that regardless of the amount in the vials, the odor dispensers had a stable mean release rate of approximately 2 mg/day as long as the wicks remained saturated (unpublished data). Control traps contained identical odor dispensers without (*R*)-(+)-γ-decalactone. The positions of the treatment and control traps were switched every two days within each replicate to avoid position effects. The field trapping experiment lasted for 40 days after the appearance of the first *O. eremita* adults (July 9), corresponding to a full estimated field season. Trap catches were analyzed with a binomial test (Siegel and Castellan, 1988), against the null hypothesis that beetles would be equally attracted to treatment and control traps.

RESULTS

Based on our olfactory evaluations of 120 male and 58 female hermit beetles encountered in or outside tree hollows, 38% of the males but none of the females emitted enough of the characteristic odor to be detectable by the human nose. We did not observe any obvious calling postures from either sex. According to our subjective evaluation, none of the beetles found during the 2001 season had the same extreme emission rates that we had sometimes noted during previous seasons. Handling of the beetles often resulted in substantially increased odor release from males, but never from females. A comparison of gas chromatograms of odor extracts collected from males and females revealed the presence of a specific compound released in large amounts by male beetles (Figure 1). Independent evaluation by five different test persons of the effluents from a GC showed that this compound in all cases was associated with the characteristic *O. eremita* odor.

A prominent signal at m/z 85 in the mass spectrum of the unknown male-specific compound suggested the structure of a γ-lactone, which was supported

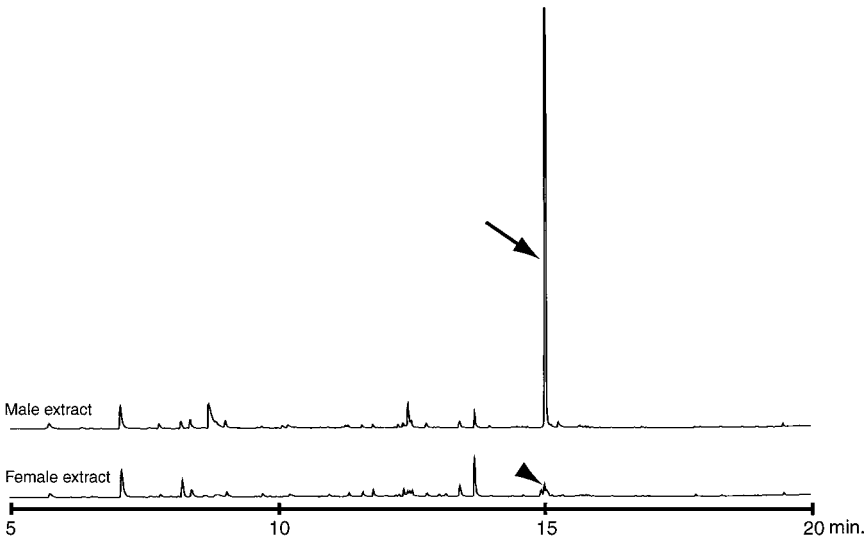

FIG. 1. Gas chromatograms of volatiles collected from five male and five female beetles during 24 hr show the presence of a compound released in large quantities from males (arrow). The peaks with similar retention time in the extract from females (arrowhead) do not represent the same compound. The male-released compound was identified as γ-decalactone by comparison with retention time and mass spectra of synthetic γ-decalactone. This peak was also associated with the characteristic fruity odor of *O. eremita* in olfactory tests with human subjects.

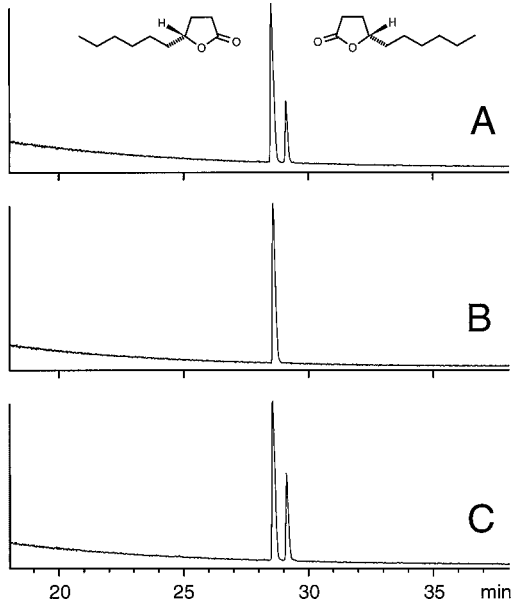

FIG. 2. Assignment of the absolute configuration of γ-decalactone from *Osmoderma eremita* by chiral gas chromatography. (A) *(R)*-(+)-γ-decalactone + racemic γ-decalactone; (B) natural γ-decalactone; (C) natural γ-decalactone + racemic γ-decalactone. For conditions see text.

by reference spectra published in the mass spectral database. Comparison of gas chromatographic retention times of unsaturated γ-lactones and mass spectra of a series of synthetic 8- to 12-carbon γ-lactones showed that both the mass spectrum and retention time of the unknown compound matched that of synthetic γ-decalactone. Coinjection of synthetic γ-decalactone with male extract on both a polar and a nonpolar column confirmed that the compound released from males was γ-decalactone. Under the employed conditions, enantiomers of γ-lactones were baseline separated ($\alpha = 1.025$: retention time of later-eluting enantiomer divided by retention time of earlier-eluting enantiomer). As can be seen in Figure 2, the *R*-enantiomer eluted first. The naturally occurring γ-decalactone showed *R*-configuration and was at least 99% pure, showing an enantiomeric excess of more than 98%. Interestingly, other γ-lactones with 8, 9, 11 or 12 carbon atoms that could also be identified in amounts of less than 1% of γ-decalactone were found to be much less pure, showing enantiomeric ratios of *R* to *S* of ca. 2:1, only.

Additional collections of volatiles from nine individual males and eight individual females showed that all males, regardless whether the odor could be detected by our noses, emitted amounts of γ-decalactone large enough to be detected by

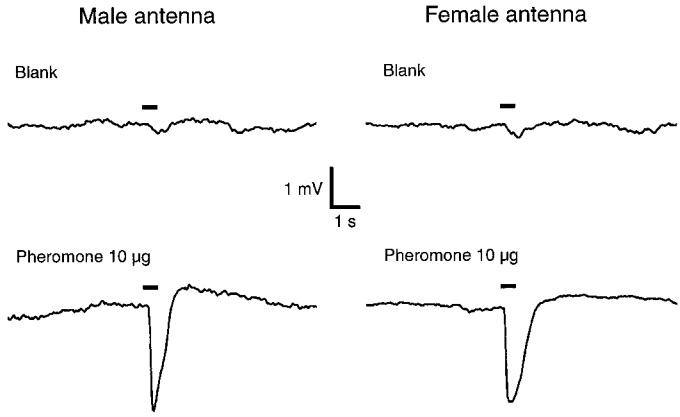

FIG. 3. Examples of electroantennographic responses from a male and a female antenna to a blank stimulus (pipette with solvent only) and to a stimulus pipette containing 10 μg of *(R)*-(+)-γ-decalactone. The differences in appearance between the electroantennograms from the male and the female antenna show typical differences between recordings and do not reflect sex-specific responses. Scale bars indicate time (1 sec) and EAG amplitudes (1 mV). The bars above the EAG traces show the stimulus duration (0.5 sec).

GC-MS (mean release rate 6.3 μg/hr, SD $\pm$ 16.8). Only in the collections from one single female, however, could γ-decalactone be detected corresponding to 0.57 μg/hr of sampling time (3 h).

Electroantennographic recordings from hermit beetle antennae showed strong responses to synthetic γ-decalactone (Figures 3 and 4). Although the response amplitudes from individual antennae varied considerably, likely depending on the quality of the contact with the recording electrode, the average dose–response recordings from male and female antennae were virtually identical (Figure 4). Microscopic examination of male and female antennae revealed no apparent sexual dimorphism. Antennae from both sexes were of similar size and appeared to have the same distribution of olfactory sensilla. On each surface of the lamellae of the antennal club, the olfactory sensilla were divided into two zones: a dorsal zone of heterogeneous appearance and a ventral smooth zone containing larger placoid sensilla. These two zones occupied similar relative surface areas in males and females, with the smooth zone containing large placoid sensilla occupying approximately one third of the total surface area.

A total of 23 hermit beetles were caught in Lindgren traps outside hollow trees during the field trapping period (Table 1). Traps baited with synthetic (*R*)-(+)-γ-decalactone caught significantly more female beetles than control traps ($P < 0.01$). In addition, several males were also caught, although not significantly more than in the control traps. Usually only single individuals were found in each

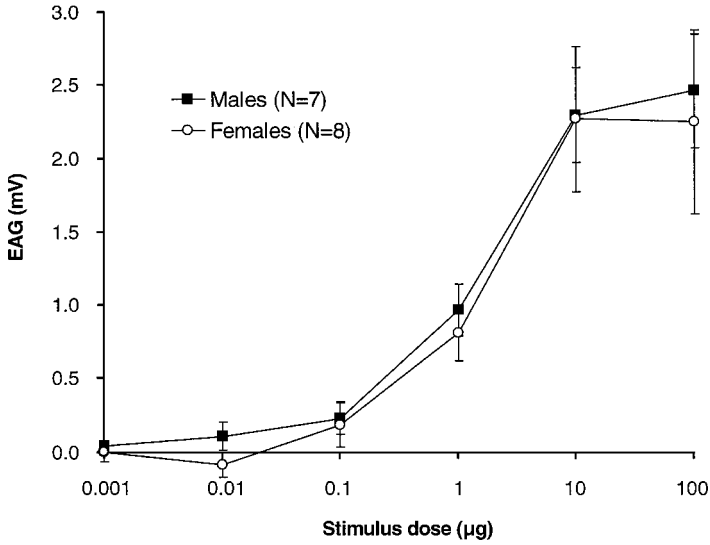

FIG. 4. Dose–response relationships of electroantennographic responses from male and female *O. eremita* beetles. The figure shows mean responses (mV) ± SE from antennae of male and female beetles to logarithmically increasing doses of *(R)*-(+)-γ-decalactone (μg) in stimulus pipettes. The dose–response curves from male and female antennae are virtually identical.

trap except for two occasions when two females, and three occasions when a male and female were found together in the same pheromone trap.

DISCUSSION

The characteristic odor of *O. eremita* is caused by *(R)*-(+)-γ-decalactone released in large quantities primarily or exclusively by males. Most females do

TABLE 1. *Osmoderma eremita* BEETLES CAUGHT DURING ONE FIELD SEASON IN TRAPS BAITED WITH (*R*)-(+)-γ-DECALACTONE AND CONTROL TRAPS[a]

	Lactone	Control	Significance
Females	15	2	$P < 0.01$
Males	5	1	Not significant
Total	20	3	$P < 0.001$

[a] The table shows the number of males and females and the total numbers of hermit beetles caught, together with an estimate of the statistical significance for each group according to a binomial test (Siegel and Castellan, 1988).

not seem to release detectable amounts of (*R*)-(+)-γ-decalactone, and the single occurrence found could be the result of contamination, either from a conspecific male or from our handling of large amounts of the compound. Antennae of males and females respond almost identically to this compound in electroantennographic recordings, corresponding with our observations that there is no apparent sexual dimorphism in their antennae. Although we have not performed electrophysiological measurements on single olfactory sensilla in this species, a comparison with investigations of ruteline and cetoniine scarabs suggests that the pheromone could be detected by the large placoid sensilla found in both sexes of *O. eremita* (Leal and Mochizuki, 1993; Hansson et al., 1999; Larsson et al., 1999, 2001; Kim and Leal 2000; Stensmyr et al., 2001).

Our trap catches show that (*R*)-(+)-γ-decalactone is a pheromone sufficient for attracting *O. eremita* in the field, although the presence of additional pheromone components cannot be excluded. The seemingly low number of adult *O. eremita* beetles caught in our traps appears, in fact, to be substantial, considering the very low density of beetles estimated to be flying around in the area (compare Ranius and Hedin, 2001). Sightings of *O. eremita* beetles outside of tree hollows usually only happen between 1–5 times per season, in spite of extensive daily visits to the experimental areas (personal observations). Based on the field trapping results, we tentatively describe (*R*)-(+)-γ-decalactone as a male-released sex pheromone of *O. eremita*. The lack of sexual dimorphism in the olfactory system indicates that the pheromone may in reality be exploited to some degree by both sexes, however, although the number of males caught in the traps was too low for statistical significance.

This is the first time the use of a lactone as a male-released scarab pheromone has been described. Lactones showing close structural relations to γ-decalactone have been identified as female-released sex pheromones in several scarab species of the subfamily Rutelinae, although in this subfamily all lactones have been unsaturated, with a double bond adjacent to the ring (see Leal, 1998, for review). Pheromones identified in other scarab subfamilies have not been of the lactone type (Leal, 1998; Rochat et al., 2000; Ruther et al., 2001). The phylogenetic relationship between *Osmoderma* and other phytophagous scarabs is unclear, but the genus should likely belong to the subfamily Cetoniinae, or alternatively to the Trichiinae, or form a related taxon of its own (Browne and Scholtz, 1998). In any case, our results also represent the first identification of a pheromone in either of these taxa.

Cetoniinae and Trichiinae are closely related to the Rutelinae, suggesting that the use of a lactone as a pheromone is a homologous trait whose expression has shifted primarily or exclusively to males. A common explanation for male-released pheromones in insects is that the males advertise a resource with a patchy distribution (Landolt and Philips, 1997; Schlyter and Birgersson, 1999). We propose that in *O. eremita* the unusual function of a lactone as a male-released pheromone is due to its potential as a guide for females to tree hollows, which are an essential and

patchily distributed resource for this species. Males may, thus, attract females dispersing from their natal trees by advertising a suitable habitat. An unintended but unavoidable side effect is that other males may also exploit the same signal to find tree hollows or to intercept approaching females. Similar scenarios are common among dung-rolling coprophagous scarabs, where males often attract females by releasing chemical signals after monopolizing a private dung hoard (Tribe, 1975; Burger et al., 1983, 1995; Edwards and Aschenborn, 1988; Burger and Petersen, 2002).

The high pheromone release rates that we measured from male beetles in the present study are in contrast to the much lower amounts usually released from or found in females of other scarab species (Leal et al., 1994, 1997; Leal, 1998; W. S. Leal, personal communication), although in some ruteline species, females may contain microgram quantities of pheromone (Leal et al., 1993). Relatively high release rates or gland contents seem to be common among male-released insect pheromones (Cardé and Baker, 1984; Chang et al., 1989; Dussourd et al., 1991; Romel et al., 1992; Innocenzi et al., 2001). Female insects, being the limiting sex, typically do not compete among themselves to obtain matings. Several factors, including energy conservation, the need to be inconspicuous to predators, and selection of responsive mates, likely work together to keep the release rates of female sex pheromones low (Greenfield, 1981; Cardé and Baker, 1984). Male insects, on the other hand, could use high release rates as a means of competition for females, and in the case of *O. eremita*, the extreme release rates observed indicate that pheromone release may be under strong sexual selection.

Evolutionary origins of scarab pheromones are not known, but have been attributed to defense compounds that have evolved into pheromones (Haynes et al., 1992; Haynes and Potter, 1995; Leal, 1998; Ruther et al., 2001). Many scarab pheromone compounds are exactly the same as or similar to food-related scarab attractants, indicating that the pheromones may have originated from ingested food odorants (Larsson, 2001; Stensmyr et al., 2001). The emitting sex may over time have switched to *de novo*-synthesized compounds to exploit an already existing sensory apparatus and search behaviors in the receiver; an evolutionary explanation commonly suggested also as the origin of bark beetle pheromones (Schlyter and Birgersson, 1999). These hypotheses are by no means mutually exclusive. We found that handling of male beetles often resulted in greatly increased release of pheromone, suggesting that the pheromone could have defensive properties if this is indeed an adaptive behavior. Whatever its origin, the main role of *(R)-(+)-*γ-decalactone for *O. eremita* is now likely pheromonal, since its expression is limited primarily or exclusively to males. Earlier one of us (W.F.) identified γ-decalactone (and some homologs as well as δ-decalactone) in workers and queens of the leaf-cutting ants *Acromyrmex crassispinus*, *A. disciger*, *A. niger*, and *A. subterraneus*, where the compounds may serve as components of the defense chemistry. It is interesting to note that also in the ant species the enantiomeric compositions of

the lactones varied with chain length (W. Francke and W. Borchert unpublished, results).

The existence of an attractive pheromone released in large quantities is likely to have an impact on the dispersal and colonization patterns of *O. eremita*, and should, thus, form an integral part of our future understanding of its dispersal. The pheromone system of *O. eremita* has likely evolved in the old-growth forests that once were its original habitat. The fragmented landscape of today, with long distances between stands of suitable trees, poses an enormous problem for beetles. Beetles that disperse out of the stands have a low chance of finding other hollow trees and will most probably end in the matrix between stands. Using a pheromone as a guide to trees could have both positive and negative effects on the long-term persistence of *O. eremita*. On one hand, it could lower the chances of colonization or recolonization of empty suitable habitats far away from other occupied trees. On the other hand, an efficient pheromone system will result in few, if any, dispersing beetles leaving the stands and ending in the matrix. The pheromone system could, therefore, also delay the extinction of remote local populations of *O. eremita*. In the future, the pheromone system may be used in monitoring or to attract the species to restored habitats near existing populations when natural colonization fails.

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(*Z*,*Z*)-6,9-HENEICOSADIEN-11-ONE,[1] LABILE SEX PHEROMONE OF THE WHITEMARKED TUSSOCK MOTH, *Orgyia leucostigma*

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Abstract—The whitemarked tussock moth (WMTM), *Orgyia leucostigma* (J. E. Smith), is a major pest of coniferous and deciduous trees in eastern Canada. Chemical identification of its sex pheromone depended primarily on GC-EAD and HPLC analysis, with confirmation of behavioral activity by wind tunnel and field tests. We identified (*Z*,*Z*)-6,9-heneicosadien-11-one (*Z*,*Z*-6,9-ket) at 4–5 ng/female as the only essential sex pheromone component. Also detected in female extracts were (*Z*)-6-heneicosen-11-one (*Z*6-ket) at 2.5 ng/female, (*Z*,*E*)-6,8-heneicosadien-11-one (*Z*,*E*-6,8-ket) at about 0.5 ng/female, and a trace amount of (*Z*,*E*)-6,9-heneicosadien-11-one. Traps containing as little as 1 μg of *Z*,*Z*-6,9-ket attracted males at low population levels, indicating it is a potent sex attractant. Traps baited with *Z*6-ket attracted few males, and in wind-tunnel bioassays it was at least 100-fold less attractive to males than *Z*,*Z*-6,9-ket. No improvement in trap catch occurred with the addition of *Z*6-ket in various binary mixtures with *Z*,*Z*-6,9-ket, including the female ratio, and a ternary mixture of *Z*,*Z*-6,9-ket, *Z*6-ket, and *Z*,*E*-6,8-ket in the 9:5:1 ratio detected in females was no better than *Z*,*Z*-6,9-ket alone. We attribute the presence of *Z*,*E*-6,8-ket and *Z*,*E*-6,9-ket in female extracts to the spontaneous and rapid

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[1]IUPAC rule A-1 has removed the e from henicosane and related hydrocarbons (see http://www.acdlabs.com/iupac/nomenclature/79/r79_34.htm).

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stereospecific isomerization of *Z,Z*-6,9-ket at room temperature. Male flight began at sunset but peaked during the second half of the night.

Key Words—Lepidoptera, Lymantriidae, *Orgyia leucostigma*, whitemarked tussock moth, (*Z,Z*)-6,9-heneicosadien-11-one, (*Z,E*)-6,8-heneicosadien-11-one, (*Z*)-6-heneicosen-11-one, sex pheromone, GC-EAD, wind tunnel.

INTRODUCTION

The whitemarked tussock moth (WMTM), *Orgyia leucostigma* (J. E. Smith) (Lepidoptera: Lymantriidae), occurs across North America from British Columbia to Florida, being most widely distributed in the eastern half of the continent (Ferguson, 1978). Typically, one generation is produced per year, but up to three generations can occur in the southern part of its range. The apterous females lay eggs on their cocoons as a conspicuous frothy white mass. Larvae are polyphagous, feeding on both deciduous and coniferous trees. They can reach high numbers, causing substantial defoliation and widespread economic damage. In 1998, over 20,000 ha of prime timber, primarily balsam fir, was destroyed in Nova Scotia and another 400,000 ha was severely defoliated, forcing the province to launch a $6 million (Canadian) aerial spray program (Solutions: The Canadian Forest Service Newsletter, p. 6, Spring/Summer 1999). WMTM is one of 25 major forest insect pests recognized by the Canadian Forest Service.

Because of its importance, especially in eastern Canada, we initiated the identification of the sex pheromone of WMTM almost 30 years ago. At that time, the only known pheromone in the genus was the (*Z*)-6-heneicosen-11-one (*Z*6-ket) component of the Douglas-fir tussock moth (DFTM), *O. pseudotsugata* (McDunnough), a western species (Smith et al., 1975). In flight-tunnel bioassays, *Z*6-ket was attractive to male WMTM but much less so than conspecific females (Grant, 1977). Similarly, *Z*6-ket in sticky traps attracted WMTM males but was less attractive than females.

Several of the *Orgyia* (and *Dasychira*) tussock moth species are sympatric and, although some are capable of pheromone cross stimulation and hybridization in the laboratory (Grant et al., 1975), there is no evidence of interbreeding populations, providing some indication of a more complex and species specific pheromone bouquet for these moths. In 1978–1979, we isolated a single potent attractant from WMTM females by HPLC that, based on its retention characteristics, was clearly not *Z*6-ket (unpublished data). A glass rod dipped in a solution of the HPLC isolate initiated intense and immediate sexual wing fanning behavior from caged males, but the substance was unstable, completely losing its bioactivity overnight at room temperature. Antioxidants and acid/base buffering were ineffective in preventing activity loss. Bioactivity could be maintained in the isolate for a week or more at -20°C, and for more than one year when kept in liquid nitrogen. The instability

and low levels of the putative pheromone component present in female extracts precluded any spectroscopic studies and structural assignment of this ephemeral substance.

In 1995, our collaborative studies on DFTM with gas chromatographic–electroantennographic detection (GC-EAD) established that a potent component of DFTM's pheromone was a diene henicosa-11-one, present in nanogram amounts per female (Gries et al., 1997). Chemical synthesis of several potential candidates established (*Z*,*E*)-6,8-heneicosadien-11-one (*Z*,*E*-6,8-ket) as the central component of DFTM's pheromone blend. The importance of a diene ketone as a key pheromone component in an *Orgyia* species encouraged us to continue our identification efforts with the sex pheromone of WMTM. The results of those efforts are reported here.

METHODS AND MATERIALS

Chemical Analysis of Gland Extracts. Insects were reared at the Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada, and shipped as pupae to Simon Fraser University, Burnaby, British Columbia, Canada. Pupae and female adults were maintained on C:D photoperiod at 24°C. Pheromone glands of female adults 1–2 days old were excised 1 hr prior to photophase, the peak calling period (Grant et al., 1975), and extracted with hexane at 0°C for 5 min. The extract was used immediately or kept in a freezer at −20°C.

Gas chromatographic (GC) analyses were carried out on a Hewlett-Packard 5890 gas chromatograph equipped with DB-1 or DB-210 fused silica columns (30 m × 0.32 mm ID, 0.25-mm film thickness) (J&W Scientific). The instrument was operated in the splitless mode with a delay of 0.5 min and injector temperatures of 260°C for DB-1 and 220°C for DB-210. The operating conditions were as follows: DB-1, 150°C for 1 min, 15°C/min to 200°C, isothermal for 1 min, 2°C /min to 250°C, and then isothermal: DB-210, 150°C for 1 min, 5°C/min to 200°C and then isothermal. GC-chemical ionization mass spectrometry (GC-CI-MS) analyses were performed on an HP 5985B GC-MS equipped with a DB-1 column, using isobutane as the chemical ionization source (Liu, 1999). GC-EAD analyses (Arn et al., 1975) were carried out as previously described (Gries et al., 1997).

High-performance liquid chromatography (HPLC) analyses at Simon Fraser University were carried out on a Waters LC 625 chromatograph with a Rheodyne 9125 50-μl injector, a Waters 486 UV detector at 220 nm (which shows absorption for conjugated dienes), and an HP 3396 Series II integrator. Reverse-phase LC utilized a Nova-Pak C_{18} column (4.6 × 250 mm) and HPLC grade acetonitrile at a flow rate of 1.0 ml/min. Quantities present in female extracts were estimated by comparison with synthetic samples run at known concentrations under identical conditions.

*Z*6-ket (98% purity) was purchased from Phero Tech Inc., Delta, British Columbia, Canada. *Z*,*E*-6,8-ket was prepared as previously described for the DFTM (Gries et al., 1997). *Z*,*E*-6,8-ket was a single peak on HPLC. It is unstable on GC and, thus, cannot be assigned a purity in the normal manner. (*Z*,*Z*)-6,9-Heneicosadien-11-one (*Z*,*Z*-6,9-ket) was prepared immediately before use by oxidation of the alcohol (Gries et al., 1997; Wimalaratne, 1998).

Lure Preparation. (*Z*,*Z*)-6,9-Heneicosadiene-11-ol (*Z*,*Z*-6,9-OH) was shipped to Sault Ste. Marie and converted to *Z*,*Z*-6,9-ket, as follows. *Z*,*Z*-6,9-OH (5 mg, 0.016 mmol) in CH_2Cl_2 (1 ml) was oxidized by PCC (10 mg, 0.05 mmol) buffered with K_2CO_3 (5 mg, 0.025 mmol) and stirred for 30 min at room temperature. The reaction was monitored by TLC silica gel using 10% ethyl acetate in hexane as eluent, and visualized by spraying with an acidic solution (10% H_2SO_4) of 1% $Ce(SO_4)_2$ and 1.5% molybdic acid and heating on a hot plate. Ether was added to the reaction mixture, and the solution was passed through a short column (Pasteur pipet) of 1 g Florisil, which was previously washed with ether. The remaining residue was washed with ether (3 × 2 ml) and filtered through the same column. The column was then eluted with an additional 5 ml of ether. The eluent was evaporated, and the crude product was taken up in hexane. It was loaded on a 10% $AgNO_3$-SiO_2 column (1 g in a Pasteur pipete, Silica gel 60, 230–400 mesh, E. Merck), and eluted with 0.5% ether in hexane yielding five fractions, each 0.5–1 ml in volume. The separation was monitored by TLC.

HPLC analysis at the Great Lakes Forestry Centre was conducted on a Waters Delta Prep 4000 LC system equipped with a computer and Millennium 2010 software, and an autoscan 996 photodiode array detector. Fractions were passed through Acrodisc filters (0.45 μm) and 10-μl aliquots injected onto a Waters reverse-phase analytical column (150 mm × 3.9 mm ID) packed with Nova-Pak C_{18} material (4 μm, 60 Å). An isocratic profile using 100% acetonitrile was applied at room temperature with a flow rate of 1 ml/min for 15 min, with detection at 220 nm. Peak heights, measured as absorbance at 220 nm, were converted to milligrams per milliliter with conversion factors obtained for the standard, *Z*,*E*-6,8-ket. These analyses were performed in triplicate. Semipreparative HPLC was applied to those fractions identified from analytical HPLC containing *Z*,*Z*-6,9-ket. Purification of *Z*,*Z*-6,9-ket required a maximum of 30 min at room temperature, during which time <5% *Z*,*E*-6,8-ket would be produced from *Z*,*Z*-6,9 isomerization (Wimalaratne, 1998). Repeated cycles of semipreparative HPLC provided quantities sufficient for bioassay, which were stored at −70°C in acetonitrile.

Lure solutions for field experiments were loaded into 2 ml open polyethylene vial closures (No. 60975d-3, Kimball Glass, Vineland, New Jersey, USA) containing a plug of bleached, nonsurgical cotton batting (Padco, Fisher Scientific Canada). Lures were prepared in a laboratory fume hood immediately before a test. They were carried to the field on Dry Ice in a portable cooler and inserted into Pherocon II, diamond-shaped sticky traps (Great Lakes IPM, Michigan, USA)

that had been positioned earlier. A fresh pair of disposable latex gloves was used to handle each change of lures. For each replicate, all traps were replaced with new traps, and fresh lures were installed at sunset for the beginning of the next test. Catches were counted the next morning. Treatment positions were changed at least once during an experiment to reduce position effects.

Field Tests. Because *Z*,*Z*-6,9-ket rapidly isomerizes, preliminary tests were conducted in 1998 and 1999 to determine the best time to deploy traps to attract WMTM males. From July 27 to August 7, 1998, traps baited with 5–15 μg of *Z*,*Z*-6,9-ket were positioned early in the morning (06:00 hr) at a site in Sault Ste. Marie harboring WMTM. Additional traps were positioned in the evening ($\sim$21:00 hr) at another site. On July 28, two traps each baited with a newly emerged WMTM female held in a screen cage were deployed at 17:00 hr. They were checked for male catch at 22:30 hr and again at 08:30 hr the next morning. In 1999, four traps baited with 40 μg *Z*,*Z*-6,9-ket were deployed in an arboretum on July 24, at 21:30 hr and checked at regular intervals until 23:00 hr, and then at 08:30 hr the next morning. This was repeated the next evening with four new traps and fresh lures. Traps were checked at regular intervals until 23:30 hr, and then at 08:30 hr the next morning.

Subsequent field tests from 1999 to 2001 were conducted at two sites. The first was a narrow stand of mixed deciduous trees and shrubs along the edge of Algoma University campus in Sault Ste. Marie. Traps were placed about 1.5 m above ground and at about 25-m intervals along a path that ran through the stand. The second site, also in the city, consisted of a similar narrow stand of mixed deciduous trees and shrubs along the edge of an open field at White Pines school. Both locations were within a 15-min drive from the laboratory where the lures were prepared. Because population levels of WMTM were low at sites large enough to contain sufficient treatment replicates, tests were limited to determining the attractiveness of *Z*,*Z*-6,9-ket by itself, and whether its activity was improved by the presence of the other ketones (*Z*6-ket and *Z*,*E*-6,8-ket) identified in the female pheromone extracts. Several binary mixtures of *Z Z*-6,9-ket and *Z*6-ket were tested, including the female ratio ($\sim$2:1), as well as a ternary mixture of *Z*,*Z*-6,9-ket, *Z*6-ket, and *Z*,*E*-6,8-ket in the female ratio (9:5:1).

Treatments were arranged in a complete block design and replicated 10 times in all but one experiment, which had seven replicates. Results were subject to an analysis of variance or a nonparametric Kruskal-Wallis test followed by an analog of the Student-Newman-Keuls (SNK) multiple comparison test (Zar, 1984) to identify significantly ($P \leq 0.05$) different means. Blocks with zero counts for all treatments were not included in the analysis. Means are reported with their standard errors (SE).

Wind-Tunnel Tests. Various samples of *Z*,*Z*-6,9-ket, *Z*6-ket, and *Z*,*E*-6,8-ket were screened individually in a wind tunnel to provide additional information about their effects on male behavior. The wind tunnel was 2 m long with a

1-m × 1-m cross section (Sanders, 1982). The ceiling was covered with a black and white striped canvas to provide males with a visual cue. The airspeed was about 0.35 m/sec. Bioassays were conducted at 23 ± 1°C and ambient relative humidity. Males were 1–2 days old and held in continuous light. Prior to bioassay, males were transferred individually to wire screen cages and conditioned in the wind tunnel for at least an hour. The number of males tested for an experiment varied, depending on availability of laboratory-reared insects.

The following lures were tested. Lure A consisted of 20 or 200 ng of a *Z*,*Z*-6,9-ket sample (stored at −70°C) that was unattractive in field tests conducted about 1 month after synthesis. Subsequent HPLC analysis indicated that it was approximately a 40:60 mixture of *Z*,*Z*-6,9-ket and *Z*,*E*-6,8-ket. Lure B incorporated 20 ng of a sample of *Z*,*Z*-6,9-ket that had been prepared a year earlier and kept at −70°C. The sample contained about 25% *Z*,*E*-6,8-ket based on HPLC retention time. It was not used for field tests. Lure C consisted of 2 or 20 ng of a *Z*,*Z*-6,9-ket sample that was attractive in field tests (Table 1, data for year 2001). It was kept at −70°C and evaluated about two months after the field tests. HPLC analysis of this sample prior to bioassay indicated no ketone impurities were present. Lure D, a field-type lure (i.e., a polyethylene vial closure with cotton batting), contained

TABLE 1. CAPTURE OF MALE WHITEMARKED TUSSOCK MOTHS IN STICKY TRAPS BAITED WITH KETONE COMPONENTS IDENTIFIED IN FEMALE PHEROMONE EXTRACTS (SAULT STE. MARIE, ONTARIO)

Location (date)	Lure components	Quantity (μg)	Catch (mean ± SE)[a]	Statistical test
Algoma Univ. (3 nights, July 22–24, 1999)				
	Z,*Z*-6,9-ket	12	3.1 ± 1.2 a	(*N* = 8, Kruskal-Wallis with SNK)
	Z,*Z*-6,9-ket + *Z*6-ket	12 + 20	2.1 ± 0.5 a	
	*Z*6-ket	20	0.1 ± 0.1 b	
Algoma Univ. (3 nights, July 28–August 2, 2000)				
	Z,*Z*-6,9-ket	10	1.6 ± 0.4 a	(*N* = 10, Kruskal-Wallis with SNK)
	Z,*Z*-6,9-ket + *Z*6-ket	10 + 5	0.7 ± 0.2 b	
	*Z*6-ket	10	0 c	
	Blank		0 c	
White Pines school (5 nights, August 20–31, 2000)				
	Z,*Z*-6,9-ket	20	4.4 ± 0.9 a	(*N* = 7, ANOVA)
	Z,*Z*-6,9-ket + *Z*6-ket	20 + 0.2	4.0 ± 0.9 a	
	Z,*Z*-6,9-ket + *Z*6-ket	20 + 2	2.3 ± 0.7 a	
Algoma Univ. (4 nights, August 20–September 1, 2001)				
	Z,*Z*-6,9-ket	1	1.4 ± 0.4 a	(*N* = 8, Kruskal-Wallis with SNK)
	Z,*Z*-6,9-ket + *Z*6-ket	1 + 0.1	2.0 ± 0.8 a	
	Z,*Z*-6,9-ket + *Z*6-ket	1 + 1	0 b	

[a] Means within an experiment and followed by different letters indicate that treatments are significantly different ($P \leq 0.05$).

TABLE 2. CAPTURE OF MALE WHITEMARKED TUSSOCK MOTHS IN STICKY TRAPS BAITED WITH *Z*,*Z*-6,9-KET AND BLENDS WITH OTHER KETONE COMPONENTS IN RATIO IDENTIFIED IN FEMALE PHEROMONE EXTRACTS (ALGOMA UNIVERSITY, SAULT STE. MARIE, ONTARIO, AUGUST 3–7, 2000)

Lure components	Quantity (μg)	Catch (mean $\pm$ SE, $N = 10$)[a]
Z,*Z*-6,9-ket	20	1.4 ± 0.5 a
Z,*Z*-6,9-ket + *Z*6-ket	$20 + 10$	0.7 ± 0.3 a
Z,*Z*-6,9-ket + *Z*6-ket + *Z*,*E*-6,8-ket	$20 + 10 + 2$	0.5 ± 0.2 a

[a] Means followed by the same letter indicate no differences among treatments, ANOVA ($P > 0.05$).

12 μg of a *Z*,*Z*-6,9-ket sample that was also attractive in field tests (Table 1, July 1999 data). Lure E consisted of 20 and 200 ng doses of *Z*6-ket, which had been used in field tests (Tables 1 and 2). The final lure tested used 200 ng of *Z*,*E*-6,8-ket.

With the exception of lure D, the lure substrate consisted of 2- $\times$2-cm glass plates (histological slides) prepared by treating them in a fume hood with 1–10 μl of the test solution. As soon as the solvent evaporated, the glass plate was put into a small screen cage to facilitate handling, and then stored in a Petri dish until used. Lure D (field-type) was prepared similarly. All lures were used immediately after preparation. They were suspended from a wire support about 40 cm above the floor of the wind tunnel. Fresh lures were prepared each day.

The following behavioral events were recorded: (1) the number of males that became sexually excited (wing-fanning behavior) following exposure to the pheromone plume and the time elapsed before they did so; (2) whether or not released males "locked" onto the pheromone plume; and (3) the amount of time males took to reach the lure from the release point (about 160 cm away) and the nature of their flight to the lure. Data for the time required to reach the lures were analyzed by the nonparametric Kruskal-Wallis analysis of variance test (Zar, 1984). A normal approximation of the binomial test using Minitab statistical software, (release 13.1, Minitab Inc., State College, Pennsylvania, USA) was used to compare two proportions.

RESULTS

Identification of Sex Pheromone Components. GC analyses of the WMTM female extracts indicated the presence of three EAD–active components (Figure 1), one of which was readily identified as *Z*6-ket by matching retention times of a synthetic sample on DB-1 and DB-210, as well as GC-CI-MS (*m*/*z* 309 $M+H^+$, 291 $M+H^+$-H_2O, 169 $C_{10}H_{21}CO^+$, 167 $C_{10}H_{19}CO^+$). The second EAD-active

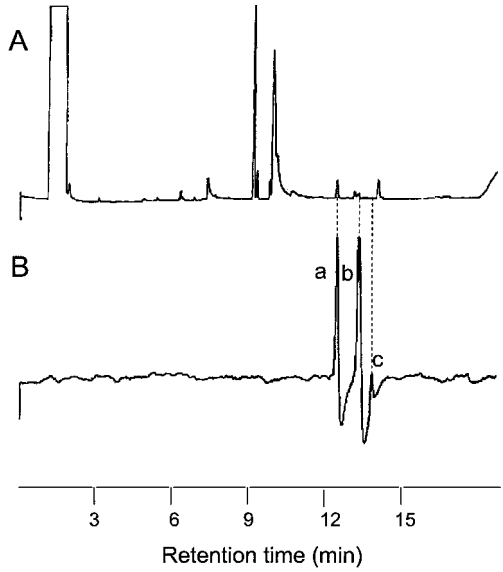

FIG. 1. Comparison of GC and EAD analysis of whitemarked tussock moth (WMTM) female extract on DB-1. (A) GC-FID response to female extract (0.5 FE injected); (B) EAD response of male WMTM antenna to female extract (0.2 FE): (a) *Z*6-ket; (b) *Z*,*E*-6,8-ket from isomerization of female produced *Z*,*Z*-6,9-ket; and (c) *Z*,*E*-6,9-ket presumably from isomerization of female produced *Z*,*Z*-6,9-ket.

eluting component indicated a C_{21}-dienone by GC-CI-MS (*m*/*z* 307 $M+H^+$, 169 $C_{10}H_{21}CO^+$). The *m*/*z* 169 fragment was indicative of an acylium ion fragmentation of an 11-one, which meant the two double bonds were on the same side chain of the carbonyl. The retention characteristics and GC-CI-MS spectrum closely matched that of *Z*,*E*-6,8-ket.

Our early studies had established that *Z*,*E*-6,8-ket was not the primary WMTM pheromone component and that the critical component was thermally unstable, resembling *Z*,*Z*-6,9-ket prepared for our study of DFTM (Gries et al., 1997). *Z*,*Z*-6,9-Ket undergoes a thermal sigmatropic isomerization to *Z*,*E*-6,8-ket with a half-life of approximately 12 hr at room temperature (Wimalaratne, 1998), and undergoes isomerization under GC conditions to both *Z*,*E*-6,8-ket and (*Z*,*E*)-6,9-heneicosadien-11-one (*Z*,*E*-6,9-ket), the third EAD-active component observed in the GC-EAD.

HPLC of fresh WMTM female extract [5 female equivalents (FE)] indicated *Z*,*E*-6,8-ket (9.2 min, 0.5 ng/FE), *Z*,*Z*-6,9-ket (12.8 min, 4.5 ng/FE), and <0.1 ng of *Z*,*E*-6,9-ket (11.2 min) (Figure 2) (Liu, 1999). GC analysis showed *Z*6-ket to be present at 2.5 ng/FE (retention indices: DB-1, 2232; DB-210, 2589).

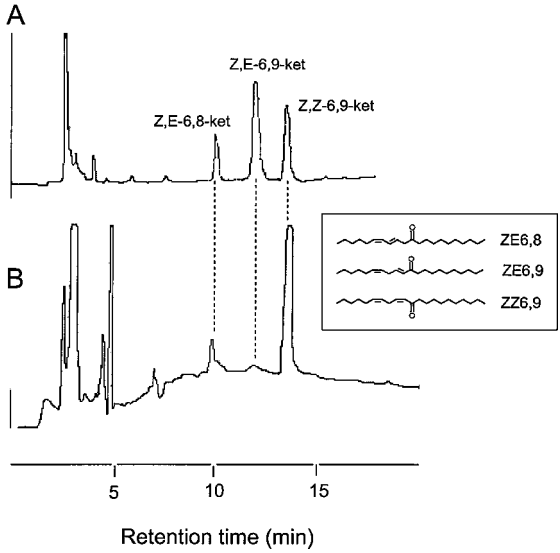

FIG. 2. HPLC analysis (220 nm) of (A) synthetic standards *Z*,*E*-6,8-ket, *Z*,*E*-6,9-ket and *Z*,*Z*-6,9-ket and (B) female extract of whitemarked tussock moth extract (5 FE). The detection wavelength was chosen to provide maximum absorption for conjugated dienes, hence the *Z*6-ket component was not detected.

Field Tests. In preliminary tests in 1998, traps baited with *Z*,*Z*-6,9-ket and deployed at dusk (20:45–21:30 hr) began to catch males shortly thereafter, whereas similarly baited traps deployed in the early morning (06:30 hr) did not. With female-baited traps, a total of 17 males were caught in two traps by 22:30 hr and an additional 27 males by 08:30 hr the next morning. In 1999, four traps baited with *Z*,*Z*-6,9-ket caught only 1 moth from 21:00 to 23:30 hr, but an additional 18 moths were caught by 08:30 hr the next morning. On the following night, 3 moths were caught by 23:00 hr, and an additional 6 moths by 08:30 hr the next morning. Thus, peak moth flight apparently occurred sometime after 23:00–23:30 hr. These observations also indicated that *Z*,*Z*-6,9-ket lures retained activity well after they were deployed. No traps baited with *Z*,*Z*-6,9-ket were attractive 24 hr later. Hence, in all subsequent tests, traps baited with *Z*,*Z*-6,9-ket were deployed at dusk and fresh lures were used for each night of trapping.

In tests conducted from 1999 to 2001, traps baited with 1–20 μg of *Z*,*Z*-6,9-ket were attractive to WMTM males, while traps baited with 10–20 μg *Z*6-ket were significantly less attractive and caught only a few moths (Table 1). Adding *Z*6-ket to *Z*,*Z*-6,9-ket in various ratios, including the female ratio (1:2), did not enhance the attractiveness of *Z*,*Z*-6,9-ket; often it reduced the catch slightly, although this reduction was significant in only one test (Tables 1 and 2). A test of the

ternary blend of the three ketones (*Z*,*Z*-6,9-ket, *Z*6-ket, and *Z*,*E*-6,8-ket) in a ratio approximating that found in female extracts (9:5:1, respectively) was no more attractive than *Z*,*Z*-6,9-ket alone (Table 2). Overall, moth catches were low, reflecting low population levels. Repeated searches for larvae or cocoons with egg masses at both test sites were unsuccessful over the 3-year period.

Wind-Tunnel Bioassays. At the 20-ng dosage, all samples of *Z*,*Z*-6,9-ket (lures A, B, and C) elicited sexual wing-fanning behavior from 91% or more of the males, and the majority (85–95%) of them "locked on" to the pheromone plume (Table 3). The proportion of males reaching the pheromone source varied directly with the purity of the *Z*,*Z*-6,9-ket sample, however.

With lure A (containing 60% *Z*,*E*-6,8-ket), almost all males that had locked on to the pheromone plume from both the 20- and 200-ng lures failed to move forward beyond their release point. Male flights typically consisted of wide lateral swings (almost to the walls of the tunnel) with little forward progress. Some males, still in the plume, drifted backward until they exited the downwind end of wind tunnel. Others left the plume and flew to the walls or ceiling of the tunnel. As a consequence, less than 10% of the males that became excited reached this lure (20 and 200 ng dosages combined). Response to lure B, containing less *Z*,*E*-6,8-ket than lure A, was similar.

In contrast, at least 75% of the males excited by lure C (no *Z*,*E*-6,8-ket detected) at the same (20 ng) or lower (2 ng) dosage reached it, which was significantly better than lures A and B ($P < 0.01$ in all comparisons). Generally males moved steadily forward from the release point with a characteristic darting, zigzag flight and arrived at the lure after 28–32 sec. This time was similar, on average, for all males that successfully reached the other test lures, B, D, and E ($H = 1.24$, $df = 4$, $P = 0.8$) (Table 3). Although the overall responses to lure C at the 0.2-ng dosage were lower than those with the higher dosages, more than 50% of the males succeeded in reaching the lure.

The response of males to lure D, the attractive field lure, was similar quantitatively and qualitatively to those responding to lure C at the 20- and 2-ng dosages. There was no difference in the time it took males to reach lure D ($H = 0.94$, $df = 2$, $P = 0.6$).

WMTM males were also attracted to the *Z*6-ket lure, lure E, but they were less responsive to it (Table 3). Even with a dose 100-fold greater than *Z*,*Z*-6,9-ket (200 ng vs. 2 ng or 20 ng vs. 0.2 ng), a lower proportion of males locked on to the pheromone plume ($P < 0.001$ and < 0.05, respectively) or reached the lure ($P = 0.06$ and < 0.01, respectively), although the time required to do so (34 vs. 26–28 sec) was similar. Otherwise the flight and wing fanning behavior of WMTM males to these compounds was essentially the same.

When males arrived at any of the above attractive lures, they typically landed and walked over them while fanning their wings, but no copulatory behavior was observed. Bouts of walking and wing fanning on the lures usually lasted 1–3 min

TABLE 3. RESPONSE OF MALE WHITEMARKED TUSSOCK MOTHS IN FLIGHT TUNNEL TO LURES CONTAINING VARIOUS SAMPLES OF *Z,Z*-6,9-KET OR *Z*6-KET

Lure Chemical	Dosage[a]	Males tested (*N*)	Wing fanning		Locking on plume [%(*N*)][c]	Reaching lure [%(*N*)][c]	Time to reach lure (sec) (*N*)
			% (*N*)	Time (sec)[b]			
A *ZZ*-6,9-ket	20 ng[d]	14	93 (13)	3.8 ± 1.3	85 (11)	8 (1)	
	200 ng[d]	11	100 (11)	1.8 ± 0.3	64 (7)	9 (1)	
B *ZZ*-6,9-ket	20 ng[e]	23	91 (21)	10.0 ± 2.7	90 (19)	38 (8)	36.4 ± 8.4 (8)
C *ZZ*-6,9-ket	0.2 ng	24	75 (18)	11.4 ± 2.8	56 (10)	56 (10)	26.1 ± 6.8 (10)
	2 ng	25	88 (22)	4.1 ± 0.9	95 (21)	75 (18)	28.1 ± 2.1 (18)
	20 ng	29	100 (29)	3.0 ± 0.6	93 (27)	79 (23)	32.5 ± 3.9 (23)
D *ZZ*-6,9-ket	12 μg	23	91 (21)	5.1 ± 1.5	76 (16)	62 (13)	42.5 ± 9.1 (13)
E *Z*6-ket	20 ng	36	64 (23)	2.4 ± 0.9	26 (6)	17 (4)	33.8 ± 4.0 (4)
	200 ng	20	75 (15)	7.8 ± 3.6	53 (8)	53 (8)	34.0 ± 5.5 (8)

[a] With the exception of lure D, lure substrate consisted of 2- ×2-cm glass plates treated with the test compound. Lure D was a field-type lure consisting of polyethylene cap with a plug of cotton batting to receive the test compound.
[b] Time elapsed before males began wing fanning.
[c] Percentage of those males that become excited (i.e., wing fanning).
[d] Amount applied was nominally 20 and 200 ng. Later HPLC analysis indicated sample was only about 40% *Z,Z*-6,9-ket and 60% *Z,E*-6,8-ket.
[e] Containing 25% *Z,E*-6,8-ket.

but could be as short as 5–10 sec. When not on the lure, males would buzz around it intensely, sometimes recasting and reapproaching the lure several times.

The lure consisting of 200 ng of *Z*,*E*-6,8-ket did not elicit any sexual wing fanning or other overt behavior from any of the males tested ($N = 20$).

DISCUSSION

Our results show that *Z*,*Z*-6,9-ket is the essential sex pheromone component of the WMTM. It is present in pheromone extracts of WMTM females at about 4–5 ng/female, along with 2.5 ng/female of *Z*6-ket, about 0.5 ng/female of *Z*,*E*-6,8-ket, and a trace amount of *Z*,*E*-6,9-ket. During synthesis of potential DFTM pheromone candidates, we had synthesized *Z*,*Z*-6,9-ket (Gries et al., 1997), a material that we recognized would be unstable due to the presence of the skipped diene system conjugated to the ketone. We attribute the presence of the small amount of *Z*,*E*-6,8-ket and *Z*,*E*-6,9-ket in WMTM females to the fast stereospecific isomerization of *Z*,*Z*-6,9-ket to these isomers at room temperature (Wimalaratne, 1998) during the female calling time and the time taken for isolation prior to HPLC separation. The instability of *Z*,*Z*-6,9-ket was surprising, not only undergoing the expected acid and base catalyzed isomerizations, but also a solvent-independent sigmatropic isomerization with a half-life of about 12 hr (Wimalaratne, 1998). Previously it was found that WMTM pheromone extracts lost substantial behavioral activity over time (Grant, 1975). This loss of activity is now accounted for by the rapid isomerization of the *Z*,*Z*-6,9-ket component. The residual behavioral activity of those extracts was probably due to the remaining *Z*6-ket component, which can sexually stimulate WMTM males (Grant, 1977).

Both wind-tunnel and field tests confirmed that *Z*,*Z*-6,9-ket is attractive to male WMTM. The facts that the field populations were low and that as little as 1 μg in traps was effective indicate that this diene ketone is a potent sex attractant. We were unable to increase its activity by adding *Z*6-ket in various ratios, including that detected in female extracts. Indeed, there was a tendency for reduced catch, at least with a higher ratio of *Z*6-ket. Adding *Z*,*E*-6,8-ket to form a ternary blend of these components that matched the female ratio also did not enhance catch. As suggested, the presence of *Z*,*E*-6,8-ket in female extracts is probably an artifact due to the spontaneous and rapid isomerization of *Z*,*Z*-6,9-ket. In the wind tunnel, the purest sample of *Z*,*Z*-6,9-ket was the most attractive. Less attractive lures in both wind-tunnel and field tests contained *Z*,*E*-6,8-ket, albeit at relatively high levels. By itself, *Z*,*E*-6,8-ket had no behavioral activity.

Traps baited with *Z*6-ket alone caught few moths, although it was clearly attractive in the wind tunnel and in previously reported field tests (Grant, 1977). The lack of catch may be attributed to low population levels, a lower dosage of *Z*6-ket than tested earlier, and the fact that it is a weaker attractant than *Z*,*Z*-6,9-ket. Our wind-tunnel tests indicated that it is at least 100-fold less attractive to WMTM

males than *Z*,*Z*-6,9-ket. *Z*6-Ket is also a weak attractant for the DFTM when compared to its binary blend with synergistic *Z*,*E*-6,8-ket (Gries et al., 1997). Its role in female WMTM remains unclear.

The discovery that WMTM males began their flight around sunset was unexpected. Previously, it was reported that males were attracted to traps in the early morning, before and after sunrise (Grant, 1977). This was based on the observation that new males appeared in traps that had been emptied after sunrise. In that study, however, traps were positioned during the day and not checked during the evening, so that any flight then would have been overlooked. Our observations on arrival time of males at pheromone-baited traps in the present study indicate that, although male flight began near sunset, peak flight occurred in the later half of the night, which is consistent with the earlier study and with the peak calling time of females (Grant et al., 1975). Nonetheless, the period of male sexual activity is unusually broad.

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EVIDENCE FOR A SHORT-RANGE SEX PHEROMONE IN FEMALE *Maladera matrida* BEETLE

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Abstract—Laboratory studies with live and frozen *Maladera matrida* female and male beetles showed that males were attracted to chemical substances emanating from the females at dusk. Beetles exhibited sexual activity (including mating) at dusk towards frozen females but not towards frozen males. Frozen females that had been extracted with methanol together with either hexane or dichloromethane or with a mixture of all three solvents did not elicit male sexual activity. Activity was fully restored when a concentrate of the extract was applied to the previously extracted frozen females. Males also responded with vigorous sexual activity to frozen males to which female extract had been applied. Deterrent chemicals appear to be absent from the male body. Males exposed to females that had been frozen during the morning displayed weak sexual activity, indicating that females lack active semiochemicals. Differences between dusk and morning extracts were found with respect to more than 20 compounds, some of which were present in much higher concentrations at dusk than in the morning, while others were not detected in the morning extract. The active component(s) of the short-range sex pheromone of female *M. matrida* is (are) presumably to be found among these compounds.

Key Words—*Maladera matrida*, beetle, Scarabaeidae, Coleoptera, sex pheromone, sexual activity.

INTRODUCTION

In 1983, the scarab beetle, *Maladera matrida* Argaman (Coleoptera, Scarabaeidae, Melolonthinae), was discovered in Israel and described as a new species (Argaman, 1986, 1990). A study of chemical communication was launched as soon as it was

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realized that this species is a severe pest, both during the larval stage—when it attacks plant roots and subterranean crops (peanut, potato, sweet potato, carrot)—and during the adult stage—when it feeds on flowers and foliage (Ben-Yakir et al., 1995).

Field studies showed clearly that the optimal bait for long-range attraction of males consisted of live females together with food (cut-up peanut leaves). Male responses to females alone or food alone were low. Attraction of females to live females and food was much lower, probably because females were attracted solely by the volatiles released from the peanut leaves (Yarden and Shani, 1994; Falach and Shani, 2000a).

(*Z*,*E*)-α-Farnesene was identified as a bioactive volatile chemical emitted by the females triggering an electroantennodetector (EAD) response in both male and female antennae (Yarden et al., 1996). However, preliminary field studies could not confirm its role as an aggregation pheromone, and this discrepancy between laboratory and field studies remains to be resolved (Yarden, Falach, and Shani, unpublished results).

Field observations showed that adult *M. matrida* beetles emerge from the soil at dusk to feed, aggregate, and mate on foliage (Harari et al., 1994; Yarden and Shani, 1994). The males emerge a few minutes before the females, which subsequently join the males to make a 1:1 ratio in the aggregations. Both field studies (Yarden and Shani, 1994) and preliminary laboratory observations (Falach and Shani, unpublished results) indicate that it is the male beetles that are attracted to the females and not *vice versa*. The long-range attraction of males (and to a lesser extent of females) to a combination of females and food, and the observed aggregation of both sexes in the field led us to hypothesize that there are two separate (and possibly parallel) stages in the sexual attraction and aggregation behavior. Females are likely first attracted to plant volatiles released when previously emerged males feed, and then males are attracted to females after a short-range (contact?) sex pheromone is released. An alternative scenario would be long-range attraction of males to feeding females, followed by release of a short-range (contact?) sex pheromone by the females.

Little is known of the short-range chemically mediated behavior of *M. matrida*. Falach and Shani (2000b) reported that male *M. matrida* were not attracted to live or frozen males. In fact, males retracted into their holes when presented with frozen males. The current study was conducted to investigate the short-range attraction and behavior of males toward females. For *M. matrida*, it is challenging to conduct a field study or observe the beetles at dusk, even if they are confined in a net cage. We, therefore, conducted a laboratory study on frozen females, since—like live specimens—they contain the chemicals thought to have biological activity and are closer in shape to live females than any other available option (shoelace model, dummy beetles).

In this work, we demonstrate that chemical components emanating from the female beetles trigger male sexual activity towards the females.

METHODS AND MATERIALS

Rearing of Beetles. Third-instars of *M. matrida* were collected in the field and reared individually in 5-ml plastic vials containing humid sand. Larvae were fed wheat roots. Adult beetles were sexed and reared in sand in groups (up to 100 beetles of the same sex in a 1-liter plastic container, since no difference in behavior or longevity was found between large and small groups) and fed with rose flowers. The vials and the containers were kept in an incubator under a 16L:8D photoperiod at 30 and 26°C and 60% ± 1% relative humidity.

Laboratory Behavioral Studies. In *M. matrida*, male sexual behavior towards the female includes a variety of activities, with mating the final crucial step. The behavior of the males was characterized in terms of five behavioral patterns: mating, mating attempt, mating disturbance, touching a female, and wing fanning. The first three have a direct relationship to the sexual behavior of the males toward the females, while the latter two are not necessarily related to the sexual ritual and are, therefore, not included in the results. Behavior was designated as "mating" when beetles took up a mating posture (mounting of the male on the back of the female) followed by copulation for 30–40 min. For mating, both excited males and receptive females are required. The case of female refusal to the mounting male was designated "mating attempt." There is no indication whether this behavior was due to discrimination of the female against the male or whether the female was simply not yet receptive. The third type of behavior, designated "mating disturbance," took place when a male beetle tried to push a mating male from a female beetle. Some of the behavioral patterns have been photographed (Falach and Shani, 2000b).

In each experiment, 10 living adult males (marked—to distinguish them from the females—with white correction fluid, which did not affect their behavior) were placed on bedding sand in a glass container (30 × 30 × 30 cm) at least 3 hr before the introduction of five live or five frozen females or five frozen males. Females that had been frozen the same evening by exposure to −13°C for 10–15 min (designated "fresh frozen") were thawed a few minutes prior to the experiment, while females that had been frozen 1–2 days previously were thawed 3 hr prior. The behavioral studies conducted with frozen, rather than live, females were designed to eliminate effects of nonreceptive or selective females on the number of incidents of mating attempts.

All experiments were conducted at dusk (except where otherwise stated), and replicated five times. Each replicate lasted 50 min.

Chemicals. *n*-Hexane, dichloromethane, and methanol AR grade (Frutarom, Haifa, Israel) were used after checking that no deterring effects were observed after evaporation. For GC-MS derivatization *O*-methoxy amine HCl salt (Supelco, Bellefonte, Pennsylvania, USA), pyridine (Aldrich, Milwaukee, Wisconsin, USA), and *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical Co., Rockford, Illinois, USA) were used as reagents.

GC-MS Identification. Solvents were evaporated by gently blowing N_2 (99.95% pure) to reduce the sample volume to 0.5 ml. The components of each extract were separated and identified by high resolution GC-MS (Hewlett Packard 5890 Series II chromatograph coupled with an AutoSpec E High Resolution Mass Spectrometer, HR-MS). Compounds were separated by utilizing a 30-m $\times$ 0.32-mm $\times$ 0.25, μ m DB-1 capillary column (J&W Scientific), temperature programmed from 100°C (hold 1 min) to 280°C (hold 15 min) at 6°C/min.

Mass spectra were obtained by using two ionization modes: electron impact (EI) and chemical ionization (CI). The electron energy in both was 70 eV. The reagent gas used in CI was methane. Individual compounds were identified by: (1) exact mass analysis through high resolution mass measurements, (2) comparison of MS spectra and retention times with those of known compounds, (3) matching spectra with those of similar compounds found in the database (NIST Library), and (4) oximation and silylation for identification of keto and hydroxy groups.

Surface or Cuticle Extraction. Adult beetles (females or males) were extracted at dusk with various combinations of three organic solvents of different polarities: *n*-hexane, dichloromethane, and methanol. Each extraction was accomplished by immersing 12 beetles in 7–8 ml of solvent for 10–12 min and then repeating the procedure once.

Application of Extract Concentrate. The three solvent extracts were combined and evaporated by gently blowing N_2 (99.95% pure) onto the solvent to reduce the volume to 60 μl. A sample of 10 μl (2 female equivalents) of the concentrate was applied to each extracted, frozen female or male, which was then allowed to dry for 90 min before the onset of the experiment.

Statistical Analysis. All results were tested by χ^2 test. All calculations were done manually ($P < 0.05$).

RESULTS AND DISCUSSION

Live Versus Frozen Females. In a previous study, we showed that males did not respond to live or frozen males (Falach and Shani, 2000b). The first experiment of the current study was aimed at examining the behavior of males towards live versus frozen females. Semiochemicals produced by the female at dusk serve to attract males and arouse them to vigorous sexual behavior even when present in frozen specimens (Table I). The higher scores obtained for the frozen females may

TABLE 1. RESPONSE OF *Maladera matrida* MALES TO FROZEN OR LIVE FEMALES

Behavior	Behavioral (responses) towards females[a]		χ^{2b}
	Frozen	Live	
Mating	7.6 ± 1.1	2.2 ± 1.1	14.9*
Mating disturbance	5.0 ± 3.0	0.0 ± 0.0	25.0*
Mating attempt	7.2 ± 1.5	1.2 ± 1.1	21.4*

[a] Values are means $\pm$ SE for 10 males responding to 5 females. This study was replicated five times with different insects in each replicate.
[b] Test statistics followed by * indicate significant difference between mean responses to frozen females and live females by behavioural patterns (χ^2 test, $df = 1$, $P < 0.05$).

be due to their inability to escape the male and to the shorter duration of the mating period, which lasted for only 5–10 min (with live females it lasted for 30–40 min). The differences in male response to frozen and live females and the reproducibility of the data (Table 1 and further results) support our argument for working with frozen specimens. To verify that the duration of freezing did not adversely affect the biological activity of the female semiochemicals, the mating response of males to females frozen for 24 or 48 hr was compared with that to fresh-frozen females (Table 2). The responses to females frozen for 24 or 48 hr prior to the experiment were not significantly different from those to fresh-frozen females.

Removal of Biological Activity by Immersion of Frozen Females in Solvents. The next set of experiments was designed to test the sexual behavior of males toward frozen females that had been extracted in various combinations of three organic solvents: hexane, dichloromethane, and methanol. Previous work (Falach and Shani, 2000b) showed that the solvent itself had no negative effect on the male response. Extraction with hexane or dichloromethane alone, both of which

TABLE 2. EFFECT OF DURATION OF FREEZING ON RESPONSE OF *Maladera matrida* MALES TO FROZEN FEMALES

Behavior	Behavioral (responses) towards females[a]					
	Frozen 24 hr	Fresh frozen	χ^2	Frozen 48 hr	Fresh frozen	χ^2
Mating	7.2 ± 0.8	7.6 ± 0.9	0.1	8.4 ± 1.1	7.4 ± 1.5	0.3
Mating disturbance	8.4 ± 6.3	8.4 ± 1.3	0.0	5.8 ± 1.8	6.4 ± 2.4	0.1
Mating attempt	2.8 ± 1.9	3.6 ± 1.9	0.5	3.8 ± 2.6	2.6 ± 3.7	1.1

[a] Values are means $\pm$ SE for 10 males responding to 5 females. This study was replicated five times with different insects in each replicate**.** Responses to females frozen for 24 hr or 48 hr were not significantly different from responses to fresh-frozen female beetles (χ^2 tests, $df = 1$, $P < 0.05$).

TABLE 3. RESPONSE OF *Maladera matrida* MALES TO FROZEN FEMALES EXTRACTED WITH DIFFERENT SOLVENT COMBINATIONS

Extraction solvent			Behavioral Response								
			Mating[b]			Mating disturbanc[b]			Mating attempts[b]		
Hexane	DCM[a]	Methanol	Extracted[c]	Nonextracted	χ^2	Extracted[c]	Nonextracted	χ^2	Extracted[c]	Nonextracted	χ^2
+	+	+	0.0 ± 0.0	7.8 ± 1.3	39.0[d]	0.0 ± 0.0	8.4 ± 3.5	42.0[d]	0.6 ± 1.3	9.8 ± 5.5	40.7[d]
+	+	−	2.4 ± 1.1	6.0 ± 1.0	7.7[d]	0.8 ± 1.3	3.0 ± 1.9	6.3[d]	1.4 ± 1.3	1.8 ± 1.1	0.3
+	−	+	0.4 ± 0.5	6.8 ± 1.1	28.4[d]	0.4 ± 0.9	7.2 ± 0.4	30.4[d]	0.0 ± 0.0	1.0 ± 1.0	5.0[d]
−	+	+	0.4 ± 0.5	6.8 ± 0.4	28.4[d]	0.0 ± 0.0	6.8 ± 1.6	34.0[d]	0.0 ± 0.0	0.6 ± 0.5	3.0
+	−	−	6.6 ± 2.9	5.0 ± 0.7	0.1	3.4 ± 3.2	6.2 ± 3.8	2.4	4.4 ± 1.8	2.8 ± 1.6	1.8
−	+	−	5.4 ± 1.7	7.8 ± 2.0	2.1	3.8 ± 2.6	6.4 ± 1.5	3.3	1.0 ± 1.2	1.8 ± 0.4	1.1
−	−	+	3.2 ± 1.3	6.8 ± 3.8	6.5[d]	2.0 ± 2.0	5.6 ± 5.5	8.5[d]	1.2 ± 0.8	2.8 ± 0.4	2.3

[a] DCM = dichloromethane.
[b] Values are means ± SE for 10 males responding to 5 females. This study was replicated five times with different insects in each replicate.
[c] Five females extracted by solvent.
[d] Indicates test statistic corresponding to significantly different responses to extracted vs. non-extracted females. (χ^2 tests, $df = 1$, $P < 0.05$).

TABLE 4. RESPONSE OF *Maladera matrida* MALES TO FROZEN FEMALES SUBJECTED TO VARIOUS TREATMENTS

Behavior	Behavioral responses[a]					
	Nonextracted Females (I)	Extracted females (II)	Extracted females plus application of FE[b] (III)	χ^2		
				I/II	II/III	I/III
Mating	7.2 ± 2.4	1.6 ± 1.1	7.8 ± 2.3	17.8*	20.4*	0.1
Mating disturbance	5.0 ± 2.4	0.0 ± 0.0	4.0 ± 2.6	25.0*	20.0*	0.6
Mating attempt	1.6 ± 1.1	0.4 ± 0.5	4.6 ± 3.0	3.6	17.6*	7.3*

[a] Values are means $\pm$ SE for 10 males responding to 5 females. This study was replicated five times with different insects in each replicate. *Values are significantly different according to χ^2 tests ($df = 1$, $P < 0.05$).

[b] Two female equivalents (FE) in 10 μl of dichloromethane were applied topically onto each female 3 hr before the experiment.

remove hydrocarbons (Cojocaru, Falach, and Shani, unpublished results; Falach and Shani, 2000b), did not lead to a significant reduction in any behavior patterns compared with nonextracted females (Table 3). A combination of each one of the two with methanol, on the other hand, did lead to a reduction in sexual ritual patterns. This indicates that the components of the hydrocarbon fraction play a limited role in sexual activity and, hence, that hydrocarbons are probably not involved in the sex pheromone components of the beetle. At most, cuticular hydrocarbons exhibit some activity when present in combination with polar components. Methanol alone removed some of the activity, hinting at a role for polar component(s). Application of all three solvents completely removed all male sexual activity toward the females, as did application of methanol together with hexane or dichloromethane. It, therefore, follows that methanol is the critical solvent.

Application of Concentrated Extract to Frozen Females to Restore Biological Activity. To prove that we were studying an extractable complex of behavioral chemicals, we restored the male response to frozen extracted females by applying female extract to the extracted specimens (Table 4). No difference was found between nonextracted (column I) and extracted females that had been rescued by application of extract (column III). Based on the number of females extracted and the number of females tested, we calculated that each female was applied with 2 female equivalents (FE) of extract. When extracted females were treated with more than 2 FE, the behavior response decreased (data not shown). These experiments demonstrated that the extracts contain active compounds and that these compounds can be removed from the female.

Application of Concentrated Female Extract to Frozen Males. The ultimate proof that the female extracts have biological activity was provided by the response of live males to frozen males treated with these extracts. As mentioned,

TABLE 5. RESPONSE OF *Maladera matrida* MALES TO FROZEN MALES EXPOSED TO VARIOUS TREATMENTS AND TO NON-EXTRACTED FEMALES

Behavior	Behavioral responses[a]				χ^2					
	Extracted[b] males plus application of FE[c] (I)[d]	Nonextracted males plus application of FE[c] (II)[d]	Nonextracted females (III)[d]	Extracted[b] males (IV)[d]	I/II	I/III	I/IV	II/III	II/IV	III/IV
Mating	4.4 ± 1.8	4.4 ± 3.0	4.6 ± 1.1	0.0	0.0	0.0	22.0*	0.0	22.0*	23.0*
Mating disturbance	1.4 ± 1.9	1.0 ± 1.2	2.2 ± 0.8	0.0	0.3	0.9	7.0*	2.3	5.0*	11.0*
Mating attempt	2.6 ± 1.3	2.0 ± 2.5	1.6 ± 2.1	0.0	0.7	0.2	6.5*	0.2	10.0*	8.0*

[a] Values are means $\pm$ SE for 10 males responding to 5 males or 5 females. This study was replicated five times with different insects in each replicate.
[b] Extraction with all three solvents.
[c] Two female equivalents (FE) in 10 μl of dichloromethane were applied topically onto each male 3 hr before the experiment.
[d] No significant differences between mean responses were found among the three first columns, but responses for extracted males (last column) were significantly different from responses for all other treatments (χ^2 tests, $df = 1$, $P < 0.05$).

TABLE 6. RESPONSE AT DUSK OF *Maladera matrida* MALES TO FEMALES FROZEN IN THE MORNING OR AT DUSK VERSUS RESPONSE TO LIVE FEMALES

	Behavioral responses[a]					
	Morning-frozen females	Live females (dusk)	Dusk-frozen females	χ^2		
Behavior	(I)	(II)	(III)	I/II	II/III	I/III
Mating	2.6 ± 1.7	2.6 ± 1.7	9.2 ± 2.6	0.0	18.5*	18.5*
Mating disturbance	0.2 ± 0.4	1.6 ± 1.5	4.8 ± 3.3	5.4*	8.0*	21.2*
Mating attempt	0.0 ± 0.0	1.4 ± 1.1	1.8 ± 1.9	7.0*	0.3	9.0*

[a] Values are means $\pm$ SE for 10 males responding to 5 females. This study was replicated five times with different insects in each replicate. Test statistics followed by * represent pair of means that are significantly different (χ^2 tests, $df = 1$, $P < 0.05$).

TABLE 7. CHEMICAL COMPONENTS FOUND (ALMOST) EXCLUSIVELY IN DUSK EXTRACT OF FROZEN FEMALES

			% area[a]							
			Hexane		DCM		Methanol		Total	
Compound	Formula	MW	D	M	D	M	D	M	D	M
n-Docosane	$C_{22}H_{46}$	310			3.1				3.1	
11-Methylpentacosane	$C_{26}H_{54}$	366			0.5				0.5	
12-Methyloctacosane	$C_{29}H_{60}$	408	0.6		0.1				0.7	
2-Methyltriacontane	$C_{31}H_{64}$	436	0.6						0.6	
4-Methyltriacontane	$C_{31}H_{64}$	436	1.9						1.9	
n-Dotriacontane	$C_{32}H_{66}$	450	0.2						0.2	
7-Methyldotriacontane	$C_{33}H_{68}$	464	0.2						0.2	
11-Methylheptatriacontane	$C_{38}H_{78}$	534	0.6	vs					0.6	
Pentadecanoic acid	$C_{15}H_{30}O_2$	242					0.2		0.2	
Linoleic acid	$C_{18}H_{32}O_2$	280					vs		vs	
Oleic acid	$C_{18}H_{34}O_2$	282		vs	4.0		9.1		13.1	vs
Oleyl palmitate	$C_{34}H_{66}O_2$	506					1.2		1.2	
Oleyl oleate	$C_{36}H_{68}O_2$	532					0.2		0.2	
Dodecyl acetate	$C_{14}H_{28}O_2$	228			9.6	0.6			9.6	0.6
Oleyl alcohol	$C_{18}H_{36}O$	268	1.0	2.3	1.5	2.7	46.2	2.8	48.7	7.8
Cetyl acetate	$C_{18}H_{36}O_2$	284			3.1				3.1	
Oleyl acetate	$C_{20}H_{38}O_2$	310		1.9	9.1	0.2			9.1	2.1
Tremulone	$C_{29}H_{46}O$	410					1.1		1.1	
Stigmasterol	$C_{29}H_{50}O$	414					1.4		1.4	
Fridoolean-3-one	$C_{28}H_{56}O_2$	424					0.6		0.6	
Fridelan-3-one	$C_{30}H_{50}O$	426	3.7						3.7	
Cholesteryl acetate	$C_{29}H_{48}O_2$	428					3.3		3.3	

[a] Percentage of area of each component from the total chromatogram.
DCM = dichloromethane, D = dusk extract, M = morning extract, vs = very small, >0.1%.

live males did not react to frozen males and, in fact, when presented with frozen males, they retreated to their holes after only a few minutes on the ground, as they do not have food to eat or females to mate with. Extracted or nonextracted frozen males treated with female concentrate (2 FE) elicited mating behavior from the live males (Table 5). This indicates that the shape and color of the beetle might not be important in the chemosexual communication of *M. matrida* and that it is the female chemicals that are the crucial element in the attraction and excitation of males. The identical response of the live males towards extracted and unextracted treated males suggests that there are no deterrent chemicals on the male body. Support for this assumption was found when male extracts were applied to frozen females and the active sexual behavior of males was identical to that of males toward frozen females without male extracts (results not shown).

Extracts Prepared at Dusk versus Morning Hours. Since the active period of the beetle is known to be limited to 45–90 min at dusk, we compared male response to females frozen in the morning with their response to females frozen at dusk (Table 6). Only low activity was elicited by the morning specimens. The factor that reduces male sexual activity is presumably the absence or low concentration of female specific compounds. It is likely that the sex pheromone of the beetle is released by the female only during the active period and that it is one or a few of more than 20 compounds, some polar and some apolar (or less polar), that occur exclusively or in greater abundance in the female extracts prepared at dusk (Table 7). By examining each component separately and in combination with others, it should be possible to identify the chemical component(s) of the short-range sex pheromone of *M. matrida*. Studies in this direction are now in progress.

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CUTICULAR LIPIDS OF THE BOOKLOUSE, *Liposcelis bostrychophila*: HYDROCARBONS, ALDEHYDES, FATTY ACIDS, AND FATTY ACID AMIDES

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Abstract—The booklouse, *Liposcelis bostrychophila*, is an increasingly common pest of stored food products worldwide. We report here the cuticular lipid composition of this pest (the first report of the hydrocarbons of any member of the Order Psocoptera and the first report of fatty acid amides as cuticular components for any insect). No unsaturated hydrocarbons were present. A homologous series of *n*-alkanes (C_{21}–C_{34}), monomethyl alkanes (3-, 4-, 5-, 7-, 9-, 11-, 12-, 13- and 15-methyl-) with a carbon chain range of C_{28}–C_{42}, and dimethyl alkanes (3, 7-; 9, 13-; 11, 15-; 13, 17-; 9, 21-; 11, 19-; and 13, 21-) with a carbon number range of C_{31}–C_{43} were identified. The relative abundances of these hydrocarbons were low, comprising approximately 0.0125% of total biomass. The amides were a homologous series (C_{16}–C_{22} in chain length), with the major amide being stearoyl amide. In addition to the amides, free fatty acids ($C_{16:1}$, $C_{16:0}$, $C_{18:2}$, $C_{18:1}$, and $C_{18:0}$ in chain length) and three straight chain aldehydes (C_{15}, C_{16}, and $C_{17:1}$ in chain length) also occurred as cuticular components. These findings are discussed in terms of the chemical and physiological ecology of this species.

Key Words—Psocoptera, chemical ecology, physiological ecology, mass spectra, fatty acid, fatty amide, aldehyde, hydrocarbon, cuticular lipids

INTRODUCTION

The booklouse, *Liposcelis bostrychophilia* Badonnel (Psocoptera: Liposcelididae), occurs worldwide (Lienhard, 1990) and is often considered a pest in households and various segments of the food industry (Turner, 1994). These are tiny (approximately 1 mm in length), wingless, light brown insects that are thelytokously

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parthenogenetic (hence no males have ever been found). They are capable of survival and reproduction on a wide variety of food items, although they are most often found on flour and other farinaceous products. The life cycle includes eggs, four nymphal stages, and adult females. The species does best at moderately high relative humidities (65–80%) and at temperatures between 20°C and 30°C (Turner, 1994). Other than these limited data on population parameters (reviewed in more detail in Turner, 1994), little is known of the biochemical and physiological properties of these insects (Ali, 1999), and their chemical ecology has not been studied. Liposcelidids are known, however, to be highly resistant to entomopathogenic fungi or bacteria (Turner, 1988; J.C.L. unpublished observations), suggesting that they might possess unusual cuticular lipids responsible for their relative immunity. Fungal infections are initiated by conidial attachment and germination on the cuticular surface. Accordingly, surface compounds are logical candidates in a search for antifungal defenses.

We report here the cuticular lipid composition of *L. bostrychophila* and discuss our findings with regard to the ecological parameters of its lifestyle. This is the first report of cuticular hydrocarbons in the order Psocoptera (common name: psocids) as well as the first report of fatty acid amides as cuticular lipid components of any insect.

METHODS AND MATERIALS

Insects. Cultures of *L. bostrychophila* were obtained from contaminated laboratory cultures of the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), originally collected from Manhattan, Kansas, USA and maintained for several years. Psocid cultures were maintained at room temperature (ca. 28°C), ca. 80% relative humidity, under ambient light, and fed on insect-free wheat that had been previously damaged by rusty grain beetles (ensuring particles of wheat of a size most suitable for consumption by these tiny insects). Psocids to be used for experiments were collected from stock cultures using the method of Turner (1990).

Chemical Analyses. Insects were frozen at −5°C for 24 hr, and gently sieved through stacked 40 and 50 mesh sieves to remove most culture contaminants. The larger psocids collected on the 50 mesh sieve were used for extractions. Cuticular lipids were extracted by immersing the insects in three successive 0.25-ml portions of hexane for 10 sec each time. The combined extracts from each sample were concentrated under a stream of N_2, and the total extract was analyzed by mass spectrometry as described below. Hydrocarbons were then isolated by chromatography on a 3-cm-long silica gel column (BioSil A, Bio-Rad Laboratories, Richmond, California, USA) as described earlier (Howard et al., 1978). The psocids are very

small insects (individual psocids weigh ca. 60 μg), containing insufficient hydrocarbon for single insect analyses, so samples consisted of ca. 2 mg of insects per replicate (ca. 333 psocids), and three replicates were analyzed. Compositional data were converted to percent values of the total volatile portion of the extract. An estimate of the total quantity of extractable hydrocarbons was calculated by calibration with an internal standard (eicosane, 57 ng/μl). This estimate was then used to calculate the hydrocarbons as a fraction of the total mass of psocids extracted.

Electron impact mass spectral analyses were conducted using a Hewlett-Packard 5890A gas chromatograph (GC) (Hewlett-Packard, Inc., San Fernando, California, USA) fitted with a DB-5 column (15 m long $\times$ 0.25 mm ID; J & W Scientific, Folsom, California, USA) interfaced to a Hewlett-Packard 5971 mass selective detector (MSD) and a Hewlett-Packard HP G1034C data system. Helium was the carrier gas, with a column head pressure of 0.75 kg/cm^2. Electron impact mass spectra were obtained at 70 eV. The GC was programmed at 100°C/min, 5°C/min to 320°C, 320°C for 20 min. The splitless injector was set at 275°C and the GC-MSD interface was at 280°C. Retention times of each hydrocarbon component and equivalent chain length values (ECL) were obtained by comparison with known *n*-alkane standards (Howard et al., 1978). Individual components were identified from their EI-MS fragmentation patterns (Jackson and Blomquist, 1976; Nelson, 1978) in conjunction with ECL values. Fatty acids and amides were tentatively identified from their characteristic mass spectra (Budzikiewicz et al., 1967). An authentic sample of stearoyl amide was synthesized from stearoyl chloride and ammonium acetate (Fieser and Fieser, 1967), and fatty acids were converted to their methyl esters by reaction with (1,1)-*N*,*N*-dimethylhydrazine (McDaniel and Howard, 1985). GC-MS analysis of the synthetic amide and fatty acid esters (Ryhage and Stenhagen, 1963) were then conducted to verify preliminary identifications. Aldehydes were detected by their conversion to *N*,*N*-dimethyl hydrazones and identified as such from their characteristic EI mass spectra. Comparisons to authentic standards were not made.

Verification that the lipids obtained were indeed of cuticular origin was obtained by gently wiping several insects with a 7-μm polydimethylsiloxane bonded-phase fiber in a Supelco (Bellafonte, Pennsylvania, USA) solid-phase microextraction (SPME) holder and then analyzing the absorbed lipids by GC-MS using the parameters listed above, with the exception that the fiber was desorbed for 2 min at 280°C with the septum purge closed before beginning the temperature program.

Identification and Voucher Specimens. Verification of species identity was obtained by submitting specimens to Dr. Edward L. Mockford, Illinois State University, Normal, Illinois, USA. Voucher specimens are in Dr. Mockford's personal collection, accession number ELM6168.

RESULTS

An unusual assortment of cuticular lipids was found in *L. bostrychophila*, including hydrocarbons, fatty acids, fatty primary amides, and aliphatic aldehydes. Direct evidence that these components were all cuticular in origin was obtained from the SPME analysis: all components seen by hexane extraction were also seen in the SPME sample, albeit in greatly reduced quantities. A homologous series of *n*-alkanes (C_{21}–C_{34}), monomethyl alkanes (3-, 4-, 5-, 7-, 9-, 11-, 12-, 13- and 15-methyl-) with a carbon chain range of C_{28}–C_{42}, and dimethyl alkanes (3, 7-; 9, 13-; 11, 15-; 13, 17-; 9, 21-; 11, 19-; and 13, 21-) with a carbon number range of C_{31}–C_{43} were identified (Table 1). The amount of extracted hydrocarbons was low, comprising approximately 0.0125% of total biomass. Most components occurred as less than 3% of the total volatile portion of the extract, with only two peaks each representing more than 10% of the total (C_{29} and the isomeric mixture of *X*-methyl-C_{31}). No unsaturated hydrocarbons were found.

Free fatty acids accounted for ca. 10–40% of total volatile lipids and were tentatively identified from their characteristic mass spectra (Budzikiewicz et al., 1967). Molecular ions were present, and *m*/*z* 60 was also always present. Conversion of the fatty acids to methyl esters produced diagnostic mass spectra (Ryhage and Stenhagen, 1963) that allowed tentative identifications of the common fatty acids, *Z*-9-$C_{16:1}$, $C_{16:0}$, $C_{17:0}$, *Z*,*Z*-6,9-$C_{18:2}$, *Z*-9-$C_{18:1}$, $C_{19:0}$, and $C_{20:0}$. Comparison of the retention times of the derivatized acids to that of a standard methyl ester mixture allowed tentative assignment of double bond locations. In all cases, the psocid methyl esters had identical retention times and mass spectra to those of known standards.

The primary fatty acid amides were clearly detectable in the crude extracts. They too have characteristic mass spectra, with a base peak at *m*/*z* 59, an intense ion, at *m*/*z* 72, an M-43 ion, and weak, but usually detectable, molecular ions (Table 2, Figure 1) (Budzikiewicz et al., 1967). These amides occurred as a homologous series from C_{16} to C_{22}, with the even carbon number components being the major compounds. Synthetic stearoyl amide had identical properties to the C_{18} psocid amide. The remaining amides had ECL values differing by integer values from that of stearoyl amide, indicating that their carbon chains were not branched.

The aliphatic aldehydes were discovered serendipitously as unexpected *N*,*N*-dimethyl hydrazone peaks in the crude reaction mixture of the methyl esters (McDaniel and Howard, 1985). The C_{15} and C_{16} aldehydes were saturated, with ECL values differing by one unit, whereas the C_{17} aldehyde had a double bond in an undetermined location. Figure 2 illustrates the mass spectra of the *N*,*N*-dimethyl hydrazones of each of these aldehydes, with a prominent molecular ion and an *m*/*z* 86 McLafferty rearrangement ion as the base peak. The free aldehydes were buried under other peaks and hence were not detected in the initial analysis of the crude extract.

TABLE 1. CUTICULAR HYDROCARBONS OF *Liposcelis bostrychophila*

Compound	ECL[a]	CN[b]	Diagnostic EI-MS ions (m/z)	Percent (mean ± SD)[c]
C_{21}	21.00	21	296	0.2 ± 0.1
C_{22}	22.00	22	310	0.5 ± 0.3
C_{23}	23.00	23	324	0.5 ± 0.3
C_{24}	24.00	24	338	1.5 ± 1.0
C_{25}	25.00	25	352	3.1 ± 2.4
C_{26}	26.00	26	366	4.4 ± 3.5
C_{27}	27.00	27	380	5.6 ± 3.7
3-MeC_{27}	27.72	28	365, 379(M-15)	1.6 ± 1.5
C_{28}	28.00	28	394	0.9 ± 0.3
3-MeC_{28}	28.72	29	379	0.1 ± 0.1
C_{29}	29.00	29	408	10.4 ± 1.8
9-;11-MeC_{29}	29.28	30	140, 308, 407 (M-15); 168, 280, 407 (M-15)	2.1 ± 1.0
7-MeC_{29}	29.38	30	112, 336, 407 (M-15)	0.8 ± 0.4
5-MeC_{29}	29.48	30	85, 365, 407 (M-15)	2.2 ± 1.6
3-MeC_{29}	29.72	30	393, 407 (M-15)	2.6 ± 1.9
C_{30}	30.00	30	422	3.6 ± 3.1
3, 7-DiMeC_{29}	30.09	31	127	0.3 ± 0.2
9-;11-;12-MeC_{30}	30.25 to 30.32	31	140, 323;168, 295; 183, 281	1.0 ± 0.3
4-MeC_{30}	30.54	31	71, 393	0.3 ± 0.2
3-MeC_{30}	30.72	31	407	0.3 ± 0.2
C_{31}	31.00	31	436	6.8 ± 3.3
9-;11-;13-;15-MeC_{31}	31.22 to 31.32	32	140, 337, 435 (M-15); 169, 309, 435 (M-15); 196, 280, 435 (M-15); 224, 252, 435 (M-15)	10.5 ± 3.6
7-MeC_{31}	31.38	32	112, 365, 435 (M-15)	3.6 ± 1.9
5-MeC_{31}	31.48	32	85, 393, 435 (M-15)	1.3 ± 0.8
11,15-;13, 17-DiMeC_{31}	31.52 to 31.54	33	168, 323, 239, 252, 449 (M-15); 196, 295, 267, 224, 449 (M-15)	3.4 ± 1.1

TABLE 1. CONTINUED

Compound	ECL[a]	CN[b]	Diagnostic EI-MS ions (m/z)	Percent (mean ± SD)[c]
9, 21-DiMeC$_{31}$	31.64	33	140, 351, 168, 323, 449 (M-15)	1.9 ± 0.9
3-MeC$_{31}$	31.72	32	421, 435 (M-15)	3.6 ± 3.1
C$_{32}$	32.00	32	57	3.2 ± 1.9
3, 7-DiMeC$_{31}$	32.09	33	435, 127, 365, 449 (M-15)	2.6 ± 3.3
11-MeC$_{32}$	32.28	33	168, 323	1.0 ± 0.3
11, 15-DiMeC$_{32}$	32.54	34	168, 323, 239, 267	0.7 ± 0.3
Unknown 1	32.87	–	141, 364	0.3 ± 0.2
C$_{33}$	33.00	33	57	1.5 ± 1.8
11-;13-;15-;17-MeC$_{33}$	33.20 to 33.28	34	168, 336, 463 (M-15); 196, 308, 463 (M-15); 224, 280, 463 (M-15); 252, 463 (M-15)	3.9 ± 0.7
11,15-;13, 17-DiMeC$_{33}$	33.52 to 33.54	35	168, 351, 239, 281, 477 (M-15); 196, 323, 252, 267, 477 (M-15)	5.1 ± 0.8
3-MeC$_{33}$	33.72	34	449	0.3 ± 0.1
C$_{34}$	34.00	34	57	1.0 ± 1.1
3, 7-DiMeC$_{33}$	34.09	35	463, 127, 393	0.5 ± 0.4
17-MeC$_{35}$	35.20	36	252, 281	0.8 ± 0.1
13, 17-DiMeC$_{35}$	35.52	37	196, 351, 267, 280	1.1 ± 0.3
11, 15-DiMeC$_{37}$	37.54	39	168, 379, 267, 309	0.7 ± 0.1
13-MeC$_{39}$	39.23	40	196, 393	0.2 ± 0.2
11,19-;13, 21-DiMeC$_{39}$	39.40 to 39.50	41	168, 435, 295, 309; 196, 407, 323, 280	2.2 ± 0.4
Unknown 2	39.60	–	196	0.2 ± 0.1
13-MeC$_{41}$	41.23	42	196, 421	0.2 ± 0.1
11,23-;13, 21-DiMeC$_{41}$	43.40 to 43.50	43	168, 463, 280, 351; 196, 435, 308, 323	1.0 ± 0.5

[a] ECL = equivalent chain length.
[b] CN = carbon number.
[c] $N = 3$.

TABLE 2. FATTY ACID AMIDES OF *Liposcelis bostrychophila*

Amide	% of total amides (mean ± SD)[a]	Diagnostic EI-MS ions (*m/z*)
$C_{15}H_{31}CONH_2$	5.8 ± 1.8	59, 72
$C_{16}H_{33}CONH_2$	0.6 ± 0.1	59, 72
$C_{17}H_{35}CONH_2$	76.3 ± 5.9	59, 72, 240 (M-43), 283 (M^+)
$C_{18}H_{37}CONH_2$	0.5 ± 0.2	59, 72
$C_{19}H_{39}CONH_2$	15.5 ± 6.2	59, 72, 268 (M-43), 311 (M^+)
$C_{20}H_{41}CONH_2$	1.4 ± 0.9	59, 72
$C_{21}H_{43}CONH_2$	Trace[b]	59, 72

[a] Calculated from *m/z* 59 extracted ion analysis, $N = 2$.
[b] Less than 0.1 percent, but detectable.

DISCUSSION

Despite the burgeoning literature on cuticular hydrocarbons of insects, as well as a lesser literature on other cuticular lipid constituents of insects, large groups of taxa remain unexplored. The Psocoptera represent one such example. As this report shows, novel compounds remain to be discovered in these unstudied taxa. The cuticular hydrocarbons of *L. bostrychophila* contain a diversity of saturated

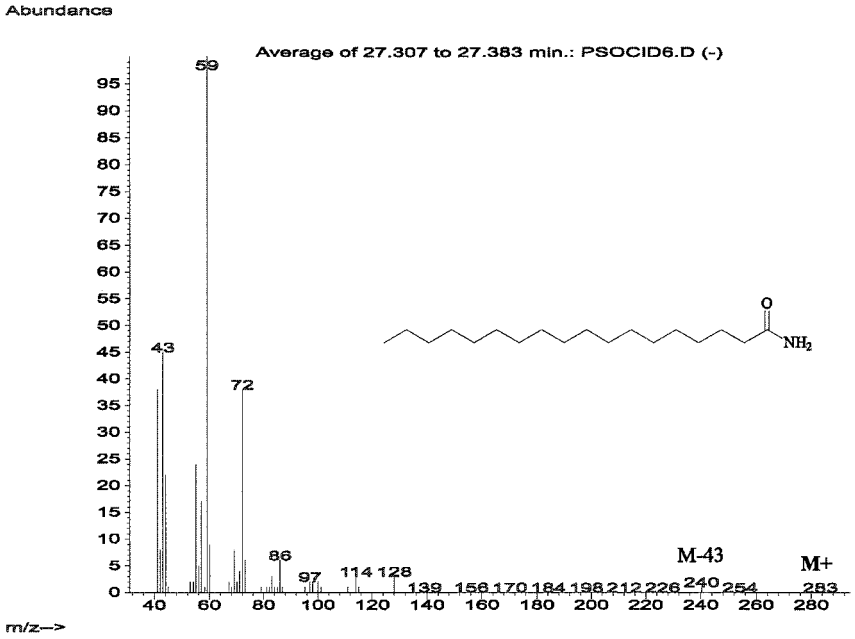

FIG. 1. EI mass spectrum of stearoyl amide isolated from the cuticle of *Liposcelis bostrychophila*.

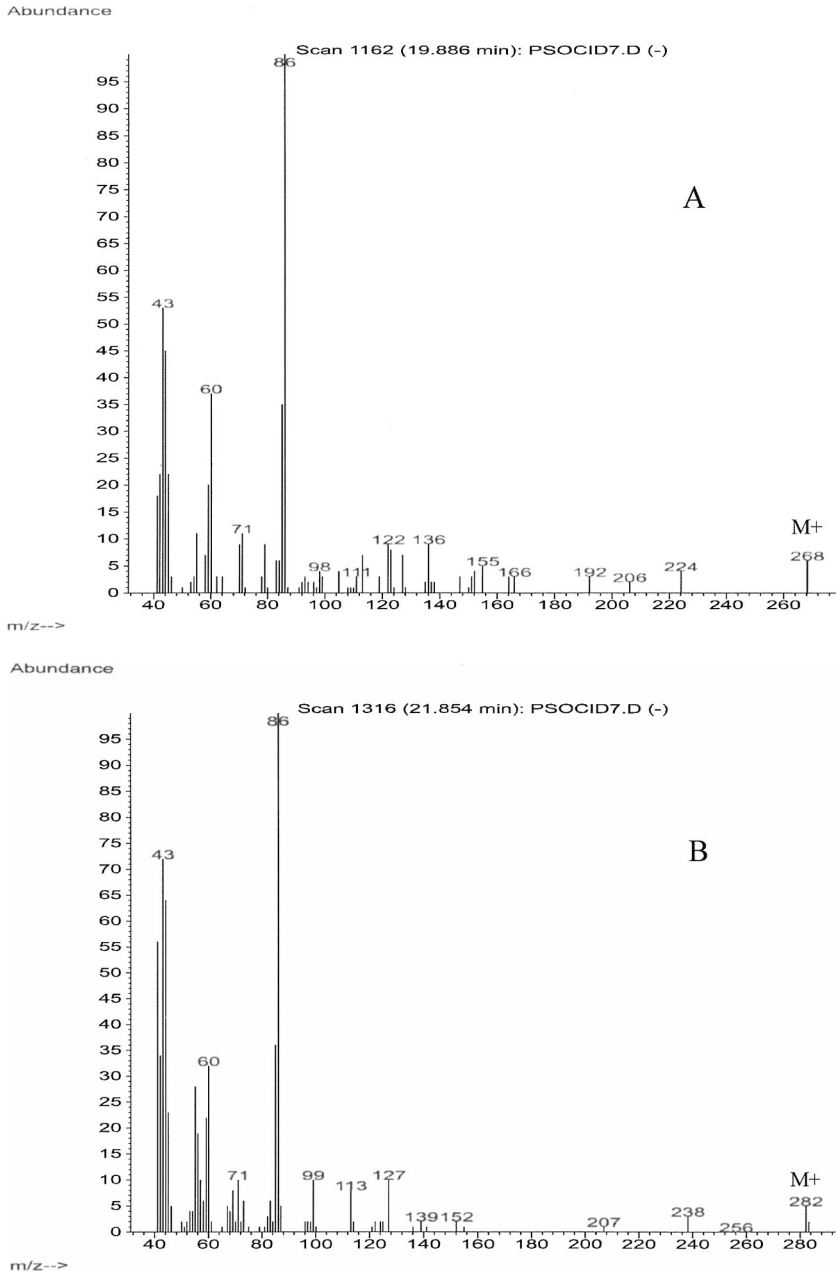

FIG. 2. EI mass spectrum of *N*,*N*-dimethylhydrazones of pentadecanal (A), hexadecanal (B) and heptadecenal (C) isolated from the cuticle of *Liposcelis bostrychophila*.

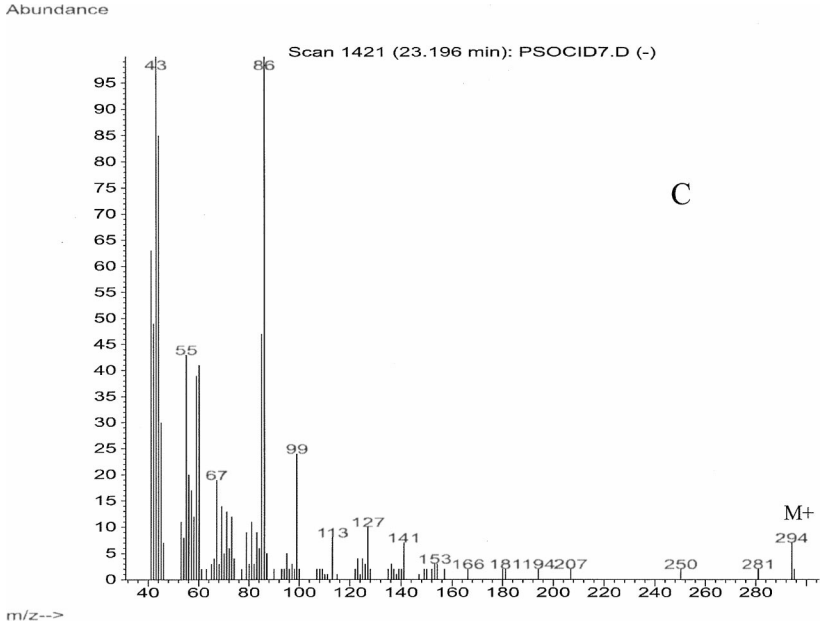

FIG. 2. Continued.

alkanes of medium to long chain length. Although the particular complex of hydrocarbons found is unique to this species, the individual components are all known from previous insect studies (Howard, 1993; Nelson, 1993). No unsaturated hydrocarbons were detected, but there are many other insect taxa where unsaturated hydrocarbons are also missing. Of particular note, the hydrocarbons recovered from *L. bostrychophila* were not the same as those from the rusty grain beetle, which has numerous unsaturated hydrocarbons (Howard, 1992), thus ruling out any possibility that the psocid hydrocarbons were contaminants from the rearing media.

The small amount of hydrocarbons (ca. 0.01% of total biomass) on this psocid is similar to the quantities found on subterranean termites (0.0001–0.01%) (Howard et al., 1978). These abundances may be compared to the few other studies where lipid has been expressed as a function of biomass: values of 0.13–1.8% for a flesh fly (Jackson et al. 1974) and five species of desert inhabiting tenebrionid beetles with values of 0.02–0.18% (Hadley, 1978). It is difficult to make exact comparisons among these taxa, however, as they differ greatly in size (and consequently in surface-to-volume ratio) and ecological habitat. Cuticular hydrocarbons are used by many insects as a barrier to the loss of internal water (Edney, 1977; Gibbs, 2002). In habitats with high humidity, however, an abundance of hydrocarbons may be less critical to a species and the metabolic energy needed to produce them can be used for other basic physiological needs.

For many insect species, cuticular hydrocarbons are important in a variety of semiochemical roles, including recognition and courtship functions (Howard, 1993). Although psocids, being parthenogenetic, do not need gender recognition cues, they occur in moderate to large aggregations, and cuticular compounds may play a role in maintenance of these aggregations.

Polar lipids are also cuticular constituents of many insects (Buckner, 1993). Of these, free fatty acids are common, with even carbon number components ranging from 14 to 20 being most prevalent. Although these acids are rarely the major cuticular component, they can be present in fairly high abundance [49% of the cuticular lipids of the adult stonefly *Pternonarcys californica* (Armold et al., 1969) and up to 47% of the cuticular lipids of the adult flesh flies, *Sarcophaga bullata* (Jackson et al., 1974)]. However, little is known of the functional significance of these free fatty acids.

Unlike the free fatty acids, long-chain aldehydes are not commonly known components of insect cuticular lipids (Buckner, 1993). The majority of such compounds reported have ranged in carbon number from C_{22} to C_{34}, they usually occur with long-chain alcohols, and they occur in low abundance. Such aldehydes are known, however, as major components in wax particles secreted by whitefly adults (Homoptera) (Nelson and Blomquist, 1995; Nelson et al., 1997). Shorter chain aldehydes (C_{14}–C_{16}, unsaturated C_{18}) have been reported as cuticular components of the cockroach, *Periplaneta americana* (Gilby and Cox, 1963). The psocid aldehydes are structurally similar to those of *P. americana*, with the exception that no evidence of methyl-branched aldehydes was found, and they occur in much lower concentrations than in the roaches. The functions of these various cuticular aldehydes are unknown.

Our finding of free primary fatty acid amides as insect cuticular components is the first such instance that we are aware of. Indeed, amide structures of any kind in insects (other than in peptides) are rare in the literature. Several recent papers have dealt with the amide volicitin and its homologs found in the oral secretions of the beet armyworm (Alborn et al., 2000 and references therein), but these are secondary amides and have numerous other functional groups that undoubtedly contribute to their biological activity.

As noted earlier, the physiological and chemical ecology of these psocids are virtually unknown. The stored grain ecosystem, either in silos or in processed grains, is a somewhat dusty environment. Perhaps the unusual cuticular lipid composition of these insects protects these insects from surface abrasion in the dusty stored grain habitats in which they are frequently found. The insects'cuticular acids, aldehydes, and amides are all relatively low-molecular-weight compounds with some degree of fluidity that may aid in sloughing off dust. This, coupled with the insects' ability to efficiently extract atmospheric moisture (Knülle and Spadafora, 1969; Rudolph, 1982) are likely to be important evolutionary adaptations to the stored grain environment.

We have found *L. bostrychophila*, unlike the majority of insect species, to be unaffected by three entomopathogenic fungi of broad host range: *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces fumosoroseus* (J.C.L., unpublished data). Earlier workers had reported that liposcelidids were highly resistant to other entomopathogenic fungi or bacteria (Turner, 1988). Could the unusual array of polar lipids found on the cuticle of *L. bostrychophila* be responsible for this resistance to fungal infection? Insect resistance to fungal pathogens has been linked to fungistatic aldehydes on the stink bug *Nezara viridula* (L.) (Sosa-Gomez et al., 1997) and short-chain fatty acids (Koidsumi, 1957; Smith and Grula, 1982) in cuticular lipids of Lepidoptera. The aldehydes and fatty acids found on this psocid are longer in chain length than the aldehydes and acids referenced above, and the amides might synergize the putative fungistatic properties of these aldehydes and acids. Indeed, fatty acid amides were among the first surface-active agents to be used by the detergent industry to enhance the detergent properties of sulfate and sulfonate detergents (Schwartz and Perry, 1949). In addition, surfactants have been shown to inhibit enzymes and to enhance the absorption of toxicants through cell membranes (Schwartz et al., 1958). Surfactants also produce "slippery" surfaces, which may disrupt the hydrophobic interactions that are critical for successful fungal attachment to host insects (Boucias et al., 1988). It is, thus, conceivable that the amides, in combination with the fatty acids and aldehydes, present an inhospitable niche to these opportunistic fungi. At this point, the roles of the various cuticular constituents of *L. bostrychophilia* remain speculative. Experiments in progress may elucidate some of the functional roles of these compounds.

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CHEMICAL COMPOSITION OF PRECLOACAL SECRETIONS OF TWO *Liolaemus fabiani* POPULATIONS: ARE THEY DIFFERENT?

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Abstract—The chemical composition of secretions of precloacal pores from two populations of the lizard *Liolaemus fabiani* (Puilar and Punta-Brava) were compared. This is an endemic species from the Atacama Salt Flat (26°46'S 68°14'W; 2400 m) in northern Chile, restricted to the internal lakes of the salt flat. Interpopulational differences in the chemical composition of the secretions were expected considering that populations have genetic differences and are subject to different thermal conditions. By using GC-MS, a total of 44 compounds were found belonging to three categories: *n*-alkanes, long-chain carboxylic acids, and steroids. Six compounds were found in all the individuals studied: tetradecanoic, pentadecanoic, hexadecanoic, hexadecenoic, octadecanoic, and octadecenoic acids. The secretions of both populations had similar types and proportions of the different compounds, except for cholesterol and hexanoic acid, which were more abundant in the Puilar population. These differences can be ascribed to the different environmental conditions to which populations are subjected. The adaptive meaning of these differences is discussed.

Key Words—Precloacal secretion, lizard, *Liolaemus fabiani*.

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INTRODUCTION

The ability to discriminate among chemical signals has been reported for different lizard species, and clearly has important consequences under different ecological contexts (see Mason, 1992; Font, 1996; Cooper, 1998). One of these, intraspecific chemical communication, has received significant attention, particularly in relation to the relevance it has for the better understanding of social behavior (e.g., Bull et al., 2000). Which chemical compounds are involved in this communication is less clear. Proteins have been proposed as the main type of compound involved, based on the intraspecific variability in the protein composition of secretions of femoral glands, which are known to have pheromonal properties (Alberts, 1990, 1991; Mason 1992; Alberts et al., 1993). However, more evidence supports the idea that the major compounds involved in chemical communication are lipid in nature (Cooper and Garstka, 1987; Mason and Gutzke, 1990; Bull et al., 1999; LeMaster and Mason, 2001).

Liolaemus (Tropiduridae) is a lizard genus with more than 150 species (Etheridge, 1995). For some species, there is evidence of intraspecific chemical communication (Labra and Niemeyer, 1999; Labra et al., 2001a,b), although the source of semiochemicals is not clear. Precloacal secretions produced by males have been proposed as a potential source (Labra et al., 2002). Analysis of the chemical composition of precloacal secretions of 20 *Liolaemus* species and nine individuals of *L. bellii* indicated interspecific and intrapopulational differences in the chemical composition of these secretions (Escobar et al., 2001). Therefore, it seems that species and individuals can be characterized by a particular chemical composition (Escobar et al., 2001; Labra et al., 2001b). Can populations of a species be characterized by the chemical composition of their precloacal secretions? In this study, the lipid fractions of precloacal secretions of two populations of *L. fabiani* were compared. This is an endemic species from the Atacama Salt Flat in northern Chile (Veloso and Navarro, 1988) restricted to the internal lakes in the salt flat. We studied Puilar and Punta-Brava populations, located approximately 40 km apart, since previous studies indicate that these populations show genetic differentiation, albeit not sufficient to consider them as different species (A. Veloso, personal communication). In addition, they show differences in their physiological thermoregulation, associated with the fact that they are exposed to different thermal conditions (Labra et al., 2001c). Therefore, it is expected that the chemical composition will differ between populations.

METHODS AND MATERIALS

Sample Collection. During October 1999, 15 males of *L. fabiani* were captured at the Atacama Salt Flat (26°46'S; 68°14'W; 2400 m); 9 from Puilar (snout–vent

length (SVL) = 73.6 ± 0.84 mm; mass (W) = 9.7 ± 0.39 g), and 6 from Punta-Brava (SVL = 67.8 ± 1.14 mm; W = 8.3 ± 0.48 g). To collect secretions, males were placed backwards and pores were pressed gently with a forceps. The yellow-reddish greasy secretions were weighed, and secretions of each individual were dissolved in 400 μl purified *n*-hexane (see below) and placed individually in glass ampoules that were sealed and kept at −18°C until use.

Solvent Purification. The *n*-hexane (Merck, chromatography grade) used for dissolving the precloacal secretions was previously purified by stirring over H_2SO_4 overnight, decanting, and stirring over K_2CO_3 for 2 hr. The solution was filtered, dried ($MgSO_4$), and redistilled before use. Glass ampoules used to keep secretions were heated to 150°C for four days before use.

GC-MS Analysis of Secretion Samples. Chemical characterization of the secretion of each individual was performed by directly submitting 50 μg of each individual's secretion dissolved in *n*-hexane (ca. 20 μl) to the derivatization procedure. Derivatization was performed by treating 50 μg of the secretion in v-vials with excess (5 μl) of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and heating the closed vial to 90°C for 60 min. Thereafter, vials were cooled on ice, and excess MSTFA eliminated under a stream of dry nitrogen. The solution was concentrated to 2 μl and injected in the GC-MS.

Analyses were performed on a capillary column (Ultra 2, 25 m × 0.2 mm ID) directly coupled to a mass detector with an integrated data system (GC model HP-5890, MD model HP-5972). Ionization by electron impact (70 eV) was carried out at 280°C. The GC oven was programmed to remain at 50°C for 10 min, then to increase up to 280°C at 5°C/min, and to remain at 280°C for 45 min. The presence or absence of a given compound in the chromatographic profile of each individual was determined by coinjection of commercial standards and comparison of retention times and mass spectra. GC peaks of coinjected compounds were considered coincident if retention times did not differ by more than ±0.03 min. When standards were not available, spectra were compared with a library database by a reverse search technique, which verifies that main peaks in the reference spectrum are present in the unknown spectrum (Pesyna et al., 1976). Spectra were considered coincident if the similarity index was higher than 95% (Pesyna et al., 1976). Alternatively, mass fragmentation patterns were used to determine tentative chemical structures.

Statistics. Student's *t* test was used to compare the total amount of secretions between populations. Mann-Whitney tests were carried out to compare the quantity (normalized chromatographic areas) of each compound between populations. Similarity coefficients of the presence and absence of compounds, reflecting the number of compounds shared by pairs of individuals, were calculated according to Lynch (1990):

$$S_{AB} = 2(AB)/A + B$$

where A equals the total number of compounds in individual A, B equals the total number of compounds in individual B, and AB equals the total number of compounds shared by individuals A and B. This index goes from 0, when no compounds are shared, to 1, when compositions are identical (see also Alberts et al., 1993). Indices obtained for individual populations and between populations were compared with a one-way ANOVA, followed by an *a posteriori* Tukey test. Data are presented as mean $\pm$ standard error (SE). Finally, in order to reveal trends of differentiation between populations an ordination technique was performed (principal component analysis; PCA), considering the absence/presence of the different compounds (Zar, 1984). The PC scores of individuals from each population were compared with the Mann-Whitney test, since they did not have a normal distribution. An additional analysis of clusters (not reported here), using the UPGMA (Unpaired Group Mean Average) algorithm applied to a similarity matrix calculated with Euclidean distance, corroborated the pattern obtained by the PCA ordination technique.

RESULTS

Populations had similar amounts of precloacal secretions ($t = 0.237$; $P = 0.82$). Values for the Puilar population were 0.71 ± 0.13 mg (range 0.20–1.60; $N = 9$), and for Punta- Brava 0.67 ± 0.12 mg (range 0.20–1.00; $N = 6$).

Forty-four compounds were identified in the secretions of *L. fabiani* (Table 1). Six of these were shared by all individuals analyzed: tetradecanoic, pentadecanoic, hexadecanoic, hexadecenoic, octadecanoic, and octadecenoic acids. The 44 compounds can be ascribed to three families: *n*-alkanes representing 12.9% (SE = 2.8; $N = 15$); long-chain carboxylic acids representing 72.6% (SE = 3.9), and steroids representing 14.4% (SE = 4.8) of all compounds detected. Hexadecanoic acid was the most abundant carboxylic acid, while cholesterol was the major steroid. Additionally, hexadecanoic acid was the most abundant among all detected compounds. *n*-Alkanes ranged from C_{10} to C_{29} and carboxylic acids from *n*-C_3 to *n*-C_{22}.

In the Puilar population, *n*-alkanes represented 12.4% (SE = 4.2; $N = 9$), long-chain carboxylic acids 71.7% (SE = 5.4), and steroids 15.7% (SE = 6.8) of compounds detected. Similar results were found in the Punta-Brava population, in which *n*-alkanes represented 13.6% (SE = 3.7; $N = 6$), long-chain carboxylic acids 73.9% (SE = 5.9), and steroids 12.4% (SE = 6.8) of compounds detected. There were no differences between populations in the normalized area of the different compounds ($P > 0.05$), including those compounds shared by all the individuals: tetradecanoic ($U = 21$; $P = 0.48$), pentadecanoic ($U = 15$; $P = 0.16$), hexadecanoic ($U = 19$; $P = 0.35$), hexadecenoic ($U = 24$; $P = 0.72$), octadecanoic ($U = 24$; $P = 0.72$), and octadecenoic acids ($U = 21$; $P = 0.48$). The main

TABLE 1. CHEMICAL COMPOSITION OF PRECLOACAL SECRETIONS OF 15 INDIVIDUALS OF TWO POPULATIONS OF *Liolaemus fabiani* (PUILAR AND PUNTA-BRAVA)

		Normalized chromatographic areas (%)[a]														
		Puilar									Punta - Brava					
Compound	Im[b]	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6
n-Alkanes																
Decane	A	nd	nd	nd	0.45	nd	nd	nd	0.27	nd	nd	nd	nd	nd	nd	nd
Undecane**	A	nd	nd	nd	0.14	nd	0.64	nd	0.36	nd	0.33	0.25	0.11	nd	nd	nd
Dodecane**	A	nd	nd	nd	0.29	nd	1.05	0.41	0.65	nd	0.79	0.54	0.19	nd	0.19	nd
Tridecane**	A	nd	nd	nd	0.17	nd	1.07	0.50	0.79	nd	0.86	0.76	0.22	nd	0.25	0.15
Tetradecane**	A	nd	nd	nd	0.44	nd	1.34	1.32	1.17	nd	nd	1.30	0.58	nd	nd	nd
Pentadecane	A	nd	nd	0.12	0.57	0.16	0.73	0.74	0.61	nd	0.85	0.67	0.44	nd	nd	0.20
Hexadecane	A	nd	0.25	nd	1.31	0.21	0.97	1.13	0.78	nd	1.24	0.98	0.87	nd	0.28	0.22
Heptadecane	A	nd	nd	0.66	2.81	0.38	2.40	2.63	2.09	nd	2.70	2.09	1.92	0.45	0.78	0.73
Octadecane	A	nd	0.61	0.47	4.05	0.33	3.68	4.10	3.93	nd	3.96	3.33	2.96	1.32	1.33	1.42
Nonadecane	A	nd	0.28	nd	2.28	nd	2.21	2.12	2.51	nd	2.96	1.81	2.19	1.33	1.01	1.06
Eicosane**	A	nd	nd	nd	2.43	nd	2.22	2.26	2.96	nd	5.25	1.78	2.24	1.07	1.06	1.50
Heneicosane*	A	nd	nd	nd	1.97	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Docosane**	A	nd	nd	nd	1.73	nd	3.07	1.62	3.41	nd	3.44	2.10	2.51	nd	nd	1.27
Tricosane**	A	nd	nd	nd	1.61	0.67	2.41	0.92	2.30	nd	3.58	1.34	0.97	nd	0.50	0.70
Tetracosane	A	nd	nd	nd	1.43	1.21	nd	0.91	nd	nd	nd	0.95	1.00	nd	nd	nd
Pentacosane	A	nd	nd	1.91	2.08	nd	nd	0.85	2.26	nd	nd	1.21	0.94	nd	nd	nd
Hexacosane	A	nd	nd	nd	1.75	0.45	nd	nd	nd	nd	nd	nd	0.88	nd	nd	0.60
Heptacosane	A	nd	nd	2.34	3.20	0.48	nd	0.46	1.44	1.32	nd	0.89	nd	nd	nd	nd
Octacosane*	A	nd	nd	nd	1.77	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Nonacosane*	A	nd	nd	nd	2.13	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Carboxylic acids																
2-hydroxy-propanoic	B	3.65	5.54	6.21	nd	6.47	2.49	3.36	2.54	nd	5.86	1.46	1.71	3.56	1.10	0.28
Hexanoic	B	0.22	nd	nd	0.02	1.47	0.37	nd	0.30	nd	nd	nd	nd	nd	nd	nd
Octanoic**	B	nd	nd	nd	0.16	nd	0.55	0.39	0.28	nd	0.41	nd	0.20	nd	0.30	nd
Nonanoic	B	nd	nd	0.21	0.15	0.13	0.28	0.21	0.28	nd	0.38	0.22	0.21	nd	nd	nd

TABLE 1. CONTINUED

Compound	Im[b]	Normalized chromatographic areas (%)[a]															
		Puilar									Punta - Brava						
		1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	
Decanoic	B	nd	nd	0.29	0.32	0.17	0.32	0.15	0.29	nd	nd	0.16	0.21	nd	nd	0.17	
Dodecanoic	B	0.82	1.46	1.21	3.49	2.41	3.12	2.60	2.87	1.51	2.28	3.07	2.17	1.99	nd	nd	
Tetradecanoic*	B	3.74	4.42	5.40	11.11	6.82	8.06	7.60	9.17	10.22	7.75	9.67	8.57	10.54	6.66	6.89	
Pentadecanoic*	C	2.07	1.83	2.83	5.30	3.24	4.07	3.99	5.35	7.98	5.09	5.68	5.13	6.26	4.00	5.15	
Hexadecanoic*	A	13.19	14.23	20.72	25.27	20.76	20.67	21.97	22.92	46.64	18.95	27.69	27.62	36.36	20.05	24.97	
Hexadecenoic*	C	6.36	5.88	8.96	12.73	12.94	10.73	9.92	11.46	9.73	11.86	10.03	11.54	11.24	9.48	6.91	
Heptadecanoic**	B	0.88	0.87	1.42	2.43	1.14	1.58	nd	2.10	2.30	nd	1.82	1.01	1.88	1.39	1.40	
Heptadecenoic**	C	1.43	1.33	2.26	nd	2.93	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Octadecanoic*	A	4.56	4.85	8.85	1.34	7.10	5.47	6.98	5.62	9.72	4.33	6.84	6.57	8.71	5.67	4.38	
Octadecenoic*	A	8.68	11.45	13.96	2.21	19.81	11.57	12.78	11.31	10.58	9.75	13.35	17.03	15.28	11.59	8.80	
Nonadecanoic*	B	nd	nd	nd	nd	0.31	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Eicosanoic	B	nd	nd	1.98	nd	1.16	4.48	1.18	nd	nd	nd	nd	nd	nd	nd	nd	
Eicosenoic*	B	nd	nd	nd	nd	1.06	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Docosanoic	A	nd	nd	nd	nd	0.72	nd	0.51	nd	nd	nd	nd	nd	nd	nd	nd	
Butanedioic**	B	0.68	0.83	0.49	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Hexanedioic*	B	nd	nd	nd	nd	nd	nd	0.21	nd	nd	nd	nd	nd	nd	nd	nd	
Steroids																	
Colesterol**	A	47.90	41.33	19.74	2.38	7.25	4.45	7.74	nd	nd	7.08	nd	nd	nd	1.80	nd	
Ergosterol*	B	5.82	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
$3\beta,5\alpha$-cholestanol*	B	nd	4.85	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Unknown	C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	32.56	33.19	

[a] *Compounds that were highly related to factor 1 of PCA (scores higher than 1.2). **Compounds that were highly related to factor 2 of PCA (scores higher than 1.2). Normalized chromatographic areas expressed as percent of total detected compounds.

[b] Identification methods (Im): A = comparison with authentic compounds; B = GC-MS comparison between recorded and library. C = Fragmentation patterns in the mass spectra (as trimethylsilyl derivatives unless otherwise stated): hexadecenoic acid, MS: m/z 55 (50), 73 (100), 117 (63), 129 (49), 185 (6), 194 (10), 236 (8), 267 (2), 311 (53), 326 (M^+,8); heptadecenoic acid, MS: m/z 55 (40), 75 (100), 84 (14), 96 (20), 117 (60), 129 (31), 145 (17), 221 (7), 250 (4), 281 (10), 325 (25), 340 (M^+, 3); pentadecanoic acid, MS: m/z 55 (40), 57 (23), 73 (100), 75 (75), 117 (77), 130 (31), 132 (40), 145 (19), 299 (31), 314 (3), 393 (M^+, 2); unknown , MS: m/z 55 (6), 69 (100), 81 (46), 92 (11), 121 (9), 137 (11), 149 (5), 191 (3), 410 (M^+).

exceptions were hexanoic acid ($U = 12$; $P = 0.035$) and cholesterol ($U = 10$; $P = 0.039$), which were more abundant in secretions of the Puilar population.

The mean similarity coefficient within Puilar was 0.641 ± 0.024 ($N = 36$), and 0.771 ± 0.021 ($N = 15$) within the Punta-Brava population, while the coefficient between populations was 0.673 ± 0.013 ($N = 105$). There are significant differences in the coefficients ($F_{(2,153)} = 4.951$; $P = 0.008$). The Punta-Brava population showed the highest coefficients and differed significantly from values of the Puilar population ($P = 0.005$) and of the pooled data ($P = 0.002$).

According to results of PCA (Figure 1), individuals of Punta-Brava appeared highly grouped, while individuals of Puilar population were dispersed, as was detected by the analysis of similarity coefficients. All individuals of the Punta-Brava population were significantly and positively correlated with factor 1,

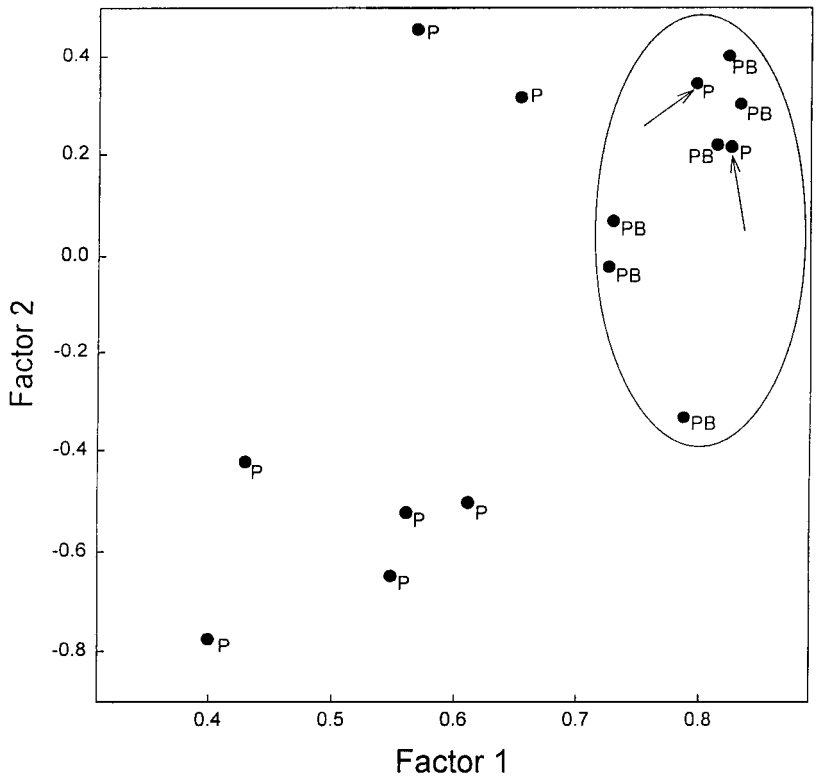

FIG. 1. Principal component analysis (PCA) of 15 individuals from two populations of *Liolaemus fabiani*. Data of presence–absence of the 44 compounds detected in the precloacal secretions. P = Puilar; PB = Punta-Brava populations. Arrows signal the two individuals of Puilar falling within the group of Punta-Brava individuals.

but there was no clear pattern for the Puilar population. The first two factors explained 64.83% of the variance (factor 1: 47.37% of the total variance; factor 2: 17.36% of the variance). The comparison of the PC scores of individuals of both populations indicated that there were significant differences between populations in factor 1 ($U = 8$; $P = 0.025$), showing highest values in the Punta-Brava population. Populations did not differ in PC scores of factor 2 ($U = 19$; $P = 0.35$). Table 1 shows those compounds that had the highest effect in factors 1 and 2.

DISCUSSION

The main question posed was whether populations of *Liolaemus* species can be characterized by the chemical composition of their precloacal secretions. The results do not allow a clear answer: while the two populations cannot be characterized by a specific chemical composition of the secretions, certain compounds are predominant in one population, and both populations differed significantly in chemical variability, with individuals from Punta-Brava showing less than those from Puilar (Figure 1).

The lipid fractions of the precloacal secretions of *L. fabiani* males have the same three families of chemical compounds as reported for other lizard species: *n*-alkanes, carboxylic acids, and steroids (Chauhan, 1986; Mason and Gutzke, 1990; Alberts et al., 1992; Escobar et al., 2001). On the other hand, in a previous study where pooled secretions of *L. fabiani* were analyzed, *n*-alkanes were not detected (Escobar et al., 2001). In contrast, in the present study, we found these compounds ranging from C_{10} to C_{29}. Considering the high intraspecific variability in their quantity (Table 1), it is possible that in the previous study individuals lacking *n*-alkanes were included in the specific pool of secretions.

The function of the most abundant compounds (hexadecanoic acid and cholesterol) as semiochemicals involved in intraspecific chemical communication has not been ascertained. It is noteworthy that they have been reported previously as present in secretions with pheromonal properties (Alberts et al., 1992; Escobar et al., 2001). Why are these compounds so abundant in both populations? We postulate that at least cholesterol constitutes an unreactive apolar matrix that delivers the compounds that are the true semiochemicals. The properties of this matrix should reflect the environment where lizards occur. Thus, it is expected that under higher environmental temperatures, the matrix would contain a larger proportion of compounds of lower volatility and higher chemical stability in order to counteract the higher tendency towards volatilization and decomposition of the semiochemicals released. Puilar has higher air temperatures than Punta-Brava (Labra et al., 2001c), and indeed the concentration of cholesterol is higher in this population than in the Punta-Brava population. In addition, the precloacal secretions from the Puilar population contained higher amounts of hexanoic acid than the Punta-Brava population. Interestingly, this is the most volatile compound in its class and in a

high temperature environment should be produced in higher amounts in order to compensate for its loss to the environment.

Studies on *L. fabiani* indicated genetic differences between the two populations studied here (A. Veloso, personal commmunication), consistent with their isolation from each other within the salt flat, and the different thermal environmental conditions to which both populations are exposed (Labra et al., 2001c). We expected to find large chemical differences between populations, however, only small ones were found. A hypothesis to account for this fact is based on factors involved in the evolution of signals used in communication. Johnstone (1997) has proposed that this evolution is triggered or accelerated by the presence of other signal emitters such as sympatric congeneric species that use similar signals. This situation may occur with semiochemicals of squamates, where they serve as reproductive barriers for congeneric and sympatric species (Ford, 1982; Ford and Schofield, 1984; Cooper and Vitt, 1986). Since *L. fabiani* does not have sympatric congeneric species, it might be expected that chemical signals in this *Liolaemus* species, in the absence of competitive signals, would show a slow rate of change and hence only small differences between populations. Notwithstanding this, other compounds important as pheromones, such as skin lipids (i.e., Mason and Gutzke, 1990), or compounds present in feces (Labra et al., 2002) may evolve at a faster rate than compounds present in precloacal secretions, and hence reveal differences between populations. New interpopulational comparisons of secretions of precloacal glands, skin, and feces are needed to test these hypotheses.

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INTERSPECIFIC VARIATION IN TERPENOID COMPOSITION OF DEFENSIVE SECRETIONS OF EUROPEAN *Reticulitermes* TERMITES

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Abstract—Sixteen terpene compounds were isolated from the soldier defensive secretions of seven European termite taxa of the genus *Reticulitermes* (Isoptera, Rhinotermitidae). We describe species-specific mixtures of monoterpenes (α-pinene, β-pinene, limonene), sesquiterpenes (germacrene C, germacrene A, germacrene B, β-selinene, δ-selinene, γ-selinene, (E)-β-farnesene, γ-cadinene, nerolidol), diterpenes (geranyl linalool, geranyl geraniol, geranyl geranial), and one sesterterpene (geranyl farnesol). Compounds were purified by HPLC and their structures determined by means of MS spectrometry, or 1D and 2D NMR spectroscopy. Comparison of two different analytical approaches, GC-MS and HPLC with subsequent NMR spectroscopy, revealed Cope rearrangement of germacrene A, germacrene B, and germacrene C to the respective β-elemene, γ-elemene, and δ-elemene under GC conditions, thus demonstrating the limits for this analytical approach. The species-specific compound composition provides insight into taxonomy and species origin of European *Reticulitermes*.

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The biological significance of the species-specific composition of *Reticulitermes* defensive secretions is briefly discussed.

Key Words—*Reticulitermes*, Isoptera, Rhinotermitidae, subterranean termites, soldier defensive secretions, terpenes, HPLC, NMR, GC-MS, Cope rearrangement.

INTRODUCTION

Most of the 2000 known termite species have a soldier caste that is morphologically distinguished from the worker caste. Soldiers of the families Rhinotermitidae and Termitidae are equipped with a caste-specific frontal gland, the secretion of which is expelled through a pore on the top of the head (Grassé, 1982). The soldier caste usually assumes a role in defense of the termite colony, and there is abundant evidence that the frontal gland secretion is employed to chemically defend the colony against predators or competitors (e.g., Quennedey and Deligne, 1975; Prestwich, 1979a,b, 1983; Deligne et al., 1981; Grassé, 1982, 1986; Kaib, 1985). These secretions are composed of species-specific mixtures of compounds, including alkanes, ketoaldehydes, vinylketones, lactones, sesquiterpenes, cyclic diterpenes, and diterpene alcohols (reviewed in Grassé, 1984, 1986; Prestwich, 1988).

Species of *Reticulitermes* (Isoptera, Rhinotermitidae) are found throughout most of North America and Europe. The frontal gland secretions of several North American species have been analyzed, and monoterpenes (α-pinene, β-pinene, limonene, camphene, and myrcene), sesquiterpenes (γ-cadinene, cadinene aldehyde, himachalene, and bisabolene), and one diterpene alcohol (geranyl linalool) have been identified (Bagnères et al., 1990; Nelson et al., 2001). The composition of the secretion varies among taxa and geographically. In combination with cuticular hydrocarbon profiles, frontal gland secretion profiles have been used for extensive chemotaxonomic analyses of this genus in North America (e.g., Haverty et al., 1996, 1999; Haverty and Nelson, 1997; Nelson et al., 2001). Recent studies have shown that there are still numerous undescribed species of *Reticulitermes* in the United States (Jenkins et al., 2000; Nelson et al., 2001).

In Europe, the taxonomy of *Reticulitermes* has similarly been subject to years of investigation including morphological, chemical, and molecular studies (e.g., Becker, 1970; Clément, 1978, 1981, 1984; Bouillon, 1981; Bagnères et al., 1988; Clément et al., 2001). Currently, six *Reticulitermes* species and one subspecies have been described from Europe—with the accurate phylogenetic placement of newly discovered species yet to be determined. The chemistry of the defensive secretion of European *Reticulitermes* species until now has only been partially investigated. Earlier studies focused on the main compound of the secretion, geranyl linalool, which is present in all European *Reticulitermes* species and functions as an effective

deterrent and toxin against predators (Parton et al., 1981; Zalkow et al., 1981; Baker et al., 1982; Clément et al., 1988; Bagnères et al., 1990; Lemaire et al., 1990). The existence of minor compounds in the frontal gland secretions has not received much attention.

Here, we examine the chemical composition of soldier frontal gland secretions of European *Reticulitermes* species from different geographic locations, to gain further insight into relatedness, taxonomic position, and origin of each species.

METHODS AND MATERIALS

Insects. Termite colonies were collected in their natural habitat from infested logs and kept in the laboratory in containers with moist soil and wood. *Reticulitermes santonensis* and *R. grassei* were collected in Ile d'Oléron in May 2000 (Charente Maritime, France). *R. lucifugus* and *Reticulitermes*. sp. nov. were collected near Marseille (Bouches du Rhône, France) in April 2001, and *R. banyulensis* were collected near Bézier (Aude, France) in June 2000. *R. lucifugus corsicus* was collected in Corsica in June 2001, and *R. balkanensis* near Athens, Greece, in October 2000. As it is difficult to distinguish these species morphologically, verification of species identity was carried out via the hydrocarbon profiles of workers (Bagnères et al., 1988, 1990, 1991; Clément et al., 2001). Voucher specimens of the known European species were deposited at the National Museum of Natural History in Paris, France.

GC and GC-MS Analyses. Termite soldiers used for the chemical analyses were removed from colonies immediately before extraction. Five different colonies per species were investigated separately to account for colony variation. Whole body extracts of soldiers were used. Ten soldiers per colony were covered in 1 ml *n*-pentane for 2 min, the extract was removed, concentrated under nitrogen, and 2 μl were immediately injected into a GC-FID (for compound quantification) or GC-MS (for compound identification), respectively.

GC analysis was performed on a Delsi Nermag DN 200 GC, FID, with splitless injection on a fused silica capillary column (25 m, 0.25-μm film thickness, CpSil 5 WCOT, Chrompack). The temperature program ran from 38°C to 40°C at 0.5°C/min, then increased with a rate of 5°C/min to 320°C. Helium served as the carrier gas. The injector temperature was maintained at 250°C and the detector temperature at 280°C. Compounds were all quantified via GC using camphene and humulene as internal standards. Quantities were calculated as mean amount per frontal gland averaging the results from five colonies per species.

GC-MS analysis of the extracts was performed on a Hewlett Packard 5890 GC coupled to a Hewlett Packard quadrupole 5889A MS in electron impact mode

(70 eV). The samples were injected splitless on the same fused silica capillary column and with the same GC temperature program as described above. Most compounds could be identified with this approach, comparing retention time and mass spectra with those of authentic materials. Determination of the enantiomeric identity of compounds by chiral GC were not be carried out due to technical constraints.

As we dealt with whole body extracts and to ensure we only analyzed compounds that truly originated from the frontal gland, we also collected pure frontal gland secretion from individual soldiers by holding them with forceps and using a small piece of filter paper to soak up the secretion exuding from the frontal pore. The filter paper was extracted in 500 μl *n*-pentane for 2 min, the extract was taken up, concentrated under nitrogen, and 2 μl were immediately injected into the GC and analyzed as above.

NMR Analysis. Some of the compounds tentatively characterized by GC-MS proved to be unstable at high temperatures, and their structures were, therefore, likely to have been rearranged during GC-MS analysis. Several compounds also had highly similar mass spectra, and authentic references for confirmation of retention time and mass spectra were not available in all cases. To confirm MS data for those compounds, they were also analyzed by NMR spectroscopy, given that sufficient material could be obtained.

Compounds first were isolated and purified by HPLC. For each species, whole bodies of 300 soldiers from one colony were extracted in 3 ml $CHCl_3$. An aliquot of the extract (0.5 ml) was taken up, concentrated to 20 μl under nitrogen, and injected in a Hewlett Packard 1050 HPLC, with a HP 1100 Diode Array Detector from 200 to 400 nm wavelength. The column used was a RP-18 Ultrasphere ODS, 5 μm, 4.6 $\times$ 250 mm. Run duration was 60 min with an eluent mixture of 85:15 CH_3CN–H_2O at a flow rate of 0.8 ml/min in an isocratic mode. Each HPLC peak was collected as a separate fraction at the end of the column, and transferred into *n*-pentane by diphasic chemical extraction. This procedure was repeated six times until the entire sample of 3 ml was purified and fractionated. Corresponding fractions were pooled. A 2-μl sample of each HPLC fraction was analyzed by GC, as described above, to confirm the purity and to correlate HPLC and GC peaks. The isolated and purified compounds were stored at -20°C for NMR analysis.

The compound samples were transferred into NMR solvent by first completely evaporating the pentane under nitrogen, and subsequently adding 130 μl of $CDCl_3$. The samples were transferred with a glass micropipet into a capillary NMR tube for microquantity samples. The minimum sample size required for acceptable spectra with this method was in the range of a few micrograms. Microquantity NMR analyses (^{1}H, ^{13}C, COSY, HMQC, HMBC) were performed with a Bruker AVANCE 500 (500 MHz) using chloroform as internal standard.

RESULTS

By using GC-MS and 1D/2D microquantity NMR-spectroscopy, we identified species-specific mixtures of monoterpenes (α-pinene, β-pinene, limonene), sesquiterpenes (germacrene C, germacrene A, germacrene B, β-selinene, δ-selinene, γ-selinene, (E)-β-farnesene, γ-cadinene, nerolidol), diterpenes (geranyl linalool, geranyl geraniol, geranyl geranial), and a sesterterpene (geranyl farnesol) from the soldier frontal gland secretion of seven European *Reticulitermes* taxa (Table 1, Figure 1). The NMR and GC-MS spectra were consistent with literature (Table 2, Joulain and Konig, 1998), however, enantiomeric identity was not determined.

Terpenoid Identification. As confirmed by the control experiment (analysis of pure secretion of each species), all of the described compounds originate from the frontal gland secretion of the respective species and not from other body parts. The monoterpenes α-pinene (**1**), β-pinene (**2**), and limonene (**3**) were identified from *R. santonensis* using GC-MS (Figure 1, Table 2). Compounds (**4–6**) proved difficult

TABLE 1. TERPENOID COMPOSITION OF SOLDIER DEFENSIVE SECRETIONS OF EUROPEAN *Reticulitermes* TERMITE SPECIES[a]

			Quantity (μg/soldier)						
Peak	Compound[b]	R_t(min)	*R.* sp.nov.	*R.balk.*	*R.l.*	*R.l.c.*	*R.g.*	*R.b.*	*R.s.*
1	α-pinene	7.41	ND	ND	ND	ND	ND	ND	1.88
2	β-pinene	8.68	ND	ND	ND	ND	ND	ND	2.51
3	limonene	10.47	ND	ND	ND	ND	ND	ND	1.39
4	germacrene C	20.3	1.9	ND	0.4	0.08	ND	ND	ND
5	[germacrene A]	21.7	1.8	0.73	0.15	ND	ND	ND	ND
6	[germacrene B]	22.5	0.2	ND	ND	ND	ND	ND	ND
7	β-selinene	22.91	ND	ND	0.2	ND	ND	ND	ND
8	δ-selinene	23.15	ND	ND	ND	0.33	ND	ND	ND
9	γ-selinene	23.26	ND	ND	0.2	ND	ND	ND	ND
10	E-β-farnesene	23.93	0.2	0.5	ND	ND	ND	ND	ND
11	γ-cadinene	24.1	ND	ND	0.5	0.15	2.1	ND	ND
12	nerolidol	25.38	ND	ND	0.12	0.17	ND	ND	ND
13	geranyl linalool	35.02	30.0	8.53	6.28	8.13	11.22	8.23	0.37
14	geranyl geraniol	38.28	ND	ND	ND	ND	ND	ND	1.09
15	geranyl geranial	38.46	ND	ND	ND	ND	ND	ND	0.83
16	geranyl farnesol	46.19	ND	ND	ND	ND	ND	ND	46.0

[a] *R.* sp.nov.: *Reticulitermes* new species. *R.balk.*: *Reticulitermes balkanensis*. *R.l.*: *Reticulitermes lucifugus. R.l.c.*: *Reticulitermes lucifugus corsicus. R.g.*: *Reticulitermes grassei. R.b.*: *Reticulitermes banyulensis. R.s.*: *Reticulitermes santonensis.*

[b] Compounds are listed in GC peak elution order. Shown are GC retention times (R_t) in minutes and mean quantity in μg per soldier. (ND: not detected or detected at a level <0.05 μg; $N = 5$, standard deviation ranging from 1.9% to 8.2% of means). Compound names in brackets indicate identification is considered tentative.

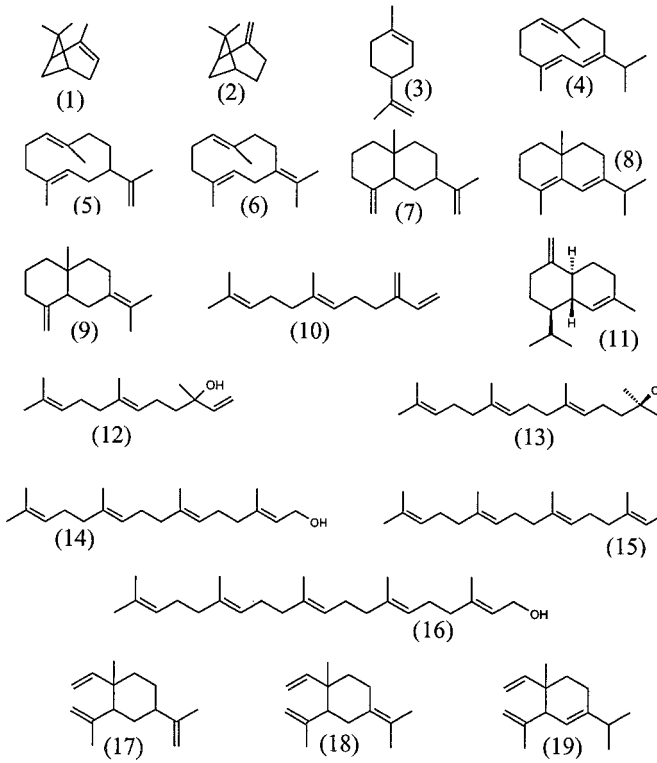

FIG. 1. Chemical structures of terpene constituents identified from European *Reticulitermes* termite defensive secretions, and structures of reference compounds (**17, 18, 19**). (**1**) α-pinene, (**2**) β-pinene, (**3**) limonene, (**4**) germacrene C, (**5**) germacrene A, (**6**) germacrene B, (**7**) β-selinene, (**8**) δ-selinene, (**9**) γ-selinene, (**10**) E-β-farnesene, (**11**) γ-cadinene, (**12**) nerolidol, (**13**) geranyl linalool, (**14**) geranyl geraniol, (**15**) geranyl geranial, (**16**) geranyl farnesol, (**17**) β-elemene, (**18**) γ-elemene, (**19**) δ-elemene.

to characterize. Their mass spectra corresponded to those of δ-elemene, β-elemene, and γ-elemene, but it is well known that elemenes can be the result of structural rearrangements under GC conditions (Cope rearrangement of germacrenes to elemenes). By using microquantity NMR spectroscopy of the isolated compound (**4**) we confirmed this compound as germacrene C (Table 2). Preliminary NMR spectroscopy of compound (**5**) showed that two of the three exomethylene signals were lacking, indicating that this compound might be germacrene A, but we did not have enough material for a full characterization. Sufficient amounts of compound (**6**) for complete NMR analysis could not be isolated either. We only obtained enough material to confirm the absence of exomethylene signals and verify the characteristic resonances of the germacrene skeleton. Therefore, the identification

TABLE 2. MASS SPECTROMETRY AND NMR SPECTROSCOPY DATA[a] FOR TERPENOID COMPOUNDS FROM EUROPEAN *Reticulitermes* TERMITE DEFENSIVE SECRETIONS AND FOR REFERENCE COMPOUNDS

Monoterpenes

α-Pinene (**1**): MS (EI, 70 eV), *m/z* (rel. int.): 136 (7), 121 (7), 105 (33), 93 (95), 91 (100), 77 (58), 67 (20), 55 (13), 43 (47), 41 (27).

β-Pinene (**2**): MS (EI, 70 eV), *m/z* (rel. int.): 136 (9), 121 (13), 105 (7), 93 (100), 91 (47), 77 (40), 69 (29), 53 (11), 41 (76).

Limonene (**3**): MS (EI, 70 eV), *m/z* (rel. int.): 136 (16), 121 (20), 107 (20), 93 (69), 91 (34), 79 (42), 69 (94), 67 (100), 53 (29), 41 (24).

Sesquiterpenes[b]

Germacrene C (**4**): ^{1}H NMR ($CDCl_3$) : δ 6.26 (1H, d, $J = 9.2$ Hz), 5.24 (1H, d, $J = 9.2$ Hz), 4.98 (1H, dd, $J = 11.4$ Hz, $J = 5.0$ Hz), 2.59 (1H, ddd, $J = 12.3$ Hz, $J = 5.6$ Hz, $J = 1.7$ Hz), 2.43 (1H, m), 2.41 (1H, m), 2.25 (1H, dt, $J = 12.0$ Hz, $J = 3.4$ Hz), 2.19 (1H, dq, $J = 12.6$ Hz, $J = 4.0$ Hz), 2.14 (1H, ddd, $J = 13.1$ Hz, $J = 4.7$ Hz, $J = 2.7$ Hz), 2.02 (1H, qd, $J = 12.6$ Hz, $J = 4.1$ Hz), 1.94 (1H, td, $J = 12.8$ Hz, $J = 4.8$ Hz), 1.77 (1H, td, $J = 12.4$ Hz, $J = 4.5$ Hz), 1.60 (3H, s, br), 1.12 (3H, d, $J = 6.8$ Hz), 1.11 (3H, d, $J = 6.8$ Hz), 1.18 (3H, d, $J = 1.2$ Hz).

[Germacrene A (**5**): complete NMR data not available, for MS-data see explanation in foot notes and text]

[Germacrene B (**6**): complete NMR data not available, for MS-data see explanation in foot notes and text]

β-Selinene (**7**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (62), 189 (47), 177 (27), 161 (50), 147 (49), 133 (58), 121 (54), 105 (100), 93 (98), 79 (72), 67 (62), 53 (36), 41 (53).

δ-Selinene (**8**): ^{1}H NMR ($CDCl_3$): δ 6.16 (1H, s, br), 2.32 (1H, hept, $J = 6.5$ Hz), 2.23 (1H, m), 2.09 (2H, t, $J = 11$ Hz), 2.06 (1H, m), 1.82 (1H, m), 1.71 (3H, s), 1.65 (1H, m), 1.51 (1H, m), 1.46 (1H, m), 1.40 (1H, t, $J = 9.3$ Hz), 1.38 (1H, m), 1.09 (3H, d, $J = 3.5$ Hz), 1.07 (3H, d, $J = 3.5$ Hz), 0.97 (3H, s). MS (EI, 70 eV), *m/z* (rel. int.): 204 (75), 189 (82), 161 (100), 147 (18), 133 (40), 119 (33), 105 (51), 93 (42), 81 (27), 69 (13), 55 (20), 41 (32).

γ-Selinene (**9**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (58), 189 (100), 177 (27), 161 (45), 147 (34), 133 (71), 119 (40), 107 (71), 93 (80), 81 (55), 69 (33), 55 (25), 41 (36).

E-β-Farnesene (**10**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (2), 161 (4), 133 (9), 120 (7), 93 (24), 69 (100), 55 (9), 41 (36).

γ-Cadinene (**11**): ^{1}H NMR ($CDCl_3$): δ 5.58 (1H, s, br), 4.69 (1H, q, $J = 1.5$ Hz), 4.58 (1H, q, $J = 1.5$ Hz), 2.41 (1H, ddd, $J = 12.8$ Hz, $J = 3.7$ Hz, $J = 3.0$ Hz), 2.22 (1H, d hept, $J = 6.9$ Hz, $J = 3.3$ Hz), 2.08 (1H, m), 2.06 (1H, m), 2.03 (1H, m), 2.02 (1H, m), 1.80 (1H, dt, $J = 12.5$ Hz, $J = 3.0$ Hz), 1.78 (1H, tm, $J = 11.5$ Hz), 1.72 (3H, s, br), 1.66 (1H, tm, $J = 10.9$ Hz), 1.51 (1H, qd, $J = 12.0$ Hz, $J = 5.8$ Hz), 1.25 (1H, tt, $J = 10.9$ Hz, $J = 3.1$ Hz), 1.15 (1H, qd, $J = 12.7$ Hz, $J = 4.1$ Hz), 0.96 (3H, d, $J = 6.9$ Hz), 0.77 (1H, d, $J = 6.9$ Hz). ^{13}C NMR δ 153.5 (C-2), 135 (C-8), 122.6 (C-7), 103.3 (C-14), 47.2 (C-5), 45.3 (C-6), 44.4 (C-1), 36.5 (C-3), 30.7 (C-9), 26.7 (C-4), 26.4 (C-11), 25.9 (C-10), 24.0 (C-15), 21.6 (C-12), 15.3 (C-13). MS (EI, 70 eV), *m/z* (rel. int.): 204 (27), 189 (7), 161 (100), 133 (24), 119 (40), 105 (54), 93 (44), 79 (33), 69 (25), 53 (18), 41 (38).

Nerolidol (**12**): ^{1}H NMR ($CDCl_3$) : δ 5.94 (1H, dd, $J = 17.3$ Hz, $J = 10.9$ Hz), 5.24 (1H, d, $J = 17.3$ Hz), 5.16 (1H, t, $J = 7.4$ Hz), 5.11 (1H, t, $J = 7.4$ Hz), 5.08 (1H, d, $J = 10.7$ Hz), 2.08 (4H, m, H-5 and H-9), 2.02 (4H, t, $J = 8.3$ Hz, H-6 and H-10), 1.71 (3H, m), 1.62 (6H, s, H-3 and H-15), 1.34 (3H, s). MS (EI, 70 eV), *m/z* (rel. int.): 204 (2), 189 (4), 161 (17), 147 (8), 136 (17), 119 (18), 107 (31), 93 (69), 81 (29), 69 (100), 55 (35), 41 (78).

TABLE 2. CONTINUED

Diterpenes

Geranyl linalool (**13**): ^{1}H NMR ($CDCl_3$) : δ 5.86 (1H, dd, $J = 17.3$ Hz, $J = 10.8$ Hz), 5.25 (1H, dd, $J = 17.3$ Hz, $J = 1.2$ Hz), 5.16 (1H, t, $J = 7.1$ Hz), 5.12 (2H, t, $J = 7.1$ Hz, H-8 and H-12), 5.08 (1H, dd, $J = 10.8$ Hz, $J = 1.2$ Hz), 2.21 (6H, t, $J = 7.4$ Hz, H-5, H-9 and H-13), 2.02 (4H, t, $J = 7.8$ Hz, H-6 and H-10), 1.71 (3H, s), 1.65 (6H, s, H-16 and H-20), 1.61 (3H, s), 1.58 (2H, t, $J = 9.6$ Hz), 1.31 (3H, s). ^{13}C NMR δ 145.2 (C-16), 135 (C-7 and C-11), 131 (C-2), 124.6 (C-4), 124.4 (C-8), 124.3 (C-12), 111.8 (C-17), 72.3 (C-15), 42.3 (C-14), 39.9 (C-6 and C-10), 28.1 (C-18), 26.9 (C-9), 25.9 (C-3), 22.9 (C-13), 17.9 (C-1), 16.2 (C-19 and C-20). MS (EI, 70 eV), *m/z* (rel. int.): 290 (1), 272 (5), 257 (2), 229 (2), 203 (6), 187 (7), 161 (11), 147 (9), 134 (18), 119 (34), 107 (36), 93 (60), 81 (54), 69 (100), 55 (29), 41 (65).

Geranyl geraniol (**14**): ^{1}H NMR ($CDCl_3$): δ 5.45 (1H, t, $J = 6.8$ Hz), 5.15 (3H, d, $J = 6.2$ Hz, H-4, H-8 and H-12), 4.18 (1H, t, $J = 6.2$ Hz), 2.01 (6H, t, $J = 7.8$ Hz, H-5, H-9 and H-13), 2.02 (6H, t, $J = 7.6$ Hz, H-6, H-10 and H-14), 1.71 (6H, s, H-1 and H-18), 1.62 (9H, s, H-3, H-19 and H-20). ^{13}C NMR δ 124.2 (C-4 and C-12), 123.5 (C-16), 59.5 (C-17), 39.8 (C-5, C-9 and C-13), 26.8 (C-6, C-10 and C-14), 25.7 (C-3), 16.2 (C-1, C-18, C-19 and C-20). MS (EI, 70 eV), *m/z* (rel. int.): 272 (7), 257 (5), 203 (5), 187 (9), 161 (13), 147 (11), 133 (18), 119 (36), 107 (29), 93 (53), 81 (55), 69 (100), 55 (27), 41 (56).

Geranyl geranial (**15**): ^{1}H NMR ($CDCl_3$): δ 10.02 (1H, d, $J = 8.1$ Hz), 5.91 (1H, d, $J = 8.1$ Hz), 5.15 (3H, t, $J = 6.9$ Hz, H-4, H-8 and H-12), 2.26 (6H, m, H-5, H-9 and H-13), 2.08 (6H, t, $J = 7.6$ Hz, H-6, H-10 and H-14), 1.71 (3H, s), 1.64 (3H, s), 1.63 (6H, s, H-19 and H-20), 1.62 (3H, s). ^{13}C NMR δ 128 (C-16), 124.2 (C-4, C-8 and C-12), 40.6 (C-5, C-9 and C-13), 26.8 (C-6, C-10 and C-14), 26 (C-1), 17 (C-3, C-18, C-19 and C-20). MS (EI, 70 eV), *m/z* (rel. int.): 288 (2), 270 (5), 252 (2), 203 (4), 185 (7), 159 (11), 145 (18), 133 (22), 119 (25), 105 (27), 93 (36), 81 (73), 69 (100), 55 (34), 41 (64).

Sesterterpenes

Geranyl farnesol (**16**): ^{1}H NMR ($CDCl_3$): δ 5.45 (1H, t, $J = 6.9$ Hz), 5.15 (4H, t, $J = 7$ Hz, H-4, H-8, H-12 and H-16), 4.18 (2H, t, $J = 5.8$ Hz), 2.08 (8H, t, $J = 6.7$ Hz, H-5, H-9, H-13 and H-17), 2.01 (8H, t, $J = 7.3$ Hz, H-6, H-10, H-14 and H-18), 1.71 (3H, s), 1.64 (12H, s, H-3, H-23, H-24 and H-25), 1.60 (3H, s). MS (EI, 70 eV), *m/z* (rel. int.): 340 (2), 203 (2), 187 (3), 161 (9), 147 (9), 135 (13), 119 (15), 107 (18), 93 (33), 81 (58), 69 (100), 55 (18), 41 (40).

Reference compounds[c]

β-Elemene (**17**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (4), 189 (27), 161 (35), 147 (49), 133 (33), 121 (47), 107 (67), 93 (96), 81 (100), 68 (69), 55 (38), 53 (32), 41 (49).

γ-Elemene (**18**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (9), 189 (14), 161 (29), 147 (13), 133 (22), 121 (100), 105 (40), 93 (69), 79 (27), 67 (25), 55 (20), 41 (36).

δ-Elemene (**19**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (4), 189 (5), 161 (38), 121 (100), 105 (20), 93 (85), 77 (18), 69 (4), 55 (11), 41 (27).

[a] NMR analyses carried out to confirm MS data where required and given that sufficient material could be isolated.

[b] MS data for germacrenes A, B, and C (compounds **5**, **6**, and **4**) correspond to those of β-, γ-, and δ-elemene (compounds **17**, **18**, and **19**) (Cope rearrangement, for further explanation see text).

[c] Authentic references analyzed for Cope rearrangement verification.

of compounds (**5**) and (**6**) as germacrenes A and B is considered tentative (Figure 1, Table 2).

The sesquiterpenes β-selinene, δ-selinene, and γ-selinene (compounds **7–9**), (*E*)-β-farnesene (**10**), γ-cadinene (**11**), and nerolidol (**12**) were identified by

GC-MS. Additional NMR-analyses for some of the compounds confirmed the identification (Figure 1, Table 2). Based on GC-MS data and UV absorption at 254 nm (HPLC) due to conjugation of the double bonds, δ-selinene (**8**) was clearly distinguished from β-selinene (**7**) and γ-selinene (**9**). Importantly, this is the first report of nerolidol (**12**) from the genus *Reticulitermes*.

The diterpene geranyl linalool (**13**) was identified in all taxa by MS and NMR analysis (Figure 1, Table 2). We also identified the oxygenated diterpenes geranyl geraniol (**14**) and geranyl geranial (**15**), as well as the sesterterpene geranyl farnesol (**16**) from the defensive secretion of *R. santonensis*. This is the first report of compounds (**14–16**) from insect defensive secretions.

Interspecific Variation. The monoterpenes (**1**), (**2**), and (**3**) were only found in *R. santonensis*, in amounts of up to 2.51 μg/frontal gland, and none of the other species contained monoterpenes (Table 1). The sesquiterpenes **4**, **5**, and **6**, were each isolated from *R*. sp. nov. Germacrene A (**5**) was also found in *R. balkanensis* and *R. lucifugus*, and germacrene C (**4**) also in *R. lucifugus* and *R. lucifugus corsicus*. The germacrenes occurred in amounts ranging from 0.08 to 1.9 μg/gland (Table 1). Selinenes were restricted to the *R. lucifugus* group: δ-selinene (**8**) was found in *R. lucifugus corsicus*, while β-selinene (**7**) and γ-selinene (**9**) occurred in *R. lucifugus*. Selinenes occurred in amounts of less than 1 μg/gland (Table 1).

(E)-β-Farnesene (**10**) was common to *R. balkanensis* and the new species *R.* sp. nov. γ-Cadinene (**11**) was identified in *R. lucifugus*, *R. lucifugus corsicus*, and in large quantity (2.1 μg/gland) in *R. grassei* (Table 1). It was the only sesquiterpene found in *R. grassei*. Nerolidol (**12**) is present in both *R. lucifugus* and *R. lucifugus corsicus*. *R. banyulensis* did not contain any sesquiterpenes, nor did *R. santonensis*.

Geranyl linalool (**13**) was the major component in all species, with quantities ranging from 6.28 to 30.0 μg/gland, except for *R. santonensis*, where the amount averaged 0.37 μg/gland (Table 1). Instead, *R. santonensis* was the only species that contained geranyl geraniol (**14**) and geranyl geranial (**15**) in amounts of 1.09 and 0.83 μg/gland, and geranyl farnesol (**16**) as the major component with 46.0 μg/gland (Table 1).

DISCUSSION

Analysis of soldier frontal gland secretions of seven European *Reticulitermes* taxa revealed a mixture of terpenoid compounds, including monoterpenes, sesquiterpenes, diterpenes, and one sesterterpene. This finding is consistent with the fact that the defensive secretions of North American *Reticulitermes* species consist of terpenoid mixtures (Bagnères et al., 1990; Lemaire et al., 1990; Nelson et al., 2001). Terpenoid compounds are commonly found in nature, functioning as pheromones, defensive compounds, feeding deterrents, or fungicides (e.g., Juettner and Bogenschuetz, 1983; Lemaire et al., 1990; Boeve et al., 2000; Bartelt et al., 2001; Nelson et al., 2001; Shinoda et al., 2002; Wheeler et al., 2002).

Terpenoids are often difficult to characterize, requiring complementary methods for complete structure elucidation (Joulain and Konig, 1998; Bartelt et al., 2001; Hanson, 2001). GC-MS can prove insufficient for characterization of germacrenes, which under GC conditions, rearrange to the respective elemenes (Cope rearrangement) (Konig et al., 1996; De Kraker et al., 2001). Such structural rearrangements might explain differences in bioactivity sometimes encountered between natural extracts and synthetic compounds, if structure determination is based exclusively on GC-MS.

We, therefore, preferred to use HPLC at room temperature for the purification and isolation of "difficult" compounds, such as germacrenes, followed by micro-quantity NMR spectroscopy. In the case of germacrene A, this approach was insufficient, due to this compound's instability, forming four different conformations even at room temperature (De Kraker et al., 2001). Low temperature NMR spectroscopy might be the method of choice to fully characterize such compounds.

Microquantity NMR spectroscopy is a comparatively new approach, requiring only microgram amounts of a component, instead of several milligrams. This could facilitate NMR analysis of natural products from insects that often is limited due to the minute amounts available. However, considering that one might need hundreds or thousands of individuals per compound for HPLC-NMR analysis and that a large percentage of the compounds can evaporate during the transfer into NMR-solvent ($CDCl_3$), even the microquantity approach can prove insufficient, as was the case for some of the sesquiterpenes we attempted to characterize.

European *Reticulitermes* species are genetically isolated from each other with little evidence of sympatry (Figure 2) and currently no evidence of hybridization. They have been characterized based on morphological, chemical, and molecular data (Clément et al., 2001). The species-specific chemical profiles of their frontal gland secretions indicate a certain correspondence with the geographic location of the species in Europe (Figure 2).

R. santonensis from southwestern France seems to distinguish itself from the other European species, being the only species to produce the monoterpenes α-pinene, β-pinene, and limonene. It does not rely on geranyl linalool as a major component, but instead contains two other diterpenes and one sesterterpene (Table 1). Its defensive secretion shows strong chemical similarities to the secretions of the North American species *R. flavipes*, which occurs in multiple phenotypes, some of which contain the same monoterpenes as *R. santonensis*, and similarly can lack geranyl linalool (Zalkow et al., 1981; Bagnères et al., 1990; Nelson et al., 2001).

The taxonomic status of the European *R. santonensis* as a species separate from the North American *R. flavipes* has been questioned since it was described in 1924 (Feytaud, 1924). An analysis of spatial distribution and reproductive cycles suggests that *R. santonensis* is exotic to Europe, likely having been introduced from North America (Vieau, 2001). *R. flavipes* introductions into Europe are indeed

known to have happened many times since the mid-19th century (Becker, 1970, 1981). The chemical similarity of the frontal gland secretions of *R. santonensis* and *R. flavipes*, coupled with the similar hydrocarbon profiles of the two taxa, supports the hypothesis (Bagnères et al., 1990; Clément et al., 2001; Nelson et al., 2001). Further evidence is provided by recent phylogenetic analyses, which revealed a close genetic relationship between *R. flavipes* and *R. santonensis* (Clément et al., 2001; Jenkins et al., 2001). What remains to be determined is whether *R. santonensis* is, in fact, a *R. flavipes* genotype now native to France, or a population derived from another taxon from North America that is a sibling species of *R. flavipes*.

R. grassei and *R. banyulensis* extend through Spain and southern France, existing sympatrically in some areas (Figure 2). Frontal gland secretions contain only geranyl linalool and one or no sesquiterpenes, thus distinguishing them from the other species *R.* sp. nov., *R. balkanensis*, *R. lucifugus*, and *R. lucifugus corsicus*. The latter two form a distinct group displaying close chemical profiles, containing as only species selinenes and nerolidol in their secretions. *R. lucifugus corsicus* has been determined as a subspecies of *R. lucifugus*, with a *trans*-Tyrrenian distribution (Figure 2) (Clément et al., 2001; Uva et al., unpublished data).

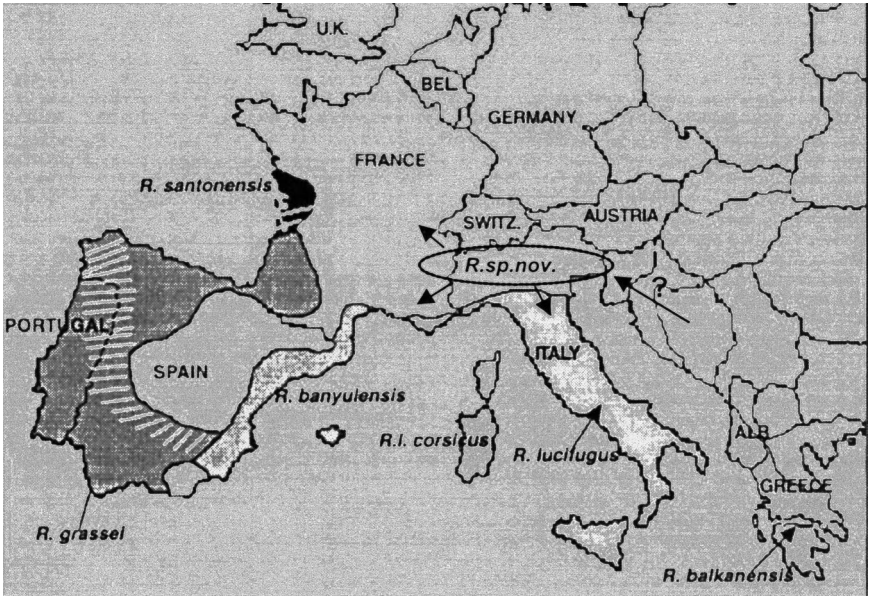

FIG. 2. Proposed endemic distribution of *Reticulitermes* species in Europe. White cross-hatched areas indicate sympatric zones between *R. grassei* and *R. santonensis* in the north and between *R. grassei* and *R. banyulensis* in the Iberian peninsula. Exact geographic distribution of the new species *R.* sp. nov. is still under investigation; arrows indicate possible origin and directions of distribution. Modified from Clément et al. (2001).

Interestingly, *R. balkanensis* from Greece shares many characteristics, such as germacrene A and especially (*E*)-β-farnesene, with the new species *R.* sp. nov. This new species is not yet fully described and has been found in urban dwellings in northern Italy and various locations in southern France (Figure 2). Our results together with other chemical, morphological, and genetic data suggest a common phylogenetic and geographic origin for *R.* sp. nov. and *R. balkanensis* (Clément et al., 2001; Uva et al., unpublished).

The biological significance of the species-specific variation in secretion profiles might lie in possible pheromonal function. There is evidence that minor compounds of the frontal gland secretion, such as monoterpenes and sesquiterpenes, play a role as alarm pheromones during colony defense, as also described for *Nasutitermes* termites (Roisin et al., 1990; Reinhard and Clément, 2002; Reinhard et al., unpublished data).

Comparative chemical analyses of *Reticulitermes* can contribute to our taxonomic knowledge of this complex genus, providing insight into species origin and evolution, and may be used as a key for species determination.

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COMBINED ROLES OF CONTACT STIMULANT AND DETERRENTS IN ASSESSMENT OF HOST-PLANT QUALITY BY OVIPOSITING ZEBRA SWALLOWTAIL BUTTERFLIES

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Abstract—Zebra swallowtail (*Eurytides marcellus*) butterflies are stimulated to oviposit by a single compound, 3-caffeoyl-*muco*-quinic acid (**1**). Analysis of the aqueous extracts of the leaves of the host, *Asimina triloba*, showed that they contained stimulant **1**, its isomer (**2**), and the flavonoids rutin (**3**) and nicotiflorine (**4**) as major components. We compared the concentrations of compounds **1**–**4** in terminal leaves (TL) and expanded leaves (EL) of the host plants at four different times throughout the growing season. In spring, the concentration of **1** was highest in TLs, and flavonoids were not detectable or present at low levels. As the season progressed, however, the concentrations of flavonoids increased, reached maxima by late summer, and then decreased as the plants started senescing. There were also significant differences in the concentrations of these compounds between TLs and ELs. In a choice assay with model leaves, we tested equivalent amounts of post-dichloromethane aqueous extracts made in spring (May) and in fall (September). September extracts received significantly fewer approaches and eggs. In greenhouse experiments with potted *A. triloba* plants, the butterflies chose some leaves to lay eggs, while others were rejected or ignored. Analyses showed that the concentrations of compound **1** were not significantly different in the three kinds of leaves. The flavonoids (**3** and **4**), however, were significantly higher in the leaves that were ignored. Multiple-choice tests using model plants suggested that concentrations of both flavonoids and stimulant were important in assessing host suitability. There was a gradual decrease in approaches as the concentration of **1** decreased. Higher amounts of flavonoids deterred egg laying even in the presence of high concentrations of stimulant **1**. At lower concentrations of **1,** the addition of low doses of flavonoids deterred egg laying. Thus, the results suggest that the butterflies use both qualitative and quantitative information about these compounds to assess host quality. This behavior may have

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evolved to take advantage of seasonal variation in the chemistry of their host, *A. triloba*.

Key Words—*Asimina triloba*, Annonaceae, *Eurytides marcellus*, Papilionidae, Graphiini, zebra swallowtail, hydroxycinnamic acid derivative, flavonoids, seasonal variation, oviposition stimulant.

INTRODUCTION

The neonate larvae of many phytophagous insects have limited mobility and their survival is influenced profoundly by the chemistry of the host plants on which the female chooses to oviposit (Zucker, 1982; Jeyabalan and Murugan, 1996; Wallin and Raffa, 1998). The chemistry of a given plant species may vary considerably in different seasons and environments (Mauffette and Oechel, 1989; Bingaman and Hart-Elwood, 1993; Bowers and Stamp, 1993; Haribal and Renwick, 2001). There is also variation in chemistry between and even within the tissues of individual plants (Van Dam et al., 1994; Alder et al., 1995; Lawler et al., 1998; Merritt, 1996). One would expect that selection would favor discrimination by ovipositing females in favor of those plants and tissues that are chemically most suitable for growth and survival of their progeny. Several studies have shown that larvae reared on plants that were chosen by female butterflies have maximal larval fitness that could be attributed to plant chemistry, predator avoidance, or other factors (Damman, 1986; Papaj 1986; Papaj and Rausher, 1987; Roininen and Tahvanainen, 1989; Lalonde and Roitberg, 1992; Barker and Maczka, 1996).

The host range of the zebra swallowtail, *Eurytides marcellus* (family Papilionidae, tribe Graphiini), is restricted to the single genus *Asimina* in the family Annonaceae (Miller, 1987). The butterfly is multivoltine, with adult abundance reaching a peak in late March in the southern part of the range, but butterflies are present in lower numbers during the rest of the growing season. *Asimina* spp. produce a single synchronous burst of leaves in spring. New foliage later in the summer is produced only in response to damage caused by other insects (Damman, 1989). Damman (1986), at his study site in Lake Delancy, Ocala, Florida, USA, found that in spring females of *E. marcellus* oviposited on leaves of *A. speciosa, A. obovata*, and their hybrids that were barely buds, while later in early summer they preferred plants with young leaves. A strong correlation was observed between oviposition and availability of young leaves at this site (Damman, 1989).

Similar oviposition behavior by *E. marcellus* was observed on *A. triloba* in the field in the northern part of its range, where the butterflies preferred to oviposit on the terminal leaf buds in early spring (M.H., personal observations). While rearing larvae of *E. marcellus* on *A. triloba* in our laboratory, we also found that neonate larvae do not develop well on randomly collected older leaves and suffered high mortality. Often in late summer, larvae grew slowly to the fourth or fifth instars

and then died or gave rise to deformed pupae. These observations suggest that the chemistry of the host plant may dramatically affect larval survival.

Recently, we reported the isolation and identification of 3-caffeoyl-*muco*-quinic acid **1** (a 3-caffeoyl-1,4,5-trihydroxycyclohexane 1-carboxylic acid), a hydroxycinnamic acid derivative, as a contact oviposition stimulant for the zebra swallowtail butterfly from the foliage of *A. triloba* (Haribal et al., 1998). We also reported the presence of another structural isomer (**2),** which was not stimulatory for oviposition, and of flavonoids in the host foliage. Furthermore, we found that the addition of a flavonoid fraction to the stimulant fraction inhibited egg laying in a choice test (Haribal and Feeny, 1998). Our preliminary examination of the chemistry of *A. triloba* extracts in various seasons showed that there was a reduction in the amount of stimulant **1** and an increase in amounts of both isomer **2** and flavonoids in leaves collected later in the season. Since contact cues are clearly involved in assessment of host suitability, we examined the role of variation in plant chemistry in host acceptance by the butterflies. We designed experiments to ask the following questions: (1) Is there variation in the host chemistry and, if so, is it seasonal? (2) Do females distinguish phenological differences based on the chemistry? (3) Is there a difference in the chemistry of the natural host leaves that are chosen or rejected by the butterflies? (4) Do females use the relative concentrations of stimulant and the flavonoids to assess host suitability?

METHODS AND MATERIALS

Plants. Foliage of *A. triloba* (L.) was collected from Marsh Creek, Ontario County, New York, USA, in September 1996 and from Selinsgrove, Pennsylvania, USA, in May 1997. We collected samples from the Cornell Plantations in Ithaca, New York, USA, for analysis of seasonal variation. In addition, potted plants of *A. triloba* were obtained from a nursery in Georgia, USA, in 1995 and maintained in the greenhouse.

Insects. Several female zebra swallowtails were collected in the Ocala National Forest, Florida, USA, in late March 1999, and their progeny were used in behavioral bioassays. The insects and their progeny were reared in a growth chamber under controlled conditions (16 L:8 D photoperiod; 30°C and 16°C; 80–85% relative humidity) on leaves of *A. triloba* collected locally (Haribal and Feeny, 1998) or on *A. speciosa* and *A. obovata* and their hybrids collected in the Ocala National Forest.

Extraction of Contact Stimulants. Leaves of *A. triloba* were extracted with boiling 95% EtOH (4 ml/g fresh wt) for about 5 min and blended in a Waring blender. The extract was filtered, concentrated under reduced pressure to remove excess EtOH, and then extracted with dichloromethane several times until no further color was extracted, to remove less polar compounds and chlorophyll. The

aqueous fraction was concentrated under reduced pressure to 5 GLE (gram leaf equivalents, fresh weight)/ml and stored in the refrigerator.

Fractionation of post-dichloromethane ("post-DCM") aqueous extracts of *A. triloba* foliage was carried out on a Waters 490 HPLC system using a C_{18} RP column (Luna, Phenomenex, 250 × 10 mm, 5 μm), eluted at a flow rate of 2 ml/min with a gradient of 1% acetic acid in water containing increasing concentrations of MeOH (0% MeOH at 0 min; 35% MeOH at 5 min; 45% MeOH at 20 min; 80% MeOH at 25 min; 100% MeOH at 35 min). The eluent was monitored at 254 nm with a Millipore photodiode array detector. Three fractions, F1–F3 were collected (Figure 1). F1 (0–22 min) contained mostly sugars and other polar components; F2 (22–29 min) contained hydroxycinnamic acid derivatives, typically mostly **1** (3-caffeoyl-*muco*-quinic acid) and compound **2** (4-caffeyol-1,3,5-trihydroxycyclohexane 1-carboxylic acid), along with other isomers present as a minor components. Compound **1** was collected as an individual component by repeated HPLC to obtain purer samples for bioassays. Fraction F3 (29–35 min) contained mostly flavonoids, based on their UV absorptions. The spectra of compounds **3** and **4** were characteristic of quercetin and kaempferol glycosides respectively.

General Bioassay Methods. Experiments were conducted in a field cage (1.9 × 1.9 × 1.9 m), set up in a greenhouse with free-flying butterflies and potted flowering plants (Haribal and Feeny, 1998). Trials were conducted on sunny afternoons between 12:00 and 15:00 hr, when temperatures were suitable for butterfly activity (29–35°C). Potted *A. triloba* saplings were made available to the butterflies for oviposition between bioassay trials.

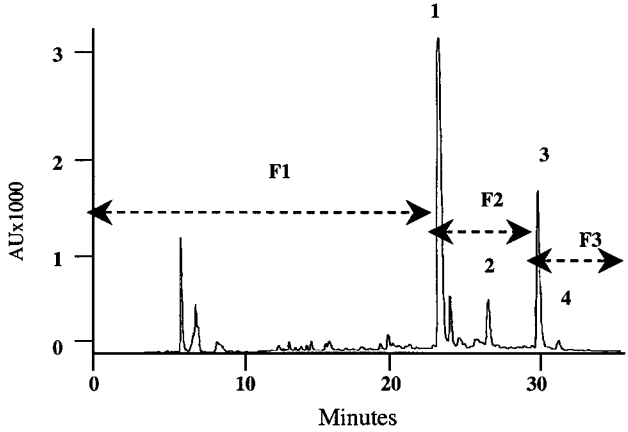

FIG. 1. HPLC chromatogram of post-dichloromethane aqueous extract of foliage of *A. triloba* (0.1 gle) collected in May 1997 from Selinsgrove, Pennsylvania, monitored at 254 nm. See text for identities of compounds **1**–**4**.

For bioassays of oviposition choice, two model plants were set 1 m apart in the cage. Each consisted of two round leaves, made of commercially available green sponge (4 cm diam. × 0.7–0.8 cm thick) and attached to horizontal wire branches that extended 15 cm symmetrically from the top of a wooden stem (40 cm tall). One leaf of each model plant was treated with an *A. triloba* extract, fraction, and/or pure compound, while the other leaf was treated with solvent alone. Each bioassay lasted for 1 hr. Model plants were rotated randomly every 15 min, and their positions were exchanged to minimize environmental and learning effects. Each female was numbered so that her behavior could be observed individually. A record was made each time a female engaged in one or more of four behaviors leading to oviposition, namely: approach, land on plant, curl abdomen, and lay egg (Haribal and Feeny, 1998). The number of females in each trial varied from 5 to 15 individuals, of which 3–7 typically engaged in oviposition activity. Generally females laid a single egg per landing. Data for each female from separate, replicated trials were pooled for analysis. The totals for each female and for all females were then calculated, and overall scores for responses to experimental and control treatments were compared.

We used mostly the post-DCM aqueous extract (0.25 GLE) of foliage collected from Selinsgrove, Pennsylvania, in May 1997 for bioassays. The post-DCM aqueous extract also contained other isomers. We have shown that the activity of compound **1** was sufficient to account for the activity of the parent post-DCM and whole ethanol extracts. The other isomers, including compound **2**, were inactive in behavioral trials (Haribal and Feeny, 1998). Fraction F3, containing **3** and **4**, from the September 1996 extract was used as the flavonoid fraction for testing deterrency. We used pure **1** isolated by repeated HPLC. We used commercial rutin (Sigma Chemicals) (as **3**) for experiments with pure flavonoid samples.

Variation in Flavonoids and Hydroxycinnamic Acid Derivatives of A. triloba *Foliage in Relation to Season and Leaf Maturity.* For quantitative analysis of the major hydroxycinnamic acid derivatives (**1** and **2**) and flavonoids (**3** and **4**), samples of *A. triloba* were collected from the Cornell Plantations (Ithaca, New York) on four dates during the 1998 growing season (May 29, July 1, August 10, and September 19). On each date, randomly chosen samples, five replicates each, were taken of young, terminal leaves (TL) from the tips of the growing points of the leaf clusters and of older, expanded leaves (EL) at positions 7 or 8 from the same clusters. A different set of plants was sampled on each sampling date. The terminal leaves ranged in length from <5 cm (in the spring) to almost 8 cm. They were tender and fleshy with a high water content (78%). The expanded leaves were 15–25 cm long and had a lower water content (58%).

Approximately 230 mg (wet weight) of leaf tissue from each sample were extracted with 5 ml of boiling ethanol and blended with a tissue tearer. Five ml of water were added to precipitate chlorophyll and other less polar compounds, and the resultant mixture was centrifuged. The supernatant (6 ml) was evaporated

almost to dryness, redissolved in water, and passed through a 4-μm filter, yielding a final concentration of 0.1 GLE/ml. Aliquots of 0.01 GLE were then injected onto the HPLC for quantitative analysis of the four major components.

Oviposition Responses of E. marcellus *Females to Extracts of Early and Late Season Foliage of A. triloba*. Aqueous post-DCM extracts were prepared from foliage of *A. triloba* collected from Marsh Creek, Ontario County, New York, in September 1996, and near Selinsgrove, Pennsylvania, in May 1997. For bioassays, one leaf of one of the model plants was treated with 0.25 GLE of the May extract, and one leaf of the other model plant was treated with the same dosage of the September extract. The control leaves of both model plants received water. The extracts were analyzed by HPLC to compare their contents of hydroxycinnamic acid and flavonoid derivatives. Results were analyzed by χ^2 tests for goodness of-fit.

Chemical Correlations with Female Oviposition Choice among Leaves of Potted A. triloba *Plants.* Five potted *A. triloba* saplings, each about 0.5–0.75 m tall with 12–15 leaves, were placed randomly in the greenhouse field cage among several potted *Lantana* plants. Three *E. marcellus* females, numbered individually, were introduced into the cage and their oviposition behaviors recorded individually for about 2 hr. Each *A. triloba* leaf was then assigned to one of three exclusive categories: (1) leaves on which one or more eggs were laid (accepted), (2) leaves that were landed on by at least one female but which did not receive eggs (rejected), and (3) leaves that received no landing (ignored). Ten accepted leaves, eight rejected leaves, and four ignored leaves were collected for chemical analysis. Each leaf was extracted separately with 95% ethanol, as described earlier and diluted with an equal volume of water. The extracts were centrifuged to remove leaf debris, waxes, chlorophylls, and other water insoluble compounds, after which 0.05 GLE of each sample were analyzed by HPLC for hydroxycinnamic acid (**1** and **2**) and flavonoid derivatives (**3** and **4**).

Responses of E. marcellus *Females to Stimulatory Extracts Supplemented with Flavonoids.* In an initial experiment, the four leaves of two model plants were treated with four different concentrations (0.25, 0.125, 0.063, and 0.031 GLE) of the post-DCM aqueous extract of *A. triloba* foliage, collected in May 1997 (cf. Figure 1). This parent extract was known to be stimulatory to butterflies and contained low amounts (0.048 mg/g) of total flavonoids. The 0.25 GLE and 0.063 GLE treatments were paired on one model plant, and the remaining two concentrations on the other.

This experiment was repeated twice with the following modifications. We first collected the flavonoid fraction, F3, from the post-DCM aqueous extract of *A. triloba* foliage collected in September 1996, which contained relatively high levels of **3** and **4**. In the first replicate experiment, we added an amount of this flavonoid fraction equivalent to 0.112 mg of rutin to the highest dose (0.25 GLE) of the May extract and applied this solution to the model leaf. The second replicate

experiment was identical in design except that we increased the flavonoid dose to the equivalent of 0.186 mg rutin. In both of these experiments, the remaining three model leaves were treated only with May 1997 extracts, as in the initial experiment. A 4×3 contingency table was prepared to compare approaches and eggs, and data were analyzed by a χ^2 test for goodness of-fit.

Responses of E. marcellus *Females to Pure* ***1*** *and Flavonoid Fractions and to Rutin.* We designed sets of experiments to test whether a reduction in quantities of stimulant **1** or an increase in concentration of flavonoid components inhibits the females from approaching and laying eggs on model leaves. In one set (A) of four experiments (A1–A4), we tested the effects of adding a flavonoid fraction (F3), obtained from September 96 extract of *A. triloba*, to pure stimulant **1**. In experiment A1, 1.00 mg of **1** was bioassayed against 1.00 mg of **1** + F3 equivalent 0.164 mg of rutin, based on UV absorption. This content of flavonoid is typical in *A. triloba* in mid-season. In experiment A2, 1.00 mg of **1** was tested with 1.00 mg **1** + F3 equivalent to 0.273 mg rutin. In experiments A3 and A4, 2.00 mg of **1** instead of 1.00 mg were used.

In another set of four experiments (B1–B4), we compared the activities of different mixtures of pure stimulant (**1**) and commercially available rutin (**3**), which was the major flavonoid in fraction F3. In this second set, two doses of rutin (0.30 mg and 0.60 mg) were assayed with each of two doses (1.00 and 2.00 mg) of **1**.

Statistics. For analysis of hydroxycinnamic acid derivatives and flavonoids, the area under each peak on HPLC chromatograms, monitored at 254 nm, was calculated. We compared concentrations in terminal (TL) and elongated leaves (EL) at each sampling date by the Mann-Whitney-Wilcoxon paired rank test (Siegel and Castellan, 1988).

For analysis of host choice by females, the data were analyzed by calculating F-Statistics for general linear model ANOVAs, using Minitab 10Xtra software. The null hypothesis that chemistry is not different in different leaves was tested.

For analysis of bioassay results, each approach was considered as a new event. Data for several females were pooled and analyzed. For each treatment, contingency tables were prepared for the total numbers of approaches and eggs. Similar tables were also prepared for the proportions of approaches that resulted in egg laying. The data were analyzed by computing the χ^2 statistic for goodness of fit test using Minitab 10Xtra software.

RESULTS AND DISCUSSION

Variation in Flavonoids and Hydroxycinnamic acid Derivatives of A. triloba *Foliage in Relation to Season and Leaf Maturity.* On the basis of UV absorption spectra, F3 (29–35 min) was found to contain at least four flavonoids (three quercetin derivatives and one kaempferol derivative), only two of which occurred in significant concentrations (Figure 1). Compound **3,** the major component, and

compound **4** were isolated and identified as rutin and nicotiflorine, respectively, on the basis of retention time and spectral studies (UV, NMR, and ESI-MS) and comparison with literature data (Mabry et al., 1970).

We monitored local *A. triloba* plants for variation in hydroxycinnamic acid derivatives (**1** and **2**) and flavonoids (**3** and **4**) throughout the growing season of 1998, from the first flush of leaves in May until just before leaf senescence in late September. The chemical profiles of the leaves changed substantially from spring to late summer. In particular, compound **1** was the major component in early spring, dropping to lower levels in the summer. However, **2** and both flavonoids (**3** and **4**) occurred at low or undetectable levels in the spring but increased markedly as the season progressed (Figure 2, Table 1).

Comparison of the chemical profiles of young terminal leaves and of older expanded leaves revealed no consistent trends. Although there was a tendency for compound **1** to occur at higher concentrations in terminal than in expanded leaves in the spring (not significant, $P = 0.854$, Mann-Whitney-Wilcoxon test), this pattern did not hold up through the summer. In fact, in July the expanded leaves contained significantly higher concentrations of **1** than did the younger terminal leaves (Figure 2, Table 1, $P = 0.048$, Mann-Whitney-Wilcoxon test). Concentrations of **2** were higher in expanded than in terminal leaves in July ($P = 0.004$), but not in other months.

Oviposition Responses of E. marcellus *Females to Extracts of Early and Late Season Foliage of A. triloba.* The previous experiment revealed that leaf concentrations of compound **1**, the major contact oviposition stimulant for *E. marcellus*, declined sharply from spring to summer while concentrations of flavonoids, suspected of being oviposition deterrents (Haribal and Feeny, 1998), increased over the same time period. We, therefore, compared the responses of females to extracts of early and late season foliage, at the same dosage, to determine if the preferences were as expected from the differences in chemistry.

Females were found to approach model leaves treated with the May extract more frequently than leaves treated with the September extract (Figure 3, $P = 0.010$, χ^2 test). They also laid fewer eggs on the September extract than on the May extract (Figure 3, $P = 0.010$, χ^2 test), and the proportion of approaches followed by curling and egg laying differed between the two treatments (χ^2 statistic $= 138.86$ for curling, $P < 0.001$, and 39.51 for eggs, $P < 0.001$). Quantitative analysis confirmed that the May extract contained higher amounts of compound **1** (2.970 mg/g vs. 0.992 mg/g) and lower amounts of total flavonoids (0.048 mg/g vs. 0.671 mg/g) than did the September extract.

The May extract was more stimulatory to females than was the September extract. Information from contact chemoreceptors was not available to approaching females, and volatile compounds, which might provide olfactory cues, were absent from the aqueous plant extracts. Although we changed the positions of the model plants and leaves every 15 min, this may not have been sufficiently frequent to

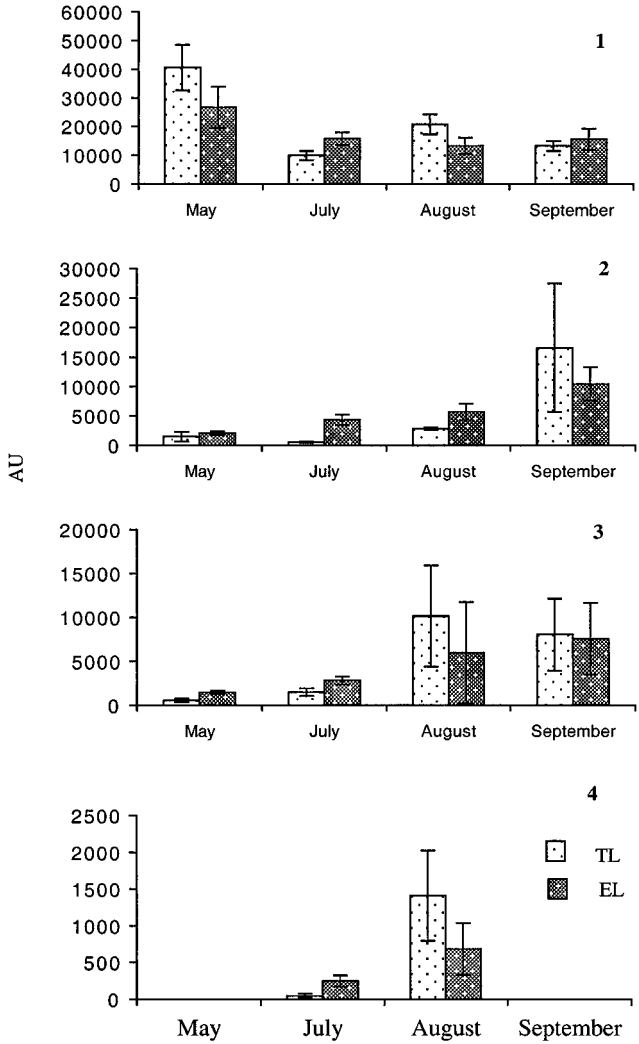

FIG. 2. Seasonal changes in concentrations of compounds **1**–**4** in foliage of *A. triloba*, as means (± SE) of AU (peak area) measured at 254 nm. TL = terminal leaves, EL = expanded leaves. For significance of differences between TL and EL concentrations at each date see Table 1.

prevent females from associating leaf position with prior experience of contact stimulants and/or deterrents. However, after the position of the model plants was exchanged during trials, females were consistently seen to make their first approach directly to the model leaf with active extract (MH, unpublished). This suggests

TABLE 1. SIGNIFICANCE OF DIFFERENCES IN CONCENTRATION OF EACH COMPOUND BETWEEN EXPANDED (EL) AND TERMINAL LEAVES (TL) OF *A. triloba* FOR EACH MONTH[a]

	P			
Compounds	May	July	August	September
1	0.854	0.048	0.881	0.500
2	0.111	0.004	0.075	0.274
3	0.028	0.500	0.726	0.354
4	NA[b]	0.028	0.290	NA

[a] EL and TL data for each compound were compared by Mann -Whitney-Wilcoxon's Paired Rank Tests.
[b] NA indicates that there were no detectable amounts of **4** in any kinds of leaves.

that the females were able to distinguish visually between the May and September extracts from a distance, even though the model leaves appeared indistinguishable to us. Both flavonoids and hydroxycinnamic acid derivatives absorb strongly in the UV, and butterflies are known to be able to detect light within this wavelength range (Takagi and Arikawa, 1998; Arikawa et al., 1999; Gronquist et al., 2001). Such visual responses might be learned through association with contact cues or conceivably could be at least partly heritable.

Chemical Correlations with Female Oviposition Choice among Leaves of Potted A. triloba Plants. When females were allowed to oviposit on *A. triloba* saplings in the greenhouse, they laid eggs on some leaves, landed on and rejected others, and, finally, ignored some of the leaves completely. When these three leaf categories were compared, significant differences were found in the content

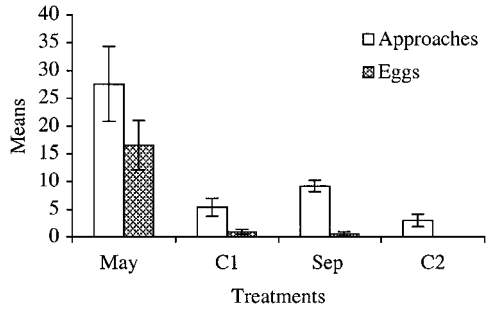

FIG. 3. Responses of females (as means of approaches and eggs ± SE, $N = 11$) to the post-dichloromethane aqueous extracts of *A. triloba* foliage collected in May 1997 from Selinsgrove, Pennsylvania, and in September 1996 collected from Marsh Creek, New York. C1 and C2: Responses to control leaves treated with HPLC-grade water.

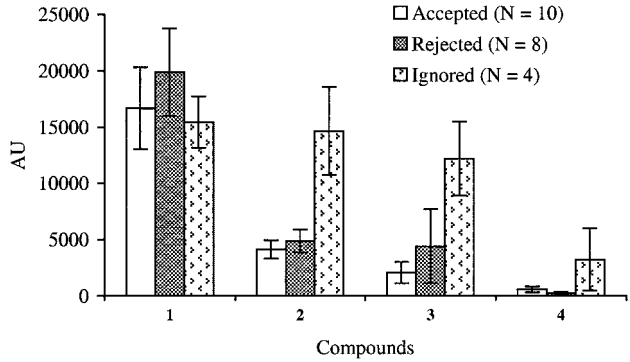

FIG. 4. Mean concentrations (± SE) of compounds **1**–**4** in *A. triloba* leaves that were accepted, landed on but rejected, or ignored by females. Data are expressed as absorption units (AU) measured at 254 nm.

of compounds **2** and **4**, but not of **1** and **3** (**1**: $P = 0.725$; **2**: $P = 0.001$; **3**: $P = 0.070$; 4: $P = 0.050$; general linear model ANOVA). However, for none of the compounds was there a difference between leaves that were accepted and those that were rejected after landing (Figure 4). Concentrations of **3** did not differ when all three leaf categories were compared, but they differed between the leaves that were accepted and those that were ignored ($P = 0.001$). Thus, the results indicate that landing is less likely to occur when higher concentrations of flavonoids are present. It is more likely, however, that these chemical differences are correlated with differences in other leaf traits that may be used as visual or olfactory cues influencing female oviposition choice.

Responses of E. marcellus *Females to Stimulatory Extracts Supplemented with Flavonoids*. The numbers of approaches and eggs laid by *E. marcellus* females on model leaves declined with decreasing doses of **1** in the post-DCM aqueous extract of May 1997 (experiment 1; Figure 5). In particular, the two lowest doses received fewer eggs than did the highest dose ($\chi^2 = 54.00$, $df = 3$, $P < 0.001$).

When results from all three experiments (1–3, Table 2) were analyzed by a χ^2 test on the 4×3 contingency table, no significant difference was found in approaches ($\chi^2 = 5.607$, $df = 6$, $P = 0.469$) among the treatments, but the total number of eggs on the three treatments differed ($\chi^2 = 21.921$, $df = 6$, $P = 0.001$). This difference in egg distribution resulted primarily from the much lower than expected number of eggs laid on the flavonoid treatment in experiment 3. When the results from only the 0.25 GLE dosages in the three experiments were compared, the addition of flavonoids reduced the number of eggs ($\chi^2 = 14.721$, $df = 2$, $P < 0.001$) on that treatment, although the approaches did not differ. While the addition of lower amounts of flavonoids (0.112 mg) to the highest dosage of

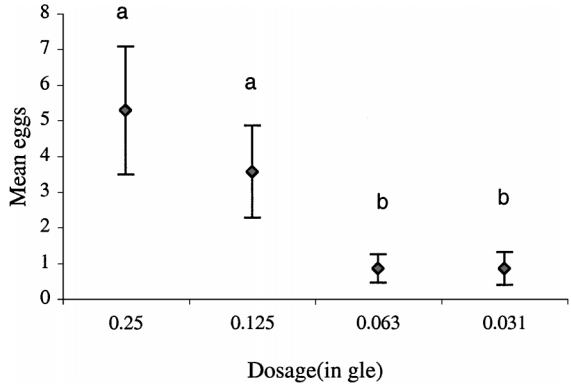

FIG. 5. Numbers of eggs (mean ±SE, $N = 7$) laid by female butterflies on model leaves treated with decreasing concentrations of post-dichloromethane aq. extract of *A. triloba* foliage of May 1997.

stimulant did not change the acceptance, the addition of higher amounts (0.186 mg) of flavonoids reduced the number of eggs. The numbers of curlings per landing also were reduced ($\chi^2 = 41.802$, $df = 2$, $P < 0.001$). This is consistent with the conclusion that contact chemoreceptors are involved in perception of flavonoids after landing.

*Responses of E. marcellus Females to Pure **1** and Flavonoids.* The addition of any amount of flavonoid to the lower dose of **1** reduced the proportion of egg laying relative to approaches (A1 and A2) ($\chi^2 = 6.785$, $df = 1$, $P = 0.009$

TABLE 2. EFFECT ON OOVIPOSITION BY *E. marcellus* FEMALES OF ADDING TWO DIFFERENT DOSES OF *A. triloba* FLAVONOIDS (F3) TO HIGHEST CONCENTRATION OF FOUR DIFFERENT DOSES OF MAY 1997 POST-DCM AQEOUS EXTRACT

Exp.		0.25 GLE extract	0.125 GLE extract	0.063 GLE extract	0.031 GLE extract	Females (N)
1	Approaches	69	54	34	32	7
	Eggs	48	33	9	7	
2[b]	Approaches	74	39	33	25	9
	Eggs	56	38	16	8	
3[c]	Approaches	50	41	35	19	8
	Eggs	12	29	18	9	

[a] Experiments 4.1–4.3 (4 × 3 contingency table).
[b] In experiment 2, flavonoid fraction of F3 equivalent of 0.112 mg rutin of was added to 0.25 GLE extract.
[c] In experiment 3, flavonoid fraction of F3 equivalent of 0.186 mg rutin of was added to 0.25 GLE extract.

TABLE 3. EFFECT OF ADITION OF FLAVONOIDS (F3) TO PURE **1** ON OVIPOSITION BY *E. marcellus*[a]

Exp.	1 mg	F3 mg[b]	Approaches	Eggs	χ^2 (P values) at $df = 1$	(Females) N
A1	1.00	0.000	56	26	6.785 ($P = 0.009$)	7
	1.00	0.164	53	12		
A2	1.00	0.000	55	24	4.682 ($P = 0.03$)	7
	1.00	0.273	36	9		
A3	2.00	0.000	84	43	2.874 ($P = 0.09$)	7
	2.00	0.164	58	38		
A4	2.00	0.000	45	29	4.666 ($P = 0.031$)	7
	2.00	0.273	37	15		

[a] Results are represented as χ^2 tests on 2×2 table of experiment A for number of approaches resulting in eggs to **1** and **1** plus flavonoid fraction from the host.
[b] Fraction F3 amount is in rutin equivalents (based on UV absorption).

and $\chi^2 = 4.682$, $df = 1$, $P = 0.03$, respectively) (Table 3). At higher amounts of **1** and lower doses of flavonoid (A3), however, the reduction in the number of eggs was not significant ($\chi^2 = 2.874$, $df = 1$, $P = 0.09$). Thus, the insects were unaffected by flavonoids in small doses in the presence of higher doses of **1**, although higher concentrations of flavonoid became inhibitory (A4) ($\chi^2 = 4.666$, $df = 1$, $P = 0.031$) (Table 3).

When the experiments were repeated using pure rutin in place of the flavonoid fraction F3 (experiment B, Table 4), the results were similar, except that the lower dose of rutin had no effect ($P = 0.408$). The results suggest that females are using combinations of concentrations of both **1** and flavonoids to assess host quality.

On the basis of these behavioral results, we can conclude the following: (1) Female zebra swallowtails can discriminate between suitable and unsuitable host plants before landing. (2) Once they land, butterflies are able to compare

TABLE 4. EFFECT ADDITION OF RUTIN TO PURE **1** ON OVIPOSITION BY *E. marcellus*[a]

Exp.	1 (mg)	Rutin (mg)	Approaches	Eggs	χ^2 (P values) at $df = 1$	(Females) N
B1	1.00	0.000	76	65	0.686 ($P = 0.408$)	7
	1.00	0.300	66	53		
B2	1.00	0.000	96	75	14.413 ($P < 0.001$)	7
	1.00	0.600	131	82		
B3	2.00	0.000	65	58	17.786 ($P < 0.001$)	7
	2.00	0.300	58	32		
B4	2.00	0.000	71	59	1.512 ($P = 0.022$)	7
	2.00	0.600	45	24		

[a] Results are represented as χ^2 tests on 2×2 table of experiment 5b for number of approaches resulting in eggs to **1** and **1** plus flavonoid fraction from the host.

chemical profiles by contact perception to further discriminate between leaves. The flavonoids appear to be perceived only after landing. (3) The female butterflies use both qualitative and quantitative information from **1** and flavonoids to assess host suitability.

Field studies by Damman (Damman, 1986; Damman and Feeny, 1988) have shown that survival of zebra swallowtail larvae is greatly dependent on the quality of food. The larvae on the plants that were chosen by ovipositing females, generally characterized by high proportion of young leaves, had highest survival rates. When larvae were transferred either to those plants on which the females landed and rejected or to those rejected outright before landing, they suffered higher mortality rates. In natural environments, female zebra swallowtails must encounter host plants at various stages of development and chemical composition. Our results indicate that they take advantage of changes in the relative concentrations of hydroxycinnamic acid derivatives and flavonoids to assess the suitability of the host for their progeny. Such changes are correlated with changes in plant chemistry elsewhere in the plant that may have a direct or an indirect effect on survival of the larvae. Survival of larvae may be influenced by more subtle chemical changes in their host plants, in addition to changes directly affecting nutrition.

A. triloba is known to contain an array of chemicals, such as tetrahydrofuran acetogenins and isobenzoquinoline alkaloids, which are known to have insecticidal properties (Tomita and Kozuka, 1965; Miller and Feeny, 1989; He et al., 1996). These may have either positive or negative effects on feeding, growth, and survival of the larvae. Recent studies have shown that adults, larvae, and pupae of zebra swallowtail butterflies sequestered tetrahydrofuran acetogenins (Martin et al., 1999). Thus, survival of their progeny may be enhanced by their choice of plants at a stage where the chemistry is optimal.

Contact oviposition stimulants have been reported for several swallowtail species in four of the seven tribes comprising the family Papilionidae. Butterflies in the tribe Papilionini typically respond to synergistic mixtures of flavonoid glycosides, hydroxycinnamic acid derivatives, bases, amphoteric compounds (e.g., proline, stachydrine), and simple cyclitols (inositols, quinic acid), although not all of these may be required by every species (Feeny, 1991; Nishida, 1995).

Several species of butterflies use flavonoids as a cue to oviposit. Some flavonoids seem to stimulate oviposition, and others either have no effect or are deterrent (Honda, 1995). For example, narirutin and naringin are structural isomers found in the same plant (*Citrus unshiu*). Narirutin was a stimulant for *Papilio xuthus* and naringin for *Papilio protenor*, both of which use the same plant for oviposition, but narirutin was inactive to *P. protenor* (Honda and Hayashi 1995) (Table 5). Probably the two butterfly species differ in their choice of season and site for oviposition, as plant chemistry varies spatially and seasonally. Several *Papilio* species, *Luehdorfia japonica* (Nishida, 1994), the monarchs, (*Danaus plexippus*)

TABLE 5. FLAVONOIDS AS OVIPOSITION STIMULANTS OR DETERRENTS FOR BUTTERFLIES

Flavonoids	s/d/i[a]	Butterfly	Source	Reference
Vicenin-2	s	*Papilio xuthus*	*Citrus unshiu*	Oshugi et al. (1991)
Narirutin	s	*P. xuthus*	*C. unshiu*	Oshugi et al. (1991),
	i	*P. protenor*		Nishida et al. (1987)
Naringin	s	*P. protenor*	*C. unshiu*	Honda (1990)
	i	*P. xuthus*	*C. natsudaidai*	
Hesperidin	s	*P. protenor*	*C. unshiu*	Honda (1986)
	s	*P. xuthus*		Oshugi et al. (1991)
Rutin	s	*P. xuthus*	*C. unshiu*	Oshugi et al. (1991)
3,5,7,4-Tetrahydroxy-8-(3-methylbut-2-enyl)flavone-7-*O*-β-glucoside (phellamurin)	d	*P. protenor*	*Phelleodendron amurense*	Honda (1995)
	d	*P. xuthus*		
Rhoifolin	i	*P. protenor*	*C. natsudaidai*	Honda (1986)
Isorhamnetin 3-*O*-glucosyl-(6"-*O*-galactoside)-7-*O*-glucoside	s	*Luehdorfia japonica*	*Heterotropa aspera*	Nishida (1994)
6,8-di-C-β-Glucopyranosylapigenin	s	*P. xuthus*	*C. natsudaidai*	Oshugi et al. (1985)
Quercetin 3-*O*-(2"-*O*-β-D-xylopyranosylrutinoside)	d	*P. xuthus*	*Orixa japonica*	Nishida et al. (1990)
Luteolin 7-*O*-(6"-*O*- malonyl)-.β-D-glucopyranoside	s	*P. polyxenes*	*Daucus carota*	Feeny et al. (1988)
Luteolin 7-*O*-β-D-glucopyranoside	i	*P. polyxenes*	*D. carota*	Feeny et al. (1988)
Quercetin 3-*O*-6"-β-D-glucopyranosyl-)β-D-galactopyranoside,	s	*Danaus plexippus*	*Asclepias curassavica*	Haribal and Renwick (1996)
Quercetin-3-*O*-(2'-*O*-β-xylosyl)-β-D-galactoside	s	*D. plexippus*	*A. incarnata*	Haribal and Renwick (1998)
Quercetin 3-*O*-(2",6"-β-L-dirhamnopyranosyl)-β-D-galactopyranoside,	s	*D. plexippus*	*A. curassavica*	Haribal and Renwick (1996)

[a] s = stimulant, d = deterrent and i = inactive.

(Haribal and Renwick, 1996, 1998), and other insects (Zucker, 1982) use flavonoid and phenolic constituents to assess the suitability of their host plants. The butterflies may have some common mechanisms for recognizing the flavonoids, but have further fine-tuned their individual responses to recognize some of them as positive cues and others as negative ones.

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CHARACTERIZATION OF FURANOCOUMARIN METABOLITES IN PARSNIP WEBWORM, *Depressaria pastinacella*

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Abstract—Although metabolites of furanocoumarins have been characterized in a wide range of organisms, to date they have been identified in only a single insect species, *Papilio polyxenes*. *Depressaria pastinacella*, the parsnip webworm, like *P. polyxenes* a specialist on Apiaceae, routinely consumes plant tissues higher in furanocoumarin content than does *P. polyxenes* and is capable of faster cytochrome P-450-mediated detoxification of these compounds. In this study, we characterized metabolites of xanthotoxin, a linear furanocoumarin, and sphondin, an angular furanocoumarin, in midguts and frass of parsnip webworms. Two metabolites were isolated and identified from webworms fed artificial diet containing xanthotoxin. LC-ESI-MS analysis resulted in the determination of a MW of 266 for the compound in the frass and one of the compounds in the midgut; ^{1}H NMR confirmed its structure as 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyacetic acid (HCHA). The second compound from the midgut had a MW of 252 and was identified by ^{1}H NMR and ^{13}C NMR analysis as 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyethanol) (HMCH). Whereas HCHA has been found in frass of *Papilio polyxenes* fed xanthotoxin, HMCH has not been reported previously in insects. Although the first step of metabolism of xanthotoxin in webworms as well as *P. polyxenes* is likely the formation of an epoxide on the furan ring, angular furanocoumarin metabolism in webworms appears to differ. The principal metabolite of sphondin was identified as demethylated sphondin (6-hydroxy-2H-furo[2,3-h]-1-benzopyran-2-one) by LC-ESI-MS and confirmed by

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^{1}H NMR and ^{13}C NMR analyses. That webworms produce metabolites of xanthotoxin in common not only with other Lepidoptera (e.g., HCHA) but with other vertebrates (e.g., HMCH) suggests a remarkable conservatism in the metabolic capabilities of cytochrome P-450s and raises the possibility that insects may share other detoxification reactions with vertebrates with respect to toxins in foodplants.

Key Words—Cytochrome P-450, insect-plant interactions, *Papilio polyxenes*, sphondin, xanthotoxin, *Depressaria pastinacella*, Furanocoumarin metabolites.

INTRODUCTION

Oligophagous insect herbivores, by virtue of feeding on three or fewer host-plant families (Bernays and Graham, 1988), tend to encounter a narrower range of phytochemicals than do polyphagous species that feed on a broader array of chemically diverse host-plant families. However, in narrowing their host-plant range and in concentrating on nutrient-rich and chemically well-defended tissues, specialists can encounter greater quantities of particular plant toxins over the course of their development. Understanding how specialists can cope with such enormous exposure to toxins is of interest in elucidating both the constraints and the benefits of oligophagy, the predominant form of feeding in insect herbivores (Bernays and Graham, 1988).

Among the phytochemicals that are both encountered and detoxified to different extents in oligophagous and polyphagous herbivores are the furanocoumarins, benz-2-pyrone compounds that owe their toxicity to their ability, in the presence of ultraviolet light, to cross-link DNA and interfere with transcription (Berenbaum, 1991). Although furanocoumarins are toxic or act as antifeedants at low concentrations to a range of polyphagous caterpillars (Berenbaum, 1995; Berenbaum and Zangerl, 1996), specialists on plant families containing furanocoumarins display considerably greater ability to tolerate high concentrations of these compounds in their diet. In all Lepidoptera examined to date, furanocoumarins are metabolized by cytochrome P-450 monooxgenases (Berenbaum, 2002); resistance to furanocoumarins is associated with faster and more efficient metabolism of these compounds.

Although metabolites of furanocoumarins have been characterized in a wide range of organisms, including humans, goats, rats, dogs, chickens, and bovine rumen microbes (Ivie et al., 1986a,b; Ivie, 1987), to date, metabolites of these compounds have been identified only in a single insect species, *Papilio polyxenes*, the black swallowtail caterpillar (Ivie et al., 1983). *P. polyxenes* feeds primarily on the foliage of furanocoumarin-containing host plants in the families Apiaceae and Rutaceae and can metabolize furanocoumarins 10–100 times faster than can generalist noctuids (Berenbaum, 1995). At least one cytochrome P-450

isozyme, CYP6B1, has been shown to contribute specifically to metabolism of furanocoumarins in this species (Ma et al., 1994).

Cytochrome P-450s have also been shown to play a role in the metabolism of furanocoumarins in *Depressaria pastinacell*a, the parsnip webworm (Nitao, 1991). Parsnip webworms feed exclusively on reproductive tissues of *Pastinaca* and *Heracleum*, two furanocoumarin-rich genera within the family Apiaceae. They owe their ability to consume this diet to high rates of cytochrome P-450-mediated metabolism (Nitao, 1991; Berenbaum and Zangerl, 1992). In Illinois, webworms metabolize xanthotoxin, a parsnip furanocoumarin, up to 300 times faster than more polyphagous species (Zangerl and Berenbaum, 1993); these caterpillars daily ingest 2% of their body weight in xanthotoxin, based on consumption rates (Zangerl et al., 1991) and fruit furanocoumarin concentrations (Zangerl et al., 1997). Sphondin, also found in parsnip, is metabolized more slowly than xanthotoxin (Berenbaum and Zangerl, 1992); this relative ranking of metabolic activity corresponds to the relative abundance in hostplants of these furanocoumarins (Berenbaum and Zangerl, 1998).

Although there are similiarities between P-450-mediated metabolism of furanocoumarins in parsnip webworms and in black swallowtails—both species, for example, metabolize linear furanocoumarins faster than angular furanocoumarins, and both species exhibit reduced ability to metabolize mixtures of furanocoumarins due to analogue synergism (Berenbaum and Zangerl, 1996)—according to Northern analysis, the furanocoumarin-inducible P-450s of these two species are not closely related (Cohen et al., 1992). Accordingly, we undertook a study to characterize the metabolites of furanocoumarins in the midgut and frass of parsnip webworms, in order to gain insight into the qualitative differences in furanocoumarin detoxification in two Apiaceae specialists. Specifically, we isolated and identified metabolites of xanthotoxin, a linear furanocoumarin, and sphondin, an angular furanocoumarin, in both frass and midgut, in order to understand the dynamics and outcome of toxin processing in this insect, which displays considerably greater tolerance, particularly for angular furanocoumarins, than does the black swallowtail, which encounters lower absolute levels, but a greater furanocoumarin diversity, across its broader hostplant range.

METHODS AND MATERIALS

Insects

Laboratory-reared parsnip webworms were offspring of insects collected from Cypress Hills Provincial Park Ski Area, Alberta, Canada, and Big Tesqueque, New Mexico, USA. Insects were raised from neonates to sixth instars on semidefined artificial diet (Nitao and Berenbaum, 1988) containing either xanthotoxin or sphondin to induce detoxification enzyme activity (Cianfrogna et al., 2002).

Field-collected webworms were from wild parsnip plants in Champaign County, Illinois, USA, and were used directly for experiments.

Chemicals

Unless otherwise indicated, chemicals were from Sigma (St. Louis, Missouri, USA). Sphondin was isolated from *Heracleum lanatum* seeds. Seeds (116 g) were blended in 500 ml ethyl acetate (EtOAc), filtered, and extracted twice again with EtOAc (200 ml each). The combined EtOAc extracts were rotary-evaporated to dryness. The entire residue (10.4 g) was redissolved in a minimum volume of warm EtOAc and applied on a vacuum liquid chromatography column (105 g silica gel, Merck 32–63 μm). The column was first eluted with 150 ml hexane, followed by 250 ml of 95:5 hexane–EtOAc. Collection of fractions (200 ml each) was initiated upon elution with 90:10 hexane–EtOAc (fractions 1 and 2), followed by 85:15 hexane–EtOAc (fractions 3–11), and 75:25, 65:35, 55:45, and 50:50 hexane–EtOAc (fractions 12, 13, 14, and 15, respectively). Fractions were monitored by HPLC (Alltech Adsorbosphere silica gel 4.6 $\times$ 150 mm column; 55% cyclohexane, 42% isopropyl ether, 3% butanol; 1.5 ml/min). Sphondin was isolated from fractions 12 and 13 by preparative HPLC using the same solvent system (Waters RCM 25 $\times$ 10 module, 25 $\times$ 100 mm Nova-Pak HR silica gel, 9 ml/min). The sphondin was recrystallized in butanol, and the crystals were washed in hexane, resulting in 27 mg of material.

Isolation of Metabolites

Detection. Furanocoumarin metabolites were detected with HPLC by comparing extracts and fractions from furanocoumarin-fed larvae with those processed similarly from webworms fed control diet without furanocoumarin. Fractions were analyzed with reverse-phase HPLC (C-18 column, Waters Novapak, 4.7 $\times$ 150 mm). For detection of both xanthotoxin and sphondin, a gradient elution was performed at a flow rate of 1 ml/min; solvent A was composed of 5% MeOH, 1.9% glacial acetic acid, and 93.1% water by volume, and solvent B was acetonitrile. Gradient conditions were 100% A initial, then linear gradient to 70% A/30% B over 15 min. Fractions from sphondin-fed larvae were also analyzed with an isocratic solvent system of 30% acetonitrile, 3.5% MeOH, 66.5% water. Analytes absorbing at wavelengths between 210 and 400 nm were monitored with a photodiode array detector (Waters 996). Fractions containing HPLC peaks unique to furanocoumarin-fed larvae were presumed to contain metabolites and were investigated further.

Xanthotoxin Metabolites from Incubation with Gut Homogenate. Midguts were removed from 310 sixth instars of *D. pastinacella* that were raised on artificial diet containing 0.1% xanthotoxin (fresh weight). Guts were dissected and cleared in

cold sucrose medium (Cohen et al., 1989), homogenized in 310 ml of 0.1 M sodium phosphate buffer (pH 7.8) containing PMSF, leupeptin, and glycerol, and stored at −80°C (Cianfrogna et al., 2002) until use. For incubation with furanocoumarin, the homogenate was thawed, and NADPH-generating system components (Cianfrogna et al., 2002) and xanthotoxin (20 mg/3.1 ml methanol) were added. After incubating for 1 hr at 30°C, the reaction mixture was frozen at −80°C and freeze-dried.

Freeze-dried residue was extracted with 100 ml 50% aqueous methanol (MeOH) and centrifuged to remove tissue. MeOH was removed by rotary-evaporation *in vacuo*, and aqueous material was extracted with 100 ml EtOAc to remove unmetabolized xanthotoxin and lipids; residual EtOAc was evaporated from the aqueous layer, and the aqueous layer was freeze-dried. Residue of the aqueous layer was dissolved in 5 ml water and loaded onto a 2.8 × 18 cm Amberlite XAD-16HP column (Supelco, Bellefonte, Pennsylvania, USA) prepared by washing with MeOH and equilibrating in water. The column was sequentially eluted with 200 ml each of water, 50% MeOH, and 100% MeOH. The 50% MeOH eluate was dried (50 mg), dissolved in 50 ml water, acidified to pH 2 with 5% HCl, and partitioned with EtOAc (3 × 50 ml). The ethyl acetate fraction was dried (4 mg), and metabolite(s) purified by reverse-phase HPLC (gradient separation as described in *Detection* above, but with a gradient of 100% to 85% A over 7 min). The 100% MeOH eluate of the XAD-16HP column was dried (36 mg), and a portion (20 mg) was subjected to reverse-phase HPLC for purification of metabolite (as described in *Detection*).

Additional metabolite material was obtained by incubating gut homogenate from 150 sixth instars of *D. pastinacella* collected from the field feeding on wild parsnip plants (Champaign County, Illinois, USA). Gut homogenate was prepared and incubated with xanthotoxin as described above. After incubation, the reaction mixture was frozen and freeze-dried to concentrate the mixture to 100 ml. An equal volume of MeOH was added, and the extract was centrifuged to remove tissue. After rotary-evaporating off the MeOH, the resulting aqueous portion was extracted with EtOAc. The aqueous layer was concentrated and applied to an XAD-16HP column, which was sequentially eluted with 200 ml each of water and 50% MeOH. MeOH from the 50% MeOH eluate was removed by rotary evaporation, and the resulting aqueous material was acidified to pH 2 with 5% HCl and extracted with EtOAc. The EtOAc extracts were combined and, after drying, were redissolved in 50% MeOH and passed through a C-8 solid-phase extraction column (Baker-10 SPE, 3 ml LD). The eluate was collected, and metabolite was purified with reverse-phase HPLC (gradient solvent system as described in *Detection*, but with a gradient of 100% to 85% A over 5 min).

Xanthotoxin Metabolite from Frass. Frass was collected from sixth instars of *D. pastinacella* that were fed artificial diet containing xanthotoxin (0.1% wet weight). After freeze-drying, frass (10.3 g) was extracted with 50% methanol (3 × 40 ml). Methanol was evaporated *in vacu*o, and the remaining aqueous solution

was divided and passed through 10 solid-phase extraction columns (Baker-10 SPE, Octyl C8, 3 ml LD). Eluates were collected and freeze-dried. A portion of the freeze-dried eluate (0.75 g) was dissolved in 50 ml water, acidified to pH 4 with 5% HCl, and partitioned with ethyl acetate (4×50 ml). The aqueous layer was further acidified to pH 2 and partitioned with 4×50 ml ethyl acetate. The ethyl acetate extract of the pH 2 aqueous phase was evaporated to dryness *in vacuo*. The residue (33 mg) was redissolved in 5% aqueous MeOH and fractionated on reverse-phase HPLC (gradient solvent system as described in *Detection*).

Sphondin Metabolite from Incubation with Gut Homogenate. Midguts were dissected from 325 *D. pastinacella* sixth instars that were raised on artificial diet containing 0.005% fresh weight sphondin. Guts were homogenized in 310 ml sodium phosphate buffer and treated as described for xanthotoxin metabolism except that incubation was performed with 10 mg sphondin. After incubation for 1 hr, the reaction mixture was freeze-dried and extracted with 100 ml 50% MeOH and centrifuged to remove tissue. The aqueous MeOH extract was rotary-evaporated to remove MeOH, and the remaining aqueous material was partitioned with 100 ml EtOAc. After drying, the EtOAc fraction was taken up in 1.5 ml 66% aqueous MeOH; insoluble material was centrifuged out, and the supernatant was passed through a C-8 solid-phase extraction column that was eluted further with 66% MeOH. The eluate was dried, and the residue was extracted with EtOAc (3×500 μl). The metabolite in the EtOAc extract (11 mg) was isolated by HPLC on normal phase as described for the isolation of sphondin.

Structure Elucidation of Metabolites

LC-ESI-MS Analysis for Insect Metabolites (Used on All Metabolites). Samples were run on a Finnigan-Thermoquest LCQ LC-MS system (AS3000 autoinjector, P4000 HPLC pump, UV6000 PDA detector, LCQ mass spectrometer) (San Jose, California, USA) all running under the Xcaliber 1.2 software system. MS was run with the ESI probe in the negative mode. The column was a 3×150-mm Inertsil reverse-phase C-18, ODS 3, 3 micron column (Metachem, Torrance, California, USA) with a Metaguard guard column. The source inlet temperature was set at 220°C, and the sheath gas rate was set at 90 arbitrary units. The MS was optimized for the detection of xanthotoxin metabolites by using the autotune feature of the software while infusing a solution of xanthotoxin in with the effluent of the column and tuning on an atomic mass unit of 215. Solvent system A was water with 0.1 M ammonium acetate, and 0.25% acetic acid, and system B was methanol with 0.1 M ammonium acetate, and 0.25% acetic acid. The column was equilibrated with 2% B at a flow rate of 0.3 ml/min. After injection, the column was held at the initial conditions for 2 min, then developed with a linear gradient to 40% B over 23 min, and then to 50% B over the next 10 min. The column effluent was monitored at 310 nm in the PDA detector. The software package was set to

collect mass data between 150 and 1000 AMUs. Generally, the most significant sample ion generated under these conditions was $[M-1]^-$.

GC-MS Analysis for Insect Metabolites (*Used Only on 6 Methoxy-2H-furo [2,3-h]-1-benzopyran-2-one*). Samples were analyzed on a Hewlett Packard 6890 Series GC attached to a 5972A MS detector (Agilent Technologies, Wilmington, Delaware, USA) running the HP Chemstation software package (A.03.00). The column was a HP-5MS 5% phenyl methyl siloxane (30 m $\times$ 0.25 mm $\times$ 25 μm film thickness) (Agilent Technologies) with a typical helium gas flow rate of 38 ml/min. Initial oven temperature was set at 50°C. One microliter of diluted sample was injected in the splitless mode, and the column was developed at a constant flow rate at 5°/min to a final temperature of 315°C. The MS was set to collect mass scans between 50 and 550 AMUs. This method was used to confirm the identity of the sphondin metabolite.

NMR Analysis of Insect Metabolites. ^{1}H NMR and ^{13}C NMR spectra were obtained with a Bruker (Billerica, Massachusetts, USA) Avance 400 spectrometer equipped with a 5-mm inverse broadband Z-gradient probe (^{13}C NMR, 100 MHz; ^{1}H, 400 MHz). NMR spectra were recorded in methanol-d4, which served as the internal reference (^{13}C, 49.0 ppm, ^{1}H, 3.30 ppm). Data were analyzed using the Advanced Chemistry Development, Inc., SpecManager 1D Processor and the HNMR and CNMR Predictor software suite (Toronto, Ontario, Canada).

RESULTS

Metabolite I from Frass of Xanthotoxin-fed Larvae [6-(7-Hydroxy-8-methoxycoumaryl)-Hydroxyacetic Acid] (HCHA). LC-ESI-MS showed the presence of one major compound and two or more minor constituents in extracts of frass from caterpillars fed diet containing xanthotoxin. The major compound comprised over 90% of the UV-detectable material in the sample. Negative ion LC-ESI-MS of the major peak gave a large mass ion $[M-H]^-$ of *m/z* 265. ^{1}H NMR δ (CD_3OD): 6.18 (1H, d, $J = 8.2$ Hz, C-2-H); 7.79 (1H, d, C-3-H); 7.36 (1H, s, C-9-H); 5.51 (1H, s, C-10-H); 3.91 (3H, s, C-12-H). There was not enough of the sample to run ^{13}C NMR. The ^{1}H NMR showed signals for the three olefinic/aromatic protons (δ 6.18, 7.79, and 7.36), one aliphatic proton (δ 5.51). The spectrum identifies a compound with the composition of $C_{12}H_{10}O_7$ corresponding to 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyacetic acid (HCHA).

Metabolite I from Midgut Metabolism of Xanthotoxin (HCHA). LC-ESI-MS showed the presence of one major compound and two or more minor constituents. Negative ion LC-ESI-MS of the major peak, whose retention time matched that of the xanthotoxin frass metabolite, gave a large mass ion $[M-H]^-$ of *m/z* 265. The amount and purity of this material were insufficient to yield conclusive ^{1}H NMR spectra—the spectra indicated the presence of other components that were contaminants in the sample. However, the major shifts shown for the xanthotoxin

frass metabolite were present in the spectra of this sample. This compound is most likely also 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyacetic acid (HCHA).

Metabolite II from Midgut Metabolism of Xanthotoxin [6-(7-Hydroxy-8-methoxycoumaryl)-hydroxyethanol)] (HMCH). Negative ion LC-ESI-MS showed the presence of one major compound with a large mass ion $[M\text{-}H]^-$ of *m/z* 251. ^{1}H NMR δ (CD_3OD): 6.20 (1H, d, $J = 8.9$ Hz, C-2-H); 7.89 (1H, d, C-3-H); 7.40 (1H, s, C-9-H); 3.91 (3H, s, C-10-H); 5.09 (1H, m, C-10-H); 3.78, 3.54 (2H, m, C-12-H). ^{13}C NMR δ (CD3OD): 163.1 (C1); 112.3 (C2); 146.9 (C3); 113.3 (C4); 122.5 (C5); 128.1 (C6); 152.7 (C7); 135.3 (C8); 148.4 (C9); 61.9 (C10), 70.9 (C11); 67.0 (C12). The ^{1}H NMR showed signals for the three olefinic/aromatic protons (δ 6.20, 7.89, and 7.40), three aliphatic protons (δ 5.09, 3.78, and 3.54), and one methoxyl group (δ 3.94). The ^{13}C NMR revealed the presence of one lactone carbonyl carbon (δ 160.4), eight aromatic carbons (δ 112.3, 146.9, 113.3, 122.5, 128.1, 152.7, 135.3, 148.4), and three oxygenated carbons (δ 67.0, 70.9, 61.9), The spectrum identifies a compound with a composition of $C_{12}H_{12}O_6$ corresponding to 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyethanol (Figure 1).

Metabolite III from Midgut Metabolism of Sphondin (6 Hydroxy-2H-furo[2,3-h]-1-benzopyran-2-one). Negative ion LC-ESI-MS showed the presence of one major compound with a large mass ion $[M\text{-}H]^-$ of *m/z* 202. ^{1}H NMR δ (CD3OD): 6.36 (1H, d, $J = 9.6$ Hz, C-2-H); 7.94 (1H, d, C-3-H); 6.89 (1H, s, C-9-H); 7.12 (3H, s, C-10-H, $J = 2.2$ Hz); 7.88 (1H, d, C-11-H). ^{13}C-NMR δ (CD_3OD): 163.4 (C1, s); 114.6 (C2, d); 146.8 (C3, d); 115.6 (C4, s); 142.1 (C5, s); 115.6 (C6, s); 148.4 (C7, s); 138.7 (C8, s); 108.7 (C9, d); 104.9 (C10, d), 147.6 (C11, d). The ^{1}H NMR showed signals for the five olefinic/aromatic protons (δ 6.36, 7.94, 6.89, 7.12, and 7.88). The signal for the tertiary methoxyl protons was not present in this spectrum. The ^{13}C NMR revealed the presence of one lactone carbonyl carbon (δ 163.4), and 10 other aromatic and olefinic carbons (δ 114.6, 146.8, 115.6, 142.1, 115.6, 148.4, 138.7, 108.7, 104.9, 147.6). The spectrum matches that of a compound with a composition of $C_{11}H_6O_4$ corresponding to a structure of 6-hydroxy-2H-furo[2,3-h]-1-benzopyran-2-one.

DISCUSSION

Two metabolites were isolated and identified from parsnip webworms fed xanthotoxin in artificial diet. Examination of the prepared extracts from both the frass and the midgut by LC-ESI-MS showed the presence of three compounds that absorbed at 237 nm and had apparent molecular weights in the range of 230–270. We were able to isolate two compounds from the midgut and one compound from the frass. LC-ESI-MS analysis resulted in the determination of a MW of 266 for the compound in the frass and one of the compounds in the midgut. The second compound from the midgut had a MW of 252. The structures, based on

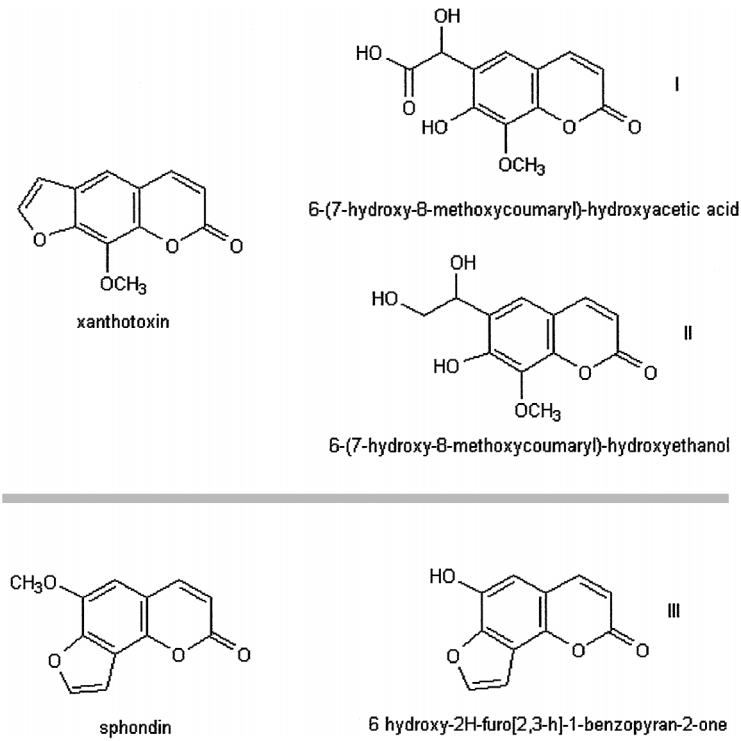

FIG. 1. Metabolites of xanthotoxin and sphondin produced by parsnip webworms (metabolites numbered as in text): metabolite I: 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyacetic acid (HCHA); Metabolite II: 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyethanol) (HMCH); and metabolte III: 6 hydroxy-2H-furo[2,3-h]-1-benzopyran-2-one.

^{1}H NMR data, are shown in Figure 1. The xanthotoxin metabolite found in both frass and midgut preparations (HCHA) has been found in frass of *Papilio polyxenes* fed xanthotoxin (Bull et al., 1984) and in the excreta of laying hens raised on a xanthotoxin-containing diet (Pangillinan et al., 1992). To our knowledge, the xanthotoxin metabolite found exclusively in the midgut (HMCH) has not been reported previously in insects but has been identified as a metabolite of xanthotoxin in humans (Schmid et al., 1980). Low levels of a third metabolite of MW 236 may also be produced in webworms fed xanthotoxin, but this compound is not accumulated in quantities sufficient for isolation and identification by NMR.

Based on the structure of metabolites characterized, we suggest that the first step of metabolism of xanthotoxin in the parsnip webworm is the formation of an epoxide on the furan ring by a cytochrome P-450 enzyme, as is postulated to occur in other organisms (Bull et al., 1984; Koenigs and Trager, 1998a,b). This epoxide

is not very stable and may degrade into the more chemically stable forms of the second midgut metabolite (II) and possibly a third metabolite with a MW of 236, as yet uncharacterized, either in the intact gut or during the extraction and analysis process. A second oxidation step, acting either on the epoxide intermediate or on one of the other metabolic intermediates, then occurs to form metabolite I, which is found in both the midgut and the frass. Whether this second step is catalyzed by the same cytochrome P-450 or by a different one is unknown.

Angular furanocoumarin metabolism in the parsnip webworm appears to differ in a fundamental way from linear furanocoumarin metabolism. We isolated and identified only a single metabolite from webworms fed sphondin in artificial diet. Two UV-absorbing peaks with spectra similar to sphondin were isolated from midguts of webworms fed artificial diet containing sphondin. One peak was shown to be unmetabolized sphondin by GC-MS and ^{1}H NMR analysis. The second peak was identified as demethylated sphondin (6 hydroxy-2H-furo[2,3-h]-1-benzopyran-2-one) (Figure 1) by LC-ESI-MS; the ^{1}H NMR and ^{13}C NMR analyses for this peak are similar to those already reported for this compound (Sadari et al., 1999). However, in black swallowtails, angelicin (isopsoralen), the unsubstituted angular furanocoumarin, is metabolized by oxidation of the furan ring double bond to generate mono- and dihydroxy acid products (Ivie et al., 1986a,b). No evidence of such a reaction in parsnip webworms was seen in this study.

The structure of furanocoumarin metabolites in parsnip webworms is consistent with the suggestion that different P-450 isozymes are responsible for metabolism of linear and angular furanocoumarins. Whereas rates of the linear furanocoumarins xanthotoxin and bergapten are highly correlated ($r = 0.954$, $N = 98$, $P < 0.001$; data from Berenbaum and Zangerl, 1998), sphondin metabolism rates are not significantly correlated with rates of metabolism of any linear furanocoumarin (all $r < 0.696$; data from Berenbaum and Zangerl, 1998). As well, although significant genetic variation exists for linear furanocoumarin metabolism in a central Illinois webworm population (Berenbaum and Zangerl, 1992), no genetic variation could be found for sphondin metabolism in this population. The fact that only a single metabolite was found is also consistent with the idea that angular furanocoumarin metabolism in this species is less sophisticated and efficient than is linear furanocoumarin metabolism, thus leading to the status of sphondin as a resistance factor in natural populations of wild parsnip (Zangerl and Berenbaum, 1993).

Parsnip webworms and black swallowtails, then, differ both quantitatively and qualitatively in how they metabolize furanocoumarins in their apiaceous host plants. Such differences are, on one hand, unsurprising given the phylogenetic distance between these taxa. However, the fact that some similarities exist across a broad range of taxa is noteworthy. That webworms produce metabolites of xanthotoxin in common not only with other Lepidoptera (e.g., HCHA) but also

with other vertebrates (e.g., HMCH) suggests a remarkable conservatism in the metabolic capabilities of cytochrome P-450s and raises the possibility that insects may share other detoxification reactions with vertebrates with respect to toxins in food plants.

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ANTIOXIDANTS IN GRASSHOPPERS: HIGHER LEVELS DEFEND THE MIDGUT TISSUES OF A POLYPHAGOUS SPECIES THAN A GRAMINIVOROUS SPECIES

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Abstract—Polyphagous grasshoppers consume plants that contain markedly greater amounts of potentially prooxidant allelochemicals than the grasses eaten by graminivorous grasshoppers. Therefore, levels of antioxidant defenses maintained by these herbivores might be expected to differ in accordance with host plant ranges. Antioxidant levels were compared in midgut tissues and gut fluids of a polyphagous grasshopper, *Melanoplus sanguinipes*, and a graminivorous grasshopper, *Aulocara elliotí*. Glutathione concentrations in midgut tissues of *M. sanguinipes* (10.6 mM) are among the highest measured in animal tissues and are twice as high as those in *A. elliotí*. α-Tocopherol levels are 126% higher in midgut tissues of *M. sanguinipes* than in those of *A. elliotí*, and remain at high levels when *M. sanguinipes* is reared on plants containing a wide range of α-tocopherol concentrations. Ascorbate levels in *M. sanguinipes* midgut tissues are 27% higher than in those of *A. elliotí*, but vary depending on the host plant on which they are reared. Midgut fluids of both species contain elevated levels of glutathione, as well as large (millimolar) amounts of undetermined antioxidants that are produced in the insects. The consumption of tannic acid decreases ascorbate concentrations in midgut tisssues and gut fluids of *A. elliotí* but has no effect on ascorbate levels in *M. sanguinipes*. The results of this study provide the first measurements of antioxidants in grasshoppers and suggest that the maintenance of high levels of antioxidants in the midgut tissues of polyphagous grasshoppers might effectively protect them from oxidative stress.

Key Words—Orthoptera, *Melanoplus sanguinipes*, *Aulocara elliotí*, ascorbic acid, glutathione, α-tocopherol.

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INTRODUCTION

Research on insect defenses against ingested plant allelochemicals has focused to a large extent on detoxification and antioxidant enzymes (Lindroth, 1991; Rosenthal and Berenbaum, 1991; Felton and Summers, 1995). More recently, the importance of the acquisition and maintenance of low-molecular-weight antioxidants as defenses against potentially prooxidant plant allelochemicals has been examined. Antioxidants that have been demonstrated to protect herbivorous insects from photooxidative stress include lutein (Carroll et al., 1997), vitamin A (Green and Berenbaum, 1994), and α-tocopherol (Aucoin et al., 1995). Ascorbic acid and glutathione are believed to be important in the defense against oxidative stress from ingested phenolic compounds (Summers and Felton, 1994; Bi and Felton, 1995, 1997; Barbehenn et al., 2001).

The primary low-molecular-weight antioxidants in plants and animals are ascorbic acid (vitamin C), α-tocopherol (vitamin E), and the tripeptide glutathione. These compounds each have essential functions in the biochemical defense and nutrition of herbivorous insects (Vanderzant et al., 1962; Dadd, 1973; Kramer and Seib, 1982; Ahmad, 1992; Felton and Summers, 1995). Ascorbic acid chemically reduces a wide variety of oxidized compounds, including reactive oxygen species (ROS), α-tocopherol radical and quinone (Packer et al., 1979; Abuja and Albertini, 2001). α-Tocopherol is the major lipophilic antioxidant in biological systems and plays a key role in protecting lipids in membranes from peroxidation (Thomas et al., 1992; van Ginkel and Sevanian, 1994; Mallet et al., 1994; Glascott and Farber, 1999). Glutathione acts as a non-enzymatic radical scavenger or as a cofactor for antioxidant enzymes, such as dehydroascorbate reductase and glutathione transferase peroxidase (Meister, 1992; Ahmad, 1992; Summers and Felton, 1993; Winkler et al., 1994; Vethanayagam et al., 1999).

Phenolic compounds can act either as antioxidants or prooxidants, depending on chemical properties of the phenol (e.g., redox potential and semiquinone radical stability) and the physicochemical environment (e.g., pH, overall redox potential, oxygen tension, oxidase and peroxidase activities, and the types and concentrations of co-occurring chemical species such as catalytic metal ions, metal-chelating substances, and ascorbate). Perhaps as a result of this complexity, there are numerous reports of phenolic compounds having prooxidant effects (Hodnick et al., 1989; Canada et al., 1990; Summers and Felton, 1994; Pardini, 1995; Sakagami et al. 1997; Nose et al., 1998; Metadiewa et al., 1999) and/or antioxidant effects (Kitagawa et al., 1992; Aruoma et al., 1993; Salah et al., 1995; Guo et al., 1996; Cao et al. 1997; Hagerman et al., 1998; Aherne and O'Brien, 2000). The variety of physicochemical factors that together determine the chemical fate of ingested phenolics necessitates the measurement of one or more of the following response variables to verify prooxidant activity: (1) oxidation products (semiquinone radicals, quinones, and/or melanin like pigments), (2) ROS (peroxides, $^{\cdot}OH$, O_2^-), or

(3) biomarkers of oxidative stress. In grasshoppers, elevated levels of melanin-like pigments are produced from ingested hydrolyzable tannins (but not from gallic acid), suggesting that a substantial fraction of these tannins are oxidized (Barbehenn et al., 1996).

Useful biomarkers of oxidative stress include oxidized lipids and proteins, as well as decreased levels of antioxidants (Lauteburg et al., 1988; Lykkesfeldt et al., 1995; Glascott and Farber, 1999). A decreased level of ascorbate, for instance, may promote oxidative damage in tissues and is associated with ulcerative colitis in the human intestine (Buffinton and Doe, 1995). In insects, lower concentrations of ascorbate have been found in *Helicoverpa zea* midgut tissues in association with other markers of oxidative stress (Summers and Felton, 1994; Bi and Felton, 1995, 1997), and lower levels of ascorbate are present in the gut tissues of *Trichoplusia ni* that consumed xanthotoxin (Timmerman et al., 1999). In other insect species, such as *Heliothis virescens*, no evidence for oxidative stress has been found from ingested phenolic compounds in tobacco (Johnson and Felton, 2001). It is unclear to what extent these different results reflect differences in experimental design, e.g., phenolic compounds ingested in artificial diets or leaves, or differences in the degree of resistance to oxidative stress in different insect species.

The seminal work of Bernays and colleagues demonstrated that fatal lesions form in the midgut epithelium of graminivorous grasshoppers after long-term consumption of tannic acid (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980). Ulcerlike lesions also form in the midgut tissues of some caterpillars from the consumption of phenolic compounds (Steinly and Berenbaum, 1985; Lindroth and Peterson, 1988) and quinones (Thiboldeaux et al., 1998). The mode of action of tannic acid has been interpreted as a nonspecific association between tannins and the gut epithelium. Midgut lesions might also result from chronic oxidative stress from the oxidation of ingested tannins in the gut lumen (producing ROS) and/or the toxicity of absorbed phenols or quinones. Quinone cytotoxicity is believed to result either from sulfhydryl arylation (leading to enzyme damage and/or decreased glutathione levels) or redox cycling (producing ROS) (Gant et al., 1988; Zheng et al., 1997). Oxidative stress in either case causes elevated levels of apoptosis or necrosis and can lead to the formation of ulcerlike lesions (Babbs, 1992; Das et al., 1997; Gardner et al., 1997; Madesh et al., 1999; Chandra et al., 2000).

Previous work on antioxidants in herbivorous insects has focused primarily on larval Lepidoptera, and no examination of antioxidants in grasshoppers has been made, to my knowledge. This study tests the hypothesis that polyphagous grasshoppers, such as *Melanoplus sanguinipes* (Melanoplinae), maintain higher levels of antioxidants than do graminivorous grasshoppers, such as *Aulocara elliotti* (Gomphocerinae), and that levels of antioxidants in polyphagous species are less susceptible to decreases than are those in graminivorous species when they consume tannic acid. The basis for expecting that these insects have evolved

different levels of defense against oxidative stress lies in the distinct differences in the allelochemical profiles of their host plants. Individual polyphagous grasshoppers commonly consume a wide range of herbaceous plants (Gangwere et al., 1976; Joern 1983; Bernays and Bright, 1993). These host plants include species that produce potentially prooxidant allelochemicals, such as phenolic compounds, furanocoumarins, and terthiophenes (Lee and Berenbaum, 1990; Ahmad, 1992; Summers and Felton, 1994; Aucoin et al., 1995). By comparison, graminivorous grasshoppers feed almost exclusively on mature grasses. Because mature grasses commonly contain small amounts and limited types of allelochemicals (Bernays and Barbehenn, 1987), grass-specialist grasshoppers might derive less benefit from maintaining high levels of antioxidants compared to polyphagous grasshoppers.

METHODS AND MATERIALS

Insects

Eggs of *M. sanguinipes* were obtained from a non diapause colony (USDA, Bozeman, Montana, USA). Insects were reared on romaine lettuce (*Lactuca sativa* var. *longifolia*) and wheat bran in the fall, as previously described (Barbehenn et al., 1996). Third-instar nymphs of *A. ellioti* were collected in central Wyoming, USA, in the spring and reared to adulthood in Ann Arbor, Michigan, USA in the same conditions as *M. sanguinipes*, with the exception that the host plant was seedling wheat (*Triticum aestivum*). Wheat was grown in ambient light for experiments on *A. ellioti*, and under a mixture of ambient and supplemental light in a greenhouse for *M. sanguinipes*. Adult females were used for all experiments, unless otherwise noted. Each experimental insect was placed in a separate ventilated plastic container (470 ml) in an incubator (28°C, 16L:8D) for 2–2.5 days. Insects were assigned at random to feed on control or treated wheat leaves. Seedling wheat leaves ($N = 4$ or 5) were cut and treated with 13 μl of 70% acetone (control) or 13 μl of a 70% acetone solution of tannic acid (Sigma, lot 64F-0049) to give a final concentration of 15% dry weight (DW). Fresh control or treated leaves were placed loosely in each container daily, and these were usually completely consumed by both species.

Sample Collection

Insects that had fed recently were selected for dissection. Each insect was chilled at −20°C for 10 min, following which the entire gut was removed from the body, rinsed in distilled water (2–3 sec), blotted dry, and the Malpighian tubules removed. Samples of foregut and midgut contents were weighed in tared microcentrifuge tubes flushed with N_2. Midgut contents included the portion of the food bolus that extended into the anterior hindgut. Ascorbate and glutathione

were extracted from samples by vigorous shaking (10 sec) in 400 μl of 5% (w/v) metaphosphoric acid (MPA) and 1 mM EDTA with 50 mg/ml hydrated insoluble polyvinylpolypyrolidone (PVP). Extracts of gut contents in MPA were incubated for a minimum of 5 min on ice, with occasional mixing, to remove tannic acid. α-Tocopherol and total antioxidants in gut fluids were extracted into 325 μl of N_2-purged (1 min/ml) ethanol. Ethanol extracts were centrifuged (8000g, 2 min), and 200-μl aliquots of supernatant solutions were mixed with 10 μl of BHT (4.0 mM final concentration) for α-tocopherol measurement. Total antioxidant capacity was measured in aliquots of ethanol extracts that were not treated with BHT. Midgut tissues, including the ceca, were blotted dry on a paper towel, weighed, and homogenized in 300 μl of 5% MPA (without PVP) or 400 μl of N_2-purged ethanol in a glass hand-held tissue homogenizer (20 strokes). Aliquots (150 μl) of tissue extracts were mixed with 10 μl of BHT (5.3 mM final concentration) for α-tocopherol measurement. Wheat used as food for the insects ($N = 3$ control or tannin-treated leaves) and lettuce were weighed and homogenized in 500 μl MPA and treated with 25 mg PVP (for ascorbate and glutathione) or in 500 μl N_2-purged ethanol (50 strokes). All samples were centrifuged (8000g, 3 mins) and stored at -75°C, with ethanol extracts under N_2. Sample sizes are presented in Tables 2–5. Reagents for all assays were purchased from Sigma-Aldrich Chemical Co., Acros Organics, or ICN Pharmaceuticals.

Water

Representative samples of wheat and lettuce leaves ($N = 3$/treatment) were weighed fresh, and then reweighed after drying at 65 or 70°C. The percent water in fore- and midgut contents and midgut tissues from control insects ($N = 4$–5) of each species was measured on weighed pieces of aluminum foil before and after drying. Fluid volumes in samples were calculated (percent water $\times$ fresh weight) in order to calculate antioxidant molarity.

Antioxidants

Ascorbic Acid. Frozen MPA extracts were thawed, mixed, centrifuged (8000g, 5 min), and kept on ice. Filtered samples (0.45 μm, Gelman GHP) were mixed with 2 M Tris buffer (pH 9.2, 26% v/v) and analyzed immediately with reverse-phase HPLC (Lykkesfeldt et al., 1995; Levine et al., 1999). Ascorbate was separated with a Vydac C-18 column (201 HS, 250 $\times$ 4.6 mm) and guard column, using a mobile phase composed of aqueous ammonium phosphate (20 mM) and EDTA (1.0 mM), adjusted to pH 3.0 with 5% MPA (containing 1 mM EDTA). The flow rate was 1.0 ml/min (35°C). Peak area was measured with a Shimadzu UV–visible detector (265 nm), and peak area was integrated with a Shimadzu C-R4A integrator. The identity of the ascorbate peak was made by analysis of ascorbate standards and confirmed by treatment of standards and

samples with ascorbate oxidase (10 EU (enzyme units)/ml neutralized sample). Peak areas were converted to nanomoles injected using ascorbate standard curves. Samples were analyzed within 1–3 months from the time of storage. Previous studies have shown that ascorbate is stable after at least 6 months of storage in similar conditions (Lykkesfeldt and Ames, 1999). In this study, ascorbate remained at 87 and 98% of initial levels in tannin-treated and control wheat leaf extracts, respectively, after a period of 12 months' storage.

Glutathione. Frozen MPA extracts were thawed, mixed, and centrifuged. Aliquots (125 μl) of sample supernatant solutions were derivatized with iodoacetic acid and 2,4-dinitrofluorobenzene (Reed et al., 1980). Glutathione (GSH) and glutathione disulfide (GSSG) derivatives were analyzed with HPLC (Reed et al., 1980). Aliquots (30 μl) of filtered derivatized samples were injected onto an Alltech amino column (250 $\times$ 4.6 mm) with a C-18 guard column. Peaks were detected at 365 nm, and peak areas were converted to nanomolos injected using glutathione standard curves. Total glutathione concentrations (GSH + 2 $\times$ GSSH) reported in this study include GSSG that was present in the original samples and that which was formed during sample preparation. Samples were analyzed within 7–11 months from the time of storage. Previous studies have shown that glutathione is stable for at least 9–12 months in acidic solutions at -60° to -80°C (Brehe and Burch, 1976; Roberts and Francetic, 1993). The stability of glutathione was not tested further in this study. Glutathione levels in *A. ellioti* were measured in insects that fed on 10% DW tannic acid-treated or control wheat. An enzymatic recycling assay, adapted for use on a microplate reader, was also used to measure glutathione in the midgut tissues of *M. sanguinipes* (Griffith, 1983). Tissue samples were collected from insects as described above, with the exception that samples were homogenized in 300 μl of 5% (w/v) sulfosalicylic acid, and samples were stored (-75°C) for $<$2 weeks before analysis.

α-Tocopherol. Samples were filtered with CR PTFE filters (0.2 μm, Gelman) to HPLC vials, and the headspace flushed with N_2. Aliquots (20 or 30 μl) were injected on a Vydak C-18 column with a guard column, and eluted with 100% methanol (1.0 ml/min, 30°C), as modified from Aucoin et al. (1990). Peaks were measured with a Shimadzu fluorescence detector (294 nm excitation, 325 nm emission wavelengths), and integrated with a Shimadzu C-R4A integrator. Peak areas were converted to nanomoles of α-tocopherol injected with α-tocopherol standard curves. Samples and standards were stored for 1–5 months. α-Tocopherol was found to be stable in extracts of *L. sativa* (99.9% recovery after a 4-month storage period) and wheat (*Triticum aestivum*) (90.9% recovery after 7 month storage).

Total Antioxidant Capacity (TAC). TAC was measured with the method of Re et al. (1999). Briefly, an aqueous solution of 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid (ABTS) was prepared with potassium persulfate to create the stable $ABTS^{\cdot+}$ radical cation. Absorbance (655 nm) of a stock solution of $ABTS^{\cdot+}$

was adjusted to 0.7 with ethanol. Ethanolic extracts that were stored at -75°C were remixed and centrifuged (8000g, 3 min, ambient temperature). Three replicate aliquots (6 μl) of samples or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards were mixed with 190 μl of the $ABTS^{\cdot+}$ solution in 96-well microplates. After a period of 9 min in the dark (ambient temperature), absorbance was measured at 655 nm with a Biorad Benchmark microplate reader. Decolorization of the $ABTS^{\cdot+}$ solution is proportional to the concentration of "antioxidants" present in a sample, and TAC was expressed as an equivalent concentration of Trolox using a Trolox standard curve. Samples were analyzed within 1–36 days from the time of storage. TAC measurements were found to be stable in wheat samples (95.8% recovery, $N = 9$) after storage for 4 months.

Effect of Host Plant on Antioxidant Levels in M. sanguinipes

Wheat grown with supplemental light in a greenhouse for *M. sanguinipes* contained higher levels of ascorbate than wheat grown in ambient light. To test the possibility that higher ascorbate levels in the midgut tissues of *M. sanguinipes* resulted from feeding for a 2-day period on wheat containing a high concentration of ascorbate, the measurement of ascorbate was repeated in the spring using wheat grown in the same conditions as that used in experiments on *A. ellioti*. To test the long-term effect of feeding on wheat or lettuce, groups of *M. sanguinipes* from the same egg source were either reared exclusively on seedling wheat and dried seedling wheat (instead of wheat bran) or reared as usual on lettuce and wheat bran. Adult females ($N = 10$) from both rearing regimes were placed individually in containers in an incubator and fed control wheat blades for 2–2.5 days. Midgut tissues were dissected and ascorbate extracted and analyzed as described above. This experiment was repeated to compare α-tocopherol concentrations in the midgut tissues of *M. sanguinipes* reared on seedling wheat or lettuce. α-Tocopherol was extracted and analyzed as described above. Representative wheat and lettuce leaf samples ($N = 3$/species) were extracted and analyzed in each experiment.

Statistical Analyses

Analyses of antioxidant concentrations and percent water were made with SAS version 8e (SAS, 2000). Antioxidant concentrations and percentages were compared using repeated measures ANOVA, with site as the repeated measure within each insect. Species, treatment, and site were used as fixed factors in the model, which included all two-way and three-way interactions. Paired comparisons were made using the differences of least-squares means with the Proc Mixed module (SAS, 2000).

RESULTS

Water Content. Similar water content was found in wheat in each experiment and in the foregut and midgut contents and midgut tissues of *A. ellioti* and *M. sanguinipes* (Table 1). Changes in water content between the foregut and midgut contents were small and would not explain the changes in antioxidant concentrations measured along the length of the gut. The lower water content in the foregut than in the food ingested was presumably due to dehydration that occurred between the time of presentation of clipped leaves and the time they were ingested.

Ascorbic Acid. When reared on lettuce, *M. sanguinipes* maintains higher levels of ascorbate in its midgut tissues than does *A. ellioti* ($P = 0.034$, Table 2). Higher ascorbate levels in the foregut of *M. sanguinipes* presumably reflect the higher levels of ascorbate in the wheat ingested in the experiment on this species (Table 2). As a percentage of levels in food, ascorbate levels in foregut fluids of *A. ellioti* (88%) and *M. sanguinipes* (93%) were not significantly different on untreated wheat ($P = 0.530$, Table 2). Tannic acid consumption significantly decreased ascorbate concentrations in *A. ellioti* but not in *M. sanguinipes*; ascorbate levels were reduced in *A. ellioti* midgut tissues by 20.5% ($P = 0.020$), in foregut fluid by 25.0% ($P = 0.051$), and in midgut fluid by 39.1% ($P = 0.001$). In *M. sanguinipes*, ascorbate levels in the midgut fluids were only 18–30% of those in *A. ellioti* (Table 2), but remained unaffected by ingested tannic acid.

Glutathione. M. sanguinipes maintains 10.6 mM glutathione in its midgut tissues, approximately twice the level of glutathione found in *A. ellioti* ($P < 0.001$) (Table 3). Results of an enzymatic assay for glutathione in the midgut tissues of *M. sanguinipes* were similar to those measured with HPLC: 11.4 ± 0.7 mM in control tissues and 13.4 ± 0.8 mM in the tissues of insects that consumed tannins. By contrast, gut fluids of *A. ellioti* contained more glutathione than those of *M. sanguinipes*; midgut fluids in *A. ellioti* that fed on tannin-treated wheat were 2.5-fold greater than those of *M. sanguinipes* ($P < 0.001$), and there is a trend

TABLE 1. WATER CONTENTS IN WHEAT LEAVES, GUT CONTENTS, AND MIDGUT TISSUES OF *A. ellioti* AND *M. sanguinipes*[a]

Species	Site	Water (%)	*N*
Triticum aestivum	Leaf	88.2 ± 0.4	12
A. ellioti	Foregut contents	75.3 ± 3.5	4
A. ellioti	Midgut contents	81.8 ± 1.1	4
A. ellioti	Midgut tissue	75.8 ± 1.4	5
M. sanguinipes	Foregut contents	77.8 ± 1.4	5
M. sanguinipes	Midgut contents	82.6 ± 0.6	4
M. sanguinipes	Midgut tissue	75.5 ± 1.1	5

[a] Data presented as mean $\pm$ SE. Clipped leaves without a water source were fed to insects.

TABLE 2. ASCORBATE CONCENTRATIONS IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species	Site	Tannic acid (%)	Ascorbic acid concentration (mM)	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	2.93 ± 0.15^{ef}	87.5 ± 4.5^{ef}	10
A. ellioti	Foregut contents	15	2.20 ± 0.24^{d}	61.2 ± 6.7^{d}	10
A. ellioti	Midgut contents	0	1.57 ± 0.19^{c}	47.0 ± 5.8^{c}	10
A. ellioti	Midgut contents	15	0.96 ± 0.09^{b}	27.8 ± 2.4^{b}	7
A. ellioti	Midgut tissue	0	2.89 ± 0.17^{e}	86.5 ± 5.2^{ef}	11
A. ellioti	Midgut tissue	15	2.30 ± 0.11^{d}	64.1 ± 3.0^{d}	10
M. sanguinipes	Foregut contents	0	5.20 ± 0.24^{g}	93.4 ± 4.3^{f}	10
M. sanguinipes	Foregut contents	15	4.79 ± 0.35^{g}	83.1 ± 6.1^{ef}	11
M. sanguinipes	Midgut contents	0	0.28 ± 0.02^{a}	5.0 ± 0.4^{a}	9
M. sanguinipes	Midgut contents	15	0.29 ± 0.21^{a}	4.9 ± 0.4^{a}	9
M. sanguinipes	Midgut tissue	0	3.67 ± 0.34^{f}	65.8 ± 6.1^{de}	10
M. sanguinipes	Midgut tissue	15	4.15 ± 0.30^{f}	72.1 ± 5.2^{de}	11

[a] Data presented as mean ± SE. Summary statistics in each column followed by one or more of the same letters are not significantly different.

[b] Ascorbic acid concentrations in wheat ingested by *A. ellioti* and *M. sanguinipes* were 3.44 ± 0.15 mM and 5.67 ± 0.20 mM, respectively. No differences were observed in ascorbic acid levels between control and treated wheat, and these values were pooled ($N = 6$/experiment). Percent of concentration in food is based on the concentration ingested in each separate experiment.

TABLE 3. GLUTATHIONE CONCENTRATIONS IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID

Species	Site	Tannic acid (%)	Glutathione concentration (mM)[a]	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	0.23 ± 0.02^{a}	43.0 ± 4.4^{a}	10
A. ellioti	Foregut contents	15	0.43 ± 0.06^{b}	80.5 ± 12.1^{b}	10
A. ellioti	Midgut contents	0	0.59 ± 0.10^{cd}	110.9 ± 19.2^{c}	8
A. ellioti	Midgut contents	15	0.83 ± 0.16^{d}	156.1 ± 29.1^{c}	7
A. ellioti	Midgut tissue	0	5.94 ± 0.38^{e}	1116.9 ± 71.4^{d}	12
A. ellioti	Midgut tissue	15	5.22 ± 0.30^{e}	980.3 ± 56.5^{d}	10
M. sanguinipes	Foregut contents	0	0.20 ± 0.02^{a}	31.8 ± 2.8^{a}	10
M. sanguinipes	Foregut contents	15	0.33 ± 0.02^{a}	53.1 ± 3.5^{ab}	10
M. sanguinipes	Midgut contents	0	0.43 ± 0.05^{bc}	69.9 ± 8.6^{ab}	9
M. sanguinipes	Midgut contents	15	0.32 ± 0.05^{ab}	52.5 ± 8.1^{ab}	9
M. sanguinipes	Midgut tissue	0	10.65 ± 0.49^{f}	1718.3 ± 79.7^{e}	10
M. sanguinipes	Midgut tissue	15	10.95 ± 0.44^{f}	1766.0 ± 70.2^{e}	11

[a] Data presented as mean ± SE. Summary statistics followed by one or more of the same letters are not significantly different. Total glutathione was calculated as $[GSH] + 2 \times [GSSG]$.

[b] Glutathione concentrations in wheat ingested by *A. ellioti* and *M. sanguinipes* were 0.53 ± 0.03 and 0.62 ± 0.04 mM, respectively. No differences were observed in glutathione concentrations between control and treated wheat, and these values were pooled ($N = 6$/experiment).

TABLE 4. α-TOCOPHEROL CONCENTRATIONS IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species	Site	Percent Tannic acid (%)	α-Tocopherol concentration (μM)	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	31.6 ± 4.7^{b}	108.5 ± 18.7^{b}	14
A. ellioti	Foregut contents	15	36.6 ± 5.3^{b}	144.6 ± 23.4^{bc}	10
A. ellioti	Midgut contents	0	35.2 ± 5.6^{b}	124.0 ± 22.2^{b}	10
A. ellioti	Midgut contents	15	24.6 ± 3.1^{ab}	94.9 ± 12.0^{b}	9
A. ellioti	Midgut tissue	0	13.2 ± 2.8^{a}	48.6 ± 12.6^{a}	9
A. ellioti	Midgut tissue	15	13.3 ± 1.6^{a}	47.1 ± 5.3^{a}	10
M. sanguinipes	Foregut contents	0	31.8 ± 3.1^{b}	96.2 ± 9.2^{b}	10
M. sanguinipes	Foregut contents	15	37.8 ± 4.1^{b}	117.2 ± 13.4^{bc}	12
M. sanguinipes	Midgut contents	0	54.8 ± 7.1^{c}	165.6 ± 21.2^{c}	11
M. sanguinipes	Midgut contents	15	41.8 ± 5.1^{bc}	128.7 ± 15.0^{bc}	12
M. sanguinipes	Midgut tissue	0	30.3 ± 4.5^{b}	91.9 ± 13.8^{b}	12
M. sanguinipes	Midgut tissue	15	29.7 ± 3.1^{b}	93.2 ± 10.3^{b}	11

[a] Data presented as mean $\pm$ SE. Tannic acid was applied to wheat at a final concentration of 15% DW. Summary statistics followed by one or more of the same letters are not significantly different.
[b] α-Tocopherol concentrations in untreated wheat averaged 32.9 ± 2.3 μM, and 30.5 ± 2.7 μM in wheat treated with tannic acid (15% DW) ($N = 9$/treatment).

towards higher glutathione levels in the midgut fluids of *A. ellioti* than in *M. sanguinipes* in insects that fed on untreated wheat ($P = 0.062$). Foregut fluids of both species contained similar levels of glutathione when they fed on untreated wheat, but increased nearly 100% in *A. ellioti* that fed on tannin-treated wheat ($P = 0.031$, Table 3). Glutathione concentrations increased by over twofold along the length of the gut in each species, with the exception of *M. sanguinipes* that fed on tannin-treated wheat. *M. sanguinipes* that ingested tannin-treated wheat maintained similar levels of glutathione along the length of the gut. Glutathione in midgut fluids of *A. ellioti* and *M. sanguinipes* were affected in opposite directions by ingested tannic acid ($P = 0.002$ for species$\times$treatment interaction), with concentration increasing in the midgut fluid of *A. ellioti* (Table 3).

α-Tocopherol. α-Tocopherol levels in *M. sanguinipes* midgut tissues were 126% greater than in those of *A. ellioti* ($P = 0.016$, Table 4). α-Tocopherol levels in the foregut and midgut fluids in both species were typically as great as, or greater than, levels in the wheat ingested (Table 4). α-Tocopherol levels in *M. sanguinipes* were significantly higher in the midgut lumen than those in *A. ellioti* ($P = 0.011$), averaging 55.7% (19.6 μM) greater in control insects and 69.9% greater in tannin-consuming insects. Tannin consumption had no significant effect on α-tocopherol concentrations in gut fluids of either species (Table 4).

Total Antioxidant Capacity. TAC was virtually the same in both species (Table 5). Tannin-treatment greatly elevated the TAC of wheat and grasshopper gut fluids ($P < 0.001$; Table 5). Surprisingly, TAC in the foregut and midgut fluids of both

TABLE 5. TOTAL ANTIOXIDANT CAPACITY (TAC) IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID

Species	Site	acid (%)	(mM Trolox)[a]	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	27.8 ± 8.1^{a}	230.0 ± 50.0^{d}	10
A. ellioti	Foregut contents	15	204.0 ± 38.5^{bc}	176.9 ± 35.5^{bcd}	7
A. ellioti	Midgut contents	0	24.6 ± 7.2^{a}	221.8 ± 51.1^{d}	10
A. ellioti	Midgut contents	15	155.6 ± 26.7^{b}	132.8 ± 27.7^{bc}	10
A. ellioti	Midgut tissue	0	14.3 ± 2.3^{a}	127.9 ± 10.5^{b}	10
A. ellioti	Midgut tissue	15	18.8 ± 2.8^{a}	16.2 ± 3.1^{a}	10
M. sanguinipes	Foregut contents	0	14.0 ± 0.5^{a}	199.3 ± 9.6^{cd}	10
M. sanguinipes	Foregut contents	15	268.0 ± 18.3^{c}	167.8 ± 10.2^{bcd}	10
M. sanguinipes	Midgut contents	0	16.0 ± 2.0^{a}	208.3 ± 17.3^{cd}	12
M. sanguinipes	Midgut contents	15	174.3 ± 21.5^{b}	111.3 ± 15.4^{b}	12
M. sanguinipes	Midgut tissue	0	10.3 ± 1.1^{a}	140.0 ± 12.7^{bc}	12
M. sanguinipes	Midgut tissue	15	23.7 ± 1.9^{a}	14.9 ± 1.0^{a}	12

[a] Data presented as mean ± SE. TAC is the extent of $ABTS^{+}$ radical reduction measured in Trolox equivalents. Summary statistics followed by one or more of the same letters are not significantly differents.

[b] TAC of wheat across all experiments was 9.2 ± 0.9 mM (control) and 141.8 ± 8.7 mM (15% DW tannic acid).

species fed untreated wheat was at least 100% higher (14–28 mM) than the TAC of the wheat ingested (7–15 mM) (Table 5).

Effect of Host Plant on Antioxidant Levels. When *M. sanguinipes* were reared on lettuce and then fed wheat containing either 3.34 or 5.67 mM ascorbate for a 2-day period, there was no significant effect on ascorbate levels in their midgut tissues, which measured 3.41 or 3.67 mM, respectively ($P = 0.600$) (Table 2, Figure 1). However, when *M. sanguinipes* were reared on wheat (containing 3.4 mM ascorbate), the ascorbate concentration in midgut tissues was decreased ($P = 0.043$) (Figure 1). Decreased levels of ascorbate were found in wheat-reared insects despite the fact that wheat contains a 4.0-fold greater ascorbate concentration than lettuce (Figure 1). Thus, when *M. sanguinipes* and *A. ellioti* are reared solely on wheat, the ascorbate levels in their midgut tissues are similar (2.70 and 2.89 mM, respectively; $P = 0.570$). α-Tocopherol also varies between the two food plants, with the concentration in *L. sativa* being 3.4-fold greater than that in seedling wheat (Figure 1). Despite the large difference in the concentrations of this antioxidant in the two host plants, *M. sanguinipes* reared on each plant maintained similar α-tocopherol concentrations in their midgut tissues (Figure 1, $P = 0.767$).

DISCUSSION

The results of this study demonstrate that a polyphagous grasshopper, *M. sanguinipes*, maintains higher levels of antioxidants in its midgut tissues than does

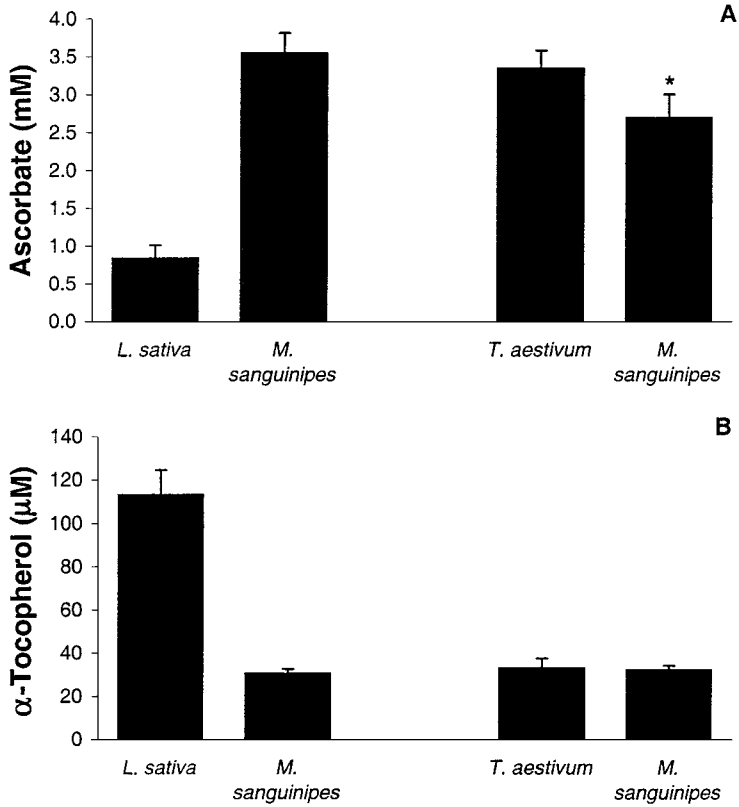

FIG. 1. (A) Ascorbate concentrations in lettuce and wheat and in *M. sanguinipes* midgut tissues from insects reared on each plant. (B) α-Tocopherol concentrations in lettuce and wheat and in *M. sanguinipes* midgut tissues from insects reared on each plant.

a graminivorous species, *A. ellioti*. Concentrations of ascorbate, α-tocopherol, and glutathione are each substantially greater in *M. sanguinipes*, consistent with the hypothesis that polyphagous grasshoppers are better defended from oxidative stress than are graminivorous species. Significant treatment effects from the consumption of tannic acid were observed in *A. ellioti*, in which ascorbate concentrations were reduced in the midgut tissues and foregut and midgut fluids. By contrast, the lack of a treatment effect on ascorbate concentrations at each of the sites measured in *M. sanguinipes* guts is consistent with the hypothesis that polyphagous species are less susceptible to oxidative stress than are graminivores. Decreased ascorbic acid in midgut tissues of tannin-consuming *A. ellioti* could result from lower levels in the gut lumen (a dietary mechanism) and/or an oxidation rate in gut tissues exceeding the insect's ability to recycle ascorbate (oxidative stress). Either

of these mechanisms could potentially lead to greater oxidative damage to the gut epithelium of *A. ellioti* than *M. sanguinipes*. Additional studies are needed to determine whether lower ascorbate levels in the midgut tissues of *A. ellioti* that consumed tannic acid for 2 days would be further aggravated in long-term feeding trials, such as those performed previously (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980).

It is clear from this study that tannin-tolerant (polyphagous) grasshoppers in general do not depend on high levels of ascorbate in their midgut fluids as a key antioxidant defense, as has been suggested for tannin-tolerant caterpillars (Barbehenn et al., 2001). Both glutathione and ascorbate show a pattern of lower concentration in midgut fluids in *M. sanguinipes* compared to concentrations in *A. ellioti*. This result is the opposite of the pattern expected if low-molecular-weight antioxidants in the gut fluids of tannin-tolerant grasshoppers represented defenses that are necessary to permit polyphagy. Based on this study and previous results suggesting that ingested tannic acid is oxidized in *M. sanguinipes* (Barbehenn et al., 1996), it appears that well-defended midgut tissues, rather than well-defended gut fluids, are a necessary component of its antioxidant defense system. For example, higher α-tocopherol concentrations in the midgut tissues of *M. sanguinipes* might produce a greater degree of protection of the epithelial membranes from oxidative damage than is available in *A. ellioti*.

Glutathione levels in the midgut tissues of *M. sanguinipes* (10.6 mM) are among the highest recorded in animal tissues, and are nearly twice as great as those in *A. ellioti*. Intracellular glutathione in animal tissues is typically in the range of 1–8 mM (Griffith, 1999), but ranges as high as 11–12 mM in liver tissues (my calculation) (Baker et al., 1990; Roberts and Francetic, 1993). Glutathione levels in both grasshoppers examined in this study are higher than the glutathione levels in the midgut tissues of larval Lepidoptera: 0.4–1.2 mM in *Lymantria dispar* (Peric-Mataruga et al., 1997), 2.2 mM in *Manduca sexta* (Aucoin et al., 1995), and 1.2 mM in *Actias luna* and *Callosamia promethea* (Thiboldeaux et al., 1998) (my calculations). High levels of glutathione might increase the rate of product formation by a variety of antioxidant enzymes, e.g., glutathione peroxidase (if present), glutathione transferase peroxidase, and dehydroascorbate reductase, or more effectively scavenge free radicals and reduce other oxidized chemicals (Winkler et al., 1994).

Glutathione levels increased from the foregut to the midgut in three of four cases examined in this study, suggesting that this antioxidant may be secreted into the midgut lumen. Previous work on a tannin-tolerant caterpillar, *Orgyia leucostigma*, also found evidence for glutathione secretion into the gut lumen (Barbehenn et al., 2001). The trend towards elevated levels of glutathione in foreguts of tannin-consuming grasshoppers may result from regurgitation of glutathione-rich midgut fluid into the foregut (Table 3). Regurgitation into the foregut is an important process for distributing digestive enzymes, and probably

also antioxidant enzymes, in grasshoppers (Evans and Payne, 1964; Ferreira et al., 1990; Barbehenn, 2002). However, additional work is needed to determine the mechanisms underlying the patterns in antioxidant concentrations in grasshopper gut fluids. Elevated glutathione levels in tannin-fed insects, for example, could potentially result from increased damage to epithelial cells (Hagen et al., 1990). Alternatively, microbial antioxidants could be included in the extracts of gut fluids (Mead et al., 1988) in cases in which they are lysed in the extracting medium. It is also possible that elevated concentrations of antioxidants in gut fluids could result from a low efficiency of absorption of ingested antioxidants relative to other nutrients.

Levels of ascorbate vary as much as 40-fold among species of wild plants (Jones and Hughes, 1983), with species of Asteraceae (including *L. sativa*) containing an average of one third the amount of ascorbate found in species of Poaceae. Similarly, α-tocopherol varies by more than 10-fold in herbaceous plants (Mallet et al., 1994). Antioxidants vary substantially not only from one plant species to another, but also from one season to another and according to abiotic factors (e.g., Luwe, 1996; Schwanz et al., 1996a,b). The antioxidant status of polyphagous grasshoppers appears to be determined by both plant and insect factors. When *M. sanguinipes* specialize on host plants with widely varying α-tocopherol levels, they are able to maintain high levels of α-tocopherol in their midgut tissues (Figure 1), consistent with a genetically determined homeostatic mechanism. By contrast, ascorbate is neither maintained at a high level independent of its level in the host plant nor are concentrations of ascorbate in the midguts of *M. sanguinipes* directly proportional to foliar ascorbate concentrations (Figure 1). This result differs from previous work comparing *T. ni* and *Depressaria pastinacella* caterpillars, which showed that concentrations of ascorbate in gut tissues vary in proportion to levels of ascorbate in the diets of *T. ni*, and to a lesser extent in *D. pastinacella* (Timmerman et al., 1999). Previous work has shown that mixed feeding by polyphagous grasshoppers results in the acquisition of a better balance of nutrients and reduces the amounts of particular allelochemicals ingested (Bernays and Bright, 1993). It is also possible that polyphagy (mixed feeding) improves the antioxidant status of polyphagous grasshoppers in comparison to host plant specialization.

The TAC of foliage and gut fluids was greatly increased by the presence of tannic acid, a result of the strong antioxidant capacity of tannins and gallic acid in the $ABTS^{\cdot+}$ assay (Hagerman et al., 1998). A decrease in TAC from the foregut to the midgut lumen in tannin-consuming grasshoppers is consistent with the chemical transformation of ingested tannic acid (Barbehenn et al., 1996). However, the twofold increase in TAC in the foregut and midgut fluids of insects fed untreated wheat compared to the TAC of the wheat itself (Table 5) is an unexpected result and suggests that grasshoppers produce compounds in gut fluids that are potential antioxidants. The comparison between foliar and gut fluid TAC is a conservative one,

i.e., wheat leaf samples were more thoroughly extracted (macerated in ethanol) than the contents of the gut lumen (briefly extracted in ethanol by shaking). The TAC of aqueous extracts of *Heliothis virescens* midgut fluid has also been found to be greater than that of aqueous homogenates of tobacco leaves, suggesting the formation or secretion of antioxidants in caterpillar gut fluids (Johnson and Felton, 2001). However, it is less clear in this case whether concentrations of phenols and antioxidants are decreased in enzymatically active leaf extracts relative to those in gut fluids. By comparison, the $ABTS^{\cdot+}$ assay in this study used anoxic ethanolic extracts kept in an oxygen-free environment, which maintains stable TAC in plant and insect samples. In both cases, further work is needed to determine the extent to which high TAC represents high antioxidant activity *in vivo*, such as by decreasing levels of ROS or biomarkers of oxidative stress.

The doubling of the total antioxidant capacity in the gut fluids of *M. sanguinipes* and *A. ellioti* represents an increase of greater than 10,000 μM Trolox equivalents. Of the antioxidants measured in this study, only α-tocopherol was found to increase in concentration in gut fluids compared to levels in wheat leaves, but this increase is only on the order of 25 μM. Substances that are likely to be in abundance in grasshopper gut fluids that are potential antioxidants include melaninlike compounds and chlorophyll degradation products. The possibility that the black-colored "tobacco juice" of grasshoppers acts as an antioxidant is supported by extensive observations of antioxidant activity in melanins produced from oxidized phenolics such as 3,4-dihydroxyphenylalanine (DOPA). Melanins chelate metal ions, are good redox buffers, and scavenge free radicals (Sarna et al., 1976; Dunford et al., 1995; Fogarty and Tobin, 1996). The possibility that melanins act as "sacrificial antioxidants" has also been suggested previously (Dunford et al., 1995; Halliwell and Gutteridge, 1999), because oxidative damage to these molecules might occur (especially at the sites of chelated catalytic metal ions) in lieu of damage to essential nutrients and cellular components. Although high-molecular-weight melanins do not remain in ethanolic supernatant solutions (C. Felix, personal communication), the ethanolic extracts of gut fluids in this study were light brown, suggesting that low-molecular-weight melaninlike substances were retained. Pure gut fluid would contain far higher concentrations of these pigments. Chlorophyll *a* is degraded in the digestive tracts of herbivores, producing large amounts of metabolites, such as pheophytin-a (Ma and Dolphin, 1999). Further work is needed to determine whether the putative antioxidant activities of these metabolites occurs in the physicochemical conditions of the grasshopper gut lumen.

In a previous study on antioxidant enzymes in *M. sanguinipes* and *A. ellioti*, no significant differences in the activities of superoxide dismutase, catalase, ascorbate peroxidase, or glutathione transferase peroxidase were observed (Barbehenn, 2002). However, the results reported here suggest that there are additional enzymes that may enhance antioxidant levels in polyphagous grasshopper species

that should be investigated. Such enzymes might include those that maintain antioxidants in a reduced form, such as glutathione reductase, dehydroascorbate reductase, and ascorbate free radical reductase, as well as enzymes involved in glutathione synthesis, such as gluthathione synthase. The results also suggest the possibility that the costs associated with the synthesis and maintenance of large amounts of antioxidants, such as glutathione in *M. sanguinipes*, are outweighed by the low benefits to graminivorous grasshoppers because of the low levels of potentially prooxidant allelochemicals in grasses.

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LINKING CHEMICAL REACTIVITY AND PROTEIN PRECIPITATION TO STRUCTURAL CHARACTERISTICS OF FOLIAR TANNINS

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Abstract—Tannins influence ecosystem function by affecting decomposition rates, nutrient cycling, and herbivory. To determine the role of tannins in ecological processes, it is important to quantify their abundance and understand how structural properties affect reactivity. In this study, purified tannins from the foliage of nine species growing in the pygmy forest of the northern California coast were examined for chemical reactivity, protein precipitation capacity (PPC), and structural characteristics (^{13}C NMR). Reactivity of purified tannins varied among species 1.5-fold for the Folin total phenol assay, and 7-fold and 3-fold, respectively, for the acid butanol and vanillin condensed tannin assays. There was about a 5-fold difference in PPC. Variation in chemical reactivity and PPC can be largely explained by differences in structural characteristics of the tannins determined by ^{13}C NMR. In particular, the condensed versus hydrolyzable tannin content, as well as the hydroxylation pattern of the B-ring and stereochemistry at the C-2–C-3 position appear to influence reactivity. Due to the large differences in chemical reactivity among species, it is necessary to use a well-characterized purified tannin from the species of interest to convert assay values to concentrations. Our results suggest that structural characteristics of tannins play an important role in regulating their reactivity in ecological processes.

Key Words—Tannins, polyphenols, ^{13}C NMR, chemical structure, phenolics, Folin-Ciocalteu, acid butanol, vanillin, astringency, nutrient cycling.

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INTRODUCTION

The importance of tannins in ecosystem function and nutrient cycling has long been recognized (Handley, 1961; Haslam, 1988), and recent studies have highlighted their significance (Northup et al., 1995, 1998; Schimel, 1996, 1998; Preston, 1999; Bradley et al., 2000; Hättenschwiler and Vitousek, 2000; Lorenz et al., 2000). Tannins comprise a significant portion of terrestrial biomass C; plant foliage may contain up to 20% tannin by weight (Preston, 1999; Yu and Dahlgren, 2000). Tannins are defined by their capacity to precipitate proteins; this property is considered to be primarily responsible for the effects of tannins on biogeochemical cycling. Some other active areas of tannin research include plant defense (Bernays et al., 1989), fodder and feed quality (Aerts, 1999), astringency in wine (Robichaud and Noble, 1990), and antioxidant properties and implications for human health (Morton, 1978, 1992; Santos-Buelga and Scalbert, 2000).

Tannins are divided into two major classes: condensed and hydrolyzable. Condensed tannins (CT), also referred to as proanthocyanidins, are polymers of three-ring flavanols joined with C-C bonds (Figure 1a). The monomer units that make up CT can be further grouped by the number of OH groups on the B-ring: procyanidins (PC) have a dihydroxy B ring, while prodelphinidins (PD) have a trihydroxy B ring. Monomer units can also have different C-2–C-3 stereochemistry (*cis* or *trans*). Linkages between monomers are typically C-4 $\rightarrow$ C-8, although C-4 $\rightarrow$ C-6 linkages may also be found, with variable chain length. Hydrolyzable tannins (HT, Figure 1b) are further grouped into gallotannins and ellagitannins that are made up of gallic acid or hexahydroxydiphenic acid esters, respectively, linked to a sugar moiety. While gymnosperms and monocots contain only CT, dicots can contain either or both types of tannins (Bate-Smith, 1977; Haslam, 1988).

There are a variety of assays for tannin determination (see reviews by Mole and Waterman, 1987a,b; Hagerman and Butler, 1989; Scalbert, 1992). Chemical methods are usually based on the formation of a colored product, and include analyses for total phenolic content and more specific analyses for particular groups of tannins (e.g., CT, HT, gallotannins, ellagitannins). Protein precipitation methods are based on the ability of tannins to form insoluble complexes with proteins via hydrogen bonding. These analytical methods are largely operationally defined, and studies comparing the results of different methods have shown inconsistent results (Hagerman and Butler, 1978; Martin and Martin, 1982; Mole and Waterman, 1987a,b; Wisdom et al., 1987; Makkar et al., 1993; Ginger-Chavez et al., 1997; Nelson et al., 1997; Jones et al., 2000; Yu and Dahlgren, 2000). However, there is little information on how specific differences in tannin structure influence assay responses.

At the core of all methods for tannin quantification is the need for appropriate standards that show a similar response per mass unit to the tannins of interest (Scalbert, 1992). However, many assays respond differently to contrasting types

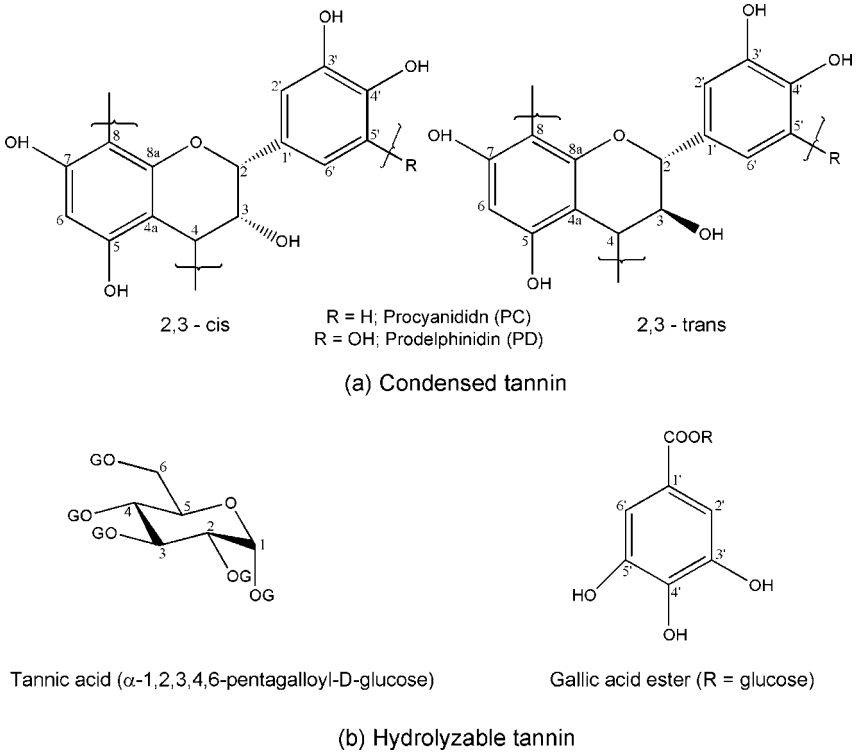

FIG. 1. Structures of (a) condensed and (b) hydrolyzable tannins.

of tannins (Wisdom et al., 1987; Nelson et al., 1997; Hedqvist et al., 2000; Stewart et al., 2000; Appel et al., 2001). These differences are probably related to structural variations, such as the nature of the monomeric units, the degree of polymerization, or the type of intermonomeric linkages. While use of commercially available standards allows comparison of results among studies, the chemical composition of these standards is likely to differ appreciably from the test species, resulting in uncertainties in quantification. Because tannins are comprised of a diverse group of polyphenols, it has been suggested that standards should be made by purifying tannins from the species of interest (Mole et al., 1989; Stewart et al., 2000).

A better understanding of the relationship between the chemical structure of tannins and their reactivity will improve our appreciation of the ecological significance of tannins (Zucker, 1983; Clausen et al., 1990; Foo et al., 1996, 1997; Ayers et al., 1997; Nelson et al., 1997). This understanding should start with factors that affect their quantification. In this study, we compared results from several common published methods for the quantification of purified tannins for

nine plant species collected from the pygmy forest ecosystem on the northern California coast (Northup et al., 1995, 1998). We compared results from the Folin-Ciocalteu assay for total phenols, the acid butanol and vanillin assays for condensed tannins, and the radial diffusion assay for protein precipitation capacity. We used solution-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy to provide structural characterization of the complex, polymeric tannins purified from plant extracts (Czochanska et al., 1980; Ayres et al., 1997; Preston et al., 1997; Foo et al., 2000). ^{13}C NMR provides direct information on PC versus PD content, *cis* versus *trans* stereochemistry, chain length for CT, and the occurrence of CT–HT mixtures. We used this technique to elucidate structural differences of the tannins that may contribute to differences in chemical reactivity and protein precipitation capacity.

METHODS AND MATERIALS

Study Area. The pygmy forest ecosystem is located on ancient [>240,000 years before present (ybp)] marine terraces along the northern California coast (Northup et al., 1995). The soils (Spodosols and Ultisols) are highly acidified, and low in fertility with nutrients concentrated in the top few centimeters of the soil in humic materials. While cypress and pines comprise the majority of the pygmy forest biomass, ericaceous shrubs make up the majority of the vascular plant species.

Collection, Purification, and Characterization of Tannins. Foliage was collected from nine species (Table 1) on pygmy forest sites in June 1998. Foliage was composited from 10 to 15 individuals of each species. Materials were freeze dried and ground in a Wiley mill to pass through a 0.5-mm screen. Tannin extraction and purification followed methods described by Schimel et al. (1996). Briefly, the plant material was washed several times with methylene chloride and then extracted with acetone–water (80:20) and 100% methanol. After rotoevaporation to remove solvents, the acetone- and methanol-extractable fractions were combined and washed with hexane. The extracted material was loaded on a Sephadex LH-20 column, washed with methanol–water (50:50) to remove low-molecular-weight phenolics and flavonoids, and then washed with acetone–water to remove the bonded tannin fraction from the column. The eluted acetone–water solution was then evaporated and freeze dried. Total carbon and nitrogen concentrations were determined by dry combustion (Carlo Erba analyzer). Ash content was determined by combusting the material at 550°C for 4 hr. Tannic acid (Fisher, Lot #922178) was also used in this study.

Folin-Ciocalteau Assay for Total Phenols (Folin). In this method (Scalbert et al., 1989), phenols reduce a phosphomolybdic-phosphotungstic reagent to produce a blue pigment. Absorbance data were collected at five concentrations (0, 5, 10, 50, 100 mg/L) of purified tannins dissolved in 50% acetone. Data were

TABLE 1. SLOPE AND REGRESSION COEFFICIENTS FOR TOTAL PHENOLS (FOLIN-CIOCALTEU) AND CONDENSED TANNINS (ACID BUTANOL AND VANILLIN) ASSAYS[a]

		Folin-Ciocalteau			Acid Butanol			Vanillin		
Common Name	Latin Name	Slope[b]	Standard error	R^2	Slope[b]	Standard error	R^2	Slope[b]	Standard error	R^2
Huckleberry	*Vaccinium ovatum*	9.23	0.20	0.997	7.90	0.09	0.999	1.01	0.01	0.999
Cypress	*Cupressus goveniana* ssp. *Pygmaea*	8.76	0.14	0.998	7.05	0.20	0.995	1.28	0.03	0.994
Salal	*Gaultheria shallon*	6.94	0.17	0.996	4.80	0.08	0.998	0.63	0.03	0.982
Rhododendron	*Rhododendron macrophyllum*	8.51	0.06	1.000	4.78	0.06	0.999	0.75	0.08	0.926
Bolander pine	*Pinus contorta* ssp. *Bolanderi*	6.54	0.09	0.999	4.10	0.30	0.967	0.92	0.05	0.979
Bishop pine	*Pinus muricata*	6.01	0.07	0.999	3.43	0.19	0.980	1.07	0.03	0.993
Labrador tea	*Ledum glandulosum*	8.50	0.11	0.999	2.79	0.03	0.999	0.85	0.02	0.998
Manzanita	*Arctostaphylos nummularia*	7.26	0.13	0.998	1.47	0.04	0.995	0.41	0.03	0.964
Wax myrtle	*Myrica californica*	7.33	0.04	1.000	1.10	0.04	0.993	0.54	0.02	0.991
Tannic acid		8.91	0.09	0.999						
Catechin								0.28	0.01	0.994

[a] Calibration curves were reproducible during several sets of analyses for the Folin assay. Both the butanol/HCl and vanillin assays are sensitive to time and temperature; although we obtained different slope values for different runs, the same relative values were obtained in multiple runs. Data reported here are from a single run.

[b] Slope = (1000 ∗ absorbance)/(mg/kg).

plotted as absorbance $\times 1000$ versus concentration in milligrams per kilogram. Linear regressions with zero intercepts were fit to these data and the slope [1000 $\times$absorbance/(mg/kg)], standard error of the slope, and R^2 values of the lines calculated. Data are presented as the slope of absorbance versus concentration, because this integrates results across all concentrations. In addition, wavelength scans (725–800 nm) of the colored product were conducted to check that the same product was formed for all tannins.

Acid Butanol Assay for Condensed Tannins. In this method (Porter et al., 1986), condensed tannins are depolymerized by heating in *n*-butanol/HCl (95:5 v/v) to produce anthocyanidin that has an intense red color. The anthocyanidins result from the oxidation of the carbocations initially formed by cleavage of the interflavanoid bond. Purified tannins were dissolved in 50% acetone. Absorbance data were collected and plotted as described above for the Folin assay. In addition, wavelength scans (500–600 nm) were run on all colored products.

Vanillin Assay for Condensed Tannins (Vanillin). In this assay (Price et al., 1978), vanillin, an aromatic aldehyde, reacts with condensed tannin A rings to form a red adduct. The reaction is specific to a narrow range of flavanols and dihydrochalcones that have a single bond at the 2,3 position and free meta-oriented hydroxy groups on the B ring (Sarkar and Howarth, 1976). Absorbance data were collected at five concentrations (0, 10, 50, 100, 250 mg/L) of purified tannins dissolved in 100% methanol. Color development was determined as described above for the Folin assay. We also ran catechin, which is commonly used as a standard in this assay.

Radial Diffusion Assay for Protein Precipitation Capacity (PPC). This assay, developed by Hagerman (1987), measures the protein precipitation capacity of tannins by the formation of insoluble protein–tannin complexes in a protein-containing agar plate. It is most suitable for ecological applications because it is not affected by the solvent (acetone), avoids the problem of varying the tannin-to-protein ratio (Scalbert, 1992), requires only small amounts of tannin (0.5 mg), and is relatively simple. Tannin solutions are pipetted into wells punched into the agar plates. The tannins diffuse into the medium and form insoluble complexes with the proteins, forming an opaque ring. The volume of agar within which the tannins precipitate is indicative of the PPC of the tannin.

To examine differences that arise from using different proteins, we used bovine serum albumin (BSA, Sigma Fraction V, Lot 60H0018) as well as gelatin (GEL, Knox Brand). Plates, prepared as described by Hagerman (1987), contained either 1.0 mg BSA/ml or 0.5 μg GEL/ml. Three concentrations of purified tannins (12.5, 25, 50 mg/ml) dissolved in 50% acetone were tested in both the BSA and GEL plates. Four replicate 20-μl aliquots of each concentration were pipetted into each well, and plates were stored for 24–36 hr at 30°C. The diameters of the opaque precipitation rings were measured in two directions, and the depth of the wells was determined using calipers. The ratio of the weight of protein precipitated

per weight of tannin (P:T ratio, grams per gram) was calculated using the volume of agar gel in which the protein-tannin complexes formed.

Solution ^{13}C NMR Spectroscopy. Purified tannins (100–200 mg) were dissolved in a mixture of 1.5 ml acetone and 1.5 ml D_2O and transferred through sintered glass filters into 10-mm-diam. NMR tubes. Spectra were obtained at 75.47 MHz on a Bruker MSL 300 spectrometer, using inverse-gated decoupling, 45° pulse, 0.4 sec acquisition time, 2.6 sec delay, and 25,000–30,000 scans. These conditions were chosen to minimize intensity distortions due to nuclear Overhauser enhancement and insufficient relaxation and should be sufficient for CH carbons (Newman et al., 1987), although ^{13}C T_1 values were not measured.

The CT spectra were used to derive average values for the proportions of PC versus PD units, *cis* versus *trans* stereochemistry (C-2–C-3) and chain length according to previous publications (Czochanska et al., 1980; Newman et al., 1987; de Bruyne et al., 1996; Foo et al., 1996, 1997, 2000; Ayres et al., 1997; Hatano and Hemingway, 1997). These studies were also used to compile Table 2, showing chemical shifts expected for polymers composed entirely of epicatechin (PC *cis*), epigallocatechin (PD *cis*), catechin (PC *trans*), and gallocatechin (PD *trans*). Note that chemical shifts vary slightly with solvent and temperature, which can also affect solution conformation. As shown, some carbons have distinct chemical shifts for terminal units. Small contributions from other possible structural variations, such as 4,6 linkages (Newman et al., 1987) are difficult to analyze solely by ^{13}C NMR, and were not considered in this study, similar to the approach used in other ecological studies (Clausen et al., 1990; Ayres et al., 1997; Bradley et al., 2000).

We also developed two new approaches to analyze PC versus PD units and to estimate the relative proportions of CT and HT in mixed preparations (see below). To facilitate this, in addition to the nine tannins prepared for this study, spectra were obtained for tannic acid (Aldrich) and from four CT prepared in a similar manner and previously described. These were from foliage of black spruce [*Picea mariana* (Mill.)], (Lorenz and Preston, 2002), salal (*Gaultheria shallon* Pursh), (Preston, 1999), sheep laurel (*Kalmia angustifolia* L. var. *angustifolia*), (Preston et al., 1997; Bradley et al., 2000), and balsam fir (*Abies balsamea* (L.) Mill (Preston et al., 1997; Bradley et al., 2000).

Determination of Procyanidin (PC) versus Prodelphinidin (PD). The PC/PD ratio for CT is usually determined from the ratio of the peak areas at 145 ppm (C-3′ and C-4′ of PC) and 146 ppm (C-3′ and C-5′ of PD). While this approach is useful qualitatively, the peaks are not completely resolved, and it is difficult to measure their relative areas accurately, especially when one structure is present in much higher proportion than the other. In addition, the method can not be used for samples containing HT, which produces an interfering peak at the same chemical shift. For these reasons, we attempted to utilize other features of the spectra to quantify the PC/PD ratio.

TABLE 2. CHEMICAL SHIFTS (ppm) FOR 4 → 8 CT POLYMERS[a] AND TANNIC ACID (α-1,2,3,4,6-PENTAGALLOYL D-GLUCOSE)[b]

	C2	C3	C4[c]	C4a	C5	C6	C7	C8	C8a	C1′	C2′	C3′	C4′	C5′	C6′
PC, 2-3 *cis* polymer															
Epicatechin units	79	72–73	37–38	102	157–158	97–98	157–158	107	157–158	131–132	115–116	144–145	144–145	115–116	119
Non-linked[d]	76–77	66–68	28–29	99–101				95–97							
PD, 2-3 *cis* polymer															
Epigallocatechin units	79	72–73	37–38	102	156–158	95–97	156–158	107	156–158	132	107–108	146	133	146	107–108
Nonlinked	76–77	66–68	28–29	99–101				95–97							
PC, 2-3 *trans* polymer															
Catechin units	83–84	73–74	38–39	106–107	156–158	96–97	156–158	109	156–158	130–131	115–116	145–146	145–146	115–116	119–120
Nonlinked	81–82	66–67	28–29	99–102				96–97							
PD, 2-3 *trans* polymer															
Gallocatechin units	83–84	73–74	38–39	106–107	157–158	96–97	157–158	109	157–158	132–133	107–108	146–147	133–134	146–147	107–108
Nonlinked	81–82	66–67	28–29	99–102				96–97							
	C1	C2	C3	C4	C5	C6	C1′	C2′	C3′	C4′	C5′	C6′	COOH′		
Tannic acid	93.4	71.9	75.3	69.5	74.1	62.9	121	110	146	140	146	110	166		

[a] Based on data from Czochanska et al. (1980), de Bryne et al. (1996), Foo et al. (1996, 1997, 2000), Hatano and Hemingway (1997), Newman et al. (1987).
[b] From Porter et al. (1989): C1–C6, glucose moiety; C-1′–C-6′, C'OOH, gallic acid moiety.
[c] The stereochemistry at C-4 in extender units is largely determined by that at C-2–C-3. If C-2–C-3 is *cis*, C-3–C-4 tends to be *trans*, and *vice versa*.
[d] Terminal (bottom) unit for C-2, C-3, C-4 and C-4a; top unit for C-8.

PC units produce peaks at 115–116 ppm for C-2′ and C-5′ (a sharp peak at 116 ppm not resolved from a broad peak at 115 ppm) and at 119 ppm for C-6′, while PD units have no intensity in this region. For PD structures, the broad peak at 106 ppm arises from three carbons (C-8, C-2′, C-6′), compared to one carbon for PC (C-8). However, the situation is complicated because for both PC and PD polymers, this region also includes C-4a in *trans* units, which were found to comprise up to 27% of the total; but not free C-8 carbon (upper terminal unit) that occurs at 95–97 ppm. As the average chain lengths were generally around 4 units, these effects tend to cancel. The 106 ppm peak is also broad, and poorly resolved from its neighbors at lower chemical shift. For tannins with close to 100% PC units (based on the 145–146 ppm region), the ratio of the area at 115–116 ppm to that at 106 ppm was 2.0 for huckleberry, 2.2 for cypress, and 2.3 for black spruce, reasonably close to the value expected for an ideal, infinitely long chain of PC, *cis* units.

Since the peak shapes were consistent for all tannins, to facilitate analysis we used the ratio of peak heights at 116 –106 ppm instead of peak areas. For the tannins with close to 100% PC units, this ratio was close to 4:1, mainly because the peak at 116 ppm is very sharp compared to those at 115 and 106 ppm. The highest ratios were found for species containing pure PC (3.96 for rhododendron and 3.95 for black spruce), while the lowest ratio was 0.28 for balsam fir with the highest PD content. Based on this analysis, we set a ratio of 4:1 for a pure PC structure. For a mixture of PC and PD units, where the fraction of PC $= x$ and the fraction of PD $= (1 - x)$, then peak A (116 ppm) $= 4\text{PC} = 4x$, and peak B (106 ppm) $= 1\text{PC} + 3\text{PD} = x + 3(1 - x) = 3 - 2x$. Then A/B, the 116/106 ppm peak height ratio would be:

$$\text{A/B} = 4x/(3 - 2x)$$

For each of the samples, A/B was measured from the spectrum, and the fraction of PC units, x, was estimated after substituting A/B into the equation. As we discuss later, both this approach and the one described in the next section, are open to further development.

Condensed versus Hydrolyzable Tannins. The remaining challenge was to estimate the proportion of HT in mixed samples. Comparison of the tannic acid spectrum with that of the mixed tannins showed distinct HT peaks at 165, 140, and 110 ppm. As we do not have any further information on the type of HT structures in our mixtures, and since these peaks occur at positions corresponding to tannic acid (Porter, 1989), tannic acid was used to represent the HT component of our samples. However, while there was a reasonably good match in the 110–165 ppm region, the 65–85 ppm region did not match either tannic acid or a commercial chestnut tannin. Variation was also found in the carboxyl region, as some samples had sharp peaks at 161.5 and 164.5 ppm in addition to the broader peak at 165–166 ppm, similar to that of tannic acid. These sharp peaks were found in

rhododendron, wax myrtle, labrador tea, and manzanita, the four obviously mixed CT–HT samples.

For the mixed tannins, we used the 90 to 172-ppm region to estimate equivalents of tannic acid (as galloyl units). In the tannic acid spectrum, the three peaks at 110, 140, and 165 ppm are assigned to C-2′ + C-6′, C-4′, and C′OOH (esterified) of the gallic acid moiety. This sum amounted to 0.4663 of the total integral from 90 to 172 ppm, compared to 0.5714 expected for four of seven gallic acid carbons. The discrepancy arises because the tannic acid is not pure pentagalloyl glucose and the relative intensities (areas) may be distorted by incomplete relaxation for some carbons.

For species with mixed tannins (rhododendron, wax myrtle, labrador tea, and manzanita), the integral for the sum of these peaks was determined, scaled up to represent the seven carbons of gallic acid (divided by 0.4663), and subtracted from the total for 90–172 ppm to get the integral area due to CT. For example, for wax myrtle, the total integral height was 151.0 mm, and the height for the three GA peaks was 33.2 mm, which was scaled up to 71.2 mm. The remaining integral due to CT was then 79.8 mm. If the gallic acid intensity represented seven carbons, and the CT intensity represented 12 of the 15 carbons, then the ratio of GA to CT units was determined by the ratio of 79.8/12 to 71.2/7, to give 1.017/0.665 or approximately a mix of 60 GA monomer units to 40 CT monomer units. In the absence of specific information on the HT moieties, we did not attempt to utilize the 60 to 85-ppm region, in which both C-2 and C-3 of CT and the glucose C-2–C-5 occur. The nominal formula weight of the tannic acid was 1701.23 g/mol, close to the value of 1728 calculated for a 1:1 mixture of penta- and tetragalloyl glucose. The value estimated from comparison of the integrals of the glucose and gallic acid regions was 5.1 galloyls per glucose.

Relationships Between Chemical Assays and NMR. Correlations between chemical and structural information were determined by linear regression analysis. In addition, forward stepwise multiple regression analysis was run to determine which structural features correlate to chemical reactivity. The chemical assays were run individually as the dependent factor (Folin, acid butanol, vanillin, BSA, GEL). The structural features were run as the independent factors including interaction terms for those features that are specific to condensed tannins (CT%, PC%, CIS%, chain length, CT ∗ PC, CT ∗ CIS, CT ∗ chain length). All statistical analyses were performed using Systat, Version 7.0 (SPSS Inc. 1997).

RESULTS AND DISCUSSION

Characterization Data. Carbon contents were similar among all of the species, ranging between 500 and 550 mg/g. Nitrogen content was low (0.7–1.4 mg/g) compared to the N content of the foliage before purification (5–20 mg/g). Ash content was below 0.1% for all samples.

Folin Assay. Folin assay reactivities varied 1.5-fold among purified tannins from the nine species, with huckleberry having the greatest absorbance to bishop pine the lowest (Table 1). Tannic acid showed the second highest reactivity, indicating that use of tannic acid as a standard for these species would result in an underestimation of total phenol content. These differences in reactivity are comparable to those seen in the reaction of monomeric procyanidins in the Folin-Denis assay conducted by Swain and Goldstein (1963) (1.8-fold). Appel et al. (2001) found a 3.6-fold difference in reactivity among tannins purified from 16 woody species.

Given that the product measured in this assay arises from the reduction of the molybdenum–tungsten reagent, the nature of the reductant (the phenol) should not influence the absorption spectra (Singleton and Rossi, 1965). Wavelength scans of the colored product confirmed this. Because we know that both color intensity and absorbance maxima are the same for all samples, differences in absorbance values for the Folin assay can be explained by differential reduction power of the tannins.

Acid Butanol Assay. Differences in purified tannin reactivity for the acid butanol assay varied 7-fold among species, with huckleberry having the greatest absorbance and wax myrtle the lowest (Table 1). This is comparable to the 9-fold difference in reactivity between purified tannins of three species reported by Nelson et al. (1997) and an 8.5-fold difference seen in foliage of different trefoil varieties (Hedqvist et al., 2000).

Several factors may explain why the acid butanol assay differed among species. First, as the acid butanol assay is specific for CT, material that contains HT should show lower reactivity. However, even the gymnosperms (bolander pine, bishop pine, and cypress) that are known to contain only CT showed a 2-fold difference in reactivity. Second, because the colored anthocyanidin product forms from interflavanoid bond cleavage, terminal units of the CT are not detected and therefore, lower yields are expected for shorter-chained CT (Porter et al., 1986). In addition, acid catalyzed interflavin bond lability may differ between CT units due to the type of interflavanoid linkages (e.g., C-4–C-8 versus C-4–C-6), substitution pattern on the A ring, and *cis* versus *trans* conformation at C-2–C-3 (Hemingway and McGraw, 1983; Porter et al., 1986; Hemingway, 1989). Third, because the colored product arises from the tannin units themselves, structural differences in these units are likely to affect both the color intensity (extinction coefficients) and the absorbance maxima of the product. Differences in absorbance can, thus, be due to differences in CT versus HT concentrations, chain length, interflavin bond lability, extinction coefficients, and absorbance maxima.

Vanillin Assay. Vanillin reactivity of the purified tannins varied 3-fold among species, with cypress having the greatest absorbance values and manzanita the lowest (Table 1). Like the acid butanol assay, the vanillin assay detects only condensed tannins, and again the colored product is made up of the CT units

TABLE 3. PROTEIN TO TANNIN (P:T) PRECIPITATION RATIOS FROM RADIAL DIFFUSION ASSAY FOR BOVINE SERUM ALBUMIN (BSA) AND GELATIN (GEL) PLATES

	BSA		GEL	
	P:T ratio (μg/μg)	Standard error[a]	P:T ratio (μg/μg)	Standard error[a]
Huckleberry	0.687	0.065	0.240	0.004
Cypress	0.724	0.060	0.258	0.008
Salal	0.581	0.021	0.181	0.006
Rhododendron	0.590	0.033	0.226	0.007
Bolander pine	0.605	0.077	0.193	0.005
Bishop pine	0.695	0.069	0.219	0.003
Labrador tea	0.533	0.014	0.165	0.006
Manzanita	0.506	0.056	0.168	0.007
Wax myrtle	0.381	0.044	0.134	0.004
Tannic acid	1.524	0.161	0.704	0.087

[a] $n = 4$.

themselves. As in the acid butanol assay, the three gymnosperms had varying responses to the vanillin assay. Catechin, which is commonly used as a standard in this method, had less color development than the purified tannin materials. Catechin is a monomer and reacts more slowly than the polymers; this use of catechin standards can overestimate tannin content (Price et al., 1978).

Radial Diffusion Assay. The protein- to -tannin (P:T) ratios of the nine purified tannins are presented in Table 3. A higher P:T ratio indicates that less tannin is required to precipitate a given mass of protein. Among the nine purified tannins, PPC varied 4.0-fold for BSA and 5.3-fold for GEL. This is comparable to the 3.3-fold difference seen in leaves of different trefoil varieties with BSA (Hedqvist et al., 2000). The P:T ratio obtained from the two kinds of gel plates are strongly correlated ($R^2 = 0.98$, $P = 0.002$); however, BSA required about three times more tannin by weight than gelatin to initiate precipitation. Hagerman and Butler (1981) also found that gelatin had a higher affinity for CT than BSA. Tannic acid precipitated two to five times more protein than any of the purified tannins, suggesting that using tannic acid as a standard in this assay greatly underestimates tannin content.

Solution ^{13}C NMR Spectroscopy. Figure 2 shows the ^{13}C NMR spectra of species containing pure CT, and Figure 3 shows those containing a mixture of CT and HT along with tannic acid; both figures include expansions for the C-2–C-3 region. Chemical shift assignments are shown in Table 2. In condensed tannins, the shift for C-3 terminal units [(C-3(term)] occurs at 65.0–69.5 ppm, with the main peak observed at 66–67 ppm, and a trailing shoulder or smaller peak at the higher chemical shift values. The region for C-3 extender units [C3(ext)] is

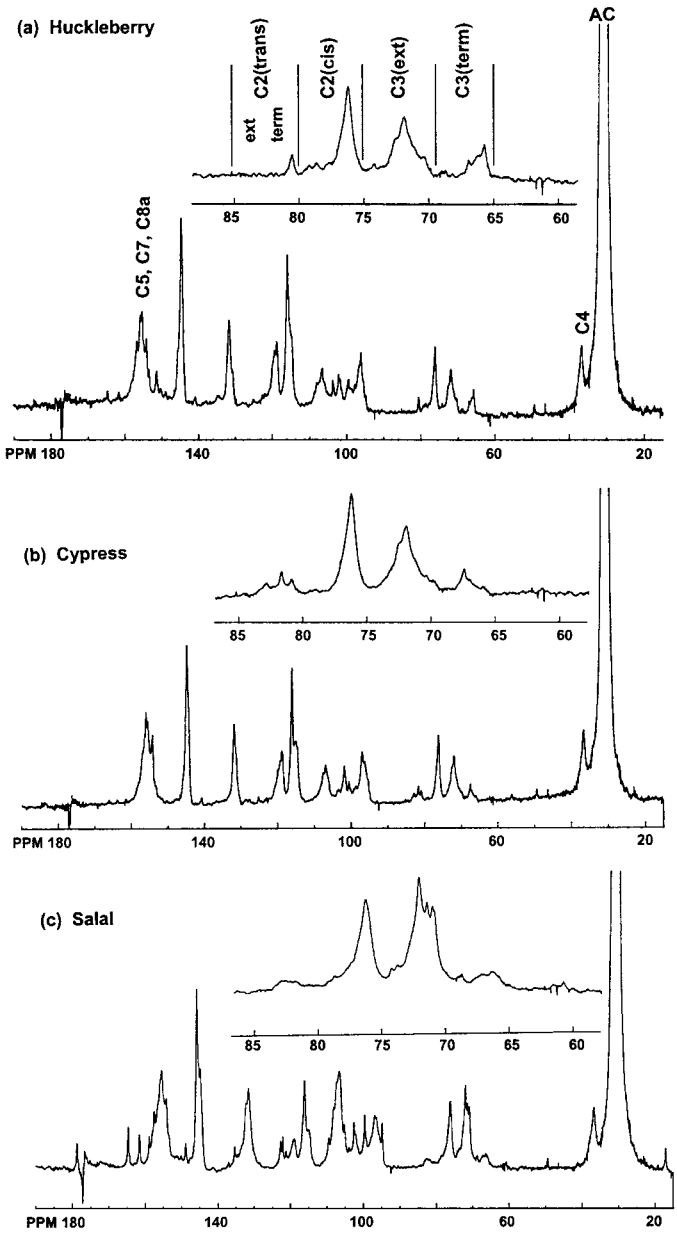

FIG. 2. Solution ^{13}C NMR spectra of purified tannins for species containing only condensed tannins with expansions of the C-2–C-3 region: (a) huckleberry, (b) cypress, (c) salal, (d) bolander pine, and (e) bishop pine. AC = acetone solvent peak.

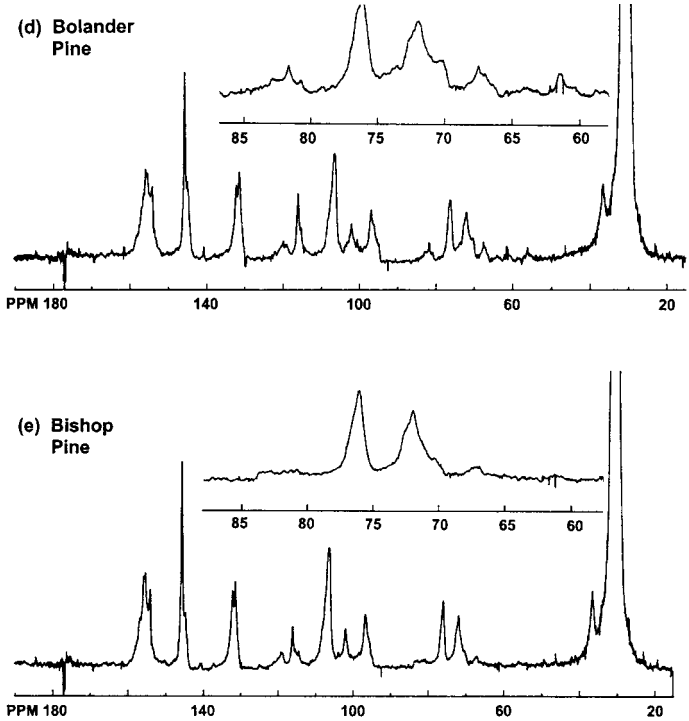

FIG. 2. Continued

69.5–75.0 ppm, with the main peak at 71.9–72.0 ppm. The average chain length is determined from the area ratio of [C-3(term) + C-3(ext)] to C3(ext). Interfering HT peaks occur at 72.6–73.8 ppm; for rhododendron (Figure 3a), these are just shoulders on the main C-3 peak at 72.0 ppm, but are increasingly prominent in the order: labrador tea < manzanita < wax myrtle (Figures 3b, 3c, and 3d, respectively).

The C-2 region occurs at 75–85 ppm, with 80 ppm being the dividing line between *cis* and *trans* structures. The main peak for *cis* structures occurs at 76.0–76.2 ppm, with a small peak or shoulder at 78–79 ppm in some spectra from C-2(*cis*) in terminal units. The latter is more obvious in rhododendron, balsam fir, salal, huckleberry, and bolander pine, but smaller in manzanita, bishop pine, and pygmy cypress, indicating that in the latter species most *cis* structures are in extender rather than terminal units. This region is also affected by the presence of HT, which causes a slight shift from 76.0–76.2 ppm to 75.8 ppm. The peak for rhododendron and manzanita is at 76.2 ppm (Figure 3a and c), labrador tea has a split peak at 75.8 and 76.0 ppm (Figure 3b), and wax myrtle is at 75.8 ppm (Figure 3d).

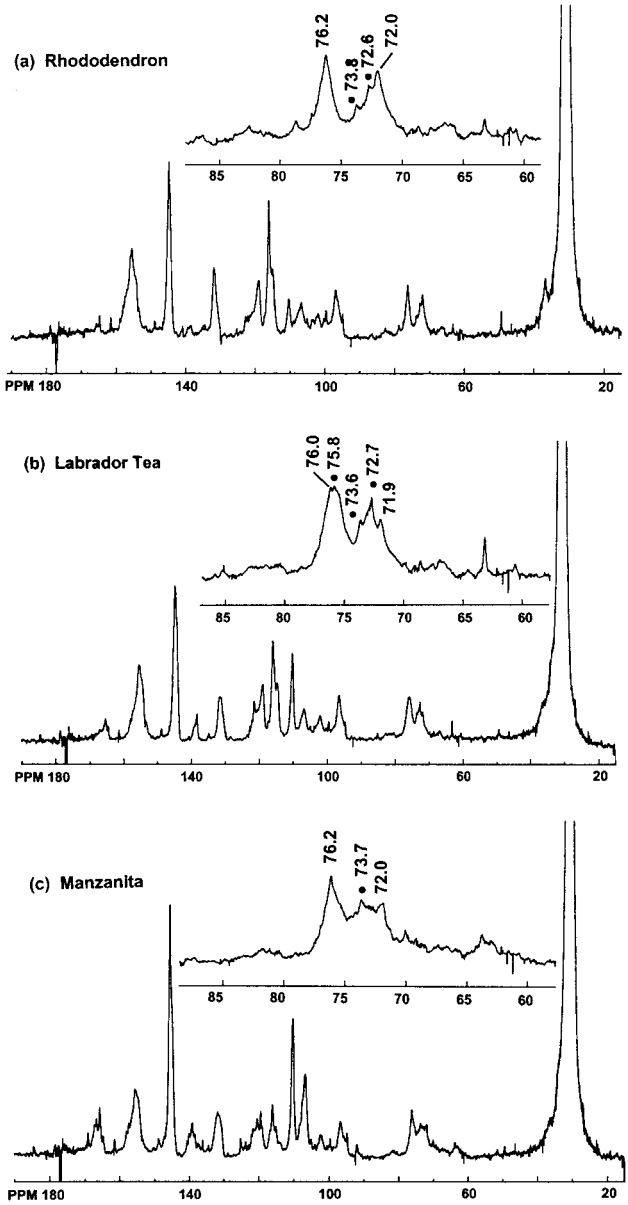

FIG. 3. Solution ^{13}C NMR spectra of purified tannins for species containing both condensed and hydrolyzable tannins with expansions of the C-2–C-3 region: (a) rhododendron, (b) labrador tea, (c) manzanita, (d) wax myrtle, and (e) tannic acid. Peaks in the C-2–C-3 region marked with • are from HT.

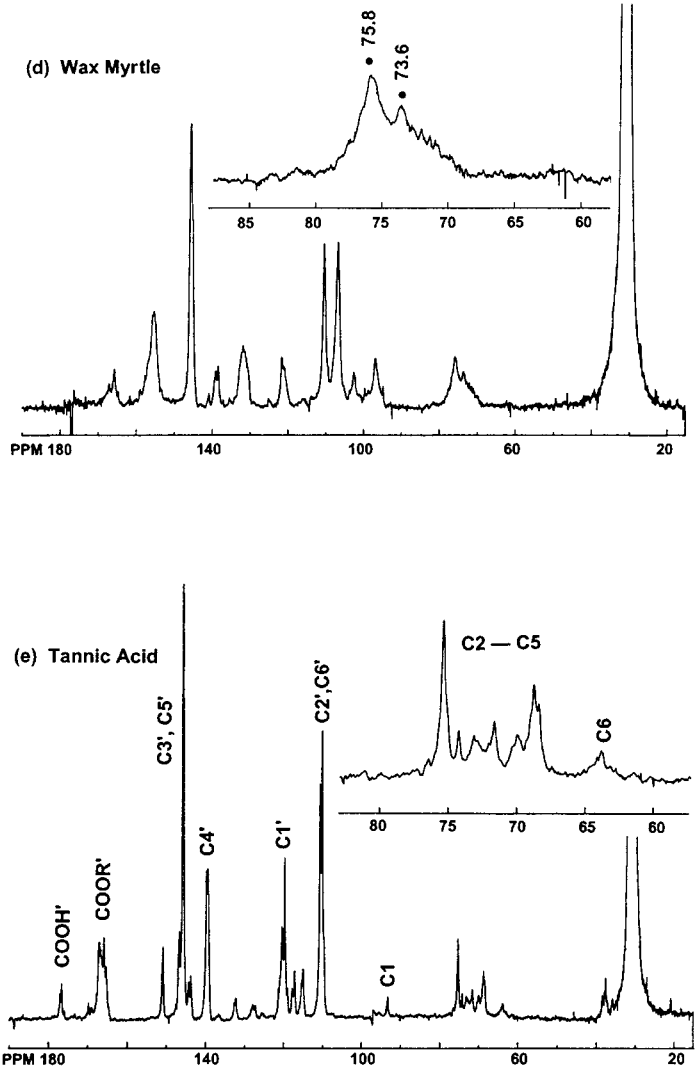

FIG. 3. Continued.

In the species we studied, the C-2(*trans*) region at 80–85 ppm is usually broad and weak, reflecting the low proportion of *trans* structures. It also often shows two regions of intensity, with 80–81.5 ppm associated with terminal units and 83 ppm with extender units. Estimates from the relative areas indicate that, for the samples in this study, a much higher proportion of the *trans* units are found in terminal

positions. This trend has also been found in other studies (Foo and Porter, 1980; Foo et al., 1996, 1997, 2000). For the huckleberry tannins, it appears that all of the *trans* units are in terminal positions.

The range of structural variation for the species containing only CT is demonstrated by the spectra in Figure 2, which include huckleberry (98% PC), pygmy cypress (96% PC), salal (48% PC), bolander pine (35% PC), and bishop pine (19% PC). Differences in the proportion of PC versus PD are evident in the 100 to 150-ppm region. There was less variation in the average chain length and the proportion of *trans* structures, and, therefore, less variation in the C-2–C-3 region (Figure 2 expansions). The signal for C-4(term) at 28–29 ppm is obscured by the acetone solvent peak, but the C-4(ext) signal is partially resolved. The C-4(ext) peak occurs at 37 ppm with, in some cases, a shoulder at 38 ppm, consistent with C-4(ext) occurring mainly in *cis* structures.

Spectra of the other four tannins (Figure 3a–d) have features indicating a mixture of CT and HT structures. As previously observed, these two classes of tannins are not separated by Sephadex LH-20 column (Foo and Porter, 1980). A commercial tannic acid is shown for comparison in Figure 3e. While other structures may contribute to HT, the peaks of our mixed tannins correspond reasonably well to those of the gallic acid moiety of tannic acid in the 110 to 170-ppm region. The expanded regions from 60 to 80 ppm in Figure 3a–d include signals both from the C-2 and C-3 of CT, and the glucose moieties of HT. The C-4(ext) peak in rhododendron (Figure 3a) is reduced to an unresolved shoulder for the species with higher proportions of HT (Figures 3b–d).

Structural information determined from the ^{13}C NMR spectra is summarized in Table 4. As expected, the three gymnosperms (cypress, bishop pine, and bolander pine) contained only CT. In addition, huckleberry and salal appear to be pure CT. Hydrolyzable tannins were present in the four remaining species (rhododendron, labrador tea, manzanita, and wax myrtle) in amounts ranging from 30 to 63%.

Our structural interpretations generally agreed with previously studied tannins of closely related species [*Vaccinium* (Czochanska et al., 1980; Foo and Porter, 1980; Ayres et al., 1997), *Pinus* (Porter and Woodruffe, 1984), *Gualtheria* (Preston, 1999)]. These studies and others (Ellis et al., 1983) indicated that PC *cis* units were the most common in nature. In our study, *cis* stereochemistry predominated; however, over half of the species contained more than 50% PD units (salal, bolander pine, bishop pine, and manzanita), and one—wax myrtle—contained exclusively PD units. We did not observe a correlation between PD units and *trans* units, as suggested by Ellis et al. (1983).

The empirical NMR methods developed for structural analysis produced results that are reasonable over the whole range of PC and PD structural mixes, agree well with the estimates from the 144 to 145-ppm region (for pure CT structures), and can be used for mixed CT–HT structures. However, both NMR methods developed in this study have potential for further development, especially the second

TABLE 4. STRUCTURAL INFORMATION OF PURIFIED TANNINS DETERMINED FROM ^{13}C NMR SPECTRA[a]

Species	% CT	A/B ratio (116/107 peaks)	%PC[b]	%CIS	Average chain length	Fraction of *trans* terminal units
Huckleberry	100	3.79	98	92	3.4	1.0
Cypress	100	3.55	96	76	4.6	0.8
Salal	100	0.94	48 (50)	78	4.4	0.5
Rhododendron	70	3.96	100	85	4.5	<0.5
Bolander pine	100	0.62	35 (38)	73	4.7	0.7
Bishop pine	100	0.29	19 (30)	73	4.2	0.5
Labrador tea	57	3.25	93	81	4.8	0.5
Manzanita	37	0.62	36	84	—	>0.5
Wax myrtle	40	0.00	0	>85	—	>0.5
Balsam fir[c]	100	0.28	18	75	4.5	0.4
Salal[c]	100	1.07	51 (50)	75	3.2	0.5
Kalmia[c]	100	3.30	93	75	1.9	0.8
Black spruce[c]	100	3.95	100	79	4.6	0.7

[a] %CT = percent condensed tannin versus hydrolyzable tannin, A/B ratio calculated as described in the methods section, %*PC* = percent procyanidin versus prodelphinidin, %CIS = percent *cis* versus *trans* stereochemistry at C-2–C-3, "—" = interference due to presence of hydrolyzable tannins.
[b] First numbers refer to values calculated by the A/B ratio while the values in parentheses were calculated by the integral method.
[c] Balsam fir and kalmia tannins from Preston et al. (1997) and Bradley et al. (2000). Salal tannins from Preston (1999). Black spruce tannins from Lorenz and Preston (2002).

that determines HT content based on a model of pentagalloyl glucose (tannic acid). It provides an estimate of gallic acid monomer units versus CT monomer units; no attempt was made at this stage to account for the glucose moiety of HT or other HT structures. For pure CT, the method for determining PC versus PD units could be expanded to use the 130 to 133-ppm region, which has two carbons for PD and one for PC units. Other possibilities include the use of integrated areas rather than peak heights, investigating the possible effects of differential ^{13}C relaxation times, and more sophisticated structural models. Further development will require both wet-chemical molecular-level analysis, more sophisticated NMR, and study of a wider range of tannins and subunits, especially for the HT. However, the approaches used here should provide a useful starting point for CT–HT mixtures, especially for preliminary investigations and where molecular-level analysis is not feasible.

Relationships between Chemical Assays and NMR Characterization. Differences in chemical reactivity among the nine tannins were examined in light of the structural information obtained from NMR. Table 5 shows results from simple linear regression analysis among all of the chemical and structural information. Forward stepwise, multiple regression analysis was also performed to examine possible structural features that affect reactivity. Spectral interference by

TABLE 5. CORRELATION MATRIX (P VALUES) FOR SINGLE-FACTOR REGRESSION ANALYSIS USING DATA FROM NINE PURIFIED TANNINS

	Folin slope	Acid Butanol slope	Vanillin slope	BSA ratio	GEL ratio	%CT	%PC	%CIS
Folin								
Butanol	0.120[a]							
Vanillin	0.492	0.032						
BSA	0.644	0.007	0.004					
GEL	0.281	0.002	0.010	<0.001				
%CT	0.888	0.014	0.023	0.004	0.030			
%PC	0.003	0.036	0.222	0.173	0.072	0.510		
%CIS	0.057	0.773	0.331	0.438	0.857	0.251	0.404	
Chain length	0.541	0.122	0.758	0.250	0.298	0.358	0.768	0.101

[a] Correlation between the Folin and butanol/HCl assays was significant ($P < 0.001$) for the five species which are pure CT (bolander pine, bishop pine, cypress, salal, huckleberry).

HT precluded information on chain length for manzanita and wax myrtle; thus, when this factor was included in the model, only seven cases were available for comparison. However, average chain length was not a significant contributor to the regression model ($P < 0.05$) and was eliminated from the model, and the forward stepwise regression was rerun with CT + PC + CIS + CT∗PC + CT∗CIS as independent variables (Table 6).

For the Folin assay, reactivity differences appear largely correlated with the PC% of the CT (Tables 5 and 6, Figure 4). When all nine species are compared, PC% and Folin reactivity are correlated ($P = 0.003$), and this trend is even stronger when only the five species with pure CT are examined ($P = 0.001$). Although it has been suggested that B-ring hydroxylation might affect reactivity in the Folin

TABLE 6. RESULTS OF FORWARD STEPWISE REGRESSION ANALYSIS RELATING ASSAY REACTIVITY TO STUCTURAL INFORMATION[a]

ASSAY	Significant factors	R^2	F	P
Folin	PC	0.893	26.34	0.002
	CIS		8.99	0.024
Acid butanol	PC	0.993	10.68	0.022
	CT∗PC		45.72	0.001
	CIS∗CT		16.11	0.010
Vanillin	CT	0.546	8.43	0.023
BSA	CT	0.721	18.11	0.004
GEL	CT∗PC	0.632	12.15	0.010

[a] Model: dependent variable = constant + CT + PC + CIS + CT∗PC + CT∗CIS.

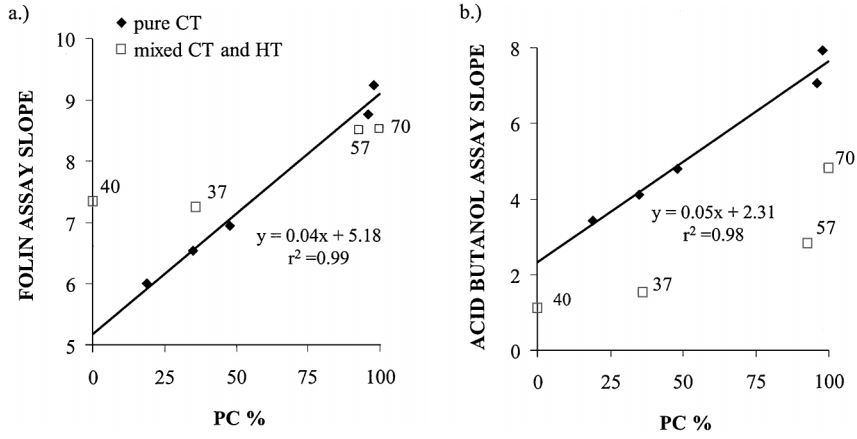

FIG. 4. Correlation between the percent procyanidin content (PC%) of condensed tannin (CT) units versus reactivity in the (a) Folin assay for total phenols and (b) acid butanol assay for condensed tannins. Slope = 1000∗absorbance/(mg/kg). Filled points are for pure CT species and the linear regression line is fit to these data. Numbers near the open points indicate the percent CT of the mixed purified tannins determined by ^{13}C NMR.

assay (Swain and Hillis, 1959), this is the first evidence supporting this hypothesis. Our results suggest a strong relationship. As mentioned above, wavelength scans of all Folin reacted materials were comparable, indicating that the colored product is the same for all tannins. It appears that PC units with two hydroxyl groups on the B ring are better at reducing the phosphomolybdic–phosphotungstic reagent than PD units with three hydroxyl groups. Previous studies on the effects of B-ring hydroxylation patterns on the antioxidant capacity of flavanoids show inconsistent trends; in some cases PC units are better antioxidants, while in other cases PD units are (Noferi et al., 1997; Wang et al., 1997; Fukumoto and Mazza, 2000; Santo-Buelga and Scalbert, 2000).

Multiple regression analysis indicates that in addition to B-ring hydroxylation, C-2–C-3 stereochemistry (CIS%) is a significant factor affecting tannin reactivity in the Folin assay. Our data suggest that tannins containing proanthocyanidins with *cis* structures are better oxidizing agents than those containing *trans* structures. This is in agreement with work by Saint-Cricq de Gaulejac et al. (1999a,b), which found a greater antioxidant capacity for epicatechin than for catechin. The *cis* versus *trans* structures are also likely to lead to different conformation of the tannin polymer. Surprisingly, we found no significant relationship between Folin reactivity and the presence of HT (Tables 5 and 6), indicating that CT and HT react similarly in this assay. In contrast, Hagerman et al. (1998) found that a CT (tannic acid) had greater antioxidant activity than a HT.

The correlation between the Folin and acid butanol assays was not significant when all nine species were included in the analysis (Table 5). As mentioned above, the acid butanol assay is specific for CT and, thus, should show lower reactivity when HT is present in the sample. The presence of HT in four of the nine species (rhododendron, labrador tea, manzanita, and wax myrtle) explains this poor correlation. Using only the five species that contain pure CT (huckleberry, cypress, salal, bishop pine, and bolander pine), produced a strong correlation between the Folin and acid butanol assays ($R^2 = 1.00$, $P = 0.001$).

For the single-factor regression analysis, reactivity in the acid butanol assay was correlated with CT% and PC% (Table 5). In this assay, colored anthocyanidin products form from the individual CT units. Because the maximum absorbance for the cyanidin carbocation is around 547 nm, and for the delphinidin carbocation it is about 558 nm, it has been suggested that CT content is overestimated in samples with a high PD content (Mole and Waterman, 1987a). To test this, we ran wavelength scans on the 100 mg/kg and 250 mg/kg solutions of tannins reacted in the acid butanol assay. At the 250 mg/kg concentration, differences in the wavelength absorbance maxima were observed, and these correlated with PC% (Figure 5). Wax myrtle and manzanita, which both have a high HT content, did not follow the trend as well. Samples with greater than 90% PC units in the CT fraction had absorbance maxima of 549 nm, close to the reported value of 547 nm (Bate-Smith, 1977). Samples with increasing PD content had maxima ranging between 553 and 556 nm. However, at the 100 mg/kg concentration, the spectra for all samples had a broad, flat peak with a maximum at 550 ± 1 nm. Even for the 250 mg/kg

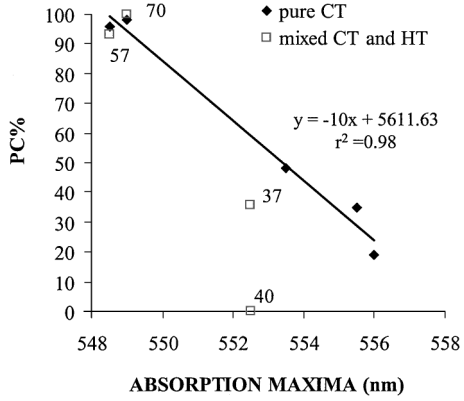

FIG. 5. Maximum absorbance values for the acid butanol assay versus PC% for solutions containing 250 mg/kg purified tannins. Filled points are for pure CT species and the linear regression line is fit to these data. Numbers near the open points indicate the percent CT of the mixed purified tannins determined by ^{13}C NMR.

solutions, the difference in absorbance values between the peak maxima and the measured absorbance at 550 nm was less than 2%. Since samples with concentrations of 100 mg/kg or less were used in the acid butanol assay, differences in peak absorbance maxima due to the number of hydroxyl units on the B ring should be negligible. Thus, reactivity in the acid butanol assay should not be attributed to differences in the absorbance maxima of cyanidin and delphinidin.

Among the nine species included in our study, we found that those with higher PC content had higher absorbance values in the acid butanol assay for equal tannin concentrations (Figure 4). This is contrary to the conclusion of Porter et al. (1986) that there are no differences in the acid catalyzed anthocyanidin yield between PC and PD units. Again, it appears that CT units with two hydroxyl groups on the B ring are more reactive than those with three hydroxyl groups.

The forward stepwise multiple regression analysis for the acid butanol assay indicates that in addition to CT% and PC%, the stereochemistry at C-2–C-3 may significantly affect tannin reactivity, being positively correlated with CIS% (Table 6). Hemingway and McGraw (1983) showed that the hydrolysis cleavage rate of 2,3-*cis* forms of extender units is nearly a factor of 2 greater than the cleavage rate of 2,3-*trans* forms. It has long been known that 4β-8 linkages are more easily cleaved than 4β-6 linkages and that substitution patterns of the A ring may influence lability (Hemingway and McGraw, 1983; Porter et al., 1986; Hemingway, 1989). We did not consider variations in linkage type or A-ring substitution, but these are unlikely to have had a major effect. The greatest range of structural variation for the CT in our study was found to be hydroxylation of the B ring (PC versus PD), with smaller differences in *cis*/*trans* stereochemistry and chain length.

We expected chain length to have an influence on the acid butanol assay because terminal units do not form a colored product and because polymer size has been shown to affect cleavage rate (Porter et al., 1986). However, there was little variability in average chain length among the nine species (huckleberry being the exception with an average chain length of 3.4 vs. 4.2–4.8). As both CT% and the PC:PD ratio have a strong effect on the reaction, we did not observe any significant differences in the acid butanol reactivity associated with average chain length.

Unlike the Folin and acid butanol assays, the vanillin assay was not significantly correlated to either B-ring hydroxylation or C-2–C-3 stereochemistry. Both single factor and multiple factor regression analysis found CT% to be the only factor significantly correlated to vanillin reactivity (Tables 5 and 6). Among the five species that contained pure CT (cypress, bishop pine, bolander pine, and huckleberry, salal), there was a 2-fold range in reactivity.

When all 9 species are analyzed, there is a significant correlation between the vanillin and acid butanol assays (Table 5); however, this relationship had a low R^2 value (0.50), and the correlation is strongly influenced by the low reactivity of manzanita and wax myrtle, which are both high in HT. Among the five species containing pure CT, results from the vanillin and acid butanol assay are not

correlated. This suggests that the positive correlation seen among the nine species is driven by differences in CT and HT content.

The lack of correlation between the vanillin and acid butanol assays for CT can be explained by the different mechanisms involved in the reactions. The acid butanol assay utilizes hydrolysis to break the interflavanoid bond, creating a colored anthocyanidin chromophore. In contrast, the vanillin assay reacts with the A ring to form a colored complex. While the acid butanol reaction does not measure terminal units and, thus, reacts less with shorter chained tannins, the vanillin is attenuated by steric hindrance as the chain length increases (Waterman and Mole, 1987a). Although both reactions involve formation of colored products that include the procyanidin units themselves, the adduct formed in the vanillin assay does not appear to be affected by the B-ring hydroxylation pattern, while the carbocation formed in the acid butanol assay does. In addition, the stereochemistry at C-2–C-3 appears to affect the acid butanol reaction but not the vanillin assay.

Results from the Folin assay were not significantly correlated with the vanillin assay, when either all species or only those with pure CT were considered. This is not surprising since reactivity in the Folin assay is correlated to B-ring hydroxylation and C-2–C-3 stereochemistry whereas the vanillin assay is not.

Protein Precipitation Assay. For the BSA protein, both single and multiple factor regression analysis indicated that CT% affects protein precipitation capacity. For the GEL protein, CT% was correlated for the single factor regression analysis, while the multiple factor regression analysis indicated that the interaction between CT and PC was the prime factor determining astringency. The positive correlation between CT% and PPC suggests that CT are more effective at precipitating proteins than HT. Traditionally, the tanning industry assigned a lower astringency to HT relative to CT (Roux et al., 1980). However some research found HT to be more astringent than CT (Bate-Smith, 1977; Dawra et al., 1988) or found no differences in astringency (Porter and Woodruffe, 1984). Our results show that tannic acid, a pure HT, has greater astringency relative to the nine purified tannins tested.

The specificity of tannin–protein interactions is believed to be a function of the size, conformation, and charge of both the tannin and protein molecules (Hagerman and Butler, 1981; Zucker, 1983). Procyanidin molecular weight (i.e., chain length) has been shown to be the predominant factor determining astringency, with B-ring hydroxylation and C-2–C-3 stereochemistry having little or no effect (Porter and Woodruffe, 1984; Kawamoto et al., 1990). This also seems to be the case among the 9 species examined here, although there is some indication that PC units may be more astringent with gelatin. While we did not see a correlation between PPC and average chain length, it may be due to low variability in average polymer size among our samples.

Protein precipitation capacity for both BSA and GEL was correlated ($P < 0.01$) with the acid butanol and vanillin assays for CT, but PPC was not correlated to the Folin assay for total phenols (Table 5). Again, this is largely explained by the

presence of HT in four of the species. Single-factor regressions indicate that the CT% was correlated to the PPC for both BSA and GEL plates. Since HT and CT react similarly in the Folin assay, samples that have HT in them are not expected to correlate between this assay and the protein precipitation assay. However, because both the acid butanol and vanillin assays measure only CT and, thus, show lower reactivity when HT is present, a correlation ($P < 0.05$) between these assays and the P:T ratios was found.

CONCLUSIONS

Our results indicate that the structural characteristics of tannins play an important role in regulating their reactivity. Among the nine species tested, purified tannin reactivity varied 1.5-fold for the Folin total phenol assay, and 7-fold and 3-fold, respectively, for the acid butanol and vanillin condensed tannin assays. Protein precipitation capacity varied about 5-fold. These differences in reactivity and PPC highlight that all tannins do not react similarly, and thereby demonstrate the need to utilize appropriate standards in order to measure tannin concentrations.

Solution ^{13}C NMR helped elucidate structural differences among the nine species studied. Five of the nine plant species examined contained both CT and HT, confirming that the standard method used to extract and purify tannins does not separate these two types of tannins. The possibility of mixtures of CT and HT must be considered when using materials as standards for assays that are specific to one type of tannin (e.g., acid butanol, vanillin).

The hydroxylation pattern of the B ring (PC versus PD units) appears to be an important characteristic influencing tannin reactivity in both the Folin and acid butanol assays. We demonstrated this for the first time and showed that differences in the acid butanol assay do not appear to be driven by a difference in the absorbance maxima of the anthocyanidins (cyanidin versus delphinidin). Contrary to expectations, tannins with a larger proportion of PC versus PD units were more reactive in both the Folin and acid butanol assays.

In the nine species studied, HT appear to have less astringency than CT. Tannic acid appears to be an exception; it has unusually high astringency. In contrast to the chemical assays, the protein precipitation assay was not strongly correlated to B-ring hydroxylation or C-2–C-3 stereochemistry of the CT units for these nine species.

Our results help to explain why previous studies that compared different assays for tannin quantification often obtained conflicting results. If the species being tested contained only CT, the acid butanol and Folin assays should be well correlated, but in samples with variable concentrations of HT, correlations between the two methods should diminish. Conversely, the presence of variable HT amounts among samples can drive the correlation between assays specific for CT, or between

these assays and protein precipitation capacity. It should be noted that additional structural features that were not characterized in this study may confer further discrepancies in reactivity. These include flavonoid linkage positions, the hydroxylation pattern of the A ring, enantiomerism, branching extent, glycosylation, and other substitutions.

Clearly, accurately quantifying tannin concentrations in plant species is challenging. Studies have shown that structural characteristics of foliar tannins can vary significantly among closely related species or even varieties and also may vary by physiological stage, season, and environmental conditions. The diversity in tannin structure clearly influences reactivity in the assays tested in this study. Besides influencing astringency, structural differences likely affect lability of bonds and oxidation capacity. These characteristics undoubtedly affect the role tannins play in ecosystem function.

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EFFECTS OF MOLASSES GRASS, *Melinis minutiflora* VOLATILES ON THE FORAGING BEHAVIOR OF THE CEREAL STEMBORER PARASITOID, *Cotesia sesamiae*

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Abstract—Olfactory responses of the cereal stemborer parasitoid *Cotesia sesamiae* to volatiles emitted by gramineous host and nonhost plants of the stemborers were studied in a Y-tube olfactometer. The host plants were maize (*Zea mays*) and sorghum (*Sorghum bicolor*), while the nonhost plant was molasses grass (*Melinis minutiflora*). In single-choice tests, females of *C. sesamiae* chose volatiles from infested and uninfested host plants and molasses grass over volatiles from the control (soil). In dual-choice tests, the wasp preferred volatiles from infested host plants to those from uninfested host plants. There was no discrimination between molasses grass volatiles and those of uninfested maize, uninfested sorghum, or infested maize. The wasp preferred sorghum volatiles over maize. Combining uninfested maize or sorghum with molasses grass did not make volatiles from the combination more attractive as compared to only uninfested host plants. Infested maize alone was as attractive as when combined with molasses grass. Infested sorghum was preferred over its combination with molasses grass. Local growth conditions of the molasses grasses influenced attractiveness to the parasitoids. Volatiles from Thika molasses grass were attractive, while those from Mbita molasses grass were not. Growing the Thika molasses grass in Mbita rendered it unattractive and *vice versa* with the

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Mbita molasses grass. This is a case of the same genotype expressing different phenotypes due to environmental factors.

Key Words—*Melinis minutiflora*, *Cotesia sesamiae*, foraging behavior, parasitoid, sorghum, maize, molasses grass, stemborers, olfactometer, plant volatiles, intercropping.

INTRODUCTION

Habitat management approaches to pest control attempt to divert insect pests away from a crop and to increase natural enemy activity (Landis et al., 2000). One component of this approach is diversification of the agroecosystem through multiple cropping, intercropping, selective retention of weeds within the crop, or conservation of wild plants at field margins (Price et al., 1980; Verkerk et al., 1998; Letourneau and Altieri, 1999). The conditions within the diversified habitat must match the behavioral and nutritional requirements of the natural enemy for effective biological control (Langer, 1996; Altieri et al., 1997; Ogol et al., 1998; Rutledge and Wiedenmann, 1999). As a result, it is important to have information on the interactions involved at the multitrophic level.

Cotesia sesamiae (Cameron) (Hymenoptera: Braconidae) is one of the common larval parasitoids of lepidopterous cereal stemborers (Noctuidae, Crambidae) in eastern Africa (Mohyuddin and Greathead, 1970; Omwega and Overholt, 1997), but parasitism is typically low. The wasp accounts for only 0.5–3% generational mortality in the spotted stemborer larvae, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae; Oloo, 1989; Overholt et al., 1994; Songa, 1999). Therefore, to exploit the potential of this parasitoid as a natural control agent, habitat management strategies are being developed to increase parasitism in cereals. Khan et al. (1997a,b) discovered that when maize is intercropped with molasses grass, *Melinis minutiflora* Beauv. (Poaceae), a nonhost plant for stemborers, not only is there a significant decrease in stemborer infestation levels in the main crop, but also an increase in stemborer parasitism by *C. sesamiae*. They hypothesized that volatile chemicals produced by the intact molasses grass plant attracted the foraging parasitoids. They particularly singled out the compound (*E*)-4,8-dimethyl-1,3,7-nonatriene and demonstrated its attractiveness to *C. sesamiae*. This compound is known to be released by plants under stress of herbivory and to recruit several species of predators and parasitoids (Dicke, 1994). Molasses grass is a native perennial grass with various beneficial attributes. It is well known as a drought-resistant pasture and hay grass and is also used to thatch houses (Fröman and Persson, 1974; Chiera et al., 1977). Furthermore, it has been shown to be repellent to ticks, tsetse flies, and mosquitoes (Thompson et al., 1978; Ibrahim and Kabuye, 1987; Barros and Evans, 1991; Prates et al., 1993; Mwangi et al., 1995).

The attraction of parasitoids to a molasses grass intercrop presents an opportunity for the exploitation of this plant to enhance the foraging activities of

C. sesamiae. No studies on the foraging behavior of this parasitoid in a diversified system have been reported. Therefore, the aim of the present study was to investigate the response of *C. sesamiae* females to volatile cues from molasses grass and uninfested as well as infested sorghum and maize. This would provide information on whether molasses grass has potential for increasing parasitism of stemborers in cereal-based farming systems if intercropped with the cereals.

METHODS AND MATERIALS

Plants

The host plants sorghum [*Sorghum bicolor* (L), cv. Seredo] and maize (*Zea mays* L. H-511) (Poaceae) were planted in plastic pots (20 cm diam). The nonhost plant molasses grass (*Melinis minutiflora*), derived from two different locations in Kenya, Mbita (longitude 34°10'E and latitude 0°25'S) and Thika (longitude 37°5'E and latitude 1°3'S), were also planted in plastic pots (25 cm/diam). from root splits. The plants were kept at the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, under cages covered with 400-μ mesh netting to protect them from natural insect attack. Sorghum was thinned to two plants and maize to one plant per pot at 7 days after emergence (DAE). This was to obtain approximately similar amounts of biomass in tests that compared the two plant species.

Insects

Busseola fusca Fuller (Lepidoptera: Noctuidae) eggs were obtained from a colony maintained at ICIPE-Nairobi and incubated at 25°C until they hatched. After hatching, larvae were starved for 1 day to eliminate weak individuals and also to stimulate voracious feeding in the remaining larvae, which were used for infestation. Third-instar *B. fusca* were also obtained from the ICIPE colony.

Cotesia sesamiae parasitoids were obtained from a laboratory colony maintained at ICIPE, which had been initiated with individuals collected from *B. fusca* in Kitale, western Kenya. They were reared on the pink stemborer, *Sesamia calamistis* Hampson (Lepidotera: Noctuidae). After parasitization, the stung stemborer larvae were maintained on an artificial diet (Onyango and Ochieng'-Odero, 1994). Parasitoid cocoons were collected in glass vials and kept in clean Perspex cages for emergence. After emergence, adult wasps were provided with a 20% honey–water solution as diet and were allowed to mate during a 24-hr period. Females used in the bioassays were 1–2-days old and naïve with regards to ovipositional experience.

Bioassay

Behavioral responses of *C. sesamiae* females to volatile compounds emitted by different plant sources were determined in a Y-tube olfactometer described by Ngi-Song et al. (1996). Airflow was set at 2.5 liters/min in each arm. Parasitoids were introduced individually into the stem of the Y tube and observed for a period of 5 min. When they crossed the "choice line" (4 cm past the intersection) and remained there for at least 15 sec, it was recorded as a choice. After five parasitoids were tested, the Y tube was turned 180° (interchange of positions of the arms) to eliminate the effect of any bias for one of the arms. Between different tests, the whole Y-tube apparatus was dismantled and washed thoroughly to remove any possible contaminants from the previous assay. The tests were conducted at 25°C ± 2°C, 60–75% relative humidity, and a light intensity of 350–450 lux.

Plant Treatment

The sorghum plants used in experiments 1 and 2 were infested at 21 DAE with three neonate larvae per plant of the African maize stemborer, *B. fusca*, by introducing the larvae into the whorl of the plants. For the other experiments (3 and 4), 5-to 6-week-old sorghum plants were used, while in all experiments where maize was the host plant, 5-to 6-weeks-old plants were used. These were infested with two third-instar *B. fusca* per plant by introducing one larva each into two holes bored into the stem of the plant, 24–36 hr prior to conducting the bioassays.

Plant Volatile Sources

Single-Choice Tests. The response of females of *C. sesamiae* to a plant volatile source versus a pot of soil (control) was observed for the following: (1) uninfested sorghum, (2) infested sorghum, (3) uninfested maize, (4) infested maize, and (5) molasses grass. The molasses grass used in this and subsequent assays (2 and 3) was originally collected from Thika.

Dual-Choice Tests. Experiments were conducted using the following plant volatile sources: (1) uninfested sorghum vs. molasses grass, (2) infested sorghum vs. molasses grass, (3) uninfested sorghum vs. infested sorghum, (4) uninfested maize vs. molasses grass, (5) infested maize vs. molasses grass, and (6) uninfested maize vs. infested maize.

For experiments 1 and 2 with sorghum as host plant, 35–50 parasitoids were tested over 5 consecutive days per wk, with 7–10 wasps per day per treatment. This was done to control for day effects. Bioassays were repeated each week with the same plants from 1 wk after larval infestation, until 5 wk after infestation. The aim of conducting the experiments in this manner was to find out if response to the different plant odors changes with time, assuming that with the

advance in time, the plants will also be growing, developing more biomass, and producing more volatiles. At the same time, larvae in the host plants will also grow and, as they get bigger, inflict more damage, leading to production of more volatiles. All bioassays using maize were only conducted once at 24–36 hr after infestation.

Volatile Combinations. The potted test plants were combined as follows: (1) molasses grass + one uninfested sorghum vs. two uninfested sorghum, (2) molasses grass + one uninfested sorghum vs. two molasses grass, (3) molasses grass + one infested sorghum vs. two molasses grass, (4) molasses grass + one infested sorghum vs. two infested sorghum.

A second series of experiments was conducted using maize as the host plant instead of sorghum. Depending on the insect supply, 10–15 parasitoids were tested per day for each treatment and the trials were conducted over several days for a total of 55–90 parasitoids per treatment.

Host Plants. Response of the parasitoids to volatiles from host plants was investigated. These included: (1) uninfested maize vs. uninfested sorghum, (2) uninfested maize vs. infested sorghum, (3) infested maize vs. uninfested sorghum, and (4) infested maize vs. infested sorghum.

Molasses Grass. Preliminary observations suggested that the attractiveness of molasses grass to *C. sesamiae* varied, depending on the source population of the plants. Thus, experiments were carried out to test the response of the *C. sesamiae* females to odors of molasses grass from two locations, Thika and Mbita. The following plant volatile sources were tested: (1) Thika molasses grass vs. soil, (2) Mbita molasses grass vs. soil, and (3) Thika molasses grass vs. Mbita molasses grass.

Interchanged Molasses Grass Plants. To test whether the environment where molasses grass was grown had an effect on production of volatiles, potted molasses grass plants from Thika were transported to Mbita and maintained there for a period of 7–8 months (called Thika–Mbita molasses grass). They were transported back to ICIPE-Nairobi for use in bioassays. Likewise, potted molasses grass plants from Mbita were transported to ICIPE-Nairobi and tested after 7–8 months (called Mbita–Thika molasses grass). Responses of *C. sesamiae* to volatiles from these interchanged plants were investigated in a Y-tube olfactometer. The following plant volatile sources were tested: (1) Thika-Mbita molasses grass vs. soil, (2) Mbita–Thika molasses grass vs. soil, and (3) Thika-Mbita molasses grass vs. Mbita–Thika molasses grass.

Data Analysis

To examine whether there was consistency in response by the parasitoid to odors produced by sorghum and molasses grass plants in experiments 1 and 2 over the experimental time period, logistic regression analysis was performed (GenStat

5.0 for Windows). The other data were analyzed using the log likelihood ratio test (G test) for goodness of fit with William's correction (Sokal and Rohlf, 1995), to test for significant preferences for an odor.

The logistic regression analysis revealed that responses of the parasitoid to odors from sorghum, infested sorghum, and molasses grass were consistent over time ($P > 0.05$). The data were, therefore, pooled, and the G test was performed to determine significant preferences for an odor.

RESULTS

Single-Choice Tests. Naïve *C. sesamiae* females consistently exhibited a preference for uninfested and for infested sorghum over the control of a pot of soil (Figure 1a,b). Molasses grass was attractive to the wasp (Figure 1c). Volatiles

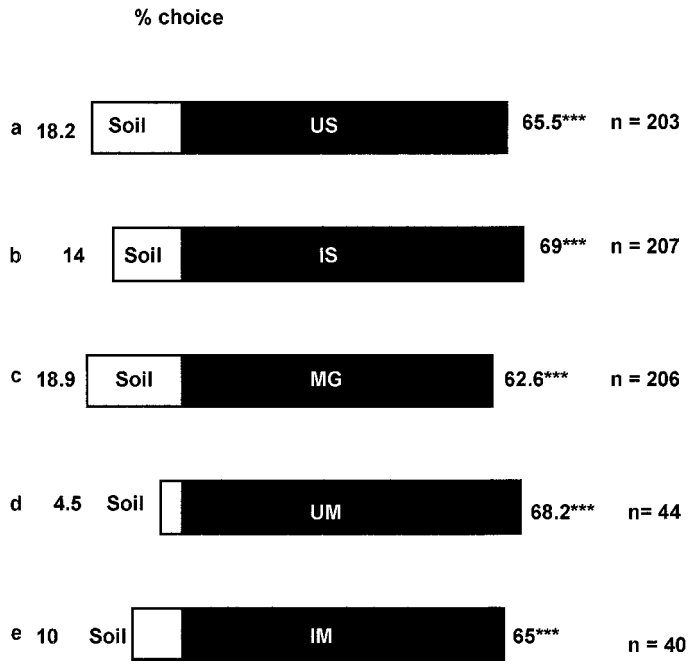

FIG. 1. Response of naïve *Cotesia sesamiae* in Y-tube olfactometer single choice tests to volatiles from uninfested sorghum (US), infested sorghum (IS), uninfested maize (UM), infested maize (IM), and molasses grass (MG) versus control (soil). N = number of parasitoids tested. Numbers next to bars indicate percentages of parasitoids making a choice for one of the presented volatile sources. Asterisks indicate significant differences within a choice test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, G test for goodness of fit. Number of wasps not responding was not included in the analysis.

from both uninfested and infested maize were also preferred over the control (Figure 1d,e).

Dual-Choice Tests. C. sesamiae did not discriminate between volatiles emitted by either uninfested sorghum plants or molasses grass (Figure 2a). However, parasitoids showed a preference for volatiles from infested sorghum over those from molasses grass (Figure 2b). Infested sorghum also attracted parasitoids more than uninfested sorghum (Figure 2c). Wasps did not discriminate between volatiles from uninfested maize and molasses grass or infested maize and molasses grass (Figure 2d,e). Volatiles from infested maize were more attractive than those from uninfested maize (Figure 2f).

Plant Volatile Combinations. No significant difference was found between the response of *C. sesamiae* to uninfested sorghum versus a combination of molasses grass + uninfested sorghum (Figure 3a). The same observation was made for this combination against molasses grass plants (Figure 3b). Wasps showed a clear preference for infested sorghum over the molasses grass + infested sorghum

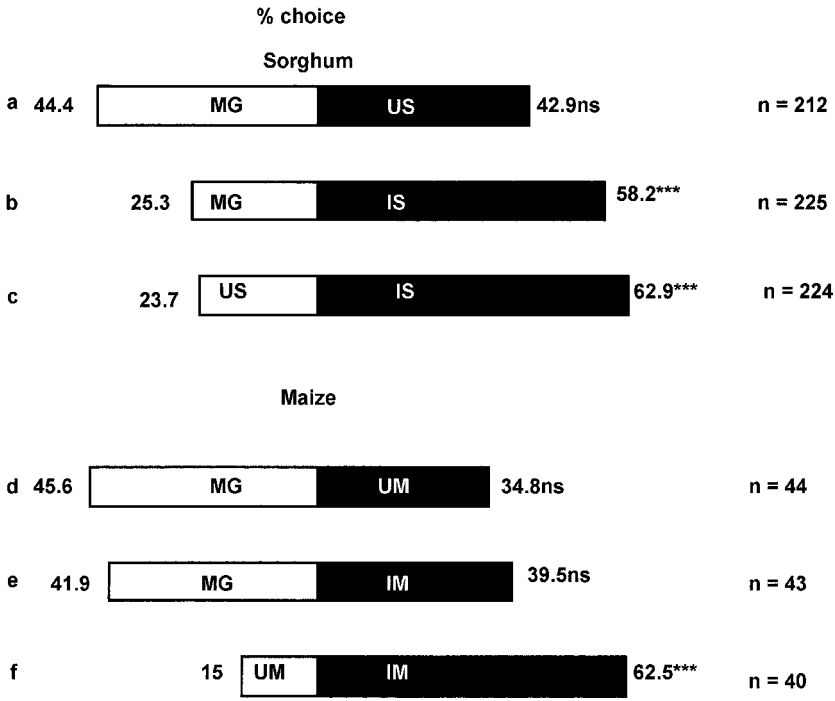

FIG. 2. Response of naïve *Cotesia sesamiae* in Y-tube olfactometer dual choice tests to volatiles from uninfested sorghum (US), infested sorghum (US), uninfested maize (UM), infested maize (IM) and molasses grass (MG). For further details see legend of Figure 1.

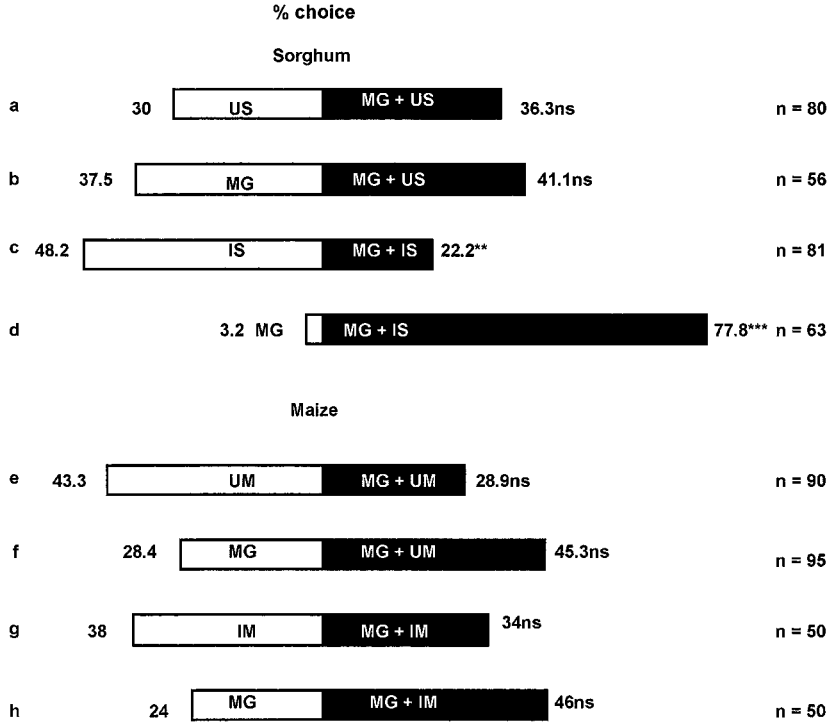

FIG. 3. Response of naïve *Cotesia sesamiae* in a Y-tube olfactometer to combinations of volatile sources from uninfested sorghum (US), infested sorghum (IS), uninfested maize (UM), infested maize (IM), and molasses grass (MG). For further details see legend of Figure 1.

combination (Figure 3c). When the same combination was presented with molasses grass, the parasitoid preferred the combination (Figure 3d). In bioassays where uninfested maize or molasses grass were presented versus a combination of molasses grass + uninfested maize, no clear preference was shown for either of the odor sources (Figure 3e,f). The wasp did not discriminate between infested maize or molasses grass when offered versus a combination of molasses grass + infested maize (Figure 3g,h).

Host Plants. When offered uninfested sorghum and uninfested maize, wasps preferred sorghum (Figure 4a). Infested plants were preferred when presented with uninfested plants (Figure 4b,c). Volatiles from infested sorghum were more attractive than those from infested maize (Figure 4d).

Molasses Grass. Wasps exhibited a preference for the Thika molasses grass over the control (pot of soil) (Figure 5a). However, when offered a choice between

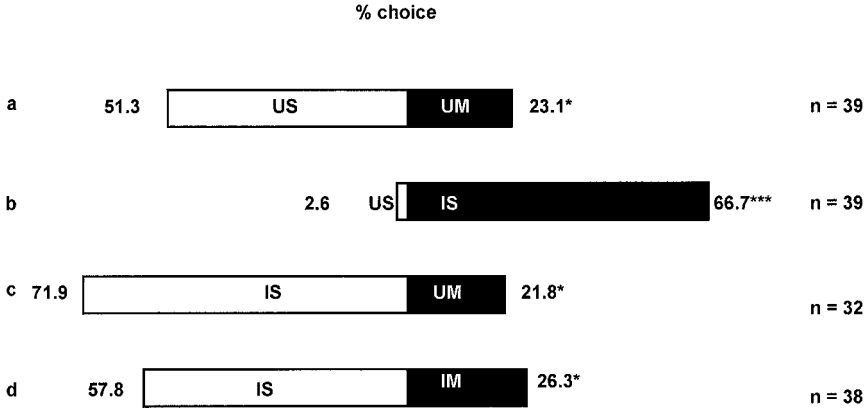

FIG. 4. Response of naïve *Cotesia sesamiae* in a Y-tube olfactometer to volatiles from stemborer host plants, uninfested sorghum (US), infested sorghum (IS), uninfested maize (UM) and infested maize (IM). For further details see legend of Figure 1.

Mbita molasses grass and soil, they preferred the soil volatiles (Figure 5b). When *C. sesamiae* individuals were offered a choice between the two populations of molasses grass, they showed a clear preference for the Thika population (Figure 5c), which was used in all the previous experiments.

The attractiveness of Thika molasses grass disappeared when these plants were grown in Mbita. Volatiles from Thika–Mbita molasses grass plants were less attractive than soil volatiles (Figure 6a). When the nonattractive Mbita molasses grass was grown in Nairobi, it became attractive. The Mbita–Thika molasses grass was preferred over both the control and the Thika-Mbita molasses grass (Figure 6b,c).

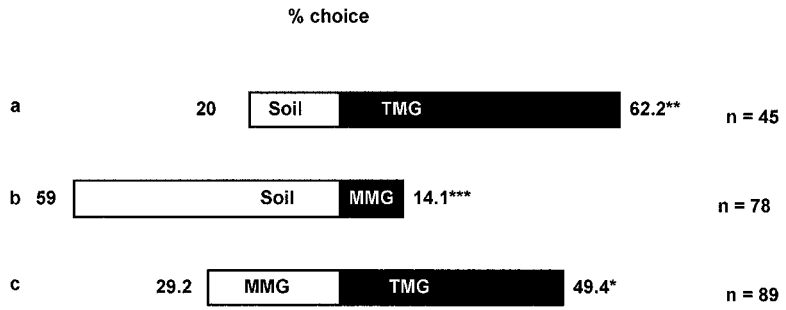

FIG. 5. Response of naïve *Cotesia sesamiae* in a Y-tube olfactometer to volatiles from molasses grass plants from two different locations in Kenya namely Thika molasses grass (TMG) and Mbita molasses grass (MMG). For further details see legend of Figure 1.

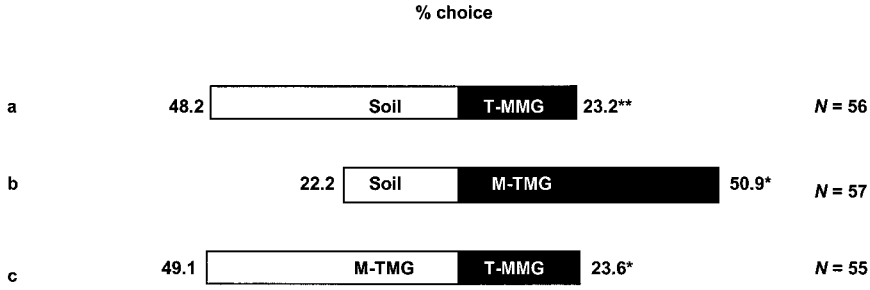

FIG. 6. Response of naïve *Cotesia sesamiae* in a Y-tube olfactometer to volatiles from Thika and Mbita molasses grass plants grown in interchanged locations, Thika molasses grass grown in Mbita (T-MMG) and Mbita molasses grass grown in Nairobi (M-TMG). For further details see legend of Figure 1.

DISCUSSION

Stemborer parasitoids are attracted to volatiles emanating from gramineous plants, which are the main host plants for their herbivore hosts (Potting et al., 1995; Ngi-Song et al., 1996; Smith and Wiedenmann, 1997; Rutledge and Wiedenmann, 1999). The same observation was made in the present study, as *C. sesamiae* was attracted to volatiles from the host plants sorghum and maize. Our results also confirm the attractiveness of some collections of molasses grass to *C. sesamiae*. Findings by Takabayashi et al. (1991) showed that the parasitoid *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae) not only responded to volatiles from uninfested host plants, but also to volatiles from nonhost food plant species. They attributed this attraction to a volatile compound (*Z*)-3-hexenol, which is commonly produced by many green plants. Khan et al. (1997a) observed attraction of *C. sesamiae* to the nonhost plant, molasses grass. The grass produced (*E*)-4,8-dimethyl-1,3,7-nonatriene, which they demonstrated was attractive to the parasitoid.

We confirmed that volatiles released by herbivore-infested maize and sorghum plants are potent attractors of parasitoids when compared to uninfested plants. This is in agreement with works by Potting et al. (1995), Ngi-Song et al. (1996), and Rutledge and Wiedenmann (1999). These chemicals, termed herbivore-induced synomones (Dicke and Sabelis, 1988), are reliable indicators of herbivore presence. In fact, studies have shown that some wasps are more sensitive to these chemicals than they are to those from hosts and host by-products (Steinberg et al., 1993; Potting et al., 1995; Du et al., 1996).

It was expected that with the advance in time, the plants would gain more biomass, thus producing more volatiles that would be more attractive to the parasitoids. At the same time, larvae in the infested plants would grow, causing more damage, thus stimulating the plants to produce greater quantities of

herbivore-induced volatiles. On the contrary, responses of the wasps to the different plant odors were consistent over time, whether the plants were young or old, or infested by young or older larvae. While investigating attraction of *C. flavipes* and *C. sesamiae* to maize infested with different stemborer species at the same density, Ngi-Song et al. (1996) found that the plants infested by larger stemborers were more attractive. They hypothesized that the attractiveness could be attributed to the larger stemborers feeding more voraciously, inflicting more injury to the plant, thus increasing quantities of volatiles produced. However, to appropriately compare the attractiveness of plants at different ages or those infested by herbivores of different ages, dual-choice tests of the same would be required.

Based on the assumption by Khan et al. (1997a)—that increased parasitism of stemborers by *C. sesamiae* in maize-molasses grass intercrops was due to attractive volatiles produced by the molasses grass—we expected the combinations of molasses grass and uninfested host plants maize and sorghum to attract more parasitoids than the sole uninfested host plants, but the field and present laboratory results do not agree. It should also be noted that there was no difference in attraction to the host plants and molasses grass in dual choice tests, except for infested sorghum (Figure 2). It seems there is no interaction or synergistic effect between the host plant and molasses grass volatiles in the combinations. The presence of molasses grass had a neutral effect on uninfested host plant volatiles for attraction of the parasitoid. A possible explanation for the contradiction between the present laboratory results and reported field results with maize as the host plant could be that molasses grass may affect long-range host finding, which would be an important factor in the field but not in the small cages used in the laboratory experiments. Additionally, Khan et al. (1997a) reported that stemborers were repelled by molasses grass. The higher parasitism found in the maize–molasses grass intercrop may have been due to a lower stemborer density rather than recruitment of parasitoids into the field. Infested sorghum alone was preferred to the molasses grass + infested sorghum combination. Although the combination was more attractive than the molasses grass alone. Infested sorghum must be producing some volatile compounds that are strong signals to the parasitoids and can be picked out easily despite the presence of the molasses grass. It should be noted that studies on the effects of intercropped plants on foraging behavior of parasitoids by testing of such plants in combinations in laboratory experiments are uncommon. This study, to our knowledge, is the first attempt on this aspect.

When offered infested sorghum versus infested maize, *C. sesamiae* showed preference for infested sorghum. While investigating the response of stemborer parasitoids to volatiles of some grasses, Ngi-Song et al. (1996) and Rutledge and Wiedenmann (1999) reported preference of *C. sesamiae* for sorghum over maize volatiles. Both sorghum and *C. sesamiae* are indigenous to Africa (Dogget, 1988; Achterberg and Walker, 1998), while maize is a relatively recent introduction (Acland, 1971). The parasitoid might have a genetic adaptation to search

in sorghum with which it shares a longer evolutionary history, thus the reported observations.

Molasses grass from Thika consistently attracted the wasp while plants from Mbita did not. Mbita is located in a hot, moist, mid-altitude region, while Thika is in a cooler and drier mid-altitude zone. Climatic differences in the two geographical zones had an effect on the growth conditions of the molasses grasses, which influenced the type of volatiles produced, leading to the differences in response to these grasses. This observation was confirmed by the bioassay on the interchanged molasses grass plants whereby the molasses grass from Thika, when grown in Mbita, was no longer attractive, while the one from Mbita grown in Nairobi was now attractive. This finding demonstrates how the environment can influence a plant genotype causing it to express different phenotypes. This information is important because it could form a basis on which to partially answer why some biological agents are successful in some regions and completely fail in others. Several other studies have also reported chemical variations in volatiles brought about by the geographic origin of the plant (Adams, 1994; Rafii et al., 1996; Halitschke et al., 2000), although these differences were not confirmed by use of bioassays as described in our study, but by chemical analytical means. Chemical analyses are needed to confirm whether there is a qualitative or quantitative difference in the odor bouquet of the two grass populations.

The study established that *C. sesamiae* was attracted to volatiles from its uninfested and infested host plants and the nonhost plant, Thika molasses grass, but not to those from Mbita molasses grass. Based on the results obtained, a rank order of the attractiveness of plants tested can be determined as follows: soil = Mbita molasses grass < uninfested maize < uninfested sorghum < infested maize < infested sorghum. Thika molasses grass seems to be at the same level of attractiveness as uninfested maize, uninfested sorghum, and infested maize, but less attractive than infested sorghum.

In conclusion, data from the Y-tube experiments are not sufficient to explain parasitoid behavior in field situations. The most plausible way to determine this is to either carry out semifield or field experiments. However, the experiments conducted in this study can be used as preliminary indicators of whether certain plants can be used in combination to enhance foraging of parasitoids. In this case, it is not recommended to use sorghum with molasses grass because infested sorghum by itself is more attractive than when combined with molasses grass. Additionally, the environment may influence the attractiveness of plants, as shown by the variable response of the parasitoids to molasses grass from two locations. Therefore, the approach can not be generalized for all crops or cropping areas. Trials should be carried out first in prospective crops or zones to validate the approach. Finally, for a better understanding of the different behavioral responses reported, comparative headspace analyses of volatiles of both the host and nonhost plants used are necessary and are in progress.

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EFFECT OF UREA FERTILIZER APPLICATION ON SOLUBLE PROTEIN AND FREE AMINO ACID CONTENT OF COTTON PETIOLES IN RELATION TO SILVERLEAF WHITEFLY (*Bemisia argentifolii*) POPULATIONS

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Abstract—The impact of urea nitrogen fertilization on silverleaf whitefly, *Bemisia argentifolii* Bellows & Perring, population dynamics was examined in field-grown cotton (*Gossypium hirsutum* L.). Five urea nitrogen treatments were tested, consisting of soil applications of 0, 112, 168, and 224 kg nitrogen per hectare, and a combined soil–foliar application of 112:17 kg nitrogen per hectare. A positive response was observed between N application rates and the measured levels of nitrate N in petioles from mature cotton leaves. Similarly, a positive response was observed between N application rates and the numbers of adult and immature whiteflies appearing during population peaks. To determine whether these positive responses were related, we measured the levels of dietary N compounds (proteins and free amino acids) that would be available for insect nutrition in cotton petioles at the different N application rates. Sampling dates and N application treatments affected levels of soluble proteins in cotton petioles, and interactions between sampling dates and treatments were significant. Across all sampling dates, the relationship between N application rates and levels of soluble proteins was linear. Sampling dates also affected levels of total and individual free amino acids. Fertilizer treatments only affected levels of total amino acids, aspartate, asparagine, and arginine plus threonine. Levels of aspartate or asparagine and the N application rates were linearly correlated. No significant correlations were observed between levels of dietary N compounds in cotton petioles and numbers of whiteflies, either adults or immatures, on the cotton plants.

Key Words—*Bemisia argentifolii*, whitefly densities, *Gossypium hirsutum*, soluble proteins, free amino acids, cotton–whitefly interactions.

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INTRODUCTION

The silverleaf whitefly, *Bemisia argentifolii* Bellows & Perring, is a serious pest of cotton in the southern United States. The insect ingests phloem sap from plants causing severe reductions in lint yield (Gerling et al., 1980; Bellows and Arakawa, 1988; Henneberry et al., 1995). In addition, copious amounts of honeydew are produced that may contaminate open bolls of cotton, resulting in decreased commercial quality of lint (Perkins, 1986; Henneberry et al., 1996). The honeydew deposited on leaves serves as a substrate for the growth of sooty molds that reduce light available at the leaf surface for photosynthesis (Yee et al., 1996).

Dietary nitrogen is regarded as a key nutritional factor for growth, reproduction, and survival of herbivorous insects (Auclair et al., 1957; Dixon, 1970; Weibull, 1987; Sandstrom and Pettersson, 1994). Nitrogen is a limiting resource for phloem-feeding insects because its concentration in phloem sap is relatively low and, thus insects have to accumulate it against a concentration gradient (Mattson, 1980). Nitrogen is present in phloem sap as free amino acids, and the sap also contains soluble proteins (Ziegler, 1975). It has been hypothesized that plant nitrogen fertilization may increase the availability of these dietary nitrogen compounds for phloem-feeding insects, which may in turn affect the population growth of these insects (Jansson and Smilowitz, 1986; Jauset et al., 1998, 2000; Godfrey et al., 1999). We recently reported a correlation between N application rates on cotton and the population dynamics of silverleaf whitefly (Bi et al., 2000). In the present study, we further examined this correlation, focusing on the effects of different nitrogen fertilization rates on the availability of dietary N forms (soluble proteins and free amino acids) to determine whether these may be responsible for the altered population dynamics observed previously.

METHODS AND MATERIALS

Experimental Plots. Cotton (*Gossypium hirsutum*, cv. Acala) was planted on May 20, 1998, at the Agricultural Experimental Station, University of California, Riverside. Five nitrogen levels were evaluated in a randomized complete block design with 5 replications. Each plot was 16 m long $\times$ 8 m wide with 3 m bare soil buffer areas between adjacent plots in the same block. Each block was separated by 4 rows of bare soil. Row spacing was 1 m, and there were eight rows in each plot. Plants were thinned to 10 cm apart at the 4 node stage of development. Nitrogen application treatments consisted of either soil applications of 0, 112, 168, and 224 kg of urea N per hectare, or a combined soil–foliar application of 112:17 kg of urea N per hectare, which corresponded to suboptimal, optimal, and supraoptimal nitrogen fertility for cotton production in the area. Soil applications of nitrogen were side-dressed with a seed planter when the plants were at the

7-node growth stage (July 6). Foliar nitrogen was applied with a hand sprayer just prior to flowering (August 27). The field was furrow-irrigated every 2 weeks prior to nitrogen fertilization and every week thereafter. The last irrigation date was October 4.

Nitrate Nitrogen Measurement. Nitrate N analysis was performed by the Analytical Laboratory of the Division of Agriculture and Natural Resources of University of California (Davis, California, USA) using a standardized protocol for cotton petioles in commercial use for cotton production (Keisling et al., 1995). A petiole from a fully matured leaf at node 3 (counted from top) was removed from each of 10 individual plants in each plot for nitrate N determinations. Sampling was done on August 14 and September 11 during the growing season. Nitrate N was extracted in 2% acetic acid and quantified by zinc reduction and conductimetric analysis (Johnson and Ulrich, 1959; Carlson, 1978).

Determination of Soluble Proteins and Free Amino Acids. Cotton petioles were used for determination of soluble proteins and free amino acids because petioles consist largely of vascular tissue, and their contents tend to reflect phloem contents (Madore and Webb, 1981). Petioles from the fifth-node main stem leaves, which were the leaves used for whitefly density estimates (Naranjo, 1996), were sampled weekly between 15:00 and 16:00 hr (Pacific Standard Time). One petiole from each of 10 individual plants growing in the middle four rows of each plot was excised, wrapped in aluminum foil, and immediately dropped into liquid nitrogen for transport to a -80°C freezer. Samples were freeze-dried with a Labconco Freeze Dry System and then ground to powder with an intermediate Thomas Wiley cutting mill for assays of soluble proteins and free amino acids.

Protein content was determined by the method of Bradford (1976). Ten milligrams of tissue powder was vigorously mixed on a vortex mixer in 1 ml of 0.1 M ice-cold phosphate buffer, pH 7.0, containing 1% polyvinylpolypyrrolidone. The resulting mixture was centrifuged at 10,000 g at -2°C for 10 min, and the supernatant was immediately used for soluble protein measurements. A 50-μl aliquot of the supernatant was mixed with 150 μl of Bio-Rad protein assay reagent (Bio-Rad, Richmond, California, USA). Absorbance of the reaction mixture was read at 595 nm with a spectrophotometer, and protein content was determined from a standard curve established using known quantities of bovine serum albumin (Sigma Chemical Co.) and the above reagent.

Free amino acids were extracted and quantified according to Yang and Sepulveda (1985) and Mitchell et al. (1992). Ten milligrams of petiole tissue powder was transferred into a microcentrifuge tube and extracted three times, each for 8 min, in 1.2 ml of 80% ethanol in an 80°C water bath, and the ethanol extracts were pooled. Two milliliters of ethanolic extract was dried under vacuum using a Savant SpeedVac vacuum centrifuge (Farmington, Illinois, USA). The dried sample was resuspended in 100 μl of a drying reagent consisting of triethylamine–absolute ethanol–HPLC grade water (1:1:1, v/v), and again dried under vacuum. Amino

acids in the extract were converted to their phenylisothiocyanate (PITC) derivatives by adding 200 μl of PITC reagent (phenylisothiocyanate–absolute ethanol–triethylamine–HPLC grade water 1:7:1:2, v/v) and allowing the derivatization reaction to take place for 10 min at room temperature. Samples were dried to remove excess reagent, resuspended in 4.0 ml of resuspension buffer [15 mM sodium acetate, 3% (v/v) acetonitrile, and 0.025% (v/v) triethylamine, adjusted to pH 7.4 with phosphoric acid], and filtered through 0.2-μm syringe filters. A 20 μl aliquot of the filtrate from each sample was injected onto a Rainin Dynamax ODS column (4.6 $\times$ 25 mm) maintained at 48°C. The column was connected to a Beckman binary gradient chromatography system, and injections onto the column were performed by a Spectraphysics SP8780 autosampler. PITC-amino acids were detected by UV absorption at 256 nm. A standard mixture of known quantities of individual amino acids (Sigma Chemical Co.) was derivatized as above and used for quantitation and identification of individual amino acids. Total levels of free amino acids were derived from the sum of all the detectable individual amino acid levels.

Determination of Whitefly Numbers. Plants were monitored weekly throughout the growing season for the presence of whitefly adults or immatures. Beginning in mid-July, adults were collected from the entire third or forth row in each plot by passing an engine-powered vacuum (Allen-Vac) (Osborne and Allen, 1999) over the plants at a consistent walking speed. In early September, when population build-up was noted, immature whitefly populations were estimated by picking the leaf from the fifth main stem node (counted from top) and counting the number of nymphs on the underside of each leaf with the aid of a microscope (Naranjo, 1996). This was done for 20 randomly chosen plants across the middle four rows within each of the 25 plots.

Statistical Analyses. Analysis of variance (ANOVA) and the least significant difference (LSD) test in one-way randomized complete block general linear models (GLM) (SAS Institute Inc. 1989) were used to analyze nitrate N levels and separate the means. A repeated measures ANOVA was used to determine soluble proteins and free amino acid levels in cotton petioles as affected by the N application rates or sampling dates, and their interactions. A regression analysis was performed for soluble proteins and free amino acids across sampling dates as affected by the N application rates using RSREG (SAS Institute Inc. 1989) to establish a line of best fit (linear, quadratic, etc.) between soluble proteins or free amino acids and N application rates. To determine the relationship between soluble proteins or free amino acids and numbers of adult or immature whiteflies, simple linear regression analyses (SAS Institute Inc. 1989) were used. Data were averaged over the replications across the blocks for the regression analyses. Numbers of whitefly adults from vacuum samples were transformed using the formula $(y + 0.5)^{1/2}$ and numbers of immature whiteflies were log $(y + 1)$-transformed before ANOVA and regression in order to normalize the data (Little and Hills, 1978).

TABLE 1. EFFECT OF NITROGEN TREATMENTS ON NO_3-N LEVELS IN COTTON PETIOLES[a]

Nitrogen treatment (ka/ha)	NO_3-N levels (ppm)	
	August 14	September 11
0	432 ± 58 c	26 ± 10 b
112	992 ± 115 b	58 ± 26 b
112 plus foliar urea	1072 ± 130 b	38 ± 8 b
168	1458 ± 187 b	70 ± 23 ab
224	2048 ± 247 a	146 ± 48 a

[a] Means in columns followed by different letter are significantly different at $P < 0.05$.

RESULTS

Nitrate Nitrogen in Cotton Petioles. Nitrogen fertilization generally increased levels of nitrate in cotton petioles on both sampling dates compared to controls (Table 1). However, the differences were not always statistically significant when compared with untreated controls. On August 14, fertilization rates of 112, 112 plus foliar urea, and 168 kg/ha enhanced the nitrate levels from 2.3- to 3.4-fold, whereas the 224 kg/ha rate boosted the nitrate level by 4.7-fold ($F = 13.8$; $df = 4, 20$; $P < 0.01$). On September 11, the nitrate N level was 5.6-fold higher than the control for the 224 kg/ha application ($F = 3.04$; $df = 4, 20$; $P = 0.04$).

Soluble Proteins and Free Amino Acids. Results of repeated measures ANOVA indicated that both sampling dates and N application rates significantly affected levels of soluble proteins in cotton petioles and that interactions between sampling dates and N treatments were significant (Table 2). Across all sampling dates, regression analysis showed that the relationship between N application rate and soluble protein level was linear ($df = 1, 297$; $R^2 = 0.0254$; $P = 0.01$). Early in the season, especially before peak cotton plant flowering (around the middle of August), N application increased soluble proteins levels ($P < 0.05$). However, later in the season, beginning on September 19, protein levels decreased at all N applications compared to the control (Figure 1).

Significant differences were also seen for total quantities of free amino acids among N application treatments and sampling dates, but interactions between sampling dates and N applications were not significant (Table 2). Sampling dates also affected levels of all individual amino acids measured. In contrast, N fertilization only significantly affected levels of aspartate, asparagine, and arginine/threonine (these two amino acids were not separated by the HPLC protocol used). Interactions between sampling dates and fertilizer treatment were significant only for the amide, asparagine (Table 2). Regression analysis indicated that a linear correlation

TABLE 2. RESULTS OF REPEATED MEASURES ANOVA ON AMOUNTS OF SOLUBLE PROTEINS AND AMINO ACIDS AMONG SAMPLING DATES AND NITROGEN TREATMENTS

Source	Statistics	Proteins	Asp	Glu	Asn	Ser	Gln	Gly	His	ArgThr	Ala	Pro	Total
Date	*F*	24.63	22.00	90.98	50.55	18.41	17.62	2.28	22.46	29.23	5.75	21.55	76.21
	P	<0.01	<0.001	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
N	*F*	7.06	3.19	1.88	5.88	1.58	2.29	0.18	0.85	2.72	1.21	2.11	2.66
	P	<0.01	0.01	0.114	<0.01	0.18	0.06	0.95	0.49	0.03	0.31	0.08	0.03
Date × N	*F*	5.84	1.00	0.88	0.81	0.77	1.45	0.89	0.72	0.91	1.16	0.98	0.50
	P	<0.01	0.48	0.691	0.81	0.86	0.04	0.67	0.915	0.637	0.236	0.513	0.99

df: date – 11, *N* – 4, error – 236.

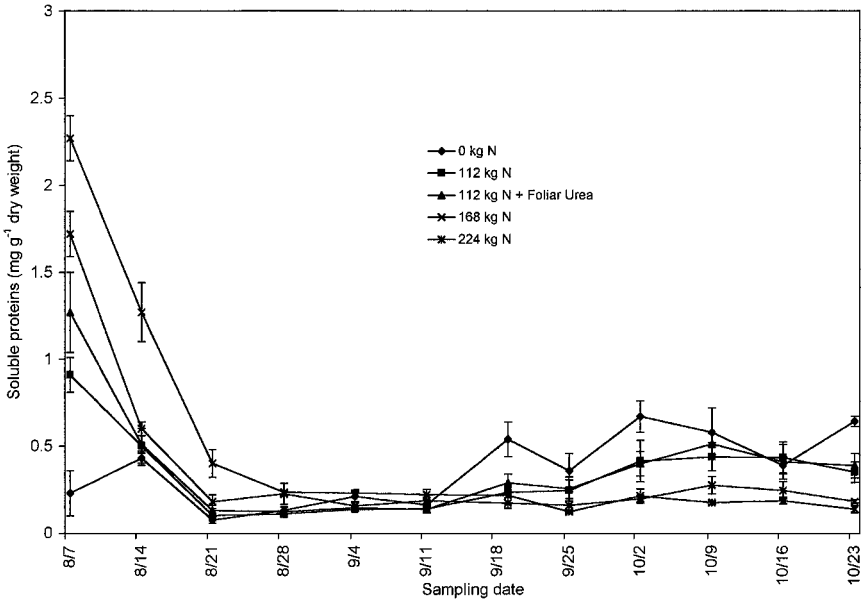

FIG. 1. Effect of nitrogen fertilizer treatments on levels of soluble proteins and in cotton petioles. Points with error bars represent means ± SE.

existed between N application rates and levels of aspartate ($df = 1, 322$; $R^2 = 0.016$; $P < 0.05$) or asparagine ($df = 1, 322$; $R^2 = 0.024$; $P < 0.05$).

Peak levels of total free amino acids in petioles occurred late in the growing season (October 23), (Figure 2). Before September 26, N application increased the level of asparagines (Figure 3), glutamine (Figure 4), and possibly arginine (this also includes threonine, Figure 5), although the results were variable on most sampling dates. Thereafter, no consistent pattern was observed, and no consistent pattern was ever observed, for any of the other measured amino acids that would be expected to be phloem-mobile, including aspartate, alanine, glycine, serine, proline, and glutamic acid.

Relationship between Whitefly Numbers and Nitrogen Parameters. Tables 3 and 4 show the relationships between numbers of adult or immature whiteflies and levels of soluble proteins, and individual or total amino acids. Correlations between numbers of adult or immature whiteflies and levels of soluble proteins were not significant ($P > 0.05$) on any sampling date except October 16 for adults ($P < 0.05$) and September 19 for immatures. Numbers of adult whiteflies were correlated ($P < 0.05$) with levels of aspartic acid, glutamine, glycine, arginine plus threonine, proline, or total amino acids on two, one, one, two, two, and two of the nine sampling dates, respectively. The numbers of adult whiteflies were not

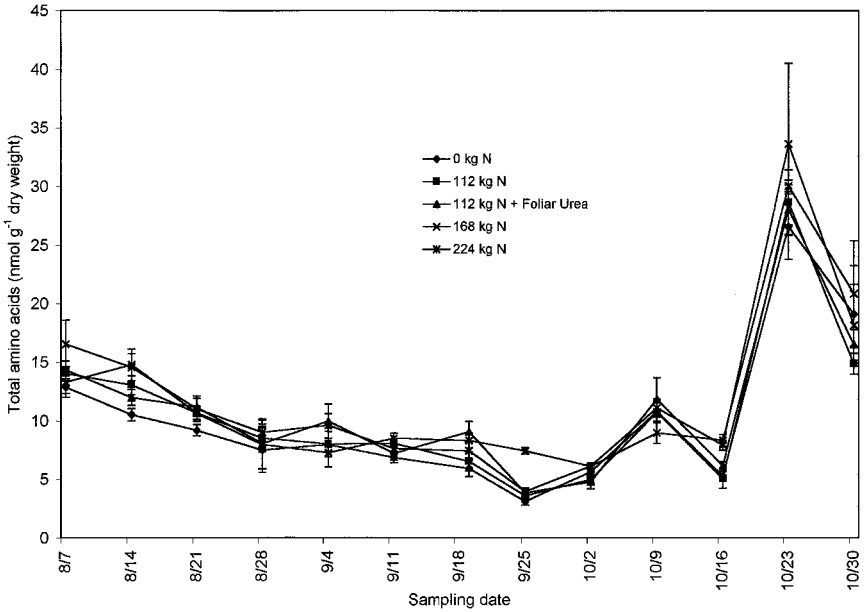

FIG. 2. Effect of nitrogen fertilizer treatments on levels of total amino acids in cotton petioles. Points with error bars represent means ± SE.

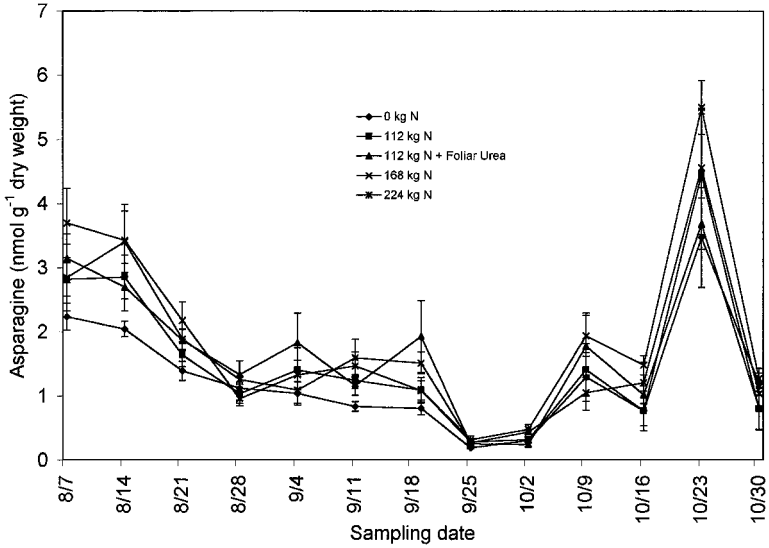

FIG. 3. Effect of nitrogen fertilizer treatments on levels of asparagine in cotton petioles. Points with error bars represent means ± SE.

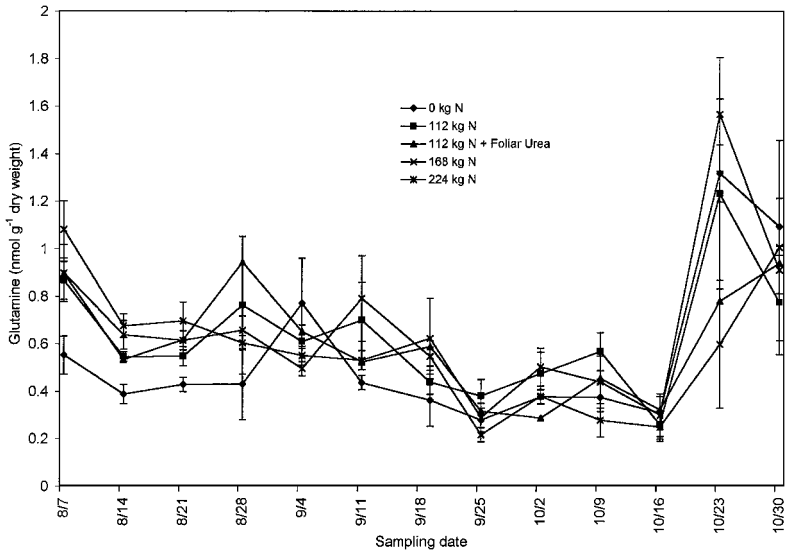

FIG. 4. Effect of nitrogen fertilizer treatments on levels of glutamine in cotton petioles. Points with error bars represent means ± SE.

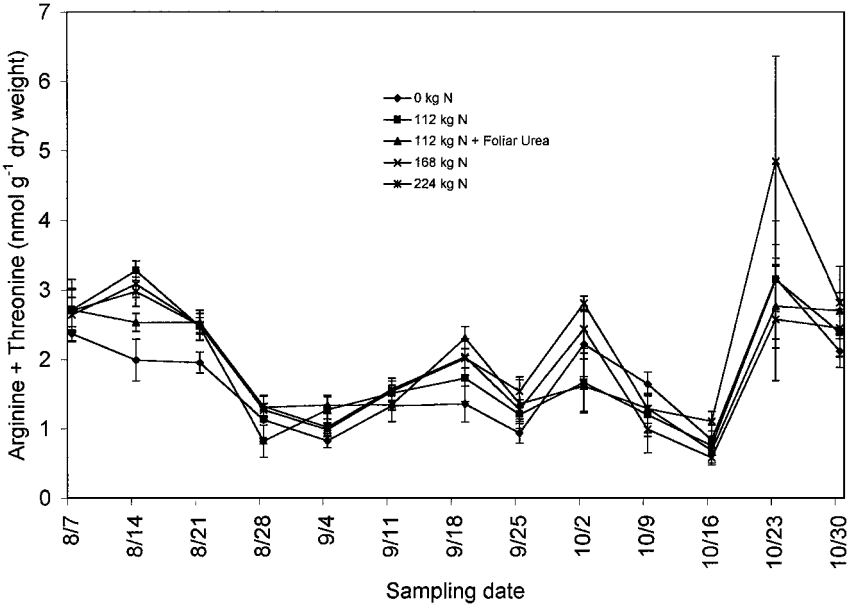

FIG. 5. Effect of nitrogen fertilizer treatments on levels of arginine plus threonine in cotton petioles. Points with error bars represent means ± SE.

TABLE 3. RESULTS OF REGRESSION ANALYSES BETWEEN NUMBERS[a] OF ADULT WHITEFLIES AND LEVELS OF SOLUBLE PROTEINS OR FREE AMINO ACIDS IN COTTON PETIOLES

Date	Regression	Proteins	Asp	Glu	Asn	Ser	Gln	Gly	His	ArgThr	Ala	Pro	Total
Sep 4	*R*	0.059	0.063	0.170	0.122	0.032	0.089	0.110	0.000	0.118	0.192	0.170	0.148
	P	0.585	0.774	0.418	0.549	0.879	0.676	0.616	0.983	0.576	0.375	0.409	0.483
Sep 11	*R*	0.107	0.032	0.032	0.192	0.148	0.210	0.259	0.032	0.228	0.032	0.071	0.447
	P	0.612	0.968	0.883	0.351	0.458	0.327	0.213	0.965	0.283	0.867	0.733	0.512
Sep 19	*R*	0.318	0.237	0.126	0.412	0.292	0.272	0.251	0.339	0.404	0.381	0.407	0.436
	P	0.122	0.242	0.539	0.040	0.154	0.196	0.203	0.099	0.046	0.057	0.040	0.034
Sep 25	*R*	0.066	0.100	0.055	0.321	0.187	0.126	0.402	0.000	0.134	ND[b]	0.104	0.055
	P	0.753	0.637	0.810	0.126	0.378	0.550	0.050	0.983	0.523	ND	0.616	0.808
Oct 2	*R*	0.147	0.247	0.371	0.646	0.197	0.421	0.173	0.253	0.451	0.313	0.412	0.615
	P	0.485	0.243	0.074	0.002	0.288	0.028	0.419	0.176	0.026	0.137	0.044	0.001
Oct 9	*R*	0.276	0.032	0.274	0.345	0.055	0.032	0.0167	0.104	0.161	0.184	0.089	0.257
	P	0.182	0.914	0.175	0.099	0.797	0.960	0.432	0.632	0.439	0.384	0.667	0.220
Oct 16	*R*	0.425	0.322	0.221	0.190	0.032	0.202	0.045	0.145	0.319	0.095	0.155	0.095
	P	0.034	0.124	0.244	0.375	0.926	0.328	0.830	0.486	0.126	0.665	0.472	0.659
Oct 23	*R*	0.333	0.197	0.138	0.241	0.270	0.145	0.045	0.095	0.104	0.224	0.110	0.055
	P	0.104	0.355	0.489	0.250	0.163	0.499	0.845	0.630	0.622	0.292	0.605	0.808
Oct 30	*R*	ND	0.118	0.255	0.114	0.077	0.141	0.071	0.100	0.095	0.152	0.286	0.000
	P	ND	0.578	0.231	0.573	0.712	0.501	0.724	0.641	0.645	0.476	0.175	0.990

[a] Densities of whitefly adults were transformed using $(y + 0.5)^{1/2}$.
[b] ND = amino acid not detectable.

correlated with levels of free asparagine, glutamic acid, histidine, or alanine in petioles on any of the sampling dates. Likewise, numbers of immature whiteflies were correlated ($P < 0.05$) with levels of aspartic acid, asparagine, glutamic acid, arginine plus threonine, alanine, proline or total amino acids on two, one, two, two, two, and one of the nine sampling dates, respectively, but were not correlated with any of the other amino acids detected in this study.

DISCUSSION

Nitrogen fertilizer strongly increased levels of petiole nitrate N, an index of nitrogen status in cotton (Table 1). The results agree with reports of numerous other investigations (e.g., Keisling et al., 1995; Weir et al., 1996). Nitrogen applications also enhanced numbers of whiteflies as reported in our previous work (Bi et al., 2000). Nitrogen fertilization also enhanced soluble protein levels in cotton petioles early in the season, but not during the late season (Figure 1). The late season decrease may be caused by protein breakdown to amino acids, as there was an increase in the levels of free amino acids during the late season (Figure 2). However, there was no correlation between petiole protein levels and numbers of either adult or immature whiteflies, in agreement with previous findings (Blua et al., 1994) that indicated that phloem protein levels in *Cucurbita pepo* plants were not positively correlated with *Aphis gossypii* (Glover) population development. Thus, although phloem-feeding whiteflies can readily ingest and assimilate cotton proteins (Salvucci et al., 1998), the levels of these soluble proteins in the plant may not have a major influence on whitefly population dynamics.

There was also no correlation between amino acid content of cotton petioles and whitefly populations, again in agreement with earlier findings (Blua et al., 1994) for *A. gossypii* and the phloem amino acids from *C. pepo*. Similarly, Sandstrom and Petterson (1994) demonstrated that the total concentration of amino acids in the phloem sap of five genotypes of pea (*Pisum sativum* L.) could not explain the differences in performance of the pea aphid (*Acyrthosiphon pisum*) feeding on them. The major amino acid concentration peak occurred during late season, corresponding with the decrease in soluble proteins. Only 10 amino acids were detected in cotton petioles in this study; the predominant ones were glutamate, asparagine, and proline. Thus, the amino acid composition of plant phloem saps, including cotton, would appear to be imbalanced with respect to the dietary requirements of phloem-feeding insects such as aphids and whiteflies. Aphids are known to use endosymbiotic bacteria to synthesize essential amino acids from phloem-derived glutamate (Sasaki and Ishikawa, 1995). Whiteflies are also known to have endosymbionts (Costa et al., 1995) and it seems likely that these are used for similar amino acid interconversion.

It has been recently suggested that multiple components should be simultaneously considered for explanations of variation in herbivore response to plant

TABLE 4. RESULTS OF REGRESSION ANALYSES BETWEEN NUMBERS[a] OF IMMATURE WHITEFLIES AND LEVELS OF SOLUBLE PROTEINS OR FREE AMINO ACIDS IN COTTON PETIOLES

Date	Regression	Proteins	Asp	Glu	Asn	Ser	Gln	Gly	His	ArgThr	Ala	Pro	Total
Sep 4	*R*	0.122	0.489	0.173	0.469	0.071	0.084	0.261	0.145	0.442	0.447	0.518	0.401
	P	0.854	0.011	0.414	0.016	0.737	0.659	0.212	0.479	0.031	0.029	0.010	0.048
Sep 11	*R*	0.335	0.463	0.197	0.370	0.200	0.105	0.031	0.161	0.235	0.266	0.235	0.340
	P	0.102	0.020	0.349	0.064	0.341	0.612	0.914	0.451	0.260	0.189	0.268	0.087
Sep 19	*R*	0.415	0.283	0.345	0.268	0.145	0.575	0.270	0.032	0.055	0.167	0.190	0.263
	P	0.039	0.159	0.092	0.176	0.502	0.003	0.200	0.838	0.798	0.371	0.377	0.214
Sep 25	*R*	0.045	0.251	0.402	0.095	0.372	0.100	0.126	0.105	0.000	0.000	0.239	0.095
	P	0.830	0.243	0.050	0.667	0.072	0.621	0.524	0.622	0.988	0.000	0.262	0.665
Oct 2	*R*	0.356	0.122	0.268	0.105	0.032	0.063	0.045	0.032	0.438	0.063	0.399	0.394
	P	0.081	0.560	0.205	0.622	0.871	0.778	0.845	0.906	0.032	0.753	0.053	0.056
Oct 9	*R*	0.028	0.122	0.226	0.184	0.089	0.187	0.045	0.045	0.176	0.045	0.045	0.770
	P	0.891	0.571	0.287	0.385	0.669	0.380	0.848	0.826	0.411	0.858	0.029	0.428
Oct 16	*R*	0.055	0.221	0.279	0.173	0.032	0.100	0.055	0.114	0.071	0.155	0.071	0.134
	P	0.795	0.300	0.186	0.415	0.862	0.614	0.797	0.595	0.744	0.462	0.735	0.531
Oct 23	*R*	0.368	0.344	0.192	0.077	0.000	0.045	0.055	0.318	0.084	0.190	0.114	0.243
	P	0.073	0.099	0.362	0.717	0.932	0.859	0.804	0.125	0.695	0.371	0.599	0.252
Oct 30	*R*	ND	ND	0.210	0.148	0.000	0.217	0.162	0.063	0.063	0.105	0.210	0.292
	P	ND	ND	0.322	0.493	0.958	0.299	0.431	0.750	0.758	0.633	0.325	0.166

[a] Densities of immature whiteflies were transformed using log $(y + 1)$.
[b] ND = amino acid not detectable.

nutrients (Busch and Phelan, 1999). Other plant nutrients such as carbohydrates and the carbohydrate–amino acid ratio (Simpson et al., 1995; Blackmer and Byrne, 1999) are known to influence insect development. We previously reported that glucose in cotton petioles had more impact on whitefly population development than petiole sucrose and fructose (Bi et al., 2000). Busch and Phelan (1999) found that nitrogen and phosphorus interactively affected development of the soybean looper, *Pseudoplusia includense*, and that phosphorus had more impact on performance of *P. includense* than nitrogen. We did not measure the phosphorus levels in cotton plants in this study. Other factors influenced by nitrogen fertilization, such as host plant resistance should also be considered. Thomas et al. (1995) reported that protease inhibitors in cotton reduced whitefly fecundity. Plant secondary metabolites such as gossypol are also assumed to be involved in cotton resistance against whiteflies (Butler et al., 1991). It has been reported that nitrogen fertilization decreased total phenolic concentrations in needles of the Scotch pine (*Pinus sylvestris*) (Kainulainen et al, 1996). Any or all of these factors would likely be operating to affect whitefly population dynamics on cotton.

In summary, nitrogen fertilization enhanced both the level of nitrate nitrogen in cotton petioles and the number of whiteflies infesting the plants. However, the underlying physiological mechanisms resulting in the enhancement of whitefly densities did not appear to be directly related to levels of nutritional N in the form of soluble proteins or free amino acids available in the phloem. Some other factor(s) seems to be operating. The economic importance of *Bemisia* in worldwide agricultural systems would certainly justify continuing studies to determine exactly what these factors may be. The strategy of using current crop management practices as a means of development of effective whitefly management programs is likely to prove fruitful and exciting in the future.

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CONSTRAINTS TO HERBIVORE-INDUCED SYSTEMIC RESPONSES: BIDIRECTIONAL SIGNALING ALONG ORTHOSTICHIES IN *Nicotiana attenuata*

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Abstract—We investigated the impact of leaf vascular connections on systemically transmitted herbivore-induced gene expression in *Nicotiana attenuata*. Although systemic signaling is clearly associated with the plant vascular system, few studies consider vascular architecture when measuring systemically induced defenses. *N. attenuata* is a plant with dispersed phyllotaxis approximating 3/8 in the rosette stage of growth. We mimicked *Manduca sexta* herbivory by introducing larval regurgitant to wounds produced with a standardized continuous mechanical wounding and investigated mRNA accumulation of genes. Herbivory in *N. attenuata* induces the expression of genes coding for a proteinase inhibitor protein (PI), threonine deaminase (TD, EC 4.3.1.19), a luminal-binding protein (BiP), and an α-dioxygenase (α-DOX). We measured the systemic response of sink leaves when orthostichous (growing at an angular distance of 0 degrees) source leaves were treated, and *vice versa*, and compared it to the systemic response of leaves growing at the maximum angular distance of 180 degrees. Vascular architecture clearly controlled the intensity of systemic mRNA accumulation within the 4-hr time frame of the experiment. In addition, we found signal translocation to be bidirectional, travelling from source to sink as well as from sink to source leaves, which argues against a phloem-based assimilate-linked signal identity.

Key Words—Herbivore-induced defense signaling, *Nicotiana attenuata*, orthostichies, proteinase inhibitors, α-dioxygenase, luminal-binding protein, threonine deaminase.

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INTRODUCTION

The characterization of the systemic signals mediating induced resistance against insect attack remains a major challenge. Many signals have been suggested: soluble compounds transported in the phloem or xylem, gases dispersed by diffusion in the apoplast, as well as electrical and hydraulic signals transmitted by the vascular system (de Bruxelles and Roberts, 2001). Although systemic signaling has long been associated with plant vasculature, few studies have directly linked the spatial and temporal patterns of defense accumulation to the plant's vascular architecture (Davis et al., 1991; Jones et al., 1993; Shulaev et al., 1995; Rhodes et al., 1999; Orians et al., 2000). Plant phyllotaxis (not distance between leaves along the stem) determines vascular architecture and the intensity of leaf interconnection. In tomato, the intensity of systemic proteinase inhibitor (PI) accumulation was found to correlate with the degree of vascular linkage between and within leaves (Rhodes et al., 1999; Orians et al., 2000). The same was true for salicylic acid transport in *Nicotiana tabacum* plants (Shulaev et al., 1995). Whereas these studies on Solanaceous plants did not include the directly linked orthostichous leaves (growing at an angular distance of 0 degrees) in acro- or basipetal directions along the stem from the treated leaf, results from orthostichous leaves in *Populu*s species seem controversial. In *P. deltoides*, systemic-induced resistance to beetle feeding was induced both acro- and basipetally (Jones et al., 1993), whereas in *P. trichocarpa* × *P. deltoides*, systemic *win* mRNA was induced in sink leaves only after treatment of the orthostichous source leaves and not in the reverse direction (Davis et al., 1991).

We extended these studies to *Nicotiana attenuata* Torr. ex Wats., a plant with many well-characterized systemically induced defenses against its insect herbivores (Baldwin, 2001) and a dispersed phyllotaxis, approximating 3/8 symmetry in the rosette stage of growth. *N. attenuata* recognizes attack from the specialist lepidopteran herbivore, *Manduca sexta*, and tailors its defense responses appropriately (Baldwin, 2001). This recognition occurs when fatty acid–amino acid conjugates from the larvae's saliva are introduced into wounds caused by larval feeding. The plant's responses to attack can be rigorously mimicked by adding minute amounts of *M. sexta* larval regurgitant to mechanically produced puncture wounds (Halitschke et al., 2001). We measured systemic transcript accumulation in leaves orthostichous to those elicited in both acro- and basipetal directions and in a leaf with no direct vascular connection to the systemically elicited leaves. Additionally, the experiment allowed us to investigate the capacity of sink leaves to function as exporters of systemic signals.

We chose four genes known to be inducible by herbivory of *M. sexta* larvae on *N. attenuata* plants (Hermsmeier et al., 2001). One codes for a PI protein, one for threonine deaminase (TD), one for an α-dioxygenase (α-DOX), and one for a luminal-binding protein (BiP). Whereas proteinase inhibitors are known to interfere with herbivore digestive enzymes (Duffey and Stout, 1996), the functional

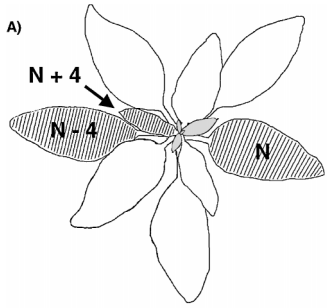
significance of the other three proteins in the context of herbivory has not been clarified. TD catalyzes the committed step in isoleucine biosynthesis by converting threonine to 2-ketobutyrate (Samach et al., 1991; Azevedo et al., 1997). The α-DOX catalyzes α-oxidation of fatty acids to hydroperoxy fatty acids and may be involved in signal generation (Sanz et al., 1998; Hamberg et al., 1999; de Leon et al., 2002). BiPs are chaperones that assist protein folding in the endoplasmic reticulum and have been shown to respond to pathogen stress (Jelitto-Van Dooren et al., 1999).

Our results underscore the impact of vascular connections on systemic defense responses and the bidirectional character of the systemic signal.

METHODS AND MATERIALS

Plant Growth. Plants were cultivated under a 32°C, 16 hr–27°C, 8 hr day–night regime with 65% constant relative humidity and 1000–1500 μmol/m^2/sec from high-pressure Na lights. Seeds from an inbred line (sixth generation) *Nicotiana attenuata* (originating from the DI ranch in southwest Utah, USA, T40S R19W, section 10, 1988) were germinated in smoke-treated soil (Baldwin et al., 1994). Ten-day-old seedlings were transferred into communal 25-liter-hydroponic boxes containing 0.292 g/liter of Peters hydrosol (W.R. Grace, Fogelsville, Pennsylvania, USA) and 0.192 g/liter of $Ca(NO_3)_2$ (Roth, Karlsruhe, Germany). After 6 days, plants were individually placed in 1-liter containers containing no-nitrogen hydroponic solution (Baldwin and Schmelz, 1994). Upon transfer, each plant was supplied with 2 ml of 1 M KNO_3 (Roth), followed by 7 vol of 0.5 ml of 1 M KNO_3 within the next 16 days. The experiment was done on day 17 after transfer, when rosette plants were in the early stage of stem elongation.

Experimental Design. Regurgitant collected from *M. sexta* larvae reared on *N. attenuata* plants was diluted 1:3 (v/v) with autoclaved water and supplied to standardized puncture wounds on the leaf surface. Plants were divided into four groups of 27 replicate plants. Plants from one group were treated on their second fully expanded (source) leaf at node N. Plants from the other groups were treated either on the sink leaf at node N+4, on the orthostichous source leaf at node N−4, or not treated at all (control group) (Figure 1). We used a fabric pattern wheel (Dritz, Spartanburg, South Carolina, USA) to create one row of puncture wounds parallel to the mid-rib, every 30 min, and immediately added 5 μl of regurgitant solution to the wound sites. A total of eight rows, four on each side of the middle rib, were generated per leaf. Leaves at nodes N−4, N, and N+4 were harvested from all four treatment groups; tip leaves (N+5 and younger) were also collected as one bulk (27 plants) sample from each plant. Leaves were frozen in liquid nitrogen 30 min after the last treatment, except for the tip leaves, which were harvested 60 min after the last wounding event (30 min after the other three leaves had been taken from their respective plants).



B)	N-4	N-3	N-2	N-1	N	N+1	N+2	N+3	N+4
C)	0	3/8	6/8	9/8	12/8	15/8	18/8	21/8	24/8
D)	0º	135º	90º	45º	180º	45º	90º	135º	0º

FIG. 1. *Nicotiana attenuata* phyllotaxis. (A) Schematic of a rosette-stage plant used in experiments. Leaves elicited in either one of the treatment groups are hatched. Tip leaves that were additionally harvested (N+5 and younger) are shaded. (B) Leaf positions as designated within this study. (C) Additive angular distances assuming 3/8 phyllotaxy (135-degree angles between leaves). Proceeding from the leaf at node N−4 to the leaf at node N+4 requires 24/8 (=3) complete rotations around the plant axis. The leaf at node N+4 is 8 nodes higher on the plant axis than the leaf at node N−4. (D) Angular distances viewed from orthostichous leaves at nodes N±4.

Molecular Techniques. Total cellular RNA was isolated from leaves (bulked from 27 plants for each treatment group) according to Pawlowski et al. (1994). Gel electrophoresis of RNA, northern blotting, probe labeling, and hybridizations were performed as described in Hermsmeier et al. (2001). GenBank accession numbers of the template sequences are AW191811 (TD, EC 4.3.1.19), AW191821 (α-DOX = PIOX), and AW191822 (BiP). The PI probe coded for a 571-bp fragment of a *N. attenuata* PI gene repeat region (K. Gase, A. Patankar, D. Hui, I. Baldwin, unpublished data).

RESULTS AND DISCUSSION

Transcript accumulation of the four investigated genes demonstrates that wound-induced response patterns are controlled by the vascular architecture of the plant. Studies on *N. tabacum* had revealed 3/8 phyllotaxis for rosette stage plants and 5/13 phyllotaxis for the stem of elongated plants (Allard, 1942; Jones et al., 1959; Shirova et al., 1961). According to the 3/8 phyllotactic growth of rosettes, leaves at nodes N−4 and N+4 in the present study were orthostichous, whereas the leaf at node N grew at a maximum angular distance of 180 degrees to these two leaves. Accumulation of mRNA (Figure 2) reflected these vascular patterns although the systemic responses of PI and TD were much stronger than

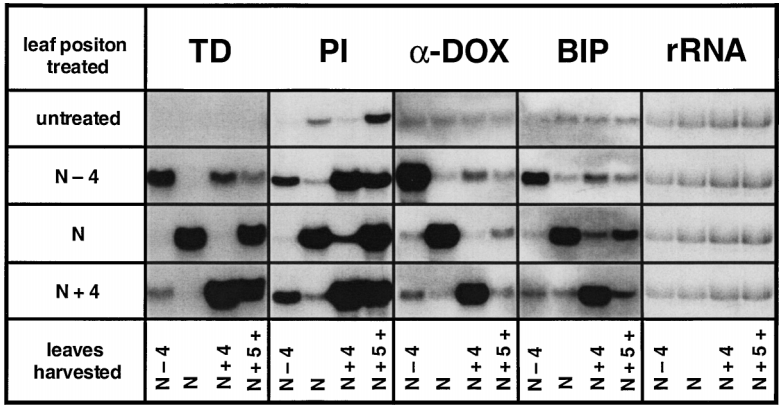


FIG. 2. Transcript accumulation in leaves at nodes N−4, N, N+4, and the tip leaves (at nodes N+5 and younger) with the leaf at node N being the second fully expanded leaf of rosette plants at the beginning of stem elongation. Twenty-seven replicate plants were continuously wounded and supplied with larval regurgitant at the leaf growing at either node N−4, N, or N+4. Only one nodal position was wounded per plant. The wounding treatment produced one row of puncture wounds every 30 min, and leaves were harvested 30 min, (for leaves at nodes N−4, N, and N+4) or 60 min (for the tip leaves) after the final treatment. Leaves from untreated plants were harvested as controls. Replicate leaves were bulked for analysis. One band of rRNA is shown to demonstrate equal loading. TD = threonine deaminase; PI = proteinase inhibitor; α-DOX = α-dioxygenase; BiP = luminal-binding protein.

those of α-DOX and BiP. Within 4 hr of elicitation, increased mRNA levels of all four genes were detected in the N−4 source leaf when it had been elicited (a local response) or when the orthostichous N+4 sink leaf had been elicited (a systemic response). Increased mRNA levels in the leaf at node N occurred only when this leaf had been elicited, not as a result of systemic signaling when leaves at nodes N+4 or N−4 had been elicited. Strong mRNA accumulations in the N+4 leaf were observed when this leaf had been elicited or when the orthostichous source leaf at node N−4 had been elicited. A weak response was also visible after elicitation of the leaf in position N for BiP and PI transcripts. Transcript accumulation in the tip leaves (N+5 and younger) represents the average response of a mixture of leaves at different angular distances of 45–180 degrees to leaves N+4 and N−4 and one leaf orthostichous to leaf N. A strong response to eliciting leaf N reflects these direct vascular connections. For PI and TD, tip leaves also responded strongly when leaves at position N+4 had been treated. This may, in part, be a gene-specific effect due to developmentally increased sensitivity to elicitation and/or a reflection of greater "cross-talk" among orthostichies in young leaves whose vascular connections are not fully matured.

Whereas these results indicate the importance of vascular architecture, the direction of vascular flow does not seem to be as important as suggested by the systemic response in source leaves after elicitation of orthostichous sink leaves. A similar result has been reported only for *P. deltoides* trees, in which induced resistance to beetle feeding was observed in source leaves 24 hr after the acropetally growing orthostichous source–sink transition leaf had been crushed with a file (Jones et al., 1993). In contrast, a basipetally translocated systemic response of orthostichous leaves in *Populu*s hybrids, measuring *win* mRNA accumulation 6–8 hr after macerating leaf lamina with pliers, was found only when the source leaf was shaded for 64 hr prior to the experiment. The shading treatment turned the source leaf into a sink (Davis et al., 1991), and the authors concluded that the direction of assimilate movement controlled systemic gene expression. Assimilate movement in plants is clearly controlled by the stage of leaf and plant development, in addition to vascular architecture, and has been studied in detail in *N. tabacum* by ^{14}C-labeling of assimilates (Jones et al., 1959; Shirova et al., 1961). We did not find that signal transmission is restricted by leaf developmental stage in *N. attenuata* rosettes, but studies considering different plant developmental stages are required.

The observed signal transduction from sink to source leaves leads to the question of the nature of the systemic signal. Electrical signals transmitted by the phloem or through hydraulic pressure pulses in the xylem can translocate in both acro- and basipetal directions in a plant (Stankovic and Davies, 1996). Alternatively, it has been proposed that chemical signals are rapidly distributed by transient xylem flow reversal (Malone et al., 1994; Malone and Alarcon 1995; Rhodes et al., 1999), and the underlying hydraulic events have been shown to be triggered by insect feeding (Alarcon and Malone, 1994). In the present study, the bidirectional nature of the signal argues against a chemical elicitor linked to the assimilate flow in the phloem, and staining experiments with *N. attenuata* indicated that there is no assimilate flow out of N+4 leaves (A. Kessler, personal communication).

Systemic signaling has been examined most intensively with regard to PI protein accumulation in tomato (Bowles, 1998; Ryan, 2000). These studies suggest that certain types of damage may lead to the release of chemical elicitors, like systemin, into the phloem only, restricting the response to sink tissues (Rhodes et al., 1999). Other treatment types induced systemic responses faster than systemin can translocate in the phloem. These responses did not depend on functional phloem tissue (Stratmann and Ryan, 1997), which argues for xylem-based electrical or mass-flow signaling. Accordingly, the type of signal (phloem- or xylem-based or both) elicited by herbivore feeding will determine the spatial pattern and kinetics of systemic responses in addition to the impact of vascular architecture itself. Moreover, within-leaf patterns of defense accumulation are strictly controlled by the vasculature (Rhodes et al., 1999; Orians et al., 2000).

Vascular connections tightly restricted by phyllotaxis have been suggested to be advantageous in the context of pathogen attack, to limit disease spreading to orthostichous leaves, and to conserve energy required for defense accumulation (Murray et al., 1982). Vascular architecture has been shown to control the response of tobacco plants to TMV for a period of several days (Shulaev et al., 1995). Since vascular restrictions cause within-plant and within-leaf resource heterogeneity, they will also affect the performance and behavior of insect herbivores. If, for example, spatial and temporal variation impairs insects' detoxification capacity or increases their foraging activity at the risk of predator attack or reduced growth rates, it will reinforce the defensive character associated with the heterogeneity of induced plant responses (Denno and McClure, 1983; Karban and Baldwin, 1997; Orians and Jones, 2001).

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DISTRIBUTION OF THE CARCINOGENIC TERPENE PTAQUILOSIDE IN BRACKEN FRONDS, RHIZOMES (*Pteridium aquilinum*), AND LITTER IN DENMARK

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Abstract—The distribution of ptaquiloside (PTA) was studied in four Danish bracken populations in order to evaluate the transfer of PTA from ferns to soil. Populations showed statistically significant differences in PTA contents of fronds and rhizomes despite large in-site variations. The highest concentrations were encountered in fronds with concentrations ranging between 213 and 2145 μg/g, while rhizomes had concentrations between 11 and 902 μg/g. PTA was present in soil materials in amounts of 0.22–8.49 μg/g but apparently with no correlation with PTA contents of fronds or rhizomes. Laboratory tests showed that water could leach PTA from bracken fronds, which is in support of the high soil contents observed at sites exposed to heavy showers just before sampling. The observed soil contents correspond to estimated soil solution concentrations of 200–8500 μg/liter, demonstrating a substantial risk of PTA contamination of surface water and groundwater.

Key Words—Ptaquiloside, bracken, *Pteridium aquilinum*, Denmark, soil.

INTRODUCTION

The bracken fern (*Pteridium aquilinum* (L.) Kuhn) is one of the most widespread plants on Earth (Fenwick, 1988). In most of Europe, the variety common bracken (*P. aquilinum* var. *aquilinum*) is typically seen on sandy acid soils. Bracken is known to contain several toxic metabolites, which cause a wide range of diseases in humans and animals, ranging from induced thiamine deficiency to cancer (Smith, 1997). The norsesquiterpene glycoside ptaquiloside (PTA) is supposed to be the main carcinogen in bracken, but other bracken constituents might be carcinogenic

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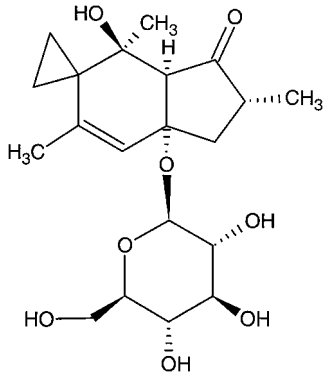

FIG. 1. Ptaquiloside. CAS numbers: 87625-62-5 and 88825-03-0.

as well (Figure 1) (Niwa et al., 1983; Smith and Seawright, 1995; Shahin et al., 1999). PTA is found in common bracken fronds in the range of 60–10,000 μg/g, and in rhizomes up to 1200 μg/g (Saito et al., 1989; Smith et al., 1994). The highest concentrations in fronds are found at the beginning of the growth season, while the highest rhizome concentrations are found at the end of the growth season (Rasmussen and Hansen, 2002).

PTA can be transferred to humans either by direct consumption or through milk contaminated via cows feeding on bracken, causing an increased incidence of gastric cancer (Hirono et al., 1972; Alonso-Amelot, 1997). PTA may also be transferred to humans through drinking water. PTA is highly water-soluble with a very low octanol–water partioning coefficient ($\log K_{ow} = -0.63 \pm 0.01$). This indicates that PTA has very poor affinity for sorption to humic matter in soils. In addition, PTA does not contain functional groups that can bind to mineral surfaces; hence, the sorption of PTA to soil material will be poor (Schwarzenbach et al., 1993; Rasmussen et al., 2003). Thus, PTA transferred to soil from dead or living plant material will be mobile in the soil environment and might readily be leached to surface and groundwaters.

The aim of this study was to investigate the variation in PTA contents in common bracken fronds and rhizomes in Denmark and to test for the presence of PTA in soil material, in order to evaluate the possibility for transfer of PTA from bracken to soil.

METHODS AND MATERIALS

Study Sites. Four Danish bracken-dominated ecosystems were chosen for the investigation (Figure 2, Table 1). At each site, three subsites (approx. 50 m^2) were delineated 50–500 m apart.

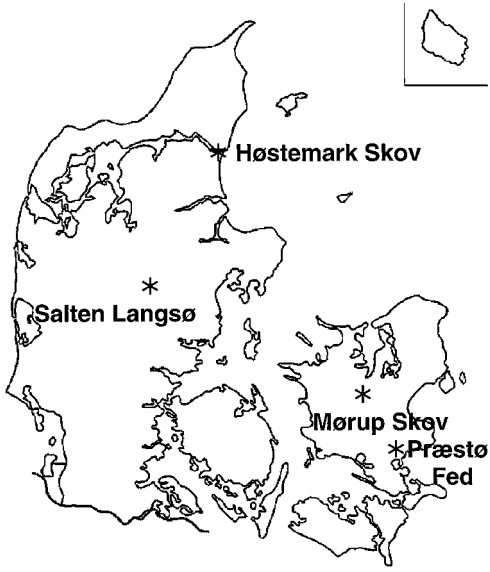

FIG. 2. Map of Denmark, study sites marked (cf. Table 1).

TABLE 1. DESCRIPTION OF INVESTIGATED SITES ACCORDING TO FAO (1990)[a]

Site	Subsite	Parent material	Drainage class	Land use	Herbal dominants	Bracken height (cm)
Præstø Fed	P1	Sandy beach	Somewhat	*Calluna*-	B,C	50–75
	P2	ridges	excessively	Heather	B,C	50–75
	P3		drained		B,C	50–75
Mørup Skov	M1	Sandy glaciofluvial	Well drained	Oak^{P}	B,R,M,Bl	150–200
	M2	deposits			B,N,R	200–250
	M3			Spruce^{S}	B,R,M	125–175
Salten Langsø	S1	Fine sandy till	Well drained		B	200–250
	S2			Fir^{D}	B,G,Bl,Be	150–200
	S3				B	150–200
Høstemark Skov	H1	Sandy beach	Poorly drained	None	B,J,L	150–200
	H2	ridges			B,J,L	150–200
	H3				B,J,L	150–200

[a] B=bracken [*Pteridium aquilinum* (L.) Kuhn], Be=beech (*Fagus sylvatica* L.), Bl=blackberry (*Rubus fruticosus* L.), C=Scotch heather [*Calluna vulgaris* (L.) Hull], D= Douglas fir (Pseudotsuga menziesii Franko.). G=wavy hair-grass [*Deschampsia flexuosa* (L.) Trin.], J=soft rush (*Juncus effusus* L.), L=lichens, M=mosses, N=nettle [*Urtica dioica* L.], P=pedunculate oak (*Quercus robur* L.). R=raspberry (*Rubus idaeus* L.), S=Norway spruce [*Picea abies* (L.) Karst].

Sampling. Several (10–30) average-sized fronds with no visible damage and approx. 1 m in total rhizome length were sampled from each subsite together with 1 liter of soil material from the litter layer (Oi horizons) in August and September, 1999. The study was carried out at the end of the growth season to investigate the PTA concentrations, that may have accumulated in the soil during the spring and summer. Samples were brought to the laboratory, washed (rhizomes only), dried (50°C, for 5 days), milled ($\leq$2 mm diam.), and stored at -20°C until analysis.

Laboratory Analysis. PTA was extracted from plant and soil material with deionized water at ambient temperature for 60 min (sample–solution ratio: 1:50 w/w). Aqueous extracts were cleaned on Polyamide 6S (Riedel-de Haën) and PTA converted to pterosin B before measurement at 220 nm on a Perkin Elmer Series 10 liquid chromatograph, equipped with a Shimadzu SPD-10A UV Detector, and a Perkin Elmer LCI-100 Laboratory Computing Integrator (Agnew and Lauren, 1991). The analytical column was a Merck LiChroCART 250-4, Lichrospher 100 RP-8 4 μm. Eluent was water–methanol (45:55 vol%); flow rate was 1.20 ml/min; sample loop was 50 μl (plant material) and 500 μl (soil samples). Quantification of PTA was performed using standards of pterosin B in the range 0.011–22.500 μg/ml ptaquiloside equivalents (Agnew and Lauren, 1991). Pure ptaquiloside was used for making standards of pterosin B for quantification. Ptaquiloside was obtained from Professor Ojika (Nagoya University, Nagoya, Japan). All samples were determined in duplicate or triplicate. Soil pH was measured in a 1:2.5 soil–0.01 M $CaCl_2$ suspension. A laboratory test of PTA leaching from bracken leaves was performed by exposing fresh mature bracken pinnae from Præstø enclosed in polyethylene bags to water aerosols [30 ml/pinnae (weight: 3.3–7.8 g)] during a period of 1.5 hr, followed by determination of PTA in the leachate collected at bottom of the bags.

Statistical Analysis. The variation within and between sites of the PTA content in fronds, rhizomes, and soil material was evaluated using one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

PTA Content in Fronds and Rhizomes. The PTA content was between 213 and 2145 μg/g in fronds and 11 and 902 μg/g in rhizomes, respectively (Table 2). The rhizome concentration was rather high compared to values reported in literature (Saito et al., 1989; Smith et al., 1994). Large subsite variations were found, resulting in site coefficients of variation between 17 and 63% for fronds and 44 and 84% for rhizomes, respectively. ANOVA performed on the PTA content in the fronds showed statistically significant differences between the sites [$P(F \geq F_{obs}) = 3.4\%$ (fronds) and 3.9% (rhizomes)]. These findings are in accordance with other investigations of PTA levels in common bracken (Saito et al., 1989; Smith et al., 1994). Ptaquiloside levels in the fronds showed a tendency to decrease the further north the site was located (Figure 2, Table 2). The opposite trend

TABLE 2. PTAQUILOSIDE CONTENTS (±SD) AND SOIL-pH[a]

Site	Subsite	Fronds (μg/g)	Rhizomes (μg/g)	Oi-horizon (μg/g)	Oi-horizon pH_{CaCl2}
Præstø Fed	P1	1476	31	1.71	2.68
	P2	1666	11	0.98	2.81
	P3	2070	23	4.97	2.78
	Average	1737±303	22±10	2.55±2.12	2.76
Mørup Skov	M1	2145	123	1.39	2.97
	M2	979	183	1.34	3.06
	M3	1088	578	0.82	2.74
	Average	1404±644	294±247	1.18±0.31	2.92
Salten Langsø	S1	458	902	0.42	2.88
	S2	1390	322	0.22	2.84
	S3	805	754	0.26	2.80
	Average	884±471	659±301	0.30±0.11	2.84
Høstemark Skov	H1	213	240	1.62	2.69
	H2	764	619	8.49	2.77
	H3	376	453	0.91	3.04
	Average	451±283	437±190	3.67±4.19	2.83

[a] PTA contents is based on air-dry weights of soil and plant material.

was found in rhizomes. This pattern may be due to differences in growth stage at the different latitudes. Although the sampling sites were not widely spread, marked differences in climate occurred from north to south, and the more northernly situated brackens might have began carbohydrate translocation from the fronds to the rhizomes at the time of the sampling. Previously, high ptaquiloside concentrations in rhizomes have been found to correlate with decreases in frond ptaquiloside concentrations and correlate with the time of carbohydrate replenishment of the rhizome system (Rasmussen and Hansen, 2003).

PTA Content in Oi Horizons. The PTA content in Oi horizons was between 0.22 and 8.50 μg/g (Table 2). Subsite variation was high with site coefficients of variation between 26 and 114%; no statistically significant differences between sites could be observed. There was no correlation between the PTA content in Oi horizons and the content in fronds or rhizomes. The large subsite variation might have been caused by natural variation in frond cover and density and in the hydrological conditions in the litter layer.

The PTA content in the Oi horizons might have been derived from the soil material itself, since most of the soil was composed of partly mineralized bracken fronds from previous years. This is unlikely, however since PTA is unstable and hydrolyzes at the low pH of the Oi-horizons (Table 2) (Saito et al., 1989). However, it was observed that the highest soil content was measured at the Høstemark and Præstø sites, both of which had been exposed to heavy rain showers within

TABLE 3. LEACHING OF PTAQUILOSIDE FROM BRACKEN PINNAE[a]

Pinnae no.	Weight (g)[b]	Water content (%)[c]	PTA in pinnae (μg/g)	Pinnae drip (μg/liter)[d]	Leachable PTA (%)[e]
1	7.84	73.9	1152	553	0.18
2	3.44	66.9	962	99	0.09
3	4.13	71.9	1530	99	0.05
4	7.21	78.4	1641	131	0.04
5	5.48	65.9	1798	734	0.23
6	3.31	71.7	1408	370	0.24

[a] PTA contents based on fresh weight of 3rd pinnae from soil surface.
[b] Weight of fresh pinnae.
[c] Loss on drying at 105°C.
[d] Concentration of PTA in leachate.
[e] Percentage of PTA in pinnae leached by exposure to 30 ml water aerosol.

12 hr before sampling. This suggests that leaching of bracken fronds by rainwater may transfer PTA from living plant tissue to soil. This hypothesis was supported by a laboratory test demonstrating that PTA could easily leach from bracken pinnae (Table 3). PTA concentrations in drip water from fronds briefly exposed to water aerosols ranged between 100 and 730 μg/liter, resulting in leaching of 0.05–0.25% of total PTA in leaves. There was no apparent correlation between the content of PTA in the pinnae and in the pinnae drip, but the test clearly demonstrated the possibility that PTA could be leached from fronds during rainfall events. Although, it is an open question why PTA, which has no known allelopathic effects, can be leached from the frond. An explanation might be that PTA, leached from the fronds, protects the fern from invertebrates or fungi found in the soil.

The concentrations encountered in the Oi horizon soil materials equal PTA concentrations of 200–8500 μg/liter (when using a Oi-horizon density of 0.1 g/cm^3, water content of 10%, and assuming all PTA to be in the aqueous phase). These concentrations are extremely high compared to threshold concentrations of industrial water contaminants, e.g., pesticides with a threshold concentration of 0.1 μg/liter (Law Announcement, 2001), but are in accordance with the PTA concentrations measured in pinnae drips (Table 3). The high PTA concentrations in the litter layer and the fact that PTA can be leached from fronds by rainwater makes possible the of contamination of drinking water. In particular, contamination of small farm wells with water intake from shallow groundwater sources may occur.

CONCLUSION

In summary, PTA was detected in all samples at all sites (fronds, rhizomes, and Oi horizons). Despite large variation in PTA content among fronds and rhizomes

from different subsites, ANOVA revealed statistically significant differences between the sites. The highest PTA concentrations were found in fronds at all sites. There was no significant difference between sites with respect to PTA content in the soil. The highest PTA concentrations in the soil were found sites, that had recently been exposed to heavy rain showers. A laboratory leaching test supported the possibility that PTA can leach from bracken leaves to soil. The high potential PTA concentrations in pinnae drips and in soil water emphasize that soil and water contamination with PTA is likely to occur and should be taken into account when investigating the potential link between gastric cancer and bracken.

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TRYPTAMINE-INDUCED RESISTANCE IN TRYPTOPHAN DECARBOXYLASE TRANSGENIC POPLAR AND TOBACCO PLANTS AGAINST THEIR SPECIFIC HERBIVORES

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Abstract—The presence of amines and their derivatives in plant tissues is known to influence insect feeding and reproduction. The enzyme tryptophan decarboxylase (TDC) catalyzes the decarboxylation of tryptophan to tryptamine, which is both a bioactive amine and a precursor of other indole derivatives. Transgenic poplar and tobacco plants ectopically expressing *TDC1* accumulated elevated levels of tryptamine without affecting plant growth and development. This accumulation was consistently associated with adverse effects on feeding behavior and physiology of *Malacosoma disstria* Hub. (forest tent caterpillar, FTC) and *Manduca sexta* L. (tobacco hornworm, THW). Behavior studies with FTC and THW larvae showed that acceptability of the leaf tissue to larvae was inversely related to foliar tryptamine levels. Physiological studies with FTC and THW larvae showed that consumption of leaf tissue from the transgenic lines is deleterious to larvae growth, apparently due to a postingestive mechanism. Thus, ectopic expression of *TDC1* can allow sufficient tryptamine to accumulate in poplar and tobacco leaf tissue to suppress significantly the growth of insect pests that normally feed on these plants.

Key Words—Forest tent caterpillar, tobacco hornworm, *Manduca sexta* L., *Malacosoma disstria* Hub., *Populus tremula* x *P. alba*, *Nicotiana tabacum* L. cv. Xanthi, tryptamine, tryptophan decarboxylase, defense.

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INTRODUCTION

Canadian deciduous forests, particularly in the northern boreal region, are often dominated by poplar and aspen, which are subject to severe defoliation by forest tent caterpillars (FTC). FTCs are certainly one of the major defoliators of deciduous trees in the interior of British Columbia (BC). This insect occurs in cyclic outbreaks that persist for 2–6 years on a 6-to 16-year interval (Robinson and Raffa, 1990). During 1990, a heavy infestation of forest tent caterpillars in northeastern BC caused defoliation of trembling aspen stands covering 206,000 ha (Wood, 1992).

Severe defoliation by insects not only reduces the growth rate of trees, but also renders them susceptible to other insects and diseases. Although evolution has provided trees with a range of chemical defenses that help protect them from insect herbivory, increasing the harvestable output from this forest system will require strategic pest management interventions. However, unlike agricultural crops, protection measures involving application of insecticides on a forest scale are not always feasible. A practical alternative in trees could be the incorporation of genetic-based resistance against insects. In recent years, ectopic expression of anti-insect compounds in transgenic plants has been implemented to control insect predation. It is now well established that expression of *Bacillus thuringiensis* (Bt) toxins, or protease inhibitors (PIs), in transgenic plants can greatly decrease insect damage (Hilder et al., 1987; Johnson et al., 1989; Perlak et al., 1990; Kleiner et al., 1995; Wang et al., 1996; Cornu et al., 1996; Meilan et al., 2000). However, in view of the ability of insect pests to develop resistance to specific control agents, the long-term control of insect pests will require a constant search for new sources of resistance.

One potential source of novel genetic resistance could be ectopic expression of genes encoding the capacity to accumulate new plant metabolites or to increase the levels of existing plant metabolites that provide protection. Tryptamine and its derivatives are among the array of neuroactive substances that have been found to act as insect oviposition-deterring and antifeeding agents and/or inhibitors of larval and pupal development. In laboratory feeding trials, tryptamine itself has been reported to inhibit development in *Tetrahymena pyriformis* (Csaba, 1993) and *Helix aspersia* (a snail) (Vehovszky and Walker, 1991), while *Drosophila* fed on an artificial diet supplemented with 75 mM tryptamine suffered a 15% reduction in reproduction due to decreased fecundity (Thomas et al., 1998).

However, few plants naturally produce the millimolar levels of tryptamine that are required for anti-insect activity (Songstad et al., 1990; Aerts et al., 1991; Thomas et al., 1995, 1998). This suggests that the use of tryptamine and/or its metabolites as a means of protecting crop plants would require development of suitably equipped transgenic plants that can accumulate high levels of tryptamine and its metabolites. This has been explored in a few model systems. Transgenic tobacco plants overexpressing tryptophan decarboxylase (TDC) accumulated elevated

levels of tryptamine, and this was correlated with a decrease in whitefly colonization (Thomas et al., 1995). Strong TDC expression in plant tissues might also be predicted to depress the concentration of essential amino acids such as tryptophan (required for insect yolk production and oogenesis). However, no change in tryptophan levels was observed in leaves of the TDC transgenic tobacco plants (Thomas et al., 1995).

In the present study, we created and analyzed *TDC1*-transgenic hybrid poplar 717 plants as a possible approach to improved pest resistance in plantation forestry. The resulting transgenics accumulate elevated levels of tryptamine, as do similarly transformed tobacco plants (unpublished). To assess the biological impact of this modified chemistry, leaf disk choice bioassays and nutritional analysis bioassays were performed using forest tent caterpillar (*Malacosoma disstria* Hbn.) and tobacco hornworm (*Manduca sexta* L.). Our results demonstrate that this simple change in the chemical profile of the transgenic plants significantly reduces feeding and growth of insect herbivores.

METHODS AND MATERIALS

Transgenic Plants. Poplar hybrid clone INRA 717 1B4 (*Populus tremula* x *P. alba*) and tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants were transformed with a *Camptotheca acuminata* tryptophan decarboxylase (*TDC1*) gene using an *Agrobacterium*-mediated transformation approach. Different *TDC1* transgenic lines accumulated elevated levels of tryptamine, ranging from 1400 to 8000 pmol/mg leaf tissue. Based on tryptamine levels, the transgenic lines were grouped into high, medium, and low gene-expressing groups within poplar and within tobacco. One transgenic line from each of the three poplar and three tobacco groups was used in the insect bioassays described here. Additional poplar and tobacco plants were transformed with an empty vector, EV (Bin19/pRT101), and used as controls in the bioassays.

Insect Material. Egg bands of the forest tent caterpillar (FTC), *Malacosoma disstria* Hub. (Lepidoptera, Lasiocampidae), were provided by Dr. Bob McCron, Insect Production Unit, Canadian Forest Service, Sault Ste. Marie, Ontario. Egg bands were surface-sterilized for 1.5 min by swirling in 5% Javex (commercial bleach containing sodium hypochlorite) and then rinsed with cold tap water for 3–5 min. The egg bands were then washed with 1% Javex, air dried, and placed into a Petri dish near the poplar stem to hasten larvae eclosion. It is well established that *M. disstria* caterpillars can detect poplar leaf volatiles and will modify their eclosion time to reflect availability of leaf material (Grisdale, 1976). Eggs hatched in 4–5 days, and the neonates were maintained for about 10 days on an artificial diet (No. 9795, BioServ Inc., Frenchtown, New Jersey, USA) supplemented with finely ground alfalfa to improve acceptability, and with a vitamin mix (No. 8045,

BioServ Inc.) for added nutritional value (Bomford and Isman, 1996). They were then fed cut leaves of wild-type poplar 717 for the remainder of their larval development.

Eggs of *Manduca sexta* L. (Lepidoptera: Sphingidae) (THW) were purchased from Carolina Biological Supply Company (Burlington, North Carolina, USA). The eggs were placed onto tobacco leaves in Petri dishes lined with moist filter paper and held at room temperature under normal day light conditions, where they hatched within 2–3 days.

Neonate Consumption and Performance. Equal-sized *M. disstria* neonates were selected for behavioral and physiological studies. Twenty-five neonates were placed into a Petri dish containing 5 leaf disks (cut with a #15 cork borer, 21 mm diam.) of one of three *TDC1*-expressing poplar 717 lines (high TDC1 #12, medium TDC1 #7, and low TDC1 #4), or from a line transformed with empty vector (EV). Ten replications (5 leaf disks/Petri dish/replication) were performed with each genotype. On day 2 and day 4, leaf disks from different treatments were collected and photographed using an IS-500 Digital Imaging System (Alpha Innotech Corporation). The leaf area consumed was quantified using Scion Image software for Windows 98. From the fourth day onward, eight larvae were removed from each Petri dish, and the remaining 17 larvae were allowed to feed on fresh leaves added from respective transgenic poplar lines. Leaves were exchanged every other day. However, instead of determining leaf area consumption (as on days 2 and 4), larval weight was recorded on days 6 and 8.

Equal-sized *M. sexta* neonates were selected for behavioral and physiological studies on wild-type and transgenic tobacco (Voelckel et al., 2001). Three neonate larvae were transferred into a single Petri dish containing 5 leaf disks (cut with a #15 cork borer) of one of three *TDC1*-expressing tobacco lines (high TDC1 #4, medium TDC1 #11, and low TDC1 #3) or from a line transformed with empty vector (EV). Ten replications were performed with each genotype. After 2 days, leaf disks from different treatments were photographed and the leaf area consumed was quantified. From the second day onward, two larvae were removed from each Petri dish and only a single larva was allowed to feed on leaves added from the respective transgenic tobacco lines. Leaves were exchanged every other day, and larval weight was recorded on days 2, 4, 6, and 8.

Antifeedant Bioassay. In the leaf disk choice bioassay, a larva was given a choice between transgenic and control leaf disks. Larvae of *M. disstria* were reared on leaves collected from wild-type poplar plants. On the day of the bioassay, equal-sized early fourth instars were selected and starved for 4 hr. Leaf disks from high (#12), medium (#7), low (#4) *TDC1*-gene expressing and transformed control (EV) poplar lines were cut with a #15 cork borer. Two control and two leaf disks from one *TDC1* transgenic line were placed into a Petri dish lined with a moistened filter paper and one larva was added to each Petri dish. The Petri dishes were placed into a clear plastic box on moistened paper towels, and the insects were

allowed to feed at 26°C for 4 hr, by which time about 50% of the control leaf disks were consumed. Twelve replications were performed for each treatment. The pattern of leaf area consumption was recorded and quantified as described above. A deterrence index was then calculated as $(C - T)/(C + T) \times 100$, where C is consumption of control leaf disks (EV) and T is consumption of TDC transgenic leaf disks (lines #12, 7, or 4) (Isman et al., 1990).

Manduca sexta larvae were reared on leaves collected from wild-type tobacco plants. On the day of the bioassay, equal-sized late third instars were selected and starved for 4 hr. Leaf disks from high (#4), medium (#11), and low (#3) *TDC1*-gene-expressing and transformed control (EV) plants were cut with a #15 cork borer. The leaf disk choice test was performed essentially as described above for *M. disstria.*

Nutritional Analysis. This bioassay was carried out in order to differentiate behavioral feeding responses from any toxic effects resulting from the activity of TDC. Early fourth instars of *M. disstria* were used to study the effect of *TDC1* expression in poplar plants on larval growth, consumption, and dietary utilization. On the day of the consumption and growth assay, early fourth instars were selected and starved for 4 hr. Larvae and leaf material were weighed, and one larva was placed into a Petri dish along with a known amount of leaf material from transgenic or control poplars. Larvae were then allowed to feed on leaves of transgenic and control plants for 4 days. More leaf material was added when larvae had consumed about 70% of the initially added leaves. At the end of the experiment, the larvae were weighed, and the residual leaves and frass were dried at 60°C for 24 hr and also weighed. Twelve replications were performed with each genotype. All nutritional indices were calculated on a dry weight basis. To determine the fresh weight–dry weight ratios, 15 leaf samples (ca. 1.2 g each) and 15 early fourth instars were dried for 24 hr at 60°C and weighed.

Once dry weights had been determined for larvae, diet, and frass, and initial dry weights calculated for larvae and diet, nutritional indices were calculated using the following formulae (Farrar et al., 1989): relative growth rate (RGR_i = weight gained (mg)/initial larval weight (mg)/number days); relative consumption rate (RCR_i = leaf consumed (mg)/initial weight (mg)/number days); efficiency of conversion of ingested food (ECI = [weight gained (mg)/leaf ingested (mg)] × 100); efficiency of conversion of digested food (ECD = [weight gained (mg)/leaf ingested – frass (mg)] × 100); and approximate digestibility {AD = [leaf ingested – frass (mg)/leaf ingested (mg)] × 100}.

Nutritional analysis with *Manduca sexta* feeding on control and transgenic tobacco plants was performed essentially as described above for *Malacosoma disstria*.

Statistics. Statistical software (Statistix 7) was used for data analysis. A one-way analysis of variance (ANOVA) was conducted, and for comparison of

means between different *TDC1* transgenic and control plants, the least significant difference (LSD) test was applied with a 0.05 rejection level.

RESULTS

Neonate Consumption and Performance. Consumption and performance studies were carried out to assess the effect of *TDC1* overexpression on growth and physiology of FTC neonates. In poplar, the average leaf area consumed by FTC was determined on days 2 and 4 from the start of the feeding experiment (Figure 1). On day 2, the average leaf area consumed from the high *TDC1*-gene-expression line (#12) was significantly lower (1.2 cm^2) than that from the empty vector (EV) control line (1.7 cm^2). The same pattern was observed on day 4. Overall consumption levels in the first 2 days are similar to those in the subsequent 2-day period because first instar FTC grow quite slowly relative to the later larval stages.

On days 6 and 8 of the experiment, larval weights were recorded (Figure 2). The average larval weight appeared to vary among the *TDC1* transgenic lines, with the highest weight gain recorded in larvae feeding on the low expression line (#4) and the lowest in the high expression line (#12), although the differences were not significantly ($P > 0.05$) different between the transgenic lines. In contrast, FTC larvae weight was greater on EV control plants than on any of the *TDC1* transgenic lines. This trend continued on day 8.

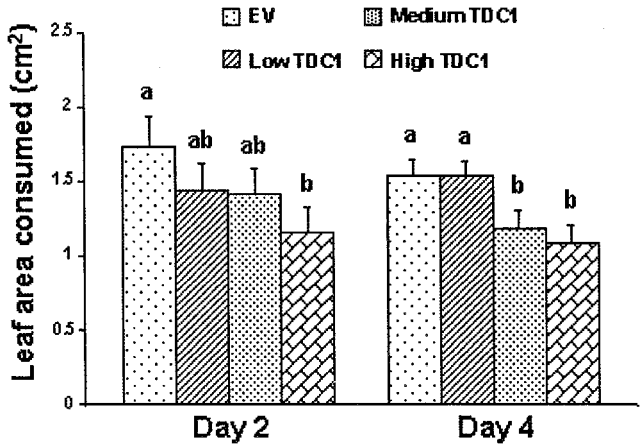

FIG. 1. Leaf area consumed by *M. disstria* neonates after 2 and 4 days of feeding on leaf disks from control (EV) and *TDC1* transgenic poplar lines (high #12, medium #7, and low #4). Each value represents mean ± SE of 10 replications. Columns having the same letters within a cluster do not differ significantly ($P > 0.05$) in LSD test.

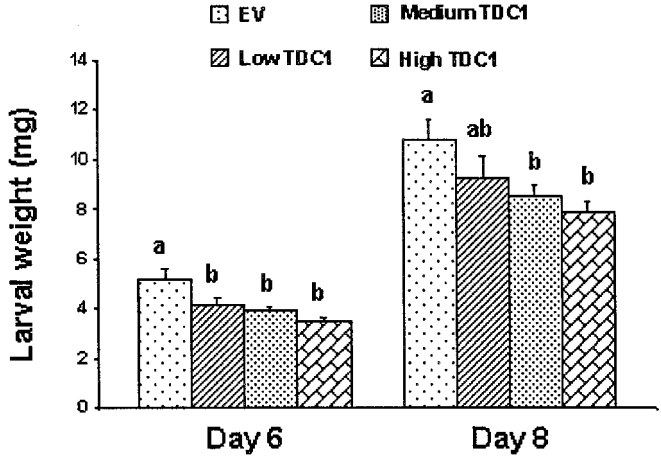

FIG. 2. Larval weight of *M. disstria* neonates after 6 and 8 days of feeding on leaves from control (EV) and *TDC1* transgenic poplar lines (high #12, medium #7, and low #4). Each value represents mean ± SE of 10 replications. Columns having the same letters within a cluster do not differ significantly ($P > 0.05$) in LSD test.

In the tobacco experiments, the average leaf area consumed by THW on day 2 from the start of the feeding experiment (Figure 3) was significantly lower on the high (2.5 cm^2) and medium (2.5 cm^2) *TDC1*-gene-expression lines compared to the empty vector (EV) control tobacco (3.0 cm^2) and low expression lines.

The average THW larval weight was recorded on days 2, 4, 6, and 8 (Figure 4). On day 2, the average larval weight of THW feeding on control and low and medium gene-expression lines were at par and higher than the weight of the larvae on the high gene-expression line. The average larval weight on day 4 was lower on the high (127.1 mg) gene-expression line as compared to the low-expression (157.7 mg) and control (148.8 mg) tobacco line. This pattern continued on days 6 and 8.

Based on these results, it appears that the larval consumption of the leaf tissue, as well as larval weight gain resulting from that consumption, are inversely correlated with the level of tryptamine in *TDC1* transgenic lines. These adverse effects of TDC1 enzyme activity on larval growth could either be behavioral (feeding deterrent) or physiological (postingestive). To distinguish these possibilities, antifeedant bioassays and nutritional analysis were conducted with fourth instars of FTC and THW.

Antifeedant Bioassay. The leaf disk choice bioassay is a short-term assay that reports the effects of diet on larval feeding, rather than on growth or vitality of the insect. The assay was carried out using third instar *M. disstria* and *M. sexta* because it has been generally observed that later instars generate less variable

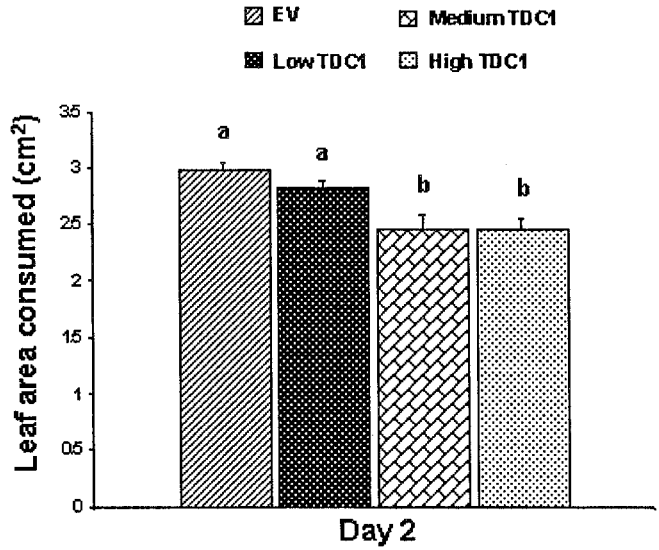

FIG. 3. Leaf area consumed by *M. sexta* neonates after 2 days of feeding on leaf disks from control (EV) and *TDC1* transgenic tobacco lines (high #4, medium #11, and low #3). Each value represents mean ± SE of 10 replications. Columns having the same letters within a cluster do not differ significantly ($P > 0.05$) in LSD test.

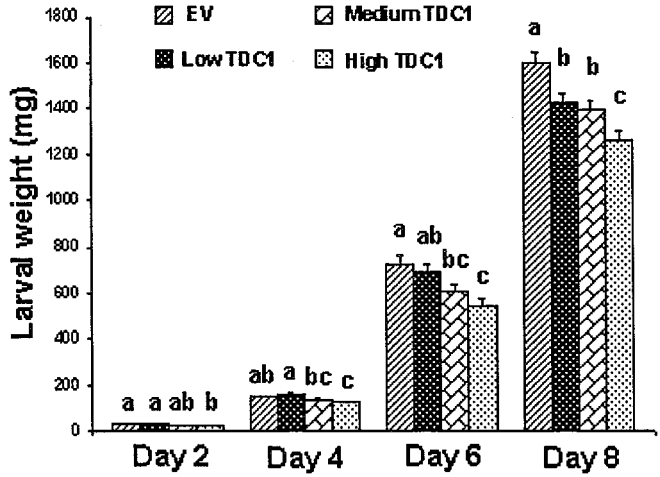

FIG. 4. Larval weight of *M. sexta* neonates after 2, 4, 6, and 8 days of feeding on leaves from control (EV) and *TDC1* transgenic tobacco lines (high #4, medium #11, and low #3). Each value represents mean ± SE of 10 replications. Columns having the same letters within a cluster do not differ significantly ($P > 0.05$) in LSD test.

TABLE 1. ANTIFEEDANT ACTION OF TDC1 TRANSGENICS ON LATE THIRD INSTAR *Malacosoma disstria* AND *Manduca sexta* LARVAE[a]

Poplar 717 transgenic line	Average deterrence (%)	Tobacco transgenic line	Average deterrence (%)
12 (high-*TDC*)	17.0 ± 8.0	4 (high-*TDC*)	33.6 ± 12.5
7 (med-*TDC*)	12.5 ± 7.9^b	11 (med-*TDC*)	27.1 ± 10.0^b
4 (low-*TDC*)	9.2 ± 6.1	3 (low-*TDC)*	6.5 ± 10.4

[a] Each value represents mean ± S.E. of 12 replications.
[b] Average values within columns do not differ significantly ($P > 0.05$) in LSD test.

results in choice bioassays compared to neonates (Xie and Isman, 1992; Kleiner et al., 1995).

The assay was performed using either one of high (#12), medium (#7), or low (#4) gene-expression lines as a treatment and empty vector (EV) poplar hybrid 717 line as a control, and the results expressed as a deterrence index. The highest average deterrence ($17.0 \pm 8.0\%$) was observed with the high gene-expression line (#12) and the lowest ($9.2 \pm 6.1\%$) with the low gene-expression line (#4, Table 1).

A similar gradient of deterrence was observed in leaf disk choice bioassays carried out with transgenic and control tobacco plants. The high gene-expression line (#4) showed the highest ($33.6 \pm 12.5\%$) and low gene-expression line (#3) the lowest average deterrence ($6.5 \pm 10.4\%$) (Table 1).

Nutritional Analysis. The results of nutritional experiments on fourth instar FTC and THW are presented in Tables 2 and 3. The RGR_i, ECI, and ECD of FTC larvae feeding on the leaves of high *TDC1*-expressing poplar line (#12) were reduced in comparison to larvae feeding on leaves of empty vector control (EV) line (Table 2). However, no significant reductions in the RCR_i and AD were observed.

TABLE 2. EFFECT OF *TDC1* OVEREXPRESSION ON GROWTH, FEEDING, AND DIETARY UTILIZATION BY *Malacosoma disstria* LARVAE AFTER FEEDING FOR 4 DAYS ON LEAVES OF *TDC1* TRANSGENIC AND CONTROL POPLAR PLANTS[a]

Plant	Tryptamine (pmol/mg leaf tissue)	RGR_i (mg/mg/day)	RCR_i (mg/mg/day)	ECI (%)	ECD (%)	AD (%)
EV (Control)	903	0.37^b_a	2.93_a	14.09_a	24.70_a	65.20_{ab}
4 (Low)	1485	0.30_{ab}	2.97_a	10.74_{ab}	19.48_{ab}	60.94_b
7 (Medium)	2175	0.27_{bc}	2.26_a	12.82_a	19.05_{ab}	72.62_a
12 (High)	4002	0.21_c	2.70_a	8.33_b	13.87_b	64.03_{ab}

[a] Abbreviations are as defined in the text. Each value represents mean of 12 replications.
[b] Means followed by the same letters within columns do not differ significantly ($P > 0.05$) in LSD test.

TABLE 3. EFFECT OF *TDC1* OVEREXPRESSION ON GROWTH, FEEDING, AND DIETARY UTILIZATION BY *Manduca sexta* LARVAE AFTER FEEDING FOR 4 DAYS ON LEAVES OF *TDC1* TRANSGENIC AND CONTROL TOBACCO PLANTS[a]

Plant	Tryptamine (pmol/mg leaf tissue)	RGR_i (mg/mg/day)	RCR_i (mg/mg/day)	ECI (%)	ECD (%)	AD (%)
EV (Control)	334	2.05_a[b]	5.55_a	32.07_{ab}	80.93_a	48.46_a
3 (Low)	1820	1.71_{ab}	5.04_a	33.57_a	61.77_{ab}	57.45_{ab}
11 (Medium)	5037	1.52_{ab}	5.15_a	26.03_{bc}	46.20_b	61.39_b
4 (High)	8480	1.02_b	5.59_a	23.40_c	35.29_b	67.13_b

[a] Abbreviations are as defined in the text. Each value represents mean of 12 replications.
[b] Means followed by the same letters within columns do not differ significantly ($P > 0.05$) in LSD test.

Similarly, the RGR_i, ECI, and ECD of THW larvae feeding on the leaves of high *TDC1*-expressing tobacco line (#4) were lower compared to larvae feeding on leaves of empty vector (EV) line, whereas the AD was in a reverse order (Table 3). No significant differences in the RCR*i* were observed between transgenic and control lines.

DISCUSSION

The interaction between plant indole derivatives and insects has been shown to result in altered insect behaviors, including feeding and reproduction (Argandona et al., 1980; Corcuera, 1984; Aerts et al., 1992). In this study, we used a molecular approach to generate poplar and tobacco *TDC1* transgenics with elevated levels of tryptamine. The transgenic lines with the highest accumulation of tryptamine consistently showed an adverse effect on feeding behavior as well as physiology of forest tent caterpillars (FTC) and tobacco hornworms (THW) in insect bioassays performed with both neonates and fourth instars, at least in bioassays of short duration. Increased levels of tryptamine in TDC transgenic tobacco plants had previously been correlated with a marked decrease in whitefly pupal emergence on the transgenic plants (Thomas et al., 1995).

In no-choice feeding assays conducted over several days, both FTC and THW neonates consumed less leaf tissue and gained less weight on the foliage of plants with high *TDC1* expression and high tryptamine levels as compared to control (empty vector, EV) plants. While this demonstrates that transgenics with elevated levels of tryptamine display anti-insect activity, it does not distinguish between feeding deterrence and toxicity as a mechanism.

The mode of action of the anti-insect effect in the transgenics was investigated with fourth instar FTC and THW. Leaf disk choice assays make it possible to

dissect out potential antifeedant activity (Schoonhoven, 1982; Xie et al., 1994), while measurement of the nutritional indices reveals possible toxic effects.

These experiments revealed that the initial acceptability of the leaf tissue to FTC and THW larvae is substantially reduced as the tryptamine levels in the tissue increased. The relative deterrence indices of the high and low *TDC1* expression lines of both poplar and tobacco are also consistent in this regard (Table 1). It is interesting that *Drosophila* detected and avoided tryptamine at 75 mM concentration (Thomas et al., 1998), whereas the concentration of tryptamine in the high *TDC1* expressing lines used here was maximally 8 mM and 4 mM in tobacco and poplar lines, respectively. Herbivorous insect species clearly differ in their sensitivity to the amine profiles of their host plants.

Feeding trials with tryptamine and its derivatives have shown that these indole metabolites can act as insect antifeedants in other, nontransgenic plants. The indole metabolites tryptamine, 5-hydroxytryptamine, and methoxytryptamine, were found to possess antifeedant properties against adult female brown planthoppers, *Nilaparvata lugens* (Sogawa, 1971). In insect bioassays with wood extracts from *Virola calophylla*, a plant known to accumulate 5-methoxy-*N*,*N*-dimethyltryptamine and 2-methyl-6-methoxytetrahydro-β-carboline, antifeedant effects against the cotton boll weevil (*Anthonomus grandis*) were attributed to these metabolites (Miles et al., 1987).

In contrast to the short-term choice test (4 hr in duration), no significant differences were detected in the relative rate of consumption (RCR_i) in the long-term nutritional experiments (4 days in duration). Lepidopteran larvae are known to habituate to feeding deterrents upon sequential or continuous exposures (Bomford and Isman, 1996). However, a significant reduction was observed in the efficiency of conversion of ingested food (ECI) in FTC larvae feeding on the leaves of high *TDC1* transgenic poplar lines as compared to control lines. The ECI is an overall measure of an insect's ability to utilize food that is ingested for growth, and it is a product of both the AD and ECD. The AD represents the portion of food assimilated, and ECD reflects the efficiency with which the assimilated food is converted to body substance. Parallel differences in reduction of ECI and in the growth rate of the insects suggest interference with the physiology of food conversion as a mechanism of action.

While the FTC larvae feeding on high *TDC1*-expression poplar plants showed a lower ECD, no differences were observed in the AD between the same larvae. This demonstrates that the reduction in ECI was due to a significantly lower efficiency of conversion of the digested food to body tissues, i.e., growth, rather than to problems with digestion and absorption from the gut. The lower growth rate of FTC larvae feeding on high *TDC1* plants may, therefore, be due primarily to a higher metabolic cost encountered when feeding on leaves containing elevated levels of tryptamine. The development times and growth rates of gypsy moth larvae feeding on a diet containing phenolic glycosides were also strongly affected, consistent with the

higher metabolic cost associated with dealing with toxic metabolites (Hemming and Lindroth, 2000).

In addition to toxic metabolites, physical factors and bioavailability of plant nutrients can affect the growth rate of insects. Comparative behavioral studies of *Chrysomela scripta* (Coleoptera: Chrysomelidae) adults feeding on mature and immature leaves of *Populus* x *euramericana* illustrated that low nitrogen content and high amounts of fiber and tannins in mature leaves resulted in longer developmental times and lower prepupal weights in larvae feeding on mature leaves (Harrell et al., 1982). Eastern tent caterpillars were shown to grow faster and process food stored in their gut more rapidly at high temperatures (35°C) as compared to larvae maintained at lower temperatures (20°C) (Schroeder and Lawson, 1992).

The situation observed in tobacco differed somewhat from that in poplar. THW larvae feeding on the leaves of high *TDC1* tobacco plants also suffered a reduction in their larval growth rate (RGR_i), ECI, and ECD, but, unlike poplar, the approximate digestibility was increased in insects feeding on the high *TDC1* tobacco line. This implies that the observed differences in ECI must be due to a marked reduction in the efficiency with which the digested food was converted to body substance. The higher AD may reflect the fact that the high *TDC1* tobacco tissue contained almost double the amount of tryptamine accumulated in the high *TDC1* poplar line, which would place a correspondingly heavy metabolic demand on the consuming insects as they coped with tryptamine detoxification.

FTC and THW larvae did not reject outright the transgenic leaf tissue in the no-choice growth test, even though it is clearly deleterious to their growth. In such experiments, it is not possible to determine if growth inhibition is a consequence of reduced feeding or vice versa. The nutritional analysis experiments segregate feeding behavior from postingestive physiological mechanisms. Our results suggest it is the latter that accounts for the growth inhibitory effects. However, the exact mode of toxicity of tryptamine and related amines remains to be elucidated. Derivatives such as N_ω-methyltryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, 5-methoxytryptamine, and 3-*N*,*N*-dimethylaminomethylindole (gramine) have all been shown to reduce the survival of nymphs of *Rhopalosiphum maydis* (corn aphid) 48 hr after feeding on a synthetic diet supplemented with the pure compounds (Corcuera, 1984). These compounds possessed LD_{50} values ranging from 2.3 to 3.8 mM in these tests, which is in the same range as the tryptamine concentrations measured in the high *TDC1* lines tested here.

It is clear that the elevated levels of tryptamine accumulated in the *TDC1* transgenic poplar and tobacco leaf tissues are capable of reducing the growth rate of specific herbivore insects. The behavioral and physiological studies suggest that the origin of this effect is probably a form of chronic toxicity, at least under laboratory conditions. Although the inhibition is not dramatic, small differences in feeding rates could have important ecological consequences. Modest preference differences in combination with environmental stresses to larvae could contribute

additively or synergistically to increased insect mortality under field conditions. For example, in a comparative study using larvae of the diamondback moth (*Plutella xylostella*), survival of larvae confined on leaf disks of cabbage (2518 genotype) in the laboratory was much greater (80% of controls) than it was on whole plants in the field (0.36% of controls) (Eigenbrode et al., 1990).

Whether *TDC* should be considered for development as a potential insect resistance gene will depend upon several factors, including its performance under field conditions. Possible differences between gene expression in juvenile and mature tissues need to be explored, and further studies are required to test the long-term stability of transgene expression. Field trials with transgenic poplars and spruce at different locations have demonstrated that expression of some transgenes can be stable in trees over time (reviewed in Ellis et al., 2001). Finally, the ecological consequences of adding *TDC* to the array of genetic defenses available in the poplar genome need to be carefully examined. Elevated tryptamine levels can be expected to have positive and/or negative impacts on other organisms that depend on their interactions with poplar to successfully complete their life cycle.

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ANTIFEEDANT EFFECTS OF PROTEINASE INHIBITORS ON FEEDING BEHAVIORS OF ADULT WESTERN CORN ROOTWORM (*Diabrotica virgifera virgifera*)

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Abstract—Low-molecular-weight peptidyl proteinase inhibitors (PIs) including leupeptin, calpain inhibitor I, and calpeptin were found to be potent antifeedants for adult western corn rootworm (WCR) against the phagostimulation of cucurbitacin B (Cuc B) or a corn pollen extract (CPE). Leupeptin was the strongest ($ED_{50} = 0.36$ and 0.55 nmol/disk for Cuc B and CPE, respectively) among PIs tested with an antifeedant potency much stronger than the steroid progesterone ($ED_{50} = 2.29$ and 5.05 nmol/disk for Cuc B and CPE, respectively), but slightly less than the reference alkaloid, strychnine ($ED_{50} = 0.17$ and 0.37 nmol/disk for Cuc B and CPE, respectively). All active PIs contain a di- or tripeptidyl aldehyde moiety, indicating that PIs exert their antifeedant effects by covalent interaction with putative sulfhydryl (SH) groups on taste receptors as do these PIs with cysteine proteinases. However, opposite inhibition potency against Cuc B versus CPE by two thiol-group reducing agents, DTT and L-cysteine, and the results with other cysteine-modifying reagents obscure the net functional role of SH groups at WCR taste chemoreceptors. Surprisingly, the model phagostimulant for diabroticites, Cuc B, was more easily counteracted by these feeding deterrents than the stimulants present in CPE. Three-dimensional structure–antifeedant relationships for the PIs suggest that a novel taste chemoreception mechanism exists for these peptidyl aldehydes or that they fit partially into a strychnine binding pocket on protein chemoreceptors. Favorable economic benefit may be achieved if PIs are discovered to be useful in adult WCR control, since both pre- and postingestive sites would be targeted.

Key Words—Antifeedants, *Diabrotica virgifera virgifera*, cucurbitacin B, corn pollen, proteinase inhibitors, leupeptin, strychnine, steroids, sulfhydryl reagents, taste receptors, structure–activity relationships.

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INTRODUCTION

Primary metabolites in plants, especially sugars and free amino acids, have critical roles as nutrients as well as phagostimulants (feeding stimulants) for many insect species. However, the dominant focus in the scientific literature on plant secondary metabolites directing insect–plant interactions has reduced enthusiasm for continued study on the joint role of plant nutrients as essential cues for host-plant specialization. More plant secondary compounds act as antifeedants (phagodeterrents or feeding deterrents) than phagostimulants, and these phytochemicals usually exert their inhibitory effects on insect feeding in a dose-dependent manner (Schoonhoven, 1982; Bernays and Chapman, 1994). Nonetheless, some antifeedant chemicals for generalist insects function as phagostimulants for insect specialists that are adapted to host plants rich in the respective phytochemicals. Thus, the acceptance or avoidance of a food plant is determined by integration of all positive and negative signals through associated sense cells along with the modulatory factors that vary according to the physiological state of an insect (Renwick, 1999; Bernays and Chapman, 2000). Berenbaum (1986) has reasoned that an antifeedant not associated with postingestive toxicities has little evolutionary impact as a plant defensive compound on host-plant selection by insects. Poor correlations of antifeedant potency with internal toxicities of phytochemicals have been noted (Bernays, 1990, 1991; Mullin et al., 1997; González-Coloma et al., 2002). Nevertheless, preingestive detection by herbivores of nonhost antifeedants limits host-plant range, especially for polyphagous insects (Bernays and Chapman, 1994).

Feeding deterrents are perceived by insects primarily through molecular ligand interactions at taste receptors. Inhibition of sugar stimulation by an antifeedant is the most frequently cited interaction at insect taste cells. However, one or more chemoreception mechanisms may act to achieve the preingestive detection of antifeedants by an insect by: (1) stimulating a deterrent taste cell, (2) inhibiting the response to phagostimulants, or (3) evoking irregular (bursting) activity of chemoreceptor cells (Schoonhoven, 1982; Mitchell and Harrison, 1985; Dethier and Bowdan, 1989; Chapman et al., 1991; Messchendorp et al., 1998).

Diabroticite beetles share similar thresholds of sensitivities to various cucurbitacins irrespective of their degree of Cucurbitaceae specialization, with cucurbitacin B (Cuc B) being the most phagostimulatory (Metcalf and Metcalf, 1992; Tallamy et al., 1997). In contrast to these phagostimulants, four adult diabroticite species [*Diabrotica virgifera virgifera* LeConte, western corn rootworm (WCR); *D. barberi* Smith & Lawrence, northern corn rootworm; *D. undecimpunctata howardi* Barber, southern corn rootworm; and *Acalymma vittatum* (Fabricius), striped cucumber beetle] showed widely different sensitivities to antifeedants varying greatly in structures, although the alkaloids strychnine and hydrastine were uniformly antifeedant to all diabroticites tested (Eichenseer and Mullin, 1997). Mullin

et al. (1997) found remarkable differences between adult WCR and Colorado potato beetle in sensitivities to antifeedants from varying chemical classes, especially alkaloids and terpenoids. However, all of the established γ-aminobutyric acid (GABA) and glycine neuroreceptor antagonists were antifeedant for these beetle species, suggesting a shared molecular mechanism for antifeedant chemoreception exists in these Chrysomelidae.

Corn pollen is among the most preferred foods for diabroticite beetles, and simple mixtures of its dominant short-chain, neutral amino acids explain much of the phagostimulatory effect (Hollister and Mullin, 1999). These free amino acids strongly elicit action potentials from two galeal taste neurons of the five-cell sensillum of adult WCR, of which two of the four chemosensory cells also potently respond to cucurbitacin B and another to antifeedant alkaloids like strychnine in a dose-dependent manner (Chyb et al., 1995). The net outputs of the taste sensilla to an increasing dose of antifeedants relative to phagostimulants negatively correlates with the amount of feeding (Chyb et al., 1995; Hollister and Mullin, 1999; cf. Mullin and Kim, 2001).

A GABA/glycine-like chemoreceptor model was proposed to account for the neuropharmacological specificity of WCR gustation (Mullin et al., 1992, 1994), since free amino acid agonists stimulate feeding and alkaloid and terpenoid antagonists selective for this neuroreceptor are antifeedant. Moreover, the pregnane steroid 5α-pregnan-α-ol-20-one, which is a strong modulator of GABA binding at vertebrate $GABA_A$-receptors (Purdy et al., 1990), antagonized the potent phagostimulation of cucurbitacins (Mullin et al., 1994), suggesting a mutual interaction at a steroid binding site occurs. More recently, G-protein coupled receptors have been shown to mediate sugar perception in taste neurons of *Drosophila* (Clyne et al., 2000; Ishimoto et al., 2000).

Many cysteine modification agents such as *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), and cysteine are known to inhibit or suppress chemosensory responses at taste neurons (Frazier and Heitz, 1975; Shimada, 1975; Norris, 1981; Bryant, 1990). These and many insect antifeedants are proposed to exert their effects through covalent interactions with thiol or sulfhydryl (SH) groups on chemoreceptor proteins (Norris, 1981, 1986).

Proteinase inhibitors (PIs) such as *p*-hydroxymercuribenzoic acid (PHMB), leupeptin, and antipain significantly inhibited feeding and fecundity of alfalfa weevil (Elden, 1995, 2000). However, it was not clear whether the reduction of foliar feeding was due to a preingestive (antifeedant) or postingestive (metabolic feedback) effect of the PIs. Several di- or tripeptidyl cysteine proteinase inhibitors such as calpain inhibitor I, calpain inhibitor II, leupeptin, and calpeptin strongly inhibited azocaseinolytic proteolysis in the oral secretion and midgut fluid from adult WCR (Kim, 2002). Inhibition of proteolysis by these aldehyde PIs results from the covalent modification of an SH group at the active-site cysteine

essential to cysteine proteinase activity (Otto and Schirmeister, 1997; Leung et al., 2000).

The purpose of this study was to investigate the feeding effects of proteinase inhibitors on adult WCR, comparing their effects with representatives of other chemical classes including alkaloids (strychnine) and steroids (cucurbitacins, pregnanes) previously associated with the GABA/glycine taste model for rootworm feeding behavior. In addition, the involvement of putative SH groups in rootworm taste chemoreception was investigated through use of some selective SH-modifying reagents after peptidyl aldehyde inhibitors of cysteine proteinases were found to be rootworm antifeedants. Basic structure–antifeedant relationships for these peptide inhibitors were also defined by computerized overlay and docking of three-dimensional molecular models.

METHODS AND MATERIALS

Insects. A nondiapausing laboratory strain of western corn rootworm (WCR) was purchased from French Agricultural Research, Inc. (Lamberton, Minnesota, USA). Beetles were received as pupae in soil, and the emerged adults were placed into a plastic shoebox ($30 \times 17 \times 9$ cm). Adult beetles were fed sliced store-bought acorn squash fruit and water *ad libitum* and were maintained at $25 \pm 1^{\circ}$C and a 16L:8D photoperiod in an M-31 Environmental Growth Chamber (Chagrin Falls, Ohio, USA).

Corn Pollen Extraction. Sweet corn (*Zea mays* L.) var. Bodacious was used for preparation of a corn pollen extract (Hollister and Mullin, 1999). Two grams (fresh weight) of corn pollen from storage at -80°C were mixed with 10 ml of buffer (30 mM tripotassium citrate, pH 6.0) in an Erlenmeyer flask (125 ml), vortexed for 1 min, and then left at 4°C in the dark for 1 hr. After centrifuging the extraction mixture at 10,000*g* for 15 min at 4°C, the supernatant was decanted off, and the pollen was washed with another 10-ml buffer, and centrifuged as before. The pooled extract was then centrifuged at 10,000*g* for 15 min at 4°C, and the supernatant (corn pollen extract) stored at -20°C until use.

Feeding Bioassay. The antifeedant effect of test chemicals on beetle feeding was measured using a two-choice regenerated cellulose disk bioassay. After starvation for 12 hr, two beetles (undetermined sex) were placed into a 100 × 20-mm Petri dish containing two regenerated cellulose disks (26.5 mm^2, 1.0-μm pore size; Schleicher and Schuell, Daytona Beach, Florida, USA) suspended at opposite ends of the dish on a minute pin 2 mm above a paraffin layer covered with a moistened filter paper. Cucurbitacin B (Cuc B, 4 μl, 0.2 nmol/disk) or corn pollen extract (CPE, 4 μl, 0.4-mg pollen equivalent/disk) was used as a feeding stimulant. Cuc B in methanol or CPE in buffer (30 mM tripotassium citrate, pH 6.0) was applied with a Hamilton repeating dispenser (Reno, Nevada, USA) to

the disk. One disk was treated with a phagostimulant alone and the other disk was treated with a phagostimulant plus a putative antifeedant compound. The test antifeedant was applied in an appropriate solvent (usually water or methanol) after the phagostimulant application had dried. After a 24-hr feeding period, dry disk areas were determined to the nearest 0.1 mm^2 with an Optomax V image analyzer (Analytical Measuring Systems, Saffron Walden, UK) and then subtracted from the mean disk area of 10 disks not offered to beetles, to give the area consumed. Three to six different concentrations per antifeedant were tested, and each dose was replicated 5–23 times. Previous exposure to Cuc B reduced the behavioral sensitivity of beetles with increasing age (J.H. Kim, personal observation), and thus, beetles were not used in more than four different bioassays if Cuc B was used as a phagostimulant.

Leupeptin-Treated Pollen Feeding Bioassay. A multiple choice test was used to measure the antifeedant effect of leupeptin on corn pollen feeding by beetles. Leupeptin was dissolved in water, and 100 μl of leupeptin (0, 2.5, 5, 7.5 or 10 nmol/mg pollen) were applied onto 30 mg pollen in an aluminum foil bowl (1×0.5 cm). Pollen was carefully mixed using a tiny spatula and then was provided as a slurry to beetles. Fifteen pollen-containing bowls ($N = 3$ for each dose) were randomly distributed in a plastic shoe box ($30 \times 17 \times 9$ cm) and 48 adult WCR were allowed to feed on pollen over a 29-hr period. Water was supplied *ad libitum* using moistened paper tissues.

Chemicals. Calpain inhibitor I and calpain inhibitor II were purchased from both Sigma (St. Louis, Missouri, USA) and ICN (Irvine, California, USA). Calpeptin was purchased from ICN, benzyloxycarbonyl-leu-leu-norleucine-aldehyde was from Bachem (King of Prussia, Pennsylvania, USA) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was from Eastman (Rochester, New York, USA). All other chemicals were purchased from Sigma-Aldrich. Cucurbitacin B was isolated according to Eichenseer and Mullin (1996).

Data Analysis. The antifeedancy of a compound was calculated using an antifeedant index: $AI = [(C - T)/(C + T)] \times 100$ (Blaney et al., 1984), where C indicates the area of the phagostimulant-only disk consumed (in mm^2) and T is the consumed area of the other disk containing phagostimulant and antifeedant. An $AI = 0$ indicates that control disks were not distinguished from treatment disks by beetles, while $AI = 100$ indicates no consumption of the treatment disk at the applied dose.

ED_{50} (dose for 50% inhibition of phagostimulation) was calculated based on the AI values at each dose using a simple linear regression model with log-transformed doses (inverse prediction). Analyses were performed over the linear response range (at least three doses) of the dose–response curves (JMP 3.1, SAS Institute).

An antifeedant discrimination threshold is defined as the dose of antifeedant causing significant differences between control and treated disk consumption at

$P < 0.05$ level. The Wilcoxon rank-sum test was used to determine significant differences of disk consumption between control and treatment at each dose.

RESULTS

Strychnine, one of the most potent alkaloid antifeedants known for adult WCR (Mullin et al., 1994, 1997), was the strongest feeding deterrent against Cuc B or CPE among all chemicals tested in the present study (Tables 1 and 2, Figures 1 and 2). Nevertheless, the antifeedant potency of low-molecular-weight peptidyl proteinase inhibitors (PIs) was evident for adult WCR feeding. For leupeptin and calpain inhibitor I, a dose of 1 nmol/disk was the lowest concentration where significantly less of the phagostimulant plus antifeedant disk was consumed over the phagostimulant-only disk, while 5 nmol/disk was required for calpeptin, Z-leu-leu-nle-aldehyde, antipain, calpain inhibitor II and TLCK (Table 1). E-64, pepstatin A, and elastatinal did not inhibit feeding within the dose range tested (0.1–10 nmol/disk).

The ED_{50} value for leupeptin was less than 1 nmol/disk against both Cuc B and CPE (Table 2). Three other PIs, calpain inhibitor I, calpeptin and Z-leu-leu-nle-aldehyde, had ED_{50} values less than 2 nmol/disk. The ED_{50}s of strychnine, progesterone, and proteinase inhibitors for CPE were generally higher than those for Cuc B except for calpeptin and TLCK. Antipain had a similar ED_{50} value against Cuc B phagostimulation to that of calpain inhibitor I or calpeptin, but these two PIs had lower ED_{50}s against CPE than antipain. TLCK showed a weak antifeedant potency compared to other PIs.

Progesterone was the most potent antifeedant among steroids. Beetles ate significantly ($P < 0.05$) more of Cuc B or CPE-only (control) disks than progesterone-treated disks at concentrations equal to or higher than 1 or 5 nmol, respectively (Table 1). Consistent antifeedant effect by the five other steroids occurred only at a concentration of 10 nmol/disk against Cuc B (Table 1). Neither 5α-pregnan-3α-ol-20-one nor 5α-pregnane-3α21-diol-20-one (up to 10 nmol/disk) inhibited phagostimulation by either Cuc B or CPE. Four other steroids, 4-androstene-3,17-dione, fucosterol, estriol, and dipterocarpol, also did not inhibit feeding on Cuc B-treated disks.

The antifeedant threshold doses for progesterone, leupeptin, and calpain inhibitor I were all around 1 nmol/disk against Cuc B phagostimulation (Table 1), but differences in the slopes of the dose–response lines were observed (Figure 2). The dose–response slope for progesterone was less potent and shallower (more heterogeneous) than those for leupeptin and calpain inhibitor I, giving 6.4- and 9.2-fold higher ED_{50} values than those for leupeptin with Cuc B and CPE, respectively, as a phagostimulant (Table 2). Antifeedant potencies for all four compounds were slightly higher with Cuc B than with CPE (Figure 2), although no significant

TABLE 1. ANTIFEEDANT DISCRIMINATION THRESHOLDS FOR NONDIAPAUSE ADULT WESTERN CORN ROOTWORM ON CUCURBITACIN B- OR CORN POLLEN BUFFER EXTRACT TREATED DISKS

Compound	Antifeedant threshold (nmol/disk)[a]		N^b
	Cucurbitacin B	Corn pollen extract	
Strychnine (0.05–5)[c]	0.1	0.5	14
Proteinase Inhibitors (0.1–10)			
Leupeptin	1	1	14
Calpain inhibitor I	1	1	14
Calpeptin	5	5	14
Z-leu-leu-nle-aldehyde[d]	nt[e]	5	7
Antipain	5	5	7
Calpain inhibitor II	5	5	14
TLCK	5	5	7
E-64	(-)[f]	(-)	14
Elastatinal	(-)	(-)	7
Pepstatin A	(-)	(-)	7
Steroids (0.25–10)			
Progesterone	1	5	14
5α-pregnan-3α-ol-20-one	(-)	(-)	12
5α-pregnane-3α,21-diol-20-one	(-)	(-)	12
5α-pregnan-3α-ol-11,20-dione	10	nt	5
20-hydroxyecdysone	10	nt	5
Cortisone acetate	10	nt	5
Fusidic acid	10	nt	5
Deoxycholic methylester	10	nt	5
4-androstene-3,17-dione	(-)	nt	5
Fucosterol	(-)	nt	5
Estriol	(-)	nt	5
Dipterocarpol	(-)	nt	5
Thiol modifiers			
DTNB[g] (1–10)	5	10	14
DTT (5–50)	10	(-)	23
L-Cysteine (10–100)	(-)	10	17
D-Cysteine (10–50)	(-)	(-)	7
N-Ethylmaleimide (10–50)	(-)	(-)	7

[a] Minimum concentration required to significantly reduce feeding on treatment versus cucurbitacin B (0.2 nmol/disk) or corn pollen buffer extract (0.4-mg pollen equivalent) control in the two-choice bioassay.
[b] Replication number.
[c] Dose range tested.
[d] Z: benzyloxycarbonyl, nle: norleucine.
[e] Not tested.
[f] No inhibition within dose range tested.
[g] DTNB: 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE 2. FEEDING DETERRENCY OF PROTEINASE INHIBITORS ON ADULT WESTERN CORN ROOTWORM IN COMPARISON TO STRYCHNINE AND PROGESTERONE

Compound	ED_{50} (nmol/disk)[a]			
	Cucurbitacin B[b]	Slope	Corn pollen extract[b]	Slope
Alkaloid				
Strychnine	0.17 (0.04, 0.34)[c]	38.5	0.37 (0.26, 0.54)	54.0
Steroid				
Progesterone	2.29 (1.58, 3.58)	41.1	5.05 (2.82, 13.8)	39.8
Proteinase inhibitors				
Leupeptin (acetyl-Leu-Leu-Arg-aldehyde)	0.36 (0.02, 0.91)	43.0	0.55 (0.06, 1.20)	51.3
Calpain inhibitor I (acetyl-Leu-Leu-nle-aldehyde)	1.17 (0.29, 2.11)	43.3	1.24 (0.43, 2.12)	40.1
Calpeptin (Z-Leu-nle-aldehyde)[d]	1.82 (0.44, 3.42)	49.2	1.33 (0.65, 2.04)	47.6
Z-Leu-Leu-nle-aldehyde	Not tested		1.39 (0.60, 2.47)	64.8
Antipain (1-carboxy-2-phenylethyl carbamoyl Arg-Val-Arg-aldehyde)	1.16 (0.58, 2.30)	31.3	2.58 (1.57, 5.00)	69.7
Calpain inhibitor II (acetyl-Leu-Leu-Met-aldehyde)	2.61 (1.41, 4.07)	50.3	3.78 (2.91, 5.02)	79.1
TLCK (Tos-Lys-CH_2Cl)	6.50 (2.28, 118.5)	36.7	4.96 (2.31, 20.3)	41.0

[a] Dose for 50% inhibition of phagostimulation. See Methods and Materials for calculation.
[b] Cuc B = 0.2 nmol/disk; CPE = 0.4-mg pollen equivalent/disk.
[c] Numbers in parenthesis represent 95% confidence intervals.
[d] Z: benzyloxycarbonyl, nle: norleucine.

differences were found at any doses between antifeedant indices with Cuc B versus CPE except for 0.1 nmol/disk of strychnine ($P < 0.043$, Wilcoxon rank-sum test).

Major differences in inhibition potency were found for thiol (SH) compounds on Cuc B versus CPE phagostimulation. Feeding stimulation by Cuc B was significantly inhibited by DTT at a dose of 10 nmol/disk, but not by L-cysteine, while the reverse was demonstrated for feeding on CPE (Table 1). The lowest concentrations at which significant feeding deterrence were seen for DTNB in the presence of Cuc B and CPE were at 5 and 10 nmol/disk, respectively. Other thiol modifiers, *N*-ethylmaleimide and D-cysteine, did not inhibit beetle consumption over the dose range tested (10–50 nmol/disk).

Corn pollen consumption was inhibited in a dose-dependent manner by pretreatment of corn pollen with leupeptin (Figure 3). At a dose of 2.5 nmol leupeptin/mg pollen, corn pollen consumption was inhibited by 43% compared to the control (27.7 mg pollen consumption) and more than 83% inhibition was observed at ≥ 5 nmol leupeptin/mg pollen.

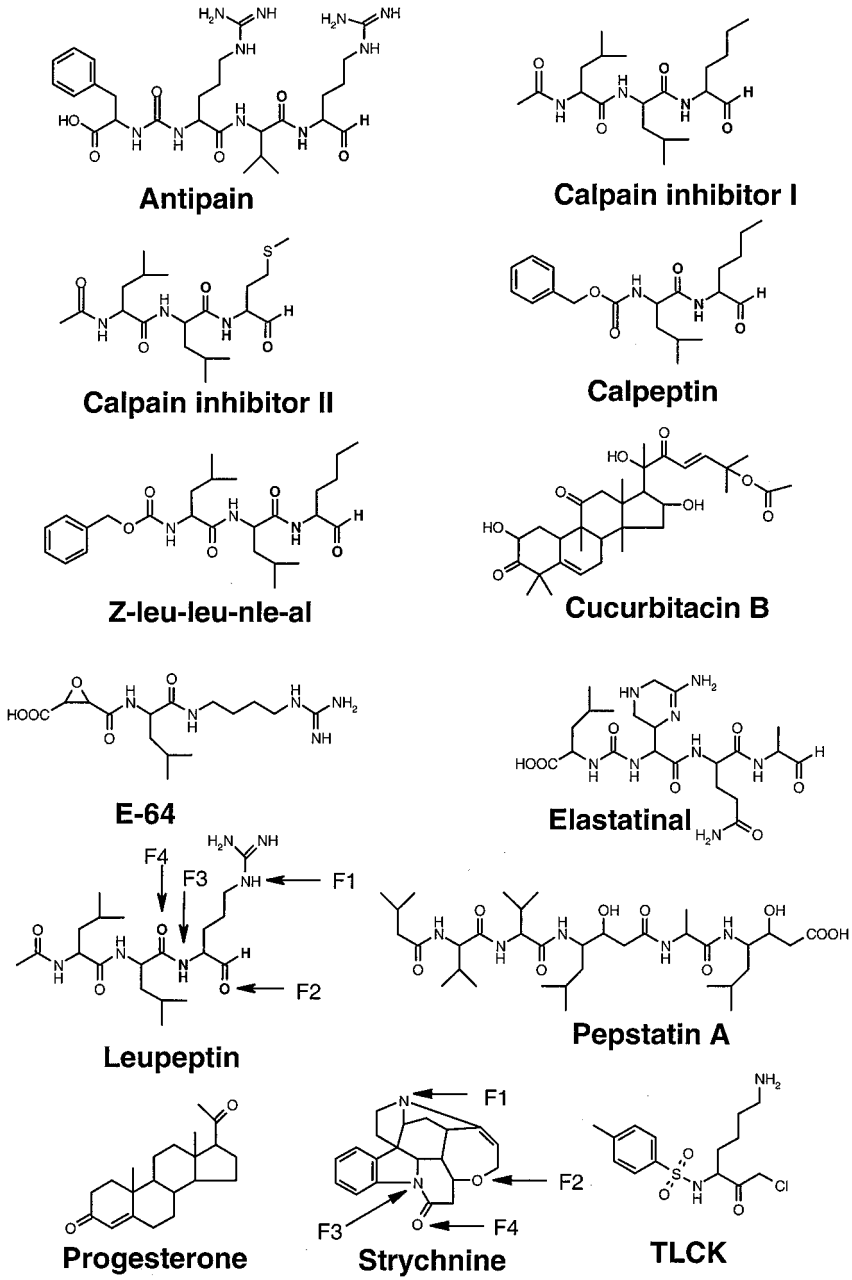

FIG. 1. Chemical structures of proteinase inhibitors, an alkaloid (strychnine), a steroid (progesterone), and a phagostimulant (cucurbitacin B) used in this study. The four atomic centers are indicated as F1–F4 (see Figure 4).

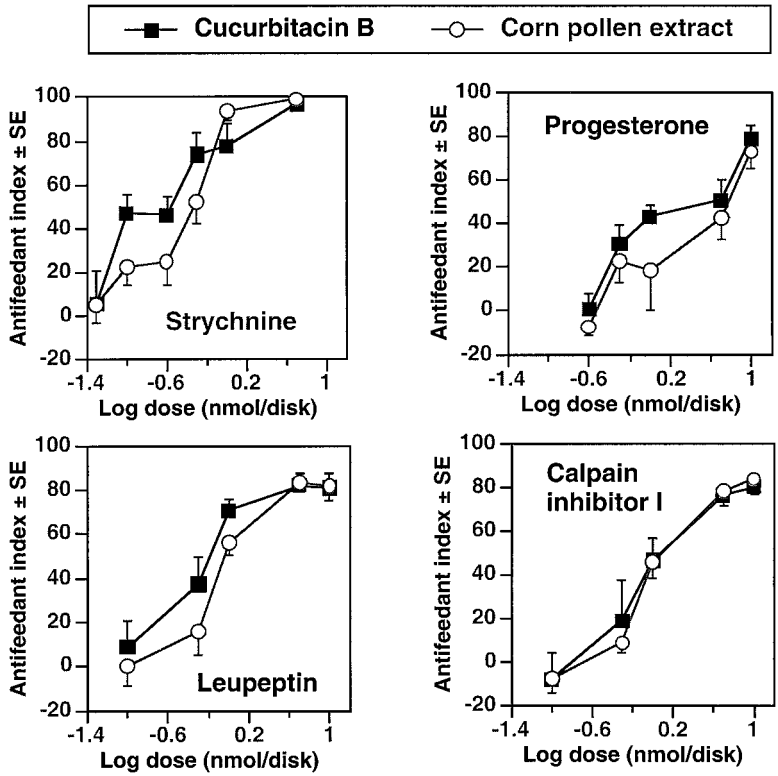

FIG. 2. Dose–responses of adult WCR for four antifeedants in combination with cucurbitacin B (0.2 nmol/disk) or the corn pollen extract (0.4-mg pollen equivalent/disk). An antifeedant index (AI) of 0 indicates control disks were not distinguished from treatment disks by beetles, while AI = 100 indicates no consumption of antifeedant-treated disks at the specified dose.

DISCUSSION

This is the first report on the strong antifeedant effect of low-molecular-weight peptidyl proteinase inhibitors (PIs) for adult WCR. Based on ED_{50} values, leupeptin (both a cysteine and serine proteinase inhibitor) was 2.1- and 1.5-fold weaker as an antifeedant with either Cuc B or CPE phagostimulation, respectively, than strychnine (Table 2). The antifeedant threshold for leupeptin applied to corn pollen was about 75 nmol/30 mg pollen (2.5 nmol/mg pollen, Figure 3), which was consistent with that from the regenerated cellulose disk feeding bioassays [1 nmol leupeptin/0.4-mg pollen equivalent (=2.5 nmol/mg pollen), Table 1].

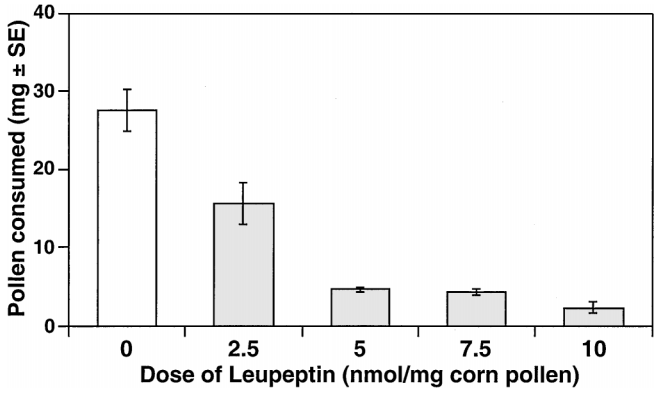

FIG. 3. Inhibition of corn pollen consumption by leupeptin. A multiple choice test was used to measure the antifeedant effect of leupeptin-treated corn pollen ($N = 3$ for each of four doses plus a solvent control).

While it is possible that a postingestive effect could influence feeding behavior on PI-treated pollen or cellulose disks over a 24-hr feeding period, chronic feeding studies on leupeptin-treated pollen at 67% of the above dose required at least 5 days to demonstrate a significant reduction of WCR feeding (Kim, 2002). Both adult WCR (Koiwa et al., 2000) and southern corn rootworm (Fabrick et al., 2002) are susceptible to the postingestive growth and development inhibition by larger molecular weight peptidyl cysteine PIs from soybean and potato, respectively. Additional studies measuring electrophysiological inputs from taste cells in parallel with behavioral outputs are needed to understand pre- and postingestive interactions between phagostimulants and antifeedants for adult WCR.

The antifeedant threshold for strychnine against 0.2 nmol/disk of Cuc B was 0.1 nmol/disk, which was lower than that of a previous study (Eichenseer et al., 1998), probably because the concentration of Cuc B used in the present study was lower than the previous one (0.5 nmol/disk). Mullin et al. (1992, 1994) found that one of the pregnane steroids (5α-pregnan-3α-ol-20-one) antagonizes Cuc B phagostimulation in a squash flower disk bioassay with an antifeedant ED_{50} of about 130 nmol/disk. However, in the present study, this steroid did not inhibit either Cuc B or CPE phagostimulation, probably due to the lower dose range tested (1–10 nmol) and/or the different matrix used for a bioassay (squash blossom disk versus regenerated cellulose disk).

We expected that feeding stimulation by Cuc B (major component in Cucurbitaceae host plants for WCR) would be stronger than that by CPE, because stimulatory amino acids alone were thought to be primarily responsible for corn pollen feeding (Hollister and Mullin, 1999), but even high concentrations of amino acids did not reach phagostimulation elicited by Cuc B (Kim, 2002). However,

both Cuc B (0.2 nmol/disk) and CPE (0.4-mg pollen equivalent) were equivalent in stimulating greater than 70% of disk consumption by two beetles over a 24-hr period in a no-choice bioassay (data not shown). The present study also showed that Cuc B was more sensitive to three structurally distinct antifeedant compounds (strychnine, progesterone, and leupeptin) and gave shallower slopes for its dose–response lines than CPE except for progesterone (Figure 2, Table 2), suggesting that total phagostimulation elicited by CPE (0.4-mg pollen equivalent) is greater than that by Cuc B (0.2 nmol/disk).

CPE contains a complex mixture of chemicals. The combination of amino acids, sugars, flavonol glycosides, probably lipids (Lin and Mullin, 1999), and still unknown factors interact together in an antagonistic, additive, or synergistic manner, resulting in a net positive feeding stimulation (Kim, 2002). These complex interactions between feeding stimulants and inhibitors in corn pollen make it difficult to interpret if common or independent taste receptor types are responsible for taste ligand interactions. Nonetheless, the fact that no significant differences were found between antifeedant indices for Cuc B and CPE phagostimulation (Figure 2) suggests that three different antifeedant classes of compounds exert their effects through a similar taste neuroreceptor site.

Eichenseer et al. (1998) demonstrated that the antifeedant dose–response curve for alkaloids was steeper with L-alanine than with Cuc B as the phagostimulant. They suggested that alkaloids such as strychnine directly bind to amino acid sites, but allosterically interact through a distinct steroidal site, resulting in a shallower dose–response for the Cuc B interaction than for amino acids. The steroid, progesterone, which is assumed to interact at binding sites for the tetracyclic triterpenoid, Cuc B, due to their similar structures (Figure 1), was 6.4-fold less potent against Cuc B phagostimulation than the PI, leupeptin.

The importance of sulfhydryl (SH) groups in taste transduction mechanisms for insects as well as vertebrates has been reviewed (Norris, 1986). Antifeedants may react covalently with active thiol or amino groups or interact through other types of bonds (e.g., ionic, hydrogen, hydrophobic) at sensory receptors (Norris, 1986; Lam and Frazier, 1987; Mullin et al., 1994). Here, we found that some structurally related aldehydic PIs, known to inhibit cysteine proteinases by covalent binding to an active site thiol (McConnell et al., 1998), are potent antifeedants for WCR. For example, calpain inhibitor I (*N*-acetyl-leu-leu-norleucine-aldehyde), calpain inhibitor II (*N*-acetyl-leu-leu-met-aldehyde), and leupeptin (*N*-acetyl-leu-leu-arg-aldehyde) are tripeptidyl homologs, each having at its C-terminus a norleucine-, methionine-, or arginine-aldehyde, respectively, with a hydrophobic *N*-acetylated terminus comprised of two leucine residues. Calpeptin has similar molecular features to calpain inhibitor I except for a benzyloxycarbonyl-group replacing the *N*-acetylated leucine terminus. All aldehyde-containing PIs tested were strong antifeedants except for elastatinal, which has no hydrophobic carbon chain between an aldehyde and its nearest peptide bond (Figure 1). Whether the

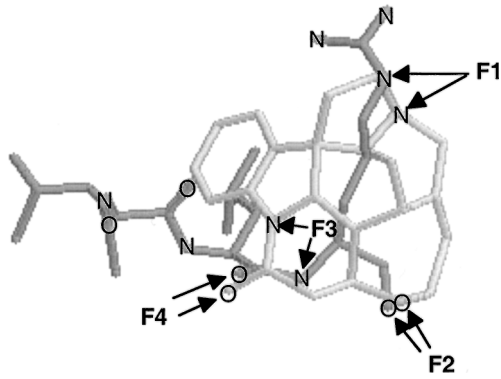

FIG. 4. 3-D molecular fit between strychnine and leupeptin. Distance between atomic centers F1–F4 is less than 1Å except for F3 (1.7Å) using Chem 3D (CambridgeSoft, Massachusetts, USA) energy minimization and fit algorithms. See Figure 1 for the four fit sites.

aldehyde group or a di- or tripeptidyl arrangement of PIs is critical to eliciting an antifeedant effect is presently under investigation.

All antifeedant PIs (antipain, calpain inhibitors I and II, calpeptin, leupeptin, and Z-leu-leu-nle-aldehyde) share a similar spatial arrangement of atoms at F2, F3, and F4 (Figure 1, see leupeptin), but lack structural similarity to known phagostimulants or antifeedants for adult WCR. It is possible that a novel antifeedant chemoreception mechanism exists for these peptidyl aldehyde inhibitors, which is distinct from the GABA/glycine taste receptor hypothesis (Mullin et al., 1994). On the other hand, co-docking of strychnine and leupeptin (Figures 1 and 4) demonstrated closely fitted homologous atoms (F1–F4), suggesting leupeptin and other PIs could fit partially into a strychnine binding pocket on taste neurons of adult WCR (cf. Mullin and Kim, 2001).

DTNB, which is frequently used to measure cysteine residues in proteins, was the only nonproteinase inhibitor and SH-modifying reagent that effectively inhibited the phagostimulation of Cuc B or CPE. Alternatively, two thiol-group reducing agents, DTT and L-cysteine, showed opposite inhibition potency against Cuc B versus CPE (Table 1). Unlike L-cysteine, D-cysteine did not inhibit phagostimulation elicited by CPE, which makes it more difficult to explain the role of SH groups in taste chemoreception for adult WCR. It also remains to be shown if putative SH groups at WCR taste receptors are disabled by a covalent interaction with peptidyl aldehydes.

Adult WCR regurgitate an oral secretion prior to or during feeding on a phagostimulant-treated disk and different types of enzymes including cysteine proteinases in this oral secretion can modify chemicals on the disk (Kim, 2002). However, it is presently unclear how interactions among oral secretion components,

SH reagents, and the complex mixture of corn pollen chemicals produce behavioral outputs different from those elicited by Cuc B alone.

Use of antifeedants in pest management has several advantages over classical synthetic pesticides, such as target insect specificity, limited residual activity and environmental impact, and low probability of resistance development. However, much interdisciplinary effort is needed for the determination of the chemical basis for insect–host plant associations, which can allow exploitation of natural products as well as synthetic compounds for targeted pest control (Frazier and Chyb, 1995). If low-molecular-weight peptidyl PIs are discovered to be useful in adult WCR control, favorable economical benefit and long-term efficacy may be achieved since both pre- and postingestive sites (Kim, 2002) would be targeted.

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PHENOLIC COMPOUNDS ON THE POD-SURFACE OF PIGEONPEA, *Cajanus cajan*, MEDIATE FEEDING BEHAVIOR OF *Helicoverpa armigera* LARVAE

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Abstract—A methanol extract of the pod surfaces of *Cajanus cajan*, a feeding stimulant for fifth-instar *Helicoverpa armigera*, was shown to contain four main phenolic compounds. Three of these were identified as isoquercitrin, quercetin, and quercetin-3-methyl ether, by comparing UV spectra and HPLC retention times with authentic standards. The fourth compound was isolated by semipreparative HPLC and determined to be 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid (stilbene) by NMR spectroscopy and mass spectrometry. Quercetin, isoquercitrin, and quercetin-3-methyl did not affect the selection-behavior of fifth-instar *H. armigera*. However, larvae were deterred from feeding on glass-fiber disks impregnated with the stilbene. Furthermore, larvae exposed to quercetin-3-methyl ether consumed significant amounts of both disks. In a binary-choice bioassay, a combination of quercetin-3-methyl ether and the stilbene on one disk and pure quercetin-3-methyl ether on the other disk resulted in increased consumption of both glass-fiber disks by larvae. In contrast, consumption was reduced if the combination was presented to larvae on one disk with purified stilbene on the other disk. *Cajanus cajan* cultivars that varied in their susceptibility to *H. armigera* were surveyed for the presence of the four phenolic compounds. An absence of quercetin and higher concentrations of isoquercitrin than the cultivated variety characterized pod surface extracts of pod-borer-resistant cultivars. In addition, the ratio of the stilbene to quercetin-3-methyl ether was greater in the pod-borer-resistant cultivars. These findings

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are discussed in relation to the identification of chemical characters that can be used for crop improvement.

Key Words—*Cajanus cajan*, podborer, *Helicoverpa armigera*, feeding behavior, stilbene, quercetin-3-methyl ether, quercetin, isoquercitrin.

INTRODUCTION

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a major pest of pigeonpea, *Cajanus cajan* (L.) Millsp. (Fabaceae: Papilionoideae) (Lateef and Reed, 1990; Ranga Rao and Shanower, 1999). Larvae cause substantial damage to plants and reduce the yield of grain by feeding upon flowers and pods (Shanower et al., 1999).

Previous experiments have shown that acetone extracts from the pod surface of a variety of *C. cajan* (ICPL 87) susceptible to pod-borers stimulated the feeding of third-instar *H. armigera* (Shanower et al., 1997). More recently, we showed that hexane, methanol, and water extracts of *C. cajan* (ICPL 87) pods also stimulate feeding of fifth instars, with the methanol extract being most stimulatory (Green et al., 2002). Food selection behavior of *H. armigera* larvae is known to be affected by secondary compounds present in other cultivated species of legumes, such as chickpeas (*Cicer* sp.), in which some compounds act as feeding stimulants, whereas others deter feeding (Simmonds and Stevenson, 2001). Secondary compounds on the surface of pods of *C. cajan* may also modulate the feeding of larvae of *H. armigera*. We now report on the isolation and characterization of some compounds from a methanol extract of the pod surfaces of *C. cajan* (ICPL 87) and describe their effects on the feeding behavior of larvae of *H. armigera*. A comparison of the presence and concentration of these compounds is made among different cultivars of *C. cajan* that vary in their susceptiblity to predation by the pod-borer. The implications of these data in understanding the factors that determine why the cultivated genotype of *C. cajan* (ICPL 87) is susceptible to predation by pod-borer larvae are discussed.

METHODS AND MATERIALS

Preparation of Extracts. A methanol extract from a known surface area of freshly collected, 7- to 10-day-old, *C. cajan* pods (ICRISAT accession: ICPL 87) was prepared (at ICRISAT in November 1998) by dipping individual pods into the solvent for 120 sec. The extract was concentrated under reduced pressure and redissolved in methanol so that each 100 μl of solvent contained the equivalent extract from 3.46 cm^2 of pod surface, the surface area of a glass-fiber disk. Thus, the concentration of different compounds in an aliquot could be equated to an

area of pod surface to ensure that insects were presented with naturally occurring concentrations during feeding bioassays.

Isolation and Identification of Compounds from Pod Surface of Pigeonpea (ICPL 87). Extracts were analyzed and compounds isolated by HPLC (Waters 600E pump; 717 autosampler and 996 photodiode array detector). Aliquots (20 μl) were injected onto a reverse-phase column (Merck, Lichrospher 100; 250 mm long, 4 mm ID, RP-18; 5-μm particle size, temperature 30°C) and eluted at 1 ml/min using the gradient 25% A: 75% B at $t = 0$ min to 100% A at $t = 20$–30 min, where A is methanol in water and B is 2% acetic acid. Three compounds present in the extract of the pod surfaces were identified by comparison with HPLC profiles of authentic standards (>95% purity, Apin Ltd., Oxford, UK). A fourth, unknown, compound was isolated by manually collecting it as it eluted from a semipreparative reverse-phase column (Merck, Lichrospher 100, 250 mm long, 10 mm ID, RP-18; 10-μm particle size, temperature 30°C), with the flow rate set to 4.7 ml/min. The eluent was then dried under reduced pressure.

NMR Spectroscopy and Mass Spectrometry. Both 1D and 2D ^{1}H NMR spectra of **4** were acquired in $CDCl_3$ at 30°C on a Bruker 400 MHz instrument. ^{13}C NMR data were obtained from the indirectly detected dimension in HSQC and HMBC experiments. Spectra were referenced to residual solvent resonances at $\delta_{H/C}$ 7.25/77.0 ($CDCl_3$) relative to TMS. Negative ion first-order MS were recorded using LC-MS (Thermo-Finnigan LCQ) with an electrospray ionization (ESI) source.

Quantification. Known concentrations (1, 10, 100, 250, and 500 ppm) of each of the phenolic compounds were injected onto an analytical column and eluted according to the method described above. Peak areas were integrated and compared with the authentic standards (Apin Ltd.), the isolated compound, and an extract of the pod surfaces of ICPL 87 at a known concentration (i.e., 100μl contained the extract from 3.46 cm^2 of pod surface). These data were used to calculate the concentration of compounds in the pod surface extracts.

Larval Behavior. Authentic standards (>95% purity; Apin Ltd.) or the isolated compound (>95% purity) were used in the behavioral bioassays. Glass-fiber disks (Whatman GF/A grade, 2.1 cm diam., 3.46 cm^2) were impregnated with 100-μl aliquots of either a methanol extract of the pod surfaces of ICPL 87 ($N = 15$) or a naturally occurring concentration of one of the four phenolic compounds present in the methanol extract ($N = 10$, for each compound). Individual larvae from a control group ($N = 10$) were each presented with two blank disks (no extract or compound). Once dry, disks were weighed and placed into individual 9-cm-diam. plastic Petri dishes with a weighed, untreated disk. Disks were moistened with 100 μl of distilled water, as previous experiments had shown that larvae were less likely to feed on a dry disk (Green et al., 2002). Subsequently, one fifth-stadium *H. armigera*, selected from a colony reared at the Royal Botanic Gardens, Kew, UK, according to the methods of Armes et al. (1992) was added to each dish.

Each insect was 24–36 hr into the fifth stadium and had been without food for 2 hr (Simmonds et al., 1990).

Dishes were placed into a growth cabinet 12 hr light at 27°C, 12 hr dark at 20°C, 70% relative humidity. After 24 hr, insects were removed from the dishes and disks were left to dry for a further 24 hr prior to being reweighed to determine the amounts of control and treated disk eaten. The mean amounts of both the control and treated disk eaten by larvae were calculated for all the insects. Feeding indices (FI) were calculated from the amounts of control (C) and treated disks (T) eaten: $FI = [(C - T)/(C + T)] \times 100$ (Simmonds et al., 1990). Since feeding indices are calculated for each replicate within a treatment, they can be distorted by inclusion of replicates in which larvae consume small amounts of disk material. Therefore, only replicates in which larvae had consumed at least 1 mg of one of the disks were used for the purposes of calculating a FI. This also ensured the inclusion only of those insects taking large bites from a disk, as opposed to those that had merely rasped the surface of the disks without consuming disk material.

Data Analyses. The amounts of glass-fiber disk eaten by larvae exposed to different treatments and the feeding indices associated with each treatment were compared with the Mann-Whitney test (MW) (Minitab, v. 12). The quantities of the control and treated disk eaten within a treatment were compared with the Wilcoxon signed rank test (WSR) (Sokal and Rohlf, 1987).

Comparison of Concentration of Compounds in Other C. cajan Cultivars. Methanol extracts of a known area of pod surface were prepared from different varieties of *C. cajan* displaying resistance (ICPL 332) or moderate resistance (ICPL 7203-1, ICPL 84060, and ICPL 187-1) to damage caused by podborer larvae. The level of resistance was measured as the proportion of undamaged pods recorded on plants grown in experimental field plots at ICRISAT. These plants were exposed to naturally occurring insect populations (Sharma et al., 2001). The extracts were prepared as described for ICPL 87 and analyzed by HPLC. The concentrations of the four phenolic compounds in the extracts were calculated as described above.

RESULTS

Identification of Compounds from Pod Surface of Pigeonpea (ICPL 87). Methanol extracts of the pod surfaces of ICPL 87 contained isoquercitrin (**1**) (R_t = 13.8 min), quercetin **(2)** (R_t = 17.1 min), and quercetin-3-methyl ether **(3)** (R_t = 18.1 min), respectively (Figure 1).

The UV spectrum of compound **4** (R_t = 23.2 min) recorded in acidic MeOH was typical of a stilbene (λ_{max} = 260, 304, 325sh nm) (Gorham, 1989). Its ^{1}H NMR spectrum contained resonances for a double bond in the *E* configuration (δ 7.82 and 6.82, both 1H, d, J = 15.9 Hz), a monosubstituted phenyl ring (δ 7.52,

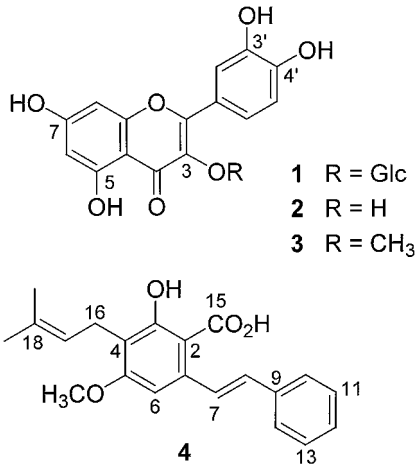

FIG. 1. Structure of phenolic compounds present in a methanol extract of the pod surfaces of *Cajanus cajan*: isoquercitrin **(1)**, quercetin **(2)**, quercetin-3-methyl ether **(3)** and 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid **(4)**.

2H, d, $J = 7.2$ Hz; 7.37, 2H, t, $J = 7.5$ Hz, 7.28, 1H, m), and a single aromatic proton at δ 6.65 (s) indicating the presence of a pentasubstituted phenyl ring. Long-range $^3J(^1\text{H}–^{13}\text{C})$ correlations observed in HMBC data between the double-bond protons and carbon atoms of both phenyl rings (Table 1) confirmed that **4** was a stilbene. The substituents of the pentasubstituted phenyl ring were identified as carboxyl (δ_C 174.0), hydroxyl (δ_H 11.52, br s), prenyl (δ 3.38, 2H, d, $J = 7.1$ Hz; 5.21, 1H, m; 1.79, 3H, d, $J = 0.9$ Hz; 1.68, 3H, d, $J = 0.9$ Hz), and methoxyl (δ_H 3.94, s). Their relative locations were determined from long-range $^1\text{H}–^{13}\text{C}$ correlations in HMBC data (Table 1) and dipolar $^1\text{H}–^1\text{H}$ connectivities from 1D ROE experiments. ESI-MS data (negative mode) for **4** showed a deprotonated molecule at m/z 337 $[\text{M}-\text{H}]^-$ and a fragment ion at m/z 293 $[\text{M-H-44}]^-$, consistent with the loss of CO_2. UV, NMR, and MS data allowed the structure of **4** to be confirmed as 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid ($C_{21}H_{22}O_4$), a phytoalexin reported previously from leaves of *Cajanus cajan* challenged with *Botrytis cinerea* (Cooksey et al., 1982). ^{13}C NMR resonance assignments for this compound were not given in the earlier report and are summarized in Table 1 together with a complete set of ^{1}H NMR resonance assignments acquired in $CDCl_3$ (previous data given only in acetone-d_6 and C_6D_6).

Compound Concentrations in Extracts. Of the four main compounds in the methanol extract of the pod surfaces, **3** occurred at the highest concentration (880 ppm; 2.46 μg/cm^2 of pod surface), followed by **4** (560 ppm; 1.57 μg/cm^2), **1** (60 ppm; 0.17 μg/cm^2), and **2** (50 ppm; 0.14 μg/cm^2).

TABLE 1. ^{1}H AND ^{13}C NMR RESONANCE ASSIGNMENTS (δ) AND COUPLING CONSTANT DATA FOR COMPOUND **4** IN $CDCl_3$ AT 30°C

Carbon	^{13}C	^{1}H	HMBC correlations
1	141.7		
2	102.9		
3	162.4		
4	116.8		
5	162.3		
6	103.3	6.65 (s)	C-1[a], C-2, C-4, C-5, C-6, C-15[a]
7	130.4	7.82 (d, $J = 15.9$ Hz)	C-1[a], C-6, C-8[a], C-9,
8	131.1	6.82 (d, $J = 15.9$ Hz)	C-1, C-7[a], C-9[a], C-10, 14,
9	137.2		
10, 14	126.8	7.51 (d, $J = 7.2$ Hz)	C-8, C-12,
11, 13	128.8	7.37 (t, $J = 7.5$ Hz)	C-9,
12	127.9	7.28 (m)	C-10, 14
15	174.6		
16	22.2	3.37 (d, $J = 7.1$ Hz)	C-3, C-5, C-17, C-18
17	121.9	5.21 (m)	
18	132.0		
19	17.9	1.79 (d, $J = 0.9$ Hz)	C-17, C-18, C-20
20	26.0	1.68 (d, $J = 0.9$ Hz)	C-17, C-18, C-19
OCH_3	55.8	3.94 (s)	C-5,
3-OH		11.52 (br s)	C-3, C-4, C-2

[a] Weak correlations indicative of two and four bond couplings.

Larval Behavior. A methanol extract of ICPL 87 at the naturally occurring concentration per unit area of glass-fiber disk stimulated feeding of fifth-instar *H. armigera* (Table 2). Comparison of the total amount eaten by larvae exposed to either compound **1** or **2** with the total amount consumed in the control shows that neither of these compounds stimulated feeding. In contrast, larvae exposed to **3** and **4** consumed as much of either the compound-treated and/or control disk as occurred when larvae were exposed to the methanol extract (Table 2). However, the effect of **3** and **4** on the food selection behavior of larvae varied. For example, the stilbene showed potent antifeedant activity, whereas **3** did not affect selection behavior.

As the phagostimulatory activity of the methanol extract of ICPL 87 could not be explained by the activity of compounds **1–4** when tested on their own, a combination bioassay was undertaken with the two compounds (**3** and **4**) that resulted in appetitive behavior (Table 3). In this binary-choice bioassay, compound **3** or **4** was applied to one of the disks and exposed to larvae along with a disk treated with both **3** and **4**. The concentration of each compound was the same as occurred in the methanol extract of ICPL 87. Very few larvae exposed to **4** versus the **3** + **4** combination consumed the disks. So the antifeedant activity of **4** masked

TABLE 2. FEEDING BY FIFTH INSTAR *H. armigera*, ON GLASS FIBER DISKS DURING CHOICE BIOASSAYS: EFFECTS OF METHANOL EXTRACT AND COMPOUNDS **1 – 4** ON FEEDING

Extract or compound on treated disk (ppm)	Amount eaten, (mg, mean ± SEM)			Feeding Index (mean ± SEM)[b]	N^c
	Control disk	Treated disk	Control + Treated		
Water control	0.4 ± 0.11 b	0.5 ± 0.13 c	0.9 ± 0.16 b		
ICPL 87 methanol extract	0.3 ± 0.11 b	4.8 ± 1.06 * a	5.1 ± 1.08 a	−88 ± 4.7 c	10
1 (60)	0.3 ± 0.15 b	0.5 ± 0.37 c	0.8 ± 0.48 b		
2 (50)	0.6 ± 0.33 b	0.5 ± 0.29 c	1.1 ± 0.61 b		
3 (880)	2.7 ± 1.05 a	2.2 ± 0.66 b	4.9 ± 1.35 a	6 ± 18.4 b	7
4 (560)	3.2 ± 0.86*a	0.9 ± 0.38 c	4.1 ± 1.03 a	62 ± 16.7 a	7

[a] **1** to **4** correspond to the compounds shown in Figure 1.
[b] Where no feeding-index is shown, <5 larvae consumed, on average, >1 mg of both disks.
[c] N = the number of replicates used to calculate the feeding index.
The amounts of control and treated disk eaten, within a treatment, were compared with the Wilcoxon signed rank test. $^*P < 0.05$. The amounts of disk eaten and feeding indices were compared between treatments using the Mann-Whitney test, different letters in a column indicate significant differences. $P < 0.05$.

the appetitive activity of **3** (Table 3). Larvae exposed to disks treated with **3** and **3** + **4** consumed both disks: thus, in this combination the ability of the stilbene (**4**) to modulate the selection behavior of the larvae has been lost. However, the combinations tested do not enable us to explain the phagostimulant activity of the methanol extract of ICPL 87.

Comparison of Concentration of Compounds in Other C. cajan Cultivars. The pod surface extracts of the resistant *C. cajan* varieties were characterized by

TABLE 3. EFFECTS OF COMBINATIONS OF COMPOUNDS **3** AND **4** ON FEEDING BY *H. armigera* LARVAE[a]

Compounds on disks		Amount eaten, (mg, mean ± SEM)			Feeding Index, (mean ± SEM)[b]	N^c
Treatment 1	Treatment 2	Treatment 1	Treatment 2	Treatment 1 + Treatment 2		
3	**3** and **4**	2.5 ± 0.42 a	3.1 ± 0.70 a	5.6 ± 0.58 a	1 ± 16.2	10
4	**3** and **4**	0.6 ± 0.32 b	0.9 ± 0.28 b	1.4 ± 0.57 b		

[a] **3** and **4** correspond to the compounds shown in Figure 1. Compounds were applied to disks at their naturally occurring concentrations.
[b] Where no feeding-index is shown, <5 larvae consumed, on average, >1 mg of both disks.
[c] N = the number of replicates used to calculate the feeding index.
The amounts of disk eaten were compared between treatments using the Mann-Whitney test, different letters in a column indicate significant differences. $P < 0.05$. There were no differences between the amounts of control and treated disk eaten within each of the two treatments (Wilcoxon Signed Rank, $P > 0.05$).

TABLE 4. CONCENTRATION OF COMPOUNDS PRESENT IN POD SURFACE EXTRACTS OF *C. cajan* CULTIVARS WITH DIFFERENT SUSCEPTIBILITY TO POD-BORER PREDATION

	Degree of resistance to podborer feeding[b] and concentration (ppm),[c] of compounds in different accessions of *Cajanus cajan*				
	Susceptible	Moderate resistance			Resistant
Compound[a]	ICPL 87	ICPL 7203-1	ICPL 84060	ICPL 187-1	ICPL 332
1	60	253	379	206	412
2	50	ND	ND	ND	ND
3	880	2284	893	774	1092
4	560	1852	738	745	923

[a] **1**, **2**, **3** and **4**, correspond to the compounds shown in Figure 1.
[b] Data from Sharma et al. (2001).
[c] ND = not detected.

the absence of compound **2** and by concentrations of compounds **1** and **4** that differed from those in the extracts of the susceptible cultivar, ICPL 87 (Table 4). The ratio of compound **3** to **4** in the pod-surface extract of the susceptible cultivar (ICPL 87) was 1.6, whereas the ratio in extracts from the less suceptible genotypes varied from 1.2 (ICPL 7203-1, ICPL 332, ICPL 84060) to 1 (ICPL 187-1). A decrease in the proportion of the feeding stimulant **3**, compared to the antifeedant stilbene **4**, could, therefore, contribute to the resistance of the *C. cajan* genotypes to pod-borer larvae (Table 4).

DISCUSSION

Of the phenolic compounds identified in the methanol extract of the pod surfaces of ICPL 87, quercetin (**2**) is the most widespread in the plant kingdom. This compound is found in many higher plants and frequently occurs in glycosylated forms, such as isoquercitrin (**1**) and rutin (Harborne et al., 1999). Quercetin-3-methyl ether (**3**) has a more restricted distribution and occurs mainly in the leaves, leaf resin, and flowers of the Compositae, Cistaceae, Cyperaceae, and Cactaceae (Harborne et al., 1999). The stilbene (**4**) has previously been reported only from the leaf surfaces of *Cajanus cajan* that had been challenged with the fungus, *Botrytis cinerea* (Cooksey et al., 1982). However, we found that the pod surfaces produced this compound in the apparent absence of fungal infection.

The data presented above show that larvae of *H. armigera* are able to perceive the methanol extract of the pod surfaces, as they consumed more of the disks impregnated with the methanol extract than untreated glass-fiber disks. While a methanol extract of the pod surfaces stimulated feeding, the response of larvae to the phenolics in the extract varied. For example, exposure of larvae to the stilbene

(**4**) resulted in larvae feeding on the untreated disk, while the other compounds did not affect selection behavior. The fact that larvae did not feed on disks treated with isoquercitrin (**1**) and quercetin (**2**) may have been because they were at too low a concentration to elicit a feeding response from larvae of *H. armigera*. Isoquercetrin (**1**) has been reported to stimulate feeding of *Bombyx mori* (L.) (Hamamura et al., 1962), although it is common for quercetin and derivatives of quercetin not to affect the feeding behavior of Lepidoptera larvae (Lindroth and Peterson, 1988; Faini et al., 1997).

Protection of grain legumes from larvae of Noctuidae, by modulating the levels of phenolic compounds, has been proposed for developing genotypes of chickpea that produce concentrations of isoflavonoids that are deterrent and growth inhibitory to *H. armigera* (Simmonds and Stevenson, 2001). Similarly, development of cultivars of groundnut that produce concentrations of quercetin glycocides and phenylpropanoids deterrent to larvae of *Spodoptera litura* (Fabricius) (Stevenson, 1993) have been suggested. Other phenolic compounds, such as schaftoside (an apigenin-*C*-glycoside) deter feeding by insects, such as the brown planthopper [*Nilaparvata lugens* (Stal)] (Grayer et al., 1994) and affect the growth of planthopper nymphs (Stevenson et al., 1996). Rutin (quercetin-3-*O*-rhamnosyl [1→6] glucoside) similarly deters feeding by *Heliothis zea* (Boddie) and *Helicoverpa armigera* at concentrations in excess of 10^{-3} M (Blaney and Simmonds, 1983).

Single compounds, in isolation, are unlikely to explain completely the interaction between larvae of *H. armigera* and pigeonpea. The response of larvae to combinations of compounds was complex. In a binary choice test, the response of larvae to one disk impregnated with both compound **3** and **4** was determined by the compound, either **3** or **4**, present on the other disk: stilbene (**4**) inhibited feeding on both disks, whereas quercetin-3-methyl ether (**3**) stimulated feeding. This result shows that quercetin-3-methyl ether (**3**) could modulate the antifeedant activity of the stilbene (**4**) at concentrations found in the susceptible cultivar, ICPL 87. However, the larval responses of **1–4** do not explain the phagostimulatory activity of the methanol extract of ICPL 87. It is clear from our results that plant host recognition of pigeonpea by larvae of *H. armigera* is not attributable to any of the individual phenolic compounds isolated in this study. Synergism between two or more compounds can determine host selection by gravid female Lepidoptera (Feeny et al., 1988; Roessingh et al., 1991; Carter et al., 1998). Thus, the interactions between phenolic and/or other compounds could similarly affect the feeding of larvae of *H. armigera* on pigeonpea.

The levels of identified compounds were found to vary among the *C. cajan* cultivars that vary in their susceptibility to pod-borers. Quercetin (**2**) was absent from all but the susceptible cultivar (ICPL 87). The ratios of quercetin-3-methyl ether (**3**) to the stilbene (**4**) were consistent among the three *C. cajan* cultivars (ICPL 332, ICPL 7203-1, and ICPL 84060) that are the least susceptible varieties to pod damage by larvae of *H. armigera* (Sharma et al., 2001) and were less

than occurred in the susceptible variety (ICPL 87). Therefore, an increase in the amount of quercetin-3-methyl-ether, relative to the stilbene, could inhibit feeding inhibition due to the stilbene once the ratio exceeds 1.2.

In conclusion, although a crude methanol extract of the pod surfaces of *C. cajan* stimulates feeding, it also contains phenolics that deter feeding (stilbene, **4**), stimulate consumption (quercetin-3-methyl ether, **3**), or that have no effect on selection or consumption (isoquercitrin, **1** and quercetin, **2**). Further studies are necessary to investigate the effects of different ratios and combinations of these phenolic compounds with the other (nonphenolic) compounds in pod surface extracts on food selection by larvae. Overall, data showed that the absolute amounts of stilbene (**4**) and its concentration relative to other phenolic compounds, especially quercetin-3-methyl ether (**3**), in pigeonpea genotypes could influence predation by larvae of *H. armigera*. Thus, there is a potential for using cultivars that produce high proportions of stilbene, relative to the other phenolics, in selective breeding programs for developing pigeonpea genotypes that are both less susceptible to fungal infection (Cooksey et al., 1982) and more resistant to pod-borers.

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EFFECT OF IRIDOID GLYCOSIDE CONTENT ON OVIPOSITION HOST PLANT CHOICE AND PARASITISM IN A SPECIALIST HERBIVORE

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Abstract—The Glanville fritillary butterfly *Melitaea cinxia* feeds upon two host plant species in Åland, Finland, *Plantago lanceolata* and *Veronica spicata*, both of which produce iridoid glycosides. Iridoids are known to deter feeding or decrease the growth rate of many generalist insect herbivores, but they often act as oviposition cues to specialist butterflies and are feeding stimulants to their larvae. In this study, two iridoid glycosides (aucubin and catalpol) were analyzed by micellar electrokinetic capillary chromatography. We measured the spatial and temporal variation of iridoid glycosides in natural populations of the host plants of *M. cinxia*. We also analyzed the aucubin and catalpol content in plants in relation to their use by ovipositing females, and in relation to the incidence of parasitism of *M. cinxia* larvae in natural populations. The mean concentrations of aucubin and catalpol were higher in *P. lanceolata* than in *V. spicata*, and catalpol concentrations were higher than aucubin concentrations in both host species. *Plantago lanceolata* individuals that were used for oviposition by *M. cinxia* had higher aucubin concentrations than random plants and neighboring plants. Additionally, oviposition and random plants had higher catalpol concentrations than neighboring plants, indicating that ovipositing females select for high iridoid glycoside plants or that oviposition induces iridoid glycoside production in *P. lanceolata*. Parasitism by the specialist parasitoid wasp *Cotesia melitaearum* occurred most frequently in larval groups that were feeding on plants with low concentrations of catalpol, irrespective of year, population, and host plant species. Therefore,

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parasitoids appear to avoid or perform poorly in host larvae with high catalpol content.

Key Words—Åland, aucubin, catalpol, *Cotesia melitaearum*, iridoid glycosides, oviposition, *Melitaea cinxia*, parasitism, *Plantago lanceolata*, *Veronica spicata*.

INTRODUCTION

Secondary compounds in plants often vary considerably both qualitatively and quantitatively among plant individuals and populations, as well as temporally and spatially (e.g., Puttick and Bowers, 1988; Jones and Firn, 1991; Bowers and Stamp, 1992; Bowers et al., 1992; Herms and Mattson, 1992; van Tienderen, 1992; Adler et al., 1995; Darrow and Bowers, 1997). Chemical defense by secondary compounds necessarily affects herbivores, but generalist herbivores and pathogens are generally more severely affected than specialist herbivores (e.g., Bernays and De Luca, 1981; Bowers and Puttick, 1988; Puttick and Bowers, 1988; Zangerl and Berenbaum, 1993; Koricheva et al., 1998). In this paper, we focus on iridoid glycosides, which are one important group of secondary defensive compounds produced by plants.

Iridoids are optically active cyclopentanoid monoterpenes, which can be divided into four distinct groups according to differences in chemical structure: iridoid glycosides, nonglycosidic (aglycone) iridoids, secoiridoids, and bisiridoids. Several hundred different structures of iridoid glycosides have been identified (El-Naggar and Beal, 1980; Boros and Stermitz, 1990, 1991), making them the most numerous iridoids. In this study, we are concerned with two iridoid glycosides, aucubin and catalpol, which are found in the plant families Apocynaceae, Bignoniaceae, Buddleiaceae, Callitrichaceae, Cornaceae, Eucommiaceae, Globulariaceae, Hippuridaceae, Lentibulariaceae, Loganiaceae, Orobanchaceae, Plantaginaceae, Scrophulariaceae, and Verbenaceae (El-Naggar and Beal, 1980). Catalpol is biosynthetically derived from aucubin (Damtoft et al., 1983).

Iridoids are deterrent to or decrease the growth rate of many generalist insect herbivores (Bernays and De Luca, 1981; Bowers and Puttick, 1988; Puttick and Bowers, 1988), and iridoid glycosides in nectar have been shown to deter nectar thieves (Stephenson, 1981, 1982). There is some indication that herbivory may induce the production of iridoid glycosides in *Plantago lanceolata* leaves, but variation in iridoid glycoside concentration with leaf age appears to be much greater (Darrow and Bowers, 1999; Stamp and Bowers, 2000). The iridoid glycosides aucubin and catalpol have been shown to be oviposition cues for the specialist butterfly *Junonia coenia* (Pereyra and Bowers, 1988). Moreover, iridoid glycosides are feeding stimulants to specialist butterfly larvae (Bowers, 1983, 1984; Adler et al., 1995), which grow and survive less (Bowers, 1984; Bowers and Puttick, 1988) or even refuse to feed on a diet without the stimulant iridoid glycosides

(L'Empereur and Stermitz, 1990b). However, Camara (1997a) has shown that there is a cost of chemical defense in the specialist *J. coenia* feeding on food with high iridoid glycoside concentration. Of the two focal iridoid glycosides in this study, catalpol is more toxic to generalist herbivores than aucubin (Bowers and Puttick, 1986, 1988; Bowers, 1991, 1992).

Specialist herbivores sequester iridoids, presumably to use as defense against natural enemies. Melitaeini butterflies sequester iridoids from their host plants as larvae and, at least in some cases, retain the toxic or noxious compounds as adults (Bowers, 1980, 1991; Bowers and Puttick, 1986; Stermitz et al., 1986; Gardner and Stermitz, 1988; Belofsky et al., 1989; Bowers and Farley, 1990; L'Empereur and Stermitz, 1990a,b; Bowers and Collinge, 1992; Dyer, 1995). The sequestration of iridoids by caterpillars has been shown to deter generalist predators like birds (Bowers, 1980), and *Junonia coenia* caterpillars fed with diets containing a high concentration of iridoid glycosides are rejected by several ant species (Dyer and Bowers, 1996; Camara, 1997c), stink bugs and predatory wasps (Stamp, 1992), and spiders (Theodoratus and Bowers, 1999).

The development of parasitoids can be influenced by plant secondary compounds eaten by their herbivorous hosts (Awmack and Leather, 2002). For instance, the alkaloid tomatine in host diet reduces rate of eclosion, size, and longevity of the generalist parasitoid *Hyposoter exiguae* (Campbell and Duffey, 1979, 1981), and nicotine, another alkaloid, in the diet of the host *Manduca sexta* reduces the survival and increases the development time of the parasitoids *Cotesia congregata* and *Hyposoter annulipes* (Barbosa et al., 1986; Thorpe and Barbosa, 1986). Specialist parasitoid wasps cannot avoid the plant secondary compounds sequestered by their hosts. In fact, specialist natural enemies may avoid generalist competitors by using chemically defended hosts. However, there are few studies of the effects of plant-derived chemicals in the host on the performance of specialist parasitoid wasps. Barbosa et al. (1986) demonstrated that the specialist parasitoid *C. congregata* performs better with nicotine than does the generalist parasitoid *H. annulipes*, but there are insufficient data on other species to generalize. It is likely that there is some physiological cost to metabolizing or otherwise enduring the compounds sequestered by the host; hence, the performance of immature parasitoids, even those specialized to use only chemically defended hosts, may depend at least to some extent on the concentration of the sequestered compounds. If this is so, then variation in secondary compounds among plant individuals may cause variation in successful parasitism of herbivores by specialist parasitoids.

The focal herbivore species in this study is the melitaeine butterfly *Melitaea cinxia*, which feeds on two host plant species, *Plantago lanceolata* and *Veronica spicata*, in our study area on the Åland Islands, in Southwest Finland (Hanski, 1999). Both host plant species contain aucubin and catalpol. The possible role of iridoid glycosides as oviposition cues or larval feeding stimulants has not been studied. However, larvae refuse to feed on artificial diet without host plant material

added, which suggests a role for iridoid glycosides in larval feeding (M. Camara, personal communication).

Iridoid glycoside concentrations are known to be genetically controlled in *P. lanceolata* (Marak et al., 2000) and to vary among populations, among individuals, and among genotypes within populations. Iridoid glycoside concentration also varies with the developmental state of the plant, including leaf and plant age, and attributes of the environment such as time of day, weather, soil nutrient conditions, and presence of arbuscular mycorrhizal fungi (Teramura, 1983; Bos et al., 1986; Bowers, 1991; Bowers and Stamp, 1992, 1993; Bowers et al., 1992; Fajer et al., 1992; van Tienderen, 1992; Stamp and Bowers, 1994; Gange and West, 1994; Adler et al., 1995; Darrow and Bowers, 1997). Here, we report on a study in which we measured the spatial and temporal variation of aucubin and catalpol in the host plants of *M. cinxia* in natural populations. We analyzed the aucubin and catalpol content in plants with respect to oviposition by female *M. cinxia* and in relation to the incidence of parasitism of larvae in natural populations.

METHODS AND MATERIALS

Study Area and Focal Species. The main Åland Islands in Southwest Finland have ca. 1000 km^2 of land area. Open meadows and pastures, which contain the host plants of *Melitaea cinxia,* form a fragmented habitat. The mean and median patch sizes are 1502 m^2 and 300 m^2, respectively (for a thorough description of the study area see Nieminen et al., 2003).

Melitaea cinxia (L.) (Lepidoptera: Nymphalidae) uses two plant species as hosts in Åland, *Plantago lanceolata* L. and *Veronica spicata* L. Both plant species are currently considered to be in the family Plantaginaceae (see Judd et al., 1999; Olmstead et al., 2001). *Plantago lanceolata* is widespread in Åland, occupying ca. 4000 meadows. *Veronica spicata* has a more restricted distribution, occurring in ca. 630 meadows. It has many populations in the northern, northwestern, and western parts of Åland but is completely absent from eastern Åland. Between these areas, there are sporadic occurrences of *V. spicata*. *Melitaea cinxia* lays eggs on the two host plants in June. The larvae live gregariously in silken webs until the last instar the following May [for a detailed description of the life cycle see Hanski (1999)]. On average, ovipositing females prefer *V. spicata* to *P. lanceolata* in *V. spicata*'s main distribution area, have no oviposition preference in the middle part of Åland, and prefer *P. lanceolata* in the east where only *P. lanceolata* is present (Kuussaari et al., 2000).

Cotesia melitaearum (Wilkinson) (Hymenoptera: Braconidae) is a specialist parasitoid of checkerspot butterfly larvae. In the Åland Islands, it uses *M. cinxia* as a host, living as small populations in several tightly clustered habitat patch networks (Lei et al., 1997; van Nouhuys and Hanski, 2002). The parasitoid is an important

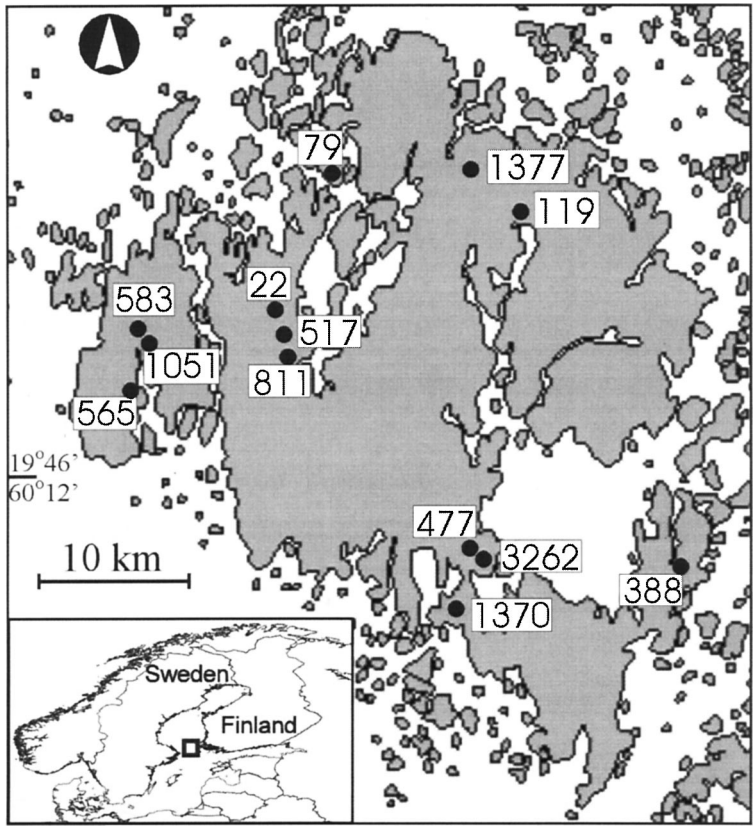

FIG. 1. Locations of sample sites in the Åland Islands. Inset map shows the position of Åland Islands in North Europe.

mortality agent for the butterfly, because the host larvae develop gregariously and there are two to three parasitoid generations per host generation (year). The parasitoid has been observed to cause local extinctions of host populations (Lei and Hanski, 1997).

Plant Samples. We took leaf samples from 13 habitat patches in different parts of Åland (Figure 1) at three times during the growing season in 1999 (Table 1). We selected habitat patches that were geographically representative and occupied by the butterfly. Randomly selected plant individuals were sampled from all parts of the habitat patches. Each *P. lanceolata* sample was an entire plant individual. *Veronica spicata* are larger, hence we collected two randomly selected stems from each plant, separating the leaves from the stems in the field.

TABLE 1. HOST PLANT INDIVIDUALS FOR WHICH IRIDOID GLYCOSIDE CONCENTRATIONS WERE ANALYZED[a*]

	Early summer, random		Mid-summer						Late summer, random	
			Oviposition		Neighboring		Random			
Patch	*Pl**	*Vs*	*Pl*	*Vs*	*Pl*	*Vs*	*Pl*	*Vs*	*Pl*	*Vs*
22		17		3	4	14	17	16	17	18
79			2	1			6	4		
119			2	1	15	5	4	6		
388	26	—					18	—	18	—
477				3	4		3	1		
517				2		12	3	5		
565			2	2	5	8	4	6		
583			24	4	19	10	4	4		
811							8	2		
1051							18	20	18	18
1370			1	—	9	—	10	—		
1377			3	2		14	4	6		
3262			2		5	2	6	4		

[a] Sample sizes from different habitat patches at different times of the season; for locations of the patches see Figure 1. **Pl* = *Plantago lanceolata*, *Vs* = *Veronica spicata*,—= corresponding host species missing from the habitat patch, empty cell = no material sampled.

We collected leaves into paper bags in the field and then left them in open plastic boxes between sheets of tissue paper to dry. In the laboratory, we stored plant samples in a freezer until analysis. After pretreatment, the extracts were analyzed as quickly as possible. If storage was necessary, they were stored at 4°C until analysis.

Host Plants Used for Oviposition. We collected data on host plant individuals used by egg-laying females from 10 habitat patches (Table 1, Figure 1) in order to study the factors that affect the oviposition plant use by female *M. cinxia*. We selected a random starting point within each patch, and around that point all host individuals were systematically searched for egg batches until at least two were found, or all plants had been investigated. If a patch had more than one separate subarea with host plants, the same procedure was repeated in each. If there was only one area with host plants, we searched the area from the random starting point until at least six egg batches were found, or all plants had been investigated. Plants that contained egg batch(es) or freshly hatched first instars are called oviposition plants. Fewer leaves were sampled from individual oviposition plants than from the other plants in order to avoid *M. cinxia* larval mortality due to starvation. These samples consisted of several leaves of different ages to avoid any potential bias due to leaf age.

In order to compare the characteristics of plants receiving eggs with those that did not, we sampled the 10 nearest host plants to each oviposition plant. These plants are called neighboring plants. Additionally, we sampled ten randomly selected plant individuals of each species from different parts of the patch. These plants are called random plants. Random plants were selected by throwing a stick (with one end sharpened) into the air, then throwing it again from the place where it dropped, and then sampling the host plant individual nearest to the sharp end of the stick. None of the random plants had eggs on them. These plants were also used for the measurement of seasonal and geographical variation in iridoid glycoside concentrations (the percentage of iridoid glycosides in dry weight) explained above.

We sampled oviposition, neighboring, and random plants for the determination of iridoid glycoside concentration as described above, except oviposition *P. lanceolata* plants of which maximally half of the leaves were sampled. Before taking leaf samples, we measured the following plant parameters: size (maximum diameter and height), number of stems for *V. spicata*, and number of leaves for both species (for *V. spicata* in two randomly selected stems), freshness (scale: 1 = completely fresh, 2 = withering, 3 = severely withered), and hairiness of the plant (scale: 1 = no hairs, 2 = moderately hairy, 3 = very hairy), growth form (mainly horizontal or erect leaves), flowering status, surrounding vegetation height, and percentage coverage of bare ground/rocks, lichen/moss, host plants, low herbs, low grasses, tall herbs, tall grasses, and scrub within a 30-cm radius. We also measured the location of the egg batch on the oviposition plant and the size of the leaf on which the egg batch was laid. We analyzed iridoid glycosides from all oviposition plants, but only a random subset of neighboring and random plants. The sample sizes from different habitat patches are given in Table 1.

Larval Samples. To detect whether *M. cinxia* larvae sequester iridoid glycosides from their food, we sampled prediapause *M. cinxia* larvae in July 1999 and postdiapause larvae in May 1999. We also analyzed postdiapause larvae reared on *P. lanceolata* in the laboratory in 2000. Larvae were starved for at least a day before freezing, dried at 50°C, and pretreated with the hot water extraction method optimized by Suomi et al. (2001).

Parasitism Rate. We measured the association of aucubin and catalpol concentration in individual plants with parasitism of *M. cinxia* larvae feeding on them in natural populations. In autumn 1999, we took leaf samples from all 34 plants with larval groups on them in patch 576, and all 22 plants with larval groups on them in patch 583. The samples were stored as described above, and the percent aucubin and catalpol in each sample was measured using the same procedure as described below. In spring 2000, we searched each of these larval groups for parasitoid cocoons. In autumn 2000, we took leaf samples from all plants with nests in four more populations occupied by the parasitoid (a total of 133 samples from patches 576, 583, 875, and 1071), and again searched for cocoons in the

following spring. Each population was known to be occupied by the parasitoid *C. melitaearum*. *Veronica spicata* occupied by larvae were sampled by collecting one stem (containing young and old leaves). *Plantago lanceolata* were sampled by collecting one young, one medium, and one old leaf. A few larval groups in each population were on plants that were too small to be sampled.

Reagents and Apparatus. We determined aucubin and catalpol concentrations using standards donated by Dr. S. R. Jensen (Department of Organic Chemistry, Danish Technical University, Lyngby, Denmark) with either an Hewlett-Packard ^{3F}CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) or a Beckman P/ACE 2000 capillary electrophoresis system (Beckman Instruments Inc., Palo Alto, California, USA) with a Compaq Prolinea 5100e computer system. The compounds had been extracted from plant material with ethanol (Damtoft et al., 1997), purified by RP-HPLC, and identified by UV detection at wavelengths 206 and 254 nm. We pretreated the plant and larval samples by hot water extraction in a heating block for test tubes.

Disodium tetraborate (borax, $Na_2B_4O_7 \cdot 10H_2O$) and standard solutions of 0.10 M and 1.0 M NaOH were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS, 99% pure) was supplied by BDH (Poole, UK). CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid, $C_8H_{17}NO_3S$] was purchased from Sigma (St. Louis, Missouri, USA). The water used in the experiments was first distilled and then purified further with a Water-I instrument (Gelman Sciences, Ann Arbor, Michigan, USA) until its resistance was 18 MΩ.

Iridoid Glycoside Concentrations. In addition to iridoid glycosides, the plant samples contain hundreds of other substances, some of which can interfere with the analyses. We used a sample pretreatment to extract the iridoid glycosides before analysis. Most researchers have used methanol or ethanol as extraction solvents for iridoid glycosides (Gardner and Stermitz, 1988; Camara, 1997c; Damtoft et al., 1997). However, hot water extraction (HWE) is an excellent pretreatment method for the isolation of iridoid glycosides because no organic solvents are needed, the extraction is simple, and the extraction of aucubin and catalpol is more quantitative than when extracted using alcohols (Suomi et al., 2000). In the optimization of the pretreatment methods for heat-dried plant (Suomi et al., 2000) and larval samples (Suomi et al., 2001), we compared the results with those obtained by employing the more commonly used alcohol extraction technique. For larval samples, the hot water extraction was slightly more quantitative than extraction with methanol, while for plant samples the HWE was significantly more efficient than the reference method.

Iridoid glycosides generally tend to be hydrolyzed and subsequently undergo rearrangement even under mildly acidic conditions (Bianco, 1990). Thus, they must be treated and analyzed under strictly basic conditions. Still, some structures are more unstable and may hydrolyze even under basic conditions or if heated (Inouye, 1991). Usually, iridoid glycosides are analyzed with chromatographic techniques

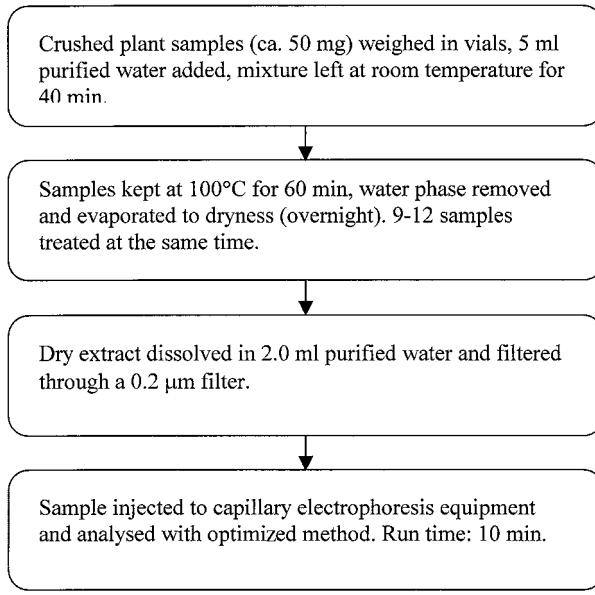

FIG. 2. Scheme of the pretreatment of plant samples. The weight of the sample was measured with an accuracy of 0.1 mg.

(gas chromatography, liquid chromatography, or thin-layer chromatography), but capillary electrophoresis (CE) with high speed and resolution was employed in this study. There are several different capillary electromigration techniques, of which micellar electrokinetic capillary chromatography (MECC) is suitable for neutral compounds like catalpol and aucubin. The separation in CE is based on different mobilities of the analytes in a high electric field across a narrow, fused silica capillary that is filled with an electrolyte solution. The mobilities depend on the size and charge of the compound. In MECC, the separation of neutral analytes is mainly based on partition between a micellar pseudostationary phase and the electrolyte solution.

A diagram of the hot water extraction procedure (Suomi et al., 2000) is shown in Figure 2, and the CE analysis conditions are listed in Table 2. We measured linearity using solutions of pure aucubin and catalpol prepared in purified water. The solutions were stored in a refrigerator when not in use. We observed no degradation of the molecules during a 4-month period. The same samples were analyzed with both CE instruments, verifying that the results were comparable.

In the Hewlett-Packard electrophoresis experiments, the concentration of micellar forming agent SDS was optimized. In the Beckman experiments, the concentrations of both components of the electrolyte solution were optimized.

TABLE 2. MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY (MECC) ANALYSIS CONDITIONS FOR QUANTITATIVE DETERMINATION OF AUCUBIN AND CATALPOL

Parameter	Hewlett-Packard 3F CE	Beckman P/ACE 2000
Capillary dimensions	50 μm ID, 375 μm OD, 41.5 cm to detection window, total length 50.0 cm	50 μm ID, 375 μm OD, 40.0 cm to detection window, total length 47.0 cm
Electrolyte solution	50 mM CHES, 120 mM SDS, pH 9.4	30 mM borax, 140 mM SDS, pH 9.4
Sample injection	Pressure: 50 mbars $\times$ 5 sec	Pressure: 35 mbars $\times$ 10 sec
Voltage and current	+15 kV, 35 μA	+15 kV, 90 μA
Analysis time	<5 min	<7 min
Limit of detection	Catalpol: 30 μg/ml Aucubin: 20 μg/ml	Catalpol: 20 μg/ml Aucubin: 20 μg/ml
Detection	200 nm, reference 250 nm	200 nm
Linearity	50–300 μg/ml for both compounds	50–400 μg/ml for both compounds
Analysis temperature	25°C	25°C
Cooling of the capillary	By air flow	By fluoro-organic fluid flowing outside the capillary
Detector	Diode array detector (spectra could be taken)	Single wavelength detector (six possible wavelengths)

The electrolyte was changed to borax to facilitate manual integration of analyte peaks.

Between analyses, the capillary was rinsed with the electrolyte solution for 8 min, and after three successive analyses it was rinsed with 0.1 M NaOH (2 min), water (5 min), and electrolyte solution (2 min). This was done to avoid blockage. The capillary was regenerated each morning by rinsing it with 0.1 M NaOH (10 min), water (15 min), and electrolyte solution (15 min). This was also done when the capillary was changed.

Statistical Methods. Iridoid glycoside concentrations were square-root transformed to normalize their distributions. For normally distributed data, we used one- or two-way analysis of variance, analysis of covariance, linear regression, *t* test, or Pearson correlation. To study the effects of plant category and freshness with their interaction, we used parametric ANOVA on ranks, because we were not able to normalize the iridoid glycoside distributions in this case. We analyzed the effects of the two iridoid glycosides on different variables using multivariate analysis of variance (MANOVA) because the iridoid concentrations are correlated. We also report the results of separate univariate ANOVA F tests for catalpol and aucubin with Bonferroni correction for significance (adjusted critical P value is 0.025). For testing goodness of fit in 2×2 tables, we used Fisher's exact test. We measured

the association between parasitism (presence/absence) and plant iridoid glycoside concentration using logistic regression. Year, population, host plant species, and the iridoid glycoside concentration were included in the logistic model as explanatory variables. We used SAS 6.0, Statistix 7, and Systat 8.0 for the statistical analyses.

RESULTS

Spatial and Temporal Variation in Iridoid Glycoside Concentrations in Host Plants. Figure 3 shows the mean aucubin and catalpol concentrations of both host plants for various classifications of the data. There was a significant positive correlation between aucubin and catalpol concentrations in both hosts (all samples included), but the correlation was stronger in *P. lanceolata* ($r = 0.761$, $P < 0.001$, $N = 347$) than in *V. spicata* ($r = 0.496$, $P < 0.001$, $N = 277$). The mean concentrations of aucubin and catalpol were higher in *P. lanceolata* than in *V. spicata* (all samples: Table 3; Figure 3A), and catalpol concentrations were higher than aucubin concentrations in both host species (all samples: Table 3; Figure 3A).

In early summer, the concentrations of aucubin and catalpol in randomly selected individuals of the two plant species did not differ between species (Table 3, univariate test; Figure 3B). However, because aucubin was slightly lower in *V. spicata* and catalpol slightly higher than in *P. lanceolata*, the overall iridoid makeup was different (Table 3, multivariate test; Figure 3B). Both aucubin and catalpol concentrations were higher in *P. lanceolata* than in *V. spicata* in mid- and late summer (Table 3; Figure 3B).

In randomly selected *P. lanceolata* individuals, there was no difference during the season in aucubin concentrations, but catalpol concentrations were higher in late and mid-summer than in late spring (Table 3; Figure 3B). In random *V. spicata* individuals, there was no difference during the season, either in aucubin or in catalpol concentrations (Table 3; Figure 3B).

Aucubin concentrations in random *P. lanceolata* differed among four habitat patches in mid-summer (Table 3; Figure 3C). There was no evident spatial pattern in this variation, because only patches 1370 (highest mean concentration) and 1051 (lowest mean concentration) differed significantly from each other (patches 22 and 388 did not differ from any other patch; Figure 1). There was also a significant difference in catalpol concentrations between the highest (patch 1370) and the lowest (388) mean concentration, and between patches 1370 and 1051 (Table 3; Figure 3C). In late summer, there were no differences among three habitat patches sampled (22, 388, and 1051) in aucubin or catalpol concentrations (Table 3; Figure 3D).

In random *V. spicata*, aucubin concentrations differed among five patches in mid-summer (Table 3; Figure 3C). Again, there was no apparent spatial pattern in

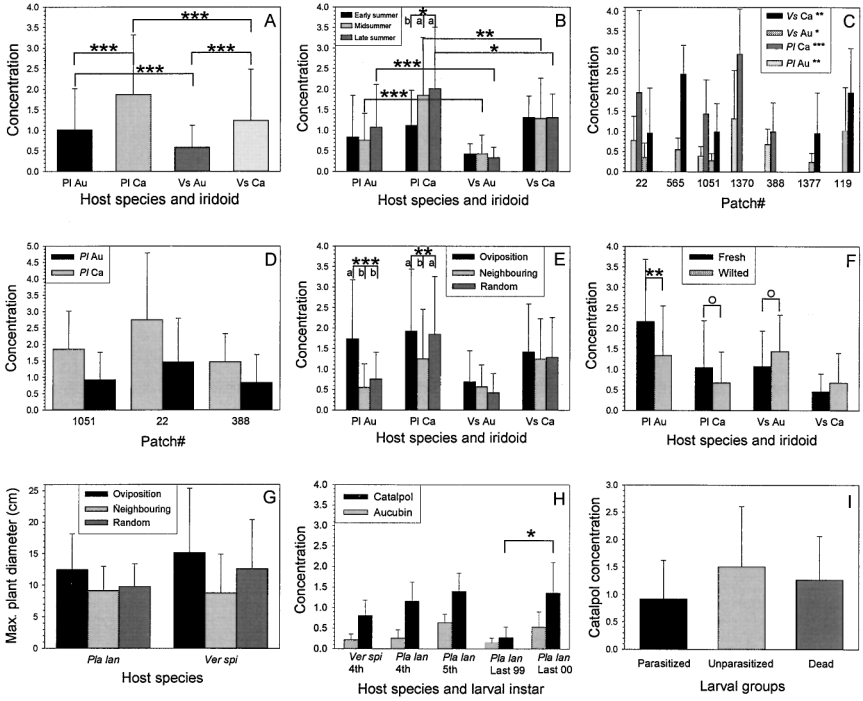

FIG. 3. (A) Average aucubin (Au) and catalpol (Ca) concentrations with standard deviations in all samples of *Plantago lanceolata* (Pl) and *Veronica spicata* (Vs). (B) Concentrations at three times during the growing season in random plants. (C) Concentrations in several habitat patches in mid-summer (random plants). See Figure 1 for the locations of the patches. (D) Concentrations in some habitat patches in late summer (only *P. lanceolata* samples; random plants). (E) Concentrations in oviposition, neighboring, and random plants in mid-summer. (F) Concentrations in completely fresh and somewhat wilted plant individuals in mid-summer (oviposition, neighboring, and random plants). (G) Average sizes with standard deviations of oviposition, neighboring, and random plants of both host plant species. The size measure used was the maximum diameter of the plant individual. *Plantago lanceolata* individuals used for oviposition were larger than neighboring plants, but random plants did not differ from oviposition or neighboring plants ($F = 3.24$, $df = 119$, $P = 0.043$). (H) Average aucubin and catalpol concentrations in *M. cinxia* larvae of different instars and fed with different hosts (99 = year 1999, 00 = year 2000). (I) Average catalpol concentrations with standard deviations in host plants with *M. cinxia* larval groups either parasitized by *C. melitaearum*, unparasitized or dead during winter. (See Table 3 for exact results of statistical tests. $^{\circ}P = 0.05$-0.025; $^{*}P < 0.025$; $^{**}P < 0.01$; $^{***}P < 0.001$; a and b indicate statistically significantly different comparisons (Tukey's test).)

TABLE 3. SUMMARY OF STATISTICAL TEST RESULTS[a]

Comparison	Panel	Test statistic	*df*	*P*
P. lanceolata versus *V. spicata*[b]				
Multivariate	A	20.4	2, 622	<0.001
Aucubin	A	37.3	1, 623	<0.001
Catalpol	A	30.7	1, 623	<0.001
Aucubin versus catalpol[b]				
P. lanceolata	A	$T = 19.2$	346	<0.001
V. spicata	A	$T = 15.9$	277	<0.001
P. lanceolata versus *V. spicata* in early summer[c]				
Multivariate	B	6.15	2, 40	0.005
Aucubin	B	2.43	1, 41	0.13
Catalpol	B	1.74	1, 41	0.19
P. lanceolata versus *V. spicata* in mid-summer[c]				
Multivariate	B	9.46	2, 175	<0.001
Aucubin	B	19.0	1, 176	<0.001
Catalpol	B	8.59	1, 176	0.004
P. lanceolata versus *V. spicata* in late summer[c]				
Multivariate	B	10.4	2, 86	<0.001
Aucubin	B	20.7	1, 87	<0.001
Catalpol	B	5.80	1, 87	0.018
Seasonal change in *P. lanceolata*[c]				
Multivariate	B	4.00	4, 360	0.003
Aucubin	B	1.64	2, 180	0.20
Catalpol	B	4.49	2, 180	0.013
Seasonal change in *V. spicata*[c]				
Multivariate	B	1.34	4, 248	0.26
Aucubin	B	1.09	2, 124	0.34
Catalpol	B	0.59	2, 124	0.56
Between patches in *P. lanceolata* in mid-summer[c]				
Multivariate	C	4.99	6, 118	<0.001
Aucubin	C	4.28	3, 59	0.008
Catalpol	C	6.13	3, 59	0.001
Between patches in *P. lanceolata* in late summer				
Multivariate	D	1.50	4, 100	0.21
Aucubin	D	1.66	2, 50	0.20
Catalpol	D	2.92	2, 50	0.063
Between patches in *V. spicata* in mid-summer[c]				
Multivariate	C	3.15	8, 98	0.003
Aucubin	C	3.35	4, 49	0.017
Catalpol	C	4.21	4, 49	0.005
P. lanceolata between oviposition, neighbor, and random				
Multivariate	E	16.7	4, 396	<0.001
Aucubin	E	19.3	2, 198	<0.001
Catalpol	E	6.26	2, 198	0.002
V. spicata between oviposotion, neighbor, and random				
Multivariate	E	1.50	4, 304	0.20
Aucubin	E	1.56	2, 152	0.21
Catalpol	E	0.07	2, 152	0.94

TABLE 3. CONTINUED

Comparison	Panel	Test statistic	*df*	*P*
Between fresh and wilted *P. lanceolata*[b]				
Multivariate	F	4.50	2, 117	0.013
Aucubin	F	8.94	1, 118	0.003
Catalpol	F	4.39	1, 118	0.038
Between fresh and wilted *V. spicata*[b]				
Multivariate	F	2.28	2, 102	0.11
Aucubin	F	4.09	1, 103	0.046
Catalpol	F	2.62	1, 103	0.11
Between fourth instar larvae on *P. lanceolata* and *V. spicata*				
Multivariate	H	1.66	2, 18	0.22
Aucubin	H	0.33	1, 19	0.58
Catalpol	H	3.36	1, 19	0.08
Between last instar larvae in 1999 and 2000				
Multivariate	H	8.05	2, 7	0.015
Aucubin	H	4.41	1, 8	0.07
Catalpol	H	10.4	1, 8	0.012

[a] Panel = panels as in Figure 3; test statistic = F test results from univariate ANOVA (Bonferroni-adjusted critical P value is 0.025), if no other test statistic is mentioned, or multivariate ANOVA (= Pillai test results).
[b] All samples.
[c] Random plants (in early and late summer only random plants could be sampled).

the differences, as only patches 119 (highest mean concentration) and 1051 (second lowest mean concentration) differed from each other, but not from patches 565, 22, or 1377 (Figure 1). Catalpol concentrations also varied among habitat patches but without any spatial pattern (Table 3; Figure 3C). Only patch 565 (highest concentration) differed from patches 1051 (third lowest mean concentration) and 22 (lowest mean concentration), while the other pairwise differences were not significant.

Hosts Used for Oviposition. Thirty-six egg batches or groups of newly hatched larvae were found on *P. lanceolata* and 18 on *V. spicata* in 10 habitat patches (Table 1). *Plantago lanceolata* individuals that were used for oviposition by *M. cinxia* in the field had significantly higher aucubin concentrations than random plants and neighboring plants (Table 3; Figure 3E). Furthermore, oviposition and random plants had higher catalpol concentrations than neighboring plants.

Fresh *P. lanceolata* individuals had higher aucubin and catalpol concentrations than somewhat wilted individuals (Table 3; Figure 3F). Eggs did not appear to be laid preferentially on either fresh or wilted plants (Fisher's exact test: oviposition plants versus neighboring plants $P = 0.30$, oviposition plants versus random

plants $P = 0.54$). In a two-factor ANOVA on ranks, the effects of plant category and freshness on aucubin concentrations were significant, but their interaction was not. Only the effect of plant category on catalpol concentrations was significant in a two-factor ANOVA on ranks. Therefore, female butterflies appear to select higher iridoid glycoside concentrations independently of the freshness of *P. lanceolata* individuals.

Iridoid glycoside concentrations did not differ significantly between fresh and somewhat wilted *V. spicata* plants (Table 3; Figure 3F). Neither plant type, freshness, nor their interaction was significant in two-factor ANOVAs. Fresh plants were used for oviposition in proportion to wilted plants (Fisher's exact test: oviposition plants versus neighboring plants $P = 0.36$, oviposition plants versus random plants $P = 1.0$).

Small *P. lanceolata* individuals had higher aucubin concentrations than large ones (size measure was square-root transformed maximum diameter of the individual host plant; coefficient $= -0.252$, Student's $t = -3.29$, $P = 0.001$, $R^2 = 0.08$). Plants used for oviposition were larger than neighboring plants, but random plants did not differ from oviposition or neighboring plants ($F = 3.24$, $df = 119$, $P = 0.043$; Figure 3G). Aucubin concentrations were higher in oviposition plants independent of plant size, as their interaction was not significant in an analysis of covariance. Plant size was not associated with catalpol concentrations (coefficient $= -0.064$, Student's $t = -1.00$, $P = 0.32$, $R^2 = 0.01$).

There were no significant associations between iridoid glycoside concentrations and the hairiness of leaves or the flowering status of individuals in either of the species.

Larval Samples. The average aucubin and catalpol concentrations in fourth instar larvae in August 1999 were 0.26 and 1.16% of dry weight in *P. lanceolata*-feeding larvae ($N = 14$), and 0.22 and 0.81% in *V. spicata*-feeding larvae ($N = 7$). The differences in iridoid glycoside concentrations were not significant (Table 3; Figure 3H). The average aucubin and catalpol concentrations in the last instar *P. lanceolata*-feeding larvae were 0.16 and 0.27% of dry weight in May 1999 ($N = 5$), and 0.53 and 1.36% in May 2000 ($N = 5$). The difference was different only for catalpol (Table 3; Figure 3H), but due to the small sample size, the results are suspect.

Parasitism Rate. Plants on which larval groups of *M. cinxia* occurred ($N = 189$) were sampled in the autumn of 1999 and 2000. During the subsequent winters, 62 of these larval groups died. Twenty of the remaining 124 groups were parasitized by *C. melitaearum*. Parasitism occurred most frequently in larval groups that were feeding on plants with a low concentration of catalpol (logistic regression, effect of catalpol concentration $F_{1,178} = 4.43$; $P = 0.04$, coefficient $= -0.23$; Wald's $\chi^2 = 26.29$, $P < 0.001$; Figure 3I). This was true irrespective of year, population, and host plant species. We found no association between parasitism and aucubin concentration.

DISCUSSION

Chemical Analyses. The concentrations of aucubin and catalpol in *Plantago lanceolata* and *Veronica spicata* were reliably and quickly determined by MECC. Hot water extraction used for the isolation of the compounds was reproducible and simple. In addition, it avoided the use of organic solvents, which burden the environment. Because the amounts of iridoid glycosides isolated from plant samples were so high, the limit of detection did not cause any problems. In fact, most extracts had to be diluted so that they could be reliably quantified.

Iridoid Glycoside Concentrations in Plants. Aucubin and catalpol concentrations have been intensively studied especially in *P. lanceolata*, which allows comparisons of our results from Finland with studies mainly performed in the United States. For example, the biomass and iridoid glycoside content of *P. lanceolata* were analyzed four times during one growing season in five different populations by Darrow and Bowers (1997). They found no correlation between the iridoid glycoside content and biomass, but both biomass and iridoid glycoside concentration varied among sampling dates and among populations (concentrations increased during the season). Our results showed differences in aucubin concentration among populations in mid-summer. The differences in the mid-summer may be important for ovipositing females, as well as for newly hatched larvae, which are vulnerable to various adverse environmental effects.

In several American studies on *P. lanceolata*, the average content of catalpol in the leaves has been lower than that of aucubin (Darrow and Bowers, 1997, 1999; Stamp and Bowers, 2000), although in one study the reverse was observed (Bowers, 1991). In our data from Finland, catalpol concentrations were higher than aucubin concentrations in mid- and late summer and overall. In both American studies in which plants were sampled from the field, the aucubin concentration was higher [ca. 1.6–2.7% in Bowers (1991), ca. 0.5–5% in Darrow and Bowers (1997)] than in Finland (ca. 0.6–2.2% in this study). Catalpol concentrations were similar to our results in those studies [ca. 0.4–3.6% in Bowers (1991), ca. 0.2–2.2% in Darrow and Bowers (1997), ca. 0.7–2.0% in this study]. Interestingly, in the two American studies in which laboratory-reared plants were used, catalpol concentrations were extremely low (ca. 0–0.6%) (Darrow and Bowers, 1999; Stamp and Bowers, 2000). The iridoid glycoside concentrations in *V. spicata* have not been studied previously. We found that the iridoid glycoside concentrations did not differ over the season, but aucubin did differ among populations.

Stamp and Bowers (1994) found that iridoid glycoside concentrations in *P. lanceolata* increase over the growing season in dry years and decrease in wet years. Therefore, temporal variation in catalpol concentrations in our data could be related to the wet summer of 1998, with low iridoid glycoside concentrations in host plants remaining in the following spring, and to the dry summer of 1999, with high iridoid glycoside concentrations remaining in the next spring. The mid-summer

of 1999 was extremely dry, and many host plants were either completely dry or at least wilted during the oviposition period of *M. cinxia*. However, fresh plants were not used for oviposition more often than wilted ones, even though fresh plants contained more aucubin. As wilting of host plants is detrimental for the newly hatched larvae, it would be beneficial to avoid such plants for oviposition. It is, of course, possible that the host plants wilted after oviposition, but before our sampling.

Role of Iridoid Glycosides in Oviposition. Melitaea cinxia females lay eggs more frequently on *P. lanceolata* with high iridoid glycoside concentrations than on individuals with low concentrations. Females of the butterfly *Junonia coenia* are known to select for *P. lanceolata* with high aucubin and catalpol concentrations for oviposition (Pereyra and Bowers, 1988), and our results suggest the same for *M. cinxia* searching for *P. lanceolata* as the host plant. However, there was no difference in iridoid glycoside concentration among *V. spicata* individuals used for oviposition and random *V. spicata*. Both spatially and temporally, *V. spicata* had more stable iridoid glycoside concentrations than *P. lanceolata*, and the aucubin and catalpol concentrations were also significantly lower. These characteristics may make the identification of high iridoid glycoside plants among *V. spicata* individuals difficult for ovipositing females. There may also be some as yet undetected iridoids, which would explain the host selection, and we are currently searching for them in these plant species.

There is also the possibility that butterflies do not choose iridoid glycoside-rich *P. lanceolata* but that the presence of herbivore eggs induces increased production of iridoid glycosides by the plant. We do not yet have any evidence for this, but iridoid glycoside production can apparently be induced by herbivory in *P. lanceolata* (Darrow and Bowers, 1999; Stamp and Bowers, 2000), and there are examples of induction of plant defense by eggs in other plant herbivore systems (Agrawal, 2000). If this were the case, then perhaps the iridoid glycoside concentrations of neighboring plants, which one might expect to be similar to the oviposition plants, have lower iridoid glycoside concentrations because they have not been induced. Yet another possibility is that the iridoid glycoside levels in the chosen plants are high due to attacks by *M. cinxia* larvae or some other herbivores in the spring or previous summer, which again may cause induced defense.

Larvae of some *Euphydryas* species (close relatives to *M. cinxia*) and *Junonia coenia* sequester catalpol selectively from *P. lanceolata* (Bowers and Puttick, 1986; Bowers and Collinge, 1992), and *E. anicia* even metabolizes another iridoid glycoside into catalpol (Gardner and Stermitz, 1988). Catalpol is especially important to the chemical defense of larvae because it is more toxic to generalist herbivores than aucubin (Bowers and Puttick, 1986, 1988; Bowers, 1991, 1992). *Melitaea cinxia* larvae appear also to sequester catalpol more efficiently than aucubin, as the catalpol–aucubin ratio in *P. lanceolata*-feeding larvae in late summer of 1999 was higher in larvae (6:1) than in the plants (2:1; Figure 3). Interestingly,

M. cinxia larvae appear not to sequester catalpol more efficiently than aucubin from *V. spicata*, as the catalpol–aucubin ratio in *V. spicata*-feeding larvae in late summer of 1999 was the same in larvae (4:1) as in the plants (4:1; Figure 3). This finding requires further research because, as we have seen, plant iridoid glycoside concentration is variable, and the plants used for iridoid glycoside determinations were different individuals from those fed upon by the larvae.

The average catalpol concentration in the *P. lanceolata*-feeding fourth instar larvae was only 1.2% of dry weight, and the maximal concentrations detected in *M. cinxia* larvae so far are ca. 2% of dry weight (Suomi et al., 2001), as opposed to the maximum of 9.3% in *P. lanceolata* in our study. Bowers and Collinge (1992) observed notably higher average catalpol concentrations for larvae of *J. coenia* with a maximum of 3% dry weight in fifth instars. However, the iridoid glycoside concentrations in larvae and adult butterflies seem highly variable. For example, Gardner and Stermitz (1988) found mean catalpol concentrations of 3.1% and 0.8% dry weight in *Euphydryas anicia* males in successive years, and Suomi et al. (2001) noticed that the average catalpol concentrations in last instar *M. cinxia* larvae were 0.3% and 1.4% of dry weight in the springs of 1999 and 2000. These results call for further research, as we have so far not sampled a large number of larvae.

Role of Iridoid Glycoside in Parasitism. As shown above, *M. cinxia* females appear to use *P. lanceolata* with higher iridoid glycoside concentrations for oviposition than is expected by random choice of plant individuals. Moreover, larvae effectively sequester catalpol from *P. lanceolata*, which is probably related to their defense against natural enemies. It has been suggested that natural enemies (predators in this case) are the driving force for the oviposition host preference by female *Junonia coenia*, a species with high iridoid glycoside concentrations in larvae (Camara, 1997b). Van Nouhuys and Hanski (1999) showed that *Cotesia melitaearum*, a specialist parasitoid of *M. cinxia* in Åland, successfully parasitizes larval groups feeding on *V. spicata* more often than larval groups, which feed on *P. lanceolata*. They suggested several possible explanations for this difference, one of which was plant chemistry, but as olfactory cues to locate host larvae. It may be, as iridoid glycosides are used both in plant and herbivore defense against their respective natural enemies, that high concentrations in larvae may function as deterrents to ovipositing *C. melitaearum* or may cause mortality of parasitoids developing within relatively well defended hosts. *Cotesia melitaearum* are specialist parasitoids, only using hosts feeding on iridoid producing plants (Lei et al., 1997; van Nouhuys and Hanski, 2003), so one may expect them to be adapted to high iridoid concentrations. Indeed, laboratory studies using artificial diet suggest that high iridoid glycoside concentration does not reduce acceptance of hosts by *C. melitaearum* adults or the survival of developing *C. melitaearum* larvae (Lei and Camara, 1999).

Nevertheless, our result, that parasitoids are found in *M. cinxia* larval groups feeding on plants with low catalpol concentration, lends support to the latter

hypothesis. A low rate of successful parasitism of larvae feeding on especially high iridoid glycoside *P. lanceolata* might explain why female *M. cinxia* select the latter plants for oviposition. This does not happen with *V. spicata* as the host plant, perhaps because the iridoid concentrations may not be high enough to deter parasitoids and because larvae feeding on *V. spicata* do not appear to be able to accumulate catalpol as efficiently as those feeding on *P. lanceolata*. If such selectivity by the ovipositing butterfly would reduce parasitism by *C. melitaearum* on larvae feeding on *P. lanceolata*, it might also reduce consumption by generalist invertebrate predators and decrease competition with generalist herbivores.

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EFFECTS OF *Acacia* CONDENSED TANNINS ON URINARY PARAMETERS, BODY MASS, AND DIET CHOICE OF AN *Acacia* SPECIALIST RODENT, *Thallomys nigricauda*

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Abstract—The aim of this study was to investigate the dietary and physiological effects of condensed tannin ingestion on foregut fermenters, using *Thallomys nigricauda*, a folivorous rodent, as a model. We initially investigated the variability in physiological parameters, such as daily body mass (DM_b), daily feed intake, daily fecal energy loss (FE), daily energy intake (DEI), daily urine pH, and daily urinary ammonia and urea concentrations, in response to different diets with low condensed tannin levels. This experiment was conducted to identify which physiological variables showed the least variation in the absence of tannin. In a second experiment, we investigated the response of the same dietary and physiological parameters to the effects of high dietary condensed tannin ingestion in *T. nigricauda*. We hypothesized that DM_b, daily feed intake, FE, and DEI of *T. nigricauda* would be adversely affected by high dietary tannin content. We predicted that detoxification activity by *T. nigricauda* would increase at higher tannin levels. Ingestion of tannins affected the nutritional status of *T. nigricauda*, as shown by a decrease in body mass at high tannin levels. We also found that fewer ammonium ions were excreted in the urine by *T. nigricauda*, as would be expected if this were a means of regulating metabolic acidosis. The urine produced was more alkaline. This result indicates that *T. nigricauda* is not metabolizing these allelochemicals. Urea production was initially reduced, indicating conservation of bicarbonate ions that will neutralize blood acidity if there

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is detoxification. A diet choice experiment showed that tree rats avoid high tannin diets, even to the extent that they lose body mass on an alternative diet. This last-mentioned result is noteworthy because previous studies of the effects of tannins on herbivorous mammals have shown that there is physiological control rather than behavioral avoidance of the negative effects of tannin ingestion.

Key Words—Tannins, digestion, herbivory, food selection, *Thallomys nigricauda*, folivorous rodent.

INTRODUCTION

Tannins reduce dietary quality in several ways (Rhoades, 1979; Foley and McArthur, 1994). They affect physiological, nutritional and metabolic aspects of the herbivorous consumer (Mole et al. 1990; Foley and McArthur, 1994). By binding to dietary and endogenous proteins, including digestive enzymes, tannins reduce the digestibility of protein (Foley and Hume, 1987; Robbins et al., 1991; Hanley et al., 1992; Chung-MacCoubrey et al., 1997). Tannins have also been implicated in reducing fiber digestion in mammalian herbivores (Barry et al., 1986; Robbins, 1993; Chung-MacCoubrey et al., 1997; Dearing, 1997) and are potentially toxic because they are absorbed across the alimentary tract (Robbins et al., 1991; Dearing, 1997). By creating toxicity, tannins potentially reduce dietary intake and, consequently, growth (Freeland and Janzen, 1974; Robbins et al., 1987; Owen-Smith et al., 1993; Foley and McArthur, 1994).

Considerable research has been conducted on the effects of tannins on herbivores and the associated ecological implications (reviewed in Foley et al., 1999). Despite this body of research, the dietary and physiological effects of tannins are not clearly understood (Hagerman et al., 1992; Foley and McArthur, 1994; Foley et al., 1999). The widely accepted view of tannins as digestibility reducers (Feeny, 1976; Rhoades and Cates, 1976) has not been demonstrated in all cases (Robbins et al., 1987; Foley et al., 1999). Tannins have been shown to promote rather than inhibit digestive enzymes in some cases (Mole and Waterman, 1985). Tannins may also impose greater costs to the consumer as toxins rather than as digestibility reducers (Robbins et al., 1987; Dearing, 1997).

The effectiveness of tannins as antiherbivore defenses has been questioned because of the many counterdefenses of herbivores (Robbins et al., 1987). For example, some mammalian herbivores produce salivary tannin-binding proteins (TBPs) that show a high affinity for tannins and preferentially bind to them (Robbins et al., 1987; Mole et al., 1990; Owen-Smith, 1993). Foregut fermenters may have an additional line of defense against tannins. In these species, the principal fermentation chamber is a modified part of the stomach (Alexander, 1993), and detoxification, which may be microbial (Crawley, 1983; Foley and McArthur, 1994), occurs in the forestomach. Because this occurs early in the digestive process, enzymatic digestion in subsequent parts of the gastrointestinal tract is not hampered by poisonous

substances (Freeland and Janzen, 1974). It has also been proposed that foregut fermenters are able to avoid metabolic acidosis, an imbalance in the acid–base balance of the blood that occurs as a consequence of detoxification (Foley, 1992), by producing TBPs (Dearing, 1997). Another form of response by mammalian herbivores to tannins lies in their excretion when conjugated to small molecules, e.g., glucuronic acid (Foley and McArthur, 1994). By countering the effects of tannins, herbivores may exhibit little or no external evidence of physiological stress, indicating that the costs of physiological defense may be low (Thomas et al., 1988).

A quantitative measure of the biological effectiveness of tannins is fundamental to understanding their ecological and evolutionary role in herbivore–plant interactions (Hanley et al., 1992, Cork et al., 1999). As yet, no techniques or equations to evaluate the role of tannins as plant defenses have been validated (Robbins et al., 1987), and models pertaining to herbivore–plant interactions cannot be developed further (Cork et al., 1999). The effects and, in particular, the costs incurred to the animal through tannin ingestion must be quantified (Foley and McArthur, 1994; Foley et al., 1999).

The aim of this study was to investigate the dietary and physiological effects of condensed tannin ingestion on foregut fermenters, using *Thallomys nigricauda*, a folivorous rodent, as a model. Black-tailed tree rats are distributed throughout arid and semiarid regions of South Africa, Botswana, and Namibia (De Graaff, 1981; Skinner and Smithers, 1990). They are arboreal and inhabit *Acacia* woodlands, most notably those dominated by *A. erioloba* and *A. reficiens* (De Graaff, 1981; Skinner and Smithers, 1990). Their natural diet consists mostly of leaves, seeds, pods, and twig tips of *Acacia* tree species, as well as leaves of *Boscia albitrunca* and husks of *Ziziphus mucronata* (De Graaff, 1981; Skinner and Smithers, 1990; C. T. Downs, personal observation). They may supplement their diet with insects and gum (De Graaff, 1981; Skinner and Smithers, 1990). They must cope with high levels of condensed tannins that occur in *Acacia* leaves and gum (Wrangham and Waterman, 1981; Rohner and Ward, 1997); thus, we expect this rodent to be a useful model of physiological responses of a foregut fermenter (Perrin and Curtis, 1980; Perrin and Maddock, 1983) that has to contend with a diet high in condensed tannin on a daily basis.

We conducted two experiments on the effects of low and high condensed tannin diets on the physiology of *T. nigricauda*. One was an investigation of the variability in the following dietary and physiological parameters in response to different diets with low tannin levels: daily body mass (DM_b), daily feed intake, daily fecal energy loss (FE), daily gross energy intake (GEI), daily energy assimilated (DEA), daily urine pH, and daily urinary ammonia and urea concentrations. We hypothesized that there would be no difference in dietary and physiological parameters of *T. nigricauda* among diets of similar condensed tannin content. The second experiment was an investigation of the same dietary and physiological parameters of *T. nigricauda* in response to the effects of high dietary condensed

tannin ingestion. We predicted that DM_b, daily feed intake, FE, and DEI would be adversely affected by high dietary condensed tannin content. We further predicted that detoxification activity by *T. nigricauda* would increase at higher condensed tannin levels. Ammonium ions are excreted by arboreal marsupials as a means of regulating metabolic acidosis (Foley and Hume, 1987). This results in the production of acidic urine (Foley and Hume, 1987). Foley (1992; see also Foley et al., 1995) argues that these effects should be observed in other animals because the end-products of allelochemical metabolism are always strong organic acids (see also Guglielmo et al., 1996). Foley et al. (1995) also argues that the creation of large acid loads challenges acid–base homeostasis and that animals need, as a result, to control the rates at which these acid loads are formed and excreted, and maintenance of acid–base metabolism may be linked to the regulation of feeding. Dearing (1997) proposed that urea production is reduced to conserve bicarbonate ions and thereby neutralize blood acidity. As a consequence of increased detoxification, Dearing (1997) predicted that there would be a decrease in urine pH, higher urinary ammonia concentrations, and lower urinary urea concentrations.

In a third experiment, to determine whether there is behavioral avoidance of condensed tannins, we tested the diet choice of *T. nigricauda* for diets containing high and low concentrations of condensed tannins.

METHODS AND MATERIALS

Experimental Animals. Animals were captured during autumn near Windhoek, Namibia, under permit of the Department of Environment and Tourism, Namibia. Rodent box traps, made of galvanized steel, and baited with peanut butter and oats, were used to live-trap individuals. Rodents were housed individually in glass terraria ($50 \times 35 \times 40$ cm) in a constant environment (CE) room (25°C; RH = 35% relative humidity; 12L:12D photoperiod) at the University of Natal, Pietermaritzburg (UNP), South Africa. The maintenance diet consisted of sorghum, apple, and spinach or lettuce. Occasionally, the diet was supplemented with rabbit chow and ProNutro (National Brands Ltd.). Water was available *ad libitum*.

Experimental Design. In experiment A, nonreproductive adults were randomly assigned, regardless of sex or age, to one of two groups ($N = 10$). Individuals were kept singly in stainless steel metabolic cages ($40 \times 40 \times 25$ cm) with perforated Perspex lids and raised floors of wire mesh (0.5×0.5 cm) in a CE room under the same conditions as above. Beneath each floor was a plastic tray containing liquid paraffin for urine collection. Trays were covered with fine wire mesh to facilitate the separate collection of feces. Each group received a different test diet: a sorghum variety produced in South Africa (sorghum S.A.) or Zimbabwe (sorghum Zim). The test diet was administered during the 3-day acclimatization period and the 7-day trial period. Apple was provided throughout the experiment because

no free water was available. Experiment B followed the same basic procedure as experiment A, with the exception that after an initial experimental diet treatment of sorghum S.A. for 4 days, animals were fed pelleted *Acacia raddiana* leaves of high condensed tannin content for a further seven days.

Because these animals are predominantly nocturnal in the wild and emerge at dusk to forage (De Graaff, 1981), they were fed weighed amounts of food daily at 18:00 hr. Body masses and mass of remaining food were also recorded then. Urine and feces were collected at the same time. Urine was collected with a syringe, which was sealed with wax (to prevent evaporation) in 2-ml plastic Eppendorf vials and then frozen. Feces were oven-dried at 60°C for a period of at least one week to obtain a dry weight.

Energy content of feed (sorghum varieties, condensed tannin pellets, and apple) and feces were determined by bomb calorimetry (Grodzinski and Wunder, 1975). The Kjeldahl method was used to determine dietary protein content (Grodzinski and Wunder, 1975). Dietary fat content was determined by using the Soxhlet method with diethyl ether as the solvent (Grodzinski and Wunder, 1975). Analyses were conducted (on a dry weight basis) by the Department of Animal and Poultry Science, UNP. It is widely recognized that *Acacia* leaves (including *A. raddiana*) are high in polyphenols and that these consist primarily of condensed tannins (see, e.g., Wrangham and Waterman, 1981; Cooper and Owen-Smith, 1985; Palo et al., 1993; Rohner and Ward, 1997, Ward and Young, 2002). Consequently, we measured condensed tannin content of all experimental diets by using a standard acid–butanol assay (Waterman and Mole, 1994). Quebracho was used as a standard condensed tannin as recommended by Hagerman and Butler (1989) and was provided by A.E. Hagerman.

Daily intakes were calculated as the difference between food given and food remaining. Mass-specific daily energy assimilation (DEA) was determined gravimetrically as the difference between gross energy intake (GEI) and daily fecal energy loss (FE), divided by daily body mass (DM_b) (Downs and Perrin, 1990). Energy content of urine was ignored because it constitutes only 1% of total energy ingested (Godzinski and Wunder, 1975). Mass-specific FE was calculated by dividing fecal energy content by DM_b.

Urinary urea concentrations (for five individuals on trial days 3, 5, and 7) were determined by an enzymatic colorimetric test with salicylate by using a commercial urease kit, Urea S (Boehringer Mannheim). Absorbencies were measured on a spectrophotometer (Varian) at 600 nm. Urinary ammonia concentration (for the same five individuals on trial days 2, 4, and 6) was determined using a chemical kit for the UV method (Boehringer Mannheim). Absorbencies were measured on a spectrophotometer (Varian) at 340 nm. Urine pH (for the same individuals on trial days 2, 4, and 6) was measured at 21.9°C by using a Crison pH meter. All absorbencies were obtained to 0.001% accuracy. All urine samples were diluted fivefold with distilled water.

A repeated-measures analysis of variance (Statistica, Statsoft Inc., 1996) was used to compare individual results for each diet treatment over time. Individual results were tested for significance ($P < 0.05$) in terms of diet, days, and an overall effect, i.e., taking both days and diet into account.

Diet Choice Experiment. Individuals were weighed daily in the morning, before fresh food was provided. Any uneaten food was collected and weighed before fresh food was placed into the cages. Each *T. nigricauda* was provided daily with approximately 3 g each of high tannin pellets and low tannin pellets, placed in separate feeders in the metabolic cage. As a water source, fresh apple was provided daily. Pellets were produced at the University of Natal's Ukulinga farm. Low condensed tannin pellets consisted of milled sorghum [0% condensed tannin QE (= % tannin per unit dry mass in quebracho equivalents as determined by the acid–butanol assay) (Waterman and Mole 1994, Ward, unpublished results)) (gross energy 16.204 MJ/kg, 8.19% protein, 2.86% fat, 3.19% crude fiber] while high condensed tannin pellets consisted of milled sorghum and *Acacia* leaves [10–20% QE Ward, unpublished results) gross energy 17.344 MJ/kg, 13.72% protein, 6.04% fat, 8.73% crude fiber]. Although the sorghum and *Acacia* leaves were milled, the high tannin pellets had a higher fiber content. However, the level in the high tannin pellets was comparatively low compared to other food typically consumed by herbivores.

The amount of food consumed each day was recorded by subtracting the mass of food remaining from the mass of food provided each day. Energy intake from food was calculated by multiplying the mass of food eaten each day by the energy content of the food type (obtained by bomb calorimetry, Department of Animal and Poultry Science, UNP).

RESULTS

The two sorghum varieties had similar energy, fat, fiber, and water contents (Table 1). However, sorghum Zim had a higher percentage protein (13.54%) than sorghum S.A. (8.83%) (Table 1). Tannin assays indicated that both varieties contained no measurable condensed tannin. The *Acacia* pellets were higher in fat and fiber but had lower water content than the sorghum varieties (Table 1).

Experiment A: Comparison of Sorghum Varieties. Each of the experimental groups of *T. nigricauda* consisted of five males and five females ($N = 10$). Results of repeated-measures ANOVA indicated that DM_b, FE, and GEI were significantly different overall, taking into account diet and days ($F_{(6,108)} = 5.97$, $P < 0.05$; $F_{(6,108)} = 9.66$, $P < 0.05$, and $F_{(6,108)} = 90.61$, $P < 0.05$ respectively). However, DM_b was not different between diets ($F_{(1,18)} = 0.25$; $P > 0.05$). Daily energy assimilated was not different overall, taking diet and days into account ($F_{(5,90)} = 1.48$, $P > 0.05$). Body mass was lower for those individuals on sorghum

TABLE 1. COMPOSITION OF EXPERIMENTAL DIETS FED TO *Thallomys nigricauda*

Diet	Protein (%)[a]	Energy (kJ/g)[b]	Fat (%)[a]	Fiber (%)[a]	Water content (%)[a]
Sorghum S. A.	8.83	16.31	3.71	3.71	11.60
Sorghum Zim	13.54	16.94	3.78	3.78	10.47
Acacia pellets	13.72	17.34	6.04	8.73	4.47
Apple	1.38	15.87	1.00	5.09	22.50

[a] Percentage of dry mass.
[b] Gross energy content (dry mass basis).

Zim than on sorghum S.A. and declined over the trial, while body mass for individuals on sorghum S.A. increased with time. Fecal energy loss (FE) was extremely variable but low. Daily urine pH, urinary ammonia, and urea concentrations were not different overall, taking into account diet and days ($F_{(2,6)} = 1.53$, $P < 0.29$; $F_{(2,8)} = 1.30$, $P < 0.32$; and $F_{(2,8)} = 1.59$, $P < 0.26$, respectively) (Table 2). Daily urine pH, urinary ammonia, and urea concentrations were not different between diets ($F_{(1,3)} = 9.61$; $P < 0.05$; $F_{(1,4)} = 1.09$, $P < 0.36$; and $F_{(1,4)} = 0.32$, $P < 0.60$, respectively). Daily urine pH, which was alkaline, remained fairly constant, showing only a slight increase over the trial.

Experiment B: Comparison of Low Tannin Sorghum S.A. and High Tannin Acacia *Pellets.* Because sorghum S.A. showed little effect on physiological parameters, it was used for this comparison in experiment B to determine whether changes in physiological parameters could be attributed to differences in dietary tannin levels. In experiment B, the 4 days of sorghum (low tannin) diet were compared with the first 4 days on the *Acacia* pellet (high tannin) diet. Results of repeated-measures ANOVA indicated that DM_b FEI, GEI, and DEA were significantly different overall, taking diet and days into account ($F_{(3,27)} = 7.702$; $P < 0.05$; $F_{(3,27)} = 3.796$,

TABLE 2. PHYSIOLOGICAL PARAMETERS OF *T. nigricauda* MEASURED AT BEGINNING AND END OF EXPERIMENT FOR TWO DIET TYPES

	Sorghum S.A.		Sorghum Zim Initial	
Variable	Initial	Final	Initial	Final
Body mass (g)	74.79 ± 4.52 ($N = 10$)	75.51 ± 4.26 ($N = 10$)	73.68 ± 4.95 ($N = 10$)	71.24 ± 4.72 ($N = 10$)
GEI (mJ/g day)	0.99 ± 0.074 ($N = 10$)	1.150 ± 0.078 ($N = 10$)	0.82 ± 0.102 ($N = 10$)	0.92 ± 0.086 ($N = 10$)
DEA (mJ/g day)	0.89 ± 0.073 ($N = 10$)	1.030 ± 0.071 ($N = 10$)	0.75 ± 0.099 ($N = 10$)	0.80 ± 0.080 ($N = 10$)
Urine pH	8.36 ± 0.52 ($N = 3$)	6.48 ± 0.34 ($N = 4$)	9.06 ± 0.25 ($N = 3$)	10.33 ± 0.29 ($N = 4$)

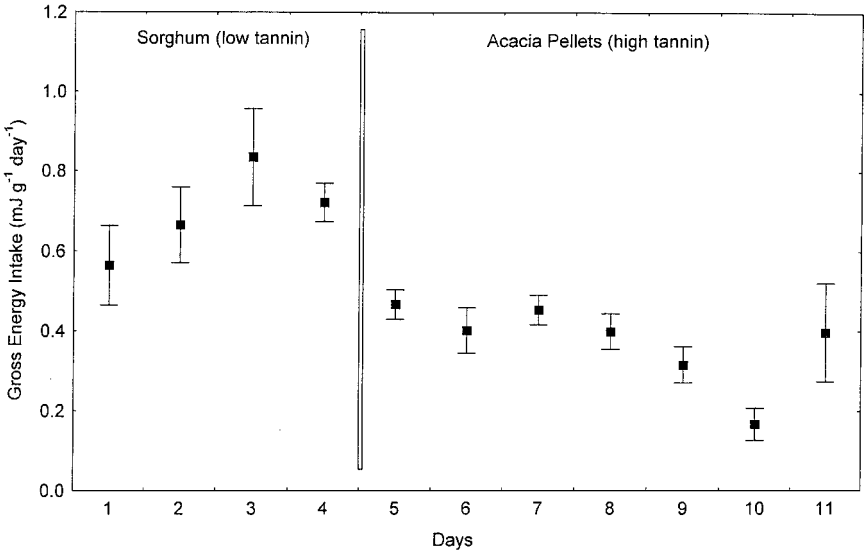

FIG. 1. Gross energy intake of *T. nigricauda* fed sorghum S.A. (low tannin) and then pelleted *Acacia raddiana* (high tannin) (mean ± SE).

$P < 0.05$; $F_{(3,27)} = 3.800$, $P < 0.05$ and $F_{(3,27)} = 3.802$, $P < 0.05$, respectively; Figure 1), showing that tannin ingestion negatively affected nutritional status. There was a mean decline of 4.1% in body mass between the last day of the sorghum diet and the last day of the tannin diet (Table 3). The difference in body mass between the last day of the sorghum diet and the last day of the tannin diet was significant (paired t test: $t = 5.31$, $P < 0.001$). Fecal energy was minimal and was not different between diets. Consequently, the percentage of daily energy assimilated for both the sorghum (99.96%) and tannin (99.49%) diets was high. Initial and final means of experiment B for DM_b, GEI, DEA, daily urine pH, daily urinary ammonia, and urea concentrations are shown in Table 3. Daily urine pH, urinary ammonia, and urea concentrations did not differ overall, taking into account diet and days ($F_{(3,6)} = 1.156$, $P > 0.05$; $F_{(3,6)} = 0.345$, $P > 0.05$; and $F_{(3,6)} = 1.017$, $P > .05$, respectively). However, daily urine pH and urinary urea concentrations were different between diets ($F_{(1,2)} = 28.651$; $P < 0.05$; and $F_{(1,2)} = 36.459$, $P < 0.05$, respectively). There was no difference in urinary ammonia between diets (paired t test on final day's data for each diet: $t = 0.68$, $P = 0.273$).

Diet Choice Experiment. Body mass of *T. nigricauda* decreased over the experimental period (repeated-measures ANOVA, within subjects design, $F_{(6,54)} = 3.38$, $P < 0.05$). However, the overall decrease in body mass was only 0.76%.

TABLE 3. PHYSIOLOGICAL PARAMETERS OF *T. nigricauda* MEASURED IN EXPERIMENT B

Variable	Sorghum S.A. initial	*Acacia* pellets final
Body mass (g)	74.95 ± 3.16	71.70 ± 2.77
GEI (mJ/g day)	0.564 ± 0.099	0.397 ± 0.122
DEA (mJ/g day)	0.564 ± 0.099	0.398 ± 0.123
urine urea (mmol/liter)	0.681 ± 0.275	0.491 ± 0.120
urine ammonia (mmol/liter)	1.280 ± 0.023	0.402 ± 0.063
Urine pH	6.48 ± 0.34	7.96 ± 0.184

Gross energy intake (GEI, kilojoule per gram per day) reflected body mass trend and changed over the experimental period (repeated-measures ANOVA, within subjects design, $F_{(5,45)} = 9.78$, $P < 0.05$). Pellet choice showed a definite avoidance of high tannin pellets for the duration of the experiment. The amount of high tannin food eaten (grams) was lower than the amount of low tannin food eaten (repeated-measures ANOVA, $F_{(5,45)} = 2.17$, $P < 0.05$, Figure 2). Similarly, when expressed as the amount of energy intake per gram of body mass, amount of high condensed tannin food eaten was lower than amount of low condensed tannin food eaten (repeated-measures ANOVA, $F_{(5,45)} = 2.28$, $P < 0.05$).

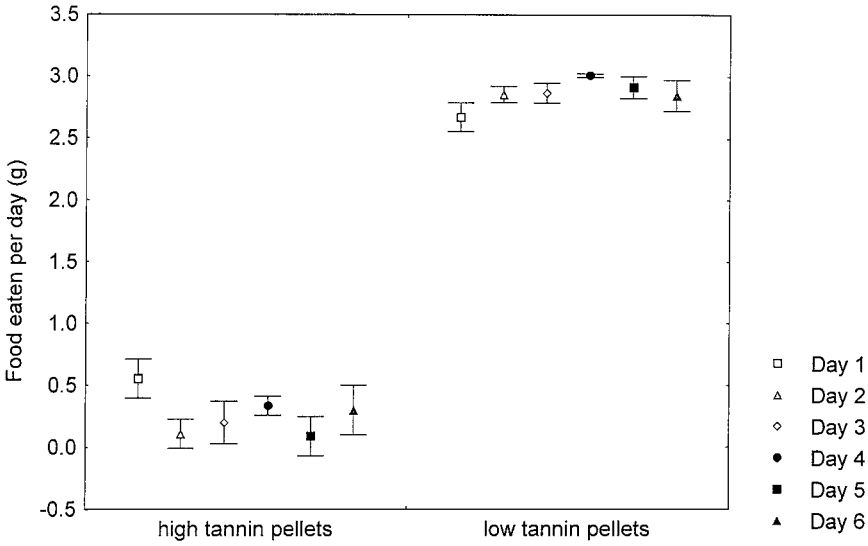

FIG. 2. Amount of high tannin versus low tannin food eaten daily by *Thallomys nigricauda* in the choice experiment (mean ± SE).

In the previous experiments, when fed only high tannin pellets, *T. nigricauda* had eaten these. However, when offered a choice between these and low tannin pellets, all individuals showed a definite preference for food low in tannin (Figure 2), despite the higher nutritional value of the high tannin pellets. These results indicate that condensed tannin concentration affected *T. nigricauda* food choice. As animals selectively avoided high condensed tannin pellets, it appears that the pelleting process had no effect on the relative condensed tannin content.

DISCUSSION

Dearing (1997) and Foley et al. (1999) proposed that urea production of rodents on a high tannin diet would be reduced in order to conserve bicarbonate ions and thereby neutralize blood acidity. We predicted that lower urine pH, higher urinary ammonia concentrations, and lower urinary urea concentrations would occur as a consequence of increased detoxification when tree rats were fed high tannin diets. We also hypothesized that DM_b, daily feed intake, FE, and DEI of *T. nigricauda* would be adversely affected by high dietary tannin content. Ingestion of tannins did affect the nutritional status of *T. nigricauda,* as shown by a decrease in body mass at high tannin levels (Table 3). Furthermore, and importantly, GEI declined significantly (Figure 1), as did DEA. On its own, a decline in DEA might be ascribed to the higher fiber content of the *Acacia* diet relative to that of the sorghum diet. This is unlikely, however, because animals typically increase their intake rather than decrease it when a diet is high in fiber in order to maintain energy intake (Robbins, 1993). Instead, we found that the rodents decreased their intake of *Acacia* leaves (Table 3) in experiment B. Because no other food was simultaneously available to them, this is a clear indication of behavioral avoidance. Additionally, they had lower intakes of the high tannin diet in the diet choice experiment (Figure 2). This behavioral avoidance with a clear cost (decreased body mass, Tables 2 and 3) must be a response to the high condensed tannin content of the food. This result is significant because, in a review of the literature, Foley et al. (1999) found that mammals generally do not exhibit behavioral avoidance of tannins; rather there is physiological avoidance or control. Ingestion of tannin is, indeed, costly in this species, which is surprising in light of their specialization on *Acacia* species that have high condensed tannin concentrations (Rohner and Ward, 1997; Ward and Young, 2002). Behavioral avoidance of tannins by tree rats indicates that there is indeed a cost to the ingestion of tannins, even in these specialists; in contrast, results from other studies that suggest that ingestion of tannins may be noncostly (Thomas et al., 1988). Furthermore, this result also indicates that the tree rats can detect condensed tannins, perhaps by taste; the evolution of this unique detection ability is

tantalizing preliminary evidence of the close coevolution of the tree rats and *Acacia* trees.

Two possible confounding factors for this result are: (1) the higher fiber in the *Acacia* diet than in the sorghum diet. This is unlikely to have affected our results because fecal energy very low in all three diets (Table 2), indicating that most energy was being used. (2) lack of time for gut microflora to adapt to the diet. This too is unlikely because the animals were fed similar diets prior to the experiment. Furthermore, detoxification enzymes require only 3 days for induction (Sipes and Gandolfi, 1986; see also Dearing et al., 2000); our experiment was run for 7 days and measurements were first taken after 3 days.

We found that in both experiments performed here, as did Torres-Contreras and Bozinovic (1997), in experiments carried out *Octodon degus* that when individuals are given a choice, they selected the low tannin diet. However, it is difficult to determine if tannin effects are traded off against intake of nutrients, rather than with other factors including nutrient concentration or fiber content (Foley et al., 1999). Little is known about the detoxification by-products (Dearing, 1997). However, Foley et al. (1999) expect there to be significant costs of detoxification of absorbed plant secondary metabolites, but indicate that it is difficult to demonstrate these costs. Consequently, some mammals may select a lower tannin diet to negate the effects of tannins on dietary quality, particularly in terms of protein and energy input and toxic potential (Foley et al., 1999). Selection of a diet lower in nutrients and tannin may actually increase the overall amount of nutrients and energy per unit time compared with a diet high in nutrients and tannin.

Although *T. nigricauda* appears to be unable to maintain body mass on an *Acacia* diet, there was a physiological response to high tannin content, as seen in the initial reduction in urea production. This indicates that the rodents are conserving bicarbonate ions, which will neutralize blood acidity if there is detoxification, as suggested by Dearing (1997). However, the reduction in excreted ammonium ion concentration in the urine suggests that they do not metabolize these allelochemicals and, therefore, are not using this as a means of regulating metabolic acidosis (Foley and Hume, 1987; Foley, 1992). The fact that urine pH was alkaline is additional evidence for a lack of detoxification activity at this level of tannins, contrary to the evidence provided by the reduction in urea production. As mentioned this is unlikely due to insufficient time for the induction of detoxification enzymes (Sipes and Gandolfi, 1986; Dearing et al., 2000). Further studies are needed to ascertain whether tree rats utilize other avenues of defense against condensed tannins, such as salivary-binding proteins (Robbins et al., 1987, 1991).

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QUALITATIVE AND QUANTITATIVE VARIATION IN MONOTERPENE CO-OCCURRENCE AND COMPOSITION IN THE ESSENTIAL OIL OF *Thymus vulgaris* CHEMOTYPES

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Abstract—*Thymus vulgaris* has a chemical polymorphism with six different chemotypes that show marked spatial segregation in nature. Although some populations have a single chemotype in majority, many have two or three chemotypes. In this study we analyze the quantitative variation among *T. vulgaris* populations in the percentage of oil composed of the dominant monoterpene(s) for each chemotype. In general, phenolic chemotypes (thymol and carvacrol), which occur at the end of the biosynthetic chain, have a significantly lower proportion of their oil composed of their dominant monoterpene than nonphenolic chemotypes (geraniol, α-terpineol, and linalool). This is due to the presence of high amounts of precursors (γ-terpinene and paracymene) in the oil of phenolic chemotypes. The essential oil of the nonphenolic thuyanol chemotype has four characteristic monoterpenes that together make up a lower proportion of the oil than the single dominant monoterpene of the other nonphenolic chemotypes. For all chemotypes, the percentage composition of the dominant monoterpene decreased significantly at sites where the chemotype is not the majority type. This decrease is correlated with a significant increase in either the proportion of the two precursors for the thymol chemotype or the monoterpenes characteristic of the other chemotypes at the site. The latter result suggests that a plant with dominant genes

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is responsible for the production of different monoterpenes can produce several molecules.

Key Words—Lamiaceae, Mediterranean, adaptation, essential oil composition, monoterpene production, polymorphism.

INTRODUCTION

The Mediterranean flora is well known for its abundance in aromatic plants, with an estimated 49% of genera containing aromatic species in this climate zone (Ross and Sombrero, 1991). Members of the Lamiaceae from this region have attracted a great deal of attention due to the diversity of monoterpenes produced by different species (reviews in Richardson, 1992; Stahl-Biskup, 2002; Thompson, 2002). This diversity occurs at three levels: among genera, among species within genera, and as polymorphic variation within species. Indeed, different genetically based types, or chemotypes, have been reported in *Mentha spicata* (Kokkini and Vokou, 1989), *Origanum vulgare* (Vokou et al., 1993), *Rosmarinus officinalis* (Granger et al., 1973), and *Thymus vulgaris* (Granger and Passet, 1973). The presence of intraspecific chemotype variation is, in fact, probably quite common in the genus *Thymus* (Stahl-Biskup, 2002).

Secondary compounds, including terpenes have multiple ecological functions. The most notable of these lie in the defense they provide against herbivores and parasites (Simeon de Bouchberg et al., 1976; Bryant et al., 1991; Shonle and Bergelson, 2000). A number of other functions also may exist, such as the mediation of plant competition via allelopathic effects on other plant species (e.g., McPherson and Muller, 1969), pollinator attraction (Bergstrom, 1978; Beker et al., 1989), leaf decomposition (Grime et al., 1996), soil microbe activity (Vokou and Margaris, 1984), and climatic adaptation (Seufert et al., 1995). However, studies of patterns of variation in secondary compound production in relation to ecological variation remain rare.

Many species in the genus *Thymus* show evidence of polymorphic variation in monoterpene production (Stahl-Biskup, 2002). A species in which chemotype variation is particularly striking is the Mediterranean wild thyme, *T. vulgaris.* In southern France, this species has six genetically distinct chemotypes that can be distinguished on the basis of the dominant monoterpene produced in glandular trichomes on the surface of the leaves (Passet, 1971; Vernet et al., 1986). Each of the six chemotypes, geraniol (G), α-terpineol (A), thuyanol-4 (U), linalool (L), carvacrol (C), and thymol (T), is named after its dominant monoterpene. The six monoterpenes are all produced from geranyl pyrophosphate via a series of changes in configuration and hydroxylation and have fairly similar molecular structures (Figure 1). A major distinction is the phenolic nature of carvacrol and thymol, and the nonphenolic nature of the four other monoterpenes. Presence of the dominant

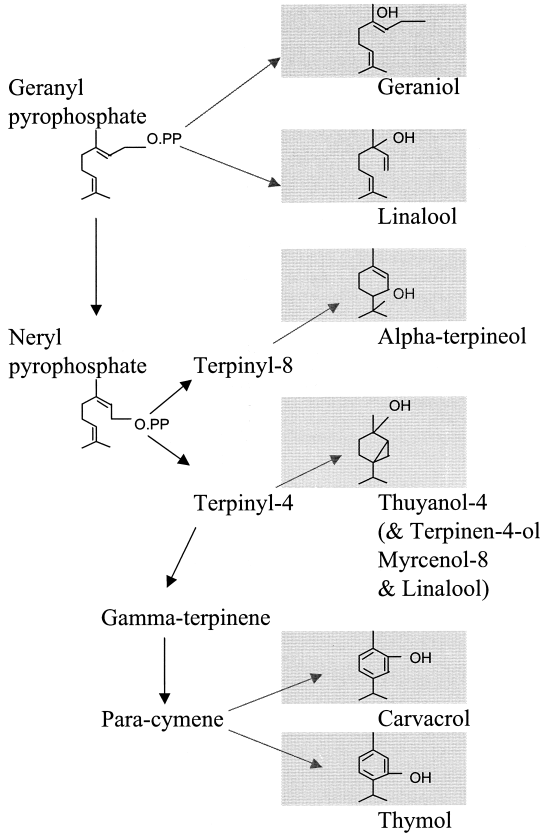

FIG. 1. The predicted biosynthetic chain of monoterpene production in *Thymus vulgaris* (modified from Passet, 1971).

monoterpene is controlled by an epistatic series of five biosynthetic loci (Vernet et al., 1986).

Granger and Passet (1971) and Passet (1971) reported that the thuyanol chemotype, unlike the five other chemotypes that have a single predominant monoterpene in their essential oil, is characterized by the occurrence of four monoterpenes (thuyanol-4, terpinen-4-ol, myrcenol-8, and linalool). They also suggested that in the oil of the thymol and carvacrol chemotypes, the dominant monoterpene co-occurs with relatively high levels (up to 30% of the oil) of two precursors—γ-terpinene and para-cymene. Granger and Passet (1971) provided an estimate of the percentage of the oil of each chemotype that is composed of the dominant monoterpene. For geraniol, linalool, and α-terpineol chemotypes, the dominant monoterpene occurs at levels up to 90–95% of the oil. The thuyanol

chemotype contains up to 60–65% of a combination of thuyanol-4 and terpinen-4-ol (with some linalool and myrcenol-8). The carvacrol chemotype contains up to 85% carvacrol, whereas the thymol chemotype contains no more than 65% thymol. There has been no statistical comparison of monoterpene variation among chemotypes or sites for each chemotype, despite the fact that the different chemotypes show marked spatial segregation in relation to a complex climatic–edaphic gradient. The two phenolic chemotypes are confined to low-elevation areas close to the Mediterranean sea (i.e., relatively mild winters and shallower, stonier soils), and the four nonphenolics are more abundant in areas with colder winters and deeper soils (Granger and Passet, 1973; Vernet et al., 1977a,b; Gouyon et al., 1986).

This paper has three objectives. First, we compare the production of the dominant monoterpene among chemotypes of *T. vulgaris*. Second, for each chemotype, we contrast the proportion of the dominant monoterpene in two different types of sites—those where it is the most frequent chemotype and those where it is either part of a mixed-chemotype population or where it is rare. Third, we quantify correlated variation among monoterpenes in plants of each chemotype. The results are discussed in relation to the potential effects of ecological and genetic backgrounds on monoterpene production.

METHODS AND MATERIALS

Plant Material. In May 1997 and 1998, leaf material was sampled from plants growing in natural populations in two distinct areas in order to cover the range of environmental variation observed in sites where *Thymus vulgaris* occurs in southern France (see Table 1 for sampling details). The first area (sites 1–13) is the St. Martin-de-Londres valley in the Hérault department of the Languedoc region east of the Rhône valley (Figure 2). All six chemotypes have been reported from this area, and previous work has demonstrated the occurrence of localized spatial variation in their relative abundance (see review by Thompson et al., 1998). Second, we sampled plants from lowland and upland sites (14 and 15) in the Drôme department in the Rhône-Alpes region where previous sampling (Granger and Passet, 1973; J. Lamy, personal communication) suggested the absence of phenolic chemotypes. All plants were sampled at the end of the flowering period to minimize phenological differences among samples.

Chemical Analyses. Analytical gas chromatography was carried out on a Delsi 121C apparatus fitted with a flame ionization detector and a CP Wax 51 fused silica capillary column (25 $\times$ 0.3 mm; 0.15-μm film thickness). The temperature was programmed from 50°C at 5 min, rising 3°C/min to 220°C. N_2 was used as a carrier gas. For GC-MS analysis, a CP Wax 51 fused silica WCOT capillary column (60 $\times$ 0.3 mm; 0.25-μm film thickness) and 52 CB stationary phase was employed. The initial temperature was programmed at 50°C for 5 min, rising 3°C/min to 240°C, with He as a carrier gas, and the column coupled to an HP mass spectrometer.

TABLE 1. NUMBER OF *Thymus vulgaris* PLANTS SAMPLED AND ANALYZED (IN PARENTHESES) AT 15 SITES

Population	Sample size	Majority chemotypes	
		Previous work[a]	This study
1. Cazevieille	30 (28)	Carvacrol	Carvacrol (27), thymol (1)
2. Tourriére	30 (28)	Carvacrol	Carvacrol (25), thymol (3)
3. Pic St Loup - Nord	25 (25)	Carvacrol	Carvacrol (23), thymol (2)
4. Hubac-haut	20 (20)	Carvacrol + Linalool	Carvacrol (18), thymol (2)
5. Colline geraniol	20 (17)	Geraniol	Geraniol (16), linalool (1)
6. Aeroport	25 (23)	Linalool/U	Thuyanol-4 (13), linalool (10)
7. Fraicinede	35 (31)	Linalool/Thuyanol-4/ α-terpineol	Thuyanol-4 (16), linalool (8), (α-terpineol (5), carvacrol (1), thymol (1)
8. La Borie	6 (6)	α-terpineol	α-Terpineol (6)
9. Cabane de la Plaine	30 (28)	Linalool	Linalool (22), thymol (4), geraniol (2)
10. Gardiol	15 (15)	Thymol/Linalool	Thymol (15)
11. Hortus-januq	15 (15)	Thymol	Thymol (13), α-terpineol (2) carvacrol (1)
12. Ferriéres les Verreries	35 (33)	Thymol	Thymol (31), Carvacrol (2)
13. Pompignan-haut	20 (16)	Thymol	Thymol (16)
14. Lowland Drôme	20 (15)	Linalool	Linalool (7), geraniol (5), carvacrol (3)
15. Upland Drôme	35 (32)	Linalool	Linalool (27), geraniol (3), α-terpineol (2)

[a] From raw data used by Gouyon et al. (1986) (populations 1–13) and J. Lamy (personal communication) (populations 14 and 15).

Samples were injected at 240°C and identified according to their retention time indices and mass spectra in comparison with those of known compounds or with published spectra.

Data Presentation and Analysis. The essential oil of *T. vulgaris* can contain more than 30 different monoterpenes. In this study, plants with more than 10% of their oil containing unidentified compounds were not included in comparisons of monoterpene production (hence the difference between number of sampled plants and number for which extraction was successful in Table 1). The goal here is not to show all the diversity in monoterpene presence, since many compounds only occur as trace elements. In fact, 11 monoterpenes (geraniol, α-terpineol, thuyanol-4, terpinen-4-ol, linalool, myrcenol-8, 1,8 cineole, γ-terpinene, paracymene, carvacrol, and thymol) regularly occur as more than 10% of the oil and form the basis of the variation in composition among sites. β-Caryophylene is frequently present as 5–10% of the oil and is included in our presentation of variation among and within chemotypes.

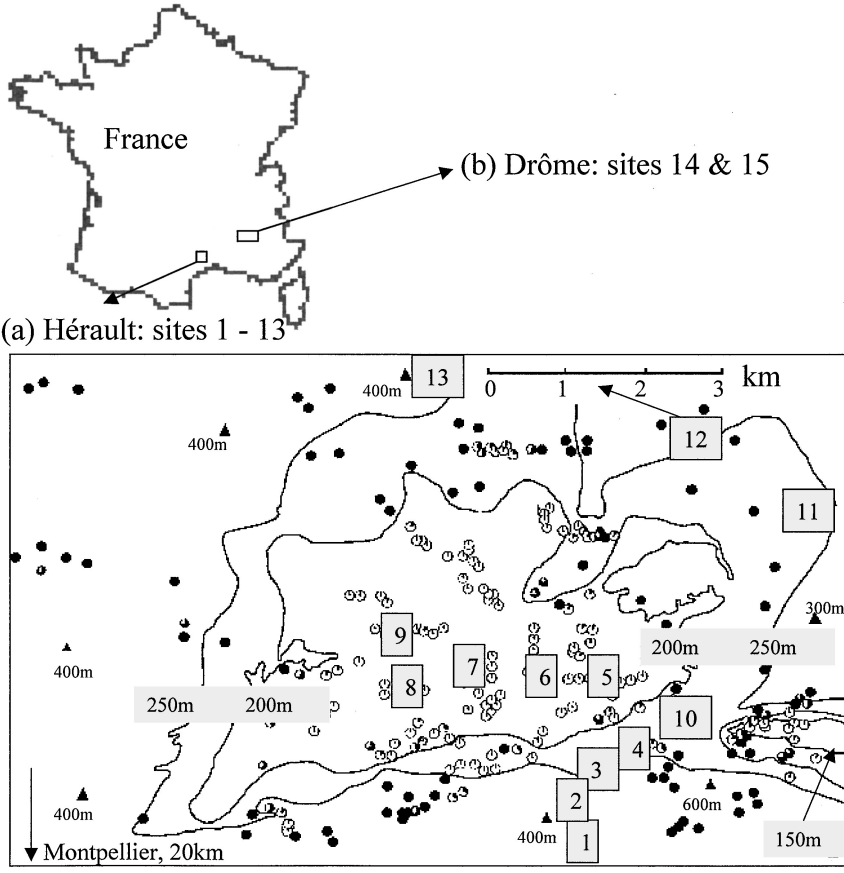

FIG. 2. Location of the study sites. (a) Closed circles represent phenolic populations and open circles represent nonphenolic populations. (b) Phenolic chemotypes are rare or absent in natural populations.

To analyze the composition of the major monoterpenes in the essential oil of *Thymus vulgaris*, we made a number of simplifications. First, the percentage composition of all the major nonphenolic monoterpenes is reported as the sum of each monoterpene and its acetate. Second, myrcenol-8 is reported as the sum of four different forms (ac-*Z*-2, ac-*E*-2, *Z*-2, and *E*-2) of methyl-2 methylene-6 octadiene-2,7-ol (see Granger et al., 1972). Third, the composition of the thuyanol-4 molecule is the sum of the *cis* and *trans* forms. Fourth, plants that had equal proportions of carvacrol and thymol were classed as carvacrol since they must have the dominant gene coding for this monoterpene.

The number of plants of each chemotype sampled at different sites varied due to the differences in their spatial distribution in southern France. For all six chemotypes, we regrouped plants into two classes (home and away) that reflect their abundance at a given site: "home" included all plants of a given chemotype in sites where it represented the majority type, and "away" included all plants of a given chemotype in sites where it was a minority type. These two classes were established based on previous surveys and data in the present survey.

From the populations we sampled, we obtained a total of 26 geraniol plants (16 at home sites and 10 at away sites), 15 α-terpineol plants (6 and 9), 75 linalool plants (49 and 26), 100 carvacrol plants (93 and 7), 88 thymol plants (75 and 13), and 29 thuyanol plants in two sites where no one chemotype predominated. Statistical analysis of variation between home and away sites for the thuyanol chemotype was not possible. In order to include the thuyanol chemotype in a statistical comparison of the percentage of the oil composed of the dominant monoterpene, we excluded plants sampled in away sites for the five other chemotypes and performed one-way ANOVA of variation in the dominant monoterpene across chemotypes.

All statistical analyses were carried out by using SAS version 8 (SAS, 1999). Analysis of variance (ANOVA) of mean values across chemotypes and sites were performed with PROC GLM on arcsine square-root transformed data. Where significant differences were observed among chemotypes, mean values were compared with the Scheffé pairwise comparisons test. Correlation analyses for particular compounds within chemotypes were carried out using PROC CORR in SAS (1999). In all tests, significance levels were adjusted to account for multiple comparisons following the method proposed by Rice (1989).

RESULTS

Variation among Chemotypes. When the thuyanol chemotype was considered to be a mix of terpinen-4-ol, thuyanol-4, myrcenol-8, and linalool, we found significant ($F_{5,262} = 45.9$, $P < 0.001$) variation among chemotypes. The geraniol, α-terpineol, and linalool chemotypes had higher (Scheffé means test at $P < 0.05$) mean values of their dominant monoterpene than did the thuyanol, carvacrol, thymol chemotypes (Figure 3a). Within each of these two groups, there were no significant differences in mean values. If the precursors for the two phenolic monoterpenes are included as part of the dominant monotepene, i.e., the mean value for the carvacrol (or thymol) chemotype is the sum of carvacrol (or thymol), paracymene, and γ-terpinene, there is significant variation among chemotypes ($F_{5,262} = 24.3$; $P < 0.001$) with the same two statistically different (Scheffé means test at $P < 0.05$) groups of chemotypes (Figure 3b).

Variation within Chemotypes. Two-way ANOVA was carried out on the proportion of the oil made up by the dominant monoterpene in each of the different

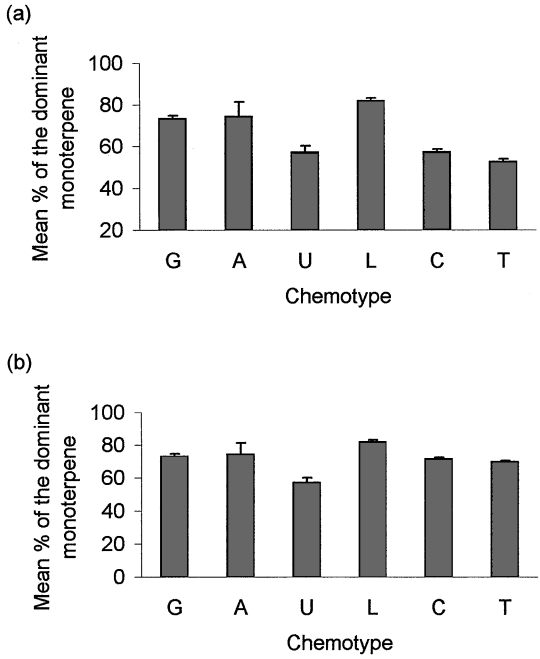

FIG. 3. Mean percentage (±SE) of the dominant monoterpene in the essential oil of the different *Thymus vulgaris* chemotypes in southern France. The thuyanol chemotype is considered to have four characteristic monoterpenes (thuyanol-4, terpinen-4-ol, myrcenol-8, and linalool), whose proportions are summed to give single value per plant. Phenolic chemotypes are considered as either (a) the single monoterpene or (b) the monoterpene plus precursors (λ-terpinene and paracymene). Chemotypes are: G, geraniol; A, α-terpineol; U, thuyanol; L, linalool, C, carvacrol; and T, thymol.

chemotypes at home sites, where the chemotype was the dominant form, and away sites, where the chemotype was either rare or in mixed-chemotype populations. We found variation between sites ($F_{1,294} = 87.21$; $P < 0.001$) and differences among chemotypes ($F_{4,294} = 46.29$; $P < 0.001$), but no interaction between chemotype and site ($F_{4,294} = 1.61$; $P = 0.17$). For all five chemotypes, the dominant monoterpene occupied a higher fraction of the oil at home sites, where that chemotype was the most abundant form, compared to away sites, where the chemotype was rare or in mixed populations (Figure 4).

The decrease in the dominant monoterpene at the away sites is accompanied by increases in one or more of the other main monoterpenes (Table 2) and by an increase in the proportion of the range of minor monoterpenes not analyzed by statistical comparisons. As a consequence, the 11 predominant monoterpenes tend to occupy a smaller fraction of the total oil for most plants sampled in away sites. As

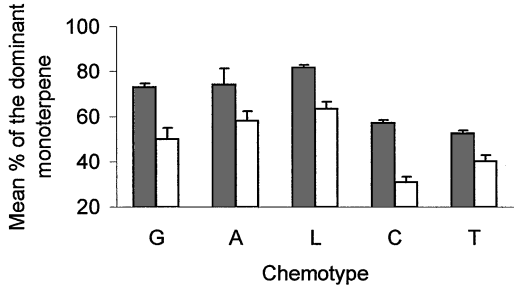

FIG. 4. Mean percentage (±SE) of the dominant monoterpene in the essential oil of five *Thymus vulgaris* chemotypes in southern France at home sites where that chemotype is the majority type (closed bars) and away sites where that chemotype is rare or in a mixed-chemotype population (open bars). Chemotypes are: G, geraniol; A, α-terpineol; L, linalool, C, carvacrol; and T, thymol.

we now show, this decrease is correlated with an increase in the monoterpene(s) present in co-occurring chemotype(s). To illustrate this pattern we present the results on a chemotype-by-chemotype basis.

Geraniol. In plants of the geraniol chemotype at away sites, there is less geraniol in their oil and more linalool (Table 2). For the geraniol chemotype, we obtained 10 plants from sites where this chemotype was uncommon or rare. Six plants, sampled in sites 9 and 15 (Table 1), which are dominated by linalool plants, had a relatively high proportion (>20%) of linalool in their oil. The four other plants (population 14) co-occur with both the linalool and carvacrol chemotypes (Table 1), and all four plants contained relatively high amounts (5–10%) of phenolic compounds.

α-Terpineol. For the α-terpineol chemotype, we obtained nine plants from away sites to compare with the six plants in the home population (Table 2). Five plants had relatively high amounts of a combination of thuyanol-4, terpinen-4-ol, cineole, and linalool and came from site 7 where the thuyanol chemotype occurs with linalool. Two plants had relatively high amounts of linalool (with no thuyanol-4 or terpinenol-4-ol) and came from site 15, where linalool is the dominant chemotype. Finally, one plant had a relatively high composition of thymol and came from site 11 where the thymol chemotype is the majority chemotype.

Thuyanol. The thuyanol chemotype occurred in two populations (populations 6 and 7) in the St. Martin-de-Londres area that are close together (roughly 2 km apart) and do not have a single majority chemotype (Tables 1 and 3). If this chemotype is defined as having two dominant monoterpenes, thuyanol-4 and terpinen-4-ol, there was a difference in the percentage of the essential oil made up of these two dominant compounds between sites 6 and 7 ($F_{1,27} = 5.04$, $P = 0.04$). Individually, these two compounds showed no significant variation between the

TABLE 2. MONOTERPENE COMPOSITION IN *Thymus vulgaris* CHEMOTYPES IN HOME AND AWAY SITES

Chemotype and monoterpene	Composition at home sites (%)					Composition at away sites (%)					
	N	Mean	SE	Min.	Max.	*N*	Mean	SE	Min.	Max.	F^a
Geraniol											
Geraniol	16	73.1	1.7	58.2	84.1	10	49.9	5.1	23.5	72.7	26.85*
α-Terpineol		0.1[b]	0	0	0.7		0.4	0.3	0	2.5	0.88 ns
Thuyanol-4		0	0	0	0		0	0	0	0	—
Terpinen-4-ol		0	0	0	0		0	0	0	0	—
Linalool		4.2	1.2	0.4	15.9		18.7	3.9	3.8	40.8	21.64*
γ-Terpinene		0	0	0	0		0.7	0.5	0	5.3	3.44 ns
Paracymene		0.1	0.1	0	0.5		0.3	0.2	0	1.5	1.21 ns
Carvacrol		0.3	0.1	0	1.5		1.2	0.7	0	6.9	0.94 ns
Thymol		1.0	0.5	0	7.9		0.9	0.3	0	2.6	—
Myrcenol-8		0	0	0	0		0	0	0	0	—
Cineole-8		0	0	0	0		0	0	0	0	—
β-Caryophylene		3.9	0.6	0.8	9.6		2.8	0.35	0.3	4.2	—
α-Terpineol											
Geraniol	6	0	0	0	0	9	0.1	0.1	0	0.9	0.65
α-Terpineol		74.2	7.1	47.1	90.4		58.1	4.2	40.9	76.7	4.74(.049)
Thuyanol-4		0.3	0.1	0	0.9		2.9	1.5	0	13.9	2.73
Terpinen-4-ol		0.3	0.1	0	0.6		1.2	0.7	0	6.2	0.30
Linalool		1.6	0.4	0.5	3.2		6.2	2.1	0.9	19.2	3.26
γ-Terpinene		0.1	0.1	0	0.25		0.7	0.4	0	3.3	2.34
Paracymene		0.1	0.1	0	0.5		0.2	0.1	0	0.8	1.40
Carvacrol		0.8	0.5	0	3.0		0.3	0.1	0	0.9	0.66
Thymol		10.4	4.4	0	27.4		2.7	1.5	0.1	14.0	3.29
Myrcenol-8		0	0	0	0		0.3	0.3	0	2.4	2.4
Cineole-8		0.22	0.11	0	0.6		2.8	1.9	0	13.2	—

β-Caryophylene		1.48	0.14	1.02	2.0		2.3	0.5	1.3	6.5	—
Linalool											
Geraniol	49	1.6	0.6	0	30.0	26	1.3	0.8	0	16.0	2.44
α-Terpineol		0.5	0.2	0	5.0		2.5	0.6	0	14.2	21.04*
Thuyanol-4		0.1	0.1	0	1.6		4.2	0.3	0	20.6	35.88*
Terpinen-4-ol		0	0	0	0		2.6	0.5	0	8.5	87.97*
Linalool		81.8	1.3	45.4	93.8		63.4	3.1	32.2	83.0	42.60*
γ-terpinene		0.1	0.1	0	1.0		0.7	0.2	0	2.9	26.60*
Paracymene		0.2	0.1	0	1.9		0.6	0.2	0	3.2	10.72*
Carvacrol		0.8	0.2	0	6.8		1.0	0.3	0	6.0	0.49
Thymol		1.9	0.4	0	12.3		2.4	0.9	0	23.6	0.17
Myrcenol-8		0	0	0	0		1.8	0.4	0	7.1	—
Cineole-8		0	0	0	0		1.7	0.6	0	10.7	—
β-Caryophylene		3.4	0.3	0.9	9.5		3.4	0.6	0.5	12.3	—
Carvacrol											
Geraniol	93	0.1	0	0	0.9	7	2.9	1.8	0	10.3	38.52*
α-Terpineol		1.0	01	0	3.9		2.6	1.0	0.7	8.2	7.76*
Thuyanol-4		0.8	0.1	0	9.4		2.1	1.7	0	12.2	0.58
Terpinen-4-ol		0.7	0.1	0	7.1		0.4	0.4	0	3.0	7.2 (.009)
Linalool		3.7	0.3	1.1	27.1		8.0	3.7	3.0	29.9	28.20*
γ-Terpinene		8.7	0.7	0	26.8		5.0	1.5	0.8	13.0	1.57
Paracymene		5.4	0.4	0	15.4		11.7	4.2	0.9	34.6	6.93(.01)
Carvacrol		57.2	1.3	28.9	84.1		30.9	2.4	21.5	41.1	35.35*
Thymol		3.2	0.4	0	18.2		14.1	4.2	3.3	27.2	27.08*
Myrcenol-8		0	0	0	0		0	0	0	0	—
Cineole-8		1.0	0.1	0	3.1		0.4	0.4	0	3.1	—
β-Caryophylene		2.8	0.2	0	6.4		3.8	1.2	0	10.3	—

TABLE 2. CONTINUED

Chemotype and monoterpene	Composition at home sites (%)					Composition at away sites (%)					F^a
	N	Mean	SE	Min.	Max.	N	Mean	SE	Min.	Max.	
Thymol											
Geraniol	75	0.3	0.1	0	8.9	13	0	0	0	0	3.07
α-Terpineol		1.5	0.2	0	6.1		0.7	0.2	0	1.9	2.54
Thuyanol-4		1.1	0.1	0	3.3		0.6	0.1	0	1.6	8.12 (.006)
Terpinen-4-ol		0.7	0.1	0.	5.0		0.7	0.4	0	5.0	0.13
Linalool		3.9	0.1	1.1	8.0		5.1	1.1	1.9	13.4	2.64
γ-terpinene		7.2	0.6	0	21		14.1	1.3	8.4	23.5	15.36*
Paracymene		9.9	0.8	0	29		9.7	1.4	2.0	17.6	0.13
Carvacrol		4.1	0.5	0.8	26.8		5.0	1.0	1.6	13.5	1.17
Thymol		52.5	1.4	22.4	72.9		40.1	2.8	21.4	57.4	12.10*
Myrcenol-8		0.1	0.1	0	1.7		0	0	0	0	—
Cineole-8		0.8	0.1	0	3.8		0.6	0.3	0	3.1	—
β-caryophylene		2.7	0.2	0	6.8		4.2	0.7	0	7.8	—

[a] F values test differences between these sites. *Significant after correction of the $P < 0.05$ threshold following the Bonferonni sequential method (Rice, 1989). Values in parentheses are probability values before correction by the above method—Low proportions of the molecule prevented statistical comparisons.

[b] 0.1 represents all trace values less than or equal to 0.1

TABLE 3. MONOTERPENE COMPOSITION IN THUYANOL-4 CHEMOTYPE OF *Thymus vulgaris* IN TWO POPULATIONS

Monoterpene	Composition (%) at site 6 ($N = 13$)				Composition (%) at site 7 ($N = 16$)				
	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.	$F_{1,27}$[a]
Geraniol	0	0	0	0	0	0	0	0	—
α-Terpineol	3.8	0.4	2.0	6.5	7.4	0.8	3.2	13.9	18.04*
Thuyanol-4	28.1	3.5	12.8	52.2	19.0	2.7	1.6	39.3	4.64 ns
Terpinen-4-ol	15.1	2.7	2.2	29.1	14.7	2.3	3.1	29.6	< 0.01 ns
Linalool	14.0	2.4	3.0	32.5	8.8	1.4	2.4	20.5	3.68 ns
γ-Terpinene	1.9	0.5	0	5.0	1.5	0.3	0.1[b]	4.2	0.16 ns
Paracymene	0.8	0.3	0	5.0	0.3	0.1	0	0.8	0.12 ns
Carvacrol	2.8	1.6	0	21.4	0.5	0.3	0	4.4	3.04 ns
Thymol	1.3	0.7	0	9.3	0.5	0.2	0	3.0	1.33 ns
Myrcenol-8	13.1	1.2	5.7	18.7	3.7	1.3	0	12.9	26.40*
Cineole-8	0.4	0.1	0.1	1.0	3.4	1.4	0	21.2	4.66 ns
β-Caryophylene	3.0	0.6	0.8	7.5	7.9	1.5	2.4	20.3	9.88*

[a] *Significant after correction of the $P < 0.05$ threshold following the Bonferonni sequential method (Rice, 1989)—Low proportions of the molecule in the essential oil prevented statistical comparisons.
[b] 0.1 represents all trace values less than or equal to 0.1.

two sites (Table 3). If the thuyanol chemotype is considered to have four main monoterpenes (thuyanol-4, terpinen-4-ol, myrcen-8-ol, and linalool), we found a significant ($F_{1,27} = 30.3$, $P < 0.001$) difference in percentage composition due to 10% less thuyanol-4, 10% less myrcen-8-ol, and 5% less linalool at site 7, where a greater proportion of the oil contained α-terpineol, myrcenol-8, and β-caryophylene (Table 3).

For the 54 plants of all chemotypes present at sites 6 and 7, we analyzed correlated variation among the four different monoterpenes that characterize the thuyanol-4 chemotype (i.e., thuyanol-4, terpinen-4-ol, myrcenol-8, and linalool). For the six potential correlations, we found negative correlations between linalool and both thuyanol-4 ($r = -0.49$, $P = 0.002$) and terpinen-4-ol ($r = -0.45$, $P = 0.007$) and a positive correlation between thuyanol-4 and myrcenol-8 ($r = 0.62$, $P < 0.001$).

Linalool. When plants of the linalool chemotype occur at away sites ($N = 26$) they produce less linalool and more α-terpineol, thuyanol-4, terpinen-4-ol, and one or both of the two phenolic precursors (paracymene and γ-terpinene), but not the dominant phenolic monoterpenes (Table 3). Sixteen plants at away sites had thuyanol-4 and terpinen-4-ol as the most predominant secondary constituent of their oil. These plants came from sites 6 and 7 where the thuyanol chemotype is relatively abundant. One plant with high levels of α-terpineol and one plant with thymol as an important secondary constituent of the oil came from site 7,

where the α-terpineol and thymol chemotypes occur. Three plants had geraniol as a secondary constituent of the oil and occurred in sites 5 and 14, where the geraniol chemotype is present. Finally, five plants had a mix of phenolic monoterpenes and their precursors as secondary components of the oil and came from site 14 where the carvacrol chemotype is present. We found no difference in the percentage of the oil composed of the linalool monoterpene in comparisons of the two away sites (6 and 7) or the two home sites (9 and 15). Thus, the only variation in percentage composition of the oil of the linalool chemotype was between home and away sites.

Carvacrol. When carvacrol plants are found at sites where this chemotype is rare, they produce less carvacrol and more of either geraniol, α-terpineol, linalool, or thymol (Table 3). Seven carvacrol plants were obtained from sites where this chemotype was rare. Although the two plants (2 and 3) with the highest amount of thymol came from site 12, a site dominated by the thymol chemotype, the five other plants showed no correlation between local chemotype and monoterpene composition.

We found a significant ($F_{3,89} = 17.49$, $P < 0.001$) difference in the percentage of carvacrol for plants of this chemotype among the four sites (1–4) where this chemotype occurred as the majority type. This difference was due to their being two groups of populations (Figure 5a): populations 1 and 2 had higher proportions of carvacrol than populations 3 and 4 (Scheffé means test at $P < 0.05$).

Thymol. Thirteen thymol plants were detected at "away" sites where they showed a reduction in the percentage of the dominant monoterpene in their oil (Table 3). However, they did not increase in one or more of the other dominant monoterpenes and only the precursor, γ-terpinene, showed an increase in proportion at away sites that compensates for the decrease in thymol in the oil (Table 3). Nevetheless, one plant at site 7, where thuyanol-4 is the most abundant chemotype, was the thymol plant with the highest concentration of thuyanol-4 in its oil. Likewise, two plants from site 9, where linalool is the dominant chemotype, had relatively high amounts of linalool.

We found a significant ($F_{3,71} = 3.33$, $P < 0.05$) difference in the percentage of thymol in plants of the thymol chemotype among the four sites (10–13) where this chemotype occurs as the majority type. This difference was due to a lower proportion of thymol (and a higher proportion of precursors) at site 13 compared to the three other sites (Schemske means test at $P < 0.05$) (Figure 5b).

Correlations among Phenolic Monoterpenes and Precursors. The production of the phenolic monoterpenes (thymol and carvacrol) and their precursors (para-cymene and γ-terpinene) had a significant positive correlation between the two precursors paracymene and γ-terpinene and a negative correlation between the production of the monotepene characteristic of the chemotype and the two precursors for both the thymol and carvacrol chemotypes (Table 4). The carvacrol chemotype, but not the thymol chemotype, showed a negative correlation between the production of thymol and carvacrol.

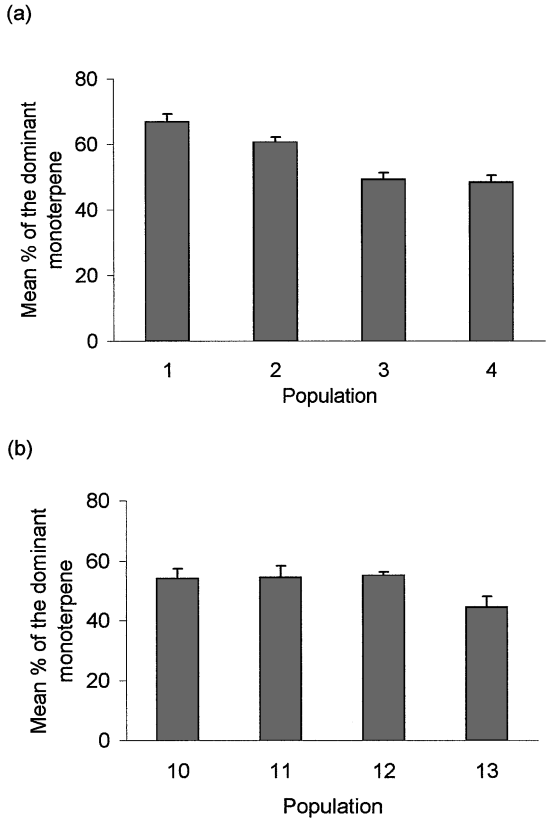

FIG. 5. Mean percentage (±SE) of the dominant monoterpene in the essential oil of the two phenolic chemotypes carvacrol (a) and thymol (b) of *Thymus vulgaris* chemotypes in four populations in southern France where the chemotype is the majority type.

DISCUSSION

Variation among Sites. The most striking result of this study of secondary compound variation in *Thymus vulgaris* is the significant difference in the composition of the essential oil between sites where a chemotype is the most abundant chemotype (i.e. home sites) and those where it is rare or occurs as part of mixed-chemotype populations (i.e., away sites). We observed this trend in five chemotypes. Two potential causes can be invoked to explain this variation.

First, environmental differences among sites may cause differences in monoterpene production (e.g., Lincoln and Lagenheim, 1976; Mihaliak et al., 1989; Muzika, 1993). Hence, we cannot rule out environment as a potential cause

TABLE 4. PEARSON CORRELATION COEFFICIENTS FOR VARIATION IN MONOTERPENES PRODUCED BY CARVACROL AND THYMOL CHEMOTYPES OF *Thymus vulgaris*

Chemotype	Monoterpene	Monoterpene[a]			
		λ-terpinene	Paracymene	Carvacrol	Thymol
Carvacrol ($N = 100$)	γ-terpinene		0.360*	−0.497*	−0.001 NS
	Paracymene			−0.472*	0.053 NS
	Carvacrol				−0.449*
	Thymol				
Thymol ($N = 88$)	γ-terpinene		0.530*	−0.096 NS	−0.808*
	Paracymene			−0.300 NS	−0.585*
	Carvacrol				−0.101 NS
	Thymol				

[a] * $P < 0.0001$, NS: not-significant at corrected threshold for multiple comparisons following Rice (1989).

for the decrease in the production of the dominant monoterpene in *T. vulgaris* chemotypes at sites where they are a minority. At such sites, the low abundance of plants of the rare chemotype may be due to the local environment, which may also cause plants of a given chemotype that can survive in such sites to produce relatively low proportions of their dominant monoterpene.

An important point here is the significant variation in percentage of the dominant monoterpene among different home sites for the carvacrol and thymol chemotypes. This variation is correlated with marked ecological differences among sites. For the four carvacrol populations, the percentage of carvacrol declines in populations 3 and 4, which are geographically the closest to the nonphenolic populations in the St. Martin-de-Londres valley (Figure 2). These two populations occur on the north-facing slope of the valley, whereas populations 1 and 2 occur on the south-facing slope outside the valley in a landscape where carvacrol is the dominant chemotype across large expanses of the landscape (Vernet et al., 1977a,b; Gouyon et al., 1986). The ridge that separates the two groups is known to separate different ecological zones—inside the valley, where nonphenolic populations predominate (see discussion by Gouyon et al., 1986; Thompson, 2002), winter minimum temperatures are much lower and soils are deeper and less stony than outside the valley. The decline in the proportion of thymol at site 13 is also associated with ecological variation, this site being drier, hotter, and on stonier soils than the three other sites where thymol was found to be the majority chemotype.

The second potential cause of variation in dominant monoterpene composition is that genetically based differences among plants of a given chemotype may occur among sites. Many species show genetically based differences in secondary metabolite formation (Hanover, 1966; Murray and Lincoln, 1970; Lincoln and Lagenheim, 1976; Mihaliak et al., 1989; Mithen et al., 1995; Shonle and

Bergelson, 2000), including *Thymus vulgaris* (Vernet et al., 1986). However, in *T. vulgaris*, such differences have been presented only in terms of the occurrence of different chemotypes. No attention has been paid to the co-occurrence of monoterpenes within chemotypes (other than the thuyanol chemotype—see below), and intermediate types have been dismissed as being rare and/or of little significance (Granger and Passet, 1973). If the variation between home and away sites is due to genetic differences, then such differences would provide evidence that genetically based variation in monoterpene production may have a quantitative component within each of the thyme chemotypes.

A result that supports the idea of genetically based variation in dominant monoterpene composition among home and away sites in *T. vulgaris* concerns the identity of the monoterpenes that occur as a high proportion of the oil at away sites. In almost all cases, the decline in proportion of the characteristic dominant monoterpene at away sites is correlated with a significant increase in a monoterpene present in the co-occurring chemotypes. If a chemotype is absent from a population, its monoterpene never occurs as a secondary constituent of the oil.

This result was particularly apparent in the four nonphenolic chemotypes and in the carvacrol chemotype, and it shows a relationship with the epistatic chain of monoterpene production. In *T. vulgaris*, the presence of the dominant monoterpene is controlled by an epistatic series of five loci (Vernet et al., 1986). A plant with a dominant G allele will have the geraniol chemotype, regardless of whether it has dominant or recessive alleles at the other loci. A plant that is homozygous recessive at the G locus (i.e., *gg*) with a dominant A allele will have the α-terpineol chemotype. A plant that is homozygous recessive *gg/aa* with a dominant U allele will have the thuyanol chemotype. The sequence continues in the order geraniol, α-terpineol, thuyanol, linalool, carvacrol. If all five loci are homozygous recessive, thymol is the dominant monoterpene. Our data illustrate that the potential relationship between monoterpene variation and the chain of five loci may not be more complex than previously thought, since it is clear that plants may produce significant proportions of more than one dominant monoterpene.

Four points are important here: (1) When geraniol plants were sampled in populations dominated by the linalool chemotype, the decrease in geraniol in their oil was accompanied by an increase in linalool. (2) When α-terpineol plants were sampled in a population dominated by the thuyanol and linalool chemotype, the decrease in α-terpineol in their oil was accompanied by an increase in linalool or thuyanol-4. (3) When linalool plants were sampled in a population with the thuyanol chemotype, the decrease in linalool in their oil was accompanied by an increase in thuyanol-4. (4) When carvacrol plants were sampled in a population dominated by the thymol chemotype, the decrease in carvacrol in their oil was accompanied by an increase in thymol, compared to an increase in linalool in a population dominated by this chemotype. The thymol chemotype showed, in

almost all cases, a decline in thymol associated with an increase in the composition of its precursors, and not the presence of one of the other characteristic monoterpenes. The latter result is not surprising since thymol is at the end of the chain.

In most cases, a decline in the percentage composition of the dominant monoterpene was associated with a significant increase in the presence of a monoterpene produced by a dominant allele at a locus that would normally not be expressed in the chemotype (e.g., an L allele in a geraniol plant or a U allele in an α-terpineol plant). Three explanations can be provided to account for this pattern. First, the presence of a "secondary" dominant gene may not be completely switched off by the dominant gene of the chemotype prior in the chain, allowing for limited production of a secondary monoterpene. Second, modifier genes at loci other than that coding for the dominant allele of a particular chemotype may allow for the production of secondary monoterpenes. Third, rather than being due to epistasis, the genetic control of monoterpene production may be a consequence of gene dosage effects along a chain of loci. For example, if the G locus produces more geraniol than the A locus produces α-terpineol (and so on down the chain) then a geraniol plant with a dominant A allele may produce small amounts of α-terpineol.

Three lines of evidence support the dosage idea: (1) the biosynthesis of linalool is more complex than that of geraniol due to transposition within the molecule (Passet, 1971); (2) the gene chain is almost identical to the biosynthetic chain, and phenolics have a significant proportion of their oil in the form of precursors, and (3) heterozygotes at the C locus are thought to produce intermediate amounts of carvacrol. Under this hypothesis, one would predict an increase in the production of linalool across a range of genotypes of the geraniol chemotype: from *GG/ll* (no linalool), to *GG/Ll*, *Gg/Ll*, and *Gg/LL*. The amount of linalool will be dependent on the genotype at the two loci and on the frequency of the dominant alleles in the population. A similar line of reasoning can be proposed for the presence of secondary monoterpenes in the oil of the other chemotypes. This scenario is further complicated by the fact that there may be two genes involved in the synthesis of geraniol (Vernet et al., 1986). The different loci may interact via dosage effects that vary across the loci and allow for variable amounts of monoterpenes in the oil.

Incomplete specificity of enzymes catalyzing different parts of the biosynthetic chain may also influence secondary monoterpene production in thyme. Carvacrol and thymol chemotypes at away sites were occasionally observed to produce small proportions (10–12% of the oil) of one or other of the four nonphenolic monoterpenes that are normally produced earlier in the chain. The explanation for this is that the enzyme catalyzing the formation of the precursor to the two phenolic monoterpenes, γ-terpinene (Granger et al., 1965), is also known to allow the production of such small proportions of the four monoterpenes that

characterize the four nonphenolic chemotypes of *T. vulgaris* (Alonso and Croteau, 1992).

The pattern of variation among home and away sites and the presence of plants with a blend of monoterpenes rather than a single dominant monoterpene is of particular interest in the context of the ecological functions of monoterpenes in thyme and the causes of spatial variation in chemotype frequency. The chemotypes show marked habitat segregation (Gouyon et al., 1996; Thompson, 2002), herbivore-specific variation in palatability (Linhart and Thompson, 1999), differences in parasite load (J. Amiot, unpublished data), and have different effects on the germination of associated species (Tarayre et al., 1995). A particular chemotype in an away site may be less suited to the local environment, and the secondary production of other monoterpenes could favor the persistence of plants at a given site. In addition, at away sites, plants of each chemotype have a greater fraction of their oil as a range of minor monoterpenes. The possibility that having more than one monoterpene is advantageous in some situations is worth investigation.

Variation among Chemotypes. Our results provide a statistical confirmation of the data presented by Granger and Passet (1971), who found that the mean values of the proportion of dominant monoterpenes in the α-terpineol, geraniol, and linalool chemotypes exceed those of the thuyanol, carvacrol, and thymol chemotypes. This variation among chemotypes has two main components.

First, the thuyanol chemotype shows marked variation in the relative composition of its characteristic monoterpenes (thuyanol-4, terpinen-4-ol, myrenol-8, and linalool) and has a lower proportion of its oil composed of these dominant monoterpenes than the other nonphenolic chemotypes. This suggests that a single enzyme is probably responsible for the production of the thuyanol-4 and terpinen-4-ol monoterpenes (see also Passet, 1971). Since the proportion of linalool is negatively correlated with the production of the above two molecules, it is possible that their production results from a different substrate and depends on a different enzyme. The molecular structures of these two molecules and probable biosynthetic pathway leading to their formation (Passet, 1971) support this idea. There are many known examples where a single enzyme catalyzes the secondary metabolism of multiple products and of enzymes of secondary metabolism that can recognize more than one substrate (see review by Pichersky and Gang, 2000). The thuyanol chemotype of *T. vulgaris* would appear to be an example of this phenomenon. Whatever the cause, the variability in the composition of the thuyanol chemotype makes it a difficult chemotype to define (see also Passet, 1971; Granger and Passet, 1971). It is also interesting to note that as well as being the sole chemotype characterized by a mixture of different monoterpenes, the thuyanol chemotype is also the only one that never seems to occur in monochemotype populations (Thompson, 2002).

Second, the two phenolic chemotypes also have lower proportions of their oil composed of their dominant monoterpenes than the nonphenolic chemotypes

that have a single monoterpene as the characteristic component of their oil. This is due to the fact that the precursors, paracymene and γ-terpinene, occur as components of the oil in the phenolic chemotypes, particularly in thymol plants. The longer chain of reactions leading to the production of thymol and carvacrol would seem to reduce the proportion of the oil composed of these monoterpenes. The correlated variation among thymol, carvacrol, and the precursors para-cymene and γ-terpinene in each of the carvacrol and thymol chemotypes is interesting in this context (Table 4). First, in both chemotypes, there was a positive correlation between the production of the precursors γ-terpinene and paracymene, supporting the claim that their production is closely linked (Granger et al., 1965; Passet, 1971) and that both compounds are precursors to thymol and carvacrol. Second, there was a negative correlation between the production of thymol (and carvacrol) and the two precursors for plants of both chemotypes, suggesting incomplete transformation of the precursors. The consistency of the results suggests that both paracymene and γ-terpinene are precursors and that paracymene is not an end product. Finally, in the carvacrol chemotype, but not the thymol chemotype, there was a negative correlation between the production of thymol and carvacrol. Since carvacrol plants can be heterozygous (*Cc*), there can be much variation in the relative composition of the two phenolic molecules in the carvacrol chemotype, with heterozygotes producing less carvacrol than plants that are homozygous *CC*. The variation in correlation structure among the two chemotypes is a logical result of the genetic control of phenolic monoterpene production.

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NOVEL DETECTION OF FORMYLATED PHLOROGLUCINOL COMPOUNDS (FPCs) IN THE WOUND WOOD OF *Eucalyptus globulus* AND *E. nitens*

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Abstract—This study characterized the chemical responses of *Eucalyptus globulus* and *Eucalyptus nitens* to artificial inoculation with a basidiomycete decay fungus. Nine-year-old trees responded to mechanical wounding or inoculation with the decay fungus by producing new wound wood characterized by the presence of dark extractives 17 months after wounding. Analysis of crude wound wood extracts by HPLC coupled to negative ion electrospray mass spectrometry revealed the presence of a complex mixture of many unidentified formylated phloroglucinol compounds (FPCs), in addition to a diverse range of other polyphenolic compounds (hydrolyzable tannins, proanthocyanidins, flavanone glycoside, stilbene glycosides). Prior to this study, FPCs have only been reported from leaves and buds of *Eucalyptus* spp. Unequivocal evidence for the presence of macrocarpal A and B, and sideroxylonal A and B in the crude extracts was obtained, as well as evidence for a wide range of as yet unreported FPCs. Subsequent preliminary *in vitro* fungal and bacterial bioassays did not support an antimicrobial role for FPCs in host-pathogen interactions in eucalypts.

Key Words—*Eucalyptus*, wood extractives, HPLC, negative ion electrospray mass spectrometry, antimicrobial, antioxidant.

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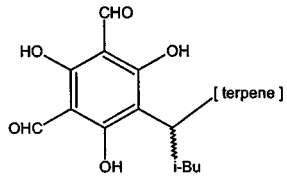
INTRODUCTION

Trees respond to fungal invasion by the production of various anatomical and biochemical barriers at the lesion margin, thus compartmentalizing the spread of damage and decay in living sapwood (Shigo and Marx, 1977; Liese and Dujesiefken, 1990). The defense mechanisms contributing to the compartmentalization process vary among tree species, but in general, putative defense mechanisms commonly reported include the accumulation of induced inhibitory secondary metabolites (Barry et al., 2001; Baum and Schwarze, 2002), the initiation of cell wall alterations (e.g., suberization) (Pearce, 1990), as well as occlusion of xylem cells (e.g., tylosis formation within vessels) (Barry et al., 2000). Detailed reviews on the compartmentalization response of many woody tree species have been written by Pearce (1996), Schwarze et al. (1999), and more recently by Yamada (2001).

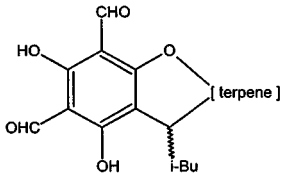
Much of the accrued knowledge concerning host–pathogen interactions in the xylem has been drawn from studies involving broad-leaved angiosperms. In comparison, the state of knowledge on the host resistance mechanisms of *Eucalyptus* L'Heritier (Myrtaceae) is still rudimentary, despite being one the world's most important and widely planted forest species (Turnbull, 2000). In addition, the few studies that have examined host responses of *Eucalyptus* have predominantly focused on the histological aspects, and little consideration has been given to characterizing either the constitutive or induced biochemical defense mechanisms (e.g., Wilkes, 1986; Barry et al., 2000). Instead, chemical analyses have been generally focused on elucidating the chemistry of healthy bark (Kim et al., 2000), leaves (Close et al., 2001), healthy wood (Cadahia et al., 1997), and kino, a polyphenolic exudate (Hillis and Yazaki, 1975). The only detailed study of the chemistry of extant tissues at wounding or infection is that reported by Barry et al. (2001). Here, the decay fungi were observed to elicit increased levels of hydrolyzable tannins in the extant tissue of *Eucalyptus nitens*. As part of a wider investigation examining host responses to wounding and fungal invasion in *E. globulus* and *E. nitens*, this study aimed to determine the chemical constituents present in the wound wood (defined as the tissue formed after wounding and located immediately adjacent to the wounding site). The biochemistry of wound wood has been poorly studied in both hardwoods and softwoods, and it has never been studied in any *Eucalyptus* species.

This paper details the detection of a group of secondary metabolites referred to as formylated phloroglucinol compounds (FPCs) in a novel location, i.e., the wound wood of *E. nitens* and *E. globulus*. We also examined the possible antimicrobial role of FPCs in host–pathogen interactions in eucalypts. FPCs are a group of secondary metabolites unique to plants in the family Myrtaceae, especially *Eucalyptus* species, and have previously been reported in buds and leaves only (Takasaki et al., 1994a,b; Osawa et al., 1996). Detection of FPCs in wood has not been published although euglobals have been detected in *E. globulus* healthy

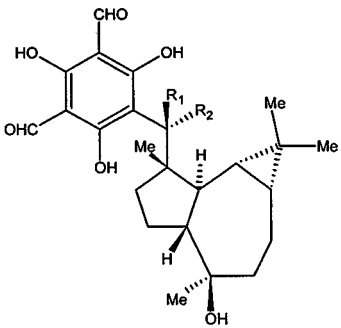
sapwood (M. Takasaki, personal communication). FPCs are mono- to tetraformylated phloroglucinol-based derivaties with an attached terpene moiety in almost all cases, and to date, various monoterpenes and sesquiterpenes have been found linked to the formylated phloroglucinol ring. The generic structures of two main classes of FPCs—macrocarpals and euglobals (I and II, respectively in Scheme 1,)—consist of standard terpenes linked to an isopentyl diformyl phloroglucinol group. For example, macrocarpals A and B (III and IV) are different stereoisomers around



(I) General structure of macrocarpals

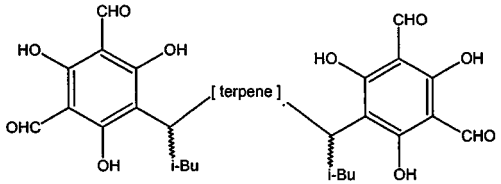


(II) General structure of euglobals



(III) Macrocarpal A (R_1=H, R_2 =isobutyl)

(IV) Macrocarpal B (R_1=isobutyl, R_2 =H)



(V) Proposed general structure for di-substituted FPCs.

SCHEME 1. Structures of compounds.

the isobutyl group of the common sesquiterpene alcohol globulol. Other FPCs including the various known sideroxylonal isomers (Satoh et al., 1992) are simply two isopentyl diformyl phloroglucinol without any attached terpene group. We provide evidence for the detection of novel FPC structures, including triterpene FPCs and a range of disubstituted FPCs.

METHODS AND MATERIALS

Experimental Site and Wounding Treatments. The experimental site growing both *E. globulus* and *E. nitens* (9-year-old trees) was in Lewisham, Tasmania (42°49′ S, 147°37′ E. 20 m as1). White et al. (1998) provide details of site establishment, fertilizer addition, and pest control of the Lewisham site. In late summer of 2000, six trees each of *E. nitens* and *E. globulus* were randomly selected for wounding treatments. At breast height (*ca*. 1.5 m), a 10-mm-diam. hole was drilled 3 cm into the outer sapwood. A second hole with identical dimensions was drilled 30 cm above the first and inoculated with decay fungi previously isolated from an *E. nitens* white-rot decay column. Inoculation involved inserting 2- $\times$1-cm^2 rectangular pieces of a 2-week-old culture growing on malt extract agar into the drill hole and sealing with a layer of lanolin wax. The decay fungus used for the inoculation is an unidentified basidiomycete known as isolate D. The fungus is described in Barry (2001).

Approximately three months later, three trees each of *E. nitens* and *E. globulus* were harvested. Billets of *ca.* 50 cm in length, which included the drill wounds, were dissected with a bandsaw by cutting longitudinal sections through the wound. Chisel shavings (60–90 mg) of the wound wood that had been produced adjacent to the drilling wound (Figure 1), as well as samples of healthy sapwood and heartwood obtained from the same corresponding tree, were prepared for LC-MS analysis as follows. The chisel shavings were extracted twice with 750 μl of 70% aqueous acetone over 48 hr in the dark at 4°C. The extract was transferred to a 1.5 ml microcentrifuge tube and centrifuged (1300 rpm for 2 min) to remove solids. Samples were stored at -20°C and analyzed within one week after extraction. This procedure was repeated with the remaining three trees, approximately 17 months after wounding.

Chromatographic and Spectrometric Analysis

HPLC-UV. Injections of 5 μl of sample were analyzed by HPLC with ultraviolet detection (HPLC-UV) with the following system. HPLC analyses were carried out on a Waters Alliance 2690 with a Waters 996 Photodiode Array Detector (Waters Corporation, Milford, Massachusetts, USA). The column was a Waters Nova-Pak C-18 column (150 mm $\times$ 3.9 mm) fitted with an Alltech Econosphere

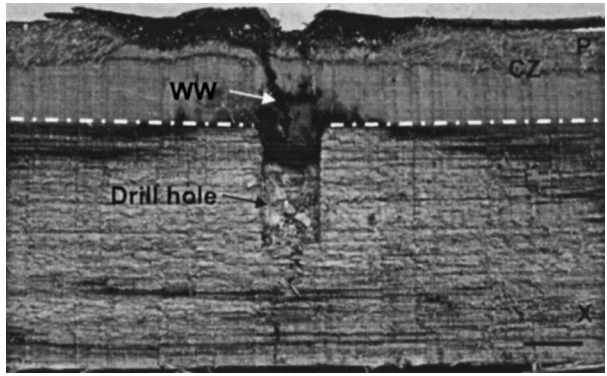


FIG. 1. Radial longitudinal section showing typical response to drill and inoculation treatment after 18 months in 9-year-old *E. globulus* and *E. nitens*. Note the boundary of healthy tissue and decayed tissue (dashed line). Scale bar = ~1 cm. P = phloem, CZ = cambial zone, WW = wound wood, X = xylem.

5-μm C-18 guard cartridge (Alltech, Melbourne, Australia). The flow rate was 0.8 ml/min. The solvent system was: solvent A, 2% acetic acid in methanol; solvent B, 2% acetic acid in distilled water; solvent C, 100% hexanes. The solvent gradient was as follows: 10% solvent A/90% solvent B; ramped to 85% solvent A/15% solvent B at 25 min; then to 100% solvent A at 35 min; then to 80% solvent A/20% solvent C at 45 min. The original conditions were reestablished by ramping to 100% solvent A for 2 min followed by 15-min requilibration. The photodiode array detector was monitored from 240 to 445 nm at a sampling rate of 1 spectrum/sec and at a resolution of 2.4 nm. The HPLC sample chamber was maintained at 5°C throughout the analyses.

HPLC-MS. The HPLC column and conditions were described as above. The HPLC column outlet was coupled directly to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source (Finnigan MAT, ThermoQuest, Co., San Jose, California, USA). The instrument was operated in the negative ion mode, scanning from m/z 125 to 1500 with an automatic gain control target value of 2×10^7 and maximum ion injection time of 100 msec. Operating conditions were as follows: sheath gas 90 psi, auxiliary gas 50 psi, ESI needle voltage 4.5 kV, capillary temperature 270°C, and capillary voltage-30 V. Data-dependent MS-MS spectra were routinely acquired from the most intense ion in the spectrum, with collision energy of 30% and a peak isolation width of 4 D.

Identification of FPCs. HPLC-ESI-MS with negative ion detection is a highly sensitive method for detection and identification of individual FPCs in a crude extract (Eschler et al., 2000; Close et al., 2001). Using data from previous investigations of *E. nitens* foliage (Close et al., 2001), the majority of peaks within the HPLC time range 25–45 min were initially identified from their negative

electrospray MS and MS-MS data as FPCs. Their identities were later confirmed with various purified FPCs standards that included euglobal Ib, euglobal Ic, euglobal III, macrocarpal A and B, and sideroxylonal A and B. Standards were weighed and dissolved in 100% acetone at concentrations of 0.10 mg/100 μl. For confirmation of proposed novel triterpene FPCs and disubstituted FPCs, accurate mass measurements were carried out on a Kratos ISQ mass spectrometer fitted with a Desorption Chemical Ionization (DCI) probe. The probe was rapidly heated to 500°C after insertion into the ion source. The instrument was operated in the electron ionization (EI) mode at 70 eV with an accelerating voltage of 5.3 kV. Perfluorokerosene was used as an internal reference, and peak matching was undertaken on the target ions at a resolution (10% valley definition) of 10,000.

Semiquantification

Total Soluble Extract in Wound Wood. To obtain dry weights of the total soluble extractives present in the 1.5 ml solvent, 400 μl of the sample were placed into a preweighed glass vial (using a 5-point balance scale), then dried under a stream of nitrogen at room temperature and placed in an oven (30°C) to remove all moisture before reweighing glass vials. Subsampling was necessary, as the extractives could not be easily reconstituted. Typical yields of dry weight of total soluble extract (as a percentage of fresh weight of tissue) ranged from 15% to 45% for wound wood and 2% for healthy sapwood extracts.

Total Levels of FPCs. HPLC chromatograms of crude wound wood extracts showed that the separation of FPCs into individual compounds was difficult, as found in other studies (Akano et al., 1981; Eschler and Foley, 1999). Consequently, semiquantification of individual FPCs was not attempted due to the large number of individual isomers and consequent complex chromatograms with many unresolved peaks. Instead, FPCs were measured as a single class of compounds to provide an estimate of the total levels of FPCs (expressed as percentage of dry weight of soluble extract) present in wound wood. The sums of the FPC peak areas (at 280 nm) as a single group were used in the semiquantification calculations. Response factors calculated from an average of macrocarpal A and macrocarpal B standards were applied for the quantification of FPCs. The highly variable nature of wound wood as well as the small sample size precluded the use of statistical analysis to determine differences in either the qualitative and quantitative FPC composition between treatments.

Antimicrobial Bioassays

Semifractionation of Crude Wound Wood Extract. Semipreparative separation of the extract was possible due to the large time interval that separated the significantly less polar fraction of the extract (predominantly containing FPCs) from the polar fraction of extract (containing the other classes of compounds).

Crude wound wood extract from *E. globulus* containing relatively high FPC levels (3.4% of dry weight of soluble extract) was used. As only a crude separation was required, a more rapid solvent system was developed. The same equipment used in the HPLC-UV analyses of the crude wound wood extract, as previously described, was used for the semipreparative separation.

The flow rate was 0.8 ml/min. The solvent system was: solvent A, 2% acetic acid in methanol; solvent B, 2% acetic acid in distilled water; solvent C, 100% hexanes. The solvent gradient was as follows: 75% solvent A/25% solvent B at 2 min; ramped to 100% solvent A at 4 min; then to 80% solvent A/20% solvent C at 5 min and held until 11 min. The first fraction (fraction I), containing condensed tannins, hydrolyzable tannins, and flavonoids was collected between 0 and 5 min. The second fraction (fraction II), containing predominantly FPCs was collected between 5 and 10 min. Injections of 100 μl were made, and a total of 1.6 ml was processed. All solvents collected from the semipreparation separation were removed with a rotary evaporator. The extract was redissolved to original concentrations with 70% acetone.

Antifungal Bioassay. Twenty microliters each of fraction I, fraction II, original crude wound wood extract, and a control (70% aqueous acetone) was spotted directly onto the center of separate silica TLC plates (2×5 cm). The TLC plates were placed in laminar flow for 2 hr to remove all traces of solvent. A dense spore and mycelium suspension of three fungal cultures (*Cladosporium cladosporiodes, Ganoderma weberianum, and Trametes zonata* grown on 2% malt extract agar (Oxoid) and incubated at 20°C in the dark) was prepared by flooding 14-day-old plates with 3% malt extract aqueous solution and agitating the plate with a glass hockey stick to dislodge the conidia and mycelium. An even layer of the fungal suspension was applied onto the previously prepared TLC plates. Following incubation at 22°C under high humidity for 5 days, plates were examined for zones of inhibition.

Antibacterial Bioassays. These were performed following a modified Kirby-Bauer disk diffusion procedure (Bauer et al., 1966): 10 μl each of fraction I, fraction II, original crude wound wood extract, and a control (70% aqueous acetone) was applied to individual paper disks, allowed to dry, and placed on nutrient agar plates seeded with either *Escherichia coli*, *Erwinina* sp., *Xanthomonas* sp. (Gram-negative bacteria) or with *Streptococcus aureus or Bacillus subtilis* (Gram-positive bacteria). All tests were carried out in triplicate, and plates were incubated at 37°C. After 48 hr of incubation, the diameters of the zones of inhibition were measured.

RESULTS

Seventeen months after injury, all drill hole wounds had fully occluded and the vascular cambium had been reestablished. Decay columns (>10 cm in length)

were associated with all drill holes inoculated with the decay fungus. In both species, the wound wood was characterized by the presence of varying amounts of dark extractives (defined as nonstructural components of wood), regardless of the whether fungal colonization had occurred or not (Figure 1).

Analysis of New Wound Wood. A diverse range of polyphenolic compounds including hydrolyzable tannins (both gallo- and ellagitannins), proanthocyanidins, flavanone glycosides, stilbene glycosides, and formlyated phloroglucinol compounds (FPCs) were detected in the crude wound extracts of both *E. nitens* and *E. globulus*. In particular, FPCs were noted as a complex series of peaks eluting late in the HPLC chromatogram (Figure 2). The presence of sideroxylonal A and B was unequivocally confirmed by comparison of HPLC-UV and MS data standards. Coinjections with FPC standards unequivocally confirmed the presence of macrocarpals A and B in the crude wound wood extract.

Tandem Mass Spectral Data of Macrocarpal and Euglobal Standards. Macrocarpals and euglobals of the same terpene series are indistinguishable by molecular

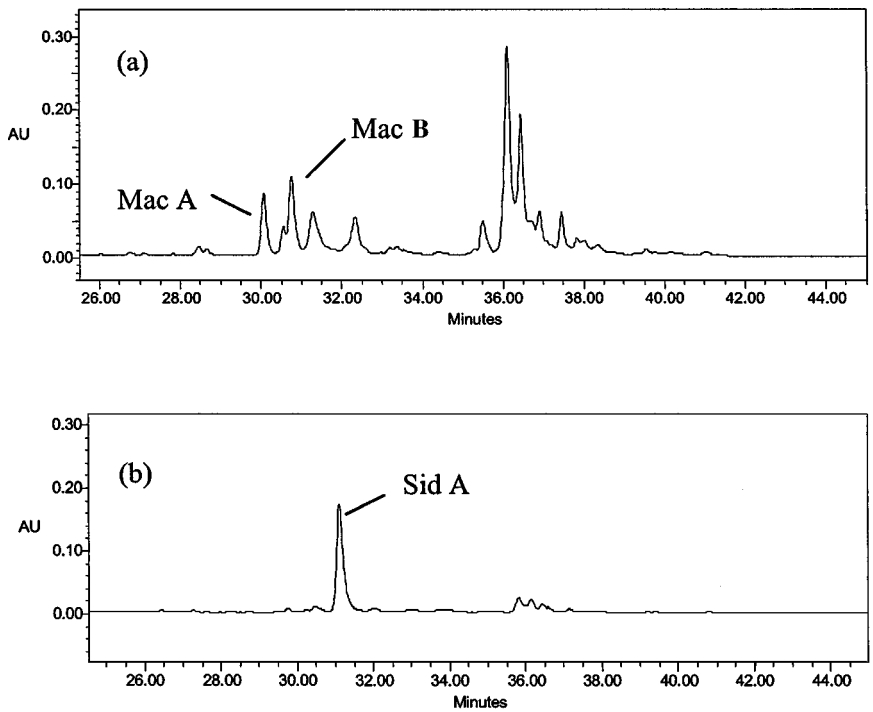


FIG. 2. Examples of HPLC-UV chromatograms of wound wood extract (70% acetone) of *Eucalyptus globulus* (a) and *E. nitens* (b) measured at 280 nm indicating major FPCs including sideroxylonal A (Sid A), macrocarpal A (Mac A) and macrocarpal B (Mac B).

weight (since they have the same degree of unsaturation despite the bond to the phenolic ring in euglobals). Tandem mass spectrometry, however, provides valuable characteristic spectra to distinguish between the two FPCs classes. In the present study, the sesquiterpene macrocarpals for which standards could be obtained gave an intense product ion at m/z 207, with a weaker product ion at m/z 250 (Figure 3). On the other hand, euglobals Ib, Ic, III, and V were characterized by an intense ion at m/z 249, and in some cases a less intense ion at m/z 207. For example, under negative ESI, the MS of euglobal IC gave m/z 385 (100%) while MS-MS of the m/z 385 ion showed m/z 249 (100%), 250 (11.6%), and 248 (10.6%). We suggest that the radical cation at m/z 250 in the macrocarpals can be formed by a single hydrogen transfer from the isopentyl diformyl phloroglucinol to the terpene with charge retention on the phloroglucinol ring. In the euglobals, there is already one less phenolic hydrogen, and a second bond has to be broken to remove the terpene group, resulting in a prominent even electron ion at m/z 249.

Wound Wood Dominated by FPCs of the Macrocarpal Series. Comparison of HPLC-UV and MS-MS behavior of the FPCs present in wound wood with that described for the euglobal and macrocarpal standards indicated that the FPCs found in wound wood were predominantly of the macrocarpal series and not of the euglobal series. More specifically, the overwhelming majority of compounds of mol wt 386, 454, and 472 gave MS-MS spectra dominated by m/z 207 and 250 product ions (Figure 3). Only a few minor peaks were characterized by an intense ion at m/z 249, consistent with their assignment as euglobals. Moreover, comparison of HPLC-UV and MS-MS data of the four available euglobal standards with the few euglobals detected in the wound wood crude extract showed the latter to be different from the euglobal standards.

Evidence for New FPCs. The number of coeluting peaks detected at mol wt 386, 454, and 472 in the wound wood extracts far exceeded the number of reported compounds, indicating the presence of many new FPCs at these specific masses. In addition, complex groups of compounds of molecular weight series other than 386, 454, and 472 were also detected in the specific mass chromatograms. Significantly, tandem MS investigation of these compound series showed that they also appeared to fit the macrocarpal pattern. For example, Figure 4 shows the MS^2 and MS^3 experiments on a major group with intense $[M\text{-}H]^-$ ions at m/z 703. The major product ion of this at m/z 453 was isobaric with the $[M\text{-}H]^-$ ions of the sesquiterpene hydrocarbon FPCs. Moreover, from a MS^3 experiment, its subsequent third generation ions were essentially identical to the product ions of the major mol wt 454 macrocarpals present in the samples (Figures 3b and 4c). Given that the first loss from 703 to 453 (250 daltons) corresponds in mass to an isopentyl diformyl phloroglucinol group after a single proton transfer, these compounds would appear to be the first reported terpenes with two of these groups attached (V). Accurate mass measurement by electron ionization confirmed a formula of $C_{41}H_{52}O_{10}$ for the mol wt of 704 (found 704.3559, calculated 704.3561).

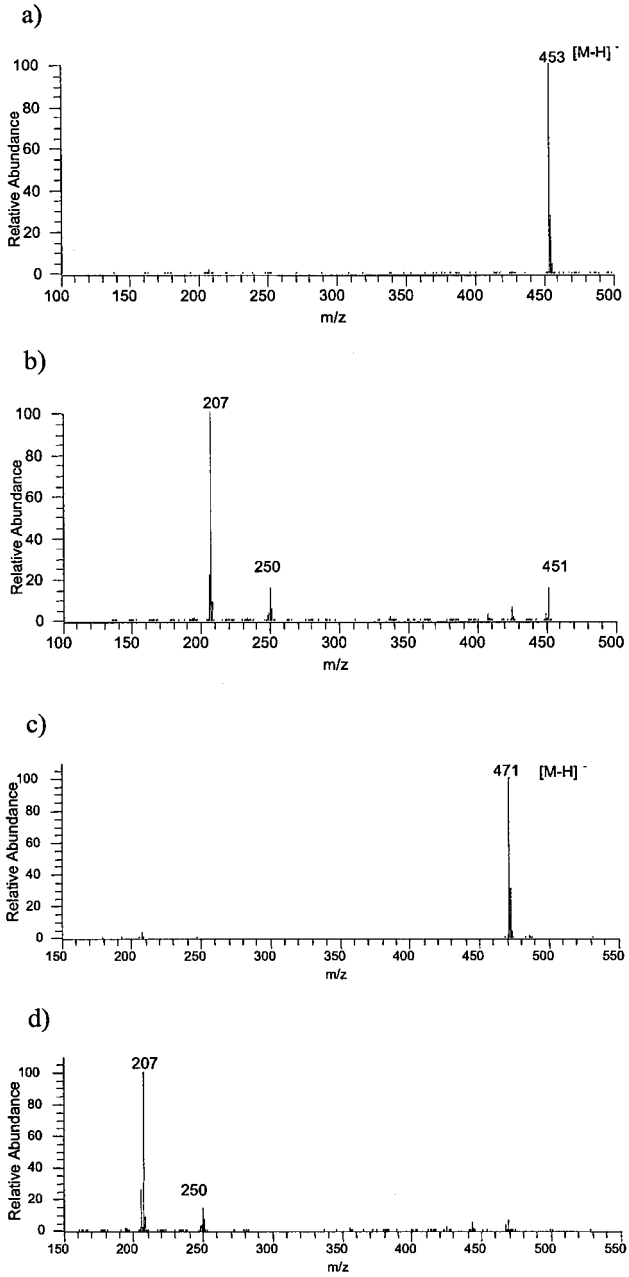


FIG. 3. MS and MS-MS of typical 454 sesquiterpene hydrocarbon FPC from wound wood extract and macrocarpal A. (a) Negative ion electrospray mass spectrum of 453; (b) MS-MS product ions of $[M\text{-}H]^-$ ions at m/z 453; (c) negative ion electrospray mass spectrum of macrocarpal A; and (d) MS-MS product ions of $[M\text{-}H]^-$ ions at m/z 471 of macrocarpal A.

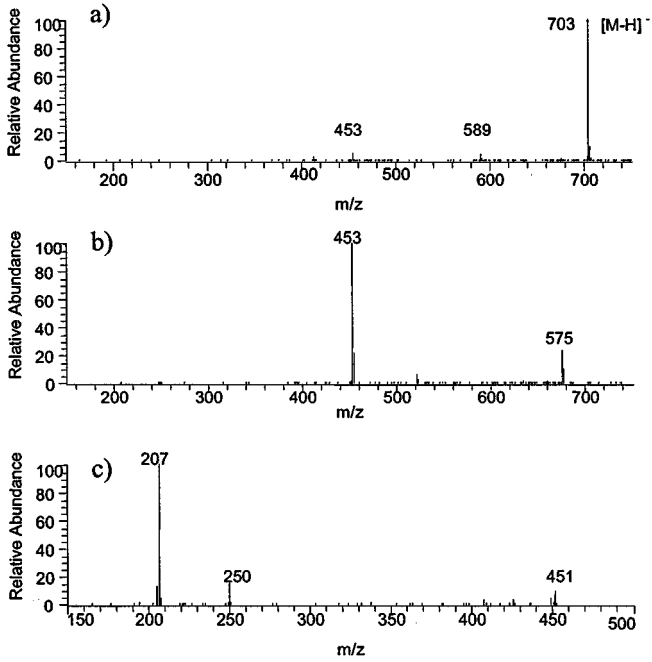


FIG. 4. Putative disubstituted sesquiterpene hydrocarbon FPC MS, MS^2, and MS^3 data from *E. globulus* wound wood extract. (a) Negative ion electrospray mass spectrum; (b) MS^2 product ions of $[M\text{-}H]^-$ ions at m/z 703; and (c) MS^3 product ions of m/z 453 ions derived from m/z 703.

Other new compound series with mol wt 658 and 676 were tentatively assigned as coming from triterpene hydrocarbon and triterpene alcohol FPCs, respectively, also the first report of this type of FPC (Figure 5). Accurate mass measurement by electron ionization confirmed a formula of $C_{43}H_{62}O_5$ for the molecular weight of 658 (found 658.4617, calculated for 658.4598). Other series with molecular weights 590 and 608 were tentatively assigned as sesterterpene (C_{25}) hydrocarbon and sesterterpene alcohol FPCs, respectively.

Further minor putative disubstituted groups were tentatively identified at molecular weights of 636, 722, 908, and 926 as monoterpene hydrocarbon bis-FPCs, sesquiterpene alcohol bis-FPCs, triterpene hydrocarbon bis-FPCs, and triterpene alcohol bis-FPCs, respectively, on the basis of their MS-MS fragmentation behavior. Proposed combinations of terpene and diformyl phloroglucinol moieties are listed in Table 1.

Semiquantification of FPCs. Total FPC content found in *E. globulus* wound wood was more variable (ranging between 0.5 and 4% of the soluble crude extract)

TABLE 1. TERPENE-CONTAINING FPC MOLECULAR WEIGHTS AND PROPOSED TERPENE CONSTITUENTS[a]

Terpene	Monosubstituted FPC	Disubstituted FPC
Monoterpene hydrocarbons	386	**636**
Monoterpene alcohols/ethers	**404**	654 (n.d)
Sesquiterpene hydrocarbons	454	**704**
Sesquiterpene alcohols	472	**722**
Sesterterpene hydrocarbons	**590**	840 (n.d)
Sesterterpene alcohols	**608**	858 (n.d)
Triterpene hydrocarbons	**658**	**908**
Triterpene alcohols	**676**	**926**

[a] Previously unreported FPCs are in bold type. FPCs, except those not detected (n.d), were present in wound wood.

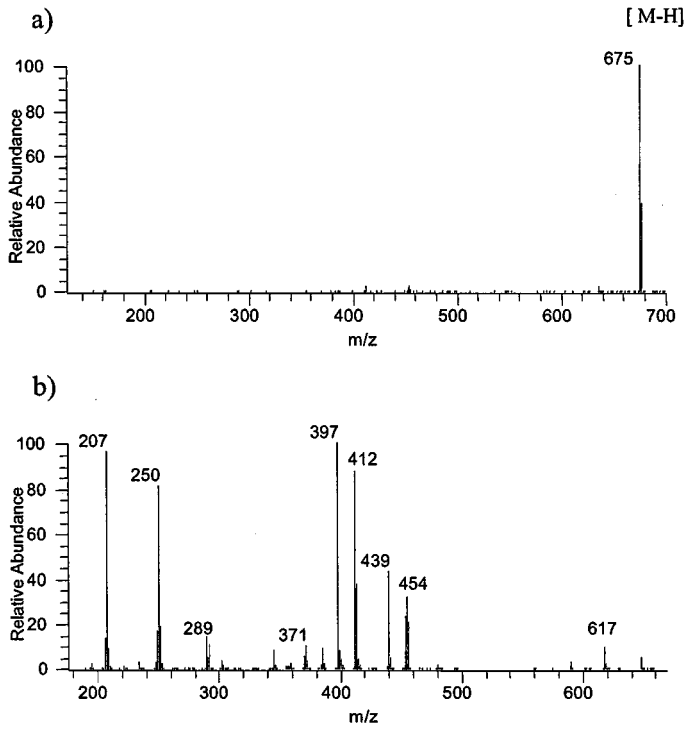


FIG. 5. Putative triterpene hydrocarbon FPC MS and MS/MS data from *E. globulus* wound wood extract. (a) Negative ion electrospray mass spectrum; and (b) MS-MS product ions of $[M\text{-}H]^-$ ions at m/z 675.

TABLE 2. COMPARISON OF TOTAL FPC CONTENT IN WOUND WOOD BETWEEN *Eucalyptus globulus* AND *E. nitens*

Harvest date	Species[b]	
	E. globulus	*E. nitens*
3 months after treatment	0.32 ± 0.29 (2)	0.02 ± 0.02 (2)
18 months after treatment	2.06 ± 0.75 (5)	0.43 ± 0.14 (4)

[a] Mean ± SE. Sample size is shown in parentheses.
[b] Weight of FPCs expressed as % of dry weight of soluble extract.

than total FPC content present in *E. nitens* wound wood (ranging between 0.1 and 0.7% of soluble crude extract) (Table 2). The FPC composition of wound wood extracts obtained from inoculated treatments and those obtained from mechanical wounding only did not appear to be markedly different. A total of 14 and 13 FPC series of different molecular weights were detected by MS in the wound wood crude extracts of *E. globulus* and *E. nitens*, respectively (Table 3). As illustrated by the HPLC-UV chromatograms (Figure 2), the FPC composition of *E. globulus* wound wood was distinctly different from that of *E. nitens*. The latter species was dominated by sideroxylonal. No FPCs were found in the sapwood and heartwood of either species or in the healthy phloem of *E. nitens*. In one of two replicates of *E. globulus* healthy phloem, FPCs at relatively minor levels were detected (Table 3).

Preliminary Antimicrobial Bioassays. Fungal bioassays with original crude wound wood extracts were observed to inhibit the growth of three species: *C. cladosporiodes*, *G. weberianum*, and *T. zonata* (Figure 6). Assays with fraction I preparations, which contained condensed and hydrolyzable tannins, flavanone glycosides, and stilbene glycosides, were similarly found to inhibit fungal growth. In contrast, bioassays using fraction II preparations, containing largely FPCs, appeared to have no antifungal effect (Table 4). In complementary antibacterial bioassays, all three fraction types inhibited the growth of Gram-positive bacteria. In these cases, antibacterial activity was most obvious in assays with original crude wound wood extracts (Table 5). Inhibition zones were not evident with Gram-negative bacteria.

DISCUSSION

The detection of FPCs in the wound wood of both *E. nitens* and *E. globulus* was unexpected. From macroscopic observations, the wound wood appeared to be effective at restricting the outward spread of fungal decay into new healthy tissue, even 17 months after inoculation. Therefore, we initially hypothesized that the

TABLE 3. COMPARISON OF FPCS DETECTED IN WOOD AND BARK EXTRACTS OF *Eucalyptus globulus* (EG) AND *E. nitens* (EN) AS DETERMINED BY HPLC-ESI-MS

	Tissue types Eg/En			
Individual mol wt.	Wound wood En	Sapwood En	Heartwood En	Phloem En
386	+/+	−/−	−/−	−/−
404	+/+	−/−	−/−	−/−
454	+/+	−/−	−/−	+/−
500	+/+	−/−	−/−	−/−
472	+/+	−/−	−/−	+/−
590	+/+	−/−	−/−	−/−
608	+/+	−/−	−/−	−/−
636	+/+	−/−	−/−	−/−
658	+/+	−/−	−/−	+/−
676	+/+	−/−	−/−	+/−
704	+/+	−/−	−/−	+/−
722	+/+	−/−	−/−	−/−
908	+/+	−/−	−/−	−/−
926	+/+	−/−	−/−	+/−

+, detected; −, not detected.

FPCs found in wound wood might possibly be inducible secondary metabolites involved in host–pathogen interactions. The accumulation of flavonoids and related polyphenols in the lesion margin of the host plant has long been implicated in plant defense against antimicrobial invasions. Many examples of this relationship can be found in a recent review by Harborne and Williams (2000).

Subsequent antifungal bioassays conducted in this study showed original crude wound wood extract to inhibit the growth of three fungal species, even using crude extract concentrations at least 10 times less than levels found *in situ* (Table 5). However, bioassays using preparations containing a purified FPC

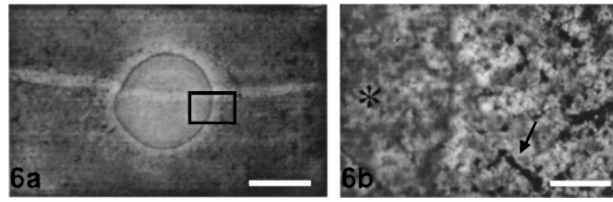


FIG. 6. Fungal bioassays on TLC plates showing antifungal activity of *E. globulus* original wound wood preparations against *Cladosporium cladosporiodes* at low magnification (6a, bar = 1 cm) and at higher magnification (6b is enlarged area of square in 6a, bar = 0.2 cm). Note interface between zone of inhibition (star) and sporulating fungal growth (arrow).

TABLE 4. ANTIFUNGAL ACTIVITY OF SEMISEPARATED EXTRACTS FROM WOUND WOOD OF *Eucalyptus globulus*

Fungal species	Antifungal activity[a]		
	E_1	E_2	E_3
Cladiosporium cladosporiodes	+	−	+
Ganoderma weberianum	+	−	+
Trametes zonata	+	−	+

[a] E_1 = fraction I, E_2 = fraction II, E_3 = original wound wood extract. +, inhibition of growth; −, no inhibition of growth.

fraction did not inhibit fungal growth. Other workers have similarly found FPCs (macrocarpals B–G) to show no antimicrobial activity towards either yeast or fungi (Yamakoshi et al., 1992). These preliminary findings demonstrate that the antifungal components of the wound wood are most likely found within fraction I, and future studies will aim to specifically identify these compounds.

Results obtained for the antibacterial assays were consistent with findings from other studies. As similarly found by Yamakoshi et al. (1992), FPCs fractions were shown to suppress the growth of Gram-positive bacteria, e.g., *S. aureus* and *B. subtilis*, but were inactive against Gram-negative bacteria. There is always a degree of uncertainty relating findings from *in vitro* bioassays to the *in vivo* situation. However, since Gram-positive bacteria are not generally associated with *Eucalyptus* tree diseases (Wardlaw et al., 2000), these results suggest that FPCs do not have an antibacterial role in the tree defense system or that Gram-positive

TABLE 5. ANTIMICROBIAL ACTIVITY OF CRUDE WOUND TISSUE EXTRACTS FROM *Eucalyptus globulus*

Microorganism	Inhibition zone (mm)[a]		
	E_1	E_2	E_3
Gram positive			
Bacillus subtilis	+	+	++++
Streptococcus aureus	+	+	++++
Gram negative			
Escherichia coli	−	−	−
Xanthomonas sp.	−	−	−
Erwinia sp.	−	−	−

[a] E_1 = fraction I, E_2 = fraction II, E_3 = original wound wood extract. The inhibition is reported as: -, without inhibition; +, d = 1.0–1.9 mm; ++, d = 2.0–2.9 mm; +++, d = 3.0–3.9 mm; and ++++, $d > 4.0$ mm, where d is diameter of the inhibition zone.

bacteria may not be important in diseases of eucalypts because they may be inhibited by the presence of FPCs. Interestingly, a higher level of inhibition was shown in bacterial bioassays using original crude wound wood extracts than in bioassays with extracts of either fraction I or fraction II. One possible explanation may be that during the preparation of the fractions, the volatile terpenes present in these extracts almost certainly would have been removed. Terpenoids such as those detected in wound wood extracts represent an important widespread inducible defense mechanism in Pinaceae, and in particular, α-pinene has been found to exhibit a high degree of biological activity towards pathogenic fungi and bark beetles (Raffa et al., 1985; Delorme and Lieutier, 1990). Future studies investigating the role of extractives present in wound wood should also aim to take into account the contribution of this group of secondary metabolites to host defense.

The role of FPCs in wound wood remains unclear. The evidence is limited for a role in antimicrobial defense. To date, the chemical structures of some 50 specific macrocarpals and euglobals have been published (reviewed by Ghisalberti, 1996; Singh and Etoh, 1997). We provide preliminary evidence for the discovery of many new FPCs. As mentioned, FPCs have been reported exclusively in buds and leaves. The FPCs found in leaves have been considered an important determinant governing plant–herbivore interactions, particularly of marsupial folivores. For example, macrocarpal G, jensenone, and sideroxylonal were shown to be highly effective antifeedants for mammalian herbivores such as the common ringtail possum (*Pseudocheirus peregrinus*), the common brushtail possum (*Trichosurus vulpecula*), and koala (*Phascolarctos cinereus*) (Lawler et al., 1999, 2000; Stapley et al., 2000). Furthermore, sideroxylonal has recently been shown to have an antifeedant role against Christmas beetles (*Anoplognathus* spp) (M. Matsuki, personal communication). From these studies examining the relationship between chemical structure and effectiveness as marsupial antifeedants, Lawler et al. (1999) proposed that there exists significant variation among different FPCs in their biological activity. If this is true, then FPCs may have multiple roles depending on composition, sites of synthesis, and storage. For example, FPCs found in the wound wood may be implicated in host resistance against insects, as found in leaves. In this case, FPCs in the wound wood may function to deter stem boring insects from entering the stem via wound sites. Alternatively, FPCs may function as antioxidants to scavenge the deleterious free radicals generated from wounding (van Doorslaer et al., 1999) and/or by white and brown rot fungi, which are thought to have a primary role in the depolymerization of wood cell material (Hirano et al., 1997). Further studies examining the role of FPCs in wound wood are required.

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IDENTIFICATION OF ODORS FROM OVERRIPE MANGO THAT ATTRACT VINEGAR FLIES, *Drosophila melanogaster*

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Abstract—Bioassays with a variety of overripe fruits, including mango, plum, pear, and grape, and their extracts showed that odors from overripe mango were most attractive to adult vinegar flies, *Drosophila melanogaster*. Combined gas chromatography–electroantennographic detection (GC-EAD) analyses of solid-phase microextraction (SPME) and Tenax extracts of overripe mango odors showed that several volatile compounds, including ethanol, acetic acid, amyl acetate, 2-phenylethanol, and phenylethyl acetate elicited significant EAG responses from antennae of female flies. Most of the volatile compounds in the extracts were identified by mass spectral and retention index comparisons with synthetic standards. In cage bioassays, lures with a blend of ethanol, acetic acid, and 2-phenylethanol in a ratio of 1:22:5 attracted six times more flies than any single EAG-active compound. This blend also attracted four times more flies than traps baited with overripe mango or unripe mango. However, in field trials, the blend was not as attractive as suggested by the laboratory bioassay.

Key Words—Overripe fruit, mango odors, SPME, GC-EAD, behavioral response, vinegar fruit fly, *Drosophila melanogaster*.

INTRODUCTION

Although the vinegar fly, *Drosophila melanogaster*, has never been considered a serious pest, it is a nuisance in households and in commercial food marketing and handling areas. Volatile chemicals associated with fermentation, such as ethanol, acetic acid, ethyl acetate, and acetaldehyde, either as single components or in mixtures, previously have been reported to attract several *Drosophila* species (Barrows, 1907; Hunter et al., 1937; West 1961). However, there have been no

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recent studies using contemporary analytical chemistry techniques to analyze fruit sources for further attractants for *D. melanogaster*.

In the present study, we report on the attraction of *D. melanogaster* to various overripe fruit sources in cage bioassays; the identification of volatiles from overripe mango that elicited electroantennographic responses from antennae of *D. melanogaster*; and the results of bioassays testing the attractiveness of a formulated blend containing those active components to *D. melanogaster*.

METHODS AND MATERIALS

Insects. Vinegar fruit flies, *Drosophila melanogaster* (wild type, Oregon), were obtained from a laboratory colony maintained at the Fruit Fly Genetics Laboratory, Iowa State University, Ames, Iowa. Adult flies were provided with a standard corn meal–agar diet containing hydrolyzed protein (ISU-Lephardt) and water until they were used in experiments.

Fruit Volatiles Collection. Six different types of overripe fruit, including mango, strawberry, banana, grape, pear, and plum, were collected from fruit sorting areas of three local grocery stores. Approximately 250 g of each overripe fruit was placed in glass jars (1 liter) for odor analysis. For fruit volatile collection, activated-charcoal-purified air was blown at 200 ml/min through a glass jar containing one type of overripe fruit, and the odors collected on a Tenax trap connected to the outlet. The trap consisted of a Pasteur pipet (5 cm long $\times$ 0.5 cm diam.) packed with 300 mg of precleaned Tenax (20–35 mesh, Alltech, Deerfield, Illinois, USA) held in place by glass wool plugs. Volatiles were continuously collected for 1–2 days, eluted with 2 ml of HPLC-grade hexane (Burdick & Jackson High Purity), and concentrated to 200 μl under a gentle nitrogen stream. Extracts (2 μl) were injected for analysis with either coupled gas chromatography–electroantennographic detection (GC-EAD) or gas chromatography-mass spectrometry (GC-MS).

For solid-phase microextraction (SPME), a SPME fiber (100 μm polydimethylsiloxane, Supelco, Bellefonte, Pennsylvania, USA) was preconditioned for 1 hr at 250°C. During collections, the fiber was exposed approx. 2–3 cm above the overripe fruit in the glass jar for ca. 12 hr at room temperature, which gave sufficient time for equilibration of all volatiles. The loaded SPME fiber was then desorbed in the injection port of either a GC-EAD or a GC-MS system.

Chemical Analyses. For GC-EAD analysis, Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a DB-5 column (30 m $\times$ 0.25 mm ID, J & W Scientific, Folsom, California, USA), and a 50:50 effluent split allowed simultaneous flame ionization (FID) and EAD of the separated fruit odors. Helium was used as the carrier gas with a flow rate of approximately 30 ml/min for both FID and EAD. Extracts were injected in splitless mode, injector temperature 250°C, and split valve delay 1 min. The temperature program was 5°C/min, then 15°C/min to 250°C. The outlet for the EAD was continuously supplied with a

purified, moisturized airstream flowing over the antennal preparation at 0.5 m/sec. A fruit fly was restrained with thin copper wires (32 gauge) for EAD recordings. An Ag–AgCl glass capillary recording electrode filled with saline (0.1 M KCl) was placed in contact with the distal segment of the antenna. The ground electrode, filled with the same solution, was placed in the eye. The EAD amplifier (a high-impedance DC amplifier with automatic baseline drift compensation) was used (Syntech, Hilversum, The Netherlands). A GC-EAD program (Syntech version 2.3) was used to record and analyze the amplified EAD and FID signals on a PC computer.

GC-MS analyses of the fruit volatiles were performed with an H-P 5890 Series II GC interfaced to a HP 5972 Mass Selective Detector (MSD). The GC-MS was equipped with either a DB-5 column (identical to the one used in the GC-EAD system described above) or a Carbowax column (30 m $\times$ 0.25 mm ID, J & W Scientific). The temperature program was the same as that described for the GC-EAD analyses, except the maximum temperature for analyses using the Carbowax column was set at 230°C. Mass spectra were recorded from 30 to 550 amu with electronic impact ionization at 70 eV. Identifications of fruit volatiles were confirmed by comparison of retention indices and mass spectra with those of authentic standards.

Chemicals. Most synthetic standards were purchased from Sigma/Aldrich (St. Louis, Missouri, USA). Purities ranged from 98% to 99.5%. α-Copaene was purchased from Fluka with 95% chemical purity.

Cage Bioassays. Trapping tests were conducted in screen cages (90 $\times$ 48 $\times$ 36 cm) either in the greenhouse or in the laboratory under conditions of room temperature (23°C $\pm$ 3°C) and daylight. About 150 mixed-sex, mixed-age flies (age differences not more than 2 days) were released into a cage containing randomly placed traps with different treatments. Traps were constructed from 18 ml clear plastic cups (Fill-Rite Inc., Newark, New Jersey, USA) covered by a white paper lid with a hole (2.5 mm diam.) drilled at the center. For experiments involving extracts or synthetic compounds, a medical cotton wick (2 cm long) was used as a dispenser. Our standard blend was comprised of 100 mg of ethanol, acetic acid, and 2-phenylethanol (in a ratio of 1:22:5) in 1 ml of water (water was critically important for vinegar fly attraction; no flies were attracted to traps loaded with chemicals alone) and was placed on the bottom of the cup. Compounds that were not water soluble were loaded onto a cotton wick and placed in a treatment cup with a second wick dosed with 1 ml of water. Controls contained only water. For the trapping tests involving a variety of overripe fruits, approx. 5 g of each fruit was used for each test. The ratio of the naturally emitted blend from mango was determined by SPME analyses of the attractive traps containing mango ($N = 3$). For testing volatiles collected on Tenax, we used approx. 50 μl of extract (out of a total volume of 200 μl) on the wick in each trap, with hexane (50 μl) for a control. Wicks were allowed to dry for 30 min before being used. For the experiment testing

the attractiveness of the synthetic blend versus overripe and unripe mango, traps were baited with 100 mg of the synthetic blend (in 1 ml water) or 5 g of chopped overripe and unripe mango. The control used only water. In the dose–response test, serial dilutions of the synthetic blend were made in water at doses ranging from 0.1 to 100 mg (total amount of three components). Treatments were tested in a randomized complete-block design. The numbers of flies caught in traps were counted 8 hr after release for each experiment. Experiments were usually conducted from 9:00 AM to 5:00 PM daily. Trap positions were rerandomized every 2–3 hr to minimize positional effects. Each experiment was replicated 3–5 times.

RESULTS AND DISCUSSION

Attraction of D. melanogaster to Overripe Fruits. Traps baited with overripe fruits were attractive to *D. melanogaster* in cage bioassays conducted in a greenhouse. Traps baited with mango caught significantly more flies than other treatments. No flies were caught in the control traps (Figure 1). A second bioassay using Tenax extracts of overripe fruits showed similar results, with traps baited with mango extracts catching significantly more flies than those baited with strawberry, plum, and grape extracts, all of which were not significantly different than the control (Figure 2).

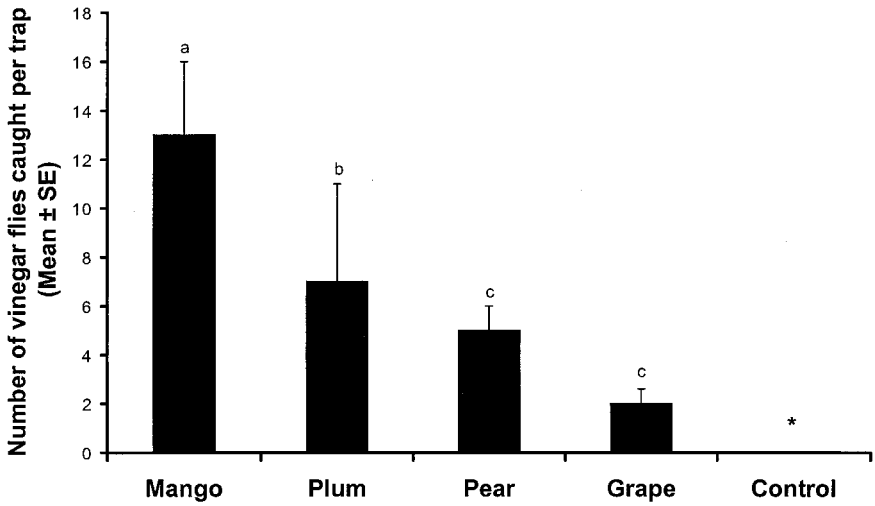

FIG. 1. Mean catches of *Drosophila melanogaster* in traps baited with a variety of overripe fruits. Different letters on top of bars indicate significant differences ($N = 3$, ANOVA followed by Fisher test, $P < 0.05$). *Control traps caught no flies and were not included in the ANOVA.

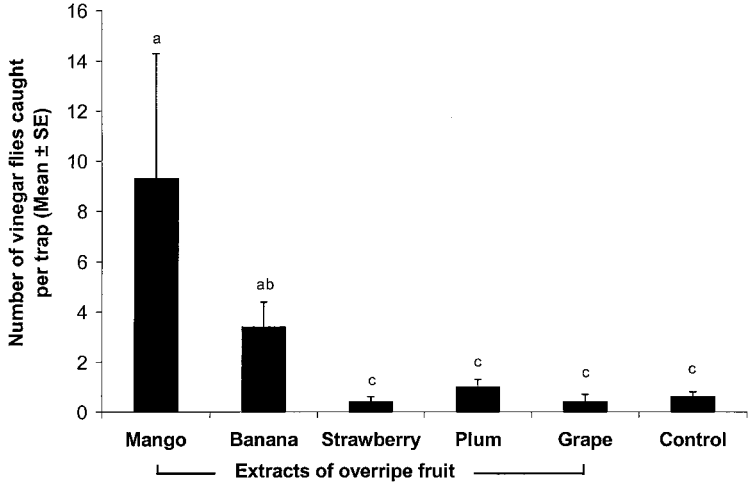

FIG. 2. Mean catches of *Drosophila melanogaster* in traps baited with solvent extracts of different overripe fruits. Different letters on top of bars indicate significant differences ($N = 5$, ANOVA followed by Fisher test, $P < 0.05$).

Analysis and Bioassay of Volatiles from Overripe Mango. GC traces of SPME or Tenax extracts of overripe mango showed more than 20 compounds in extracts, 13 of which were identified (Figures 3 and 4). These consisted primarily of monoterpene and sesquiterpene hydrocarbons (Table 1) that are considered important volatile compounds contributing to mango flavor (MacLeod and Snyder, 1985; Sakho et al., 1985; Bartley and Schwede, 1987; Winterhalt, 1991). Among them, 3-carene was consistently the most abundant volatile present, accounting for more than 75% of total terpene hydrocarbons. Another typical mangolike odor, α-copaene, found in trace amounts, has also been recorded as a minor component from a number of fruits, including citrus, guava, litchi, and peach (MacLeod and de Troconis, 1982; MacLeod et al., 1988; McInnis and Warthen, 1988).

Among the other aliphatic and aromatic hydrocarbons present in overripe mango odor, 2-phenylethanol is a common floral volatile of most roses, and various tropical fruits including mango (Wong and Siew, 1994; Ollé et al., 1998; Boulanger and Crouzet, 2001). This aromatic alcohol may be a product of fermentation of overripe fruits, because it has been identified from various fermentation products such as wine and cider (Fischer et al., 2000; Picinelli et al., 2000). Phenylethyl acetate has not been reported previously from mango.

Characterization of fruit aroma can be problematic because certain fruit odors are highly volatile and may be lost during extraction or can co-elute with the solvent used for extraction during GC analysis. Solid-phase microextraction (SPME) (Zhang and Pawliszyn, 1993) to some extent circumvents these problems and can

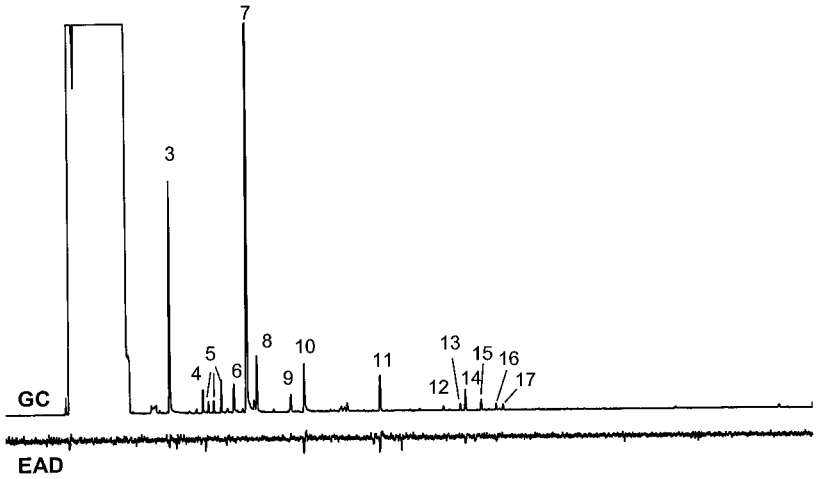

FIG. 3. GC-EAD analysis of overripe mango extract using an antenna from a female *Drosophila melanogaster*. Thirteen compounds were identified after GC-MS analysis. Number on each peak refers to compounds listed in Table 1.

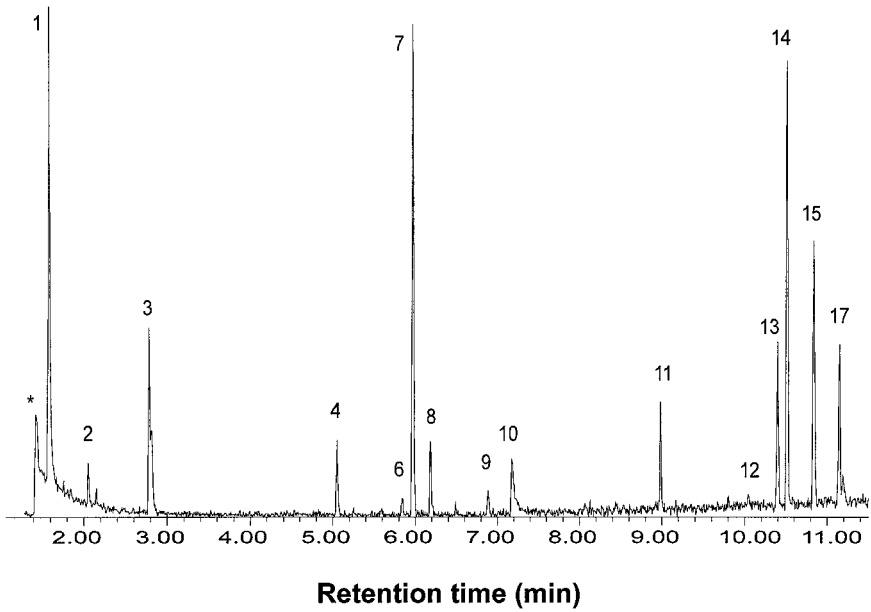

FIG. 4. Chromatogram of SPME headspace collection of overripe mango. Numbered peaks refer compounds listed in Table 1. *The compound was tentatively identified as acetaldehyde.

TABLE 1. VOLATILES IDENTIFIED FROM OVERRIPE MANGO WITH TENAX AND SPME COLLECTION AND THEIR EAG ACTIVITY

Peak	Volatile	Tenax extract	SPME collection	EAG
1	Ethanol	[a]	+	+++
2	Acetic acid	[a]	+	++
3	Amyl acetate	+	+	++
4	α-Pinene	−	+	−
5	unknowns	+	−	−
6	β-Myrcene	+	+	−
7	3-Carene	+	+	−
8	dl-Limonene	+	−	−
9	Terpinolene	+	+	−
10	2-Phenylethanol	+	+	+++
11	Phenylethyl acetate	+	+	++
12	α-Copaene	+	−	−
13	Sesquiterpene	−	+	−
14	*E*-Caryophyllene	+	+	+
15	Sesquiterpene	+	+	−
16	Sesquiterpene	+	+	−
17	Butylated hydroxytoluene (contaminant)	+	−	−

[a] Coelutes with the solvent peak.

be used to check for additional volatiles not collected by Tenax. SPME analysis of overripe mango revealed three fermentation volatiles, acetaldehyde, ethanol, and acetic acid that may have been hidden by the solvent peak of the Tenax extract in GC analyses or which were not efficiently trapped by the Tenax. Acetaldehyde, another volatile commonly associated with fermentation, also was tentatively detected from overripe mango at a low level (Figure 4).

In GC-EAD analyses, the two strongest EAD responses were elicited by 2-phenylethanol and phenylethyl acetate. In separate EAG trials with synthetic standards, ethanol and acetic acid also elicited strong EAG responses from antennae of *D. melanogaster* (Table 1).

In bioassays using synthetic standards, all compounds were tested as aqueous solutions, or with a source of water in the treatment cup, because responses were inhibited in the absence of water. Adult *D. melanogaster* were maximally attracted to an aqueous solution of three EAD active compounds, ethanol, acetic acid, and 2-phenylethanol, at their naturally emitted ratio of 1:22:5 (Figure 5). Aqueous solutions of ethanol and acetic acid were more attractive than the other single components, all of which were significantly more attractive than the controls. The attraction of *D. melanogaster* to ethanol was reported previously by Reed (1938) and West (1961), at dosages similar to those tested in the present study. Barrows (1907) showed that another *Drosophila* species, *D. ampelophila* was attracted to

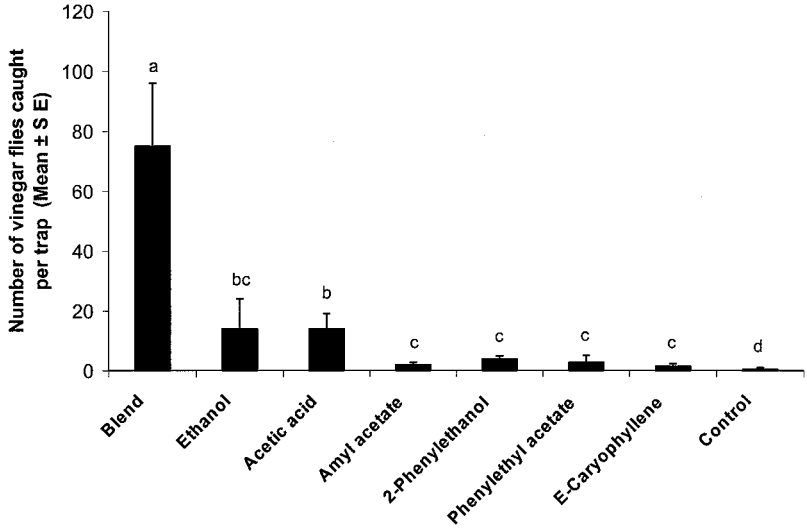

FIG. 5. Mean catches of *Drosophila melanogaster* in traps baited with standards of EAG-active compounds and the reconstructed blend identified from overripe mango. Different letters on top of bars indicate significant differences ($N = 5$, ANOVA followed by Fisher test, $P < 0.05$).

a mixture of ethanol and acetic acid. In Barrows' study, only 8% of released flies were caught, whereas our aqueous synthetic blend attracted over 70% of the flies released inside the cage, while also competing with the other treatments. The three compounds are generally considered to be fermentation-related products, although ethanol has been reported from fresh mango (TNO, 1976). 2-Phenylethanol has been reported to be attractive to several other insect species, such as cabbage looper moths (Haynes et al., 1991), pineapple beetles (Zilkowski et al., 1999), lady beetles (Nout and Bartelt, 1998), and green lacewings (Zhu et al., 1999).

The efficacy of the synthetic blend as an attractant was compared to volatiles from chopped overripe and unripe mango in a cage bioassay. Although more flies were caught in traps baited with aqueous formulations of the synthetic blend (Figure 6) than in mango-baited traps, these results must be treated with caution because the comparative release rates of volatiles from the mango baits and the synthetic lure were not determined. A subsequent dose–response test with the synthetic blend demonstrated that the highest dose tested (100 mg) was most attractive to *D. melanogaster* (Figure 7).

Although we have demonstrated the efficacy of this blend in capturing *D. melanogaster* in cage bioassays, its attraction was not as great as expected when tested under more natural conditions. Traps set on fruit shelves at a grocery store caught only 30% of the available flies, based on a 5-day observation period. Further

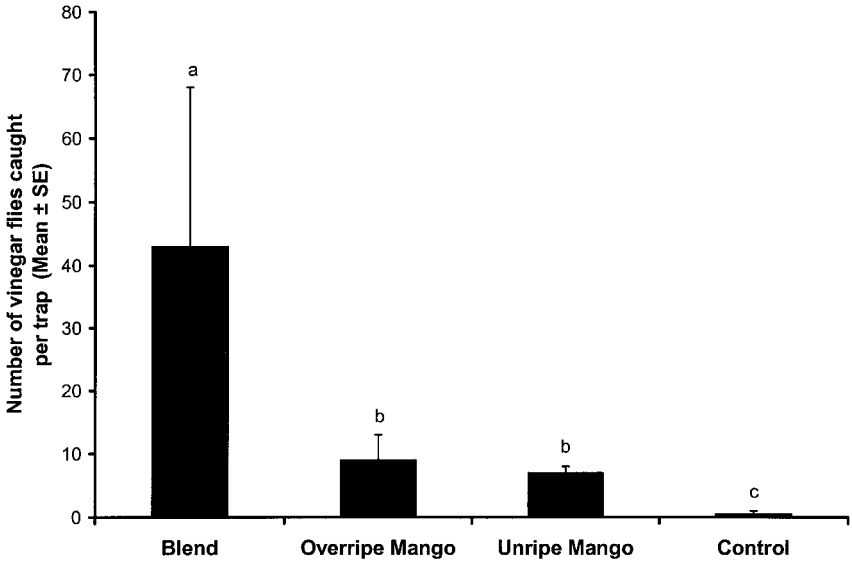

FIG. 6. Mean catches of *Drosophila melanogaster* in traps baited with synthetic mango blend, and chopped overripe or unripe mango. Different letters on top of bars indicate significant differences ($N = 3$, ANOVA followed by Fisher test, $P < 0.05$).

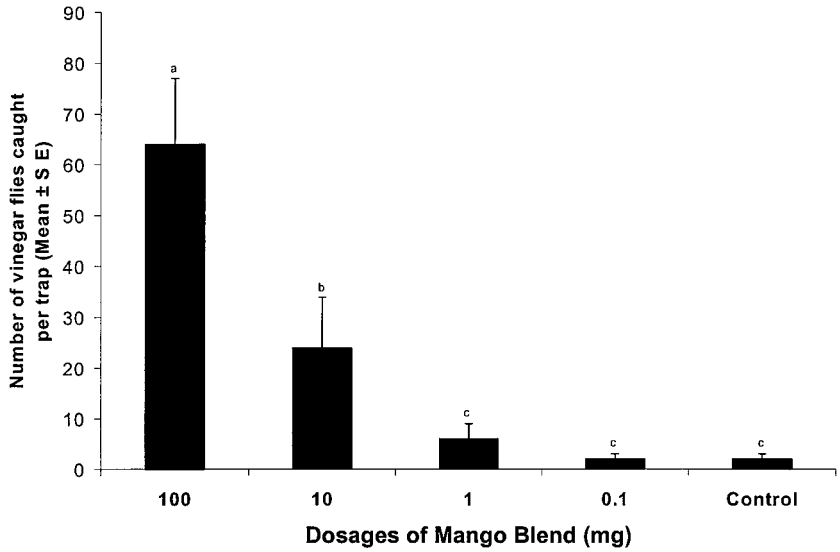

FIG. 7. Mean catches of *Drosophila melanogaster* in traps baited with different doses of the synthetic blend of overripe mango volatiles. Different letters on top of bars indicate significant differences ($N = 5$, ANOVA followed by Fisher test, $P < 0.05$).

development of this blend by the addition of synergistic compounds from other fruit sources is in progress.

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OVIPOSITION RESPONSE OF *Chironomus tepperi* TO NITROGENOUS COMPOUNDS AND BIOEXTRACTS IN TWO-CHOICE LABORATORY TESTS

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Abstract—Two-choice laboratory tests were used to investigate the oviposition response of *Chironomus tepperi* to a range of nitrogenous compounds and crude bioextracts. Responses to nitrogenous compounds varied in response to concentration. Ammonium nitrate did not influence oviposition at concentrations from 2 to 12 mg/liter. Hydroxylamine hydrochloride increased oviposition at 6 mg/liter, but had no effect at either 2 or 12 mg/liter. Sodium nitrate reduced oviposition at 2 mg/liter relative to the controls, but had no significant effect at 6 or 12 mg/liter. *C. tepperi* responded to many of the crude bioextracts, strongly avoiding oviposition in solutions containing homogenized chironomid larvae (1 final instar/100 ml; *C. tepperi* or *Polypedilum nubiferum*), and avoiding solutions conditioned by conspecific larvae at concentrations down to the equivalent of 1 final instar/100 ml over 24 hr. Homogenates of adult conspecifics had no effect on oviposition site selection. Homogenates of larval *Culex annulirostris* (Culicidae) deterred oviposition, but only at high concentrations (3 final instars/100 ml). Our results demonstrate that chemical cues from larval populations deter oviposition by *C. tepperi* females searching for newly flooded habitats where larval competition will be minimized.

Key Words—Diptera, Chironomidae, midges, *Chironomus tepperi*, oviposition, bioextracts, rice, Australia.

INTRODUCTION

The Chironomidae, or nonbiting midges, are the most widely distributed and abundant insects inhabiting freshwater ecosystems (Pinder, 1986; Cranston, 1995). Larval chironomid communities logically develop in response to two processes:

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the recruitment of larvae derived from eggs laid by airborne female midges, and the subsequent survival of these larvae that may or may not be suited to a particular aquatic environment. Considerable effort has been directed towards characterizing chironomid assemblages associated with different environments, and numerous studies, many of them laboratory-based, have examined the effects of physicochemical parameters on the survival of different chironomid species. Little attention has been given to factors that may influence oviposition site selection by female midges, and thereby influence larval recruitment. In contrast, the factors that influence oviposition site selection by mosquitoes (Diptera: Culicidae) have been extensively researched. Female mosquitoes respond, either positively or negatively, to a range of interacting physical and chemical stimuli (Bentley and Day, 1989).

Oliver (1971) remarked that little was understood about directional oviposition flight and oviposition site selection in chironomids, and comparatively few new data on oviposition site selection have been generated since publication of his review. Williams et al. (1987) found that *Chironomus riparius* Meigen avoid oviposition in water containing high concentrations of cadmium, but that the avoidance response is fairly insensitive and does not lead to females avoiding solutions containing sufficient cadmium to cause acute toxicity to early instars. Similarly, Dauble and Skalski (1983) found that *Tanytarsus dissimilis* (Johannsen) readily oviposit in solutions containing acutely toxic levels of coal liquid derivatives, but that egg mass size is reduced. Xue et al. (1994) found that ovipositing *Glyptotendipes paripes* Edwards do not discriminate between water from four different sources, despite the fact that egg hatchability is significantly higher in lake water. These studies indicate that some chironomids have the ability to detect chemical cues that identify unfavorable oviposition sites, but support the view of Davies (1976) that the level of discrimination is generally poor.

Visual cues also may be significant in oviposition site selection by chironomids. Frouz (1997) found that two species of terrestrial chironomids oviposit preferentially in open areas and areas of low vegetation. Harrison and Hildrew (1998) found that oviposition site selection by *Cricotopus sylvestris* (Fabricius) and *Microtendipes pedellus* (de Geer) favored shaded areas along lake margins, despite conditions in more open areas appearing to favor larval survival.

Chironomus tepperi Skuse is a native Australian chironomid that rapidly exploits newly flooded environments. It is an important food source for breeding waterfowl (Maher and Carpenter, 1984; Crome, 1986) and is also a serious pest of establishing rice crops in southern New South Wales (Stevens, 1998). In small experimental rice fields, *C. tepperi* has a single generation. Oviposition generally occurs only during the first few days after the fields are flooded, with a single adult emergence peak occurring approximately 17 days later. Females do not oviposit back into the fields from which they have emerged (Stevens, 1994).

This study was conducted as a first step towards determining why *C. tepperi* oviposit only into newly flooded rice fields and avoid older fields even if they have

only been flooded for a relatively short period. Ikeshoji et al. (1980) found that nutrients (in the form of organic or inorganic fertilizers) increased chironomid production from rice fields in Japan, and consequently we tested a range of nitrogenous compounds to determine their possible influence on oviposition site selection. We also tested a range of bioextracts (larval and adult insect homogenates, as well as water conditioned by *C. tepperi* larvae) to determine whether chemical cues originating from organisms already present in aquatic environments may influence oviposition behavior.

METHODS AND MATERIALS

Rearing and Collection of C. tepperi. Egg masses of *C. tepperi* were collected from temporary pools at Yanco Agricultural Institute (34°37'S 146°26'E) and cultured using the technique of Stevens (1992). Aquaria (20-liter capacity) containing 10 liters of 1 × Martin's rearing solution, (1 × MRS) (Martin et al., 1980) were lined with paper tissue prior to the addition of 3–5 *C. tepperi* egg masses. A small quantity of K9 goldfish food (Friskies Pet Care, Melbourne, Australia) was added, and the aquarium was covered with plastic film. Aquaria were maintained at 25 ± 1°C with a 15L:9D photoperiod. Aeration was provided to each aquarium by a hypodermic needle attached to an aquarium aerator by plastic tubing. Additional food was provided every 2–3 days during larval development. When adult emergence began, the plastic film was removed and replaced with a gauze-covered pyramidal frame. A 2-liter plastic jar was fitted at the apex of the frame for the collection of adults.

Test Procedure. Two-choice oviposition tests were conducted using empty 20-liter capacity glass aquaria (40 × 23 × 23 cm deep). Two translucent polystyrene cups (model B260, 315 ml capacity, 90 mm diam. at the rim; Polarcup Australia Limited, Sydney, Australia) were placed in each aquarium 5 cm from either end. One of the cups was filled to a depth of 45 mm (20 mm below the rim) with 200 ml of the control solution, while the other cup was filled to the same depth with the test solution/suspension being assessed. Several droplets of sucrose solution were placed on the walls of the aquarium midway between the cups as a food source.

Adults were anesthetized using food-grade carbon dioxide (BOC Gases Australia, Sydney, Australia), and 15 males and 15 females were removed from the collection jar and placed in a 9-cm-diam. glass Petri dish between the two test solutions. The aquarium was covered with plastic film and placed in a controlled temperature room (25 ± 1°C, 15L:9D). Aquaria were placed at right angles to the horizontal fluorescent lighting system to eliminate the possibility of a phototactic response influencing the choice of oviposition site, and the positioning of test and control solutions at either end of the aquaria was randomized across replicates.

The number of egg masses deposited by the female midges in control and test solutions was counted 24 hr after establishment. Replicates in which no egg masses were laid were discarded, since oviposition failure could occur as a consequence of an inadequate preoviposition period for a particular group of midges or, alternatively, as a consequence of excessive anesthesia. Replication of the test for each solution/suspension continued until 15 replicates were obtained, in each of which at least one egg mass was deposited.

Test Solutions/Suspensions. An initial test was conducted using 2 × MRS (i.e., double salt concentrations) against 1 × MRS to determine if there was a simple response to total dissolved solids. When no significant response was obtained, the test was repeated using 3 nitrogenous compounds [ammonium nitrate (NH_4NO_3), hydroxylamine hydrochloride ($NH_2OH.HCl$), sodium nitrate ($NaNO_3$), all Univar grade, Ajax Chemicals Ltd, Sydney, Australia]. Each compound was tested separately at three rates (2, 6, and 12 mg/liter). Compounds were dissolved in 1 × MRS, and 1 × MRS without supplementary nitrogen was used as the reference solution in the control cups.

Oviposition tests were also conducted using 10 different bioextracts prepared with either larval or adult *C. tepperi*, larval *Polypedilum nubiferum* (Skuse) (Chironomidae), or larval *Culex annulirostris* Skuse (Culicidae). Eight of these bioextracts were prepared by homogenizing either adult or larval insects in 1 × MRS, while the remaining two were 1 × MRS conditioned by *C. tepperi* larvae.

Larval and adult *C. tepperi* were obtained from the laboratory cultures described previously. *P. nubiferum* larvae were also cultured in the laboratory from locally collected eggs by using a technique based on that described by Hatakeyama (1988). Larval *Cx annulirostris* were collected from drainage channels at Yanco Agricultural Institute. All larvae used to produce bioextracts were final instars and were washed in distilled water before being manually ground in a 2-ml-capacity glass tissue homogenizer containing a small volume of 1 × MRS. The homogenate was washed into a 200-ml volumetric flask and diluted with 1 × MRS to achieve the desired test concentration. Test concentrations for larval homogenates were: *C. tepperi* and *P. nubiferum*, each 1 larva/100 ml; *Cx annulirostris*, 1 and 3 larvae/100 ml. Adult *C. tepperi* homogenates were prepared in the same way; however, the adults were not washed prior to grinding. Concentrations for *C. tepperi* adult homogenates were 1 and 5 adults/100 ml, with homogenates of male and female adults tested separately.

Test solutions conditioned by larval *C. tepperi* were prepared by placing 50 washed final instars into a 1-liter glass beaker containing 500 ml of 1 × MRS. The beaker was placed in to a controlled temperature room (25 ± 1°C, 15L:9D) with aeration, but without any food being provided. After 24 hr, the rearing solution was poured off and used immediately in the oviposition tests. This conditioned rearing solution was tested at two concentrations: 10 larvae/100 ml (i.e., undiluted), and diluted with fresh 1 × MRS to 1 larva/100 ml. In all cases, fresh

1 × MRS was used in the reference (control) cups. Test concentrations for conditioned rearing solution were chosen to reflect common *C. tepperi* densities in establishing rice crops, which often exceed 2,000 larvae/m^2 and can reach 13,000 larvae/m^2 in favorable seasons (Stevens, unpublished data). In water 5 cm deep, 10 larvae/100 ml and 1 larva/100 ml equate, respectively, to population densities of 5,000 and 500 larvae/m^2.

Statistical Analysis. As the data were counts and did not follow normal distributions, it was inappropriate to use *t* tests to determine statistical significance. Instead, data were assumed to follow Poisson distributions. This assumption did not hold for several data sets; however, this was resolved by incorporating overdispersion parameters in the model.

For each data set, a separate Poisson generalized linear model with a log-link function (McCullagh and Nelder, 1989; Schall, 1991) was fitted in ASREML (Gilmour et al., 2001) with an overdispersion parameter if required. Due to possible blocking effects associated with the replication procedure, a random effect for pairs was also fitted.

RESULTS

Results from the tests of 2 × MRS and the three nitrogenous compounds are given in Table 1. Doubling the concentration of salts in 1 × MRS did not result in a significant change in oviposition preference, indicating that slight changes in total dissolved solids (from 75 to 150 mg/liter) do not affect oviposition site selection.

Ammonium nitrate did not affect oviposition site choice at any of the concentrations evaluated, although the 6 mg/liter concentration did attract more oviposition than either higher or lower concentrations. Similar results were obtained with hydroxylamine hydrochloride, where more egg masses were laid in the 6 mg/liter concentration relative to the 1 × MRS control ($P < 0.02$). The higher and lower concentrations did not affect oviposition site selection. Sodium nitrate acted as a slight oviposition deterrent at 2 mg/liter ($P < 0.05$), but had no effect at higher concentrations.

Table 2 shows the oviposition response of *C. tepperi* to bioextracts and conditioned 1 × MRS. Bioextracts containing homogenized *C. tepperi* or *P. nubiferum* larvae strongly deterred oviposition by *C. tepperi* ($P < 0.001$), with between 89% and 93% of all egg masses produced being laid in control cups. Homogenized *Cx annulirostris* larvae also deterred oviposition ($P < 0.05$), but only at the higher concentration of 3 final instars/100 ml.

1 × MRS conditioned by the presence of *C. tepperi* larvae over a 24-hr period also acted as an oviposition deterrent, even when diluted to a concentration equivalent to 1 larva/100 ml. In contrast, homogenates of adult *C. tepperi* had no effect on oviposition site choice ($P > 0.05$), regardless of the sex of the adults used in the homogenate or their concentration.

TABLE 1. OVIPOSITION RESPONSE OF *Chironomus tepperi* TO 2 × MARTIN'S REARING SOLUTION (2 × MRS) AND DIFFERENT CONCENTRATIONS OF THREE NITROGENOUS COMPOUNDS IN TWO-CHOICE LABORATORY TESTS

Treatment	Egg masses (mean ± SE)[a]		% of egg masses in control	Significance	Treatment effect (stimulates/deters)
	control (1 × MRS)	treatment			
2 × MRS	1.87 ± 0.54	1.07 ± 0.28	63.6	nsd[b]	-
NH_4NO_3 2 mg/liter	1.33 ± 0.29	0.93 ± 0.28	58.8	nsd	-
NH_4NO_3 6 mg/liter	0.73 ± 0.30	1.60 ± 0.21	31.4	nsd	-
NH_4NO_3 12 mg/liter	2.00 ± 0.48	1.80 ± 0.51	52.6	nsd	-
$NH_2OH.HCl$ 2 mg/liter	2.27 ± 0.61	1.40 ± 0.25	61.8	nsd	-
$NH_2OH.HCl$ 6 mg/liter	0.73 ± 0.21	1.73 ± 0.33	29.7	$P < 0.02$	stimulates
$NH_2OH.HCl$ 12 mg/liter	1.93 ± 0.53	1.00 ± 0.34	65.9	nsd	-
$NaNO_3$ 2 mg/liter	1.80 ± 0.28	1.00 ± 0.24	64.3	$P < 0.05$	deters
$NaNO_3$ 6 mg/liter	0.87 ± 0.29	1.27 ± 0.25	40.6	nsd	-
$NaNO_3$ 12 mg/liter	1.40 ± 0.48	1.80 ± 0.40	43.7	nsd	-

[a] Mean of 15 replicates. Replicates with no oviposition in either cup were discarded.
[b] nsd, no significant difference ($P > 0.05$).

TABLE 2. OVIPOSITION RESPONSE OF *Chironomus tepperi* TO CRUDE BIOEXTRACTS AND REARING SOLUTION CONDITIONED BY CONSPECIFIC LARVAE IN TWO-CHOICE LABORATORY TESTS

Treatment	Egg masses (mean ± SE)[a]		% of egg masses in control	Significance	Treatment effect (stimulates/deters)
	control	treatment			
C. tepperi, 1 larva/100 ml, homogenized	2.27 ± 0.42	0.27 ± 0.15	89.5	$P < 0.001$	deters
P. nubiferum, 1 larva/100 ml, homogenized	2.40 ± 0.53	0.20 ± 0.11	92.3	$P < 0.001$	deters
C. tepperi, 10 larvae/100 ml, conditioned	1.93 ± 0.34	0.33 ± 0.21	85.3	$P < 0.01$	deters
C. tepperi, 1 larva/100 ml, conditioned	1.73 ± 0.34	0.67 ± 0.23	72.2	$P < 0.05$	deters
C. tepperi, 1♂ adult/100 ml, homogenized	1.27 ± 0.27	0.93 ± 0.27	57.6	nsd[b]	-
C. tepperi, 5♂ adults/100 ml, homogenized	1.13 ± 0.26	0.60 ± 0.16	65.4	nsd	-
C. tepperi, 1♀ adult/100 ml, homogenized	1.20 ± 0.30	1.60 ± 0.34	42.9	nsd	-
C. tepperi, 5♀ adults/100 ml, homogenized	0.80 ± 0.24	1.00 ± 0.20	44.4	nsd	-
Cx annulirostris, 1 larva/100 ml, homogenized	2.20 ± 0.38	1.60 ± 0.34	57.9	nsd	-
Cx annulirostris, 3 larvae/100 ml, homogenized	3.40 ± 0.58	1.87 ± 0.32	64.6	$P < 0.05$	deters

[a] Mean of 15 replicates. Replicates with no oviposition in either cup were discarded.
[b] nsd, no significant difference ($P > 0.05$).

DISCUSSION

C. tepperi appears to have a complex oviposition response to nitrogenous compounds, and our results for these materials are difficult to interpret. 1 × MRS contains no nitrogen compounds (Martin et al., 1980). Low levels of added nitrate (as $NaNO_3$) deterred oviposition ($P < 0.05$), while higher concentrations had no significant effect. Addition of hydroxylamine stimulated oviposition ($P < 0.02$), but only at the middle concentration tested—higher and lower concentrations did not elicit a significant response. Our data suggest that responses to nitrogenous compounds are subtle and may only occur over limited concentration ranges.

The response of ovipositing *C. tepperi* to the bioextracts we tested was less ambiguous. *C. tepperi* avoided oviposition in 1 × MRS containing homogenized conspecific larvae and similarly avoided 1 × MRS conditioned by conspecific larvae, even when the conditioned 1 × MRS was diluted to provide a concentration equivalent of 1 larva/100 ml over 24 hr. Failure of homogenized adult *C. tepperi* suspended in 1 × MRS to elicit a response from ovipositing females demonstrates that the larval factor or factors causing the deterrent effect are lost prior to adult emergence.

Homogenized *P. nubiferum* larvae produced an extremely strong avoidance response ($P < 0.001$) by ovipositing *C. tepperi*, and although water conditioned by *P. nubiferum* was not evaluated in this study, this result indicates a factor responsible for deterring oviposition in *C. tepperi* is present in at least some other chironomids. This was anticipated, since if the deterrent effect was triggered only by the presence of conspecific larvae, multiple discrete cohorts of *C. tepperi* would be expected in the field, with recolonization occurring after each successive cohort emerged. An ongoing deterrent effect caused by species such as *P. nubiferum* (which is present in fields throughout the rice season) would be necessary to ensure *C. tepperi* females restrict oviposition to newly flooded habitats, rather than to habitats characterized purely by an absence of conspecific larvae.

Our results indicate that *C. tepperi* has developed a sensitive method of oviposition site selection based on detection of compounds produced by larval conspecifics, other chironomid larvae, and possibly other aquatic dipterans. The ability to identify newly flooded habitats that are not yet inhabited by other chironomids would be of particular value to a specialized colonist species such as *C. tepperi*, as the larvae resulting from oviposition into such habitats would be exposed to minimal competition during development. *C. tepperi* has been recognized as having several other characteristics of particular value to colonist midge species, including rapid development, wide thermal tolerance, protandry (Stevens, 1998), and the capacity to reproduce without the formation of mating swarms (Martin and Porter, 1977).

Our study indicates that oviposition site selection in at least some chironomids may be more complex than previously thought. Although the results appear to be

the first experimental evidence to support the idea that oviposition site selection in colonist chironomid species may be mediated by the presence of larvae, this possibility has been hinted at by other workers. Jones (1974) found that recruitment of *Paraborniella tonnoiri* Freeman was lower when conspecific larvae were present, and in some cases there was no new recruitment until all members of the previous cohort had emerged. Paterson and Cameron (1982) studied the pitcher plant chironomid, *Metriocnemus knabi* Coquillett, and suggested that ovipositing females could detect the density of larvae already in the pitchers. McLachlan and Cantrell (1980) found that *Chironomus imicola* Kieffer was a poor colonizer of ponds containing larvae of *Polypedilum vanderplanki* Hinton, but readily colonized artificial ponds where no larvae were present.

Extensive studies have been conducted on the colonization of waterbodies by chironomids, and some of these have identified colonist taxa that appear to have only a single cohort in newly flooded environments. Matěna (1990) found that *C. melanescens* Keyl and *C. piger* Strenzke rapidly colonized experimental ponds, but are soon replaced by other taxa. Whether or not these and other colonist chironomid species respond to oviposition cues produced by chironomid larvae remains to be determined. Identification of the compounds responsible for *C. tepperi* oviposition deterrence in our short-range laboratory tests will allow field experiments to be conducted on a larger spatial scale and could potentially lead to the development of novel oviposition deterrents for use in situations where control of colonist chironomid midges is required.

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Sesamia calamistis CALLING BEHAVIOR AND ITS ROLE IN HOST FINDING OF EGG PARASITOIDS *Telenomus busseolae*, *Telenomus isis*, AND *Lathromeris ovicida*

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Abstract—In the Republic of Benin, the African pink stem borer *Sesamia calamistis* Hampson is kept under control by the two scelionid egg parasitoids *Telenomus busseolae* (Gahan) and *T. isis* (Polaszek). A third species, the trichogrammatid *Lathromeris ovicida* Risbec, although commonly obtained from egg batches, is of low importance. High egg and egg batch parasitism by *Telenomus* spp. at low host densities suggests excellent host-finding capacity. Previously it was hypothesized that pheromones produced by calling *S. calamistis* virgins play an important role in attracting the parasitoids to the area where oviposition is likely to occur. Thus, after determining the effect of age (1–4 days) on the calling rhythm of *S. calamistis*, 1-day-old virgin females were chosen to investigate the response of the parasitoids to calling and noncalling females and two empty controls, in a four-arm olfactometer. The results showed that calling started earliest and lasted longest with 1-day-old females, but was similar for 2- to 4-days-old females. The percentage of females calling was higher in early than latter scotophases. The highest percentage of active females was obtained between 8 and 9 hr after the beginning of the first scotophase. All three parasitoid species responded to calling females, while there were no differences between

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noncalling females and the empty controls. The response pattern was similar for the two *Telenomus* spp. but considerably weaker for *L. ovicida*, reflecting differing host specificity of the three parasitoid species.

Key Words—Host sex pheromone, calling behavior, egg parasitoids, *Sesamia calamistis*, host finding.

INTRODUCTION

In Benin, the African pink stem borer *Sesamia calamistis* Hampson is the most abundant noctuid stem borer species attacking maize, but pest infestations are usually not of economic importance (Shanower et al., 1991; Schulthess et al., 1997). The low infestation levels were mainly attributed to high egg parasitism by the scelionids *Telenomus isis* (Polaszek) and *T. busseolae* (Gahan): A third species, the trichogrammatid *Lathromeris ovicida* (Risbec) had no significant impact on borer infestations (Chabi-Olaye, 1992; Sétamou and Schulthess, 1995; Schulthess et al., 1997). Results from monthly surveys during 18 months revealed mean peak parasitism of over 90%, and nearly 80% of the egg batches yielded *Telenomus* spp. (Schulthess et al., 2001). Egg batch densities were always exceedingly low, and it was suggested that *Telenomus* spp. possess an excellent host-finding ability. In many Scelionidae and Trichogrammatidae, the role of long-range chemicals in host location has been intensively studied (e.g., Lewis et al., 1982; Colazza et al., 1997). The role of contact kairomones produced by ovipositing female moths for guiding *T. busseolae* and *T. isis* to host eggs has been investigated by Chabi-Olaye et al. (2001), and Chabi-Olaye et al. (2001) and Agboka et al. (2002) studied host discrimination behavior of *T. busseolae*, *T. isis*, and *L. ovicida*. In Italy, Colazza et al. (1997) showed that *T. busseolae* was attracted by pheromones of virgin female *Sesamia nonagrioides* (Lefebvre) to the area where oviposition was likely to occur. We suspect that pheromones also play an important role in the host-finding of the three parasitoids occurring in Benin on *S. calamistis*.

The aim of this study was to investigate the calling rhythm of virgin female *S. calamistis* as affected by age and the response of the three egg parasitoids *T. busseolae*, *T. isis*, and *L. ovicida* to calling and noncalling females.

METHODS AND MATERIALS

Insect Cultures. *Sesamia calamistis* eggs were obtained from a laboratory colony at the International Institute of Tropical Agriculture (IITA), Benin, and maintained on artificial diet, according to a protocol developed by Bosque-Pérez and Dabrowski (1989). Adults of the three egg parasitoid species were reared on *S. calamistis* eggs following a method developed by Chabi-Olaye et al. (1997).

Calling Behavior. One day before emergence, about 100 female pupae of *S. calamistis* were put in a plastic jar (8 cm high, 20 cm diam.) and kept at 25 ± 1°C and 75 ± 5% relative humidity. The room was illuminated during the scotophase with a dim red light (5 ± 1 lux). After emergence, moths were held under a photoperiod of 12L:12D with the photophase beginning at 8:00 PM to allow observations during the normal workday, between 8:00 AM and 12 noon. Emerged moths were isolated and kept individually in transparent glass vials (5 cm length, 2 cm diam.). Calling behavior was observed from day 1 through day 4 following emergence. In total, 20 females were used per test and the procedure was repeated twice. Observations were made only during scotophase, which was divided into 15-min intervals. Start and duration of calling period for each female and age group, as well as percentage of calling females were recorded. A female was considered to be calling when the sex pheromone gland was visible (Brady and Smithwick, 1968; Sower et al., 1972; Baker and Cardé, 1979; Castrovillo and Cardé, 1979; Raina and Klun, 1984; Mayer and Mankin, 1985; Tamaki, 1985; Babilis and Mazomenos, 1992).

Response of Egg Parasitoids T. busseolae, T. isis, and L. ovicida to Calling Behavior of S. calamistis. Experiments were carried out during the first scotophase after emergence, when calling activity was highest. Bioassays were conducted with a four-arm olfactometer, similar to that described by Scholz et al. (1997). The device has been used successfully in several studies on the host-finding behavior of Coleoptera (e.g., Scholz et al., 1997, Borgemeister et al., 1999) and Hymenoptera (e.g., Chabi-Olaye et al., 2001). Air was drawn from the outside by a vacuum pump (Neuberger, type N010 KN18). Total air flow was controlled by a flowmeter (Brooksmeter Sho-rate, 1355, R-2-15-B, sapphire float). The air was pushed through a perspex tube (25 cm long, 6 cm diam.) filled with activated charcoal, and a gas-washing bottle filled with distilled water to clean the air from volatiles and to stabilize the relative humidity. The air was then passed through a glass tube (10 cm long, 2.5 cm diam.) filled with equilibrated silica gel to adjust the relative humidity to 60–70%. The single airstream was divided and pushed through four flowmeters (Brooksmeter Sho-rate, 1355, R-2-15-D, stainless steel float) to maintain each flow at 15.2 liter/hr. The airstreams then passed through 500-ml glass jars, each either containing an odor source or serving as control, and each stream entered the arena through one of the four connected aluminum pipes. The air escaped from the arena through the center hole in the base, and the airstreams formed four distinct zones within the arena. All tube connections were made with silicon components. A single naïve mated female parasitoid was placed in the central arena of the olfactometer with the aid of a camelhair brush. Five minutes before a female was released into the olfactometer, the odor sources were introduced into the device to ensure a gradient of volatiles. The following odor sources were tested: (1) 10 calling virgin females (CVF), (2) empty jar serving as first control (C1), i.e., with only humidified air, (3) 10 noncalling virgin females (NCVF), and (4) empty

jar serving as second control (C2), i.e., with only humidified air. To reduce bias due to environmental factors, the positions of the odor sources were changed after 25, 50, and 75% of replications. For each parasitoid species, 60 females in total were tested (i.e., 60 replicates). Each observation lasted 10 min, and the total time spent in each odor field was recorded.

Statistical Analysis. Differences in percentage of calling females, start, and length of calling period according to age were analyzed by analysis of variance (ANOVA) with the general linear model (GLM) procedure of SAS (SAS Institute, 1997). In the case of significant F values, means were compared via Student-Newman-Keuls test. Percentages were arcsine transformed. Friedman two-way analysis of variance by ranks, based on time spent by female parasitoids in each odor field, was used to test for field preferences in the four-arm olfactometer experiment. The Student-Newman-Keuls test also was used to compare mean time spent per odor field. Differences between parasitoids responses were analyzed by Wilcoxon test.

RESULTS

Calling Behavior. The onset of calling by virgin female *S. calamistis* was characterized by wing vibration, vibration of the end of abdomen, and protrusion and retraction of the pheromone gland. The percentage of active females was higher during the early than latter scotophases (Figure 1); during the first scotophase, the percentage of calling females reached 50% after 5 hr, and was close to 100% between 8 and 9 hrs, after which it gradually declined to 57%. During the second to fourth scotophase, 50% called after 10 hr only, with maximum values of 58–72% at the end of the scotophase.

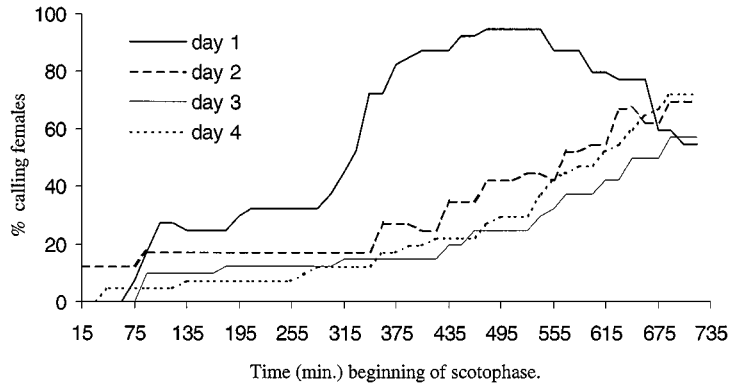

FIG. 1. Influence of age (1-to 4-day-old adults) on the calling of female *Sesamia calamistis*.

TABLE 1. INFLUENCE OF AGE OF *Sesamia calamistis* ON START AND DURATION OF CALLING PERIOD AND PERCENTAGE OF CALLING FEMALES AFTER BEGINNING OF SCOTOPHASE[a]

	Age (days)			
	1	2	3	4
Start of calling (min, mean ± SE)	256.2 ± 20.8a	387.7 ± 39.2b	453.5 ± 39.9b	471.2 ± 34.7b
Duration (min, mean ± SE)	420.0 ± 23.2a	293.0 ± 30.2b	242.3 ± 34.5b	246.2 ± 34.3b
% calling females (mean ± SE)	95 ± 5.0	80 ± 10.0	65 ± 5.0	72 ± 2.5

[a] Within a row, means followed by different letters are significantly different at $P \leq 0.05$ (Student-Newman-Keul test).

Calling started earliest ($P \leq 0.001$) and lasted longest ($P \leq 0.001$) with 1-day-old females, but was similar for 2- to 4-day-old females (Table 1).

Response of Egg Parasitoids T. busseolae, T. isis, and L. ovicida to Calling Behavior of S. calamistis. The three egg parasitoid species were attracted to calling virgin females (Table 2), whereas differences between noncalling virgin females and the controls were not significant. There was no difference in responses between *T. busseolae* and *T. isis*, but both species responded stronger to odors of calling *S. calamistis* virgins than to *L. ovicida* (Table 3).

DISCUSSION

The calling posture of *S. calamistis* females was the same as observed for other lepidopterous species (Brady and Smithwick, 1968; Sower et al., 1972; Baker and Cardé, 1979; Raina and Klun, 1984; Zhang et al., 1996; Mozuraitis et al., 2000).

TABLE 2. AVERAGE TIME SPENT (%, MEAN ± SE) BY *Lathromeris ovicida*, *Telenomus isis*, AND *T. busseolae* IN ODOR FIELDS OF FOUR-ARM OLFACTOMETER[a]

	Odor sources			
Species	CVF	NCVF	C1	C2
L. ovicida	35.2 ± 2.8a	20.4 ± 2.8b	21.4 ± 3.7b	22.5 ± 4.4b
T. isis	43.3 ± 4.2a	19.1 ± 2.2b	18.7 ± 3.8b	18.9 ± 4.1b
T. busseolae	46.1 ± 2.5a	18.3 ± 3.1b	17.7 ± 3.9b	17.9 ± 4.5b

[a] CVF = calling virgin females, NCVF = noncalling virgin females, C1 and C2 = controls with humidified air only. Within a row, means followed by different letters are significantly different at $P \leq 0.05$ (Student-Newman-Keul test).

TABLE 3. COMPARISON OF PARASITOID RESPONSES TO CALLING *Sesamia calamistis* FEMALES

Comparison	χ^2	*P*
T. busseolae vs. T. isis	1.4010	0.237
T. busseolae vs. L. ovicida	11.8609	<0.001
T. isis vs. L. ovicida	10.4172	0.001

We found that the calling behavior of *S. calamistis* females varied with age and was higher during the first day than on any subsequent day. Similar results were reported in other moth species such as the noctuids *Trichoplusia ni* (Hübner) (Sower et al., 1972) and *S. nonagroides* (Mazomenos, 1984; Babilis and Mazomenos, 1992), the pyralid *Plodia interpunctella* (Hübner) (Nordlund and Brady, 1974), the codling moth *Laspeyresia pomonella* L. (Castrovillo and Cardé, 1979), and the arctiid fall web moth *Hyphantria cunea* (Drury) (Zhang et al., 1996).

Calling behavior and pheromone production are periodic and synchronous in many lepidopterous species (Coffelt et al., 1978; Raina et al., 1986; Babilis and Mazomenos, 1992). Many studies on sex pheromone and communication revealed that pheromone emission is synchronous with the calling behavior of females (Jefferson et al., 1966; Miller and Roelofs, 1977; Raina and Klun, 1984; Babilis and Mazomenos, 1992). In noctuid moths, the production of sex pheromone occurs within one day preceding or following emergence from the pupa (reviewed by Shorey et al., 1968). This also appeared to be the case in our study, as shown by the high attraction of the parasitoids to the calling female compared to noncalling females or the control.

It is concluded that in the pest–parasitoid system under study, odor from calling *S. calamistis* females may induce in the tested parasitoid species a transition from flying to walking behavior. In numerous insect parasitoids, host sex pheromones serve as long-range chemical cues that help the parasitoid in restricting the search area and thereby direct the parasitoid to areas where host mating is in progress or where oviposition has probably taken place (e.g., Vet and Dicke, 1992; Godfray, 1994). Once the oviposition site is discovered, contact kairomones produced by the female moth are directly associated with the attack on host eggs (Chabi-Olaye et al., 2001).

Telenomus busseolae and *T. isis* are the most important parasitoids of noctuid stem borers, which lay their eggs cryptically between the leaf sheath and the stem, while *Lathromeris ovicida* has been mainly reported from crambids, such as *Chilo zacconius* Bleszynski, and the pyralids *Scirpophaga subumbrosa* Meyrick and *Maliarpha separatella* Ragonot that oviposit on the lower or upper surfaces of leaves (Polaszek, 1998; Chabi-Olaye et al., 2001). Differences in host specificity, as well as in the ecological niches occupied by their host species may explain the

stronger response pattern of *Telenomus* spp. to calling *S. calamistis* females when compared to *L. ovicida*.

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VOLATILE COMPOUNDS RELEASED BY DISTURBED AND CALM ADULTS OF THE TARNISHED PLANT BUG, *Lygus lineolaris*

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Abstract—Volatile compounds released by disturbed and calm female and male *Lygus lineolaris* were collected and analyzed. Six major compounds were present in samples from disturbed bugs and from calm females: (*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexenol, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-2,4-oxohexenal. (*E*)-2-hexenal was lacking in volatiles collected from calm males. Hexyl butyrate accounted for approximately 68% and 66% of volatiles released by agitated and calm females, and 87% and 88% of volatiles released by agitated and calm males, respectively. Blends released by disturbed insects differed quantitatively from blends released by calm insects, with amounts of compounds increasing 75–350 times in samples from disturbed insects. In static air bioassays, both females and males were repelled by natural volatiles collected from females and by five-component [(*E*)-2,4-oxohexenal excluded] and six-component synthetic blends at doses of 1 and 10 bug-hours, indicating that these volatiles may serve an alarm or epideictic function, as well as a possible role as defensive allomones. Adults also avoided hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-2-hexenol, and (*E*)-2,4-oxohexenal, but not 1-hexanol and (*E*)-2-hexenal when compounds were assayed individually in static air bioassays at doses equal to 1 bug-hour. When tested over 1 day in two-choice cage trials, adults did not prefer untreated bean plants over bean plants surrounded by vials releasing up to 8.1 mg/hr (=234 bug-hours) of the five-component synthetic blend. Therefore, the volatiles produced by disturbed adults would not be useful as a repellent for *L. lineolaris.*

Key Words—*Lygus lineolaris*, tarnished plant bug, Heteroptera, Miridae, repellent, alarm pheromone, allomone, hexyl butyrate.

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INTRODUCTION

Lygus bugs (Heteroptera: Miridae) are important pests of agricultural crops in North America (Prokopy et al., 1980; Walstrom, 1983; Hedlund and Graham, 1987; Butts and Lamb, 1991; Schaefers and Shanks, 1991; Hardee and Herzog, 1996). In particular, the tarnished plant bug, *Lygus lineolaris*, is widespread geographically (Kelton, 1975) and attacks many crops (Young, 1986). Alternatives to chemical control of this pest are needed where it has developed resistance to insecticides (Snodgrass, 1996) and where adoption of alternative management strategies for other pests has led to reduced use of insecticides (Day, 1987; Hardee and Bryan, 1997).

Like many Heteroptera (Staddon, 1979; Aldrich, 1988), adults of *L. lineolaris* produce a pungent odor when disturbed (Blumenthal, 1978; authors' personal observation). These chemicals may function as allomones to repel enemies, as alarm pheromones (Staddon, 1979; Blum, 1981; Aldrich, 1988), or as epideitic (spacing) pheromones (Prokopy, 1981). In the laboratory, the compounds released by agitated *L. lineolaris* cause disturbance and dispersal among conspecifics (authors' personal observation) and, if escape is not possible, narcosis and even death (Blumenthal, 1978; authors' personal observation).

Volatile chemicals produced by *L. lineolaris* have been previously analyzed (Blumenthal, 1978; Gueldner and Parrott, 1978; Aldrich et al., 1988), but compounds released specifically by disturbed insects have not been characterized. Our objectives were to identify, quantify, and compare the compounds released by disturbed and calm adults of *L. lineolaris*, and to test them as repellents for this insect.

METHODS AND MATERIALS

Insect Rearing. All experimental insects came from a laboratory colony reared by using modifications of several techniques for lygus bugs (Parrott et al., 1975; Bryan et al., 1976; Slaymaker and Tugwell, 1982). For oviposition and feeding, adults were given sprouted potatoes, *Solanum tuberosum*, green beans, *Phaseolus vulgaris*, cut broccoli, *Brassica oleracea* var. *italica*, carrots, *Daucus carota*, broad bean plants, *Vicia faba*, coddled wax moth larvae, *Galleria mellonella*, and sugar water. Nymphs were fed green beans and cut broccoli. Insects were reared at 21–25°C, >60% relative humidity, and on a 15L:9D photoregime. Newly molted adults were collected with an aspirator, sexed, and kept (sexes separate) on cut broccoli and green beans under the above conditions until 7–14 days old.

Volatile Collection and Analysis. Volatile chemicals were collected from disturbed and calm adults. The collection apparatus (Figure 1) consisted of a 15-cm-long × 2-cm-diam. glass tube plugged at both ends with aluminum screen (2 mm

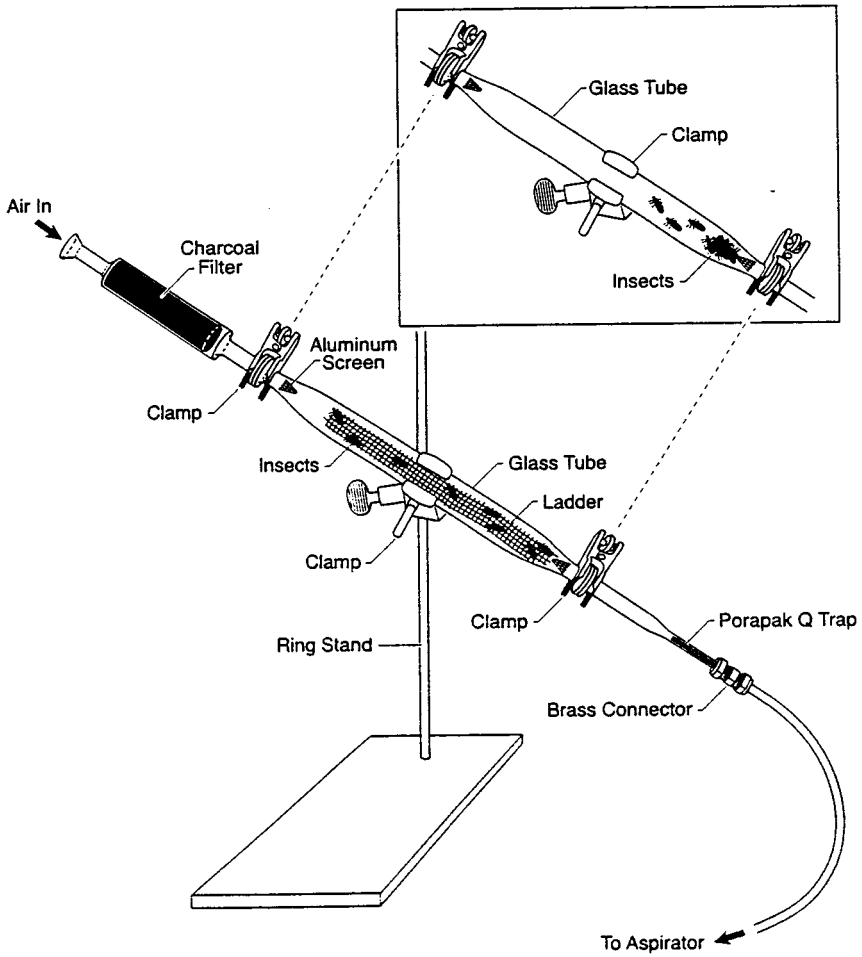

FIG. 1. Apparatus used to collect volatiles from calm and disturbed (inset) female and male *L. lineolaris*.

mesh), and suspended at a 45° angle. A charcoal filter was clamped to the intake of the tube, and a Porapak Q trap (Waters Associates, Inc., Milford, Massachusetts, USA; 50–80 mesh, 30 mm × 6 mm OD) was clamped to the outlet. The trap was attached to a water aspirator that drew air through the apparatus at 0.45 liters/min.

For collection of volatiles from disturbed bugs, approximately 50 adults of one sex were loaded into the glass tube. The top of the tube was quickly plugged with screen, and the charcoal filter was clamped in place. Air was drawn through the tube for 1 min before the Porapak Q trap was attached and the airflow adjusted.

With the apparatus tilted at 45°, bugs could crawl part way up the sides of the glass tube before falling back into a dense mass of agitated individuals constantly in contact with one another at the bottom. Volatiles were collected for approximately 1 hr between hours 2 and 10 of the photophase. Twenty-one samples totaling 1149 bug-hours (bh) were collected from females, and 13 samples totaling 766 bh were collected from males.

Similar procedures were used to collect volatiles for 6–7 hr from calm insects, except that only 10 insects of one sex were placed in the tube at one time. A ladder of aluminum screen (10 × 1 cm) inserted into the glass tube allowed insects to escape from contact with one another. Twenty-one samples totaling 1338 bh were collected from females, and 14 samples totaling 905 bh from males.

After collection, volatiles were stripped from Porapak Q traps with 3 ml of pentane, concentrated on ice to 1 ml with a stream of N_2, stored as individual samples, and analyzed with a Hewlett Packard 5830A gas chromatograph (GC) with a SP1000 column (30 m × 0.5 mm ID) (Supelco, Oakville, Ontario) and a Hewlett Packard 5890 GC with a DB-5-4 column (30 m × 0.25 mm ID) (J and W Scientific, Folsom, California, USA). Both GCs were equipped with flame-ionization detectors. Compounds were identified by comparing retention times with authentic standards and by coupled GC-mass spectroscopy (GC-MS) using a Hewlett Packard 5985B GC-MS with a DB-5 column (30 m × 0.25 mm ID) in electron impact (EI) mode. Spectra were compared with published spectra (Jennings and Shibamoto, 1980) and with spectra from authentic standards. External standards of known concentration were used to obtain area counts for quantifying captured volatiles. Samples were quantified individually, and mean amounts of compounds were calculated for disturbed and calm females and males.

Bioassays. Volatiles from disturbed females were used in initial tests of repellency. To obtain enough raw material for all bioassay doses, samples from disturbed females were combined, and the volume reduced on ice under a stream of N_2 to obtain a concentration of 1 bh/μl. This material was diluted serially with pentane. For bioassays, volatiles were delivered in 10-μl aliquots, and doses of 10, 1.0, 0.1, and 0.01 bh were tested.

Bioassays were conducted at 25°C and ambient relative humidity, between hours 2 and 10 of the photophase using a two-choice, static-air olfactometer (Vet, 1983). Volatiles or solvent were loaded onto a filter paper disk (1 cm diam.; Whatman International Ltd., Maidstone, England) and allowed to dry before being placed in an olfactometer odor chamber. Individual bugs were introduced into the central olfactometer chamber, which was closed, and they were given 2 min to choose between a solvent (10 μl pentane) -treated disk in one lateral odor chamber and a disk treated with volatiles or an untreated disk in the other. The first choice, the amount of time spent in each odor chamber, and the final position at the end of the test were recorded. Bugs that did not leave the central chamber were discarded. Ten replicates were conducted with both females and males. The

position of the olfactometer was reversed for each replicate, and within replicates doses were tested in random order.

The same procedure was used for a synthetic blend of the six major components released by disturbed females in the proportions found in collected volatiles (Table 1) at doses of 10, 1.0, 0.1, and 0.01 bh. Five compounds were purchased, and (*E*)-2,4-oxohexenal was synthesized (Piancatelli et al., 1980). A five-component synthetic blend lacking this ketoaldehyde was also tested at the above doses. The five commercially available compounds were also tested singly at doses equal to 1 bh, along with the five-component synthetic blend for reference. (*E*)-2,4-oxohexenal was similarly tested in a separate bioassay. Single component bioassays all comprised 20 replicates. For all bioassays of synthetic compounds, stimuli were delivered in 5 μl of pentane.

Cage Trials. To determine if a synthetic blend mimicking the volatiles released by agitated *L. lineolaris* could repel this insect from host plants, experiments were conducted in a large, indoor cage (91.5 cm long $\times$ 51 cm wide $\times$ 61 cm high). Stimuli were released from open glass vials (aperture diam. 24 mm) screened with hardware cloth (1.5 mm mesh). Release rates were determined by weighing each vial before and after an experiment. Two pots of flowering 5-week-old broad bean plants of similar size were placed on opposite ends of the cage, 50 cm apart. One vial was placed in each pot at the base of each plant, and the other four were spaced equidistant around the pot, 2–3 cm from the base. In the first experiment, groups of insects were tested in three different, two-choice treatments: (1) all five vials near the treated plant containing 1 ml of the undiluted five-component synthetic blend used in olfactometer bioassays [release rate per plant 6.8 ± 0.5 (mean $\pm$ SE) mg/hr], with five empty vials near the untreated control plant; (2) only the vial in the pot of the treated plant contained 1 ml of synthetic blend (release rate per plant 1.6 ± 0.2 mg/hr); and (3) all vials near both plants empty. In each of five replicates per treatment, 20 females and 20 males were released in the center of the cage 1.5–2 hr into the photophase, and their position after 23.5 hr recorded as on plant 1, plant 2, or not on either plant. The experiment was conducted at $27 \pm 4^{\circ}$C and ambient relative humidity, with a 15L:9D photoregime. Fresh stimuli, bugs, and plants were used for each replicate. Treatments were tested in random order in the same cage on consecutive days, with the positions of treated and untreated plants reversed each day.

Using the same procedure, a second, 10-replicate experiment compared the response to untreated plants and plants surrounded at their base by six vials containing 1 ml of the synthetic blend released at 8.1 ± 0.4 mg/hr per plant.

Statistics. In static air bioassays, first choice and final position of insects were compared between treatment and control stimuli using the one-tailed binomial test (Zar, 1984); times spent in odor chambers were compared using the one-tailed Wilcoxon signed rank test (Conover, 1980). The latter test was also used to compare numbers of insects on treated and control plants in cage experiments.

TABLE 1. TOTAL VOLATILES PRESENT OF SIX MAJOR COMPOUNDS RELEASED PER BUG-HOUR BY CALM AND DISTURBED FEMALE AND MALE *L. lineolaris*

	Volatiles released [ng (%)][a]			
	Females		Males	
Compound	Calm	Disturbed	Calm	Disturbed
(*E*)-2-hexenal	5.2 ± 1.2 (4.1)	770.6 ± 56.3 (2.2)	0.0 (0.0)	48.2 ± 11.0 (0.2)
1-hexanol	5.4 ± 1.4 (4.2)	406.1 ± 26.0 (1.2)	0.6 ± 0.3 (0.3)	189.7 ± 12.4 (0.6)
(*E*)-2-hexenol	2.8 ± 0.6 (2.1)	395.6 ± 26.7 (1.1)	0.2 ± 0.2 (0.1)	32.3 ± 1.9 (0.1)
hexyl butyrate	84.6 ± 23.1 (66.1)	23,653.1 ± 1,612.5 (67.5)	179.5 ± 111.0 (88.4)	27,144.2 ± 1,920.3 (87.1)
(*E*)-2-hexenyl butyrate	22.9 ± 7.5 (17.9)	8,036.5 ± 507.2 (22.9)	8.8 ± 6.8 (4.3)	1,227.8 ± 74.5 (3.9)
(*E*)-2,4-oxohexenal	7.1 ± 2.1 (5.5)	1,779.0 ± 135.0 (5.1)	13.9 ± 9.4 (6.8)	2,516.0 ± 177.2 (8.1)

[a] Values are means ± SE; percentages are in parentheses.

RESULTS

Volatile Collection and Analysis. Calm and disturbed females and disturbed males released the same array of six major volatile compounds: (*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexenol, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-2,4-oxohexenal (Table 1, Figure 2). Calm males also released the latter five compounds, but not (*E*)-2-hexenal (Table 1, Figure 2).

Increases in amounts released by disturbed over calm insects (Table 1, Figure 2) ranged from 75-fold for 1-hexanol to 351-fold for (*E*)-2-hexenyl butyrate for females, and from 140-fold for (*E*)-2-hexenyl butyrate to 316-fold for 1-hexanol for males. Hexyl butyrate was the predominant volatile, making up approximately 66% and 68%, and 88% and 87%, of volatiles released by calm and disturbed females and males, respectively. Calm and disturbed females released 2.6 and 6.5 times the amount of (*E*)-2-hexenyl butyrate released by their male counterparts.

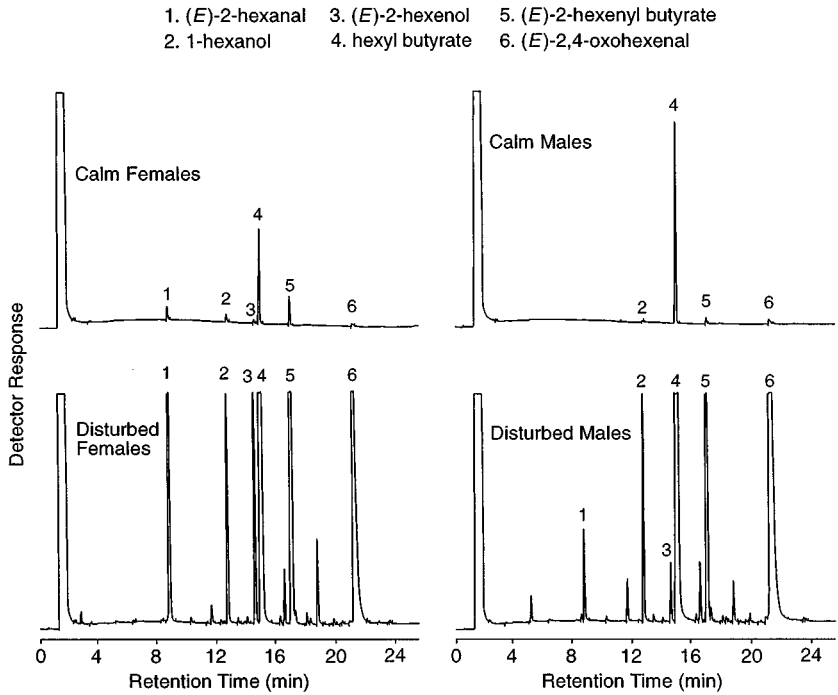

FIG. 2. Gas chromatographic traces of volatiles collected from calm and disturbed female and male *L. lineolaris.* Samples analyzed with an FID detector and an SP1000 column at 70°C for 2 min, increasing to 180°C at 4°C/min, then constant at 180°C for 30.5 min.

TABLE 2. RESPONSES OF FEMALE AND MALE *L. lineolaris* IN STATIC AIR BIOASSAYS TO NATURAL VOLATILES COLLECTED FROM DISTURBED FEMALE *L. lineolaris* AND TO SIX- AND FIVE-COMPONENT SYNTHETIC BLENDS OF VOLATILES[b]

Volatiles tested	Sex of test insects	Dose (bh)	First choice[a]		Final position[a]		Time in odor chambers (sec, mean ± SE)[a]	
			Pentane	Stimulus	Pentane	Stimulus	Pentane	Stimulus
Natural volatiles from disturbed females	Female	blank	4	6	1	6	29.7 ± 8.3	32.4 ± 9.6
		0.01	8	2	5	2	61.2 ± 9.2	11.3 ± 3.8*
		0.1	5	5	4	2	43.7 ± 10.8	21.9 ± 9.2
		1.0	9	1*	7	0*	64.7 ± 10.1	3.9 ± 1.6*
		10.0	9	1*	8	0*	56.7 ± 10.3	2.1 ± 1.0*
	Male	blank	7	3	1	5	28.5 ± 7.7	34.6 ± 6.6
		0.01	7	3	5	0*	43.0 ± 8.5	13.7 ± 3.0*
		0.1	5	5	4	2	32.1 ± 9.1	19.3 ± 5.3
		1.0	9	1*	7	0*	63.5 ± 10.4	2.5 ± 1.3*
		10.0	8	2	7	1*	69.2 ± 4.9	6.4 ± 4.0*
Six-component synthetic blend	Female	blank	5	5	5	3	40.4 ± 8.8	34.9 ± 6.9
		0.01	5	5	2	4	39.6 ± 9.7	35.9 ± 11.9
		0.1	8	2	6	1	60.7 ± 9.4	11.5 ± 4.0*
		1.0	8	2	8	1*	48.7 ± 10.0	3.9 ± 1.9*
		10.0	7	3	5	0*	59.3 ± 7.8	4.6 ± 2.4*
	Male	blank	5	5	2	3	38.5 ± 10.7	33.3 ± 9.4
		0.01	6	4	4	4	49.0 ± 10.3	29.5 ± 11.1
		0.1	8	2	6	0*	45.1 ± 9.8	13.6 ± 6.0*
		1.0	7	3	7	0*	51.0 ± 7.4	6.7 ± 2.4*
		10.0	8	2	5	0*	64.7 ± 7.2	3.1 ± 2.5*
Five-component synthetic blend	Female	blank	5	5	3	7	38.1 ± 13.6	39.3 ± 12.2
		0.01	5	5	4	3	37.2 ± 8.6	45.5 ± 9.7
		0.1	9	1*	6	0*	64.2 ± 10.8	12.9 ± 9.7*
		1.0	8	2	9	0*	93.5 ± 6.5	3.2 ± 1.7*
		10.0	10	0*	9	0*	98.8 ± 6.6	0.3 ± 0.3*
	Male	blank	5	5	2	5	30.5 ± 9.7	34.3 ± 8.1
		0.01	6	4	6	1	50.6 ± 8.0	28.5 ± 5.7
		0.1	7	3	3	2	58.5 ± 10.7	26.8 ± 8.6
		1.0	7	3	6	3	79.1 ± 11.0	14.1 ± 11.2*
		10.0	9	1*	8	1*	61.4 ± 7.3	8.5 ± 3.2*

[a] Pairs of values followed by an asterisk are significantly different, one-tailed binomial test for choice and position, and one-tailed Wilcoxon signed rank test for time, $P < 0.05$.

[b] $N = 10$ for all bioassays.

Experiments. At doses of 1 and 10 bh, females and males avoided the odor chamber that was the source of volatiles from disturbed female *L. lineolaris*, making it their first choice or final position significantly less often and spending significantly less time in it (Table 2). At doses of 0.1 and 0.01 bh, bugs seemed to avoid the chamber containing volatiles from disturbed females, but the responses were not usually significant. Responses to the solvent and blank chambers were not different, indicating that significant responses were caused by repellency of the female volatiles rather than attraction to the solvent.

Synthetic blends of five and six of the major components in natural volatiles were also repellent to both sexes at doses of 10, 1, and 0.1 bh (Table 2). Exclusion of

(*E*)-2,4-oxohexenal did not affect the repellency of the blend to adults. Responses to the single components (Table 3) confirmed that (*E*)-2,4-oxohexenal was not necessary for repellency. At doses equivalent to 1 bh, females and males were as strongly repelled by hexyl butyrate alone as by the five-component blend. Adults were also repelled by 1 bh doses of (*E*)-2-hexenol, (*E*)-2-hexenyl butyrate, and (*E*)-2,4-oxohexenal alone, indicating that there is a high degree of redundancy in the natural blend. Females and males did not avoid 1 bh doses of (*E*)-2-hexenal or 1-hexanol alone.

For all three doses tested in both cage experiments, similar numbers of adults were found on treated and untreated plants one day after their release (Wilcoxon signed rank test, $P > 0.05$). This distribution did not differ from that found when neither plant in the test cage was treated with synthetic volatiles.

DISCUSSION

Our results define the volatile blends released by both calm and disturbed adults of *L. lineolaris* of both sexes and elucidate, in part, the behavioral roles of the blend and its constituents produced by agitated adults. They differ from the results of others researchers in several respects. In steam distillations of females and males, Gueldner and Parrott (1978) found that hexyl butyrate and (*E*)-2-hexenyl butyrate made up 60–80% of the volatiles. However, they found the amounts of the two esters produced by females to be approximately equal. They also detected small amounts of (*E*)-2-hexenal. Blumenthal (1978) found that females subjected to less agitation than ours released 10,860 ng of hexyl butyrate per bug-hour, approximately a third of the estimated capacity of the scent gland reservoir. This suggests that in our experiments, most bugs virtually emptied their reservoirs. Blumenthal (1978) found that the amount of hexyl butyrate released by undisturbed insects varied from undetectable levels to 710 ng/bh. Aldrich et al. (1988) reported that hexyl butyrate and (*E*)-2-hexenyl butyrate constituted 70–80% of volatiles trapped from females and males in aerations, and in extracts of metathoracic glands. Like Gueldner and Parrott (1978), they found that females produced equal amounts of the two esters. Aldrich et al. (1988) also found (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, and (*E*)-2,4-oxohexenal in trapped and extracted volatile samples from male and female *L. lineolaris*. While (*E*)-2-hexenal was not present in volatiles collected from calm males in this work, we have detected it in other samples from undisturbed males. Chinta et al. (1994) showed that hexyl and (*E*)-2-hexenyl butyrate, (*E*)-2-hexenal, (*E*)-2-hexenol, and 1-hexanol are EAD active for *L. lineolaris*. They did not test (*E*)-2,4-oxohexenal.

In the mirid *Lopidea robiniae*, Staples et al. (2002) found that calm adults did not produce detectable volatiles, while disturbed adults emitted a six-component blend of 6 to 8-carbon aldehydes and alcohols.

TABLE 3. RESPONSES OF FEMALE AND MALE *L. lineolaris* IN STATIC AIR BIOASSAYS TO SYNTHETIC INDIVIDUAL COMPONENTS FOUND IN VOLATILES RELEASED BY DISTURBED FEMALE *L. lineolaris*[a]

Sex of test insects	Test stimulus	First choice[b]		Final position[b]		Time in odor chambers (sec, mean ± SE)[a,b]	
		Pentane	Stimulus	Pentane	Stimulus	Pentane	Stimulus
Female	blank	11	9	9	4	33.7 ± 8.4	41.2 ± 10.6
	(*E*)-2-hexenal	10	10	10	4	43.8 ± 8.7	27.2 ± 7.0
	1-hexanol	13	7	7	6	36.8 ± 9.5	30.8 ± 8.8
	(*E*)-2-hexenol	12	8	13	5*	55.1 ± 8.4	19.6 ± 6.3*
	hexyl butyrate	16	4*	15	0*	66.5 ± 7.1	8.0 ± 4.9*
	(*E*)-2-hexenyl butyrate	11	9	15	1*	45.6 ± 7.7	10.4 ± 4.0*
	five-component blend	16	4*	15	0*	68.5 ± 7.9	2.0 ± 1.3*
	blank	12	8	6	5	30.5 ± 6.8	39.2 ± 9.1
	(*E*)-2,4-oxohexenal	17	3*	9	1*	43.5 ± 6.7	10.0 ± 2.4*
	five-component blend	16	4*	12	1*	49.9 ± 7.8	8.1 ± 5.6*
Male	blank	11	9	6	8	41.9 ± 8.4	30.4 ± 6.7
	(*E*)-2-hexenal	8	12	7	6	38.1 ± 8.3	36.7 ± 7.5
	1-hexanol	11	9	7	2	38.7 ± 7.4	24.5 ± 8.2
	(*E*)-2-hexenol	11	9	10	3*	48.9 ± 9.4	15.0 ± 4.9*
	hexyl butyrate	16	4*	14	0*	71.0 ± 7.6	2.7 ± 1.4*
	(*E*)-2-hexenyl butyrate	19	1*	11	4	56.5 ± 7.6	4.4 ± 2.4*
	five-component blend	17	3*	17	2*	60.4 ± 6.9	11.0 ± 7.1*
	blank	10	10	4	9	31.6 ± 4.9	40.3 ± 5.7
	(*E*)-2,4-oxohexenal	11	9	9	4	40.9 ± 8.8	19.2 ± 6.1*
	five-component blend	17	3*	9	3	47.5 ± 6.7	13.0 ± 5.8*

[a] $N = 20$ for all bioassays. Dose = amount present in 1 bug hour (Table 1).
[b] As for Table 2.

The volatiles released at high levels by disturbed adult *L. lineolaris* probably have multiple functions, as in other heteroptera (Lockwood and Story, 1985, 1987; Gunawardena and Bandumathie, 1993; Pavis et al., 1994). In part, they may fulfill a defensive function (Staddon, 1979; Aldrich, 1988). *Lygus lineolaris* can be cannibalistic (Curtis and McCoy, 1964; Blumenthal, 1978; authors' personal observation), and the same defensive compounds could act both intra- and interspecifically in that role (Blumenthal, 1978). Another possible function is as an epideictic pheromone, reducing competition for resources (Blumenthal, 1978), as suggested for the pentatomid *Euschistus tristigmus* (Aldrich et al., 1995). A third potential function is as an alarm pheromone causing dispersal. Krall et al. (1999) hypothesized that defensive compounds released by the pentatomid *Cosmopepla bimaculata* also function as an alarm pheromone. Gregariousness or high population density, allowing rapid communication, is generally thought to be necessary for the evolution of alarm pheromones (Nault and Phelan, 1984; Blum, 1985). Adult alarm pheromones can exist in true bugs that form loose aggregations (Lockwood and Story, 1987; Leal et al., 1994; Blatt et al., 1998). Lygus bug populations can reach high densities, allowing for the alarm function of volatiles to have evolved secondarily to the defensive function (Nault and Phelan, 1984; Blum, 1985), as a result of bugs benefiting by dispersing after perceiving nearby conspecific releasing defensive compounds.

Not all compounds in a blend may perform the same function. The inactivity of (*E*)-2-hexenal and 1-hexanol when tested alone against adult *L. lineolaris* suggest that they may serve as allelochemics, rather than pheromones. Because hexyl butyrate was as repellent as the five-component blend, there was no synergistic, or even additive, role that they could have played in repellency bioassays. The apparent redundancy of bioactive compounds in the five- and six-component blends could ensure that the message from an emitting bug was active even if incomplete or there was a problem with perception. A similar fail-safe function has been hypothesized for redundant compounds in the blends that induce attack by bark beetles on host trees (Borden et al., 1990) and cause avoidance of nonhost species (Borden et al., 1998).

The volatiles released at low levels by calm *L. lineolaris* could perform different functions from the same materials released at high levels by disturbed bugs. Altering the concentration of volatiles released is one mechanism by which function can be changed (Lockwood and Story, 1985; Pavis et al., 1994). Potential roles for volatiles released at low concentrations include recognition of conspecifics or sexual recognition. In support of this hypothesis, the amounts of volatiles released by calm *L. lineolaris* were consistently lower than the lowest doses that caused repellency in static air bioassays.

The caged plant experiments indicate that the compounds released by disturbed adult *L. lineolaris* do not have a long-term effect on the behavior of conspecifics. In static air bioassays, adults avoided these volatiles, but even in a

2-min-long test, bugs sometimes repeatedly entered and left the stimulus chamber. In the two-choice cage experiments, they had an extended time in which to respond to volatiles, which were released at higher rates and longer duration than would have occurred in nature. Possibly they habituated to the volatiles (Wohlers, 1981a,b), or their need to feed or oviposit overcame their tendency to avoid contaminated resources. Such responses may be of adaptive advantage if the risk of leaving a proven food source or oviposition site is greater than the risks of predation or competition (Teerling et al., 1993). Some relatively long-term avoidance of resources treated with alarm pheromones has been seen in aphids and thrips when an untreated alternative was offered as a choice (Wohlers, 1981b; Dawson et al., 1982; Teerling et al., 1993). Wohlers (1981b), and Dawson et al. (1982) expressed some hope that these volatiles could be used as plant protectants. However, as for the western conifer seed bug, *L. occidentalis* (Blatt et al., 1998), avoidance of contaminated resources by *L. lineolaris* is ephemeral. Therefore, we conclude that the volatiles released by disturbed adult *L. lineolaris* probably have no practical application as repellents for this pest.

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PHENOTYPIC PLASTICITY OF *Rhyzopertha dominica* PHEROMONE SIGNALING: THE EFFECTS OF DIFFERENT HOSTS AND PRESENCE OF CONSPECIFIC FEMALES ON MALE PRODUCED AGGREGATION PHEROMONE

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Abstract—Male *Rhyzopertha dominica (F.)* (Coleoptera: Bostrichidae) release an aggregation pheromone consisting of a blend of two components, dominicalure 1 (D1) and Dominicalure 2 (D2). Pheromone from single insects, in different contexts, was collected and measured to determine if this signal is phenotypically plastic. Release rates were lowered when males were moved from maize grains to groundnut kernels or when moved from solitary occupation of maize grain to grain occupied by seven females. The pheromone release was increased again once these moves were reversed. The release of D1 was more affected than D2: thus, on groundnuts or in the presence of females, less pheromone was released and the proportion of D1 in the blend was lowered. Possible reasons for the modifications of the signal are discussed.

Key Words—*Rhyzopertha dominica*, Coleoptera, Bostrichidae, aggregation pheromone, dominicalure 1, dominicalure 2, phenotypic plasticity, host effect, conspecific effect, signal modification.

INTRODUCTION

The extent to which pheromone signals are honest indicators of a signaler's genes and environment is a crucial consideration when attempting to investigate the evolution of signaling systems (Smith, 1991; Johnstone, 1995). Sexual selection of signaling behavior is increasingly being linked to direct benefits acquired by the

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choosing sex, particularly when parental care may be important and there is a large influence of environmental parameters on signaling (Griffith et al., 1999). Chemical signals are technically challenging to quantify. Here, we present a successful attempt to quantify, individual by individual, the impact of two external parameters on insect aggregation pheromone signaling.

Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae) is an important insect pest of stored grain throughout warmer regions of the world (Cotton, 1956; Aitken, 1975). When on food, the male beetle produces a pheromone that is attractive to adults of both sexes (Khorramshahi and Burkholder, 1981). The pheromone consists of a blend of two unsaturated esters, (*S*)-(+)-1-methylbutyl (*E*)-2-methyl-2-pentenoate, commonly called dominicalure 1 (D1), and (*S*)-(+)-1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate, commonly called dominicalure 2 (D2) (Williams et al., 1981). Some details of factors affecting the production of the pheromone, such as food quality, male population density, and age are known from studies of groups of males (Mayhew, 1994; Mayhew and Phillips, 1994), and synthetic pheromone has been studied as a lure in traps (Sinclair and Howitt, 1984; Leos Martinez et al., 1987; Trematerra and Daolio, 1990; Fargo et al., 1994).

Aggregation pheromone is instrumental in host selection and mate location, but for *R. dominica* the relative importance of these roles for signaler and responder have not been explored. It was demonstrated recently that groups of *R. dominica* males released less pheromone when placed on groundnuts than when alone or placed on maize (Bashir, 2000). The groundnut kernels were bored into, but no reproduction occurred, suggesting that the variety used was a poor host. At least one other groundnut variety, however, is known to support reproduction (Mukherjee and Nandi, 1993). Preliminary work suggested that males kept with females also released less pheromone than those kept alone. The effect of different numbers of females was tested with up to seven females per male. Numerically, the largest effect was seen when seven females were kept with a single male.

In the current investigation, the pheromone blends emitted by individual *R. dominica* males were studied as males were switched between different environmental conditions (maize vs. groundnut or lone vs. with seven females). This has allowed us to describe the phenotypic plasticity in pheromone release by *R. dominica.* Only by measuring individual pheromone release can we determine if the modified output measured from several males results from some males switching off completely (all or nothing) or from a more subtle modification of signals by many males. It has already been shown that males placed in the same conditions exhibit intermale variation in the aggregation pheromone signals they emit (Bashir et al., 2003). Intermale variation in the attractiveness of signals has been demonstrated by Birkinshaw and Smith (2000) for another bostrichid beetle, *Prostephanus truncatus* (Horn). Variation in signaling between males could arise if there are trade-offs between signaling and alternative reproductive strategies. However, since the variation was continuous and not all or nothing, Birkinshaw

and Smith (2000) suggested that signals may be costly. We test the hypothesis that individual males modify, but do not cease, their pheromone output when placed on groundnut or when kept with seven females.

METHODS AND MATERIALS

Insects and Grain Commodities. The *R. dominica* used were originally collected from Pakistan in 1994. The insects were subsequently reared on organic English wheat grain, *Triticum vulgare* L., in a constant temperature and humidity (CTH) room set at $27 \pm 1^{\circ}$C, $60 \pm 5\%$ relative humidity on a 12-L/12-D regime. For easy removal of the newly emerged adults, subcultures were prepared using a mixture of whole wheat flour and brewer's yeast (10:1). Parental adults were removed from the subculture after 7 days, and 30 days after their removal the cultures were sieved daily through 0.71-mm sieves (Philip Harris Scientific, London, UK) to remove all newly emerged unmated adults (Ghorpade and Thyagarajan, 1980) and sexed (Dowdy et al., 1993; Mayhew and Phillips, 1994). The beetles that were to be used as pheromone sources were kept singly for 3 days on kibbled wheat grains (Longstaff and Starick, 1989) before being transferred to different treatments. At the end of the experiment, sexing was confirmed by examination of genitalia (Crombie, 1941).

The grain commodities, whole organic yellow dent maize, *Zea mays* L. (Gillet and Cook Ltd., Faversham, Kent, UK), and shelled groundnut kernels, *Arachis hypogaea* (L.) (Holland and Barrett, Chatham, Kent, UK), were conditioned in the CTH room for 24 hr before being used.

Pheromone Collection. To collect pheromone, single males, in different host grains or present with females, were placed into glass vessels of 30-ml capacity (Fisher Scientific, Loughbrough UK) through which air was drawn at a rate of l000 cm^3/min by electrical diaphragm pumps (Capex Mk II; Charles Austin). A glass round-bottomed flask (1000 ml) was also connected into the system, between the pump and collection filters, to act as a buffer against pressure variations induced by the pump. The intake air was filtered through activated charcoal, and the output air was passed through filters containing 200 mg of 50–80 mesh Porapak Q (Waters, Elstree, UK) to collect D1 and D2. Volatiles collected on the Porapak were eluted with dichloromethane (750 μl; Fisher Scientific Distol grade) and octyl acetate (5 μg) added as an internal standard. Samples were assayed by gas chromatography using a fused by silica capillary column (30 m $\times$ 0.32 mm ID) coated with CPWax 52CB (Carbowax equivalent; Varian, Walton-on-Thames, UK), with helium carrier gas and flame ionization detection. The temperature was held at 60°C for 2 min, then programmed at 6°C/min to 230°C. The injector temperature was 200°C and the detector temperature was 240°C. The sample (2 μl) was injected splitless and not concentrated to avoid loss of pheromone components. Data were captured and processed with EZChrom V.6 software. Amounts

of the two pheromone components were calculated by comparison of peak areas with that of the internal standard and application of correction factors. The latter were derived by calibration of pure synthetic components against the internal standard at the 5-μl level. Peak identity was confirmed at intervals by gas chromatography–mass spectrometry (Finnigan MAT ITD700, Thermoquest) using similar chromatographic conditions. In previous experiments, filters taken from collections from chambers without male insects did not contain detectable levels of pheromone (Bashir, 2000). To assess the accuracy and repeatability of this method for measuring D1 and D2, 5 μl of hexane holding 2.62 μg D1 and 2.86 μg D2 was applied to three Porapak filters. Each of the filters was extracted and each extract analyzed three times as described.

Pheromone Signals from Single R. dominica Male Sources Present in Maize or Groundnut. The experimental procedure adopted is shown in Figure 1. Single males were transferred into previously prepared maize grains or groundnut kernels. There were 10 male sources in each treatment. The grains/kernels were prepared by drilling approximately 4-mm-deep holes in them with a 1.5-mm-diam. drill. The beetles were moved into the drilled holes to make sure that all the beetles started to feed (bore) at the same time. The beetles were allowed to feed in the grains 4 days before the first set of pheromone collections. Pheromone was collected from one half (five) of the sources of each of the two treatments. Pheromone collections were for 24 hr. The next day, pheromone collections were made from the second half of the beetles in each treatment. This completed the first set of tests. Male sources in each of the treatments were then moved onto a fresh batch of the alternate food, i.e., beetles on maize were switched to groundnut and *vice versa*. They were left

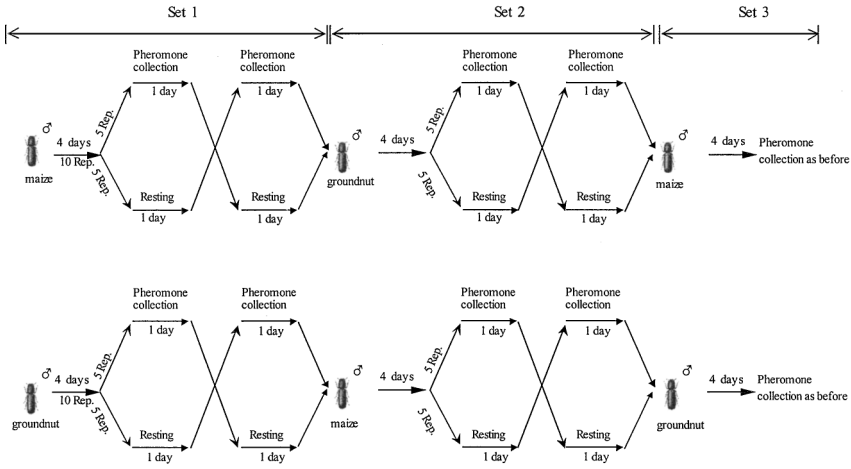

FIG. 1. Protocol for pheromone collection from adult male *R. dominica* confined initially to maize grains or to groundnuts and then moved to the other host type in the second set of pheromone collections and finally returned to the original host type for the third set.

in place for 4 days before the pheromone collection procedure was repeated, thus completing the second set of tests. Male beetles were returned to a fresh batch of their original food for a third and final pheromone collection.

Pheromone Signals from Single R. dominica Males in the Presence of Females. Except for experimental treatments, the procedures were the same as explained above (Figure 1). In one treatment, all males were kept alone, while in the other, single males were confined with seven females. Males were switched between treatments as before.

Statistical Analysis. An independent-samples *t* test was used to test the hypothesis that males produce a modified signal, rather than simply some individuals switching off completely; thus, individuals not producing any detectable signal were excluded from analysis. Where probabilities (two-tailed) were 0.05 or less they were considered statistically significant.

RESULTS

Accuracy and Repeatability of Method for Measuring Pheromone Components. Spiking the Porapak collection filters with microgram quantities of synthetic pheromone components followed by extraction and GC analysis confirmed that the method was accurate and reproducible (Table 1). Recoveries of the two components were similar at approximately 90%.

Pheromone Signals from Single R. dominica Males Present in Maize or Groundnut. In all cases, males released aggregation pheromone (Figure 2a,b). In all three sets, the mean quantities of pheromone components D1 and D2 released in 24 hr by males present in maize grains were greater than those released by males present in groundnut kernels. Likewise, the mean proportions of D1 released were greater when males were present in maize grains (Table 2).

In the first and third sets of pheromone collection, males on maize grains released on average more of the two pheromone components combined and greater

TABLE 1. RECOVERY OF PHEROMONE COMPONENTS D1 AND D2 FROM PORAPAK COLLECTION FILTERS SPIKED WITH D1 (2.87 μg) AND D2 (2.62 μg)

	Amount (μg, mean $\pm$ SD)[a]	
	D1	D2
Replicate 1	2.55 ± 0.02	2.30 ± 0.02
Replicate 2	2.54 ± 0.03	2.28 ± 0.02
Replicate 3	2.57 ± 0.02	2.32 ± 0.03
Overall mean	2.56	2.30
Recovery (%)	88	89

[a] Based on 3 analyses.

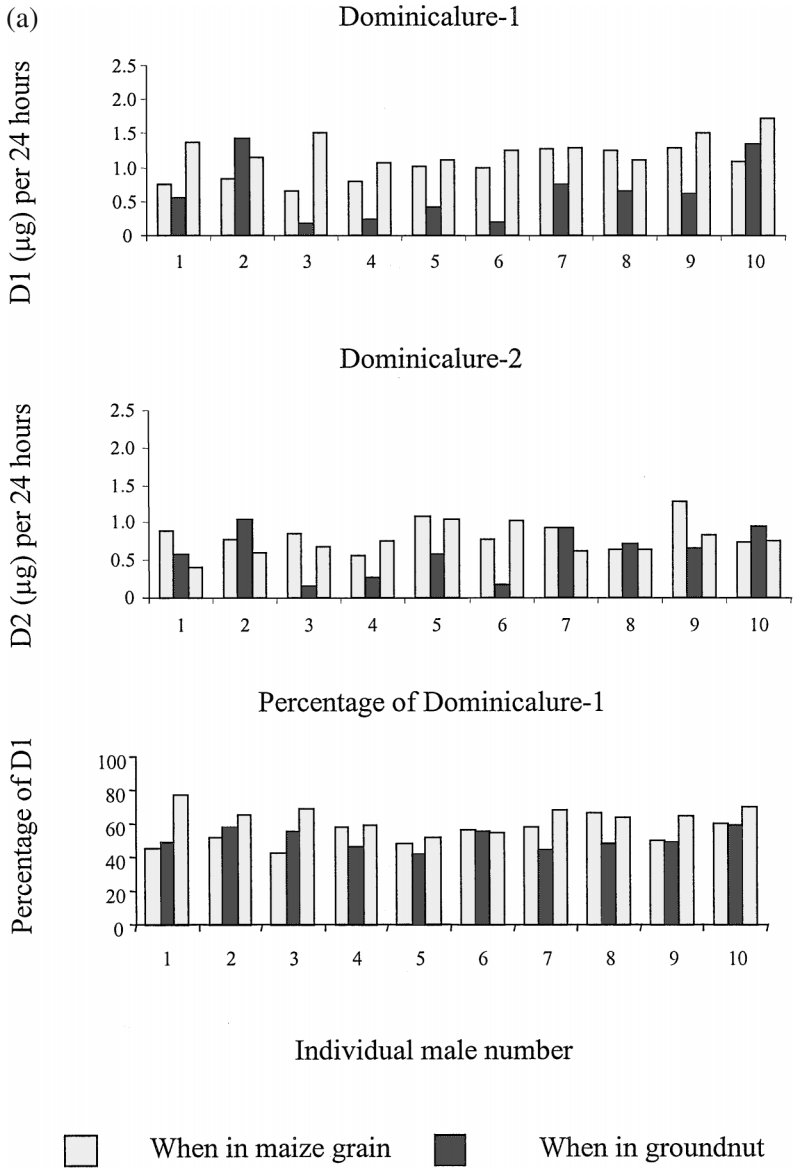

FIG. 2. (a) Quantity of the pheromone components dominicalure-1 (D1), dominicalure-2 (D2), and percentage of D1 in the pheromone blend released by individual *R. dominica* males when present in maize grains or in groundnut kernels for 24 hr. Test was initiated with males in maize. (b) As 1a, but test was initiated with males in groundnut.

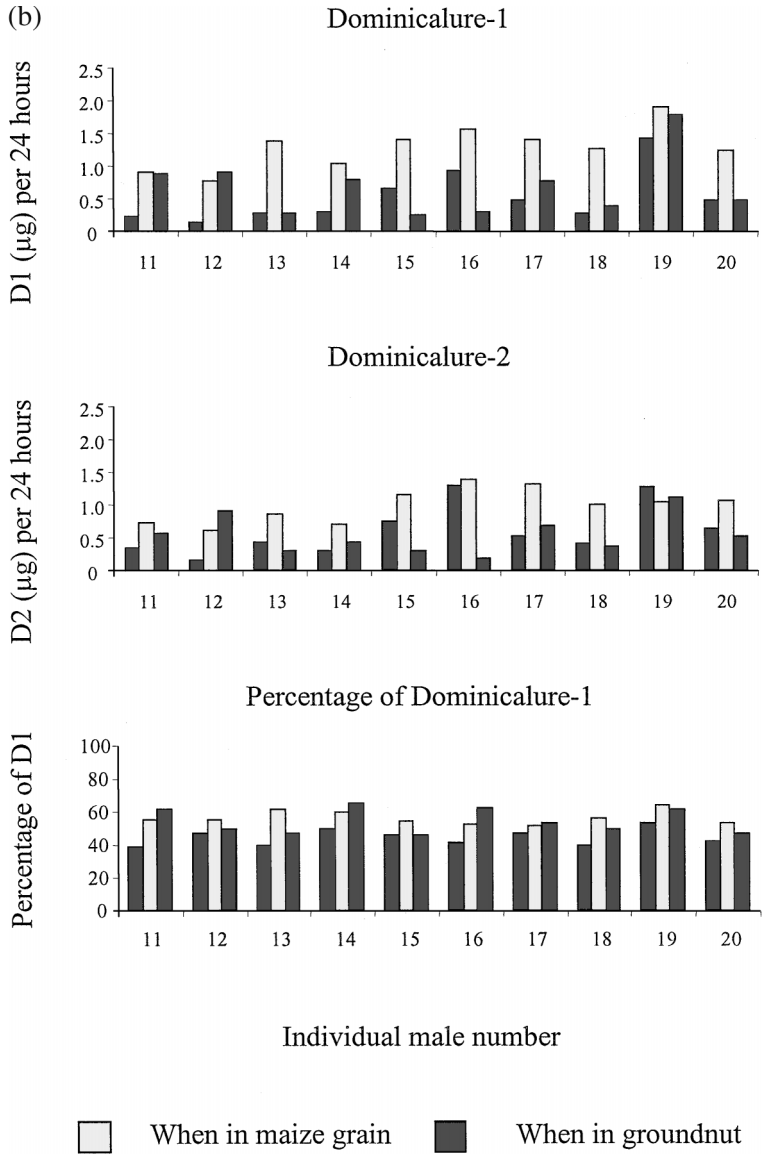

FIG. 2. CONTINUED.

TABLE 2. DOMINICALURE (D1 AND D2) IN BLEND RELEASED IN 24 HOURS BY INDIVIDUAL *R. dominica* MALES IN MAIZE GRAINS OR GROUNDNUT KERNELS AND STATISTICAL COMPARISONS BY INDEPENDENT SAMPLES *t* TEST

	Quantity (μg, mean $\pm$ SD)		
	D1	D2	D1 (%)
Set 1			
Maize	0.99 ± 0.21	0.85 ± 0.23	53.71 ± 7.24
Groundnut	0.52 ± 0.39	0.61 ± 0.40	44.79 ± 4.79
t value, *P*	3.26, <0.01	1.75, 0.10	3.25, <0.01
Set 2			
Maize	1.29 ± 0.32	0.99 ± 0.44	56.57 ± 5.80
Groundnut	0.64 ± 0.19	0.60 ± 0.22	50.55 ± 7.52
t value, *P*	3.75, <0.001	2.96, <0.01	2.68, 0.02
Set 3			
Maize	1.31 ± 0.19	0.74 ± 0.22	64.13 ± 7.52
Groundnut	0.68 ± 0.29	0.54 ± 0.47	54.54 ± 7.32
t value, *P*	3.8, <0.001	1.8, <0.09	2.89, <0.01

$N = 10$.

quantities of D1 than those on groundnut (Table 2). For both sets, the quantities of D2 released on maize, 0.85 μg and 0.74 μg, were somewhat greater than from groundnut, 0.61 μg and 0.54 μg, but the difference was not significant. In the second set of collections, all parameters—D1, D2 and the ratio—differed significantly between the two treatments.

Pheromone Signals from Single R. dominica Males in the Presence of Females. Two males ceased to signal when placed with seven females in the first set, but both of these individuals did release aggregation pheromone when moved back to this treatment in the third set. All other males released pheromone in all sets (Figure 3a,b).

In all three sets, the mean quantities of D1 and D2 produced in 24 hr by lone males were greater than those released by males with females, and these differences were significant except in the case of D2 in set 3 (Table 3). Likewise, the mean proportion of D1 to D2 released was greater for lone males and the observed differences were significant except for set 1. The proportion of D1 varied between sets and between treatments, and both the proportion and amounts of pheromone in both treatments tended to be lower in the first set than the other two.

DISCUSSION

Male *R. dominica* produce modified signals in response to changes in the external environment, changing both the amount released and the ratio of the two

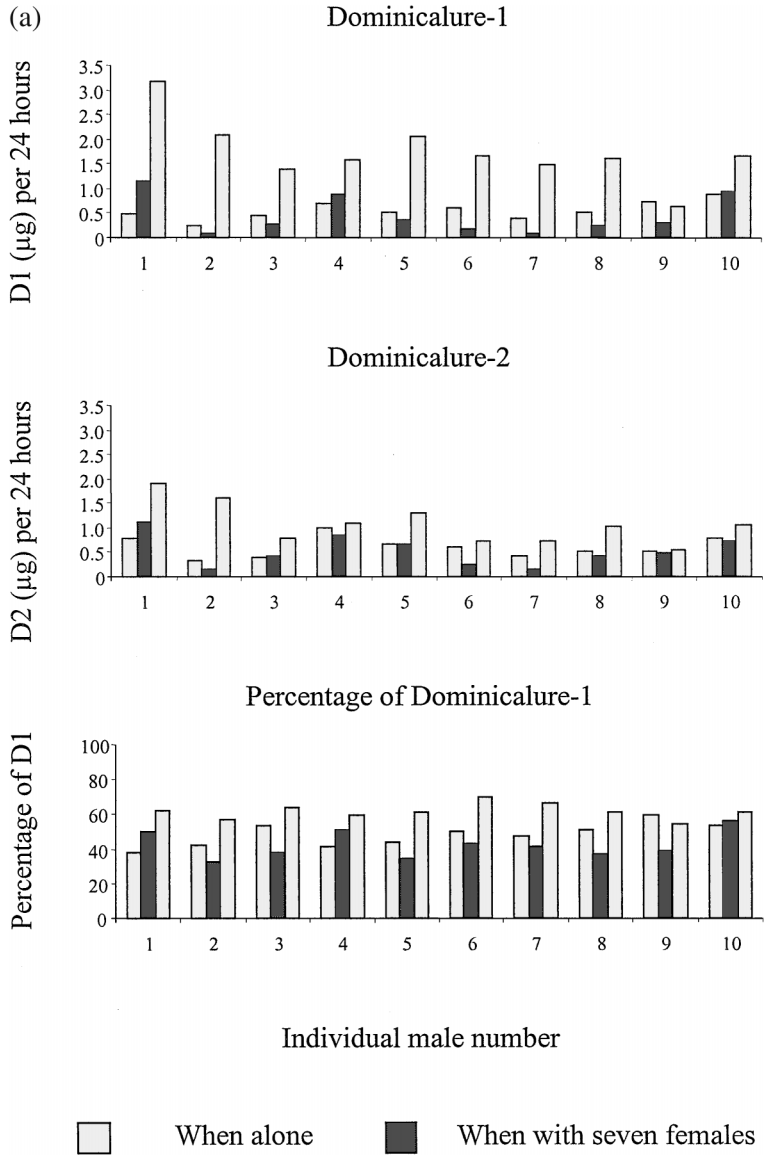

FIG. 3. (a) Quantity of the pheromone components dominicalure-1 (D1), dominicalure-2 (D2), and percentage of D1 in the pheromone blend produced by individual *R. dominica* males when present alone or with seven females for 24 hr. Test was initiated with males alone. (b) As 2a, but test was initiated with males with seven females.

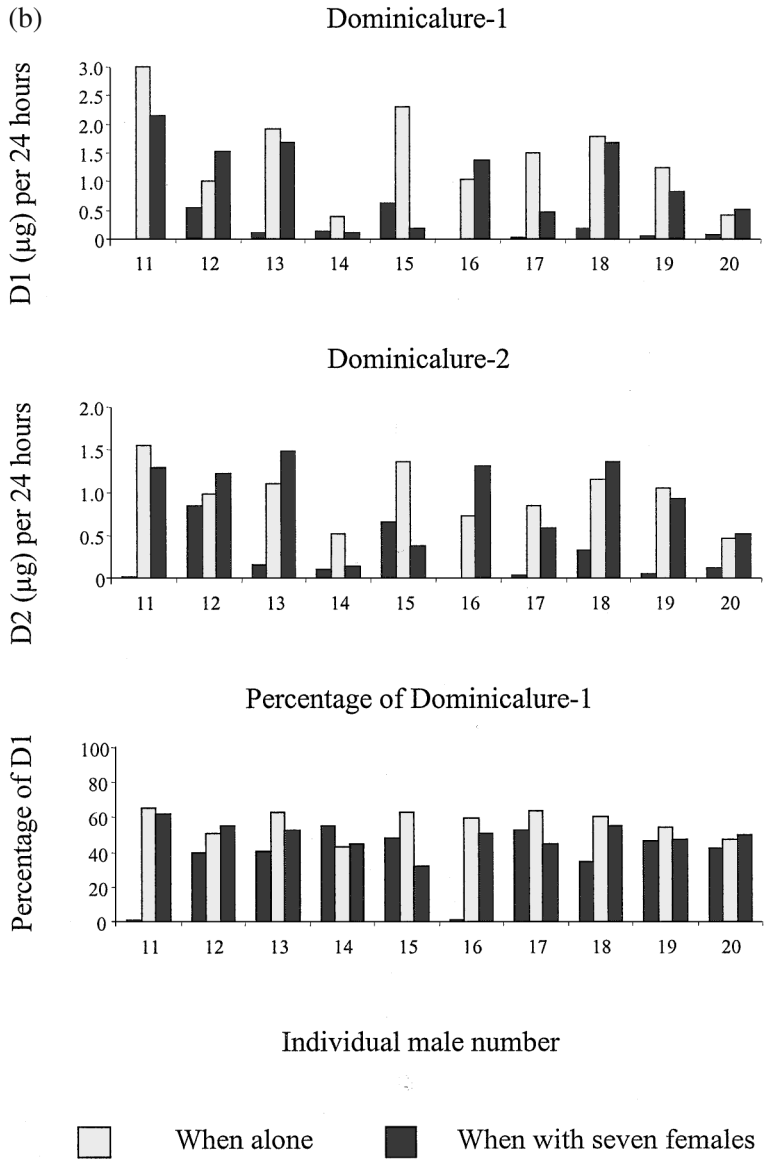

FIG. 3. CONTINUED.

TABLE 3. DOMINICALURE (D1 AND D2) IN PHEROMONE BLEND RELEASED IN 24 HOURS BY INDIVIDUAL *R. dominica* MALES PRESENT ALONE OR WITH SEVEN FEMALES AND STATISTICAL COMPARISONS BY INDEPENDENT SAMPLES *t* TEST

	Quantity (μg, mean $\pm$ SD)		
	D1	D2	D1 (%)
Set 1 (16 *df*)			
Males alone	0.55 ± 0.21	0.60 ± 0.18	48.02 ± 6.60
Males + 7 females[a]	0.22 ± 0.22	0.29 ± 0.29	43.25 ± 6.93
t value, *P*	3.42, <0.01	2.56, 0.02	0.95, 0.35
Set 2 (18 *df*)			
Males alone	1.46 ± 0.35	0.98 ± 0.82	59.87 ± 7.85
Males + 7 females	0.45 ± 0.32	0.52 ± 0.39	46.20 ± 7.78
t value, *P*	3.51, <0.01	3.00, <0.01	4.17, <0.001
Set 3 (18 *df*)			
Males alone	1.73 ± 0.43	1.07 ± 0.65	61.82 ± 4.67
Males + 7 females	1.05 ± 0.48	0.92 ± 0.72	53.25 ± 8.18
t value, *P*	2.23, 0.04	0.73, 0.48	4.08, <0.001

[a] $N = 8$, in all other cases $N = 10$.

components. Only two males ceased to signal (both when initially placed with seven females). Since pheromone was collected for 24 hr, it could be argued that males simply switched off their signal for part of the day in some treatments, rather than producing a different type of signal. This is unlikely to be the case for the change in ratio of the modified signals; previous studies have shown that the ratio of the components in the signal is particularly consistent for different times of day in the laboratory (Bashir et al., 2003).

Modification of the signal could indicate one or more of the following: (1) pheromone release is energetically costly or limited by the availability of certain nutrients, so that cheaper, modified signals are produced when the insect is exposed to unfavorable conditions; (2) modified pheromone release may communicate a different message that elicits a different response that is optimal for the signaling male at that time; and/or (3) ceasing pheromone release may be costly, e.g., if it is a waste product.

Feeding in maize resulted in more pheromone and a different ratio than feeding in groundnut. The case for pheromone production to be physically linked to host-plant chemistry is strong in some scolytid beetles, for which pheromone components are of very similar structure to host terpenoids and are at least sometimes known to be directly derived from them. Host effects on the attractiveness of signalers have been demonstrated in at least two species of *Ips* (Birgersson, 1989; Erbilgin and Raffa, 2000). Feeding on the host plant may also trigger pheromone production indirectly. Again, in scolytids (*Ips paraconfusus*), Hughes and Renwick

(1977a) found that feeding stimulated pheromone production, and this effect could also be simulated by distension of the gut with air. Pheromone production was stimulated by application of juvenile hormone, and this effect was prevented by decapitation. Based on these observations, the authors proposed that distension of the gut through feeding removes neural inhibition of the *corpora allata*. This allows release of juvenile hormone that acts on brain cells or the *corpora cardiaca* to release a brain hormone that stimulates production of the enzymes responsible for pheromone synthesis. In *Dendroctonus brevicomis*, experimental evidence suggested that ingestion of a polar constituent of host phloem in the food stimulated production of juvenile hormone, which acted directly on pheromone biosynthesis rather than through a brain hormone (Hughes and Renwick, 1977b). However, in the case of *R. dominica*, dominicalures 1 and 2 are most probably biosynthesized by an acetogenic pathway from such precursors as propionyl coenzyme A and malonyl coenzyme A (Vanderwel and Oehlschlager, 1987). It is unlikely, therefore, that pheromone production in *R. dominica* is regulated by the availability of obligate biosynthetic precursors, and it is probably under hormonal control (Vanderwel and Oehlschlager, 1987; Vanderwel, 1994).

The modification in male pheromone signaling was found to be reversible, and there was variation in the amount that signals were modified. Therefore, males may vary in their sensitivity to the influence of host type. If signals are modified because they are costly in some way, then male *R. dominica* may vary in their ability to absorb these costs, and some signalers may be constrained to be more honest signalers of host quality than others. Although not reported in this paper, during the current experiments an attempt was made to determine which aspect of the pheromone signal, i.e., quantities of components or their ratio, best correlated to the choices made by conspecifics when offered two signals. However, this test did not reveal any clear conclusions about which characteristics of the pheromone signal were best correlated to the insect choices and is omitted here for the sake of brevity, but can be found in Bashir (2000). Nevertheless, the study showed that adult *R. dominica* can distinguish between signals of differing composition. Males signaling on maize were more attractive than those signaling on groundnut, and lone males were more attractive than those kept with many females. Females were more discriminating than males in their choices between two signals.

In the future, it would be useful to demonstrate that host-type influence on pheromone signaling is not just a peculiarity of groundnuts. Other studies have already discounted the high oil content of groundnuts as a factor that might be responsible for modifying the blend through absorption (Bashir, 2000). It would be helpful to proceed by investigating a wider range of hosts as well as other conditions that might govern host suitability, such as moisture content. Ultimately, it may be feasible to manipulate such host characteristics, either physically or through the development of particular crop varieties, to produce a stored commodity on which *R. dominica* is less able to produce an attractive signal.

Males alone release more pheromone and a different ratio from males confined with seven females. Mayhew and Phillips (1994) did not observe any significant difference in the amount of pheromone released between males of *R. dominica* present alone or paired with females, but pairing was with only one female, and the study had only four replications. From our own preliminary studies, it is clear that when a male is paired with one female, there is likely to be little change in pheromone output (Bashir, 2000). In other species, the actual number of females also appears to be an important parameter, as Vité et al. (1972) found that pheromone production by *Ips calligraphus* (Germar) (Scolytidae) males ceased only when three or more females were present. The change in pheromone output of *R. dominica* could result from a trade-off between signaling and mating effort or, indeed, a response to crowding such as reduced feeding rates. However, a reliable interpretation of male pheromone release behavior will only be possible when more is known about the mating system of the species in its natural context.

Two very different changes in environment (host type and presence of females) resulted in very similar adjustments to the pheromone signal. Either both environmental changes stress the males in similar ways, leading to a similar change in the emission of pheromone, or male fitness is optimized by sending out the same alternative signal in both scenarios. Elucidation of the mechanism by which signals are modified would help determine to what extent beetles are constrained to give the signals they do and to what extent males can potentially control their pheromone emissions. If the modified signal is adaptive in itself, then it may be modified to slow the rate of recruitment to a habitat. A possible example of the opposite effect to this is the release, by males of two species of *Oryzaephilus* (Pierce et al., 1989), of an extra component to their pheromone, making it more attractive when they are maintained at a low population density. In the case of *R. domincia*, slowing of recruitment could be to the advantage of the signaler in resource-limited food patches where a more rapid increase in numbers might lead to unfavorable conditions and lower individual reproductive success. This possibility could be tested through studies on male signaling responses due to crowding and investigations of the biological success of *R. dominica* at various recruitment rates on hosts of differing suitability.

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CUTICULAR HYDROCARBONS OF *Drosophila birchii* AND *D. serrata*: IDENTIFICATION AND ROLE IN MATE CHOICE IN *D. serrata*

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Abstract—The cuticular hydrocarbon compositions of two sympatric species of Australian *Drosophila* in the *montium* subgroup of the *melanogaster* group that use cuticular hydrocarbons in mate recognition have been characterized. *Drosophila birchii* has 34 components in greater than trace amounts, with a carbon number range of C_{20} to C_{33}. *Drosophila serrata* has 21 components above trace level and a carbon number range of C_{24} to C_{31}. These two species share eight hydrocarbon components, with all but two of them being monoenes. For both species, the (*Z*)-9-monoenes are the predominant positional isomer. The hydrocarbons of *D. birchii* are *n*-alkanes, *n*-alkenes (*Z*)-5-, (*Z*)-7-, (*Z*)-9-, and (*Z*)-11-), low to trace levels of homologous (*Z*,*Z*)-7,11- and (*Z*,*Z*)-9,13-dienes; and trace amounts of (*Z*,*Z*)-5,9-$C_{25:2}$, a major component of *D. serrata*. Only one methyl branched hydrocarbon was detected (2-methyl C_{28}), and it occurred at very low levels. The hydrocarbons of *D. serrata* are dominated by a homologous series of (*Z*,*Z*)-5,9-dienes, and notably, are characterized by the apparent absence of *n*-alkanes. Homologous series of (*Z*)-5-, (*Z*)-7-, and (*Z*)-9-alkenes are also present in *D. serrata* as well as 2-methyl alkanes. *Drosophila serrata* females display strong directional mate choice based on male cuticular hydrocarbons and prefer *D. serrata* males with higher relative abundances

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of the 2-methyl alkanes, but lower relative abundances of (*Z*,*Z*)-5,9-$C_{24:2}$ and (*Z*)-9-$C_{25:1}$.

Key Words—Hydrocarbons, pheromones, sexual selection, sibling species, mass spectra, dienes, monoenes, 2-methylalkanes.

INTRODUCTION

In *Drosophila*, and in particular in members of the *melanogaster* species group, cuticular hydrocarbons have been shown to contribute to mate choice within species (Jallon, 1984) and sexual isolation between species (Cobb and Jallon, 1990; Coyne et al., 1994; Buckley et al., 1997; Coyne and Charlesworth, 1997). *Drosophila birchii* and *D. serrata* are two closely related species of the *montium* subgroup of the *melanogaster* group. Females of the two species are morphologically identical, and males are distinguishable only by the numbers of hairs on their genital arch (Ayala, 1965). Despite these morphological similarities, the species are sexually isolated (Ayala, 1965; Blows and Allan, 1998). Four lines of evidence suggest that cuticular hydrocarbons play an important role in mate recognition in the *D. serrata* complex. First, perfuming experiments (where cuticular chemicals are transferred between flies) indicated that olfaction may be an important component involved in sexual isolation (Blows and Allan, 1998). Second, the high genetic correlation between mate recognition and cuticular hydrocarbons in hybrid populations (Blows and Allan, 1998) suggested that these chemicals may be the major mechanism by which mate recognition evolves. Third, reproductive character displacement of these hydrocarbons has evolved in populations of *D. serrata* that are sympatric with *D. birchii*, demonstrating that cuticular hydrocarbons are involved in species recognition under natural conditions (Higgie et al., 2000). Finally, hybrid male cuticular hydrocarbons rapidly responded to the manipulation of sexual selection over 20 generations (Blows, 2002).

Cuticular hydrocarbons from many species of the *melanogaster* subgroup have been analyzed, and in some species cuticular hydrocarbon sexual dimorphism exists (Jallon and David, 1987). Several species of the *melanogaster* group produce alkadienes in significant amounts (Jallon, 1984; Cobb and Jallon, 1990). The alkadiene (*Z*,*Z*)-7,11-heptacosadiene in *D. melanogaster* females stimulates a dose-dependent courtship and mating response from males (Ferveur and Sureau, 1996); however, (*Z*,*Z*)-5,9-heptacosadiene, which is also found on females, does not induce courtship from male *D. melanogaster* in doses normally found on sexually mature females. Although previous work on the *D. serrata* complex has provided evidence that *D. birchii* and *D. serrata* use cuticular hydrocarbons as species recognition cues, and possibly as sex pheromones within species, the individual hydrocarbons that may be involved have not been characterized. These two species are the first members of the *montium* subgroup of the *melanogaster* group to have their cuticular hydrocarbons reported

in detail. In addition, we provide experimental evidence that *D. serrata* females choose *D. serrata* males based on at least four of these cuticular hydrocarbons.

METHODS AND MATERIALS

Insects. One population of *D. birchii* from Eungella and one population of *D. serrata* from Byfield in north Queensland, Australia, were used to characterize individual hydrocarbons. The Forster population of *D. serrata* (described in Higgie et al., 2000) was used in the mate choice experiment. All cultures were maintained in the laboratory for 1–3 years with census sizes >300 individuals.

Chemical Analysis. Hydrocarbons of 4-day-old virgin females and males of both species were separately extracted in hexane, concentrated to dryness under nitrogen, and shipped in Teflon-capped microvials to Manhattan, Kansas, USA, where they were held at -80°C until analyzed. Individual samples were made up with hexane to a volume of 10 μl and analyzed by capillary gas chromatography (GC)–mass spectrometry (MS) in both the electron impact (EI) and methane chemical ionization (CI) mode. Mass spectral analyses were conducted using a Hewlett-Packard 5790A gas chromatograph (GC) (Hewlett-Packard, Inc., San Fernando, California, USA) containing a DB-5 bonded-phase capillary column (15 m long, 0.25 mm inside diam.) (J and W Scientific, Folsom, California, USA) connected to a Hewlett-Packard 5970 mass selective detector (MSD) and a Hewlett-Packard 9133 data system. Ultrapure helium was the carrier gas, with a column head pressure of 0.75 kg/cm^2. Mass spectra were obtained at 70 eV. Analyses were done using temperature programming, with an initial temperature of 150°C, a final temperature of 320°C, a program rate of 5°C/min, and a 20-min final hold period. The splitless injector was set at 275°C and the GC-MSD interface was at 280°C. Retention times of each hydrocarbon component and equivalent chain length values (ECL) were obtained by comparison with known *n*-alkane standards (Howard et al., 1978). Individual components in the total ion-scanning mode were identified from their characteristic EI- and CI-MS fragmentation patterns (Jackson and Blomquist, 1976; Nelson, 1978; Howard et al., 1980; Harrison, 1983). Double-bond locations in alkenes and alkadienes were obtained by preparing dithiomethyl ethers and examining their EI mass spectra (Francis and Veland, 1981; Vicenti et al., 1987). We were unsuccessful in analyzing the dienes of *D. birchii* as thiomethyl ethers, and their structures were determined by EI-MS fragmentation analysis and retention time data of the parent diene only. Stereochemistry of the parent alkenes and alkadienes were established from Fourier transform infrared (FT-IR) analyses. The FT-IR vapor-phase spectra on the underivatized compounds were obtained on a Hewlett-Packard 5890 GC with a 5965B FT-IR detector and a 7958A data system. A DBX-5 bonded-phase capillary column (50 m long, 0.32 mm inside diam.) using chromatographic conditions identical to those described above was used.

For hydrocarbon quantification, newly eclosed flies were separated by sex and aged to 5 days of age in groups of 10–20 single sex flies. Five-day-old flies were extracted in groups of 10 flies and the extract chromatographed on an HP-1 bonded-phase capillary column (12 m long, 0.25 mm inside diam.) programmed from 150°C to 300°C at 5°C/min. Quantification of the hydrocarbons was done by comparing total ion current values to 100 ng/fly of octacosane as an internal standard.

Mate Choice Experiment. Individual virgin 6-day-old *D. serrata* females were placed in vials with 2 virgin *D. serrata* males, in a total of 123 such mate choice tests. Females were watched, and when a male successfully mounted, the 2 males were removed from the vial, and samples were individually prepared for analysis by gas chromatography, resulting in a total of 246 individual hydrocarbon profiles. Individual flies were soaked in 50 μl hexane for 4 min, agitated on a vortex mixer for 1 min, and 0.2 μl was then injected into the gas chromatograph. Gas chromatography was carried out on a Hewlett Packard HP6890 gas chromatograph with a 50-m 0.32-mm-ID BPX5 capillary column. The temperature program was 50 to 220°C at 15°C/min, then 220 to 320°C at 30°C/min followed by a final 15 min hold at 320°C. Ultrapure helium was the carrier gas and detection was via flame ionization.

A multivariate approach to the analysis of cuticular hydrocarbons (Blows and Allan, 1998; Higgie et al., 2000) was employed to determine the overall discrimination between rejected and chosen males, based on nine hydrocarbons. Preliminary examination of the data using up to 15 hydrocarbons had shown that the additional hydrocarbons did not increase the explanatory power of the model. Relative concentrations for the nine hydrocarbons were calculated by dividing each peak's area by the total peak area summed over the nine hydrocarbons. This created a compositional data set where the nine proportions summed to one. To break this unit-sum constraint, the proportions were transformed to logcontrasts (Aitchison, 1986). Logcontrasts were generated by dividing eight of the proportions by an arbitrarily chosen ninth proportion [in this case, that of (*Z*,*Z*)-5,9-C_{24}] and then taking the log of each of the new variables.

RESULTS

Species Differences in Cuticular Hydrocarbons. Preliminary mass spectral analysis of hexane extracts of *D. birchii* and *D. serrata* indicated no discernible compounds other than cuticular hydrocarbons, so all samples were analyzed without silica gel chromatography. The hydrocarbon profiles of both fly species were relatively simple compared to most insect species, with 34 compounds in greater than trace amounts (ca. 0.2–0.6 ng/fly) for *D. birchii* (Figure 1A) and 21 for *D. serrata* (Figure 1B). For both species, males and females had identical hydrocarbon components, although relative concentration differences were apparent for many compounds. The *D. serrata* extracts contained no discernible *n*-alkanes, and the *D. birchii* had only one 2-methyl alkane. The data used to identify the hydrocarbons of both species are presented in Tables 1 and 2 and calculated

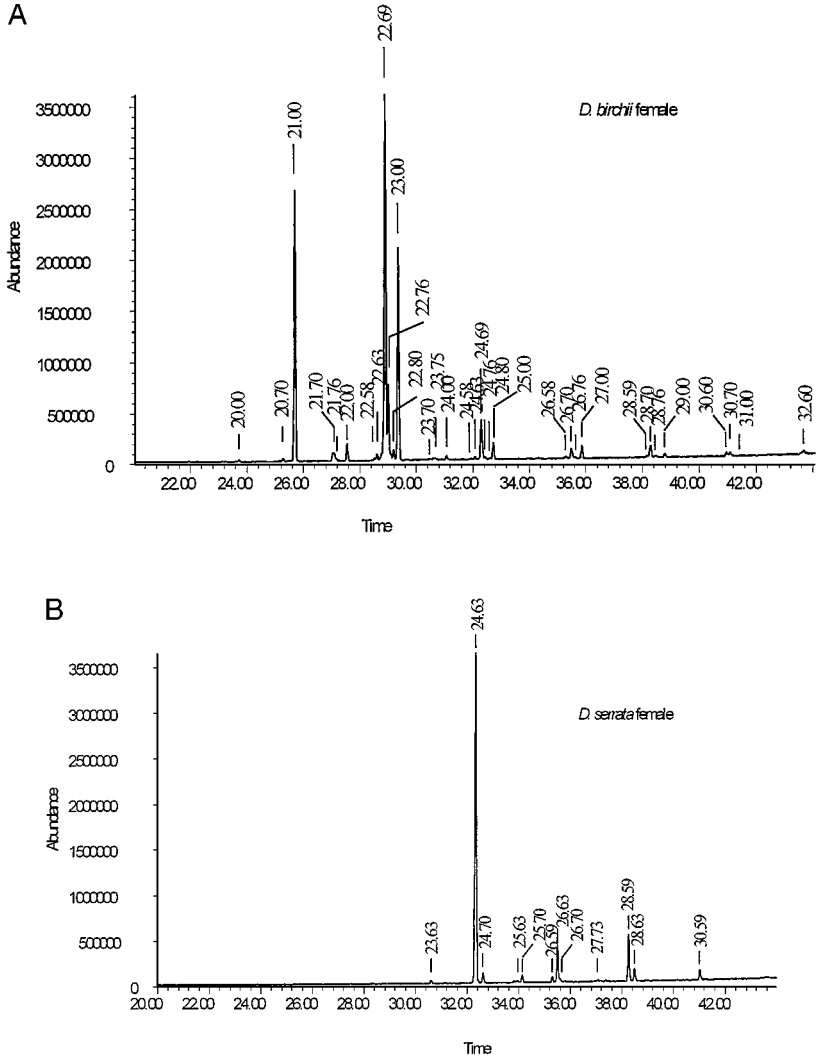

FIG. 1. (A) Total ion trace of the cuticular hydrocarbons of female *D. birchii* (A) and *D. serrata* (B). Each peak is labeled with its equivalent chain length.

equivalent chain length values for each GC peak are also indicated on Figures 1A,B. Quantitative composition data are presented in Tables 3 and 4. FT-IR analysis indicated that all alkenes and alkadienes were of *Z*-configuration [absence of a strong band at 970 cm^{-1} and presence of a weak band at 3012 cm^{-1} and a weak to moderate band at 1650 cm^{-1} (Nakanishi, 1962; Krokos et al., 2001)], as is true for the vast majority of insect unsaturated hydrocarbons (Howard, 1993). The monoenes were

TABLE 1. CUTICULAR HYDROCARBONS OF *Drosophila birchii*

Hydrocarbon	ECL[a]	CN[b]	Diagnostic ions (*m/z*)[c]
C_{20}	20.00	20	282; (281)
(*Z*)-9-$C_{21:1}$	20.70	21	294; (293, 295); [388, 173, 201]
C_{21}	21.00	21	296; (295)
(*Z*)-9-$C_{22:1}$	21.70	22	308; (307, 309); [402, 173, 215]
(*Z*)-7-$C_{22:1}$	21.76	22	308; (307, 309); [402, 145, 243]
C_{22}	22.00	22	310; (309)
(*Z*,*Z*)-7,11-$C_{23:2}$	22.58	23	320, 152, 166, 222, 236; (319)
(*Z*,*Z*)-5,9-$C_{23:2}$	22.63	23	320, 124, 138, 250, 264; (319)
(*Z*)-11-$C_{23:1}$	22.65	23	322; (321, 323); [416, 201, 215]
(*Z*)-9-$C_{23:1}$	22.69	23	322; (321, 323); [406, 173, 243]
(*Z*)-7-$C_{23:1}$	22.76	23	322; (321, 323); [416, 145, 271]
(*Z*)-5-$C_{23:1}$	22.80	23	322; (321, 323); [416, 117, 299]
C_{23}	23.00	23	324; (323)
(*Z*)-9-$C_{24:1}$	23.70	24	336; (337, 339); [430, 173, 257]
(*Z*)-7-$C_{24:1}$	23.75	24	336; (337, 339); [430, 145, 285]
C_{24}	24.00	24	338; (337)
(*Z*,*Z*)-7,11-$C_{25:2}$	24.58	25	348, 152, 166, 250, 264; (347)
(*Z*,*Z*)-5,9-$C_{25:2}$	24.63	25	348, 124, 138, 278, 292; (347)
(*Z*)-9-$C_{25:1}$	24.69	25	350; (349, 351); [444, 173, 271]
(*Z*)-7-$C_{25:1}$	24.76	25	350; (349, 351); [444, 145, 299]
(*Z*)-5-$C_{25:1}$	24.80	25	350; (349, 351); [444, 117, 327]
C_{25}	25.00	25	352; (351)
(*Z*,*Z*)-7,11-$C_{27:2}$	26.58	27	376, 152, 166, 278, 292; (375)
(*Z*)-9-$C_{27:1}$	26.70	27	378; (377, 379); [472, 173, 299]
(*Z*)-7-$C_{27:1}$	26.76	27	378; (377, 379); [472, 145, 327]
C_{27}	27.00	27	380; (379)
(*Z*,*Z*)-7,11-$C_{29:2}$	28.59	29	404, 152, 166, 306, 320; (403)
2-MeC_{28}	28.59	29	408, 393, 365
(*Z*)-9-$C_{29:1}$	28.70	29	406; (405, 407); [500, 173, 327]
C_{29}	29.00	29	408; (407)
(*Z*,*Z*)-7,11-$C_{31:2}$	30.60	31	432, 152, 166, 334, 348; (431)
(*Z*)-9-$C_{31:1}$	30.70	31	434; (433, 435); [528, 173, 355]
C_{31}	31.00	31	436; (435)
(*Z*,*Z*)-7,11-$C_{33:2}$	32.60	33	460, 152, 166, 362, 376; (459)

[a] Equivalent chain length. (DB-5 column).
[b] Carbon number.
[c] Ions in parentheses are diagnostic ions from chemical ionization mass spectral analysis; ions in brackets are of diagnostic ions from thiomethyl ethers.

all readily characterized as to location of double bond position by EI-MS analysis of their dithiomethyl ethers (Francis and Veland, 1981). In common with many other *Drosophila* species (Jallon and David, 1987), for any given carbon number, the alkenes of *D. birchii* and *D. serrata* exist as a mixture of positional isomers, with (*Z*)-9 being the major isomer for both species.

TABLE 2. CUTICULAR HYDROCARBONS OF *Drosophila serrata*

Hydrocarbon	ECL[a]	CN[b]	Diagnostic ions (*m/z*)[c]
(*Z*,*Z*)-5,9-$C_{24:2}$	23.63	24	324, 124, 138, 264, 278; (323); [460, 117, 257, 203, 343]
(*Z*,*Z*)-5,9-$C_{25:2}$	24.63	25	348, 124, 138, 278, 292; (347); [474, 117, 271, 203, 357]
(*Z*)-9-$C_{25:1}$	24.70	25	350; (349, 351); [444, 173, 271]
(*Z*)-7-$C_{25:1}$	24.76	25	350; (349, 351); [444, 145, 299]
(*Z*)-5-$C_{25:1}$	24.80	25	350; (349, 351); [444, 117, 327]
(*Z*,*Z*)-5,9-$C_{26:2}$	25.63	26	362, 124, 138, 292, 306; (361); [488, 117, 285, 203, 371]
(*Z*)-9-$C_{26:1}$	25.70	26	364; (363, 365); [458, 173, 285]
(*Z*)-8-$C_{26:1}$	25.73	26	364; (363, 365); [458, 159, 299]
(*Z*)-7-$C_{26:1}$	25.76	26	364; (363, 365); [458, 145, 313]
2-MeC_{26}	26.59	27	380, 365, 337
(*Z*,*Z*)-5,9-$C_{27:2}$	26.63	27	376, 124, 138, 306, 320; (375); [502, 117, 299, 203, 385]
(*Z*)-9-$C_{27:1}$	26.70	27	378; (377, 379); [472, 173, 299]
(*Z*)-7-$C_{27:1}$	26.76	27	378; (377, 379); [472, 145, 327]
(*Z*)-5-$C_{27:1}$	26.80	27	378; (377, 379); [472, 117, 355]
(*Z*)-8-$C_{28:1}$	27.73	28	392; (391, 393); [486, 159, 327]
(*Z*)-6-$C_{28:1}$	27.78	28	392; (391, 393); [486, 131, 355]
2-MeC_{28}	28.59	29	408, 393, 365
(*Z*,*Z*)-5,9-$C_{29:2}$	28.63	29	404, 124, 138, 334, 348; (403); [530, 117, 327, 203, 413]
(*Z*)-9-$C_{29:1}$	28.70	29	406; (405, 407); [500, 173, 327]
(*Z*)-7-$C_{29:1}$	28.76	29	406; (405, 407); [500, 145, 355]
2-MeC_{30}	30.59	31	436, 421, 393

[a] Equivalent chain length. (DB-5 column).
[b] Carbon number.
[c] Ions in parentheses are diagnostic ions from chemical ionization mass spectral analysis; ions in brackets are of diagnostic ions from thiomethyl ethers.

Characterization of the location of double bonds in the alkadienes of *D. serrata* was relatively straightforward. When alkadienes have their double bonds separated by four or fewer methylene units, reaction with CH_3SSCH_3 leads to heterocyclic products with one sulfur atom in the ring, and side chains with thiomethyl ether substituents. The mass spectra of these diastereomeric products have abundant molecular ions and strong fragment ions that are readily interpreted (Vicenti et al., 1987; Howard, 1993) (Figure 2A). All dienes in *D. serrata* had their double bonds at positions 5 and 9 and were of the *Z*-configuration (Table 2).

Although the dienes in *D. birchii* were present in high enough abundance to obtain useful EI and CI mass spectra (Table 1), we were not able to obtain thiomethylation data. Unlike monoenes, where it is well known that location of the double bond position can not be easily assigned from EI mass spectral fragmentation patterns, alkadienes that have their double bonds separated by only a few methylene units do give mass spectral patterns that are associated with double bond location (Descoins et al., 1986; Swedenborg and Jones, 1992). Figure 2B presents the EI-MS profile of (*Z*,*Z*)-5,9-$C_{25:2}$ obtained from *D. serrata*, with

TABLE 3. HYDROCARBON ABUNDANCE OF 5-DAY OLD *D. birchii*

Hydrocarbon	Amount (ng/fly, mean ± SEM)[a]	
	Female	male
C_{20}	tr[b]	tr
(Z)-9-$C_{21:1}$	tr	tr
C_{21}	125 ± 36	127 ± 40
(Z)-9-; (Z)-7-$C_{22:1}$	5 ± 1	5 ± 3
C_{22}	4 ± 1	6 ± 2
(Z,Z)-5,9-; (Z,Z)-7,11-$C_{23:2}$	3 ± 2	4 ± 2
(Z)-11-; (Z)-9-$C_{23:1}$	311 ± 92	164 ± 44
(Z)-7-$C_{23:1}$	44 ± 17	30 ± 9
(Z)-5-$C_{23:1}$	5 ± 3	1 ± 1
C_{23}	110 ± 21	90 ± 27
(Z)-9-; (Z)-7-$C_{24:1}$	tr	tr
C_{24}	tr	tr
(Z,Z)-5,9-; (Z,Z)-7,11-$C_{25:2}$	2 ± 1	4 ± 2
(Z)-9-; (Z)-7-; (Z)-5-$C_{25:1}$	12 ± 2	7 ± 4
C_{25}	17 ± 6	25 ± 4
(Z,Z)-7,11-$C_{27:2}$	5 ± 2	1 ± 1
(Z)-9-$C_{27:1}$	13 ± 4	3 ± 1
(Z)-7-$C_{27:1}$	3 ± 1	5 ± 1
C_{27}	23 ± 6	18 ± 3
(Z,Z)-7,11-$C_{29:2}$	14 ± 5	9 ± 2
2-MeC_{28}	13 ± 2	13 ± 3
(Z)-9-$C_{29:1}$	tr	tr
C_{29}	6 ± 3	6 ± 1
(Z,Z)-7,11-$C_{31:2}$	5 ± 1	3 ± 1
(Z)-9-$C_{31:1}$	9 ± 7	10 ± 2
C_{31}	tr	tr
(Z,Z)-7,11-$C_{33:2}$	1 ± 1	12 ± 3
Total hydrocarbon	730 ± 214	543 ± 156

[a] $N = 5$ groups of 10 flies each. (DB-5 column).
[b] Trace is <1 ng/fly but detectable.

major fragmentation patterns indicated. A prominent molecular ion is found at *m/z* 348 and diagnostic fragment ions at *m/z* 152/153 and 166/167 for a double bond between carbons 5 and 6 and fragment ions at *m/z* 250/251 and 264/265 for a double bond between carbons 9 and 10. Figure 2C is an EI-MS profile of what we have interpreted as (Z,Z)-7,11-$C_{23:2}$ obtained from *D. birchii*. The molecular ion at 320 is prominent, and a set of ions, equivalent to those in *D. serrata*, but shifted by 28 mass units is also prominent. We also found similar mass spectral evidence in *D. birchii* for trace amounts of (Z,Z)-9,13-$C_{23:2}$ and (Z,Z)-9,13-$C_{29:2}$ buried under the leading edge of the corresponding monoene peaks (Z)-11-$C_{23:1}$ and (Z)-9-$C_{29:1}$, respectively.

TABLE 4. HYDROCARBON ABUNDANCE OF 5-DAY OLD *D. serrata*[a]

Hydrocarbon	Amount (ng/fly, mean ± SEM)[a]	
	Female	Male
(*Z*,*Z*)-5,9-$C_{24:2}$	tr[b]	tr
(*Z*,*Z*)-5,9-$C_{25:2}$	465 ± 85	379 ± 73
(*Z*)-9-$C_{25:1}$	13 ± 3	12 ± 3
(*Z*)-7-; (*Z*)-5-$C_{25:1}$	1 ± 1	14 ± 3
(*Z*,*Z*)-5,9-$C_{26:2}$	tr	tr
(*Z*)-9-$C_{26:1}$	tr	tr
(*Z*)-8-$C_{26:1}$	tr	tr
(*Z*)-7-$C_{26:1}$	tr	tr
2-MeC_{26}	133 ± 13	101 ± 12
(*Z*,*Z*)-5,9-$C_{27:2}$	tr	tr
(*Z*)-9-$C_{27:1}$	9 ± 3	11 ± 2
(*Z*)-7-$C_{27:1}$	tr	tr
(*Z*)-5-$C_{27:1}$	tr	tr
(*Z*)-8-$C_{28:1}$	tr	tr
(*Z*)-6-$C_{28:1}$	tr	tr
2-MeC_{28}	159 ± 22	109 ± 16
(*Z*,*Z*)-5,9-$C_{29:2}$	tr	tr
(*Z*)-9-$C_{29:1}$	tr	tr
(*Z*)-7-$C_{29:1}$	tr	tr
2-MeC_{30}	14 ± 2	7 ± 2
Total hydrocarbon	794 ± 129	633 ± 111

[a] $N = 5$ groups of 10 flies each.
[b] Trace is <1 ng/fly but detectable.

At 5 days of age, there is hydrocarbon sexual dimorphism in the greater abundance of (*Z*)-11-, (*Z*)-9-, and (*Z*)-7-$C_{23:1}$; (*Z*,*Z*)-7,11-$C_{27:2}$; and (*Z*)-9-$C_{27:1}$ on *D. birchii* females (Table 3). *D. serrata* females had greater abundance of (*Z*,*Z*)-5,9-$C_{25:2}$ and the 2-methyl alkanes (2-MeC_{26}, 2-MeC_{28}, and 2-MeC_{30}), whereas males had greater abundance of (*Z*)-7- and (*Z*)-5-$C_{25:1}$ (Table 4). A summary of shared hydrocarbon components between the two species and their relative abundances for 4-day-old virgin adults is given in Table 5.

Female Choice for Male Cuticular Hydrocarbons. Single classification multivariate analysis of variance (MANOVA) indicated that chosen and rejected males differed significantly across the nine hydrocarbons ($F_{8,238} = 93327$, $P < 0.001$). A canonical discriminant analysis was then used to determine a univariate description of cuticular hydrocarbons that best distinguished between chosen and rejected males. Since the canonical discriminant analysis was conducted on logcontrasts, to determine the contributions of individual hydrocarbons to the canonical variate that distinguished between chosen and rejected males, the

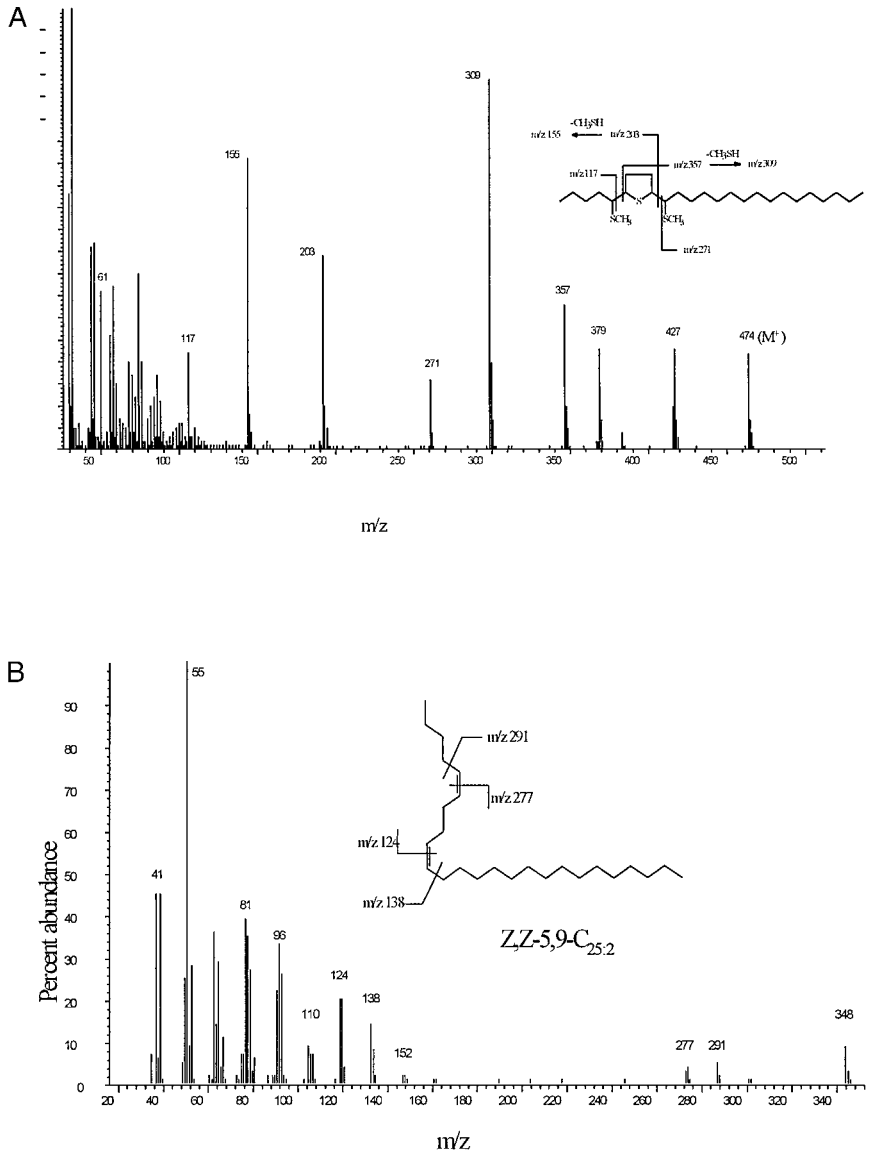

FIG. 2. (A) EI-MS of one of the heterocyclic reaction products resulting from the reaction of (*Z*,*Z*)-5,9-$C_{25:2}$ with dimethyldisulfide. (B) EI-MS of (*Z*,*Z*)-5,9-$C_{25:2}$ from *D. serrata*. (C) EI-MS of (*Z*,*Z*)-7,11-$C_{23:2}$ from *D. birchii*.

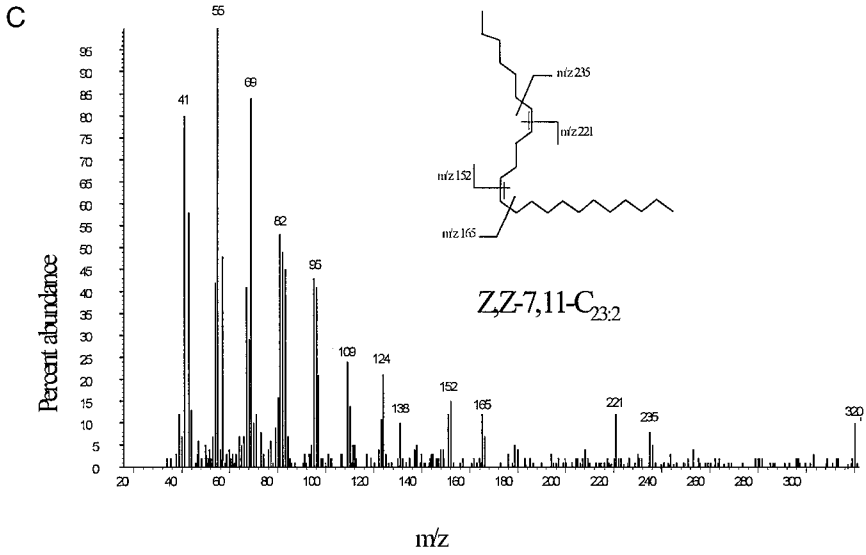

FIG. 2. CONTINUED.

canonical variate was correlated with the relative abundances of each of the nine hydrocarbons to generate a structure matrix (Table 6). Seven of the nine hydrocarbons displayed strong correlations between their relative abundance and the canonical variate. Of the nine hydrocarbons, the three 2-methylalkanes displayed elevated levels on chosen individuals, although 2-Me-C_{26} was not significantly different between chosen and rejected males as indicated by the paired *t* test. In addition,

TABLE 5. SHARED HYDROCARBONS BETWEEN *Drosophila birchii* AND *D. serrata*

Hydrocarbon	Relative hydrocarbon abundance[a]	
	D. birchii	*D. serratta*
(*Z*,*Z*)-5,9-$C_{25:2}$	trace	AA
(*Z*)-9-$C_{25:1}$	AA	A
(*Z*)-7-$C_{25:1}$	A	trace
(*Z*)-5-$C_{25:1}$	A	trace
(*Z*)-9-$C_{27:1}$	A	A
(*Z*)-7-$C_{27:1}$	trace	trace
2-MeC_{28}	trace	AA
(*Z*)-9-$C_{29:1}$	A	trace

[a] Trace is <2%; A is ca. 2–10%; AA is >10%.

TABLE 6. STRUCTURE MATRIX OF CANONICAL DISCRIMINANT ANALYSIS DISTINGUISHING BETWEEN CHOSEN AND REJECTED MALES

Hydrocarbon	Structure coefficient[a]	Paired t test
(Z,Z)-5,9-$C_{24:2}$	-0.408^{***}	$t_{122} = -2.584$, $P = 0.011$
(Z,Z)-5,9-$C_{25:2}$	-0.562^{***}	$t_{122} = -3.591$, $P < 0.001$
(Z)-9-$C_{25:1}$	-0.363^{***}	$t_{122} = -2.238$, $P = 0.027$
(Z)-9-$C_{26:1}$	-0.019	$t_{122} = -0.069$, $P = 0.945$
2-Me-C_{26}	0.289^{***}	$t_{122} = 1.667$, $P = 0.098$
(Z,Z)-5,9-$C_{27:2}$	-0.128^{*}	$t_{122} = -0.756$, $P = 0.451$
2-Me-C_{28}	0.519^{***}	$t_{122} = 3.312$, $P = 0.001$
(Z,Z)-5,9-$C_{29:2}$	0.419^{***}	$t_{122} = 2.315$, $P = 0.022$
2-Me-C_{30}	0.326^{***}	$t_{122} = 1.999$, $P = 0.048$

[a] $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(Z,Z)-5,9-$C_{29:2}$ was elevated on chosen individuals and lower abundances of (Z,Z)-5,9-$C_{24:2}$, (Z,Z)-5,9-$C_{25:2}$, and (Z)-9-$C_{25:1}$ were found on chosen individuals (Table 6).

DISCUSSION

Cuticular Hydrocarbons of Drosophila. The epicuticular hydrocarbons of the species-rich genus *Drosophila* differ qualitatively and quantitatively from the majority of insect species in having a relatively small number of hydrocarbon components (usually fewer than 30), whereas most insects examined have upwards of 100 or more (Howard, 1993). In addition, compared to other insects, a relatively small number of classes of hydrocarbons are associated with *Drosophila* spp. Unlike the majority of insects, *n*-alkanes are often found in only small amounts in any given species. 2-Methyl alkanes (and occasionally 3-methyl alkanes) are usually minor components (although the 2-methyl alkanes can be the most abundant class (Blomquist et al., 1985; Jallon and David, 1987), and only occasionally are internal methyl branched alkanes found in more than trace quantities. Instead, alkenes and alkadienes, and in rare instances alkatrienes are the dominant components of *Drosophila* hydrocarbon mixtures. Most of the unsaturated compounds have proven to be straight-chain compounds, with the exception of *D. mojavensis* where 2-methyl alkenes were found (Toolson et al., 1990). Qualitative and quantitative sexual dimorphism in hydrocarbon composition occurs for many but not all species (Bartelt et al., 1986; Jackson and Bartelt, 1986; Jallon and David, 1987; Toolson and Kuper-Simbron, 1989). Ontogenetic, temperature, and diet-related variation in *Drosophila* cuticular hydrocarbon profiles are also well known (Jackson et al., 1981; Jackson and Bartelt, 1986; Toolson et al., 1990; Markow and Toolson, 1990; Stennett and Etges, 1997).

Although numerous cuticular hydrocarbon studies have been conducted on various *Drosophila* sp. groups, many of them have been concerned primarily with major components that are thought to function as pheromonal signals [although undoubtedly, these hydrocarbons also serve an important role in desiccation resistance (Gibbs, 2002)]. The other hydrocarbon components are often either not described, or they are mentioned in only a cursory fashion with no supporting chemical or analytical data. This, of course, makes it difficult to make detailed comparisons among species and species groups. Despite this, the hydrocarbons of *D. birchii* and *D. serrata* can be partially contrasted with the various subgroups that have been previously examined. The most striking comparisons seem to be among the dienes. The *melanogaster* subgroup often has 5,9- and 7,11-dienes (Jallon and David, 1987), as does *D. birchii* and *D. serrata*. As young adults, *D. pseudoobscura* (*pseudoobscura* subgroup) possesses 5,9- and 7,11-dienes, but older adults possess 9,18-, 9,20-, and 11,20-dienes (Blomquist et al., 1985). *Drosophila mojavensis* and its sibling species *D. arizonae* and *D. navojoa*, which comprise the *mojavensis* subgroup, also have dienes with the double bonds nearly symmetrically placed at opposite ends of the chains (7,25-, 7,27-, 8,26-, and 8,28-), as well as possessing methyl alkenes (2-methyl with the double bond towards the other end of the chain) (Toolson et al., 1990; Etges and Jackson, 2001). In contrast, members of the *virilis* subgroup are characterized by 5,13-, 5,15-, and 7,15-dienes (Bartelt et al., 1986).

Despite being closely related, hydrocarbon compositions of *D. serrata* and *D. birchii* were strikingly dissimilar and may well be important components of the mechanisms providing species isolation between these two sympatric species. Of the 34 *D. birchii* compounds and 21 *D. serrata* compounds, only eight hydrocarbon structures were shared (see Table 5). Of those eight, only two, (*Z*)-9-$C_{27:1}$ and (*Z*)-7-$C_{27:1}$, were similar in relative abundance. Low quantities of 2-methyl C_{28} were also found in *D. birchii* males and females, but none of the other 2-methyl alkanes found in *D. serrata* males and females were found in *D. birchii*.

Sexual Selection on Cuticular Hydrocarbons in D. serrata. *D. serrata* females display strong directional choice for male cuticular hydrocarbons. At least four hydrocarbons are preferred in higher relative abundances, although we cannot exclude smaller potential effects from additional hydrocarbons. Of these four, 2-methyl C_{28} is the most abundant and is also found in trace amounts on the cuticle of *D. birchii*. The other three contribute less then 5% each to the total cuticular hydrocarbon content on the *D. serrata* cuticle. Individual hydrocarbons that represent major proportions of total abundance are often the subject of study and have been associated with behavioral roles in other *Drosophila* species (Antony and Jallon, 1982; Jallon and David, 1987). *D. serrata* provides an example of how some minor components of the cuticular hydrocarbon profile of individuals (especially the 2-methyl alkanes) may have important roles in mate choice within species. It is not clear what sort of receptor mechanisms are involved or what sort of central nervous system processing would be required to make these distinctions,

but clearly the females are doing so. We note that recognizing the importance of these minor compounds using standard univariant statistical approaches may have been difficult and suggest that multivariate approaches to hydrocarbon statistical analysis should possibly be more widely used.

Cuticular hydrocarbons seem to be involved in recognition, in both species and mate choice within species in the *D. serrata* complex. Similar associations between levels of mate recognition have been observed in songs of some *Drosophila* (Ewing and Miyan, 1986), but not in head width in stalk-eyed *Drosophila* (Boake et al., 1997). However, it remains to be determined whether the same individual hydrocarbons are involved in both functions. If different hydrocarbons are involved that are genetically independent, the evolution of species recognition may be able to evolve independently from how individuals choose mates from among their own species. Alternatively, if the same hydrocarbons are involved in sexual selection within species and sexual isolation between species, then a direct role for sexual selection in the generation of reproductive isolation becomes more likely.

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SEX PHEROMONE OF THE VINE MEALYBUG, *Planococcus ficus* IN ISRAEL: OCCURRENCE OF A SECOND COMPONENT IN A MASS-REARED POPULATION

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Abstract—Two pheromonal components were detected in airborne collections from the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) mass-reared on potato sprouts. The compounds were identified as (*S*)-lavandulyl senecioate (I) and (*S*)-lavandulyl isovalerate (II) by GC and GC-MS by comparison with synthetic standards. Chiral GC analysis on a cyclodextrin column established their chirality. Compound I was identified recently as the sex pheromone of *P. ficus* in California. The attraction of vine mealybug males to both components I and II was demonstrated in a Petri dish bioassay and in a flight assay in the rearing chamber. Indoors, both compounds displayed a similar level of attractiveness to the mass-reared males. However, trials in a vineyard indicated that feral males were attracted only to compound I. Reanalysis of the airborne pheromone indicated that laboratory first generation daughters of females that were collected in the vineyard produce only (*S*)-lavandulyl senecioate (I). The relative amount of (*S*)-lavandulyl isovalerate (II) increased gradually in each subsequent generation of *P. ficus* reared on potatoes. These findings indicate that feral *P. ficus* mealybugs produce and respond only to (*S*)-lavandulyl senecioate (I), whereas mealybugs that were reared in the laboratory on potato sprouts produce and respond to both (*S*)-lavandulyl senecioate (I) and (*S*)-lavandulyl isovalerate (II).

Key Words—Vine mealybug, *Planococcus ficus*, sex pheromone, (S)-lavandulyl senecioate, (*S*)-lavandulyl isovalerate, chiral GC analysis, mass rearing, bioassay.

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INTRODUCTION

The vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) is found in the Mediterranean basin, Central Asia, and South Africa and was introduced recently into the Americas. It is a serious pest of grapevine (*Vitis* spp., Vitaceae) and figs (*Ficus carica*, Moraceae) (Ben-Dov, 1988). Damage in vineyards is mainly caused by the colonization of the bunches, soiling them with a mixture of dead mealybugs and honeydew on which sooty mold develops, as well as transmission of viral diseases. Chemical control of the mealybug is difficult due to its cryptic behavior and problematic monitoring. The objective of study has been the identification of the vine mealybug sex pheromone and the development of a convenient monitoring system based on pheromone traps. The pheromone of *P. ficus* has been studied independently in California, and (*S*)-lavandulyl senecioate (I) was identified as the active component (Hinkens et al., 2001). We report here the identification of a second active component, (*S*)-lavandulyl isovalerate (II), from mealybugs reared on potato sprouts, and compare its activity with (*S*)-lavandulyl senecioate (I).

METHODS AND MATERIALS

Mass-Rearing of Vine Mealybugs. Ovisacs of the vine mealybug were collected in a vineyard (var. superior) in Gshur, southern Golan. Microscopic slides of first generation females were used to verify the taxonomic identity of the collected individuals (Cox, 1989). Mealybugs were mass-reared on potato sprouts at a constant temperature of 23°C in the dark. Male prepupae and pupae were manually separated from females. Males at the prepupa stage tend to leave the sprouts and pupate inside folded paper strips. The culture was maintained for 10–12 generations. A separate small culture was established in 2001 from female ovisacs collected in Avney Eitan, southern Golan, and maintained on potato sprouts for 4 generations.

Collection and Analysis of Pheromone. The crude pheromone of *P. ficus* was trapped on Porapak Q (50–80 mesh) in an airborne collection device, similar to that used for the collection of the *Matsucoccus josephi* sex pheromone (Mendel et al., 1990; Dunkelblum et al., 1993). Each batch consisted of 10–20 sprouts carrying about 5000 virgin females. The pheromone was collected for 3- to 4-day periods. The Poropak Q columns were eluted with hexane, and the pheromone solutions were concentrated with a nitrogen flow. Pooled samples of 20,000–30,000 female equivalents were fractionated by capillary GC on a Carlo Erba 5300 instrument. Samples were injected in splitless mode with the detector shut down onto a DB-5 30-m $\times$ 0.32-mm-ID, 0.50-μm column (J & W Scientific) maintained at 60°C for 2 min and then programmed to 160°C at a rate of 20°C/min. Ten fractions were

collected every 2 min in cooled (acetone–Dry Ice bath) capillary tubes connected to the detector outlet, starting 8 min after injection. The tubes were eluted with predistilled ether (200 μl) and hexane (200 μl). Fractions were bioassayed in a Petri dish arena as described by Mendel et al. (1990). The 12- to 14-min and 16- to 18-min fractions consistently attracted males. The pheromones of first – to fourth-generation laboratory females from the Avney Eitan rearing were analyzed only by GC-MS.

Chemical Identification. The two pheromone components were identified by GC-MS in the EI and CI mode and by comparison with standards. An AutoSpec VG GC-MS instrument, equipped with a DB-1 25-m $\times$ 0.32-mm, 0.25-ID column (J & W Scientific) was used for the analysis. The column was kept at 60°C for 2 min and then programmed to 200°C at a rate of 15°C/min. The MS analysis was conducted in the scan mode at 70 eV. Further GC-MS analyses of natural pheromone samples were conducted on an HP 6890N-MSD 5973 machine, equipped with an HP5 30-m $\times$ 0.25-mm-ID, 0.25-μm column (Agilent). The column was kept at 60°C for 2 min and then programmed to 170°C at a rate of 20°C/min. The MS analysis was conducted in the scan mode and in the SIM mode by using six indicative ions at 70 eV. A 1:1 mixture of synthetic pheromone compounds (I) and (II) was used to validate the quantitative integration of the two compounds in the SIM analysis.

The chirality of the pheromone components was determined after hydrolysis of the crude pheromone. A sample was hydrolyzed with KOH in methanol for 2 hr, diluted with water, and extracted with ether. Standards for the chiral analysis were commercial racemic lavandulol (Fluka) and (*R*)-lavandulol. The (*R*)-lavandulol was obtained from lavandin oil (steam distillate of lavandin plant). A sample of 1 g of oil, which contained ~1% of (*R*)-lavandulol and a few percent of its esters (Sukh Dev, 1982) was hydrolyzed with 25 ml 1 M KOH in methanol at room temperature for 2 hr. Hydrolysis raised the amount of lavandulol to ~5%. The reaction mixture was worked up with 100 ml water, extracted with 3 $\times$ 10 ml ether, and the combined extracts were dried with anhydrous $MgSO_4$. After filtration and removal of the solvent, the sample was fractionated by TLC on Kieselgel 60 F254 20 $\times$ 20-cm plates (2 mm thickness) with hexane 80% + ether 20% as eluent. Racemic lavandulol was used as a marker. A band with $R_t = 0.14$ was removed and extracted with ether. This sample contained mainly (*R*)-lavandulol and linalool. Chiral analysis was performed by GC on a Rt-β-DEXsm, 30-m $\times$ 0.25-mm-ID, 0.25-μm column (Restek), isothermally at 110°C in the split mode (ratio of 100:1), using an HP 6890 instrument.

Syntheses of Pheromone Components and Standards. The terpenoid C_5 esters were synthesized from the following terpenic alcohols: geraniol, nerol, racemic linalool and racemic lavandulol with valeric, isovaleric, 2-methylbutyric, tiglic, and senecioc acid chlorides in dry ether with pyridine as base at ice bath temperature. All alcohols and acid chlorides were purchased from Aldrich and Fluka. Esters

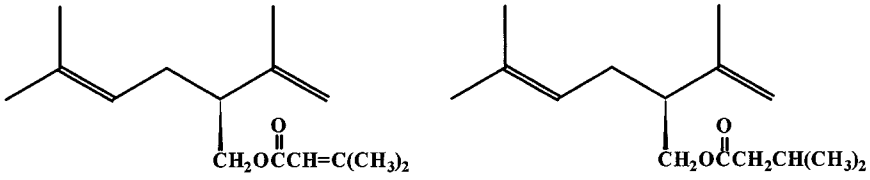
were purified by column chromatography on SiO_2, using hexane + 2% ether as eluent. The synthetic pheromone components lavandulyl senecioate (I) and lavandulyl isovalerate (II) were racemic.

Bioassays and Field Tests. The two pheromone components were bioassayed in Petri dishes, concurrently, at dosages of 3–5 ng/paper disk with untreated paper disks as control in each test. Five tests were carried out with males from the colony and with F_1 males. In each test, 25–35 males were introduced into the Petri dish at ~9 AM, and the number of males on each disk was recorded 10 times at intervals of 15 min. Flight assays were conducted in the mealybug rearing chamber, with males either from the colony or the first generation populations. Delta traps with sticky plates (10 × 18 cm) were used that were baited with lures of gray rubber septa (West Co., Pennsylvania, USA) impregnated with the two pheromone components or hexane as the control. The distance between traps was ca. 2 m due to the small size of the room. Each test included three treatments consisting of 2 traps each baited with 50 or 200 μg of the two pheromone components and hexane as the control. Traps were suspended from the ceiling, exposed for 4–9 days, and their positions were rotated daily. Field tests were conducted in an organic vineyard in Lachish, southern Judean foothills, using the same traps and lures. The test included three treatments consisting of 5 traps each that were baited with 50 or 200 μg of the two pheromone components or hexane as the control.

Data Analysis. Results from the Petri dish bioassay and flight assay in the rearing chamber were converted to percent response of males to each of the two pheromone components and control. The percentage results were aresin transformed arcsin. Field results were analyzed without any transformation. The results were analyzed by a parametric one-way ANOVA. The procedures used were PROC GLM and PROC MEAN (SAS, 1996). Differences between means were tested by Student-Neuman-Keuls multiple range test at $P < 0.05$.

RESULTS

Chemical Identification. Two active fractions were isolated from the airborne collections of the vine mealybug virgin females by capillary GC fractionation. One fraction contained lavandulyl senecioate (I), and in the second fraction we identified lavandulyl isovalerate (II) (Figure 1). Airborne collections of noninfested potato sprouts did not contain compounds I or II. The MS spectra of compounds I and II are similar (Figure 2). Both have weak molecular ions at *m/z* 236 and 238 (<1%), respectively. The main ions of I are at *m/z* 136 (17%, $M^+ - C_4H_7COOH$), 121 (35%, $M^+ - C_4H_7COOH - CH_3$), 93 (97%, $M^+ - C_4H_7COOH - C_3H_7$), 83 (100%, $C_4H_7CO^+$), 69 (35%, $C_5H_9^+$), and 55 (21%, $C_4H_7^+$). The main ions of II are at *m/z* 136 (16%, $M^+ - C_4H_9COOH$), 121 (38%, $M^+ - C_4H_9COOH - CH_3$), 93 (100%, $M^+ - C_4H_9COOH - C_3H_7$), 85 (31%, $C_4H_9CO^+$), 69 (68%,



(*S*) - Lavandulyl senecioate (I) (*S*) - Lavandulyl isovalerate (II)

FIG. 1. Structure of the *Planococcus ficus* pheromone components (*S*)-lavandulyl senecioate (I) and (*S*)-lavandulyl isovalerate (II).

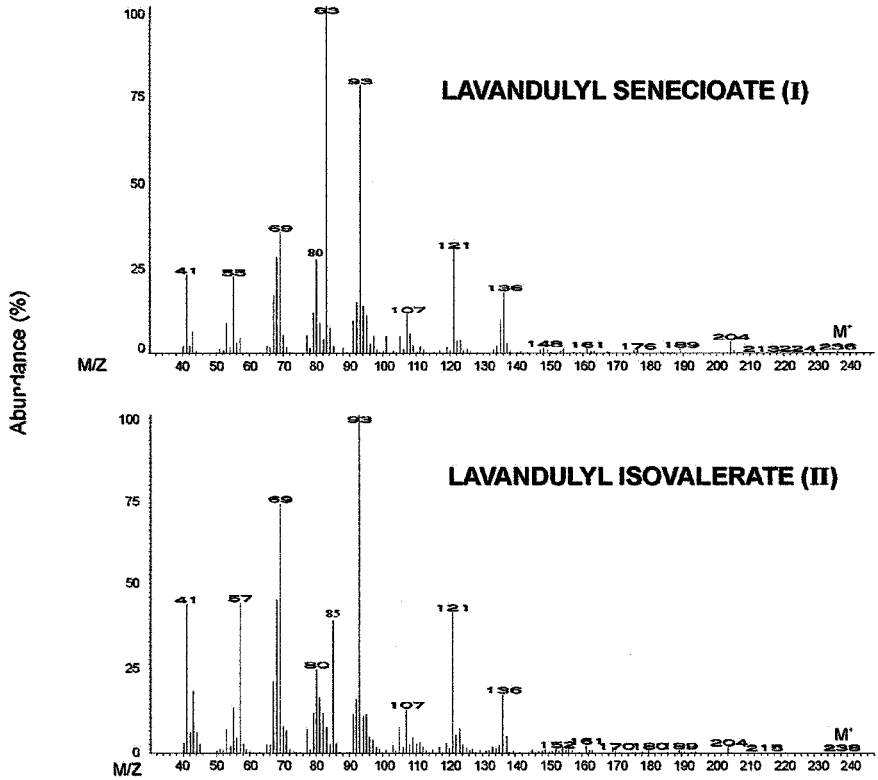


FIG. 2. EI mass spectra of pheromone components (*S*)-lavandulyl senecioate (I) and (*S*)-lavandulyl isovalerate (II).

$C_5H_9^+$), and 57 (28%, $C_4H_9^+$). The specific ions of the two compounds are due to the carboxylate part of I and II, at *m/z* 83 and 55, derived from $C_4H_7CO^+$and $C_4H_7^+$ in I, and 85 and 57, derived from $C_4H_9CO^+$ and $C_4H_9^+$ in II. The molecular ions of I and II were confirmed by CI with ammonia as reagent gas.

Components I and II are chiral. Attempts to separate racemic I and II on three chiral cyclodextrin columns: CDX-B (30 m × 0.25 mm ID, 0.25 μm), Rt-β-DEXsm (30 m × 0.25 mm ID, 0.25 μm), and Lipodex-G (25 m × 0.25 mm ID, 0.25 μm), failed. Conversely, racemic lavandulol gave a baseline separation on the Rt-β-DEXsm column. Consequently, a sample of the natural pheromone was hydrolyzed, and the resulting lavandulol was analyzed along with R-lavandulol (Sukh Dev, 1982) and racemic lavandulol on this column. The chiral GC analysis established the S-configuration of both lavandulyl senecioate (I) and lavandulyl isovalerate (II) (Figure 3).

Bioassays. Data obtained from the Petri dish and flight assays (Table 1) indicated that both components were similarly attractive to lab-reared males, although the response to lavandulyl isovalerate was higher. However, in the vineyard males were captured only in traps baited with lavandulyl senecioate (Figure 4). Because of the different response of the laboratory males (prolonged rearing) and feral

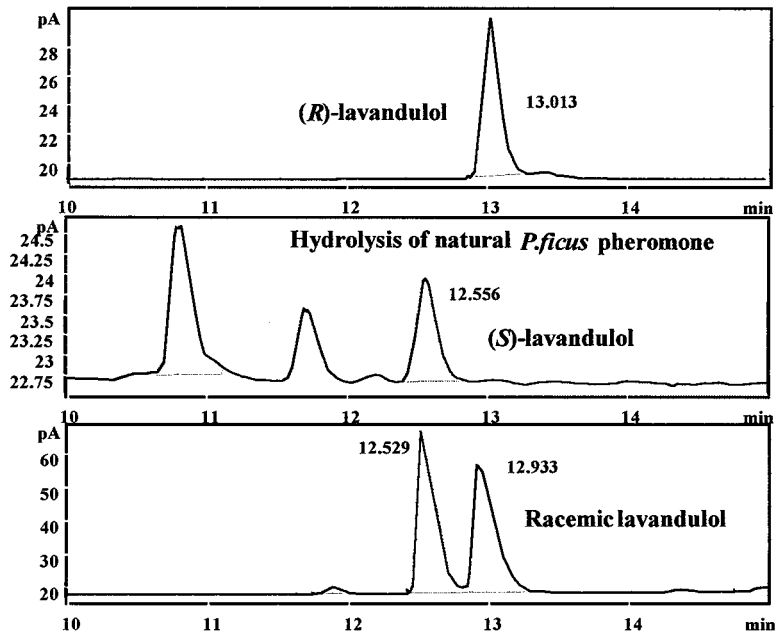


FIG. 3. Chiral GC profile of (*S*)-lavandulol from a hydrolysis of *P. ficus* pheromone compared with racemic lavandulol and (*R*)-lavandulol.

TABLE 1. RESPONSE OF *Planococcus ficus* MALES FROM CULTURE AND FIRST-GENERATION IN LABORATORY BIOASSAYS

Males	Response (%)[a]		
	Lavandulyl senecioate (I)	Lavandulyl isovalerate (II)	Control
Petri dish bioassay[b]			
Culture	42 b	57 a	1 c
First generation	91 a	9 b	0 c
Flight chamber assay[c]			
Culture	36 b	50 a	14 c
First generation	80 a	12 b	8 bc

[a] Numbers followed by different letters in a row or column are significantly different.
[b] Average of five tests with 25–35 males from the culture and from the first generation. Differences between means were tested by Student-Neuman-Keuls multiple range test at $P < 0.05$.
[c] Each treatment consisted of two traps, they were exposed for 4–9 days and rotated daily. The results with males from the rearing is an average of three tests and that with the first generation is an average of two tests. Differences between means were tested by Student-Neuman-Keuls multiple range test at $P < 0.05$.

males in the vineyard to lavandulyl isovalerate, we tested the attraction of the first-generation males to both components in the laboratory and reanalyzed the composition of the pheromone from the first- to fourth-generation females.

The laboratory and field assay results indicate clearly that the attraction of feral males differs significantly from that of the laboratory mass-reared males to the two pheromone components. The first generation males responded only to lavandulyl senecioate, in the Petri dish bioassay as well as in the flight assay (Table 1), similar to their behavior in the vineyard. The quantitative analysis of compounds I and II in the pheromone collected from four successive female generations showed a gradual increase in the amount of lavandulyl isovalerate (II) (Figure 5; Table 2). The results indicate that feral females probably emit only lavandulyl senecioate (I) (based on the first generation females), while the mass-reared females on potato sprouts produce both components I and II in an average ratio of 5:2.

DISCUSSION

It took several generations of *P. ficus* between collection of gravid females in the vineyard and the establishment of a culture on potato sprouts for the first pheromone collection. A preliminary GC-MS screening indicated the presence of two terpenoid esters, constituting a C_{10} terpene alcohol and a C_5 saturated and unsaturated acid. These esters were not present in the airborne collection of potato sprouts, but they were the main peaks in the active fractions. Some of the linalool esters that we prepared had very close mass spectra and retention times to the pheromone compounds, but they were inactive.

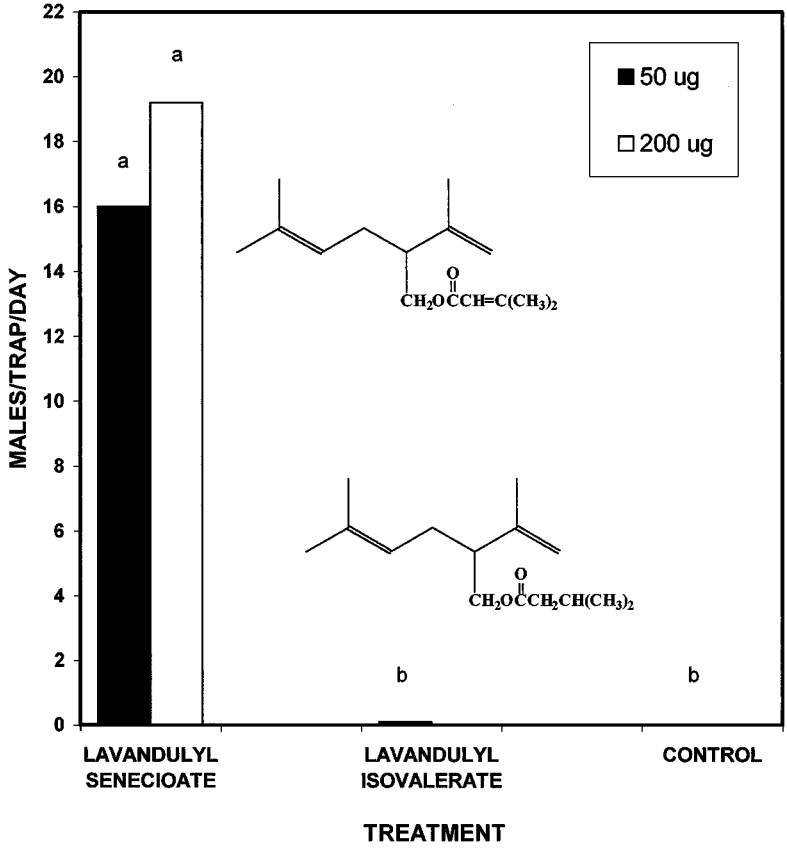


FIG. 4. Catch of *Planococcus ficus* males (mean/trap/day) in traps baited with pheromone components (*S*)-lavandulyl senecioate (I) and (*S*)-lavandulyl isovalerate (II), Lachish vineyard May 20–27, 2001. Bars superscripted by the same letters are not sigificantly different at $P < 0.05$.

Concurrent with our work, (*S*)-lavandulol senecioate (I) was identified as the sole pheromone component of *P. ficus* in the California population (Hinkens et al., 2001). Consequently, several esters of lavandulol (racemic commercial material) were prepared, and synthetic lavandulyl senecioate (I) and lavandulyl isovalerate (II) were found to have identical GC retention times and mass spectra to the two natural compounds isolated from the Israeli *P. ficus* population.

All laboratory and field tests were conducted with racemic pheromone components, which displayed high activity. Unnatural scale insect pheromone stereoisomers in general have no biological activity (Dunkelblum, 1999). Recently it has been shown that racemic lavandulyl senecioate is as active as the natural

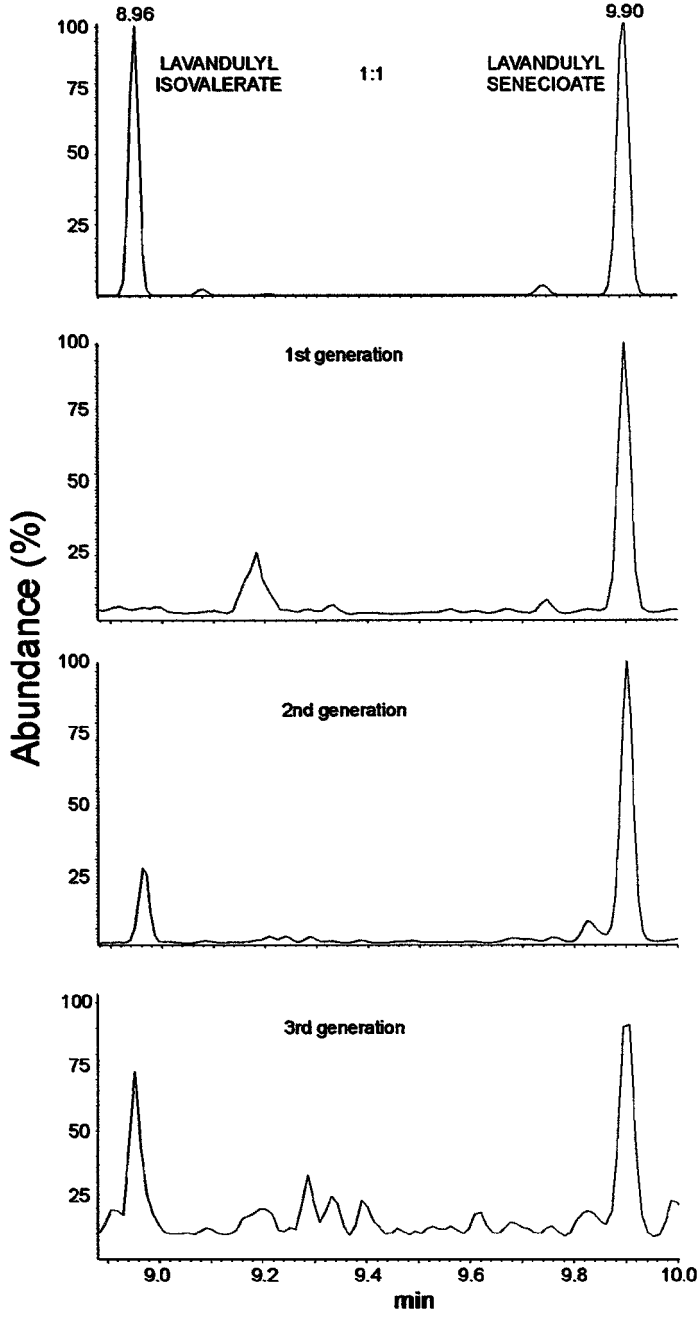


FIG. 5. GC profile of (*S*)-lavandulyl senecioate (I) and (*S*)-lavandulyl isovalerate (II) obtained from GC-MS analysis in the SIM mode of the two components in a 1:1 ratio and in the natural pheromone collected from first- to third-generation females.

TABLE 2. RELATIVE AMOUNT OF (*S*)-LAVANDULYL SENECIOATE (I) AND (*S*)-LAVANDULYL ISOVALERATE (II) IN *Planococcus ficus* FEMALES FROM CULTURE[a] AND IN FIRST- TO FOURTH-GENERATION FEMALES

Generation	Lavandulyl senecioate (I)[b]	Lavandulyl isovalerate (II)[b]
First	1	Trace
Second	1	0.2
Third	1	0.5
Fourth	1	0.5
Colony[a]	1	0.4

[a] Routine mass rearing of at least six generations on potato sprouts.
[b] Results of first- to fourth-generation females are averages of two samples, results of females from a prolonged rearing is an average of 6 samples.

S enantiomer (the *R* enantiomer did not interfere with the activity) (Millar et al., 2002).

Compounds I and II displayed similar biological activity in laboratory assays. However, with feral mealybug populations in the vineyard, only lavandulyl senecioate (I) attracted males. This surprising observation prompted us to assess the response of first-generation laboratory males to both pheromone components. These males responded to compound I alone, just as the feral ones. In the next step, we reanalyzed the pheromone composition of the first to fourth female generations. The first-generation females produced essentially only compound I. The amount of compound II increased gradually in the subsequent generations, however, and the ratio of compounds I and II, produced by the third and fourth generations was similar to that produced by females from the continuous culture (Figure 5; Table 2). The results suggest that rearing the vine mealybug in the artificial environment on potato sprouts had induced this change. In California, *P. ficus* pheromone collected from females reared on butternut squash revealed no trace of compound II (J. G. Millar, personal communication). The fact that (*S*)-lavandulyl isovalerate has not been detected in the Californian *P. ficus* population may be due to the recent introduction of the mealybug there (Gill, 1994). Such a population usually displays less genetic diversity due to the founder effect and, therefore, may lack the alleles responsible for the production of the second component.

The radical shift in pheromone production and pheromone response in a few generations was not expected. Most sex pheromones of scale insects have been collected as airborne volatiles from virgin females that were maintained on potatoes or squash and, so far, it has never been reported to affect the composition and response to the pheromone (Dunkelblum, 1999). Whenever scale insect pheromones contained more than one component, as in *Aonidiella auranti* and *Quadraspidiotus perniciosus*, all components were active in the laboratory as well as in the field (Dunkelblum, 1999).

We presume that most of the feral vineyard population of *P. ficus* in Israel produces only (*S*)-lavandulyl senecioate (I). A small proportion of the scale population probably produces (*S*)-lavandulyl isovalerate (II) in addition to I. The production of the second pheromone component II in much of the laboratory culture may be explained by selective pressures induced by potato sprouts that favor genotypes that produce both (*S*)-lavandulyl senecioate (I) and (*S*)-lavandulyl isovalerate (II). This change has created a population consisting of females that produce two active components and conspecific males able to respond to each of them. It is remarkable that the pheromone production by the females appears to shift simultaneously with male response. The existence of genetic linkage between pheromone production and male response has not been studied in scale insects, although this phenomenon has been investigated in moths, particularly in the European corn borer, *Ostrinia nubilalis* (Lofstedt et al., 1989; Roelofs et al., 1987). We intend to study the factors responsible for the development of the pheromonally distinct laboratory population of *P. ficus* and to investigate the shift in male response.

Preliminary results indicate that lavandulyl isovalerate is inhibitory to wild males. The addition of II to I reduced significantly the trap catch of *P. ficus* males in the vineyard. This finding implies a one-way mating barrier between laboratory and field populations. Further study of this issue is in progress in the laboratory and in the vineyard.

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MINOR COMPONENTS IN THE SEX PHEROMONE OF LEGUME PODBORER: *Maruca vitrata* DEVELOPMENT OF AN ATTRACTIVE BLEND

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Abstract—The legume podborer, *Maruca vitrata* (syn. *M. testulalis*) (F.) (Lepidoptera: Pyralidae) is a pantropical pest of legume crops. Sex pheromone was collected by gland extraction or trapping of volatiles from virgin female moths originating in India, West Africa, or Taiwan. Analysis by GC-EAG and GC-MS confirmed previously published findings that (*E*,*E*)-10,12-hexadecadienal is the most abundant EAG-active component with 2–5% of (*E*,*E*)-10,12-hexadecadienol also present. At least one other EAG response was detected at retention times typical of monounsaturated hexadecenals or tetradecenyl acetates, but neither could be detected by GC-MS. Laboratory wind-tunnel bioassays and a field bioassay of blends of (*E*,*E*)-10,12-hexadecadienal with (*E*,*E*)-10,12-hexadecadienol and a range of monounsaturated hexadecenal and tetradecenyl acetate isomers indicated greatest attraction of males was to those including (*E*,*E*)-10,12-hexadecadienol and (*E*)-10-hexadecenal as minor components. In subsequent trapping experiments in cowpea fields in Benin, traps baited with a three-component blend of (*E*,*E*)-10,12-hexadecadienal and these two minor components in a 100:5:5 ratio caught significantly more males than traps baited with the major component alone, either two-component blend, or virgin female moths. Further blend optimization experiments did not produce a more attractive blend. No significant differences in catches were found between traps baited with polyethylene vials or rubber septa, or between lures containing 0.01 and 0.1 mg of synthetic pheromone. Significant numbers of female *M. vitrata* moths, up to 50% of total catches, were trapped with synthetic blends but

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not with virgin females. At present there is no clear explanation for this almost unprecedented finding, but the phenomenon may improve the predictive power of traps for population monitoring.

Key Words—*Maruca vitrata*, *Maruca testulalis*, Lepidoptera, Pyralidae, sex pheromone, EAG, wind-tunnel bioassay, field trapping, (*E*,*E*)-10,12-hexadecadienal, (*E*,*E*)-10,12-hexadecadienol, (*E*)-10-hexadecenal, *Diaphania indica*.

INTRODUCTION

The legume podborer, *Maruca vitrata* (syn. *M. testulalis*) (F.) (Lepidoptera: Pyralidae) is a pantropical pest of legume crops, particularly cowpea (Jackai, 1995), pigeonpea (Shanower et al., 1999), and beans (Abate and Ampofo, 1996). The larvae feed on flower buds, flowers, and young pods (Singh and Jackai, 1988). Without control measures, flower infestation rates up to 80% were reported in cowpea in West Africa by Afun et al. (1991), and seed damage rates of 50% by Dreyer et al. (1994).

Although the basic biology of *M. vitrata* has been studied extensively (Taylor, 1967; Singh et al., 1990; Jackai et al., 1990; Onyango and Ochieng-Odero, 1993), there is a lack of information on the behavior and activity of this pest in the field, which has hindered development of IPM strategies in Africa (Jackai, 1995) and Asia (Shanower et al., 1999). Pheromone-baited traps for *M. vitrata* could provide specific devices for monitoring the activity and movements of adults that would assist researchers in this respect. They could also provide simple alternatives to pest scouting by farmers to time application of control measures.

Adati and Tatsuki (1999) reported (*E*,*E*)-10,12-hexadecadienal (*EE*10,12–16:Ald) to be an EAG-active component in extracts from female *M. vitrata* abdominal tips. Synthetic *EE*10,12–16:Ald was shown to be attractive to male moths in laboratory bioassays at a level equal to that of crude pheromone gland extracts. However, high (99%) isomeric purity was found to be critical to this attraction. The corresponding alcohol, (*E*,*E*)-10,12-hexadecadienol (*EE*10,12–16:OH), was also noted as being present at 3–4% of the aldehyde. Although no behavioral data were presented in relation to this compound, it was said to produce no increase in attraction when added to the major component. No field testing of the compounds was carried out. In this study we report laboratory and field experiments that confirm *EE*10,12–16:Ald as the major component and extend identification of the blend to include *EE*10,12–16:OH and a third candidate minor component.

METHODS AND MATERIALS

Insect Rearing. M. vitrata for laboratory cultures were obtained as pupae from the International Center for Research in the Semi-Arid Tropics (ICRISAT), Hyderabad, India; Tamil Nadu Agricultural University, Coimbatore, India; the

International Institute for Tropical Agriculture (IITA), Kano, Nigeria; IITA, Cotonou, Benin; and the Asian Vegetable Research and Development Center (AVRDC), Taiwan. The Indian, Nigerian, and Taiwanese strains were obtained during 1993–1994. The two Indian strains were merged at an early stage but the combined Indian strain and the Nigerian and Taiwanese strains were maintained separately, at NRI, Chatham, UK, until they too were merged in 1996. The resultant mixed strain was crossed with the strains from Benin in 1997, after the latter had been initially held separately for two generations.

Insects were maintained on a 12L:12D, or 14L:10D, photoperiod, and room temperatures were 27°C (light) and 24°C (dark); relative humidity was 60 ± 10%. Adults mated in 12-liter Perspex cages and were fed on 10% sugar solution. Young legume plants, hyacinth bean, *Lablab niger*, cowpea, *Vigna unguiculata*, broad beans, *Vicia faba*, runner beans, *Phaseolus coccineus*, or French beans, *P. vulgaris*, were placed in the cages for oviposition. After 2–3 days, these were transferred to transparent plastic boxes (27 × 16 × 10 cm) containing absorbent paper in which larvae subsequently developed. Beans were replaced 3–4 times a week. Insects were sexed as pupae or freshly eclosed adults and kept separately for subsequent experimental use or for mating.

Helicoverpa armigera Hbn. (Lepidoptera: Noctuidae) were obtained as pupae from ICRISAT and reared as described by Armes et al. (1992).

Pheromone Collection. Female sex pheromone gland extracts were made from virgin female moths, 2–3 days old and 6–8 hr into the scotophase. After lightly anesthetizing the moth with carbon dioxide, the ovipositor was extruded by gently squeezing the abdomen and excised with dissecting scissors directly into hexane (Analar or HPLC grade; 10 μl per ovipositor). After brief sonification and soaking for 5–10 min, the solvent was removed, the tips washed with a little more hexane, and the combined extracts stored at −20°C.

Pheromone was also collected by air entrainment. One or two virgin female moths were placed in a glass container (12 cm × 4 cm diam.) and charcoal-filtered air was drawn through the vessel at 2 liters/min during the dark period. Volatiles were trapped on filters (4 mm diam.) packed with activated charcoal (5 mg; 0.01 mm) held between stainless steel mesh screens, prepared at NRI (Grob and Zürcher, 1976; Nesbitt et al., 1979; Tumlinson et al., 1982). Volatiles were removed by washing with dichloromethane (3 × 10 μl).

Alternatively, 12–22 virgin females were placed in a 2-liter bolt-head, round bottomed flask. Charcoal-filtered air was drawn through the flask at 2 liters/min during the dark period, and volatiles were trapped on a filter made from a Pasteur pipette packed with Porapak Q (50–80 mesh; 100 mg) purified by washing with dichloromethane. Trapped volatiles were removed by washing with dichloromethane (3 × 0.5 ml).

Gas Chromatography (GC). GC analyses were carried out using Carlo Erba Mega series 5300 instruments with fused silica capillary columns (25 m × 0.32 mm

ID) coated with nonpolar CPSil5CB (methyl silicone; Chrompack, London, UK) or polar CPWax52CB (Carbowax equivalent; Chrompack) and helium carrier gas (0.5 kg/cm^2). Injection was splitless (200°C) and detection was by flame ionization detection (FID; 230°C). Temperature program conditions were used, typically held at 60°C for 2 min, then programmed at 4°C/min to 230°C, and retention times were converted to equivalent chain lengths (ECLs) relative to the retention times of straight chain acetates i.e. tetradecyl acetate has ECL of 14.00.

Electroantennography (EAG) and Gas Chromatography Linked to Electroantennography (GC-EAG). EAG preparations were made as described by Cork et al. (1990) from an intact *M. vitrata* male moth by insertion of glass microelectrodes filled with saline into the distal and proximal ends of one antenna. The electrodes were connected via silver–silver chloride electrodes to an AC/DC amplifier in DC mode (UN06, Syntech, Hilversum, The Netherlands). EAG response profiles to synthetic compounds were recorded essentially as described by Beevor et al. (1986). The test compound in hexane solution was deposited on the inner wall of a glass Pasteur pipete, solvent was removed with nitrogen (200 ml/min for 3 sec), and the stimulus was delivered directly over the antenna in nitrogen (200 ml/min for 3 sec). The interval between successive stimuli was 1 min.

Coupled GC-EAG analyses were carried out as described by Cork et al. (1990) using GC columns as above. The effluent from the analytical column was split equally using push-fit Y-tubes (Chrompack). One outlet was attached by fused silica capillary tubing (0.5 m × 0.32 mm ID coated with CPSil5CB) to the FID and the other to a glass reservoir (3 ml). Column effluent was pulsed from the reservoir with nitrogen (500 ml/min for 3 sec) at 17-sec intervals over the male *M. vitrata* EAG preparation for the duration of the GC analysis. Both GC and EAG data were captured and processed with TurboChrom 4 software (Perkin Elmer-Nelson, Beaconsfield, UK). EAG responses were considered to be significantly above background if they showed an increase of 10% or more over the mean of the responses before and after.

Coupled GC-EAG analyses of two different samples of ovipositor washings from female *M. vitrata* were also carried out with a male *H. armigera* EAG preparation as a detector for (*Z*)-11-hexadecenal (*Z*11–16:Ald) and (*Z*)-9-hexadecenal (*Z*9–16:Ald), the female sex pheromone components of this species (e.g. Nesbitt et al., 1980).

Gas Chromatography—Mass Spectrometry (GC-MS). GC-MS analyses were carried out using an ion trap detector (ITD 700; Thermoquest, Hemel Hempstead, UK) operated in electron impact (EI) or chemical ionization (CI) mode with isobutane as reagent gas. This was coupled directly (230°C) to a Carlo Erba Mega GC with fused silica capillary columns as above except for the internal diameter (0.25 mm ID). Carrier gas was helium (0.5 kg/cm^2), and injection was splitless (200°C) with the oven temperature held at 50°C for 2 min, then programmed at 6°C/min to 230°C.

High Performance Liquid Chromatography (HPLC). Pheromone gland extract was fractionated by HPLC using a column (12 cm × 4.6 mm) packed with Spherisorb ODS2, eluted at 1 ml/min with a gradient from 1:1 acetonitrile–water to pure acetonitrile over 20 min with total run time 40 min. Fractions were extracted with hexane (2 × 1 ml), and the extracts were dried with magnesium sulfate.

Synthetic Chemicals and Dispensers. Monounsaturated compounds were prepared by standard acetylenic or Wittig routes and purified as the acetates or alcohols by argentation chromatography (Houx et al., 1974) to give material of greater than 99.9% chemical and stereochemical purity. Aldehydes were formed by oxidation of the corresponding alcohols with pyridinium chlorochromate (PCC) in dichloromethane (Corey and Suggs, 1975) and were distilled before use.

*EE*10,12–16:OH was prepared by Wittig reaction of the 2-tetrahydropyranyl ether of 10-bromo-1-decanol and (*E*)-2-hexenal using potassium *tert*-butoxide in tetrahydrofuran at −20°C to give a 90:10 mixture of *Z*,*E* and *E*,*E* isomers. This was isomerized by exposing a solution in hexane containing catalytic iodine to natural sunlight, giving a 65:15:15:5 mixture of *E*,*E*, *Z*,*E*, *E*,*Z*, and *Z*,*Z* isomers. This was recrystallized three times from petroleum spirit (bp 40–60°C) at −20°C to give *EE*10,12–16:OH of at least 99.5% isomeric purity. The alcohol was oxidized to *EE*10,12–16:Ald without loss of isomeric purity using PCC in dichloromethane buffered with sodium acetate (Corey and Suggs, 1975).

Isomers of 9,11-, 11,13-, and 12,14-hexadecadienol and the corresponding aldehydes were available from previous work (Hall et al., 1980; Cork et al., 1988). The sample of (*E*,*E*,*Z*)-10,12,14-hexadecatrienal was provided by Prof. Jim Tumlinson of the USDA Laboratory at Gainsville, Florida, USA.

Dispensers for synthetic chemicals in the first bioassay series and subsequent field tests were white rubber septa (Aldrich Chemical Co. Ltd., Gillingham, Dorset UK; catalog number *Z*10,072-2) or polyethylene vials (23 mm × 9 mm × 1.5 mm thick; Just Plastics, London, UK). These were impregnated with pheromone by adding the required amount of pheromone and an equal weight of 2,6-di-*tert*-butyl-4-methylphenol (BHT) as antioxidant dissolved in 0.1 ml of petroleum spirit (bp 40–60°C) and allowing the solvent to evaporate.

Wind-Tunnel Bioassays. Bioassays were carried out 4–7 hr into the dark period using a Perspex wind tunnel (0.25 m × 0.25 m × 1.50 m long) in a room separated from the *M. vitrata* cultures maintained at 24 ± 1°C and 60 ± 10% relative humidity. A 25-W axial fan (Verity Fans Ltd., London, UK) placed 0.3 m from one end of the tunnel produced an airflow of 0.5 m/sec. Four 60-W incandescent bulbs each shielded with Wratten 87 glass filters (which only transmit wavelengths above 780 nm) were arranged around the room to provide illumination.

Lures were suspended near the upwind end of the tunnel in the middle of the cross section. Virgin male *M. vitrata* (2–5 days old) were released singly 1 m downwind from the pheromone source. The responses of males to the attractant sources were scored as follows: NR: no response, male moths did not

respond after 60-sec exposure (did not leave retaining cage); TF/W: moth took flight, or emerged from cage by walking, but did not fly upwind within 60 sec of leaving cage; UwF: upwind flight by moth, but did not reach within 25 cm of the source (this often involved plume following, but this could not always be clearly determined); SA: source approach, moth clearly followed plume and hovered in front of the pheromone source, within 25 cm, but failed to make contact; and SC: source contact, moth made contact with or landed on the pheromone source.

The first bioassay compared responses of Indian strain male *M. vitrata* to 18 synthetic blends and female ovipositor washings. The synthetic blends were dispensed from polyethylene vials or rubber septa and contained *EE*10,12–16:Ald (100 μg) with *EE*10,12–16:OH (0, 5, or 50 μg) alone or with one of the monounsaturated acetates or aldehydes, *E*9–14:Ac, *Z*10–16:Ald, *E*10–16:Ald, *Z*11–16:Ald or *E*11–16:Ald (5 μg) ($N = 10$ or 20 for each lure and blend combination). Ovipositor washings were dissolved in hexane and dispensed from triangular pieces of filter paper (1 cm^2; Whatman No. 52) and contained approximately 3 ng of natural pheromone components ($N = 30$).

In two subsequent bioassays, synthetic blends and ovipositor washings dissolved in hexane (5 μl) were always dispensed from filter paper triangles. Synthetic blends were based on 5 ng of *EE*10,12–16:Ald and ovipositor washings contained 0.3–2.0 ng of pheromone components. Blank controls, where included, consisted of hexane only.

The second bioassay used the mixed Indian/Nigerian/Taiwanese strain of *M. vitrata*; this was merged with the Benin strain prior to the third bioassay. In all bioassays, the order of presentation of treatments was varied randomly between daily test sessions of 2 hr and not all treatments were tested during every session. Three to 10 males were tested against each treatment during a session. Individual males were only tested on one day. For the first and second bioassays, males were prescreened for responsiveness by testing them against female ovipositor washings. Only those making a UwF response or better were used for testing following an interval of 30 min. Each male was tested only once. For the third bioassay, individual males were tested against three of the five treatments within a test session in order that sufficient sample sizes could be obtained from the limited supply of males available at the time.

Results of the first bioassay were analyzed statistically by fitting a log-linear model to the cross-tabulation of response category against two factors constituting pheromone blend. These factors represented the amount of *EE*10,12–16:OH and the identity of the monounsaturated compound. For the subsequent wind-tunnel bioassays, results were analyzed by 2×2 tests of independence (Fisher's exact test) between different pairs of treatments. As only responses SA and SC represented definite attraction to the source, evaluation of moths' responses was in terms of the number of combined SA and SC responses versus NR + TF/W + UwF responses.

This approach was most appropriate given the relatively small sample sizes in these cases.

Field Evaluation. Initial trapping experiments were carried out with a variety of synthetic blends of *EE*10,12–16:Ald, *EE*10,12–16:OH, and *Z*11–16:Ald at several locations in India and Nigeria during 1992–1994. These failed to produce any trap captures, and an alternative field assay, termed the "clothes line" trial, was carried out in a field of pigeon-pea at ICRISAT, Hyderabad, India, over 21 nights from September 29 to October 21, 1995. Lures containing synthetic pheromone blends were suspended 2 m apart from lengths of string supported by bamboo sticks, level with the top of the crop canopy. The same 18 blends were evaluated as those presented in the first wind-tunnel bioassay, and rubber septa and polyethylene vial dispensers were used on alternate nights. The activity of males was monitored by continuously walking the length of the string and counting approaches to within 10 cm of individual lures with the aid of a pair of night vision goggles, assisted by illumination from a low-powered head torch. Moths attracted to the lures were caught with a sweep-net to check their identity, after which *M. vitrata* males were released and other moths retained. Observations were conducted between 21:00 hr and 03:00 hr.

Subsequent trapping experiments were carried out over 2–3 months each during 1998 and 1999 in cowpea fields at the IITA research station near Cotonou, Benin. Traps were suspended from sticks using wire at a height of approximately 1 m. Synthetic lures were replaced every 2 weeks and shielded from sunlight to minimize isomerization by wrapping aluminum foil around them so as to leave only the lowermost surface exposed. Subsequent experimentation has demonstrated that such lures show no loss of attractiveness for up to 4 weeks (Downham, unpublished data). Trap catches were counted daily and trapped moths discarded at that time. Sticky card inserts in the delta traps were replaced on a weekly basis.

In the first experiment, one-, two- or three-component blends of *EE*10,12–16:Ald, *EE*10,12–16:OH, and *E*10–16:Ald (100:5:5) at 0.01 or 0.1 mg doses of *EE*10,12–16:Ald in polyethylene vial or rubber septa dispensers were compared with two virgin females confined to small wire-mesh cages and unbaited controls in sticky Delta traps (28 cm long $\times$ 20 cm wide $\times$ 14 cm high; Agrisense-BCS, Pontypridd, UK). Females were 2 days old when placed in traps and were replaced every 2 days. In each of three fields, 24 traps were set up in a 6×4 grid 10 m apart, each field forming a replicate. In each row of six, the synthetic lures consisted of either 0.01- or 0.1-mg doses in either vials or septa. Within each row, the four synthetic blends and the female-baited and control traps were positioned randomly.

The second and third experiments were carried out in randomized complete-block designs with six and five replications, respectively. Each compared the attractiveness of blends of *EE*10,12–16:Ald, *EE*10,12–16:OH, and *E*10–16:Ald in different ratios. Traps forming a replicate block were positioned 20 m apart in rectangular configurations that were identical within each experiment. Blocks were at

least 50 m apart and were usually situated in separate fields. The second experiment used sticky Delta traps, as above, while the third experiment was conducted using plastic funnel traps (Agrisense-BCS), containing DDVP insecticide strips to kill trapped moths. A previous experiment had demonstrated that this funnel trap catches two to three times as many moths as the Delta trap (Downham, unpublished data).

Statistical analysis was carried out using the total catch by each trap over the respective trapping periods. Data were transformed to $\log_{10}(x + 1)$ values before analysis of variance. Where this indicated statistically significant effects, transformed means values were separated by the least significant difference test.

RESULTS

GC-EAG and GC-MS Analyses. Analyses of ovipositor washings from virgin female *M. vitrata* moths by GC-EAG on polar or nonpolar GC columns showed a single major response and up to four minor responses (e.g., Figure 1). The latter were not observed consistently and were never shown to be unambiguously associated with peaks in the FID trace.

The major response (Figure 1, response 4) corresponded to a peak on the FID trace that amounted to 0.5–2 ng/female, and this was assumed to be the

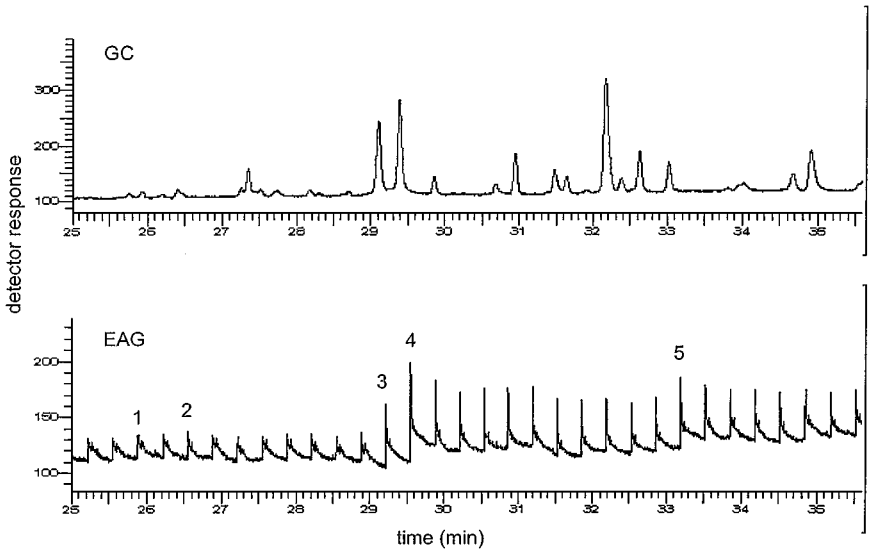

FIG. 1. Typical GC-EAG analyses of ovipositor washings from female *M. vitrata* on a polar GC column (EAG responses observed during this work are numbered).

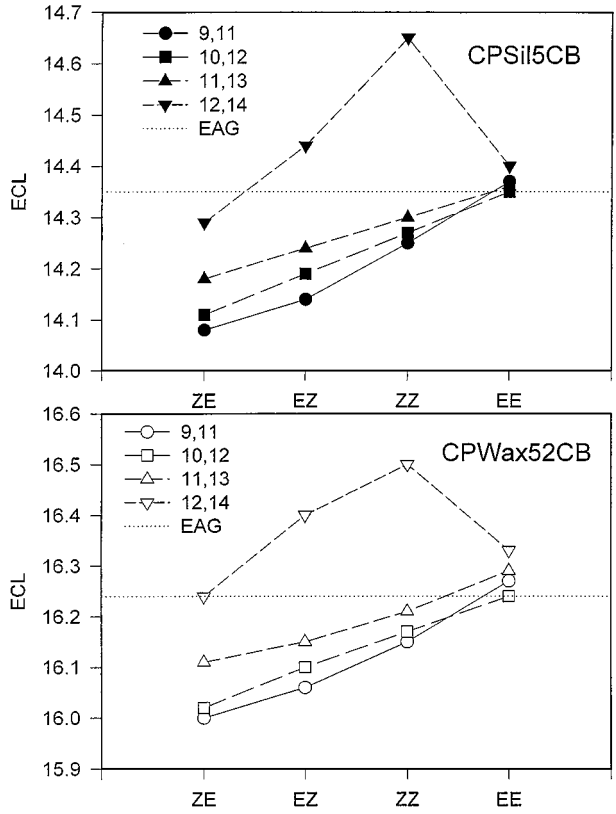

FIG. 2. Retention times (ECLs) of hexadecadienal isomers on nonpolar (CPSil5CB: 70°C for 2 min, then raised at 20°C/min to 120°C, then raised at 0.5°C/min to 230°C) and polar (CPWax52CB: 70°C for 2 min, then raised at 20°C/min to 120°C, then raised at 0.5°C/min to 230°C) GC columns.

major component of the female sex pheromone. This component was identified as (*E*,*E*)-10,12-hexadecadienal (*EE*10,12–16:Ald) by comparison of retention times on polar and nonpolar phases with isomers of 9,11–16:Ald, 10,12–16:Ald, 11,13–16:Ald, and 12,14–16:Ald (Figure 2). This identification was confirmed by comparison of the EI mass spectra of natural and synthetic materials which showed an $[M]^+$ ion at m/z 236 (3%) and base peak at m/z 67, identical with the spectra shown by Adati and Tatsuki (1999). CI mass spectra of the natural and synthetic compounds were also identical, although different from those reported by Doolittle et al. (1985) and Adati and Tatsuki (1999), presumably because these authors used a quadrupole spectrometer rather than the ion trap used here. Diagnostic ions were observed at m/z 237 (70%) for $[M+1]^+$ and m/z 85 (70%)

corresponding to the $[a]^+$ ion of Doolittle et al. (1985). The $[b]^+$ ion of Doolittle et al. (1985) at m/z 183 was barely detectable.

Similar results were obtained in analyses of volatiles collected from virgin female moths on activated charcoal or Porapak, although amounts obtained were much lower. Subsequent tests with synthetic *EE*10,12–16:Ald showed that although recovery of *EE*10,12–16:Ald from charcoal was only 44%, that from Porapak was >90%.

The most frequently observed of the minor responses in GC-EAG analyses (Figure 1, response 5) occurred at ECLs of 18.7 and 15.2 on polar and nonpolar GC columns, respectively. These corresponded with the retention times of (*E*,*E*)-10,12-hexadecadienol (*EE*10,12–16:OH), and were different from those of other isomers of 9,11-, 10,12-, 11,13-, and 12,14-hexadecadienol. On some occasions mass spectral evidence could be obtained for the presence of this compound, but the amount present was estimated to be <2% of the *EE*10,12–16:Ald.

A significant increase in the EAG response (Figure 1, response 3) was often observed in GC-EAG analyses just before elution of the major pheromone component, *EE*10,12–16:Ald. This suggested the presence of other hexadecadienal isomers, but they could not be detected by GC-MS in EI or CI mode.

A response (Figure 1, response 2) was also sometimes observed during GC-EAG analyses in the region corresponding to retention times of monounsaturated hexadecenal isomers, i.e., ECLs 14.7 and 13.8 on polar and nonpolar columns, respectively, but a monounsaturated hexadecenal isomer could not be detected by GC-MS in EI or CI mode. GC-EAG analysis of ovipositor washings from virgin female *M. vitrata* with male *H. armigera* EAG preparations gave no response at retention times corresponding to those of hexadecenal isomers on either polar or nonpolar columns. This indicated that any hexadecenal isomer present in the extract was unlikely to be the (*Z*)-11- or (*Z*)-9- isomer.

A response (Figure 1, response 1) was also sometimes observed during GC-EAG analyses at ECL 14.3 on a polar column. This region corresponds to retention times of monounsaturated tetradecenyl acetate isomers, which would have similar retention times to hexadecenal isomers on a nonpolar column. However, no evidence for the presence of a tetradecenyl acetate was obtained from GC-MS analyses.

A sample of (*E*,*E*,*Z*)-10,12,14-hexadecatrienal (*EEZ*10,12,14–16:Ald) was found to be quite stable under the conditions used for GC, GC-EAG and GC-MS analyses. This material also contained 11% *EZZ*10,12,14–16:Ald, 1.3% *EE*10,12–16:Ald, and 6.7% *EZ*10,12–16:Ald (relative to *EEZ*10,12,14–16:Ald), and, when puffed over a male *M. vitrata* EAG preparation, a significant EAG response was obtained. However, in linked GC-EAG analyses, a response to the two diene isomers was observed but there was no response to the triene isomers. The triene aldehydes have characteristic mass spectra in EI and CI mode (Tumlinson et al., 1989), but reexamination of female ovipositor washings from *M. vitrata* by

TABLE 1. EAG RESPONSES OF MALE *M. vitrata* TO FRACTIONS FROM HPLC SEPARATION OF FEMALE OVIPOSITOR WASHINGS

Fraction	Time (min)	EAG (mV)[a]
1	0–10	0
2	10–20	0
3	20–24	0.30
4	24–28	1.23
5	28–32	0.11
6	32–40	0
*EE*10,12–16:Ald (8 ng)		3.06

[a] Response to 1 μl out of 1 ml off glass.

GC-MS in both EI and CI modes failed to detect any trace of hexadecatrienal isomers.

HPLC Fractionation. Under the conditions used for HPLC fractionation, *EE*10,12–16:Ald eluted after 26.7 min, *EE*10,12–16:OH after 27 min, *Z*11–16:Ald after 28.4 min, and *EEZ*10,12,14–16:Ald after 8.5 min. A sample of ovipositor washings from 136 *M. vitrata* females was fractionated into six fractions as in Table 1, and each fraction was extracted twice with hexane to give A and B extracts.

Examination of all the fractions by GC-MS on a polar GC column confirmed that *EE*10,12–16:Ald was present in fraction 4 with traces just detectable in fractions 3 and 5. Examination of EAG-active fractions 3, 4, and 5 on both polar and nonpolar GC columns confirmed the presence of *EE*10,12–16:Ald in all three fractions. Responses at retention times corresponding to *EE*10,12–16:OH and a hexadecenal isomer were also observed in all three fractions. Retention time for the latter was in the region of those for the *Z* and *E* isomers of 10-, 11-, or 12-hexadecenal, but none could be conclusively detected by GC-MS. *EEZ*10,12,14–16:Ald or its geometric isomers could not be detected.

EAG Assays. The synthetic *EE*10,12–16:Ald (Figures 3 and 4) and *EE*10,12–16:OH (Figure 4) elicited strong EAG responses from male *M. vitrata* moths. When EAG responses to a range of monounsaturated hexadecenal isomers were compared (Figure 3), the *E* isomers of 10-, 11-, and 12-hexadecenal gave the highest responses, with *E*10–16:Ald the most active. Tetradecenyl acetate isomers elicited lower EAG responses from male *M. vitrata* compared with the hexadecenal isomers, although the (*E*)-8-, (*Z*)-9-, and (*E*)-9-tetradecenyl acetates caused responses significantly above those to the hexane blank (Figure 4).

Wind-Tunnel Bioassays. In the first bioassay series, female ovipositor washings were much more attractive to male *M. vitrata* moths than 18 synthetic blends containing *EE*10,12–16:Ald (100 μg) and *EE*10,12–16:OH (0, 5, or 50 μg) with or

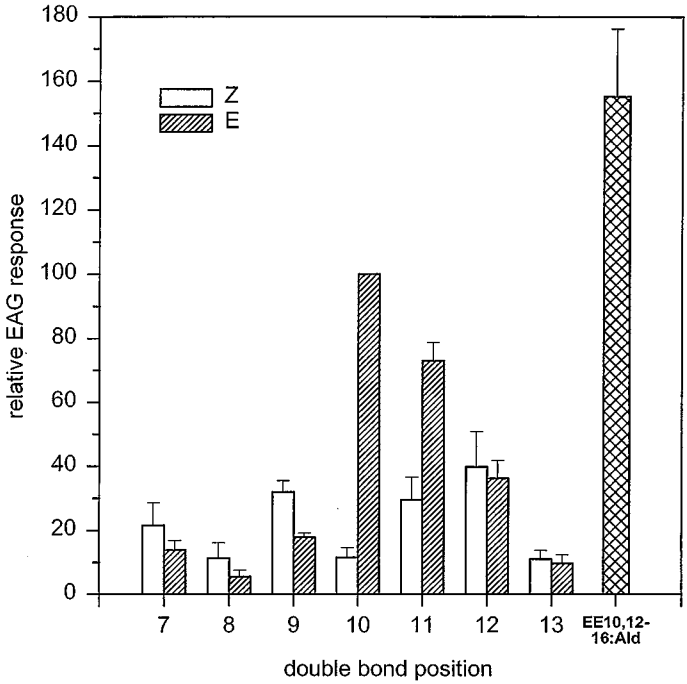

FIG. 3. EAG responses of male *M. vitrata* to hexadecenal isomers (50 ng off glass; means ± SEM of three tests on three moths; relative to response to *E*10–16:Ald = 100); response to *EE*10,12–16:Ald (5 ng) shown for comparison.

without *E*9–14:Ac, *E*10–16:Ald, *Z*10–16:Ald, *E*11–16:Ald, or *Z*11–16:Ald (5 μg) dispensed from polyethylene vials or rubber septa. While the majority (95–100%) of responses to the former were SA or SC responses (source approach or source contact), almost all responses to the synthetic blends were TF/W or UwF responses (taking flight/walking or upwind flight, with little or no plume following). However, blends including *E*10–16:Ald, either as a two-component blend with the major component alone or as a three-component blend with the major component and 50 μg (but not 5 μg) of *EE*10,12–16:OH, differed from the rest in eliciting a few (10–20%) SA, but no SC responses. Blends with *E*11–16:Ald and *Z*11–16:Ald, and the combination of the major component with 5 μg of the diene alcohol also produced some, although fewer (5–10%), source approaches. Overall, reflecting the low number of such responses, the log-linear modeling indicated no significant effect of the amount of *EE*10,12–16:OH or identity of the third blend component on response. Across all blends, there was a slightly higher proportion of UwF responses, relative to TF/W, for rubber septa dispensers than for polyethylene vials.

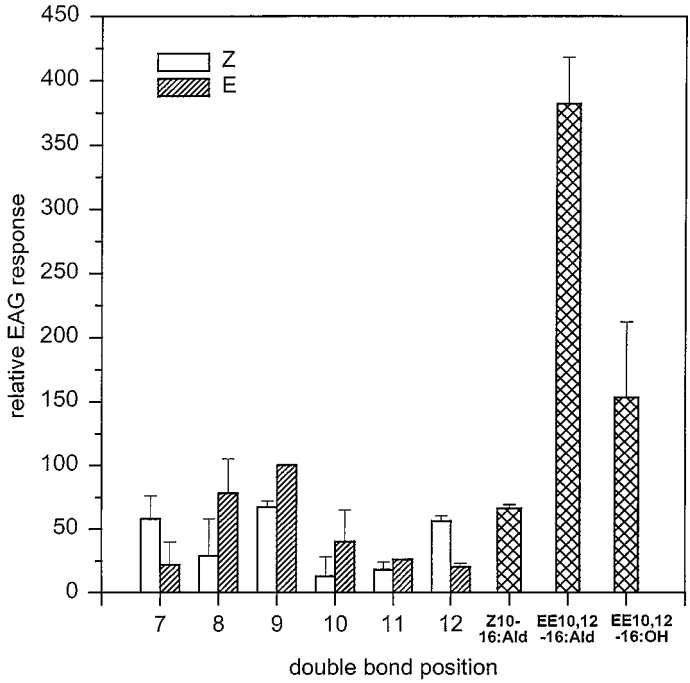

FIG. 4. EAG responses of male *M. vitrata* to tetradecenyl acetate isomers (5 ng off glass; mean ± SEM for two replicates from different insects; relative to response to *E*9–14:Ac = 100); responses to *Z*10–16:Ald, *EE*10,12–16:Ald, and *EE*10,12–16:OH (5 ng) are shown for comparison.

The difference between the dispenser types was statistically significant ($P < 0.01$, Mann-Whitney test, adjusted for ties).

In the second bioassay, the two-component blend containing *EE*10,12–16:Ald with 5% *EE*10,12–16:OH elicited significantly more SA responses than one with 50% of the diene alcohol ($P < 0.05$, Fisher's exact test, two-tailed). Addition of 5% of *E*10–16:Ald to these two-component blends did not significantly increase the proportion of SA responses, but they both gave high levels of UwF responses (Figure 5).

In the third bioassay, the blank control and synthetic blends containing *EE*10, 12–16:Ald alone or with 5% *EE*10,12–16:OH were significantly less attractive than ovipositor washings ($P < 0.05$, Fisher's exact test, two-tailed), although the two-component blend did elicit some source approaches. Addition of 5% of *E*10–16:Ald to the two component blend gave a blend not significantly different in attractiveness to male *M. vitrata* moths from the ovipositor washings, although source contact only occurred with the latter (Figure 6).

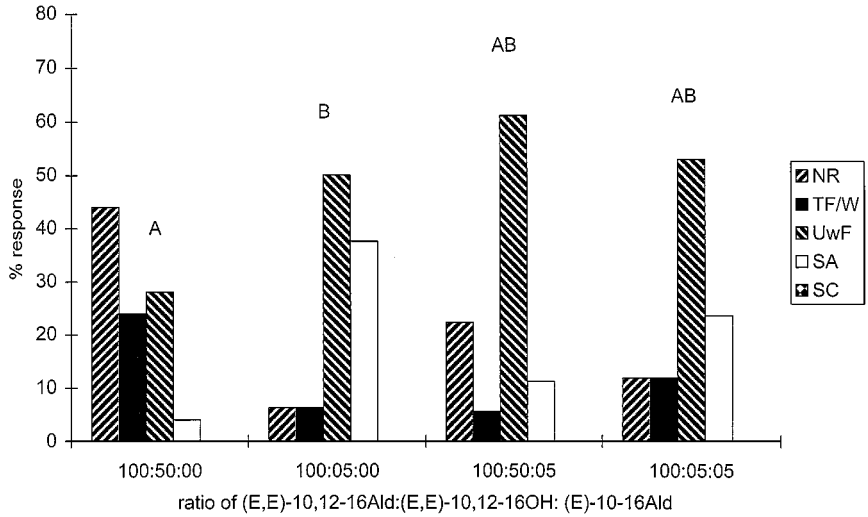

FIG. 5. Second windtunnel bioassay (June–November 1996): responses of male *M. vitrata* moths to different two- and three-component blends. (N for each blend was 16–25; common legend letters above the histograms indicate that treatments did not differ in the proportions of their category SA responses; $P > 0.05$; Fisher's exact test, two-tailed).

Field Tests. The whole range of 18 blend and dispenser combinations used in the first laboratory bioassay was evaluated in the "clothesline" trial, and results for lures eliciting at least one approach are given in Table 2. Absolute numbers of approaches did not exceed a mean of three per night, but all blends containing *E*10–16:Ald showed some attractiveness to male *M. vitrata* moths. The binary blend containing *EE*10,12–16:Ald (100 μg) and *E*10–16:Ald (5 μg) or the corresponding tertiary blend containing of *EE*10,12–16:OH (50 μg) in addition elicited more approaches than all other blends ($P < 0.05$, t test), and this was true for both types of dispenser. Of these two most attractive blends, the three-component blend elicited more approaches, although the difference was significant only with the polyethylene vial lures.

In this trial, significant numbers of another pyralid moth were attracted to blends of *EE*10,12–16:Ald (100 μg), *EE*10,12–16:OH (50 μg), and *E*11–16:Ald (5 μg). These were identified as males of the pumpkin caterpillar moth, *Diaphania indica* Saunders (Lepidoptera: Pyralidae), at the Natural History Museum, London, UK. Subsequently, Wakamura et al. (1998) reported *E*11–16:Ald and *EE*10,12–16:Ald to be components of the female sex pheromone of this species.

The first trapping experiment carried out in Benin evaluated blends of *EE*10, 12–16:Ald, *EE*10,12–16:OH, and *E*10–16:Ald in 100:5:5 ratio and showed that all three components were necessary for maximum attraction of male *M. vitrata* moths

TABLE 2. MALE *M. vitrata* MOTHS APPROACHING LURES IN CLOTHESLINE TRIAL[a]

Pheromone component			Nights	Approaches/night
*EE*10,12–16:Ald (μg)	*EE*10,12–16:OH (μg)	Monoene (5 μg)	(*N*)	(mean ± s.e.)[b]
Polyethylene vials				
100	5		10	0.40 ± 0.15 c
100		*E*10–16:Ald	10	1.60 ± 0.21 b
100		*E*9–14:Ac	10	0.30 ± 0.41 c
100	50	*E*10–16:Ald	10	2.60 ± 0.40 a
100	50	*Z*11–16:Ald	10	0.30 ± 0.14 c
Rubber septa				
100		*E*10–16:Ald	11	1.27 ± 0.23 a
100		*E*11–16:Ald	11	0.27 ± 0.13 b
100	50	*E*10–16:Ald	11	1.91 ± 0.37 a
100	50	*Z*11–16:Ald	11	0.36 ± 0.15 b

[a] ICRISAT, India, 1995; (18 blends containing *EE*10,12–16:Ald (100 μg) and *EE*10,12–16:OH (0, 5 or 50 μg) with or without *E*9–14:Ac, *E*10–16:Ald, *Z*10–16:Ald, *E*11–16:Ald or *Z*11–16:Ald (5 μg) were compared. Blends not included in the Table did not attract any males).

[b] Means within each dispenser group followed by a common letter were not significantly different ($P > 0.05$, *t* test).

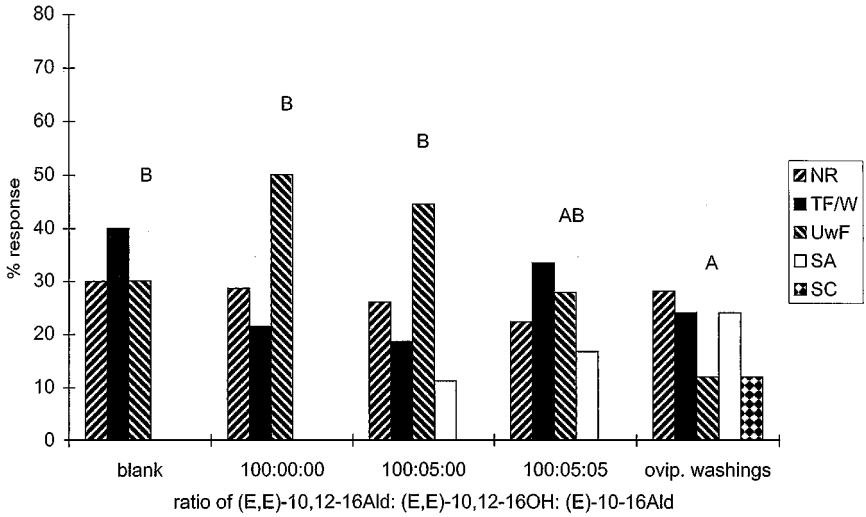

FIG. 6. Third wind-tunnel bioassay (August–October 1997): responses of male *M. vitrata* moths to one-, two-, and three-component blends, a blank control, and female ovipositor washings (*N* for each blend was 14–27; common legend letters above the histograms indicate that treatments did not differ in the proportions of their combined category SA and SC responses; $P > 0.05$; Fisher's exact test, two-tailed).

TABLE 3. CATCHES OF *M. vitrata* IN FIRST TRAPPING EXPERIMENT IN BENIN[a]

Lure[b]	Mean total catch per trap (±SE)	
	Males	Females
A		
100 : 0 : 0	7.0 ± 1.4 bc	1.3 ± 0.4 c
100 : 5 : 0	5.3 ± 0.9 c	1.8 ± 0.4 bc
100 : 0 : 5	8.9 ± 1.1 b	2.9 ± 0.6 b
100 : 5 : 5	33.1 ± 2.4 a	5.3 ± 0.9 a
2 virgin females	5.8 ± 0.8 c	0.2 ± 0.1 d
Blank, control	0.0 ± 0.0	0.0 ± 0.0
B		
0.01 mg polyethylene vial	12.3 ± 3.3	2.5 ± 0.5
0.1 mg polyethylene vial	16.8 ± 4.2	2.7 ± 0.6
0.01 mg rubber septum	13.3 ± 3.4	3.2 ± 0.9
0.1 mg rubber septum	12.0 ± 3.8	3.0 ± 1.0

[a] Catches for synthetic blends of *EE*10,12–16:Ald, *EE*10,12–16:OH and *E*10–16:Ald averaged across (A) two loadings and two dispenser types; and (B): four different pheromone blends (3 replicates; June 25–August 25, 1998). Means within a column followed by a common letter were not significantly different ($P > 0.05$, LSD following ANOVA of $\log_{10}(x + 1)$ transformed data); control omitted from ANOVA to avoid heterogeneity among the variances. In B no significant effect of dose or dispenser type on male or female catches ($P > 0.05$, F ratio, ANOVA).

[b] Blend ratio is *EE*10,12–16:Ald : *EE*10,12–16:OH : *E*10–16:Ald. Loading as amount of *EE*10,12–16:Ald.

(Table 3). When dispensed from rubber septa or polyethylene vials at loadings based on 0.1 mg or 0.01 mg of the major component, the three-component blend caught significantly more than single- or two-component blends or live females, all of which attracted similar numbers of moths. No moths were captured in unbaited control traps. Table 3A shows results for the different blends combined for both types of dispenser and at both loadings and Table 3B shows the results across all the blends for the two types of dispenser and two loadings. Although the polyethylene vial dispensers loaded with blends based on 0.1 mg of *EE*10,12–16:Ald attracted more males than other combinations of dose or dispenser, there were no significant differences among the respective treatment means (Table 3).

This experiment also provided the unexpected finding that about 20% of total catches in traps baited with synthetic lures were female *M. vitrata* moths, although virtually no females were attracted to live females or unbaited controls. Among the synthetic blends, the trends with respect to different blends, doses, and dispensers were the same as for males (Table 3).

TABLE 4. CATCHES OF *M. vitrata* IN SECOND TRAPPING EXPERIMENT IN BENIN; EFFECT OF VARYING PROPORTIONS OF MINOR COMPONENTS (6 REPLICATES; OCTOBER 20–DECEMBER 16, 1998)

Ratio of components[a]	Total catch per trap (mean ± SE)[b]	
	Males	Females
100 : 0.1 : 0.1	10.7 ± 6.1	6.8 ± 3.5
100 : 1 : 1	6.3 ± 4.1	5.8 ± 4.3
100 : 5 : 5	13.5 ± 7.9	9.5 ± 4.0
100 : 10 : 10	8.2 ± 5.0	6.8 ± 3.4
100 : 20 : 20	8.7 ± 4.3	7.5 ± 4.7
100 : 50 : 50	12.3 ± 6.3	11.0 ± 3.9
Blank, control	0.0 ± 0.0	0.0 ± 0.0

[a] *EE*10,12–16:Ald (0.1 mg):*EE*10,12–16:OH:*E*10–16:Ald.
[b] None of the means within a column were significantly different ($P > 0.05$, LSD following ANOVA of $\log_{10}(x + 1)$ transformed data); control omitted from ANOVA to avoid heterogeneity among the variances.

The second trapping experiment indicated that varying the amount of the two minor components together over a range of 0.1–50% of the major component did not have any significant effect on catches. This was true for catches of both male and female moths (Table 4). Female moths made up almost 50% of the total catch in this experiment.

The third trapping experiment compared various proportions of *E*10–16:Ald in three-component lures and showed that the blend containing 5% relative to *EE*10,12–16:Ald produced significantly higher trap catches than any of the other blends (Table 5). Catches in traps baited with three-component blends containing only 0.05% or 0.5% *E*10–16:Ald were no greater than those with single- or two-component lures. Few females were caught in this experiment.

DISCUSSION

In analyses of ovipositor washings and entrained volatiles from virgin female *M. vitrata* moths by GC linked to EAG recording from the male moth, up to five EAG responses were observed. The two most consistent responses were shown to be due to the major component, *EE*10,12–16:Ald, and *EE*10,12–16:OH at 2–5% of the major component, confirming the report of Adati and Tatsuki (1999). Of the three other possible responses, one occurred just before the response to *EE*10,12–16:Ald and may have been due to slight lack of synchrony between GC and EAG recordings. A fourth EAG response sometimes occurred at retention times

TABLE 5. CATCHES OF *M. vitrata* IN THIRD TRAPPING EXPERIMENT IN BENIN: EFFECT OF VARYING AMOUNT OF *E*10–16:ALD (5 REPLICATES; JULY 27–OCTOBER 21, 1999)

Ratio of components[a]	Total catch per trap (mean ± SE)	
	Males[b]	Females[c]
100 : 0 : 0	7.2 ± 1.8 a	0.40 ± 0.40
100 : 5 : 0	11.0 ± 0.8 a	0.60 ± 0.40
100 : 5 : 0.05	9.6 ± 2.5 a	0.40 ± 0.40
100 : 5 : 0.5	11.8 ± 3.0 a	0.80 ± 0.49
100 : 5 : 5	25.2 ± 3.1 b	0.40 ± 0.40
Blank, control	0.0	0.00

[a] *EE*10,12–16:Ald (0.1 mg) : *EE*10,12–16:OH : *E*10–16:Ald.
[b] For males, means followed by a common letter were not significantly different ($P > 0.05$, LSD following ANOVA of $\log_{10}(x + 1)$ transformed data); control omitted from ANOVA to avoid heterogeneity among the variances.
[c] Female data were not analyzed due to low catches.

corresponding to those of monounsaturated, 16-carbon aldehydes, although it was impossible to determine precise retention times from FID peaks or mass spectra because of the small amount of material present, estimated to be much less than 2% of the major component. The fifth EAG response was observed only occasionally at retention times similar to those of monounsaturated, 14-carbon acetates.

Tumlinson et al. (1989) reported the composition of the female sex pheromone of the tobacco hornworm moth, *Manduca sexta* L. (Lepidoptera: Sphingidae) and found two components essential for attraction, *EZ*10,12–16:Ald and the triunsaturated aldehyde, (*E*,*E*,*Z*)-10,12-14-hexadecatrienal (*EEZ*10,12,14–16:Ald). In earlier work on this pheromone, Starrat et al. (1979) had detected only the *EZ*10,12–16:Ald, and Tumlinson et al. (1989) and Doolittle et al. (1990) showed that the triene was destroyed under the conditions of the analysis. These authors isolated the triene by HPLC. In our hands, *EEZ*10,12,14–16:Ald was stable under conditions used for GC, GC-MS, and GC-EAG analyses, but no triunsaturated aldehyde could be detected in ovipositor washings from *M. vitrata* using these techniques or HPLC separation.

Subsequent work focused on identification of any hexadecenal isomer that might be present, and evaluation of blends of this with *EE*10,12–16:Ald and *EE*10,12–16:OH as attractants for male *M. vitrata* moths was carried out in the laboratory and field.

In a comparison of EAG responses of male *M. vitrata* moths to a range of hexadecenal isomers (Figure 3), *E*10–16:Ald gave the highest response, although high responses from the 10-, 11-, and 12-hexadecenals would be expected where

*EE*10,12–16:Ald is the major pheromone component (Roelofs, 1984). The first wind-tunnel bioassay indicated higher levels of behavioral response to blends including the *E*10-, *E*11-, or *Z*11- isomers, although these differences were not significant. However, the clothesline field trial with the same range of blends and dispensers showed blends containing *E*10–16:Ald with *EE*10,12–16:Ald were more attractive to male *M. vitrata* moths than blends with the other hexadecenals (Table 2). Subsequent laboratory bioassays provided further evidence of improved attraction to blends including *E*10–16:Ald (Figure 6), but this was not consistent (c.f. Figure 5). However, taking the clothesline trial and all the wind-tunnel bioassays together provided good evidence that *E*10–16:Ald was the hexadecenal component in ovipositor washings, and, thus, it was included in blends subsequently tested in the field trapping experiments.

Behavioral effects of *EE*10,12–16:OH were variable. In the first wind-tunnel bioassay lure, approaches to blends involving *E*10–16:Ald occurred when *EE*10, 12–16:OH was absent or present at a 50% level, but not when it was present at 5%. Similar results occurred in the clothesline trial (Table 2). However, in both experiments there was a slightly greater response level for the two-component blend of the major component plus *EE*10,12–16:OH at a 5% level, than for the major component alone. The second bioassay indicated that a two-component blend with the alcohol at a 5% level was more attractive than that containing 50% of the alcohol (Figure 5). This was also possibly true of the three-component blends, although in that case the difference was not statistically significant. The third bioassay also showed that the three-component blend containing *EE*10,12–16:OH, increased lure approaches relative to the major component alone. As a 5% level of *EE*10,12–16:OH was most consistent with the estimate of 2–5% from GC analyses, this was the amount used in the field trapping experiments.

Thus, the trapping experiments in Benin were carried out against a background of previous experiments that had given good, but not conclusive, evidence for *EE*10,12–16:OH and *E*10–16:Ald as candidate minor components of the pheromone. However, in all previous bioassays, even the best synthetic blend was less attractive than the natural pheromone.

The first trapping experiment clearly indicated that all three components, *EE*10,12–16:Ald, *EE*10,12–16:OH, and *E*10–16:Ald, were essential for optimal attraction of male *M. vitrata* moths when tested in 100:5:5 ratio, respectively (Table 3). Neither two-component blend tested attracted more males than the single component alone, but the presence of both minor components together produced a fourfold increase in catches. This blend also attracted far more moths than virgin females, presumably because the synthetic lures emitted pheromone continuously, while the females did not "call" continuously.

Surprisingly, results of the second trapping experiment indicated that amounts of the two minor components, when varied together over the range 0.1–50% of the major component, made no significant difference to catch levels (Table 4). This

was not consistent with results of the third field experiment, which showed that three-component blends with the monoene aldehyde present at 0.5% or less were no more attractive than the single- or two-component blends. If *E*10–16:Ald was present at 5%, mean catches were more than doubled (Table 5), and results of this third field experiment are reasonably consistent with laboratory analytical results that indicated the amounts of any hexadecenal isomer present in female ovipositor washings were less than 2% relative to the major pheromone component.

Since none of the lure dose or dispenser combinations tested in the first trapping experiment produced significantly different catches (Table 3), it appears that release-rate differences, within the lure dose range tested, do not have much effect on attractiveness. However, the polyethylene vial lures containing the blend based on 0.1 mg of *EE*10,12–16:Ald provided slightly greater trap catches, and they are expected to have greater longevity in traps than 0.01-mg lures. Rubber septa pheromone dispensers are anticipated to have a shorter field life than polyethylene vials based on the analyses of pheromone remaining after 2 weeks of exposure (Hall, unpublished). Thus, in subsequent experimentation polyethylene vials loaded with the three-component blend based on 0.1 mg *EE*10,12–16:Ald have been adopted as standard.

While *EE*10,12–16:Ald and *EE*10,12–16:OH have been identified as female sex pheromone components in several other lepidopteran species in the Noctuidae and Heliothinae, as well as Pyralidae (Arn et al., 2002), *E*10–16:Ald has only previously been reported as a pheromone component in *Dichrocosis (Conogethes) punctiferalis* and possibly *D. chlorophanta* (Lepidoptera: Pyralidae) by Konno et al. (1982). It was of interest that males of *D. indica* were attracted to blends containing *EE*10,12–16:Ald, *EE*10,12–16:OH, and *E*11–16:Ald in the clothesline field trial but not to binary blends of these components. Wakamura et al. (1998) subsequently reported *E*11–16:Ald and *EE*10,12–16:Ald to be pheromone components of this species, but blends of these two compounds were not as attractive as virgin female moths. The results described here would suggest *EE*10,12–16:OH could be a third pheromone component for this species.

Although there is strong circumstantial evidence for *E*10–16:Ald being a component of the female sex pheromone of *M. vitrata*, production of this compound by female *M. vitrata* has not been conclusively demonstrated, and so it remains strictly a candidate component of the pheromone. However, from a practical trapping standpoint, the development of an effective synthetic lure for *M. vitrata* is considered complete, at least for W. Africa. It consists of a polyethylene vial impregnated with *EE*10,12–16:Ald (100 μg), *EE*10,12–16:OH (5μg), and *E*10–16:Ald (5 μg). This is the first time a synthetic pheromone blend has been identified that is more attractive to male *M. vitrata* than virgin female moths under field conditions.

However, three other aspects of our findings show that pheromonal attraction in *M. vitrata* is not yet fully understood. These are the consistently weaker

responses of males to synthetic pheromones in laboratory bioassays, uncertainty over the effect of varying the relative amounts of minor blend components, and the attraction of female moths to synthetic lures in traps. In relation to the first two points, we consider it unlikely that other pheromone components remain to be identified in view of the extensive amount of work carried out, although there may be scope for some further optimization of the ratio of the three components.

The phenomenon of attraction of female *M. vitrata* to synthetic lures was striking. To our knowledge, there is only one other moth species for which captures of females in traps baited with the synthetic version of the female-produced pheromone have been reported, i.e., the noctuid, *Trichoplusia ni* (Mitchell et al., 1972; Birch, 1977). In this case, it was subsequently shown that males produce a pheromone (Heath et al., 1992) that attracts females under field conditions (Landolt, 1995). The probable explanation for the observations of Mitchell et al. (1972) and Birch (1977) was, thus, attraction of females to previously trapped males (Landolt, 1995). In recent laboratory work at NRI, the possibility of female attraction to a male-produced pheromone was not supported by the wind-tunnel results of Mondhe (2001). Our observation that moth captures in individual traps in the field were frequently exclusively female also argues against this explanation. Mondhe (2001) also found that neither *EE*10,12–16:Ald, *EE*10,12–16:OH, nor *E*10–16:Ald elicited EAG responses from female *M. vitrata* moths, so direct attraction of females to the pheromone lures seems unlikely. However, these data were limited in extent, and further investigations are required. An important further point to explain would be the variation in the proportion of female captures (see Tables 3–5). It may be that behavior of *M. vitrata* in the field is affected by factors such as host plant volatiles not tested by Mondhe (2001). Regardless of explanation, catches of females may actually improve the predictive power of traps, since catches of both sexes should more accurately reflect population events.

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FORAGING MODE AND EVOLUTION OF STRIKE-INDUCED CHEMOSENSORY SEARCHING IN LIZARDS

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Abstract—Strike-induced chemosensory searching (SICS) in lizards and snakes is a means of relocating prey by scent-trailing. The two main components of SICS are an elevated tongue-flick rate for vomerolfactory sampling after biting prey (PETF) and searching movements. In combination, these behaviors permit scent-trailing. Prey chemical discrimination, which is a prerequisite for SICS, is present in active foragers, but not in ambush foragers. Using comparative data, I show that searching movements and SICS have undergone correlated evolution with foraging mode and with prey chemical discrimination in lizards. This suggests that active foraging selects for prey chemical discrimination, which is then employed to search for escaped prey using the typical movements and tongue-flicking behaviors of active foragers. SICS in lizards is simply heightened active foraging after biting prey. In nonvenomous snakes, SICS is similar to that in lizards but is not restricted to active foragers. Only highly venomous snakes voluntarily release dangerous prey upon envenomation, pause to let the venom incapacitate the prey, and then relocate the prey by scent-trailing. PETF was observed in two ambush foragers and is not evolutionarily correlated with foraging mode or searching movements. Because it occurs in species lacking prey chemical discrimination, such PETF may be a response to gustatory cues or to internal chemicals not encountered on surfaces or trails of uninjured prey.

Key Words—Behavior, foraging mode, prey chemical discrimination, strike-induced chemosensory searching, tongue-flicking, vomerolfaction, Squamata.

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INTRODUCTION

Strike-Induced Chemosensory Searching. Venomous snakes, especially viperids, often strike, envenomate, and then release prey rather than holding it. After remaining at rest for several moments, the snake begins to tongue-flick at a high rate and relocates the prey by scent-trailing. This behavior pattern, studied first and in greatest detail in rattlesnakes, is strike-induced chemosensory searching (SICS), which allows the snake to reduce the risk of being injured by the prey and to relocate the prey after it has been incapacitated by the venom (Chiszar and Scudder, 1980a; Chiszar et al., 1983). Further studies showed that SICS occurs in a wide range of venomous snakes, including rattlesnakes and other pit vipers, Old World viperines, and elapids such as cobras and taipans (e.g., O'Connell et al., 1985; Chiszar et al., 1986a–c; Radcliffe et al., 1986).

SICS, but not voluntary release, is present in nonvenomous snakes, such as colubrids (Chiszar and Scudder, 1980b) and pythonids (Cooper, 1991a), and in many lizards (e.g., Cooper, 1994a; Cooper and Arnett, 1995). Because it is widespread among nonvenomous squamate reptiles not descended from venomous taxa, SICS must have evolved independently of envenomation. It is believed that lizards and nonvenomous snakes that do not voluntarily release prey use SICS to relocate prey that has been bitten and has escaped or been dropped (Cooper, 1993a, 1994a). The duration of SICS is much shorter in lizards and some nonvenomous snakes than in venomous snakes (Chiszar and Scudder, 1980a; Chiszar et al., 1985; Cooper, 1991a, 1993a; Cooper et al., 1994). Additional data on duration of SICS are needed for a variety nonvenomous snakes of both actively and ambush foraging species to test the hypothesis that SICS has been modified in venomous snakes to increase the probability of locating prey that is known to be nearby and is likely to remain available because it has been killed by the venom. For most lizards and at least some nonvenomous snakes, the shorter duration of SICS presumably reflects the greater likelihood that a prey not relocated rapidly has escaped (Cooper, 1993b).

Two main components of SICS are recognized, a poststrike elevation in tongue-flick rate (PETF) and scent trailing, which is accomplished by tongue-flicking the substrate to locate the trail and then following it (Chiszar et al., 1983; Cooper, 1991b). Both components occur in snakes and many lizards. Among lizards, scent-trailing has been demonstrated experimentally only in Helodermatidae and Varanidae (Garrett et al., 1996), but in many other lizards increased rates of movement associated with tongue-flicking have been observed following experimental removal of prey after it has been bitten (Table 1). These movements are presumed to indicate attempted relocation of prey by scent-trailing. The form of tongue-flicking is similar in lizards and nonvenomous snakes, but the mechanics of locomotion differ somewhat due to the limblessness of snakes. Other lizard species lacking SICS exhibit neither PETF nor searching movements following

TABLE 1. CHARACTER STATES OF PETF, SEARCHING MOVEMENTS, SICS, PREY CHEMICAL DISCRIMINATION, AND FORAGING MODE FOR LIZARDS IN WHICH SICS HAS BEEN STUDIED AND THE TUATARA *Sphenodon punctatus* AS AN OUTGROUP[a]

Taxon	PETF	SM	SICS	PCD	FM	Sources
Rhynchocephalia						
Sphenodon punctatus	–	–	–	–	AM	1–3
Iguania						
Agama agama	–	–	–	–	AM	4–5
Anolis carolinensis	–	–	–	–	AM	5–6
Crotaphyytus collaris	–	–	–	–	AM	7–8
Dipsosaurus dorsalis	+	+	+	+	H	5
Liolaemus zapallarensis	+	–	–	–	AM	5, 9
Sceloporus clarkii	–	–	–	–	AM	4, 5
Sceloporus poinsettii	–	–	–	–	AM	4, 5
Scleroglossa						
Cordylus cordylus	–	–	–	–	AM	10–12
Elgaria coerulea	+	+	+	+	AC	5, 13
Eublepharis macularius	+	+	+	+	AC	5, 10, 14
Eumeces laticeps	+	+	+	+	AC	8, 15
Gekko gecko	+	–	–	–	AM	11, 16
Gerrhosaurus nigrolineatus	+	+	+	+	AC	13, 17
Heloderma suspectum	+	+	+	+	AC	5, 18, 19
Podarcis muralis	+	+	+	+	AC	5
Tupinambis nigropunctatus	+	+	+	+	AC	5
Varanus exanthematicus	+	+	+	+	AC	5

[a] PETF: post-strike elevation in tongue-flick rate; SM: searching movements; SICS: strike-induced chemosensory searching; PCD: prey chemical discrimination; FM: foraging mode (AC: active; AM: ambush; H: herbivore). Some foraging modes are based on data for close relatives.

[b] 1, Cooper et al. (2001a); 2, Walls (1981); 3, Walls (1983); 4, Cooper (1994b); 5, Cooper (1994a,c); 6, DePerno and Cooper (1994); 7, Cooper et al. (1996b); 8, Cooper et al. (2001b); 9, DePerno and Cooper (1993); 10, Cooper (1997); 11, Cooper et al. (1995); 12, Cooper et al. (1997); 13, Cooper (1995b); 14, Cooper et al. (1996a); 15, Cooper (1992b); 16, Perry (1995); 17, Cooper (1992c); 18, Cooper and Arnett (1995); 19, Cooper et al. (1994).

removal of prey (Table 1). Two lizard species are exceptional in showing PETF, but no increase in associated movements (DePerno and Cooper, 1993; Cooper, 1994b).

These observations raise the question of the basis of variation among lizard taxa in SICS. Prey chemical discrimination, the ability to discriminate between prey chemicals and control stimuli, is important to many lizards for detection, identification, and location of prey (Cooper, 1994a,c). Other lizards lack this ability (Table 1). Comparative studies have shown that prey chemical discrimination occurs in active foragers and in herbivores, but not in ambush foragers, and that there has been correlated evolution between prey chemical discrimination and foraging mode (Cooper, 1995a, 1997, 2002, 2003). Prey chemical

discrimination is acquired when active foraging or herbivory evolves and is lost when ambush foraging evolves (Cooper, 1995a, 1997). Active foragers tongue-flick frequently while moving through the habitat searching for food, and often locate hidden prey using chemical cues (Bogert and Martin Del Campo, 1956; Evans, 1961; Auffenberg, 1984). Herbivores must move among plants to locate food and evaluate potential food by tongue-flicking. In contrast, prey chemical discrimination would be useless for locating prey in ambush foragers that wait immobile at ambush posts for prey to move into the visual field (Cooper, 1995a, 1997).

Does Foraging Mode Affect SICS? Differences in foraging mode among lizards might also explain interspecific variation in SICS. Ambush foragers may be predicted to lack SICS because they lack prey chemical discrimination based on chemical cues sampled by tongue-flicking and because their mobile prey (Huey and Pianka, 1981) are likely to have escaped if they are not visible. Active foragers are predicted to exhibit SICS because prey that has been bitten and dropped is likely to be injured and to still be nearby where it can be relocated, even if hidden, by using chemical cues. From these considerations it may be predicted that correlated evolution has occurred between SICS and foraging mode. Because foraging mode drives the evolution of prey chemical discrimination (Cooper, 1994c, 1995a, 1997) and prey chemical discrimination is a prerequisite for SICS, it may also be predicted that correlated evolution has occurred between prey chemical discrimination and SICS. Here, I present a review of the distribution of foraging mode and presence of SICS for lizard species in which SICS has been studied and a comparative study of the evolutionary relationship between SICS and foraging mode in lizards by using a statistical method that accounts for phylogenetic relationships among taxa.

Distributions of Foraging Mode and SICS. The two major taxonomic groups of squamate reptiles are Iguania and Scleroglossa, the latter including snakes (Estes et al., 1988). SICS has been studied in relatively few species of lizards, but those species span a broad taxonomic range, including representatives of the major families of Iguania and Scleroglossa (Table 1). PETF is absent in most of the iguanian species, being present only in the iguanid *Dipsosaurus dorsalis* and the liolaemid (Frost et al., 2001) *Liolaemus zapallarensis*, but is present in all scleroglossans studied except the cordylid *Cordylus cordylus*. Tongue-flicks touching substrates other than the lizard's own body did not increase after biting prey in the gekkonid *Gekko gecko*, but this species was conservatively considered to show PETF based on a postbite increase in numbers of labial licks, in which the tongue is partially protruded and wiped on the labial scales (Cooper et al., 1996a). The relationship between PETF and foraging mode would be stronger if *G. gecko* were treated as lacking PETF. The distribution of poststrike searching movements is similar to that of PETF, but searching movements are absent in *L. zapallarensis* and in the gekkonid *Gekko gecko* (Table 1).

Foraging mode in lizards is fixed in many families and in some larger groups (Cooper, 1994a,c, 1995a; Perry, 1995, 1999). Iguania consists entirely of ambush foragers plus some herbivores, but lacks carnivorous active foragers (Cooper, 1994a,c, 1995a). Within Scleroglossa, gekkonid geckos, cordylids, and a few skinks and lacertids are ambush foragers, but the remaining lizards, the majority, are active foragers (Cooper, 1994a, 1995a). Foraging modes of lizards for which data on SICS are available are shown in Table 1. The distribution of prey chemical discrimination in lizards parallels that for foraging mode exactly (Table 1).

METHODS AND MATERIALS

Evolutionary reconstructions of PETF and searching movements were made using the TRACE program in MacClade 3.01 (Maddison and Maddison, 1992). TRACE uses the phylogeny and character states in the terminal taxa to reconstruct ancestral character states under the assumption that evolution has been parsimonious. The assumption of parsimony within families is met for the data on foraging mode (Cooper, 1994a, 1995a; Perry, 1999) and for data on prey chemical discrimination (Cooper, 1994a,c, 1997). Although its applicability to intrafamilial variation of SICS remains to be demonstrated through further empirical work, search movements and SICS appear from available data to be stable in large taxonomic groups such as Anguimporpha (Anguidae, Helodermatidae, Varanidae and Serpentes) and Iguania other than the only iguanid studied (Table 1). Similarly, PETF occurs in only two of the iguanians, and in all but one of the scleroglossans. Because changes in character state have been rare, the assumption of parsimony appears to be justified.

For reconstruction of character states, the characters were assumed to be unordered. Character states were resolved by showing all most parsimonious states rather than by assuming that all changes occurred as early as possible on the phylogeny (ACCTRAN option) or were delayed until as late in the phylogeny as possible (DELTRAN). Based on previous studies, the common ancestor of Gekkonoidea (here represented by two geckos from different families) was considered to be an ambush forager lacking prey chemical discrimination (Cooper, 1995a, 1997). Reconstructions were made using *Sphenodon punctatus* as the outgroup because this species is a member of Rhynchocephalia, the sister group of Squamata. It is an ambush forager (Walls, 1981, 1983) that lacks prey chemical discrimination (Cooper et al., 2001a). It cannot be capable of SICS mediated by vomerolfaction (Cooper and Burghardt, 1990) due to lack of openings in the roof of the mouth leading to the vomeronasal organs, precluding transfer of lingually sampled chemicals to the vomeronasal organs (Schwenk, 1993).

Several chemosensory characters were used in studies defining higher taxa at and beyond the familial level (Estes et al., 1988; Schwenk, 1988). This may violate

to some extent the assumption by cladistic methods of reconstructing changes in character states that the characters being traced are independent of the phylogeny. However, the phylogeny of squamates used here is based on large numbers of characters (Estes et al., 1988), none being aspects of foraging mode and only a few (less than 3.4%) related to the structure of the tongue or to vomerolfaction, the sense that mediates prey chemical discrimination based on lingual chemical sampling in lizards and snakes (Halpern and Frumin, 1979; Cooper and Burghardt, 1990; Cooper and Alberts, 1991). Any effect of violating the assumption is likely to be trivial.

Because both foraging modes and chemosensory behaviors are often shared among taxa due to retention of character states in common ancestors, each species cannot provide an independent data point for use in correlation analyses (Brooks and McLennan, 1991; Harvey and Pagel, 1991). Tests for correlated evolution were conducted using concentrated-changes tests, which accounts for phylogenetic relationships and is appropriate for binary variables (Maddison, 1990; Maddison and Maddison, 1992). The concentrated-changes test requires fewer changes in character states to attain a reasonably high statistical power than other tests for discrete variables (Ridley, 1983; Sillén-Tullberg, 1993). Although it does not utilize information about branch lengths, it performs well in simulation studies, and its errors tend to make it overly conservative (Pagel, 1994; Lorch and Eadie, 1999).

In concentrated-changes tests, the independent variable is considered to cause changes in the dependent variable. Foraging mode was assumed to be the causative agent for changes in searching movements after biting prey and SICS. The concentrated-changes tests calculate the probability that the observed changes in searching movements and SICS would occur by chance in association with the changes in foraging mode on the phylogeny. Due to the established causal relationship and correspondence between foraging mode and prey chemical discrimination, prey chemical discrimination was considered the independent variable in tests for correlated evolution between that trait and SICS, the dependent variable.

Concentrated-changes tests assume that the phylogeny is correct. The primary phylogeny used here is based primarily on Estes et al. (1988), modified for Iguania according to Macey et al. (1997) and Schulte et al. (1998). There is some uncertainty regarding relationships of snakes, which are considered to be members of Varanoidea (Schwenk, 1993), but the analyses have been restricted to nonsnake lizards. Relationships among several iguanian families are also disputed. However, Acrodonta, represented here by *A. agama*, is the sister taxon of the remaining iguanians. Because the only controversial cases that could possibly affect the analyses involve the placement of *L. zapallarensis* and *D. dorsalis* relative to other nonacrodont iguanisans,analyses were conducted using a single preferred phylogeny and several alternative phylogenies in which the relationships among nonacrodont iguanian families were varied.

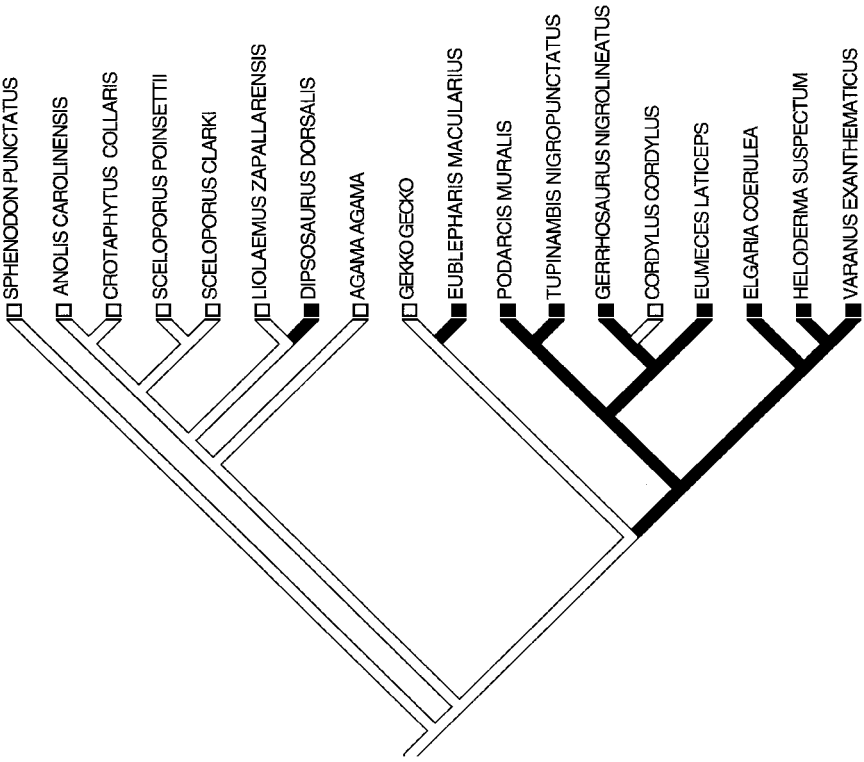

FIG. 1. The distributions of searching movements, SICS, foraging mode, and prey chemical discrimination on the preferred phylogeny of lizards are identical. Searching movements, SICS, prey chemical discrimination and active foraging (or herbivory) are present on black branches. Lizards on white branches are ambush foragers lacking the other behaviors.

RESULTS

The phylogenetic distributions of foraging mode, searching movements after biting prey, SICS, and prey chemical discrimination are identical (Figure 1). The ancestral states for lizards were ambush foraging and the absence of prey chemical discrimination, searching movements, and SICS. On the phylogeny three coincident gains and one coincident loss of active foraging, prey chemical discrimination, searching movements, and SICS occurred. Concentrated-changes tests for the phylogeny in Figure 1 showed that correlated evolution has occurred between searching movements and both foraging mode and prey chemical discrimination ($P < 0.018$ each). Identical correlations were found between SICS and both foraging mode and prey chemical discrimination.

In alternative analyses, all 11 correlations for alternative phylogenies in which the relationships among nonacrodont iguanians were varied were significant at approximately the same level ($0.016 < P < 0.018$). When *D. dorsalis* was excluded, restricting the analysis to typical active foragers and ambushers of animal prey, there were two gains and one loss of active foraging, prey chemical discrimination, searching movements, and SICS. Concentrated changes tests using all 12 phylogenies were marginally significant ($0.051 < P < 0.054$).

The distribution of PETF (Figure 2) differs from that of the other variables in that there have been only two gains and one loss and that the gains occurred deeper in the phylogenetic tree. One gain of PETF occurred in the common ancestor of *L. zapallarensis* and *D. dorsalis*, the other in the common ancestor of Scleroglossa. No gains occurred on branches where active foraging, prey chemical discrimination, searching movements, or SICS were gained or even occurred. Thus, although the single loss of PETF occurred in the same taxon in which active foraging, prey

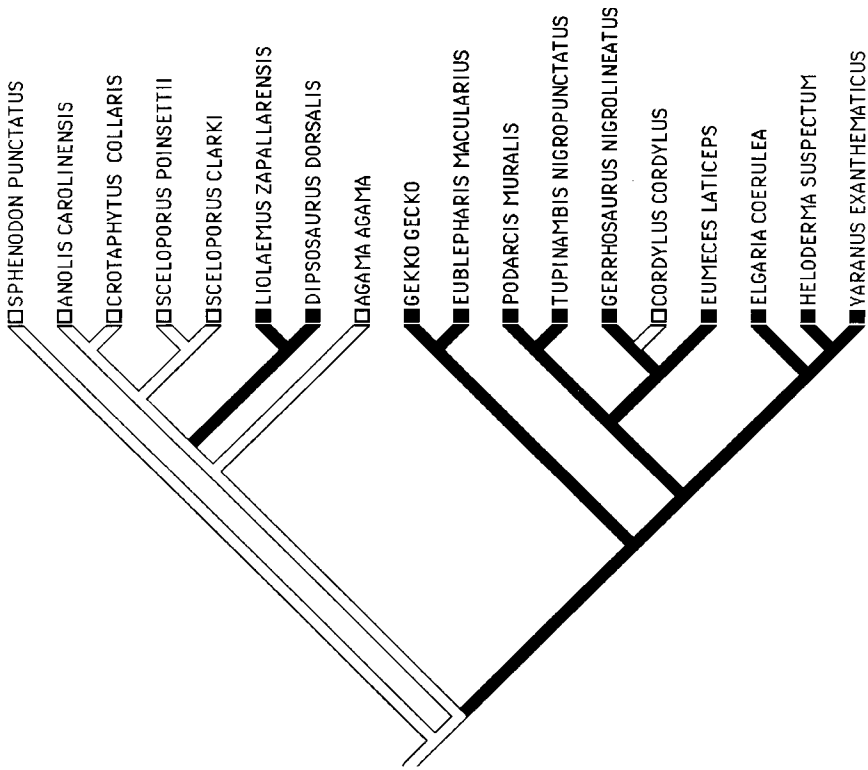

FIG. 2. The phylogenetic distribution of poststrike elevation in tongue-flick rate (PETF) differs from that of the other behaviors. PETF occurs on black branches only.

chemical discrimination, searching movements, and SICS were lost, there is no close phylogenetic correspondence between acquisition of PETF and these behaviors. No correlated evolution was detected between PETF and the remaining behaviors for any of the 12 phylogenies ($P > 0.10$ each).

DISCUSSION

Correlated evolution has occurred between SICS and foraging mode. This relationship was significant despite the small number of changes in foraging mode in the data set because each change in foraging mode was accompanied by the predicted shift in SICS on the same branch of the phylogeny. Relatively few changes in foraging mode have occurred in the evolutionary history of lizards (Cooper, 1994a,c, 1995a). Data on taxa representing additional shifts in foraging mode would be valuable, especially data for fairly strict insectivores because the findings for taxa other than herbivores were only marginally significant. Despite this limitation, the results support the predicted relationship between foraging mode and SICS. The findings appear to be robust under a wide variety of possible phylogenetic relationships among nonacrodont iguanians.

There are no data on the frequency of prey loss after biting, but laboratory observations of feeding by the skink *Eumeces laticeps* show that some prey types are frequently dropped after being bitten, including large beetles, grasshoppers, mutillid wasps, millipedes, and some caterpillars (Vitt and Cooper, 1986, unpublished data). I tentatively conclude that SICS may have evolved as a means of relocating lost prey in active foragers. Alternative hypotheses are that SICS has evolved to facilitate location of similar prey likely to be nearby in prey species that aggregate or that a combination of relocation of lost prey and search for similar prey has selected for SICS.

The phylogenetic distributions of searching movements and SICS were identical, indicating that all species exhibiting searching movements also exhibited PETF. However, PETF occurred in two species of ambush foragers that lacked searching movements and SICS. Consequently, no evidence was found suggesting that correlated evolution has occurred between PETF and foraging mode. The lack of correspondence between PETF and either foraging mode or prey chemical discrimination suggests that PETF in some cases might have a different sensory basis than prey chemical discrimination. Because it occurs after prey has been bitten, PETF might be mediated by taste in taxa that lack prey chemical discrimination mediated by vomerolfaction. In active foragers, PETF might be mediated by gustation, vomerolfaction, or both. Another possibility is that PETF in ambush foragers is a response to internal prey chemicals not normally encountered as cues to uninjured prey. Mammalian blood, for example, elicits exceptionally strong responses from the brown tree snake, *Boiga irregularis* (Chiszar et al., 1993).

In ambush foragers lacking prey chemical discrimination, PETF based on labial licking might even reflect a cleaning response rather than a chemosensory one.

SICS and searching movements have also undergone correlated evolution with prey chemical discrimination. Because searching movements and SICS occur in all of the active foragers, all of which are capable of prey chemical discrimination, the most likely adaptive scenario is that active foraging selectively favors prey chemical discrimination for identification and location of food and that once prey chemical discrimination evolves, it is used in conjunction with searching movements to relocate lost prey.

In the evolution of chemosensory relocation of prey by squamates, the critical first step appears to have been transition from ambush foraging to active foraging. The most elaborate development of chemosensory prey relocation occurs in highly venomous snakes that employ a strike-release-trail strategy (Chiszar and Scudder, 1980a; Chiszar et al., 1983) having four components (five in Cooper et al., 1994). First, the rate of tongue-flicking must increase after losing prey (PETF). Second, locomotion while tongue-flicking is necessary to locate and follow the prey's scent trail (searching movements for scent trailing). These behaviors have evolved together with prey chemical discrimination in actively foraging lizards, which tongue-flick while moving to initially locate prey, and presumably use the same behaviors to relocate lost prey. They are the evolutionary precursors of the strike-release-trail strategy, which additionally involves voluntary release of prey following envenomation and a pause before initiating scent-trailing to allow the venom to incapacitate the prey (Chiszar and Scudder, 1980a; Chiszar et al., 1983; Cooper et al., 1994).

All of the few nonvenomous snakes tested exhibit SICS regardless of foraging mode (Chiszar and Scudder, 1980b; Cooper et al., 1989; Cooper, 1991a, 1992a; Burghardt and Chmura, 1993). Loss of a link between foraging mode and SICS in snakes may be a consequence of eating large prey, limited escape ability by prey, and/or simultaneous capture and sequential consumption of several prey by predators that are capable of prey chemical discrimination. Large potential energy gain may justify searching for large, scarce prey known to be in the area and possibly injured enough to impair escape ability after being bitten. Prey that are often aggregated, such as neonatal mammals and hatchling birds, may favor chemosensory searching after losing one prey item or after eating for snakes of either foraging mode. A possible role of simultaneous prey processing is suggested by laboratory observations of constrictors biting one prey item and simultaneously constricting others (personal observations). After swallowing one prey item, the snake locates other prey by tongue-flicking and crawling.

Despite the presence of SICS in nonvenomous snakes, such snakes do not voluntarily release prey because it would be likely to escape. Venom to reliably kill prey is necessary for evolution of the final two components of chemosensory relocation of prey. The strike-release-trail strategy is feasible only if the prey

remains in the area after being released and is needed only for prey that can injure the predator, such as the typical large prey of many snakes (Radcliffe et al., 1980). Venom allows minimal contact with the prey during a brief envenomation. During the brief pause prior to scent-trailing, the venom quickly disables the prey's capacity to harm the snake and ensures that the prey will remain nearby even though it may wander out of the snake's visual field before succumbing.

In summary, chemosensory searching appears to have undergone several phases during the evolutionary history of squamates. It was absent in the common ancestor of Squamata and remained so in ambush foraging iguanian and gekkonid lizards. It evolved in actively foraging lizards and at least one herbivore. Although reversion to ambush foraging in lizards has been accompanied by loss of SICS, SICS appears to be ubiquitous in snakes, even among ambush foragers. Finally, the strike-release trail strategy has evolved in highly venomous species as a means of reducing the likelihood of being injured by prey.

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PHEROMONALLY MEDIATED SEXUAL ISOLATION AMONG DENNING POPULATIONS OF RED-SIDED GARTER SNAKES, *Thamnophis sirtalis parietalis*

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Abstract—Utilizing behavioral experiments and chemical analyses, we examined whether pheromonally mediated sexual isolation exists between denning populations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada. Simultaneous choice tests conducted during the breeding season revealed that adult males from a hibernaculum in central Manitoba displayed a strong courtship preference for females from their own population over females from a hibernaculum in western Manitoba, whereas males from the western Manitoba hibernaculum showed no such preference. In addition, trailing experiments testing the response of males from the two hibernacula to familiar and unfamiliar female trails showed similar results, demonstrating that the observed male preference is mediated through chemical cues. Subsequent chemical analysis of the female sexual attractiveness pheromone, a homologous series of long-chain saturated and ω-9 *cis*-unsaturated methyl ketones responsible for eliciting male courtship behavior and trailing behavior in garter snakes, showed significant variation in the composition of the pheromone between the two populations. Specifically, the two populations varied in the relative concentrations of individual unsaturated methyl ketones expressed by females. These results suggest that sexual isolation exists to a degree among denning populations of red-sided garter snakes due to variation in the expression of the female sexual attractiveness pheromone.

Key Words—Sexual isolation, sexual attractiveness pheromone, qualitative variation, methyl ketones, red-sided garter snake, *Thamnophis sirtalis parietalis.*

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INTRODUCTION

Studies utilizing insect models have dominated pheromone research in recent decades, resulting in the chemical identification and characterization of over a thousand pheromones (Abelson, 1985). Knowledge of the chemical structure of insect pheromones has allowed investigators to make significant advances in the understanding of how these chemical cues regulate insect behavior (Bell and Cardé, 1984; Eisner and Meinwald, 1995). In contrast to the abundance of pheromones identified in insects, few vertebrate pheromones have been isolated and identified to date. These few include pheromones for fish (e.g., goldfish—Stacey and Sorenson, 1986), amphibians (e.g., newts—Kikuyama et al., 1995; salamanders—Rollmann et al., 1999; frogs—Wabnitz et al., 1999), reptiles (e.g., snakes—Mason et al., 1989) and mammals (e.g., pig—Patterson, 1968; elephant—Rasmussen et al., 1996).

The lack of knowledge regarding the basic structure of vertebrate pheromones has hampered our efforts to understand vertebrate pheromone systems and has left many basic areas of research unexplored. For example, few studies have attempted to address intraspecific variation in the chemical composition of a vertebrate pheromone at the level of the population (e.g., Rollman et al., 2000). In insects, intraspecific variation in sex pheromone expression among geographically isolated populations is commonly observed (e.g., turnip moth—Löfstedt et al., 1986; Wu et al., 1999; corn borer—Huang et al., 1998). Such variation often results in the disruption of courtship and mating between the populations involved (e.g., pine beetle—Lanier et al., 1972; Miller et al., 1997). Similar occurrences of pheromonally mediated sexual isolation may exist within vertebrate species, but this has yet to be explored.

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is the most northern living reptile in North America (Logier and Toner, 1961). Annual aggregations of red-sided garter snakes at underground hibernacula in Manitoba, Canada, are unparalleled in scope, representing the highest concentration of snakes in the world (Gregory, 1984). Marshes, shallow lakes, and areas of poor drainage offer good summer feeding grounds for the snakes, while limestone bedrock provides hibernaculum sites where the snakes are constrained to spend up to eight months of the year to avoid the harsh winters (Aleksiuk and Stewart, 1971). A scarcity of suitable hibernation sites, coupled with a high degree of den fidelity and mating occurring almost exclusively at the dens (Gregory, 1974, 1977), has effectively divided the red-sided garter snakes in this region into geographically isolated populations defined by particular hibernacula.

Mating of the red-sided garter snake occurs directly at the hibernaculum following spring emergence (Gregory, 1974). Like most snakes (reviewed in Mason, 1992), the regulation of reproductive behaviors in the red-sided garter snake depends primarily on the production and expression of specific sex pheromones

(Mason, 1993). Of particular importance is the sexual attractiveness pheromone. Composed of a homologous series of saturated and ω-9 *cis*-unsaturated methyl ketones (Mason et al., 1989, 1990), this pheromone is sequestered in the skin lipids of females and is responsible for eliciting male courtship behavior (Noble, 1937; Garstka et al., 1982). If a male does not detect the sexual attractiveness pheromone, then courtship will not be initiated and subsequent mating will not occur (Mason, 1993).

The reliance of the red-sided garter snake on the sexual attractiveness pheromone to initiate reproductive behavior, coupled with its discontinuous population structure in Manitoba, offers a strong basis for investigating pheromonally mediated sexual isolation in this species. Here, we present a study initially designed to test the courtship preference of male red-sided garter snakes from two geographically isolated hibernacula to females from their own den versus females from the distant den. Because a courtship preference was detected for males from one of the dens, we set out to determine whether the observed preference was mediated through variation in the sexual attractiveness pheromone. This was accomplished by: (1) utilizing trailing experiments to determine whether the observed preference was mediated through chemical cues, and (2) performing chemical analyses to evaluate whether detectable variation existed in the female sexual attractiveness pheromone between the two hibernacula.

METHODS AND MATERIALS

Study Populations. Red-sided garter snakes used in this study were obtained from two hibernacula located approximately 240 km apart in Manitoba, Canada (Figure 1). The Narcisse den, located in the Interlake region of Manitoba (50°44′37″N, 97°31′26″W), houses in excess of 20,000 snakes during the winter months, whereas the Reeder den, located in western Manitoba (50°06′34″N, 101°06′32″W), shelters approximately 10,000 individuals (R.T. Mason, unpublished data). Snakes from both populations demonstrate similar temporal patterns; individuals emerge in late April, participate in a brief mating season at the hibernaculum, and then migrate to the summer feeding grounds before returning to the hibernacula in early September (Gregory, 1977). Adult females from the two populations attain an average snout–vent length (SVL) of 55–60 cm and a mean mass of 70–75 g. Adult males are smaller, attaining an average SVL of 45–50 cm and a mean mass of 35–40 g in the Narcisse population and an average SVL of 50–55 cm and a mean mass of 42–47 g in the Reeder population (Shine et al., 1999; M. P. LeMaster, unpublished data).

Behavioral Experiments

Experimental Animals. Unmated female red-sided garter snakes ($N = 11$ per hibernaculum) and sexually active adult male snakes ($N = 150$ per hibernaculum)

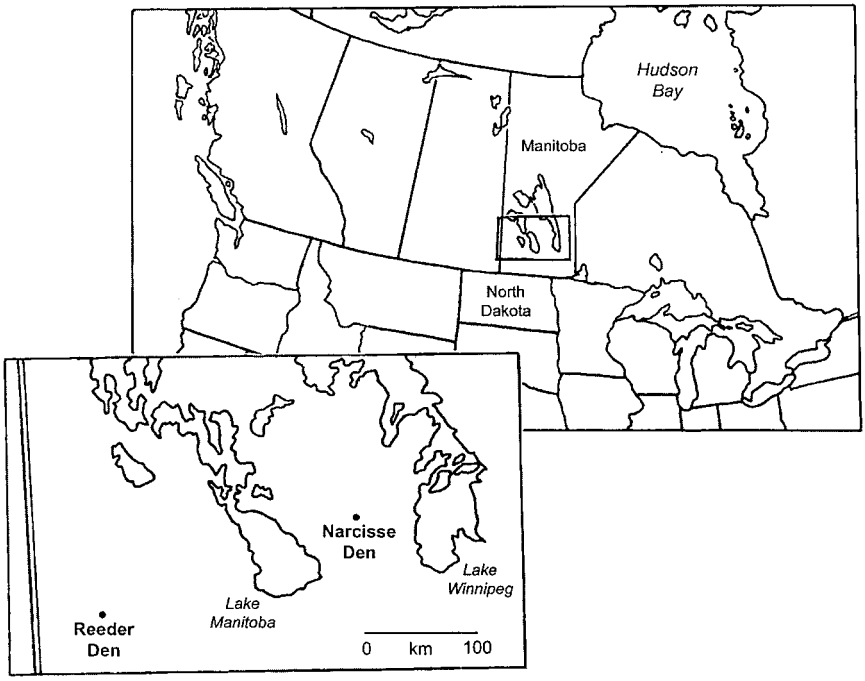

FIG. 1. Map of Manitoba, Canada, showing the location of the two populations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) used in this study. The Narcisse den population overwinters in a limestone sinkhole; the Reeder den population shelters in a shale outcrop.

were collected at the two hibernacula immediately upon emergence in May of 1998. To avoid any confounding effects due to temporal variation in mating behavior during the breeding season, snakes were collected from the two hibernacula over a single 24-hr period. Animals were segregated by sex and hibernaculum in cloth bags and returned to the Chatfield Research Station (Chatfield, Manitoba) where they were held at ambient temperature for 2 days until testing was initiated. Following testing, the animals were returned to their respective hibernacula and released.

Courtship Trials. To examine whether red-sided garter snakes preferentially court females from their own hibernaculum, we tested the courtship response of males from the Narcisse and Reeder dens to resident (same den) and nonresident (distant den) females utilizing a simultaneous choice test design (Mason and Crews, 1985). Briefly, we introduced 10 randomly chosen males from a particular den into

an outdoor arena [1 × 1 × 1 m; constructed of nylon cloth (Moore et al., 2000)] and allowed them to acclimate for 5 min. We then placed into each arena simultaneously a female from the Narcisse den and a female from the Reeder den. The two females were matched according to SVL, as male red-sided garter snakes demonstrate a strong courtship preference for females of larger size (Shine et al., 2001). In addition, tape was placed across the cloaca of each female to prevent females from mating during the trials, an event that drastically reduces further male courtship behavior (Garstka et al., 1982). Following the introduction of the females, the snakes were allowed to interact undisturbed for 5 min after which time the number of males actively courting each female was recorded.

We performed two experiments utilizing the simultaneous courtship test design to examine the courtship response of Narcisse den males (experiment 1; $N = 9$ trials) and Reeder den males (experiment 2; $N = 9$ trials) to resident and nonresident females. For each experiment, test males were replaced with new males following each trial. In addition, female pairs of similar size were only used once per experiment. Male courtship behavior was assessed using an ethogram of male garter snake mating behavior (Crews et al., 1984). For a male to be considered actively displaying courtship behavior during the trials, it was necessary for us to observe chin-rubbing by the male along the dorsal surface of the female. This behavior is only observed in a reproductive context and is, therefore, indicative of sexual behavior for males of this species. To test for significant male responses in the two experiments, the difference in the frequency of males courting females from their own hibernaculum versus females from the distant hibernaculum were analyzed by using χ^2 analyses (Zar, 1984). Level of significance for each test was set at $P < 0.05$.

Trailing Experiments. To determine whether the male snake courtship preference observed is mediated through skin-derived chemical cues, we tested the trailing response of male red-sided garter snakes when presented with resident and nonresident females on a standard Y maze previously described by LeMaster and Mason (2001). We performed two experiments testing the trailing preference of Narcisse den males (experiment 1; $N = 11$ trials) and Reeder den males (experiment 2; $N = 11$ trials). We also performed a control experiment utilizing Narcisse den males ($N = 10$ trials) at the onset of the study to verify that there was no bias in arm choice by males when no stimuli were present (both arms of the maze blank). In each experiment, unique males were used for each trial. In addition, female pairs (the same nine pairs used in the courtship experiments plus an additional two pairs) were used only once per experiment. Trials were conducted daily from 10:00-16:00 hr, when the snakes were normally active in the field (M. P. LeMaster, personal observation) and environmental conditions (e.g., temperature, lighting) remained constant throughout the experimental period. Statistical significance in male trailing behavior was analyzed utilizing one-way

binomial tests (Sokal and Rohlf, 1995). Level of significance for each test was set at $P < 0.05$.

For each trial, we randomly selected a pair of stimulus females and rubbed the dorsal surface of each on the surface of the maze. Trails were placed side by side on the base arm and then crossed over each other at the Y junction before exiting out separate, randomly chosen arms. The cloacal openings of the donor females were covered during transfer of the skin lipids so that contamination from cloacal gland secretions did not occur. We then placed a male test snake (Narcisse or Reeder den male depending on experiment) into an opaque box at the start of the base arm and allowed it to enter the maze of its own accord. Trials ended when the test snake completely entered one exit arm of the Y maze. Trials in which the test snake failed to exit the hide box after 10 mins, or trials in which the test snake fell from the maze were eliminated (<4% of trials performed). For all successful trials, we recorded the arm chosen by the test snake. We also noted whether the test snake displayed a trail contact response (TCR), an overt response characterized by a snake placing its chin in contact with the substrate and investigating with short, rapid tongue-flicks followed by subsequent movement along the preexisting trail (Brown and MacLean, 1983).

Chemical Analysis

Pheromone Collection. Adult, sexually attractive female snakes from the Narcisse den ($N = 10$) and the Reeder den ($N = 12$) were collected immediately upon spring emergence from the hibernacula in May of 1999. We focused our collection on females in a particular size range (SVL = 62.0–75.0 cm) as the sexual attractiveness pheromone of female red-sided garter snakes varies qualitatively in relation to female body length (LeMaster and Mason, 2002). The animals were killed with an overdose of brevital sodium, and each snake was then placed dorsal side down in a 500-ml glass beaker and covered with 25–50 ml of 100% hexane (Mason et al., 1989, 1990). Following a 12-hr immersion period, the animals were removed from the hexane, and the excess solvent was removed under reduced pressure by rotoevaporation at 35°C. The resulting residues were weighed on a digital scale (Mettler AT400), resuspended in fresh hexane (1–2 ml), and sealed in 9-ml glass vials with polyethylene-lined caps for storage at −20°C.

To isolate the methyl ketones composing the sexual attractiveness pheromone, we fractionated the skin lipid extracts using column chromatography as described by Mason et al. (1989). Briefly, we loaded the skin lipid extracts onto glass columns (350 mm × 22 mm ID) packed with alumina (activity III) and eluted the columns with hexane and ethyl ether solutions of increasing polarity. For each sample, the fraction containing the appropriate methyl ketones [fraction 5 (Mason et al., 1989)]

was collected, and the excess solvent was removed by rotoevaporation (35°C). The resulting methyl ketone residues were weighed on a digital scale (Mettler AT400) and resuspended in fresh hexane (1 ml). Samples were then placed in 9-ml glass vials with polyethylene-lined caps and stored at −20°C until further analysis.

Pheromone Analysis. Multicomponent pheromones, such as the sexual attractiveness pheromone of the red-sided garter snake, can show variation in both the quantity and quality of pheromone expressed. To examine variation in the quantity expressed by female snakes from the two hibernacula, we calculated the amount (micrograms) of methyl ketones expressed per unit (square centimeter) skin surface for individual females. This was accomplished by dividing the weight of the isolated methyl ketone residues extracted from a female by the total skin surface area. A general measure of skin surface area for each female was determined by multiplying the SVL of a female by its circumference at mid-body (Mason et al., 1990).

To examine variation in the quality of pheromone expressed by females from the two populations, we determined the number of unique methyl ketones expressed by individuals and compared the relative concentrations of individual methyl ketones comprising the overall pheromone profiles for the two populations. The methyl ketones present in the pheromone extracts were identified by utilizing a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 Series mass selective detector. Aliquots (1 μl) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m × 0.22 mm ID; Hewlett Packard) with helium as the carrier gas. Oven temperature was initially held at 70°C for 1 min, increased to 210°C at 30°C/min, held at 210°C for 1 min, increased to 310°C at 5°C/min, and finally held at 310°C for 5 min. Once the methyl ketones were identified, we calculated the relative concentrations of individual methyl ketones in each sample by using peak integration. Identification of compounds and peak areas were determined utilizing ChemStation software (Version B.02.05; Hewlett Packard) interfaced with the gas chromatograph–mass spectrometer.

Statistical significance in the amount of sexual attractiveness pheromone expressed on the dorsal surface of the female and the total number of unique methyl ketones expressed were examined using a Student *t* test and a Mann-Whitney rank sum test, respectively (Sokal and Rohlf, 1995). We used a randomization test (Manly, 1991) to test for a difference in the relative concentrations of individual methyl ketones composing the sexual attractiveness pheromone among the two dens. The test statistic was the Euclidean distance (S) between the average proportions of all individual methyl ketones observed. The randomization test generated a P value by comparing the observed test statistic to a simulated distribution based on 10,000 reshufflings of the data set. Level of significance for all tests was set at $P < 0.05$.

RESULTS

Behavioral Experiments

Courtship Experiments. The average SVL (± SD) of Narcisse females used in the simultaneous choice tests was 59.0 (± 8.9) cm, whereas the average SVL (± SD) for Reeder females was 60.1 (± 10.0) cm. When paired for individual trials, the average SVL difference (± SD) among females from the two hibernacula was 1.5 (± 1.2) cm. Narcisse den females were observed to be slightly larger in four of the nine trials for each experiment, while Reeder den females were slightly larger in the remaining five trials.

When presented with the females from the two hibernacula, male garter snakes responded with stereotypical courtship behaviors, including increased tongue-flick rate and chin rubbing along the dorsum of the females. Narcisse den males displayed a significant courtship preference for females from their own den over females from the Reeder den. Across the nine trials conducted, a greater proportion of males were observed courting the Narcisse den females over the Reeder den females following the 5-min interaction period $\chi^2 = 8.80$, 1 *df*, $P < 0.01$; 43.3% of males courted Narcisse den females versus 17.8% of males courting Reeder den females; Figure 2). Interestingly, Reeder den males did not demonstrate a similar courtship preference to the Narcisse den males across the nine trials conducted ($\chi^2 = 0.02$, 1 *df*, $P > 0.50$; 26.7% of males courted Reeder den females versus 25.6% of males courting Narcisse den females; Figure 2). We pooled the data for analyses after heterogeneity χ^2 tests showed no significant differences in male courtship preference among the individual trials conducted for each experiment (Narcisse males: $\chi^2 = 6.62$, 8 *df*, $P > 0.50$; Reeder males: $\chi^2 = 6.84$, 8 *df*, $P > 0.50$).

Trailing Experiments. When presented with the control maze (both arms blank), male red-sided garter snakes showed no preference for one arm over the other (binomial test; $P = 0.205$; Table 1). When tested with the simultaneous trails, males in both experiments responded with trail contact responses upon first exiting the start box, regardless of which trail a male might have initially encountered. Overall, Narcisse den males displayed a strong trailing preference for females from their own hibernaculum over females from the Reeder hibernaculum, choosing the arm with the Narcisse den female trail in 9 of the 11 trials conducted (binomial test; $P = 0.027$; Table 1). Reeder den males, however, showed no such preference among the trail types, appearing to randomly choose among the trails at the Y junction (binomial test; $P = 0.226$; Table 1).

Pheromone Analysis

Pheromone Quantity. There was no difference in the average SVL [Narcisse den females = 68.3 (± 4.9) cm; Reeder den females = 69.6 ± (6.6) cm: Student's

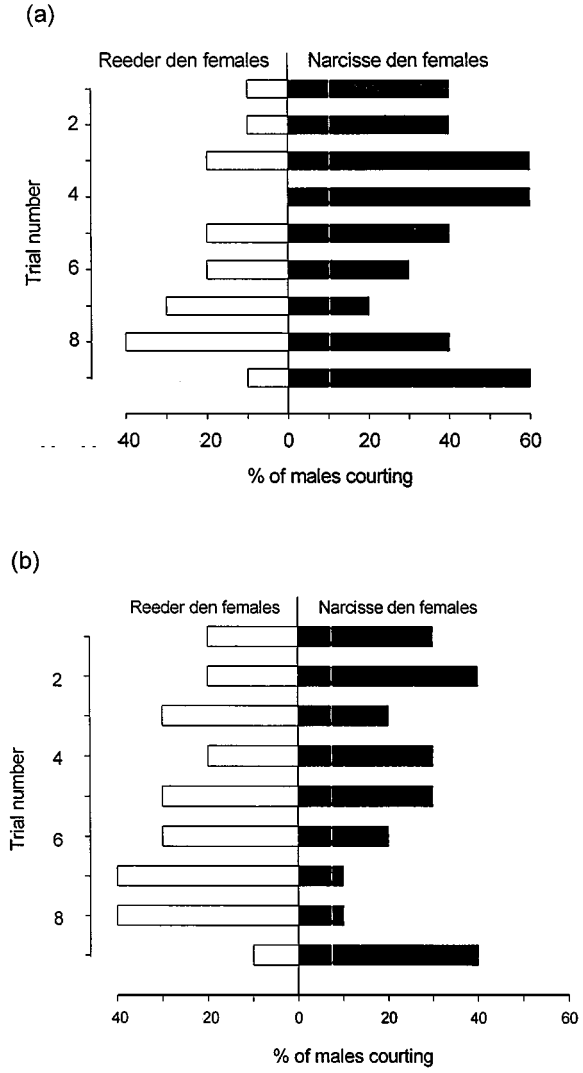

FIG. 2. Results of simultaneous choice tests in which a resident and a nonresident female red-sided garter snake (*Thamnophis sirtalis parietalis*) were placed in an outdoor arena with 10 courting males from (a) Narcisse den and (b) Reeder den. After 5 min the number of males courting each female was recorded.

TABLE 1. TRAILING RESPONSE OF MALE RED-SIDED GARTER SNAKES (*Thamnophis sirtalis parietalis*) FROM TWO HIBERNACULA TO FEMALES FROM THEIR OWN DEN VERSUS FEMALES FROM A DISTANT DEN WHEN PRESENTED SIMULTANEOUSLY ON A Y MAZE[a]

Condition	*N*	Arm treatment	Number of times selected	*P*
Control	10	Blank	6	0.205
		Blank	4	
Narcisse males tested on Narcisse versus Reeder female trails	11	Narcisse female	9	0.027
		Reeder female	2	
Reeder males tested on Narcisse versus Reeder female trails	11	Narcisse female	5	0.226
		Reeder female	6	

[a] The control experiment was performed utilizing Narcisse den males.

t test; $t = -0.522$, $P = 0.607$] or average mass [Narcisse den females = 114.7 (± 31.0) g; Reeder den females = 106.2 (± 28.7) g: Student's *t*-test; $t = -0.664$, $P = 0.514$] among the females sampled for pheromone analysis. The hexane extractions of individual females yielded an average (± SD) of 25.1 (± 8.8) mg of skin lipids per female from the Narcisse den and 22.8 (± 12.2) mg of skin lipids per female from the Reeder den. Subsequent fractionation of the lipids yielded an average (± SD) methyl ketone fraction of 4.8 (± 2.9) mg per Narcisse den female and 4.2 (± 2.4) mg per Reeder den female. Overall, the methyl ketones accounted for an average (± SD) of 20.0 (± 10.6)% of the skin lipids collected from the Narcisse den females and 19.5 (± 7.0)% of the skin lipids collected from the Reeder den females. After accounting for variation in skin surface area, we did not observe a significant difference between the females from the two hibernacula and the amount of methyl ketones extracted per unit of surface area (Student's *t* test; $t = 0.561$, $P = 0.581$; Table 2).

Pheromone Quality. Complete GC-MS analysis of the methyl ketone fractions revealed the presence of 18 unique long-chained methyl ketones (Figure 3).

TABLE 2. QUANTITATIVE AND QUALITATIVE VARIATION IN EXPRESSION OF SEXUAL ATTRACTIVENESS PHEROMONE AMONG FEMALE RED-SIDED GARTER SNAKES (*Thamnophis sirtalis parietalis*) FROM TWO DENNING POPULATIONS IN MANITOBA, CANADA[a]

Population	SVL (cm)	Mass (g)	Methyl ketone expression ($\mu g/cm^2$)	Unique methyl ketones expressed
Narcisse den ($N = 10$)	68.3 (± 4.9)	114.7 (± 31.0)	13.1 (± 7.9)	16.9 (± 0.57)
Reeder den ($N = 12$)	69.6 (± 6.6)	106.2 (± 28.7)	11.5 (± 5.1)	17.4 (± 0.52)

[a] Average (± SD) measurements are presented for each denning population.

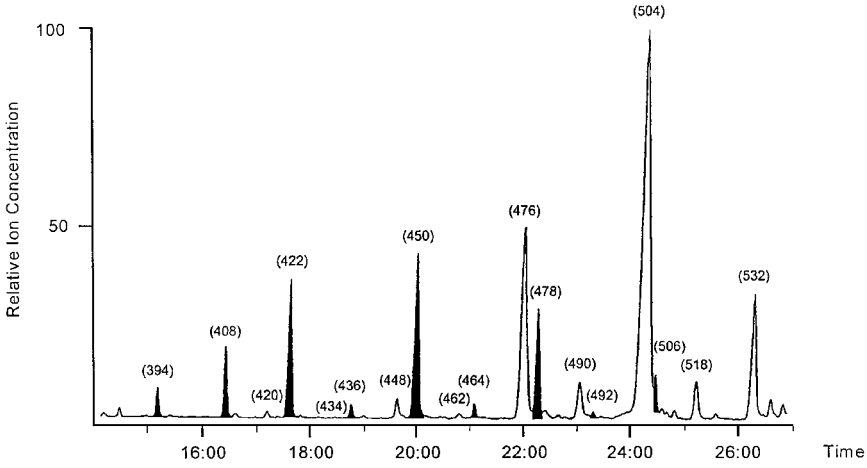

FIG. 3. Gas chromatogram of the female sexual attractiveness pheromone profile for the red-sided garter snake, *Thamnophis sirtalis parietalis*. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones.

Nine of these were identified as long-chain saturated methyl ketones, while the remaining nine had mass spectra in accord with long-chain ω-9 *cis*-unsaturated methyl ketones (Mason et al., 1990). The saturated methyl ketones ranged in size from 394 to 506 mass units, whereas the unsaturated methyl ketones ranged in size from 420 to 532 mass units. Females from the two hibernacula varied in the number of methyl ketones expressed in their skin lipids, ranging from pheromone profiles composed of 16 unique methyl ketones to pheromone profiles composed of all 18 unique methyl ketones. However, when we compared the average number of methyl ketones expressed by females from each hibernaculum, a significant relationship was not observed (Mann-Whitney rank sum test; $T = 92.5$, $P = 0.144$; Table 2).

Although the two hibernacula did not vary in the number of unique methyl ketones expressed, we did observe variation in the relative contribution of the individual methyl ketones composing the pheromone profiles for the two hibernacula (randomization test; S observed $= 0.14$, S generated $= 0.07$, $P = 0.038$; Figure 4a). Subsequent analyses showed that this difference was not due to variation between hibernacula in the relative contribution of total saturated versus unsaturated methyl ketones (t test; $t = 1.492$, $P = 0.151$). Instead, independent analyses of the two methyl ketone groups revealed that the two hibernacula varied in the relative concentration of individual unsaturated methyl ketones (randomization test; S observed $= 0.14$, S generated $= 0.06$, $P = 0.011$; Figure 4b), whereas the relative concentration of saturated methyl ketones remained stable among

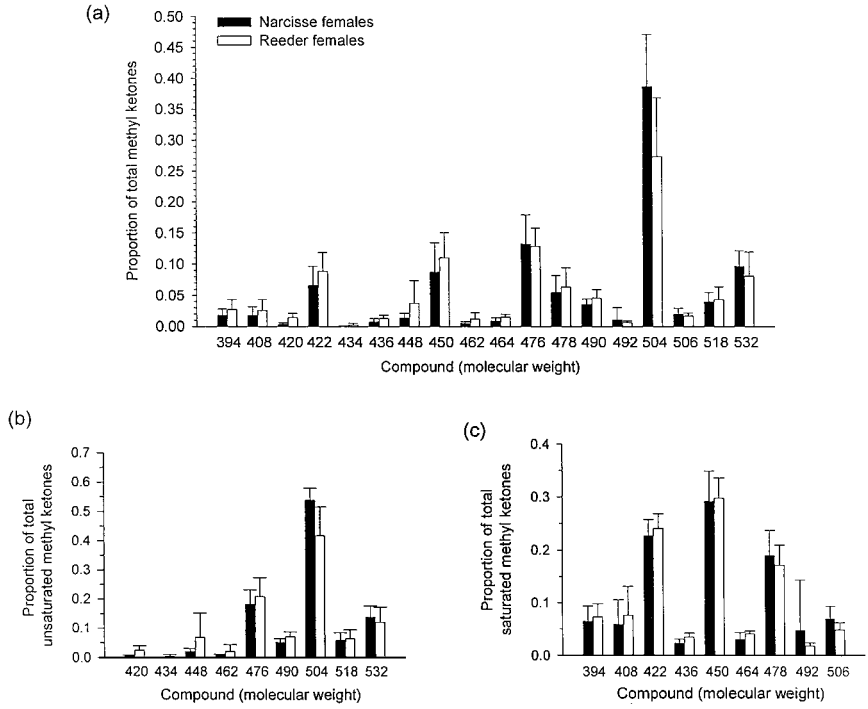

FIG. 4. Relative concentrations (± SD) of individual methyl ketones composing the sexual attractiveness pheromone of the red-sided garter snake (*Thamnophis sirtalis parietalis*) for females from the Narcisse den ($N = 10$) and the Reeder den ($N = 12$). Overall, there was significant variation in the pheromone profiles (a) with the majority of variation present in the relative concentration of individual unsaturated methyl ketones (b) and not in the individual saturated methyl ketones (c) (see text for statistical analyses).

dens (randomization test; S observed $= 0.07$, S generated $= 0.05$, $P = 0.195$; Figure 4c).

DISCUSSION

Intraspecific sexual isolation has been documented in a variety of vertebrates including fish (e.g., Ayvazian, 1993; Ziuganov, 1995), amphibians (e.g., Houck et al., 1988; Verrell and Arnold, 1989), and mammals (e.g., Pillay et al., 1995; Pillay, 2000). While multiple studies have utilized behavioral trials to explore the role of chemical cues in sexual isolation (e.g., Ovaska, 1989; Pillay et al., 1995), few studies have evaluated whether structural variation exists among populations for a particular chemical cue. For example, Rollman et al. (2000) observed biochemical

variation in a male courtship pheromone among populations of salamanders of the *Plethodon jordani-glutinosus* complex, suggesting that this pheromone might be responsible for the moderate levels of sexual isolation previously observed among populations of this species. However, behavioral trials testing the receptivity of females to pheromone extracts from various populations have yet to be performed. To our knowledge, the present study represents the first attempt to integrate behavioral experiments and chemical analyses towards understanding the role of chemical cues in sexual isolation within a vertebrate species.

The results of the courtship experiments demonstrate that sexual isolation exists to a degree among denning populations of red-sided garter snakes. Males from the Narcisse den displayed a courtship preference for females from their own den over those from the Reeder den. Interestingly, males from the Reeder den did not display a similar preference, indicating that male courtship preference in these two populations is asymmetrical. Similar instances of asymmetrical sexual isolation have been observed in other species (e.g., Krebs, 1990; Kobayashi et al., 2001), including vertebrates (e.g., Ayvazian, 1993; Pillay, 2000). Why these two denning populations of snakes show asymmetrical sexual isolation is not known. We suggest that this pattern might result from reproductive character displacement due to selective pressures to recognize conspecific mates; red-sided garter snakes in central Manitoba, unlike western Manitoba, share hibernacula with a second species of garter snake, the plains garter snake (*Thamnophis radix*). Thus, males in the Narcisse den may be more narrowly tuned to reproductive cues, allowing for discrimination among conspecific and heterospecific females, while the Reeder males, under no such pressure, are more broadly tuned. Alternatively, the pattern of sexual isolation observed in this study could represent a transient phenomenon, resulting from divergence in traits and mating preferences based on those traits through sexual selection acting within each population (Arnold et al., 1996).

Potentially, male red-sided garter snakes may utilize multiple cues to discriminate among females from various dens. Evidence suggests that both chemical and visual cues assist males of this species in selecting among potential mates during the breeding season (Shine and Mason, 2001). However, the results of the trailing experiments demonstrate that chemical cues alone are sufficient to allow males to discriminate among resident and nonresident females. When presented with the two female trails simultaneously, we observed males to display preferences similar to those observed in the courtship trials. In addition, the similarity in male preferences observed between the courtship and trailing experiments suggests that both behaviors are mediated by a similar chemical cue. In the case of the red-sided garter snake, that cue appears to be the female sexual attractiveness pheromone (Mason et al., 1989; LeMaster and Mason, 2001).

Subsequent analysis of the female sexual attractiveness pheromone from the two hibernacula demonstrated that it contains the necessary variation to function as a reliable indicator to males of a female's resident status. Specifically, females

were found to vary between dens in the relative concentration of individual unsaturated methyl ketones composing the sexual attractiveness pheromone. We were not surprised to observe that the variation was greatest with respect to the unsaturated methyl ketones. Although both unsaturated and saturated methyl ketones are necessary to elicit full male courtship, unsaturated methyl ketones appear to be the more biologically active of the two groups, eliciting a fivefold increase in male courtship behavior over saturated methyl ketones when presented in isolation (Mason et al., 1989). Thus, low levels of variation in the relative concentration of unsaturated methyl ketones between dens, as were observed in this study, are more likely to affect male courtship behavior than similar variation in saturated methyl ketones.

Why females from the two hibernacula vary in the relative concentration of individual methyl ketones is unclear. German cockroaches (*Blattella germanica*) produce a sex pheromone composed of methyl ketones (Schal et al., 1990), similar to those utilized by the red-sided garter snake. In the cockroach system, the pheromone derives its origins from fatty acid biosynthesis (reviewed in Tillman et al., 1999), suggesting that the sexual attractiveness pheromone of the red-sided garter snake is synthesized through a similar process. Thus, variation in the expression or activity levels of enzymes in the biosynthetic pathway for females from various hibernacula could result in the observed variation. In addition, the fact that males from the Narcisse den, but not the Reeder den, show a courtship preference for resident over nonresident females suggests that variation also exists among the two male detection systems. Such variation may be present at the level of the receptor neurons (e.g., Wu et al., 1999), located in the vomeronasal organ of the male snake (Halpern and Kubie, 1983) or may occur in higher processing centers, including the olfactory and accessory olfactory bulbs (Halpern, 1976). Future studies are necessary to resolve the underlying mechanism(s) responsible for variation of pheromone production and detection in this system.

The potential use of the female sexual attractiveness pheromone by male red-sided garter snakes to distinguish females from their own den over females from other dens suggests that this pheromone may represent a compound signal containing hierarchical mapping. Hierarchical mapping refers to signals in which differences in mean patterns are used to code for one question and different variants around that mean are then used to answer other questions (Bradbury and Vehrencamp, 1998). Thus, in the red-sided garter snake, the presence of saturated and unsaturated methyl ketones signifies the presence of a female, whereas variation in the relative concentration of individual unsaturated methyl ketones conveys the home locality. Other examples of compound signals containing hierarchical mapping in vertebrates can be found in electric fish (e.g., Crawford, 1992) and birds (e.g., Becker, 1982).

In summary, the results of this study demonstrate that pheromonally mediated sexual isolation exists to a degree among denning populations of red-sided

garter snakes. Male snakes from the Narcisse den displayed a courtship preference for females from their own den over females from the Reeder den, and this preference was maintained when males were presented with female trails. Further, we observed population-specific variation in the composition of the female sexual attractiveness pheromone, the pheromone responsible for mediating courtship and trailing behavior, strongly suggesting that this pheromone is responsible for the observed sexual isolation.

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(R)-ACETOIN-FEMALE SEX PHEROMONE OF THE SUMMER CHAFER *Amphimallon solstitiale* (L.)

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Abstract—Extracts of *Amphimallon solstitiale* (L.), a well known, widely distributed and rather common European scarab beetle, were analyzed by GC-MS and GC-EAD. Acetoin - (*R*):(*S*) > 9:1 - as well as 2,3-butanediol - (2*R*,3*R*):(2*S*,3*S*):*meso* = 1:1:9 - were present in extracts of both males and females. Although (2S,3S)-butanediol did not show any EAD activity, the other compounds elicited strong responses exclusively with male antennae. In contrast, several EAD active green leaf volatiles were detected equally well by male and female antennae. During preliminary field bioassays, (*R*)-acetoin was highly attractive to swarming males, whereas neither *rac*-acetoin nor the 2,3-butanediols showed activity. Therefore, (*R*)-acetoin is the female sex pheromone of *A. solstitiale.*

Key Words—Summer chafer, *Amphimallon solstitiale*, Coleoptera Scarabaeidae Melolonthinae sex pheromone (*R*)-acetoin (2*R*,3*R*)-butanediol *meso*-2,3-butanediol.

INTRODUCTION

The summer chafer, *Amphimallon solstitiale* (L.) (Coleoptera, Scarabaeidae), is distributed throughout most of Europe with a preference for dry and sandy habitats.

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The grubs undergo a two- or three-year development in the soil where they feed on roots of several plants. In middle Europe, adults occur from late June until the end of July. During this period, an intense swarming flight of the males can be observed at dusk on warm and dry days, starting around 21:00 (CET) and ending more or less abruptly towards 21:30. While the males are swarming, the females sit motionless on exposed places, presumably awaiting the males. This behavior suggests the existence of a female-produced sex pheromone involved in the mate finding process. In contrast to the cockchafers (*Melolontha* spp.), which are known to be attracted to several green leaf volatiles induced by feeding females (e.g., Ruther et al., 2000), the summer chafer does not feed during the flight period.

The aim of the present study was to identify the compounds involved in mate location of *A. solstitiale.* Whole body extracts of virgin female and male beetles were examined using GC-EAD and GC-MS. In addition, EAD activities of several green leaf volatiles (GLV), as well as other compounds known to be active in cockchafers, were tested. Field tests with the identified compounds were conducted to determine activity with swarming *A. solstitiale* males.

METHODS AND MATERIALS

Insects. Adults of *A. solstitiale* were taken from two populations in garden areas in the city of Hamburg/Germany. Virgin beetles were collected while emerging from the ground at dusk. In addition, swarming males were caught with a net, while calling females were picked from the vegetation during the males' swarming period. For natural samples, ten females or males were extracted for 2 hr with 30 ml of dichloromethane. Before analysis, the extracts were dried (Na_2SO_4) and carefully concentrated to 500 μl.

Coupled Gas Chromatography-Mass Spectrometry. Conditions for GC-MS analysis were the same as described earlier (Kozlov et al., 1996). Enantiomeric separations were achieved by using a fused silica capillary (25 m $\times$ 0.25 mm, 0.25 μm film thickness) coated with 2,3-diethyl-6-TBDMS-β-cyclodextrin (PS 086, MEGA, Legano, Italy), operated at 60°C.

Coupled Gas Chromatography-Electroantennographic Detection. GC-EAD analyses were performed on excised antennae of both sexes by using an HP 5890 GC operated under the GC-MS conditions with hydrogen as the carrier gas (2 ml/min). The EAD system and antennal preparations were the same as described earlier (Ruther et al., 2000).

Chemicals. (*R*)-Acetoin was synthesized by monobenzylation of commercially available (2R,3R)-butanediol (99% *ee*), employing sodium hydride and benzyl bromide, oxidation of the product with pyridinium dichromate, and debenzylation of the resulting ketone (H_2/Pd-C). Careful workup yielded (*R*)-acetoin in $>$ 97% *ee* and at least 98% chemical purity as determinated by (chiral) GC. Due to its high racemization rate, it was stored at -70°C until use. 1,4-Benzoquinone and the

23 green leaf volatiles used by Ruther et al. (2000) were included in the GC-EAD-tests. Apart from optically active acetoin, *rac*-acetoin and the 3 stereoisomers of 2,3-butanediol, as well as all other chemicals used in this study were purchased from Sigma-Aldrich (Steinheim, Germany), and showed chemical purities of at least 97%.

Field Tests. Funnel traps used in the field tests were identical to those described earlier (Ruther et al., 2000). Test chemicals dissolved in 500 μl CH_2Cl_2 were applied on balls of cotton wool. In a first test, carried out between July 9th and 14th 2001, 1 mg of (*R*)-acetoin was offered as the only synthetic beetle volatile released in a huge meadow in the *Stadtpark* of Hamburg, Germany. Male behavior towards the odor source was observed. In a second test, performed on July 17th 2002 at the same place, 18 traps were grouped into 3 sets with 6 traps each, and placed ca. 5 m above the ground in tall, old poplar trees. The distance between traps within one set was ca. 4 m, while the distances between the 3 sets were ca. 60 m. Each trap was baited with 5 mg of one pure compound, offered on cotton balls. In each set, 5 different compounds were used: (*R*)-acetoin, *rac*-acetoin, (2*R*,3*R*)-butanediol, meso-2,3-butanediol, and 1,4-benzoquinone, while an empty trap served as the control.

RESULTS

Extracts and GC-EAD. Acetoin as well as rac- und meso-2,3-butanediol were present in all extracts of both sexes ($N = 22$). The enantiomeric composition of the naturally occuring acetoin was determined to be (*R*):(*S*) of at least 9:1, while the 2,3-butanediol stereoisomers showed a ratio of (2*S*,3*S*):(2*R*,3*R*):*meso* = 1:1:9, respectively. EAG activity of the enantiomers was determined by using GC-EAD with a column coated with a modified cyclodextrin (Figure 1). (2*S*,3*S*)-Butanediol did not elicit any response in male or female antennae, while both enantiomers of acetoin as well as (2*R*,3*R*)- and *meso*-2,3-butanediol were highly active on male antennae. Dilution experiments showed stimulus thresholds to range in the sub-nanogram to low nanogram level. Female antennae did not respond to acetoin or to any 2,3-butanediol stereoisomer, which suggested these compounds are involved in the mate finding process. No other active compounds were found in the extracts, and no significant differences between the extracts of virgin and older beetles were observed.

1,4-Benzoquinone, absent in *A. solstitiale*, but a known semiochemical in other scarab beetles (Ruther et al., 2000; Imrei et al., 2002; Krell et al., 1999), also elicited strong responses exclusively on male antennae. In contrast, several green leaf volatiles were detected equally well by both sexes. These compounds were (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl butyrate, (±)- linalool, (*Z*)-3-hexenyl tiglate, (*E*)-2-hexen-1-ol, benzaldehyde, β-caryophyllene, 1-hexanol,

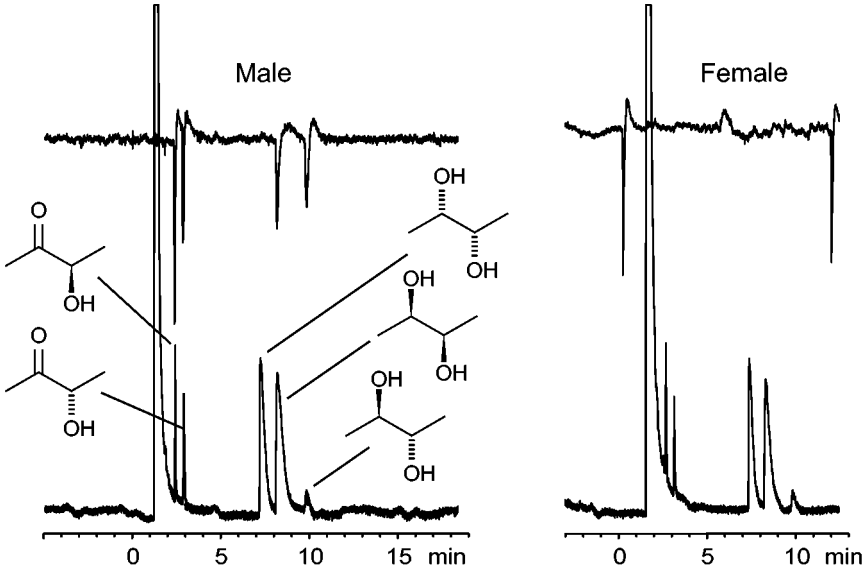

FIG. 1. GC-EAD responses by male and female antennae of *A. solstitiale* to synthetic racemic acetoin and 2,3-butanediol on an optically active stationary phase. The signals at 0 and 12.5 min (females) are due to puffs of a mixture of GLV directly into the make-up air stream of the EAD system to check the antennal preparation.

hexyl hexanoate, methyl salicylate, ethyl salicylate, benzyl alcohol, and 2-phenylethanol (Tolasch, unpublished data).

Field Bioassays. In the first test (2001), designed to check for the biological activity of (*R*)-acetoin, the compound was attractive to swarming males of *A. solstitiale*, indicated by the high flight activity around the odor source. The second experiment (2002) compared behavior releasing capacities of the compounds that had been found to be exclusively active on male antennae. Results are listed in Table 1. As can be seen, (*R*)-acetoin was the only active compound, showing high attractivity to swarming males of *A. solstitiale.*

DISCUSSION

Our study shows (*R*)-acetoin to be a sex pheromone of *A. solstitiale.* Although the ratio of acetoin enantiomers was found to be at least 9:1 (*R*:*S*), the material emanating from the beetles could be pure (*R*) since racemization may occur at room temperature. Because of this instability, the ratio of acetoin enantiomers may serve as a temporal signal in *A. solstitiale.*

TABLE 1. NUMBERS OF *A. solstitiale*-MALES CAPTURED WITH THREE SETS OF FUNNEL TRAPS

	Total numbers of captured males		
Compound (5 mg/trap)	Set 1	Set 2	Set 3
(*R*)-Acetoin	25	5	39
rac-Acetoin	0	0	0
(2*R*,3*R*)-Butanediol	0	0	0
meso-Butanediol	0	0	0
1,4-Benzoquinone	0	0	0
Empty control	1	0	0

Acetoin and the 2,3-butanediols seem to be rather widespread in scarab beetles. Recently, Nojima et al. (2002) showed (*R*)-acetoin, (2*R*,3*R*)-, and meso-2,3-butanediol to be female-produced and male antennal-specific EAD active compounds in *Rhizotrogus majalis*. In addition, acetoin (showing a bias of one enantiomer of 96:4) and a mixture of the 2,3-butanediol stereoisomers were found to be male-specific volatiles in *Scapanes australis*. In this species, a mixture of *rac*-acetoin and 2-butanol proved to be attractive to both sexes (Rochat et al., 2000, 2002).

Other EAD active substances in *A. solstitiale*, such as the beetle-produced *meso*- or (2*R*,3*R*)-butanediol have not yet been studied as possible additional pheromone components. In contrast, 1,4-benzoquinone is believed to act as an interspecific antagonist, due to its activity in other Melolonthinae, like the closely related and very similar *Rhizotrogus vernus* (Imrei et al., 2002) and the forest cockchafer *Melolontha hippocastani* (Ruther et al., 2001, 2002). Similar to the findings in *Melolontha* spp. (Ruther et al., 2001, 2002), the pheromone of *A. solstitiale* shows up in both males and females. This, as well as the agressive physiological properties of acetoin (as a carbonyl active Michael-donator it may react with many organic compounds including proteins), supports the hypothesis of the evolution of melolonthinae sex pheromones from defensive compounds (Leal, 1997).

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TO FIX OR NOT TO FIX: THE ROLE OF 2-PHENOXYETHANOL IN RABBIT, *Oryctolagus cuniculus*, CHIN GLAND SECRETION

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Abstract—The European rabbit (*Oryctolagus cuniculus*) uses the secretion of the chin gland to maintain dominance hierarchies in the wild. Recent work has investigated changes in the secretion when social status is manipulated in the rabbit. When a rabbit becomes dominant, a new compound appears in his secretion, 2-phenoxyethanol. This compound is used as a fixative in the perfume industry. This study investigates whether the compound performs a similar function in the secretion of the rabbit. 2-Phenoxyethanol is not detected olfactorially by rabbits, and slows the release rate of some of the compounds that occur naturally in rabbit chin gland secretion. We suggest that when a rabbit becomes dominant, he adds a fixative to his secretion so that his scent will persist in the environment and not dissipate. He will thus come to dominate the olfactory environment, in much the same way as he does the physical environment.

Key Words—Rabbit, *Oryctolagus cuniculus*, fixative, chemical communication, semiochemical, social structure, glandular secretion.

INTRODUCTION

European rabbits (*Oryctolagus cuniculus*) live together in social groups varying in size from 2 to 20 adults (Cowan and Bell, 1986). Strict dominance hierarchies are maintained within the social group (Mykytowycz, 1958; Lockley, 1961; Cowan and Bell, 1986), and it has been suggested that the secretion of the submandibular cutaneous (chin) gland is responsible for maintenance of this hierarchy (Mykytowycz and Dudzinski, 1966; Mykytowycz, 1968; González-Mariscal et al.,

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1993). Previous work on the secretion from the chin gland of the rabbit identified a compound that was found to vary with social status (Hayes et al., 2001). This compound, 2-phenoxyethanol, was found to be present in the secretion of all dominant males examined, but has never been detected in the secretion of subordinate animals.

We investigated two possible roles for 2-phenoxyethanol in the secretion of dominant male rabbits. The first of these is that the very presence of the compound itself in an animal's secretion is a signal to others that this is the product of a dominant animal; in other words 2-phenoxyethanol is acting as a dominance-signalling compound. 2-Phenoxyethanol is used in the perfume industry as a fixative (Winter, 1984), a compound that is added to slow the release rate of the more volatile components of a scent and increase its longevity (Whitten, 1969; Regnier and Godwin, 1977). Thus, the other proposition is that 2-phenoxyethanol may function as a fixative in rabbit chin gland secretion, slowing the release rate of the more volatile constituents, and causing a dominant animal's scent to persist in the environment. Both of these alternatives allow us to make predictions about the nature of the signal, and these predictions can then be investigated experimentally.

If 2-phenoxyethanol is acting as a direct signaller of dominance in the chin gland secretion of dominant animals, then it must be distinguishable by the rabbit. The principal method used previously to investigate such behavior in mammals is that of the simultaneous two- (or three-) choice test. In this type of test, an animal is presented simultaneously with a stimulus object and a control, and behavior towards both objects is observed. The most common behaviors that are considered to be important are approach, contact, frequency, and duration of sniffing and scent marking (Belcher et al., 1990; Price and Feistner, 1994).

Stralendorff (1986) reported that olfactory bioassays were unsuitable for detecting semiochemicals in mammals, because behavioral responses, such as sniffing of chemical signals, are rarely specific in the class. However, if treated with some caution they are considered to be the most reliable way of assessing olfactory preference in mammals (Bel et al., 1999). Indeed, sniffing behavior is often the only reliable means of investigation available in this type of analysis and has been used in many studies of the mammalian olfactory system (Belcher et al., 1990; Price and Feistner, 1994; Bel et al., 1999).

Thus, it is possible to test whether or not 2-phenoxyethanol is acting as a dominance-signalling compound in European rabbits. If it is, then in a two-choice test, where rabbits are presented with secretion containing 2-phenoxyethanol, and a secretion that is identical in every aspect, except that it does not contain this compound, there will be a difference in behavior shown towards these two alternatives. A failure to distinguish between the two choices would support the proposal that this compound is olfactorially inert, and suggest that it performs some other role in the secretion.

If, on the other hand, the function of 2-phenoxyethanol is to act as a fixative, then this is also amenable to testing. In this case, over a period of time there would be a difference in the composition of the secretion that contains 2-phenoxyethanol compared with that which does not. At least some of the components will remain in the secretion for a longer period of time in the presence of 2-phenoxyethanol, than they do if this component is not present.

Other studies have investigated the nature of fixatives, especially proteins, in the urine and scent gland secretion of mammals. Proteins are an essential part of the semiochemical message of humans (Zeng et al., 1996) and cotton-top tamarins (*Saguinus fuscicollis*) (Singer and Macrides, 1992), and it has been suggested that the role they play is that of a fixative. It has been shown that the major urinary proteins in house mice (*Mus musculus*) function to extend the longevity of semiochemically important compounds (Hurst et al., 1998), i.e., act as a fixative.

METHODS AND MATERIALS

To conduct the proposed analyses, both with and without 2-phenoxyethanol, it was necessary to have a large volume of secretion. It was not possible to use a sample of real secretion, as it was impossible to collect a sufficient quantity from animals trapped in the field. The secretion would have had to be collected from subordinate individuals, so that the secretion did not already contain 2-phenoxyethanol, and these animals produce very small quantities of secretion (0.5–1 μl per swab). A model secretion was thus prepared. In addition to this, a model secretion allowed standardization of all samples. Unless otherwise indicated, 3 μl of secretion were used for all analyses, a volume comparable to that produced by the swab of a dominant male in the breeding season.

The model secretion consisted of six of the compounds previously identified from the chin secretion of wild rabbits (Hayes et al., 2002b). The compounds used (Table 1) are those that occur in rabbit chin gland secretion from the majority of rabbits examined ($\geq$70%), and were chosen such that their retention times covered

TABLE 1. COMPOSITION OF THE MODEL SECRETION THAT WAS USED TO TEST THE ROLE OF 2-PHENOXYETHANOL IN RABBIT CHIN GLAND SECRETION

Compound name	Relative volume, μl	Approx. retention time, min
Xylenes	1 (of each isomer)	13.6–13.8
Ethylbenzene	1	14.0
Phenol	8	16.3
Mesitylene (trimethylbenzene)	10	16.5
Acetophenone	2	18.7
2-Phenoxyethanol	1	22.0
p-Cresol	1	24.0

that portion of the chromatogram where most of the variation was seen to occur (Hayes et al., 2002b). They were also chosen because they are readily available commercially in a pure form. The relative amounts of the components used for the model secretion (Table 1) were based on the compounds' relative concentration in secretion collected from wild rabbits, as determined by gas chromatography. The components of the secretion were all miscible, and thus no solvent' which might possibly interfere with its presentation, was needed to prepare the solution.

Behavioral Recognition of 2-Phenoxyethanol. Wild-type rabbits, normally living in open-pen enclosures (approximately 100 × 30 m) under natural light–dark and temperature regimes, were captured in box traps and held in an outdoor laboratory for a maximum of 24 hr. After capture, rabbits were housed individually in wire-mesh cages (measuring approximately 100 × 60 × 60 cm). Cages were lined with straw for bedding. The animals were subjected to natural light–dark and temperature conditions throughout the period of caging. Rabbits were fed daily on commercial rabbit pellets and green lucerne hay. Water was provided *ad libitum* (Ethics approval no. ARP00-08, part of ARP92-13).

The ability of rabbits to distinguish 2-phenoxyethanol was ascertained by the use of an arena (170 × 110 × 90 cm). Two pieces of filter paper (20 × 1.5 cm) were suspended from strings into the arena, so that the end of the paper was approximately 5 cm above the floor. A small amount (3 μl) of model secretion was placed onto each piece of filter paper, approximately 1 cm from the end (Figure 1).

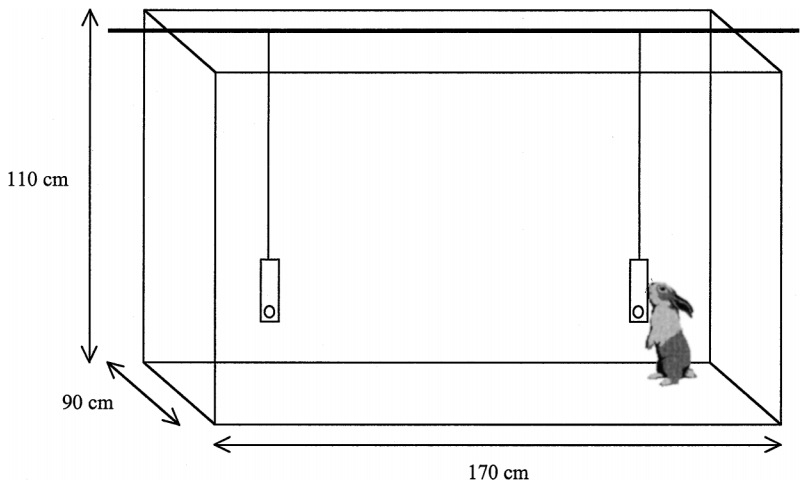

FIG. 1. Schematic diagram showing the test arena used to analyze the rabbit's sensitivity to 2-phenoxyethanol. Samples of mock secretion, either with or without 2-phenoxyethanol, were randomly placed on the hanging papers. Time spent investigating each sample was recorded over a 5-min exposure time.

The only variation in the secretion placed on each filter paper was the presence/absence of 2-phenoxyethanol. A new set of filter papers was used for testing each animal. The location of the paper with 2-phenoxyethanol was randomized between trials. Fresh air was blown into the arena between trials to prevent any localization of scent.

The arena was placed in a darkened room, and the behavior of the rabbits was observed through a window. The analysis was conducted on adult rabbits of both sexes (30 female, 11 male) and on both dominant and subordinate individuals (male: 6 dominant, 5 subordinate; female: 18 dominant, 12 subordinate). The experimental animals live in enclosures on the grounds of CSIRO, and the social status for each individual is known from regular behavioral observations.

Rabbits were placed into the arena after the filter paper was introduced, and observations begun immediately. The rabbits were observed for a total of 5 min, and the amount of time spent investigating and sniffing each of the two pieces of paper was noted. Only direct sniffing, with muzzle within 2 cm of the paper, and flaring of the nares, was timed and considered to be an investigation. A rabbit sitting stationary in that portion of the arena containing the paper was not considered as investigating the mark.

The length of time spent investigating the mark was used rather than merely whether or not the mark was investigated. This was based on the assumption that time spent investigating each mark was proportional to an animal's interest in the odor (e.g., Black-Cleworth and Verberne, 1975; Belcher et al., 1990; Price and Feistner, 1994; Kruczek, 1997; Ordinola et al., 1997; Bel et al., 1999; Swaisgood et al., 1999). The time of investigation was directly chronometered by two observers, of which one was aware, and one not aware, of the lateral position and nature of the stimulus. Investigation times were averaged between the two observers. A Wilcoxson's signed-rank test (Sokal and Rohlf, 1995) was performed on all data sets to determine whether there was a significant difference between the time spent investigating each sample.

The Effect of 2-Phenoxyethanol on the Release Rate of Volatile Compounds. The ability of 2-phenoxyethanol to slow the release rate of volatile components of the chin gland secretion of European rabbits was also tested through the use of the model secretion. Triplicate samples of model secretion, both with and without 2-phenoxyethanol (total volume $\approx 3\ \mu$l), were placed onto pieces of aluminium foil. The samples were left exposed to the laboratory air for various lengths of time (0, 0.5, 1, 5, and 24 hr; after Hurst et al., 1998).

After exposure, the foil was placed in a headspace vial, an internal standard (tetradecane, 1 μl) was added by syringe to the vial, and the vial was sealed. The vial was heated to 30°C in a heating block. Laboratory air (130 ml) was pumped through a charcoal trap, then over the sample at a flow rate of 13 ml/min. After passing over the sample, the air flowed through gas chromatography injection port liners packed with the adsorbent agent Tenax TA (35/60-mesh) ($90-2\ \mu$g). At the

end of the collection time (10 min), the liner was placed in the injector port of a gas chromatograph (Hewlett Packard 5890 Series II) equipped with a flame ionization detector and fitted with a silica capillary column (SGE, model BP-1, 25-m length, 0.22-mm ID, and 0.1-μm film thickness). The Tenax was thermally desorbed using a Programmable Temperature Vaporizer (PTV) (Hayes et al., 2002a).

Data were acquired under the following GC conditions: inlet temperature, 200°C; carrier gas, nitrogen at 23 cm/sec; split ratio, 1:20; detector temperature, 260°C; temperature program, initial temperature 45°C, initial time 5 min, rate 8°C/min, final temperature 250°C, final time 5 min. The temperature program for the PTV was as follows: initial temperature, 40°C; ramp rate, 16°C/sec; final temperature, 200°C. Multidimensional scaling ordination (MDS); ANOSIM, and SIMPER calculations were used to distinguish between samples (Clarke and Warwick, 1994; Hayes et al., 2002a,b) and were performed using the PRIMER (Plymouth Routines in Multivariate Ecological Research) (Version 4.0, 1997) software package.

Two sets of samples were run as controls to this experiment. The first was used to determine whether any of the peaks observed were due to the breakdown of 2-phenoxyethanol. For this analysis, 2-phenoxyethanol (2 μl) was applied to a piece of aluminium foil, and the samples left for 24 hr. Samples were then treated as for the model secretion samples.

Additionally, it was necessary to ascertain the usefulness of aluminium foil as a substrate for the analysis. It has been demonstrated previously (Regnier and Godwin, 1977) that different substrata retain hamster scent marks differently. It is possible that a biological substrate, more like that on which rabbits place their scent marks in the wild, would retain the secretion differently from the manner in which it is retained on aluminium foil. To determine this, samples of model secretion (3 μl), both with and without 2-phenoxyethanol were applied to commercial cotton buds. Two time intervals were examined: 1 hr and 24 hr. Triplicate samples were left for the appropriate time, and then treated as above. The results were then compared with those obtained with aluminium foil as the substrate

RESULTS

Behavioral Recognition of 2-Phenoxyethanol. There was no significant difference between the times that rabbits spent investigating each sample ($3.56 - 0.2$ sec) (Wilcoxson signed-ranks test: $T_s = 415$, $N = 41$, NS), i.e., rabbits do not show any ability to distinguish between the samples with and without 2-phenoxyethanol. This is true when all the rabbits are considered together, and also true for each sex separately (Wilcoxson signed-ranks test: females: $T_s = 211$, $N = 30$, NS; males: $T_s = 15$, $N = 11$, NS).

The Effect of 2-Phenoxyethanol on the Release Rate of Volatile Compounds. Chromatograms produced from the model secretion over time are shown (Figures 2

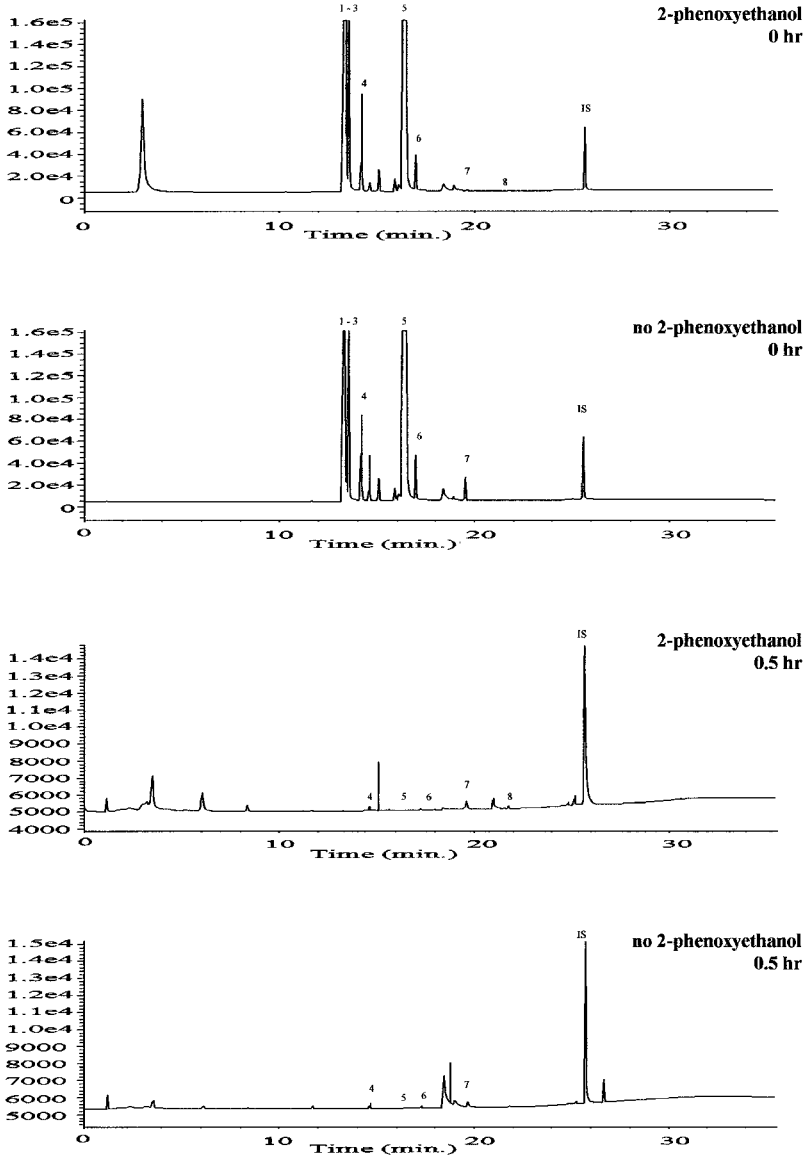

FIG. 2. The effect of 2-phenoxyethanol on the release rate of volatile compounds in model secretion. The chromatograms are shown for the zero-time and 0.5-hr readings. The samples at 1 and 5 hr were statistically indistinguishable from the 0.5-hr samples, and are not shown. The presence or absence of 2-phenoxyethanol is indicated on the diagram. Components of the model secretion are indicated by number: 1–3, xylenes; 4, ethylbenzene; 5, phenol; 6, mesitylene; 7, acetophenone; and 8, 2-phenoxyethanol. The *p*-cresol was not apparent in the chromatograms. IS is the internal standard, tetradecane.

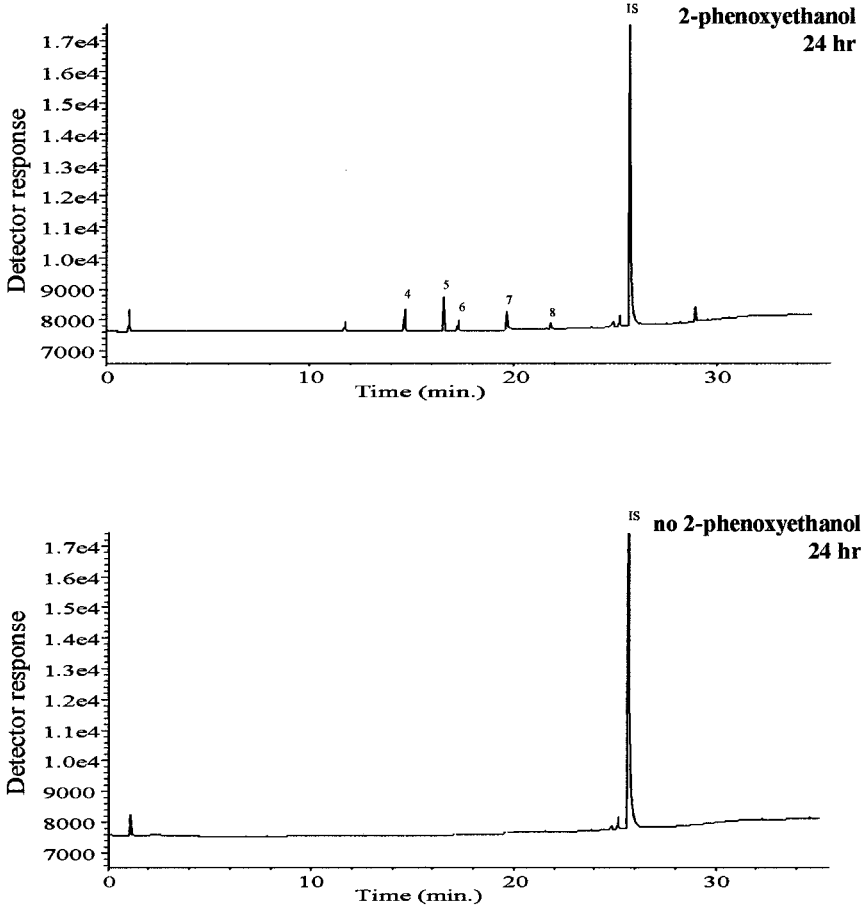

FIG. 3. The effect of 2-phenoxyethanol on the release rate of volatile compounds in the model secretion. The chromatograms shown are for the situation after 24 hr. The presence or absence of 2-phenoxyethanol is indicated. Components of the model secretion are indicated by number: 4-ethylbenzene, 5-phenol, 6-mesitylene, 7-acetophenone and 8-2-phenoxyethanol. The *p*-cresol was not apparent in the chromatograms. IS is the internal standard, tetradecane.

and 3). Peaks corresponding to the components of the secretion are numbered. Contaminants of the secretion appear as additional peaks, as well as peaks produced by water in the samples in the first 5 min of the run; however, these have no effect on the analysis as they do not co-elute with any peaks of interest.

At the zero-time reading (0 hr), the samples both with and without 2-phenoxyethanol looked the same, with very large peaks, showing high

concentrations of all of the compounds present (Figure 2). After 30 min, the concentrations of all the compounds have reduced drastically (Figure 2). The peaks produced by the three xylene isomers have completely disappeared. The samples at both 1 and 5 hr look the same and are statistically indistinguishable from each other, and from the samples, at 30 min. (ANOSIM: Global $R = 0.896$, $P < 0.001$).

It is only after 24 hr that there is an appreciable difference between the samples with and without 2-phenoxyethanol (Figure 3). After 1 day, without 2-phenoxyethanol, all the compounds have reduced to levels that are not detectable by this method. With 2-phenoxyethanol, on the other hand, all of those peaks that were present at 30 min are still observed. A Bray–Curtis similarity clustering distinguishes the 24-hr samples without 2-phenoxyethanol from all the other samples at a difference of 75%. The zero-time readings, both with and without 2-phenoxyethanol, are dissimilar to the remaining samples at 65%. A

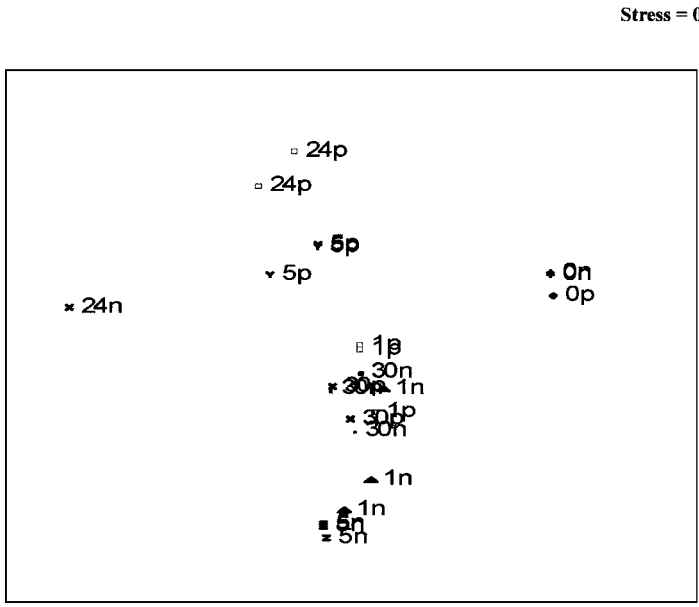

FIG. 4. The two-dimensional MDS ordination of the 30 samples of model secretion. The plot is based on $\sqrt{\sqrt{}}$-transformed abundances and a Bray–Curtis similarity matrix. Distinct clusters of samples emerge, relating to time and the presence or absence of 2-phenoxyethanol, where 0p = zero time with 2-phenoxyethanol, 0n = zero time without 2-phenoxyethanol, 30p = 30 min with 2-phenoxyethanol, 30n = 30 min without 2-phenoxyethanol, 1p = 1 hr with 2-phenoxyethanol, 1n = 1 hr without 2-phenoxyethanol, 5p = 5 hr with 2-phenoxyethanol, 5n = 5 hr without 2-phenoxyethanol, 24p = 24 hr with 2-phenoxyethanol, 24n = 24 hr without 2-phenoxyethanol.

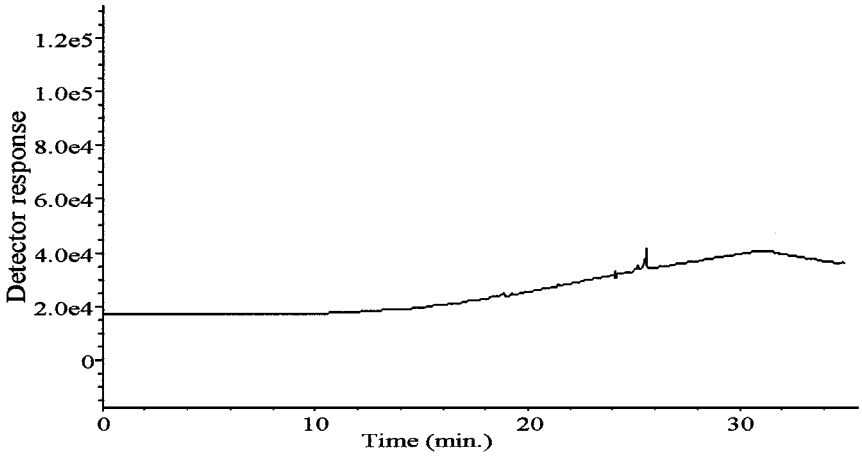

FIG. 5. The chromatogram produced by a sample of only 2-phenoxyethanol after 24 hr on an aluminium foil substrate.

multidimensional scaling (MDS) plot (Figure 4) shows that there are three distinct groups, the zero-time readings, the 24-hr samples without 2-phenoxyethanol, and all the other samples. The three 24-hr samples overlay each other exactly, as the samples are identical in composition. An analysis of similarity shows that these three groups are different (ANOSIM: Global $R = 0.896$, $N = 30$, $P < 0.001$). After 24 hr a sample of foil with only 2-phenoxyethanol shows only one peak in the chromatogram, and is statistically indistinguishable from the 24-hr sample without 2-phenoxyethanol (ANOSIM: Global $R = 0.749$, $N = 6$, $P < 0.001$) (Figures 3 and 5). After 1 hr and also after 24 hr, the chromatogram produced by model secretion placed onto a cotton swab contains the same peaks, in the same proportions, and is statistically identical to that seen when aluminium foil is used as the substrate (ANOSIM: Global $R = 0.900$, $N = 12$, $P < 0.001$) (Figure 6).

DISCUSSION

The compound 2-phenoxyethanol is present in the chin gland secretion of all dominant male rabbits, but is never found in the secretion of subordinates (Hayes et al., 2001). If the role of this compound were to act as a signal of dominance only, then it would have to be olfactorially detectable by rabbits. The behavioral experiment above provided no support for this proposal. There is no evidence that rabbits can distinguish between the samples with and without 2-phenoxyethanol when freshly mixed and deposited upon the substratum. It is not that the animals were unaware of the model secretion—in fact several individuals spent the majority of their investigation time sniffing at the scent marks. What has occurred is that the amount

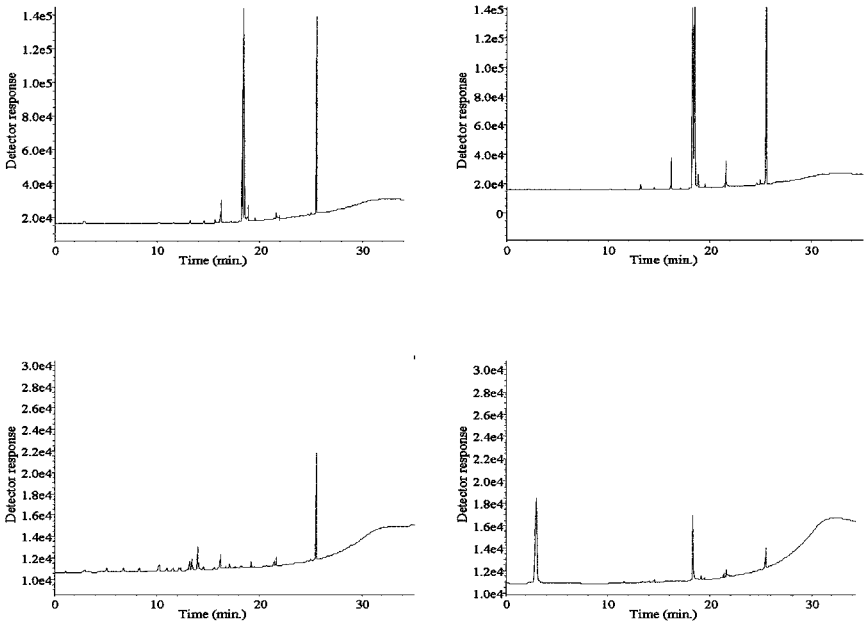

FIG. 6. The chromatogram produced by model secretion after (from top to bottom) 1 and 24 hr on a cotton bud. The left-hand column shows samples with 2-phenoxyethanol, the right-hand column shows samples without 2-phenoxyethanol.

of time spent investigating each mark is not significantly different across the group.

The other hypothesis under examination is that the compound is acting as a fixative, thereby enhancing the longevity of the dominant male's scent mark, and causing it to persist where the mark of the subordinate fades. The time-course experiment showed that 2-phenoxyethanol is able to prolong the presence of at least some of the compounds identified from the chin gland secretion of wild rabbits. The large difference between the zero-time and 30-min reading is presumably due to the initial vaporization of much of these volatile compounds. The signal from then on is statistically indistinguishable for at least 5 hr.

It is worth noting that the xylenes (ortho-, meta-, and para-) were not retained at all by 2-phenoxyethanol. It must be remembered that there are other components of the chin gland secretion that may be capable of performing a fixative function, e.g., proteins and fats (Goodrich and Mykytowycz, 1972). If the xylenes form an important part of the rabbit's message (and they are some of the compounds identified as varying in the secretion, see Hayes et al., 2001, 2002a,b), then they must be retained by a different method. Nonetheless, after

24 hr it is clear that there is a difference between the samples with and without 2-phenoxyethanol.

The first control experiment showed that the peaks that are evident in the samples with 2-phenoxyethanol, after 24 hr, are not due to the breakdown of that compound, but are instead due to the other components of the mixture. The second control experiment demonstrated that there was no difference between a biological substrate (cotton) and an artificial substrate (aluminium foil). Aluminium foil was a suitable substrate to use for the above analysis.

These experiments, support the hypothesis that 2-phenoxyethanol acts as a fixative in rabbit chin gland secretion. It is not shown to be detected olfactorially by rabbits, but it does perform as a fixative on some, although not all, of the compounds that occur naturally in the secretion. Why would an animal evolve a system where a change in social status is accompanied not by the addition of a new dominant compound, but instead by the addition of a fixative? In this way, the newly dominant animal will ensure that his signal will persist in the environment and not fade rapidly. This would seem to be a much easier mechanism to evolve—far simpler than what would be required if an entirely new communication system were being developed. Under the opposing model, a new compound would occur in an animal's secretion, and then would have to become recognized by others in the population as a signal of dominance. The fixative proposal requires only a change in one category within the population, the signaller. The rest of the animals in the population will be pre-adapted to register this suite of compounds; they are attuned to this signal that will now persist in the environment instead of being transitory. The addition of a fixative to his secretion at the beginning of a new regime is a novel way to consider the onset of dominance in the mammals.

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QUINOLIZIDINE ALKALOIDS IN *Ormosia arborea* SEEDS INHIBIT PREDATION BUT NOT HOARDING BY AGOUTIS (*Dasyprocta leporina*)

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Abstract—Quinolizidine alkaloids (QAs) are secondary compounds found in seeds of many species of plants, possibly protecting them against pathogens and seed predators. QAs were isolated from *Ormosia arborea* seeds and bioassayed against red-rumped agoutis (*Dasyprocta leporina*, Rodentia: Caviomorpha) to verify if they inhibit seed predation and food hoarding (seed dispersal). Three treatments were used: (1) seeds of *O. arborea*, (2) palatable seeds of *Mimusops coriacea* (Sapotaceae) treated with MeOH, and (3) seeds of *M. coriacea* treated with QAs dissolved in MeOH in similar concentration to that present in *O. arborea*. Palatable seeds were significantly more preyed upon than seeds treated with QAs and *Ormosia* seeds, but QAs did not influence hoarding behavior. QAs in *O. arborea* may have a strong effect in avoiding seed predation by rodents, without reducing dispersal.

Key Words—Atlantic rainforest, seed defense, seed predation, seed dispersal, Rodentia, Caviomorpha, secondary compounds, chemical defense, mimetic seeds, quinolizidine alkaloid.

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INTRODUCTION

Seed predation affects the reproductive success of higher plants. Temporal patterns of seed production (masting), seed dispersal, seed morphology such as hardness and weight, and chemical defenses are traits suggested to be related to seed defense (Janzen, 1969, 1971). Many fruits and seeds are defended by secondary metabolites toxic to insects, vertebrates, and fungi (Janzen, 1971; Janzen et al., 1986; but see Ehrlen and Eriksson, 1993).

Ormosia arborea (Vell.) Harms (Leguminosae) is a large tree that occurs both in semideciduous and evergreen forests in southern Brazil (Rudd, 1965). *O. arborea* seeds are bright red and black, and are considered as "mimetic" (van der Pijl, 1982), referring to a large group of brightly colored seeds (usually red and black, or red alone) that lack a digestible tissue reward for their putative seed dispersers. These colorful seeds are presumed to resemble arils or fleshy fruits that deceive avian frugivores. Mimetic seeds occur in several plant families, but especially in the Leguminosae (mainly in Papilionoideae and Mimosoideae) (Galetti, 2002). Although the seed dispersal systems of *Ormosia* are still unclear (Peres and van Roosmalen, 1996; Foster and Delay, 1998; Galetti, 2002), recent work has shown that *Ormosia arborea* seeds can remain for long periods on the forest floor (up to 18 months) without appreciable depletion by seed predators or pathogens (Galetti, 2002). Seeds usually fall close to the parental tree, creating a conspicuous seed shadow. The conspicuousness of the seeds on the forest floor, long exposure time, and clumped distribution make them vulnerable to potential seed predators, such as agoutis and other rodents, peccaries, and tinamous.

Ormosia seeds contain quinolizidine alkaloids (Kinghorn et al., 1988; Nasution and Kinghhorn, 1993). These substances (hereafter QAs) are a group of secondary compounds present in many seeds of legumes, that may provide protection against natural enemies (Wink, 1993a). Although the strongly deterrent action of QAs has been demonstrated in invertebrate and vertebrate metabolism (Wink, 1993a), few studies focus on the influence of alkaloids in seed predation and predator behavior (Rankin, 1978). We tested whether the presence of the QAs in *Ormosia arborea* seeds reduces seed predation or influences food hoarding by red-rumped agouti (*Dasyprocta leporina*).

METHODS AND MATERIALS

Organisms. O. arborea occurs in low density, mostly near hilltops or on steep slopes (Lorenzi, 1992). Seeds of *O. arborea* were collected in the Estação Ecológica de Caetetus, Gália, São Paulo, where a long-term study on seed dispersal of *O. arborea* is underway (Galetti, 2002). *Mimusops coriacea* (DC.) Miq. (Sapotaceae), whose seeds were used as a palatable control, is an exotic tree that occurs in Southeast Brazil, spontaneously or cultivated, mainly near beaches. The

seeds are similar to *Ormosia* in weight (1.22 $\pm$ 0.16 g versus 0.99 $\pm$ 0.16 g for *Ormosia* seeds) ($N = 30$ for both), and preliminary studies showed that agoutis ate most of them; there are no recorded QAs in Sapotaceae (Wink, 1992). Seeds were collected in São Sebastião and Campinas, São Paulo. The red-rumped agouti *Dasyprocta leporina* Husson (Rodentia: Dasyproctidae) is a relatively large brown rodent, with a blackish or reddish rump (Emmons and Feer, 1997); it occurs in many different habitats, including cerrados and evergreen forests. Agoutis are mainly frugivorous and granivorous (Henry, 1999), and are important seed dispersers and predators (Forget, 1992; Peres and Baider, 1997). They are especially important seed dispersers (by hoarding seeds) for some large-seeded neotropical plants (Asquith et al., 1999).

QA Purification and Characterization. QAs were extracted from 120 seeds using the same methodology as for free bases of pyrrolizidine alkaloids (Trigo et al., 1996). Tentative identification of QAs was done by gas chromatography–mass spectrometry (GC-MS) analysis, in a HP-6890 GC system equipped with a fused silica capillary column (HP-5MS, Sigma, 5% diphenyl–95% dimethylsiloxane, 30 m $\times$ 0.25 mm $\times$ 0.25 μm) coupled to an HP-5973 mass detector, based on their RIs and mass fragmentation (Kinghorn et al., 1988; Wink et al., 1991; Wink, 1993a; 1995). The RIs (retention index) were calculated by coinjection of *n*-alkanes following van den Dool and Kratz (1963). Confirmation of molecular weight was done by PCI using methane as the ionization gas. For quantification, alkaloids were analyzed in a GC system as above, equipped with a fused silica capillary column (DB5, J&W Scientific, 5% diphenyl–95% dimethylsiloxane, 30 m $\times$ 0.25 mm $\times$ 0.25μm) coupled with an FID. Commercial sparteine was used as an external standard. The conditions of injection in both analyses were, injector temperature 250°C, detector temperature 280°C, temperature program 70–300°C at 4°C/min, split = 20:1, carrier gas He, 1ml/min.

Seed Predation and Hoarding Behavior. Experiments were carried out in the Bosque dos Jequitibás, a 10-ha urban forest park in Campinas, São Paulo (Matthes, 1980), from February 1999 to March 2000. The area has more than 60 tame agoutis (*D. leporina*) whose behavior can be observed closely. One of us (PRG) observed agoutis for 2 years in the Bosque dos Jequitibás and never saw an *O. arborea* seed in the area; so it may be assumed that these agoutis did not have any experience with these seeds.

Three different treatments were used: (1) *O. arborea* seeds, (2) *M. coriacea* seeds treated with 10 μl MeOH, and (3) *M. coriacea* seeds treated with 10 μl of QAs in MeOH, isolated from *O. arborea* seeds (concentration of QAs equivalent to the proportion found in seeds of *O. arborea*). The solvent was evaporated under an air stream before the experiments.

The bioassay protocol was to put 10 seeds of a single treatment on the forest floor; whenever an agouti found the seeds, its behavior was recorded. The number of seeds eaten, hoarded, or rejected by agoutis was determined by direct observation.

The observations were made as follows: the observer sat at least 5 m from the station and observed for at least 1 hr after an agouti arrived. Fifteen minutes after the animal left, the observation sequence was concluded. Behavior was recorded in three categories: preyed, hoarded, or rejected (seeds still intact 15 min after the agouti visit). Each treatment was replicated 15 times.

Comparison among treatments for the proportion of seed preyed upon and hoarded were done by using a nonparametric Kruskal–Wallis test, since the data do not fit a normal distribution (Zar, 1999).

RESULTS AND DISCUSSION

Eleven QAs were found in the seeds of *O. arborea* (Table 1 and Figure 1); ormosanine (**4**) and panamine (**5**) (or its isomers) were the main alkaloids. The concentration of alkaloids found was 3.54 mg/g dry weight (about 2.95 mg per seed). QAs were not previously described in this species, but all of the known structures have been recorded in other *Ormosia* species (Kinghorn et al., 1988).

TABLE 1. QUINOLIZIDINE ALKALOIDS IN *Ormosia arborea* SEEDS

Quinolizidine alkaloids[a]	RIs	Mass fragmentation m/z (EI)[b]	Relative abundance	Concentration (mg/g dry weight)[c]
Sparteine (**1**)	1776	$[M]^+$ 234 (14), 205 (3), 193 (26), 150 (13), 137(100), 122 (15), 110 (22), 98(83)	2	0.07
Angustifoline (**2**)	2141	$[M]^+$ 234 (nd), 193 (100), 150 (13), 112 (87)	6	0.21
Lupanine (**3**)	2159	$[M]^+$ 248 (31), 193 (15), 149 (49), 136 (100), 98 (24)	1	0.04
Unknown	2462	$[M]^+$ 315 (40), 231 (42), 122 (21), 98 (55), 84 (100)	7	0.25
Ormosanine (**4**) or isomers	2487	$[M]^+$ 317 (14), 234 (32), 151 (31), 98 (51), 84 (100)	1	0.04
Ormosanine (**4**) or isomers	2498	Idem	1	0.04
Ormosanine (4) or isomers	2518	Idem	17	0.60
Ormosanine (**4**) or isomers	2543	Idem	7	0.25
Unknown	2595	$[M]^+$ 327 (7), 313 (70), 270 (34), 231 (70), 215 (42), 122 (28), 98 (100)	15	0.53
Panamine (**5**) or isomers	2618	$[M]^+$ 315 (17), 272 (7), 217 (100), 98 (43)	7	0.25
Panamine (**5**) or isomers	2679	Idem	36	1.26

[a] Tentatively identified by comparison with RIs and mass fragmentation (Wink, 1993a; Wink et al., 1991, 1995).
[b] Molecular ion confirmed by PCI.
[c] Given by GC-FID analysis.

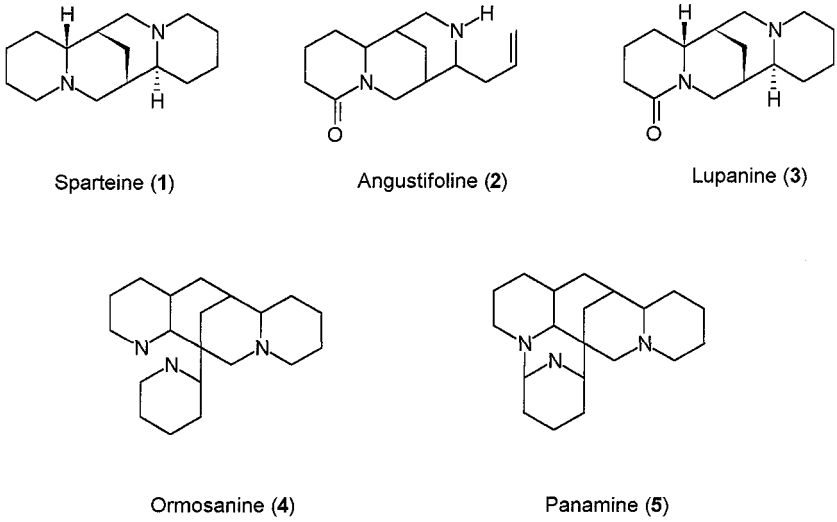

FIG. 1. Quinolizidine alkaloids present in *Ormosia arborea* seeds. Tentatively identified by comparison with RIs and mass fragmentation (Wink, 1993a; Wink et al., 1991, 1995). Stereochemical configuration does not necessarily represent the true configuration.

Agoutis usually ate only a few *Ormosia* seeds in each station. In *O. arborea* and *M. coriacea* with QA treatments, an agouti picked up a seed, put it in its mouth, gnawed for few seconds, and then rejected it. The animal repeated this behavior a few times with the same and/or another seed. Agoutis often gnawed on only 1–4 seeds, eating pieces or breaking them without ingestion. In all gnawing events, in all treatments, agoutis bit through the seeds, killing the embryo.

Statistical analysis included 12 stations of *M. coriacea* with MeOH treatment, 6 of *Ormosia* seeds, and 11 of *M. coriacea* with QA alkaloids (other stations were excluded by absence of agouti visitation). The three treatments differ significantly in seed predation proportions (Kruskal–Wallis test, $H = 11.149$, $df = 2$, $P = 0.004$) (Figure 2A). The multiple comparison test showed *M. coriacea* seeds treated with solvent only were more preyed upon than the other treatments ($P = 0.002$ when compared to *M. coriacea* with QAs, and $P = 0.027$ to *O. arborea*); the percentage of seeds preyed upon by agoutis did not differ between *O. arborea* and *M. coriacea* treated with QAs ($P = 0.664$). The presence of QAs did not influence hoarding behavior. *M. coriacea* without QAs was hoarded in a similar frequency (0–6 seeds per station) to *O. arborea* and *M. coriacea* with QAs (Kruskal–Wallis, $H = 1.570$, $df = 2$, $P = 0.456$) (Figure 2B).

These assays demonstrate that QAs reduce the proportion of seeds preyed upon even by naive agoutis. Seed predation should be even more reduced in areas where agoutis are exposed to these unpalatable seeds for long time. In

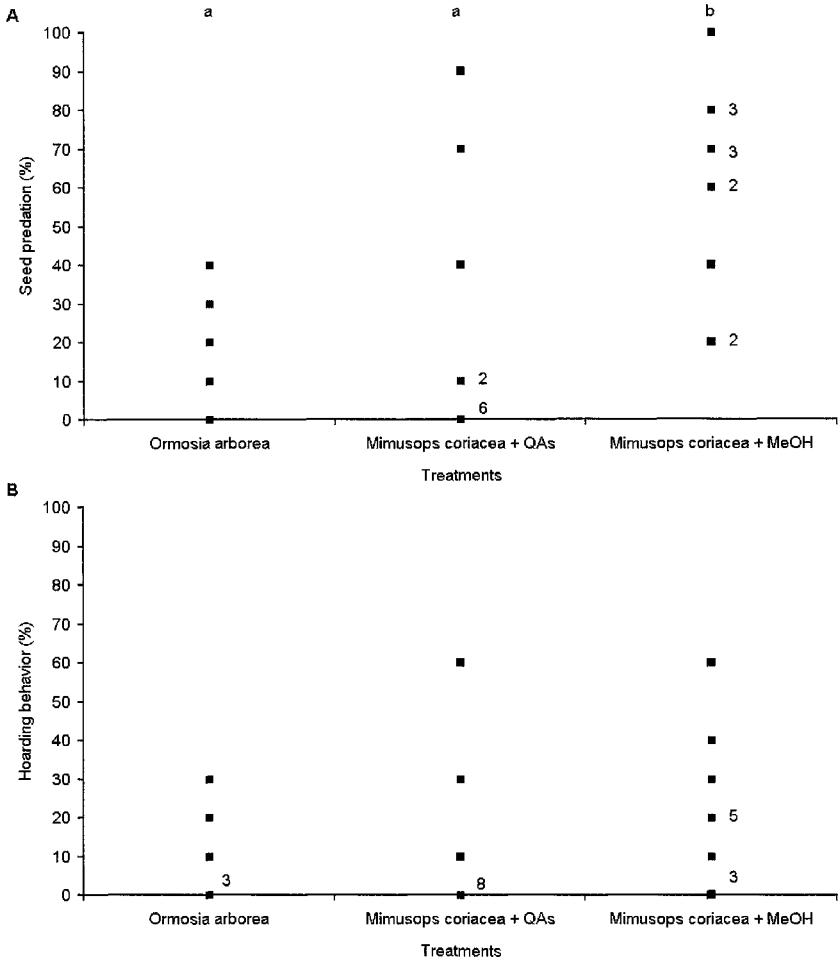

FIG. 2. Responses of *Dasyprocta leporina* in relation to *Ormosia arborea* seeds and palatable *Mimusops coriacea* seeds treated or not with QAs by (A) Seed predation; (B) Hoarding behavior. The results are shown as dispersion points, with each point being a station. The numbers on the right side of the points represent stations with similar results; if there is no number, the points represent only one station. The different letters over the dispersion data represent significant differences at the 0.05 level (Kruskal–Wallis test).

these areas, agoutis should avoid gnawing seeds with QAs by associating their features (odor, form, color) with QA presence. Wink (1993a,b) also stated that QAs have deterrence activity against vertebrates such as sheep, hares, and rabbits.

In this study, QAs did not influence the number of seeds hoarded by naive agoutis. However, after retrieval of hoarded seeds, *O. arborea* seed predation by agoutis should be deterred by QA presence. Thus, a reduction in *O. arborea* seed hoarding (and, as a consequence, seed dispersal) by agoutis should occur with their experience with QA deterrence.

Seeds such as those of *O. arborea* with long dormancy and long fruiting periods may depend on both chemical and morphological defenses in order to reduce predation and increase survival. Mechanisms that promote a reduction in the number of seeds preyed upon before or after seed dispersal, such as chemical protection, can be important for plant fitness (Janzen, 1971). The presence of secondary compounds (like QAs) in *O. arborea* seeds seems to be important in the deterrence of large rodents.

As seeds are the ultimate products of plant fitness (Janzen, 1971), it is probable that seed defense will be important, and selection, when possible, will act to improve it. We recommend comparative and experimental studies to elucidate how secondary compound evolution and seed ecology are related.

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RODENT AND RUMINANT INGESTIVE RESPONSE TO FLAVONOIDS IN *Euphorbia esula*

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Abstract—*Euphorbia esula*, common name leafy spurge, was chemically evaluated for aversive phytochemicals that appear to minimize herbivory by rodents and cattle. A middle-layer extract elicited food aversions in rats as did the petroleum ether extract of the initial methanol extract. Kaempferol-3-0-β-glucuronic acid and quercetin-3-0-β-glucuronic acid were separated and identified from the middle-layer residue. This study is the first report of quercetin-3-0-β-glucuronic acid in leafy spurge. Together these flavonoidal glucosides were mildly aversive to rats but showed less aversive activity when tested separately. The middle-layer extract produced no aversive response from cattle, while the petroleum ether extract elicited strong aversions in cattle. Flavonoidal compounds from leafy spurge that were aversive to rats, a monogastric mammal, were not aversive to cattle, a ruminant. Microbial degradation of the compounds before they reach the intestines and are absorbed into the bloodstream is likely a key advantage for cattle compared to rats.

Key Words—*Euphorbia esula*, leafy spurge, flavonoid, quercetin glucuronic acid, kaempferol glucuronic acid.

INTRODUCTION

Euphorbia esula, commonly known as leafy spurge, is a toxic, perennial, and invasive forb from Europe that is ubiquitous on the northern Great Plains of North America. It also occurs in many other areas of the continent. This plant poses a serious threat to native vegetation, wildlife, and cattle production in North America

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(Belcher and Wilson, 1989; Hein and Miller, 1992; Wallace et al., 1992; Leitch et al., 1996). Although it has high levels of protein and digestibility during its early growth stages (Fox et al., 1991), it does not appear to be significant forage for bison, cattle, elk, mule deer, or white-tailed deer (Marlow et al., 1984; Lym and Kirby, 1987; Sullivan et al., 1988). Little is known about interactions between wild rodents and leafy spurge. Rats refused to consume more than a few pellets made of leafy spurge in operant tests (Phelps and Kronberg, unpublished data), and leafy spurge elicits learned food aversions (reduced ingestion of a food following malaise that is caused by earlier ingestion of the food) in laboratory rats and cattle (Kronberg et al., 1993, 1995). Little is known about the identity of the toxin(s) that causes the food aversions in rodents and ruminants.

Previous chemical investigations of leafy spurge have identified cytotoxic diterpenoid ingenol esters (Kupchan et al., 1976; Upadhyay et al., 1978; Seip and Hecker, 1982; Sorg and Hecker, 1982), jatrophane diterpenes (Manners and Wong, 1985; Mannners and Davis, 1987), and lathyrane diterpene esters (Onwukareame and Rowan, 1992). Kaempferol-3-0-glucuronic acid (Wagner et al., 1970) and phytosterols (Farnsworth et al., 1968) were also reported as constituents of this species. Leafy spurge compounds have been studied for their skin irritant and tumor promoting activities, as well as for their antileukemic effect (Kupchan et al., 1976; Hecker, 1981; Seip and Hecker, 1982), but not until recently for their aversion-eliciting properties (Halaweish et al., 2002). The objective of this work was to identify aversion-eliciting compounds in leafy spurge that affect feeding behavior of laboratory rats and cattle.

METHODS AND MATERIALS

Initial Partitioning and Testing of Extracts. Air-dried *Euphorbia esula* (4 kg) collected in June 1995 near Velben, South Dakota (seed-ripening growth stage) were treated as follows: Ground plant material was extracted with cold 90% methanol (6×6 l) and acetone (6×6 l). The aqueous methanol extract was concentrated under reduced pressure. The resulting concentrated aqueous extract was partitioned with petroleum ether (40–60°C, 6×1 l) and chloroform (6×1 l), respectively. A thick precipitate formed at the interfacial surfaces between chloroform and the aqueous layer, especially at the first partitioning. The residue was collected separately and designated the middle layer. Each extract was concentrated under reduced pressure and finally freeze-dried.

Initial Food Aversion Trial with Rats. An aversion trial was conducted with laboratory rats as a preliminary screening of the extracts for aversive properties. Responses to the petroleum ether and chloroform extracts, middle layer, acetone extract, and the remaining portion of the original aqueous extract treatments were examined as follows: Sprague-Dawley rats (average weight 300 g) were housed

individually and had access to standard rat chow throughout the trial. During the training period, fresh water was provided at the same time each day for 5 days for only 15 min/day. On the first day of the test period, which immediately followed the training period, saccharine-flavored water (0.10% w/v) was offered for 15 min at the same time as the training period. Saccharine-flavored water was offered as a novel fluid, and intake during this period was measured. Immediately after the rats drank the saccharine-flavored water, they were gavaged with 0.15% extracts in corn oil at a dosage of 0.003% body weight. Five rats were used per treatment. On the second day, saccharine-flavored water was offered again for 15 min at the normal time and intakes of fluid were measured. These intakes were compared using a repeated measures design and PROC GLM (SAS, 1991) to determine if the rats had an aversive response. An aversive response was defined as reduced intake of the novel-tasting fluid on the day following gavage with the extracts. Treatment group was the between-animal effect, and postgavage response to the saccharine-flavored water was the within animal effect. Change in intake of novel fluid from the first to second days was also analyzed to allow for a comparison of means among treatment groups. Individual means were compared with the protected LSD procedure (SAS, 1988).

Separation of the Middle Layer. Preliminary biological screening showed the middle layer as potentially the most aversive portion. The middle layer was purified through a Sephadex LH-20 column (2×40 cm) and developed with methanol. The residue was further fractionated with a flash LC column (5×47 cm) packed with Silica gel G60 (230–400 mesh), developed with butanol:acetic acid:water (BAW; 6:0.6:4. Fifteen fractions (1 l) were collected. Fraction 5 showed 95% of the total material load on the column. Physical and color reaction suggested that Fraction 5 components were flavonoids (Mabry and Markham, 1970). Further purification of Fraction 5 with preparative TLC gave two components GL1 (8.5 mg) and GL2 (40.5 mg) with R_f 0.75 and 0.62, respectively. The aversive effect of purified GL1 and GL2 was screened again in a rat aversion trial as outlined above to confirm the effect.

Identification of Flavonoids. Flavonoids were identified by classical hydrolytic, chemical UV spectrophotometric, and NMR spectroscopic methods (Mabry and Markham, 1970; Harborne, 1973), and whenever possible by chromatographic comparisons with authentic compounds. ^{1}H NMR and ^{13}C NMR spectra were recorded in CD_3OD, and TMS was used as an internal standard. A Varian 200 MHz NMR spectrometer was used.

Acid Hydrolysis of the Glycosides. Five milligrams of each glycoside were dissolved in 1 ml of water and 1 ml of N/10 H_2SO_4, and the mixture was heated in a water bath for 2 hr. The aglycones were extracted with ethylacetate ($5 \times$ 3 ml) and used for identification. The aqueous acidic layer was neutralized with $BaCO_3$, centrifuged, then separated, concentrated, and used for the detection of the sugar. The sugar was identified on silica gel plates using the solvent system

isopropanol-ethyl acetate-water (7:2:1), and visualizing the plate with *p*-anisidine phthalate reagent (Bennini et al., 1992) followed by heating at 110°C for 5 min. The aglycone of GL2 (quercetin), as well as the middle layer and minor components of the middle layer, were tested in a separate rat aversion trial using the procedures described.

Enzyme Hydrolysis of GL1 and GL2. Two milligrams of each glycoside were dissolved in phosphate buffer 6.8, and 1 mg of β-glucoside enzyme (Sigma-Aldrich) was added. The mixture was vortexed and then kept in a water bath at 37°C for 1 hr. A few drops of trifloroacetic acid were added, and the mixture was centrifuged for 5 min at 3500 rpm. The supernatant was transferred to another vial, evaporated to dryness under N_2, and the residue used for identification of the aglycone.

Determination of the Flavonoids Concentration in Leafy Spurge Extracts. Ground leafy spurge (5 g/replicate, four replicates) was extracted with 90% aqueous methanol in a homogenizer for 5 min, and the extract was separated by centrifugation. This was repeated three times, and the extracts were combined and evaporated to dryness. The extract was purified over a solid phase extraction (SPE) column packed with 0.5 g C-18 reverse-phase silica gel. SPE columns were eluted with 5 ml methanol. The methanol extract was evaporated to dryness and used for determination of flavonoid concentrations. Flavonoid concentrations were estimated with HPLC. Quercetin aglycone was used as the external standard (Markham et al., 1998). A Varian HPLC system equipped with a PDA detector was used. A reversed phase C-18 Rannin column (4.6 $\times$ 250 mm, 10 μm) developed with MeOH:H_2O 55:45 (1 ml/min) was used for the analysis.

Screening of Middle Layer and Petroleum Ether Extracts for Aversive Response from Cattle. Three groups of yearling cattle (5/group) were trained for 1 week to eat for 30 min in the morning from feed boxes within individual pens. They had no access to feed during the night but had *ad libitum* access to water and were allowed *ad libitum* access to grass hay as a group after the morning training or testing periods. For the test period, they were given oat silage as a novel feed at the same time as for the training period. One group was given the petroleum ether extract (dosage of 0.059 g/kg body weight in gelatin capsules) after their initial consumption of oat silage on the 1st day of the test period. Another group was given the middle-layer residue at 0.176 g/kg body weight, and the control group was given empty capsules. The day after treatment, cattle were offered the same novel feed for 30 min at the same time as before, and the amount of feed consumed by each animal was recorded. Novel feed consumption on both days of the test period was compared using a repeated-measures design and PROC GLM (SAS, 1991) to determine if cattle had an aversive response to novel-feed on the 2nd day. Treatment group was the between-animal effect, and posttreatment response was the within animal effect. Individual means were compared with the protected LSD procedure (SAS, 1988).

RESULTS AND DISCUSSION

All rat treatment groups drank similar ($P = 0.32$) amounts of the novel-tasting liquid before they were gavaged with different portions of the aqueous methanol extract of *Euphorbia esula* (Figure 1). However, when they were offered the novel-tasting fluid on the 2nd day, there were clear differences among the groups ($P = 0.01$) in intake of the fluid, and the change in intake from pre- to postgavage by each group of rats differed ($P < 0.01$). Only the rats that received the acetone extract of the original aqueous methanol extract (residual) did not experience an aversion to the novel-tasting liquid. Rats that received the middle layer residue did not appear to have stronger aversion than did rats that received the chloroform extract ($P = 0.10$), but had similar aversions to the rats that received the petroleum ether extract ($P = 0.12$), and the remaining portion of the original aqueous extract ($P = 0.13$). The aversive nature of the remaining portion of the original aqueous extract was likely due to less than complete extraction of the original methanol

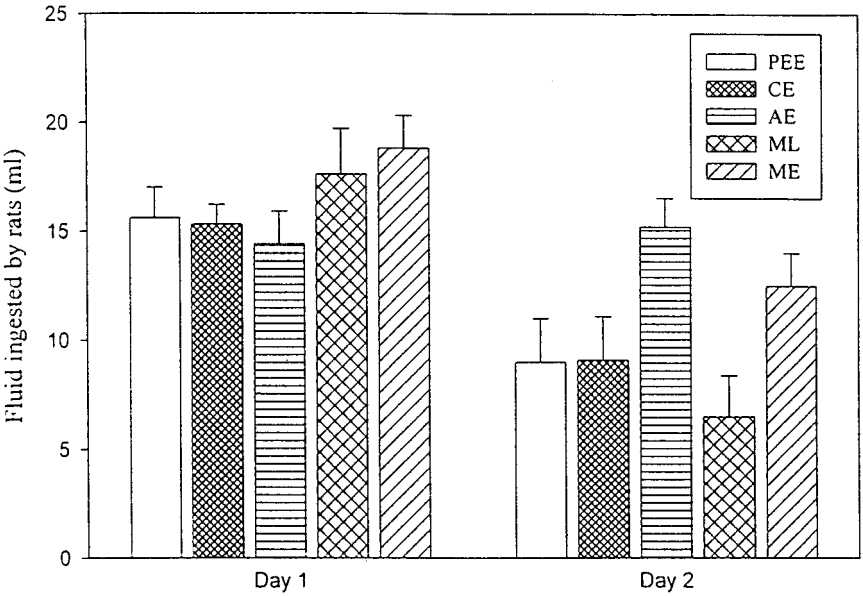

FIG. 1. Ingestion by rats of a novel-tasting fluid (mean ± 1 SE) before and after receiving different portions of a methanol extract of leafy spurge. The portions were a petroleum ether extract (PEE), a chloroform extract (CE), an acetone extract (AE), a thick viscous layer at the interface between the chloroform and aqueous layers named the middle layer (ML), and the remaining portion of the original aqueous methanol extract (ME). The only group of rats that did not develop an aversion to the novel fluid were rats treated with the acetone extract ($P < 0.01$).

extract by the solvents and process used. Although the middle layer did not appear to be more aversive than the petroleum ether, the residual or perhaps even the chloroform extract, it was partitioned and evaluated with another rat aversion trial since this material appeared to contain at least one aversive compound.

Physical and colored reaction suggested that major components of the middle layer were flavonoidal substances. Further purification with preparative TLC gave two main components with R_f 0.75 and 0.62 that were designated GL1 and GL2, respectively. Concentrations of GL1 and GL2 were 0.05 and 0.5% of total leafy spurge dry matter, respectively. The glycoside and aglycone were identified from HPLC comparison, ^{1}H NMR (see experimental), ^{13}C NMR data, and comparison of NMR data with those reported in the literature for kaempferol and quercetin (Bennini et al., 1992; Markham and Geiger, 1994). The carbohydrate part of GL1 and GL2 showed a monoglycoside structure as indicated from one ^{1}H NMR doublet at 5.48 ppm, which correlates with the anomeric carbon at 101.56 and 101.67 for GL1 and GL2, respectively. The rest of the sugar is indicated from oxycarbon at 76.25–71.42 ppm, which correlates with the proton shifts at 3.75–3.20 ppm that are assigned for C2″, C3″, C4″, and C5″, and a down field carbon shift at 168.8 ppm assigned for C6″. These showed that the sugar is an uronic acid. Acid hydrolysis of GL1 and GL2 followed by TLC examination revealed glucuronic acid by co-chromatography. The sugar in GL1 and GL2 was also hydrolyzed with β-glucosidase to indicate β-configuration for the glycosidic linkage to the aglycones. The latter were also confirmed from the coupling constant of 7.5 Hz of the anomeric doublet at 5.47 (Markham and Geiger, 1994). Attachment of the sugar moiety to position 3 was determined from the UV spectral data of the aglycones that indicated that there are free hydroxyls at positions 5, 7, and 4′ (GL1) and 5, 7, 3′, and 4′ (GL2) (Mabry and Markham, 1970).

The identities of GL1 and GL2 were revealed as kaempferol-3-0-β-glucuronic acid and quercetin-3-0-β-glucuronic acid, respectively. Kaempferol-3-0-β-glucuronic acid was identified previously from leafy spurge (Wagner et al., 1970). Our study is the first report and identification of quercetin-3-0-β-glucuronic acid from leafy spurge.

Kaempferol-3-0-β-Glucuronic acid (GL1). The UV, ^{1}H-&^{13}C NMR data compare favorably with the published data (Mabry and Markham, 1970; Wagner et al., 1970; Markham and Geiger, 1994).

Quercetin-3-0-β-Glucuronic acid (GL2). UV λ_{max} (MeOH) 354, 266sh, 253; +NaOMe: 405, 300sh, 274; +$AlCl_3$: 429, 365, 298sd, 273; AlCl3 +HCl:400sh, 360, 300sh, 269; +NaOAc: 413, 266 +NaOAc + H_3BO_3: 383, 261. ^{1}H NMR (ppm) 7.61 (1H, m, H-6′), 7.58 (1H, m H-2′), 6.76 (1H, H-5′), 6.76 (1H, H-8), 6.60 (1H, H-6), 5.48 (1H, H-1″), 3.750–3.18 (sugar protons). ^{13}C-NMR (MeOH) δ 156.10 (C-2), δ 132.56 (C-3), δ 176.58 (C-4), δ 161.04 (C-5), δ 18.60 (C-6), δ 164.20 (C-7), δ 93.12 (C-8), δ 156.23 (C-9), δ 102.96 (C-10), δ 121.04 (C-1′), δ 115.76 (C-2′), δ 143.86 (C-3′), δ 149.23 (C-4′), δ 115.65 (C-5′), δ 128.90

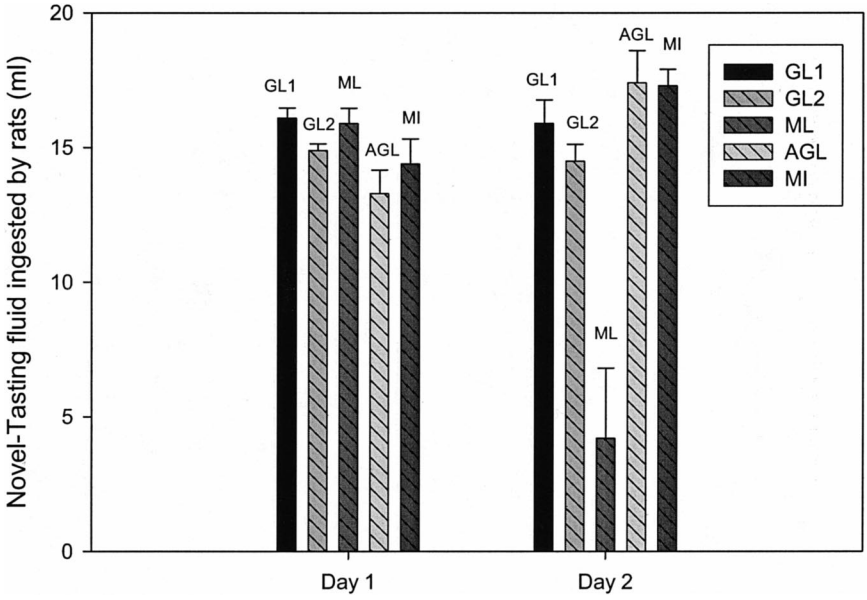

FIG. 2. Ingestion by rats of a novel-tasting fluid (mean ± 1 SE) before and after receiving kaempferol-3-0-β-glucoside (GL1), quercetin-3-0-β-glucoside (GL2), middle layer (ML), aglycone part of glycoside (AGL), and minor components of the middle layer (MI) of Euphorbia esula crude extracts. There was no difference in change in intake of the novel fluid from 1st to 2nd days for the GL1 and GL2 treatments ($P = 0.42$). The AGL and MI did not induce an aversion to the novel fluid but ML did ($P < 0.01$).

(C-6′), δ 101.67 (C-1″), δ 73.86 (C-2″), δ 76.34 (C-3″), δ 71.23 (C-4″), δ 76.04 (C-5″), and δ 169.56 (C-6″).

On the 1st day of the aversion trial to test rat response to GL1 and GL2, rats receiving GL2 drank less ($P = 0.03$) than the rats receiving GL1 (Figure 2). Change in intake of the novel-tasting fluid from the 1st to 2nd days was not different ($P = 0.42$) between the two groups with or without their first day's intake as a covariate in the model. This indicates that GL1 and GL2 had similar effects on drinking behavior, and neither of the two glucosides induced an aversion to a novel-tasting fluid ($P = 0.86$). The aglycone (quercetin from GL2) and minor components of the middle layer also did not induce a food aversion in rats as the middle layer did ($P < 0.01$ Figure 2). Although quercetin and other analogs inhibit glycolysis (Raker, 1986) and likely cause animals to feel sick, the amount of aglycone used in the aversion trial may have been inadequate to elicit a food aversion. Testing GL1 and GL2 is useful, but also creates an artificial situation if the feeding aversion is due to synergistic activity of several compounds combined.

Since the other components (minor components) of the middle layer did not elicit food aversion, we suspect that separating the two glucosides reduced their capacities to elicit food aversions. However, given the results from the trial with cattle, we did not test this hypothesis.

Cattle had similar ($P = 0.95$) intakes of novel feed on the 1st day before treatment, but those treated with the middle layer had a different ($P < 0.01$) response on the 2nd day compared to those treated with the petroleum ether extract (Figure 3). In contrast to the response of laboratory rats, the middle layer did not elicit a feed aversion. However, the petroleum ether extract induced a strong feed aversion in both cattle (Figure 3) and laboratory rats (Figure 1). An evaluation of the aversion-eliciting components of this extract was described previously in Halaweish et al. (2002). The strong contrast between the responses of rats and cattle to the middle layer is probably best explained by differences in the gastro-intestinal tract. It is known that gastro-intestinal microbes can hydrolyze flavonoid glycosides into their corresponding aglycones and sugar (Hackett, 1996). This likely eliminates the capacity of these compounds to elicit aversions in a forestomach-fermenting

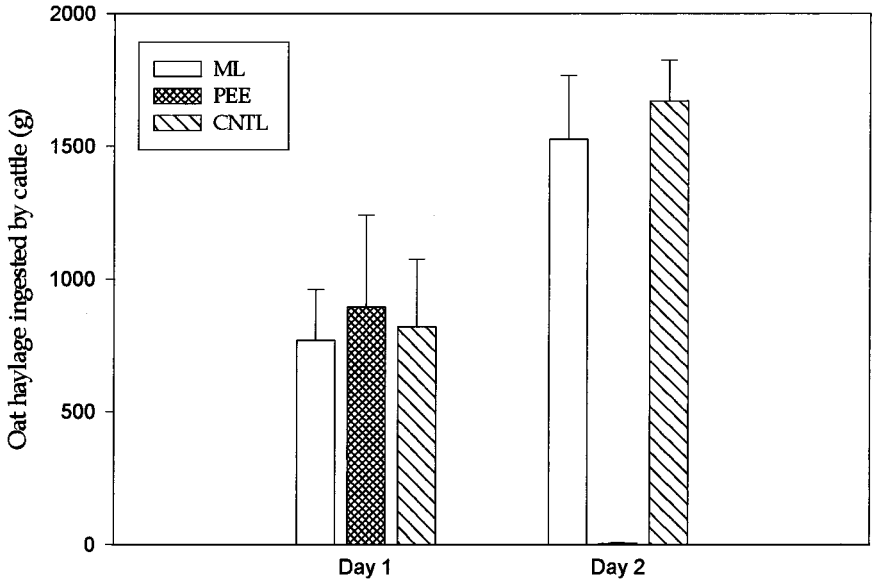

FIG. 3. Ingestion by cattle of a novel-tasting feed (mean ± 1 SE) before and after receiving different portions of the original methanol extract of leafy spurge in gelatin capsules. The portions were a thick viscous layer at the interface between the chloroform and aqueous layers named the middle layer (ML) and the petroleum ether extract (PEE). A third group of control cattle (CNTL) received an empty gelatin capsule. Cattle treated with PEE were the only group that developed an aversion to the novel feed ($P < 0.01$).

ruminant, while in hind-gut fermenters like monogastric laboratory rats the compounds are absorbed into the blood stream and can elicit aversions before microbes can degrade them. Apparently, there are one or more compounds in the petroleum ether extract of *Euphorbia esula* that can elicit food aversions in both ruminants and monogastrics, and future research to understand foraging behavior of mammals with this plant should focus first on compounds in *Euphorbia esula* that are extracted by petroleum ether.

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IN VITRO DEGRADATION OF WILLOW SALICYLATES

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Abstract—Salicylates are defensive compounds against a great variety of generalist herbivores. Salicortin and its derivatives are labile compounds that degrade immediately when cell compartmentalization is ruptured, producing a 6-hydroxy-2-cyclohexenone (6-HCH) moiety that is a strong antifeeding cue. We studied the *in vitro* degradation of willow salicylates in the presence and absence of foliar enzymes at acidic, neutral, and alkaline pHs. Higher substituted salicylates were degraded in the absence of foliar enzymes at alkaline pH and in the presence of foliar enzymes at all three pHs. Salicin and its diglucoside, on the other hand, were degraded only in the presence of foliar enzymes at acidic pH, probably by β-glucosidase activity. The main degradation products of higher substituted salicylates were salicin, 6-HCH, and catechol in both the absence and presence of enzymes, suggesting that the production of 6-HCH and catechol do not necessarily demand enzymatic activity. We propose that the degradation of salicylates begins with the cleavage of a 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety by foliar esterases and/or alkaline condition. This moiety is decarboxylated in nonenzymatic reaction to an anion of 2-hydroxy-3-cyclohexenone, which is tautomerized to the enol form and further to the keto form, 6-HCH. Hydroxyketone can be also oxidized to catechol, a substrate of polyphenol oxidases.

Key Words—Salicylates, phenolic glycosides, degradation, willow, *Salix*, herbivory, foliar esterases, β-glucosidase.

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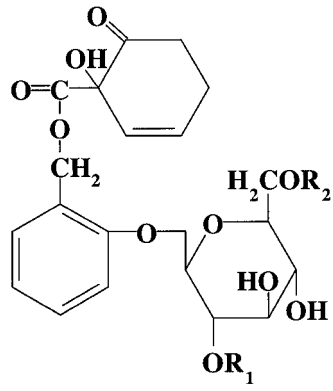
INTRODUCTION

Degradation of secondary compounds often releases aglycones or moieties that play defensive roles against herbivores. These degradation products may reduce the nutritive value of leaves, act as feeding deterrents, or they may simply be toxic substances (Scriber and Slansky, 1981). In an earlier study, salicylates, the defensive compounds of willows and poplars (Salicaceae), were degraded to catechol and salicin in the digestive tract of a generalist moth, *Operophtera brumata* (Lep.: Geometridae) (Ruuhola et al., 2001a). Catechol, unlike salicin, was an effective component in defense, acting as a strong feeding deterrent (Ruuhola et al., 2001a). Various studies suggest that catechol is derived from the 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety, a labile component of salicortin, acetylsalicortin, and tremulacin, that is released by leaf esterases when cell compartmentalization is ruptured (Pearl and Darling, 1971; Clausen et al., 1989b; Julkunen-Tiitto and Meier, 1992; Haruta et al., 2001; Ruuhola et al., 2001a). This released moiety is converted to 6-hydroxy-2-cyclohexenone (6-HCH) via decarboxylation and isomerization, reactions considered to be enzymatic (Mattes et al., 1987). However, 6-HCH is also produced in the absence of decarboxylating enzymes (Julkunen-Tiitto and Meier, 1992). We, therefore, studied in more detail the degradation of salicylates under *in vitro* conditions in the absence (dry leaves) and the presence (fresh leaves) of foliar enzymes at three different pHs, selected to correspond to the pH of plant vacuoles (pH 5.5), cytosol (pH 7.0), and the midgut of *O. brumata* larvae (pH 9.0; Berenbaum, 1980). Three willow species were selected for this study: *Salix myrsinifolia,* containing mainly nonacyled salicylates (glucose group was neither acetylated nor benzoylated) such as salicortin, *Salix pentandra,* containing acetylated salicylates, and *Salix repens,* containing both nonacyled and benzoylated salicylates (Figure 1).

METHODS AND MATERIALS

Degradation of salicylates in the absence of foliar enzymes was studied by incubating phenolics extracted from dried leaves of three willow species in potassium buffer at three pH values or in MeOH. Dry leaves (200 mg) of *S. myrsinifolia* (raised in a greenhouse, clone V8), *S. repens* (one individual collected in nature), and *S. pentandra* (raised in a greenhouse, clone RM1) were extracted by the method described in Ruuhola et al. (2001a). Extracts were evaporated to dryness in a vacuum evaporator. The dried extracts were redissolved in 20 ml 100% MeOH, divided into 1 ml aliquots, and dried with gaseous nitrogen. Samples were divided into four treatment groups: three treatments with 1 ml 0.1 M potassium buffer at pH 5.5, 7.0, or 9.0 (Sambrook et al., 1989), and one treatment with 50% MeOH (0.5 ml dH_2O + 0.5 ml 100% MeOH). Four replicates were used for each treatment. The samples were stabilized at room temperature for a few hours prior to



Salicortin: $R_1 = R_2 = H$

2'-*O*-Acetylsalicortin: $R_1 = COCH_3$, $R_2 = H$

2'6'-*O*-Diacetylsalicortin: $R_1 = R_2 = COCH_3$

Tremulacin: $R_1 = COC_6H_6$, $R_2 = H$

Acetyltremulacin: $R_1 = COCH_3$, $R_2 = COC_6H_6$

FIG. 1. Chemical structures of salicortin, 2′-*O*−acetylsalicortin, 2′6′-*O*-diacetylsalicortin, tremulacin, and acetyltremulacin. The structures of 2′6′-*O*-diacetylsalicortin and acetyltremulacin are hypothetical, based on mass-spectra of the compounds.

HPLC analysis (Ruuhola et al., 2001b). Identification of salicylates was confirmed by means of HPLC/API-ES mass spectrophotometry (Julkunen-Tiitto and Sorsa, 2001). The fragmentation voltage was 60–180 V depending on the substance. Authentic salicortin and 6-HCH samples (Julkunen-Tiitto and Meier, 1992) in MeOH and in phosphate buffer at three pH values were also analyzed. Since MS–HPLC was not able to give a mass spectrum for 6-HCH, the sample was checked by mass selective gas chromatography (GC–MS; JEOL D300) by the method described in Julkunen-Tiitto and Meier (1992).

Degradation of salicylates in the presence of foliar enzymes was studied by crushing fresh leaves of three willow species in potassium buffer at different pH values or in MeOH. Fresh leaves of micropropagated *in vitro* plants *S. myrsinifolia* (clone V6), *S. pentandra* (clone RM7), and *S. repens* (clone H251) were cut and weighed. The leaves (15–30 mg) were divided into four treatment groups: 100% MeOH or potassium buffer at pH 5.5, 7.0, or 9.0. Leaves were then homogenized in 1 ml of MeOH or 0.1 M potassium buffer with Ultra-Turrax homogenizator (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) for 30 sec. The homogenized

leaf mixtures were incubated in a water bath at +37°C for 15 min, followed by centrifugation in an Eppendorf centrifuge (Mini Spin®, Netheler-Hinz GmbH, Hamburg, Germany) (13,500 rpm) for 60 sec. Possible enzymatic reactions were stopped by adding 500 μl of 100% MeOH to 500 μl of the supernatant, and the samples were analyzed by HPLC (Ruuhola et al., 2001b). Each treatment was repeated four times.

To establish the more exact salicylate content of intact micropropagated plants, leaves were extracted in MeOH by the following method. Fresh leaves were cut and incubated at 100% MeOH (600 μl) for 15 min. Leaves were homogenized with Ultra-Turrax homogenizator for 40 sec, followed by brief centrifugation in Eppendorf centrifuge. The supernatant was carefully removed and placed in a new tube. Leaf residues were extracted twice with 600 μl MeOH, and the supernatants were combined. MeOH was dried by means of gaseous nitrogen, and the extract was redissolved in 1 ml 50% MeOH and analyzed by HPLC (Ruuhola et al., 2001b).

RESULTS AND DISCUSSION

Nonenzymatic Degradation of Salicylates. The authentic salicortin standard was stable in MeOH and in buffer solution at acidic (pH 5.5) and neutral pHs (pH 7.0), but it was degraded to salicin at alkaline pH (pH 9.0) (Figure 2A). Degradation of salicortin also produced 6-HCH and catechol; salicin was not degraded further to saligenin. The salicin standard remained unchanged in all these treatments (data not shown), confirming that neither pH nor solvent affect the quantification of salicin. The old, but authentic, sample of 6-HCH (Julkunen-Tiitto and Meier, 1992) also contained catechol and phenol, which were obviously produced during storage, indicating that 6-HCH can be converted to catechol and phenol without enzymatic activity (Figure 2B; see Mattes et al., 1987; Clausen et al., 1989b, 1991). In a water-based buffer solution, 6-HCH existed as a single isomer, while in a 50% MeOH solution, two 6-HCH peaks were found (Figure 2B). The smaller peak could be a 2-hydroxy-3-cyclohexenone, an isomer of 6-HCH detected by Mattes et al. (1987).

S. myrsinifolia contained the following individual salicylates: salicin, diglucoside of salicin, salicortin, disalicortin (Tegelberg and Julkunen-Tiitto, in press), and salicortin-2 and -3 (Julkunen-Tiitto and Sorsa, 2001; Ruuhola et al., 2001b) (Figure 3). The degradation of *S. myrsinifolia* salicylates produced salicin, 6-HCH, and catechol (Figure 3). An unknown compound was found, with a UV spectrum resembling that of 6-HCH (6-HCH-2); this compound was probably also a degradation product of salicylates. The most labile compounds in the *S. myrsinifolia* dry leaf extract were disalicortin and salicortin-2 and -3. These compounds were found both in MeOH and in buffer at acidic pH, but they degraded at neutral

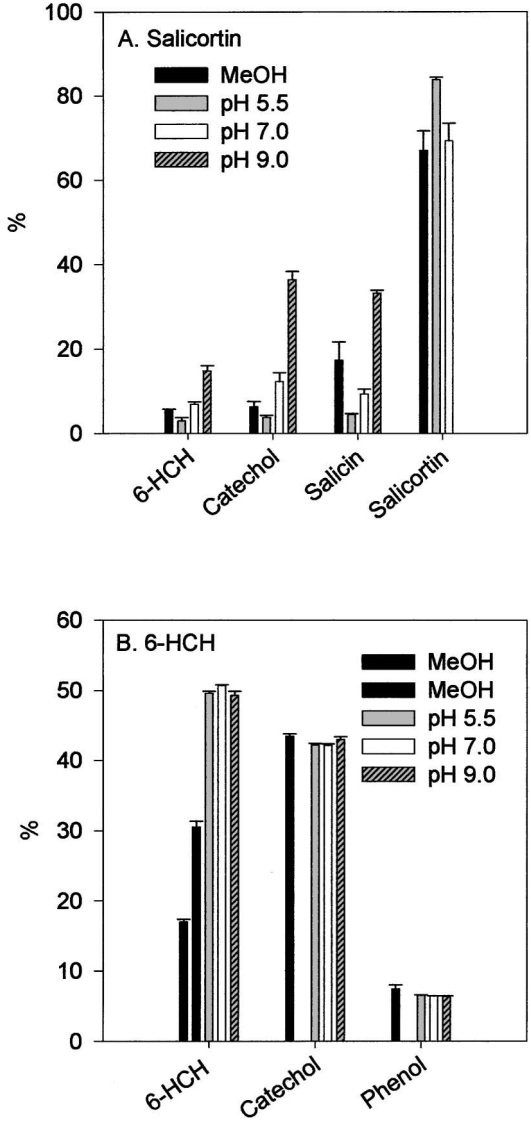


FIG. 2. Authentic samples of (A) salicortin and (B) 6-HCH in 50% methanol or potassium phosphate buffer at pH 5.5, 7.0, or 9.0. In a water-based buffer solution, 6-HCH exists as one isomer, while in MeOH solution two isomers were found.

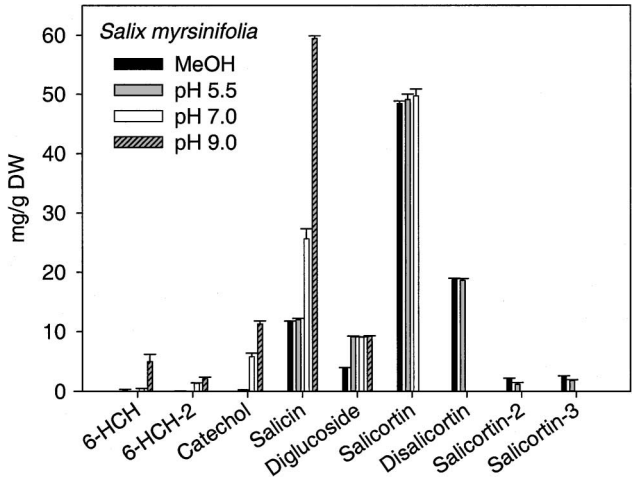


FIG. 3. Salicylates of *Salix myrsinifolia* leaf extract dissolved in 50% methanol or potassium buffer at pH 5.5, 7.0, or 9.0. *S. myrsinifolia* contained nonacyled salicylates.

and alkaline pHs (Figure 3). Like the authentic salicortin standard, salicortin in *S. myrsinifolia* leaf extract was also stable in MeOH and in buffer at pH 5.5 and 7.0, but was degraded at pH 9.0 (Figure 3). The diglucoside of salicin was not degraded at any pH, but the level of this compound was markedly lower in MeOH than in buffer solutions (Figure 3). This is probably due to the water-soluble nature of the diglucosylated compound, which leads to impaired dissolution of the compound in alcohol.

S. repens contained the same nonacyled salicylates as were found in *S. myrsinifolia*: salicin, diglucoside of salicin, salicortin, and salicortin-2 and -3, but also the benzoylated salicylates: tremuloidin and tremulacin (Figure 4). The degradation of salicylates produced salicin, catechol, and 6-HCH and its putative derivative 6-HCH-2 (Figure 4). Tremulacin started to degrade at neutral pH, and at alkaline pH a migration of the benzoyl group produced an isomer of tremulacin, tremulacin-2 (Figure 4). Pearl and Darling (1963) observed a similar migration of the benzoyl group of tremuloidin from position 2′ to position 6′ at alkaline pH or at elevated temperatures. Interestingly, the level of tremuloidin was constant in different treatments despite the degradation of tremulacin. This indicates that tremulacin is not necessarily first degraded to tremuloidin (see Clausen et al., 1989a; Julkunen-Tiitto and Meier, 1992). Instead, we propose that tremulacin might be degraded to salicortin by release of the benzoyl group of the glucose moiety and then to salicin by the release of the hydroxycyclohexenone carbonyl moiety. At alkaline pH, we found a small amount of salicortin, supporting this idea.

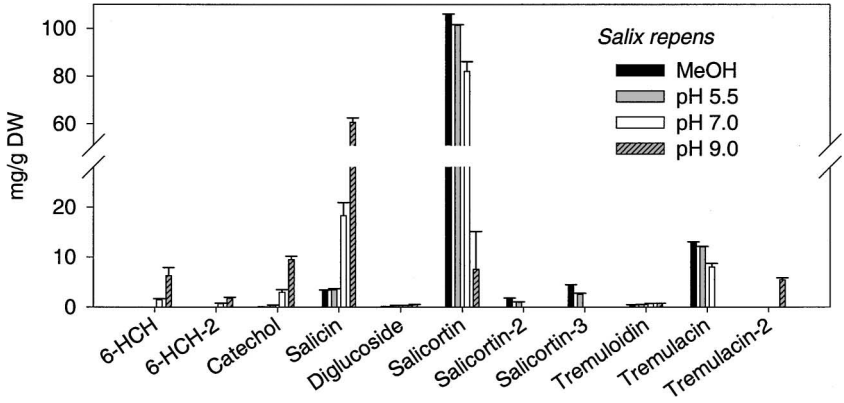


FIG. 4. Salicylates of *Salix repens* leaf extract dissolved in 50% methanol or potassium buffer at pH 5.5, 7.0, or 9.0. *S. repens* contained both nonacyled and benzoylated-salicylates.

The diversity of salicylates was greatest in *S. pentandra* (Figures 3–5). Leaves contained nonacyled salicylates (Figure 5A) and several acetylated salicylates (Figure 5B and C); a novel compound acetyltremulacin (Figure 5A) was also found. Acetyltremulacin was tentatively identified by MS–HPLC. The compound had a putative mass peak at m/z 593 $[M+Na]^+$. The mass spectrum contained a fragment ion at m/z 331, which was evidently formed by the loss of the neutral cyclohexenone saligenin ester (262 Da); it also contained a fragment ion of cyclohexenone carboxylic acid at m/z 179.

As in the two other willow species, degradation of salicylates in *S. pentandra* produced salicin, catechol, 6-HCH, and 6HCH-2 (Figure 5A). Salicortin was stable in MeOH and at pH 5.5 and 7.0, but degraded at pH 9.0 (Figure 5A). In addition, the diglucoside of salicin remained at the same level in buffer solutions at different pHs, but was markedly lower in MeOH (Figure 5A). Acetyltremulacin was a stable compound, not markedly degraded at alkaline pH (Figure 5A).

Acetylated salicylates were isomerized and degraded in buffer solutions at pH 7.0 and 9.0 (Figure 5B and C). Three isomers of diacetylsalicortin were found in MeOH and at pH 5.5 and 7.0, while at pH 9.0 only a trace of diacetylsalicortin-1 was detected and two other isomers disappeared (Figure 5C).

We also found novel degradation products of diacetylsalicortins, diacetylsalicins, which reached their highest levels at pH 9.0. 2'-*O*-Acetylsalicortin was stable both in MeOH and in buffer solution at pH 5.5, but its level decreased at pH 7.0 and the compound disappeared completely at pH 9.0 (Figure 5B). The levels of two isomers of 2'-*O*-acetylsalicortin increased at pH 7.0, and slight amounts of acetylsalicortin-1 and -3 were present at pH 9.0 (Figure 5B). The main degradation products of *S. pentandra* salicylates were acetylsalicin-3, salicin, and catechol.

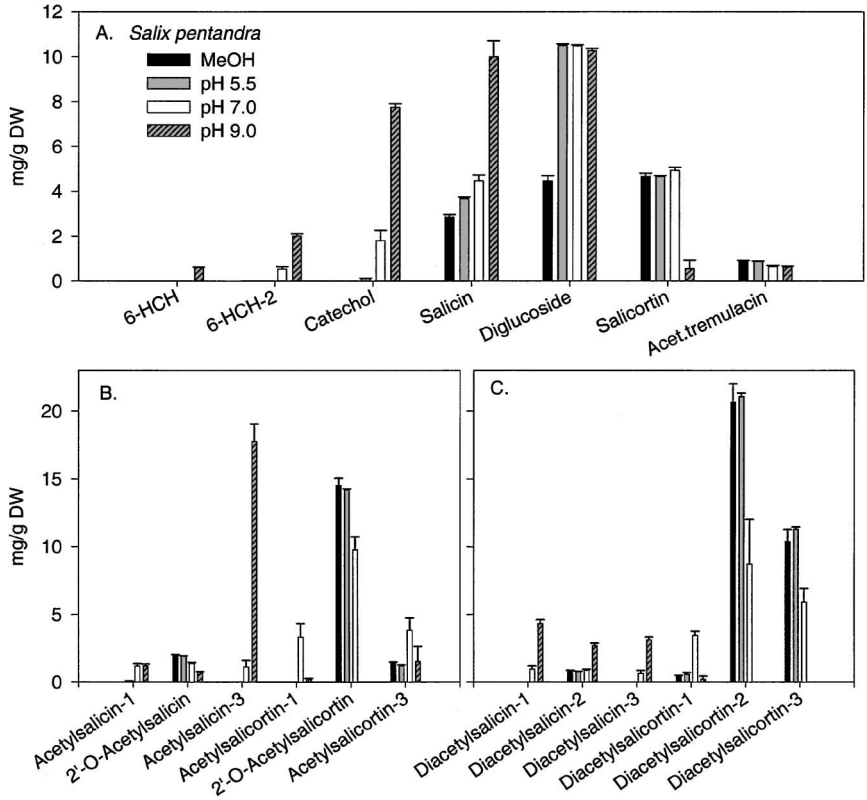


FIG. 5. Salicylates of *Salix pentandra* leaf extract dissolved in 50% methanol or potassium buffer at pH 5.5, 7.0, or 9.0. *S. pentandra* contained (A) nonacyled, (B) acetylated, and (C) diacetylated salicylates. A novel compound acetyltremulacin was also found (A).

Degradation of Salicylates in the Presence of Foliar Enzymes. The diversity of salicylates in the leaves of micropropagated willows (Figure 6) was lower than in the leaves of non-micropropagated plants (Figures 3–5). Intact leaves of micropropagated *S. myrsinifolia* contained the diglucoside of salicin, salicin, salicortin, and disalicortin (Figure 6A). Intact leaves of *S. repens* contained salicin, catechol, salicortin, tremulacin, and salicortin-2 (Figure 6B). *S. pentandra*, in turn contained the diglucoside of salicin, salicin, 2'-*O*-acetylsalicin, salicortin, 2'-*O*-acetylsalicortin, diacetylsalicortin, tremulacin, and salicortin-2 (Figure 6C). Micropropagated *S. pentandra* clone RM1 did not contain acetyltremulacin found in the leaves of clone RM7 raised in green house (Figure 5A). Also, clone RM1 did not contain acetylhydroxycyclohexenone salicortin found in our other study (Ruuhola et al., in press). This might be explained by the change in the light

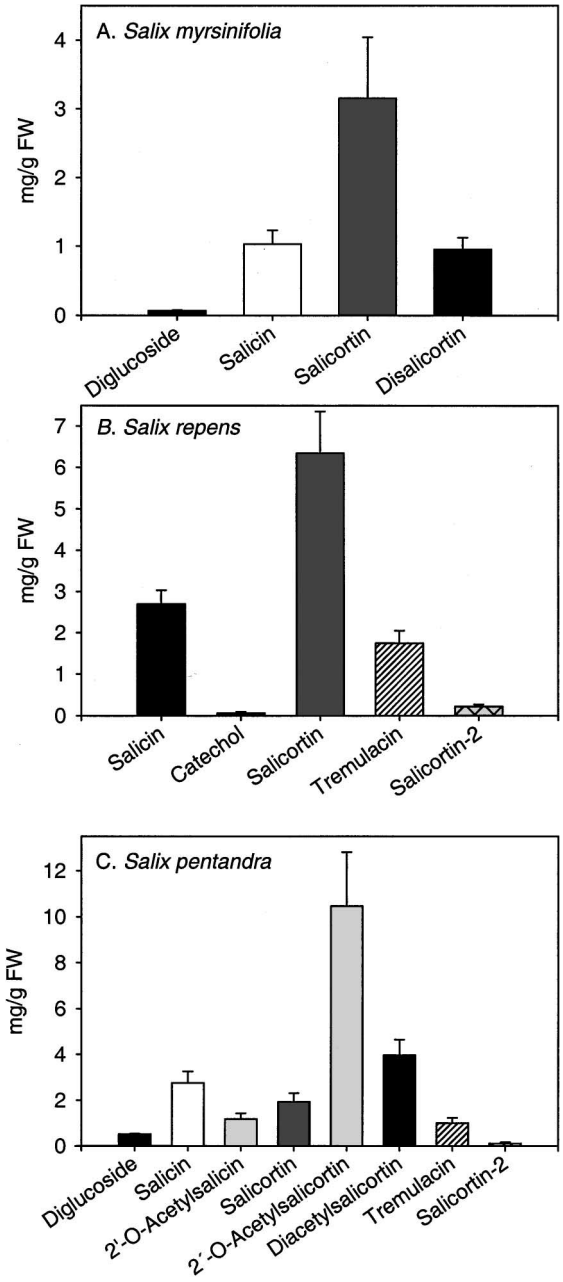


FIG. 6. Salicylates in the intact leaves of micropropagated (A) *S. myrsinifolia*, (B) *S. repens*, and (C) *S. pentandra* in MeOH.

environment; plants were transferred to a new growth chamber with higher light intensity after the previous study.

Plant leaves contain a diversity of catabolic enzymes, such as esterases and β-glucosidases, which are released from cell compartments during the homogenization of plant tissue and come in contact with their substrates. In earlier studies, salicylates were shown to be the substrates of esterases and β-glucosidases (Julkunen-Tiitto and Meier, 1992) and apparently also of polyphenol oxidases (Haruta et al., 2001). The optimum pH for the activity of these enzymes is variable and depends on plant species: optimum pH for the activities of β-glucosidases is quite acidic (around pH 5), whereas esterases are usually most active in basic solutions, and polyphenol oxidases (PPOs) in neutral solutions. In crushed willow leaves, salicylates were degraded also at acidic pH (Figure 7), which did not occur in the absence of foliar enzymes, indicating that foliar enzymes present in the leaf homogenates mediated degradation. When leaves were crushed in MeOH, salicylates were not degraded (data not shown). The degradation of salicylates produced salicin, 6-HCH, and catechol in all three willow species (Figure 7).

Salicortin and disalicortin found in intact leaves of *S. myrsinifolia* (Figure 6A) were completely degraded in crushed leaves, yielding salicin, 6-HCH, and catechol at all three pHs, while the diglucoside of salicin appeared to be relatively stable (Figure 7A). A trace of saligenin, a degradation product of salicin (Julkunen-Tiitto and Meier, 1992), was detected at pH 5.5 (Figure 7A). In crushed *S. repens* leaves, tremulacin and tremuloidin were found at all three pHs (Figure 7B); the level of salicin was also identical at the three pHs, suggesting that the conditions were not favorable for the degradation of these compounds. Salicortin and salicortin-2, however, were degraded at all three pHs.

The diglucoside of salicin present in intact leaves of *S. pentandra* was not detected in the leaf homogenate at pH 5.5, and the level of salicin was also the lowest at this pH (Figure 7C). Instead, saligenin derivative (saligenin-1) was found in the leaf homogenate at pH 5.5, suggesting that salicin and its diglucoside were degraded to saligenin by β-glucosidase with an acidic optimum pH (Figure 7C; see Julkunen-Tiitto and Meier, 1992). 2'-*O*-Acetylsalicortin and diacetylsalicortin were found in the intact leaves of *S. pentandra* (Figure 6C), but neither compound was detected in the homogenate of crushed leaves at any pH (Figure 7C). However, an unknown salicylate appeared at pH 5.5, which might be a degradation product of acetylsalicortins. 2'-*O*-Acetylsalicin and its isomers were found in the leaf homogenates at pH 5.5 and 7.0; tremulacin and tremuloidin were also detected at pH 5.5 and 7.0, whereas at pH 9.0 none of these compounds was detected (Figure 7C). Almond β-glucosidase was found to be inefficient in degrading acetylated or benzoylated salicylates, but these compounds were degraded by porcine esterases with an alkaline optimum pH (Julkunen-Tiitto and Meier, 1992). Thus, acetylated and benzoylated salicylates of *S. pentandra* might be degraded by foliar esterase. Salicortin was found at

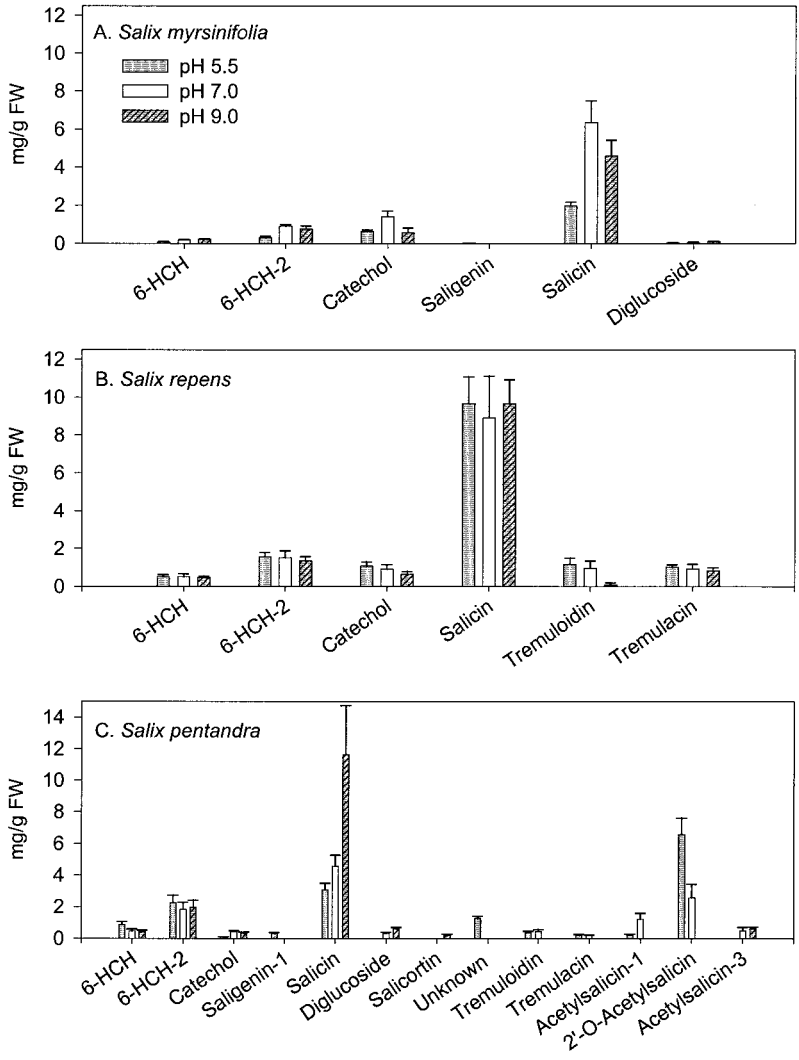


FIG. 7. Salicylates in the crushed leaves of micropropagated (A) *Salix myrsinifolia*, (B) *S. repens*, and (C) *S. pentandra* in buffer solution at pH 5.5, 7.0, or 9.0.

alkaline pH, supporting the idea that salicortin may be a degradation product of tremulacin.

Degradation Model of Salicylates. We propose that the degradation of salicortin begins with cleavage of the 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety either by esterase or simply at an alkaline pH, yielding salicin, 6-HCH, and

catechol (Figure 8). Salicin and diglucoside of salicin are degraded to saligenin by β-glucosidase. Acetylsalicortin is degraded by esterase or at an alkaline pH to acetylsalicin, which in turn is degraded to salicin. We suggest that the degradation of tremulacin may produce salicortin by cleavage of the benzoyl group from the sugar moiety.

Our degradation model supports the general idea of the degradation of salicylates, which suggests that degradation begins with the release of the 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety, producing salicin and 6-HCH (Mattes et al., 1987; Clausen et al., 1989b, 1991; Julkunen-Tiitto and Meier, 1992; Haruta et al., 2001; Ruuhola et al., 2001a). However, we propose that the production of 6-HCH is not necessarily enzymatic, as suggested by Mattes et al. (1987), instead, we suggest that the released 1-hydroxy-6-oxo-2-cyclohexenone carboxylic acid anion is decarboxylated to 2-hydroxy-3-cyclohexenone (Figure 8). The hydroxyketone tautomerizes to enol form and further to the other keto form, 6-HCH. The hydroxyketone can also be oxidized to catechol in the presence of oxygen. A similar model was presented by Pearl and Darling (1971), who suggested that the degradation of salicortin produces 1-hydroxy-6-oxo-2-cyclohexene carboxylic acid, which eliminates CO_2 and dehydrogenates to catechol in the presence of air.

Our degradation model of salicylates does not support the hypothetical model of Lindroth et al. (1988), which suggestes that the degradation of salicortin and tremulacin begins with the hydrolysis of compounds by β-glucosidase, producing cyclohexenone saligenin ester, which is then detoxified by carboxylesterases. The suggested detoxification pathway, however, releases saligenin and cyclohexenone (Lindroth et al., 1988; Lindroth, 1989), which are deleterious compounds for insects (Clausen et al., 1989b; Ruuhola et al., 2001a), rather than less toxic compounds. Furthermore, the highly labile nature of the hydroxycyclohexencarbonyl moiety of salicylates contests the degradation model of Lindroth et al. (1988).

Degradation Activates Salicylates. The activity and properties of foliar and larval enzymes, and the pH of the larval midgut, may markedly influence the toxicity of salicylates for plant-eating animals. Catechol produced in the degradation of salicylates is oxidized by polyohenoloxidase (PPO) to *o*-quinone, which is capable of reducing the nutritive value of leaves (Felton et al., 1989; Constabel, 1999; Haruta et al., 2001). In addition, 6-HCH itself is an alkylating factor (Clausen et al., 1989b).

Tremulacin reduces the growth of *Lymantria dispar* (Lep.) larvae through reduction of the approximate digestibility and food conversion efficiency (Lindroth and Hemming, 1990). However, the major function of 6-HCH and catechol in defense is apparently in deterring the feeding of generalist herbivores (Clausen et al., 1989b, 1991; Ruuhola et al., 2001a). Salicylates have also been shown to be toxic substances causing gut lesions in southern armyworm larvae (Lindroth and Petersson, 1988). In addition, saligenin reduces the growth of *O. brumata* larvae via its toxic effects rather than by feeding deterrence (Ruuhola et al., 2001a).

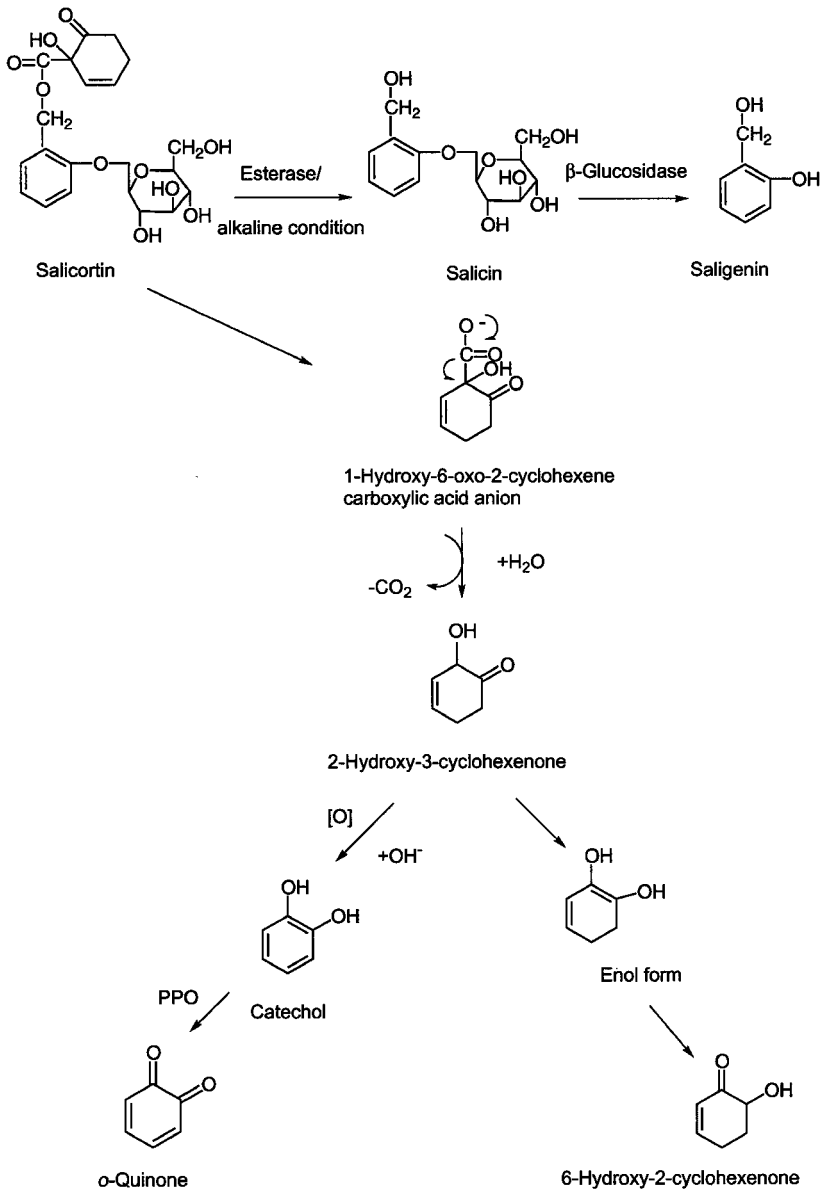


FIG. 8. Degradation model of salicortin. The degradation of salicortin by esterase or alkaline pH produces salicin, 6-HCH, and catechol. Salicin is further degraded to saligenin by β-glucosidase. The degradation of salicortin begins with a release of salicin and a 1-hydroxy-6-oxo-2-cyclohexene carboxylic acid anion. Decarboxylation of the latter produces 2-hydroxy-3-cyclohexenone, which tautomerizes to its enol form and further to 6-hydroxy-2-cyclohexenone. The enol can also be converted to catechol. Catechol is a substrate of PPOs that produce harmful $o-$quinones.

To summarize, salicylates seem to be able to reduce the growth of herbivores in three ways: by feeding deterrence, by reducing the nutritive value of leaves, or via toxic effects. These detrimental effects reduce the feeding and also the growth rate of insect larvae, which may increase their mortality from natural enemies (Clancy and Price, 1987), providing efficient protection for plants against a variety of herbivores.

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ANTIOXIDANTS IN THE MIDGUT FLUIDS OF A TANNIN-TOLERANT AND A TANNIN-SENSITIVE CATERPILLAR: EFFECTS OF SEASONAL CHANGES IN TREE LEAVES

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Abstract—The seasonal decline in foliar nutritional quality in deciduous trees also effects the availability of essential micronutrients, such as ascorbate and α-tocopherol, to herbivorous insects. This study first examined whether there are consistent patterns of seasonal change in antioxidant concentrations in deciduous tree leaves. α-Tocopherol concentrations increased substantially through time in late summer in sugar maple (*Acer saccharum*), red oak (*Quercus rubra*), and trembling aspen (*Populus tremuloides*). However, seasonal change in the concentrations of other antioxidants differed between each species: *P. tremuloides* had higher levels of ascorbate and glutathione in the spring, *Q. rubra* had higher levels of glutathione but lower levels of ascorbate in the spring, and *A. saccharum* had lower levels of both ascorbate and glutathione in the spring. To test the hypothesis that tannin-tolerant caterpillars maintain higher concentrations of antioxidants in their midgut fluids than do tannin-sensitive species, we measured antioxidants in *Orgyia leucostigma* (a spring- and summer-feeding, tannin-tolerant species) and *Malacosoma disstria* (a spring-feeding, tannin-sensitive species) that were fed tree leaves in the spring and summer. The midgut fluids of *O. leucostigma* larvae generally had higher concentrations of antioxidants in the summer than did those of *M. disstria*, and were significantly higher overall. The results of this study are consistent with the hypothesis that higher concentrations of antioxidants form an important component of the defenses of herbivores that feed on

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mature, phenol-rich tree leaves. Some limitations of the interpretation of total antioxidant capacity are also discussed.

Key Words—Ascorbic acid, α-tocopherol, glutathione, Lepidoptera, herbivore, antioxidant, tree leaves.

INTRODUCTION

Seasonal changes in foliar chemistry are well known. Numerous studies have established that the nutritional quality of tree leaves declines as they mature: protein nitrogen, sugars, phosphorus, and water decrease, while fiber, toughness, and condensed tannins increase (Feeny, 1968, 1970; Scriber, 1977; Ricklefs and Matthew, 1982; Schroeder, 1986; Ayres and MacLean, 1987; Lindroth et al., 1987; Mauffette and Oechel, 1989; Hunter and Lechowicz, 1992). Little work has been done on the seasonal changes in the levels of the three most important low-molecular-weight antioxidants in foliage, i.e., ascorbate (vitamin C), α-tocopherol (vitamin E), and the tripeptide glutathione. The levels of these substances may have important effects on the nutritive value of foliage to herbivorous insects, not only because ascorbate and α-tocopherol are essential micronutrients (Vanderzant et al., 1962; Dadd, 1973; Navon, 1978; Kramer and Seib, 1982; Meister, 1992), but also because they affect oxidative processes that occur in the gut lumens of herbivorous insects. It is well established that the oxidation of potential prooxidants (e.g., many phenolic compounds) in the gut lumens of caterpillars can produce reactive oxygen species and quinones, which can cause oxidative stress and reduce the availability of essential nutrients (Felton et al., 1989; Summers and Felton, 1994; Bi and Felton, 1995; Pardini, 1995; Thiboldeaux et al., 1998; Halliwell and Gutteridge, 1999). Antioxidants enable herbivorous insects to protect themselves from the potential effects of allelochemical oxidation in their gut lumens (Barbehenn et al., 2001) and tissues (Felton et al., 1989; Felton and Duffey, 1992; Summers and Felton, 1994; Bi and Felton, 1995; Timmerman et al., 1999). Accordingly, increased levels of antioxidants improve the fitness of herbivorous insects when they ingest potential prooxidants (Aucoin et al., 1990), and low levels of ingested antioxidants can reduce the fitness of herbivorous insects (Navon, 1978; Lindroth et al., 1991; Felton and Summers, 1993; Lindroth and Weiss, 1994), and make them more susceptible to infection by pathogens (Lindroth et al., 1991).

Malacosoma disstria Hübner (Lasiocampidae), the forest tent caterpillar, and *Orgyia leucostigma* Smith (Lymantriidae), the white-marked tussock moth, are both polyphagous Lepidoptera that feed on the three tree species examined in this study (Stehr and Cook, 1968; Baker, 1972; Nicol et al., 1997; Panzuto et al., 2001). However, they have different life-history strategies. While *M. disstria* has a single generation of larvae in the spring, *O. leucostigma* has two or more generations that feed either on young foliage in the spring or on fully mature leaves in the summer. While *O. leucostigma* larvae are unaffected by the consumption of diets containing

as much as 8% (dry weight) tannic acid, as little as 0.5% tannic acid causes lethal pupal deformities in *M. disstria* (Karowe, 1989). Higher concentrations of antioxidants in the midgut fluid of *O. leucostigma* are associated with its ability to tolerate ingested tannins by limiting phenol oxidation (Barbehenn and Martin, 1992; Barbehenn et al., 2001). In contrast, extensive phenol oxidation occurs in the midgut lumen of *M. disstria* (Barbehenn and Martin, 1994; Barbehenn et al., 2001, 2003). In this study, we tested the hypothesis that *O. leucostigma* maintains higher concentrations of antioxidants in its midgut fluid than does *M. disstria* when they feed on tree leaves, especially in the summer, when elevated concentrations of phenols are commonly present in deciduous tree leaves.

If *M. disstria* larvae are poorer at maintaining antioxidants in their midgut fluids than are larvae of *O. leucostigma* when feeding on artificial diet, we expected that *M. disstria* might rely on ingesting higher concentrations of antioxidants in spring foliage. The small number of studies that have explored seasonal variation in the levels of antioxidants in tree foliage show few consistent patterns between tree species and antioxidants (Dash and Jenness, 1985; Kunert and Ederer, 1985; Schupp and Rennenberg, 1988; Luwe, 1996). Therefore, an initial goal of this study was to establish the concentrations of the three major antioxidants and total antioxidant capacity (TAC) in the leaves of three host tree species. Ascorbic acid, glutathione, α-tocopherol, and TAC were measured in trembling aspen (*Populus tremuloides* Michaux), sugar maple (*Acer saccharum* Marshall), and red oak (*Quercus rubra* L.) in the spring and summer. Antioxidant levels and TAC were also compared in the midgut fluids of *O. leucostigma* and *M. disstria* when they fed on the leaves of these trees in the spring and summer. The findings allowed us to compare previous results obtained with artificial diets with those obtained with host plant foliage.

METHODS AND MATERIALS

Trees. Six trees of *P. tremuloides*, *Q. rubra*, and *A. saccharum* were tagged for study, including three each from two sites, the Matthaei Botanical Gardens and Keubler–Langford Park (Ann Arbor, MI). These sites are over 10 km apart and are separated by the Huron River. Selected trees were growing in the open or at the edge of forests, providing foliage that was exposed to full sun. Trees were estimated to be approximately 30–50 years old, based on their trunk diameters. Twigs with several leaves (2–4 m high) were cut from each tree using a pole pruner. Leaves with minimal damage from insects and pathogens were chosen from each tree. Samples were placed in plastic bags and stored on ice in a cooler immediately upon collection (10:00–11:30 AM). These leaves were either fed to caterpillars or were extracted during a 3-hr period in the afternoon. Trees were sampled on May 11 (spring), July 13 (summer), and August 24 (late summer) in 2000. Spring foliage was still soft and light green, but appeared to be fully expanded.

The second leaf from the tip of a twig was chosen for chemical analysis. Samples ($N = 6$ per species) were processed in a random order with respect to species and site. The petiole and midrib were removed with a razor blade, and each of the two leaf halves were weighed (± 0.1 mg) and processed separately. Leaf samples were placed in a chilled mortar and ground to a fine powder in liquid N_2. The leaf powder was mixed into a slurry with additional liquid N_2 and poured into a chilled centrifuge tube (15 ml, Corning; plug-seal cap) containing either N_2-purged ethanol (3.0 ml) or 5% (w/v) metaphosphoric acid (3.0 ml, containing 1 mM EDTA). Ethanol extracts were flushed with N_2, and all extracts were placed in a shaker (30 min, 4°C). After centrifugation ($1{,}000 \times g$, 4°C), an aliquot (1.0 ml) of each ethanol extract was mixed with 10 μl of butylated hydroxytoluene in ethanol (4 mM final concentration) in a microcentrifuge tube and flushed with N_2. All samples were stored at -75°C until analyzed. At the time of leaf extraction, a second leaf (matched for twig position and appearance) was weighed fresh (after removing its midrib and petiole), and reweighed after drying at 60 or 70°C for 4 days to determine the percent water in the leaves ($N = 6$ per species).

Insects and Midgut Fluid Collection. Eggs of *O. leucostigma* and *M. disstria* were obtained from the Canadian Forest Pest Management Institute (Sault Ste. Marie, Ontario), and larvae were reared as described previously (Barbehenn et al., 2001). Final-instar larvae were placed at random in plastic shoe boxes containing leaves from *P. tremuloides*, *Q. rubra*, or *A. saccharum*. Leaves from the same two (tagged) trees of each species were used for feeding larvae of both species in the spring and summer, and these leaves were collected from the same region of the trees at the same time of the day as were the leaves collected for antioxidant analyses. Leaves were kept hydrated by placing their twigs in water in 15-ml tubes. Insects were fed for 2–2.5 days, with fresh leaves being provided daily. Larvae were dissected on the third day: *M. disstria* on May 12 and July 12, and *O. leucostigma* on May 17 and July 5. Dissections were performed between 1 and 5 hr after larvae were placed on fresh leaves, as described previously (Barbehenn et al., 2001). The midgut contents were placed immediately in tared 2-ml microcentrifuge tubes containing 300 or 350 μl of N_2-purged (1 min/ml) double-distilled water, and the headspace in each tube was flushed with N_2. The fresh weight of the sample was recorded. Gut contents were vigorously shaken and centrifuged ($8{,}000 \times g$, 5 min, ambient temperature). Aliquots (100 μl) of the supernatant solutions were placed in 400 μl of 5% MPA with 1 mM EDTA for ascorbate and glutathione analysis. Aliquots (50 μl) of the supernatant solutions were placed in 200 μl of N_2-purged ethanol for measuring TAC. Aliquots (100 μl) of the supernatant solutions were placed in 400 μl of N_2-purged ethanol with BHT (4 mM final concentration) for α-tocopherol analysis. The headspace above each ethanolic extract was flushed with N_2, and all extracts were stored at -75°C until analyzed. An average of eight midgut samples per tree species (range = 5–12) were taken on each date. The fresh

weights of gut contents were converted to volumes based on the basis of percent water in similar samples ($N = 2$–3/species). Samples for fresh weight/dry weight (FW/DW) ratios were reweighed after drying (70°C, 2 days).

Antioxidant Analysis. Ascorbate, glutathione, and α-tocopherol were measured using high-performance liquid chromatography, as described previously (Barbehenn, 2003). Ascorbate was analyzed within 1–4 months, total glutathione (GSH + 2×GSSG) within 7–11 months, and α-tocopherol within 5–9 months from the time of storage. Antioxidant stability was examined in a previous study (Barbehenn, 2003), but was tested further for ascorbate in this study. Ascorbate remained at 90–95% of initial levels in leaf extracts from the spring and summer, and in gut fluids of *M. disstria* (fed the spring foliage of *A. saccharum*; $N = 4$) after 11–12 months of storage. However, ascorbate measured in the gut fluids of *O. leucostigma* (fed the spring foliage of *A. saccharum*; $N = 4$) was at 42% of the original levels measured after 11–12 months of storage, suggesting that ascorbate levels may have been diminished to some extent in the original measurements.

TAC was estimated in ethanol extracts of leaves and midgut fluids by measuring their ability to reduce 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) compared with Trolox standards (Re et al., 1999), as described previously (Barbehenn, 2003). Samples were analyzed within 0.5–2.5 months from the time of storage, times within which they should remain stable (Barbehenn, 2003). Ethanol extracts do not include glutathione because of its low solubility in ethanol (personal observation), but do include ascorbate, lipophilic antioxidants, and most of the phenolic compounds present in tree leaves (Swain, 1979; Pedersen, 2000).

Comparison of Antioxidant Concentrations. Antioxidant concentrations in tree leaves were compared on a moles per gram fresh weight (FW) basis. This measure incorporates seasonal changes in antioxidant concentration in the liquid fraction of the leaf, as well as changes in water content. Concentrations of antioxidants in midgut fluids were examined on a molar basis. Analyses of antioxidant concentrations and percent water were made with SAS version 8e, using PROC MIXED without a random variable (SAS, 2000). Models included tree species and season as fixed factors, along with the species × season interaction, to compare foliar antioxidant concentrations. Season was used as a repeated measure. Antioxidant concentrations in midgut fluid were compared using models that included insect species, tree species, and season as fixed effects, and included all two-way and three-way interactions. Paired comparisons of all means (presented in all figures and tables) were made using the differences of least-squares means, calculated with PROC MIXED (SAS, 2000). Antioxidant concentrations in gut fluids were log-transformed to fit a normal distribution. The overall difference between *O. leucostigma* and *M. disstria* was determined with a chi-square test (Sokal and Rohlf, 1981). The means of 24 independent measurements of antioxidant concentrations in each insect species were contrasted pairwise, and the observed number of cases

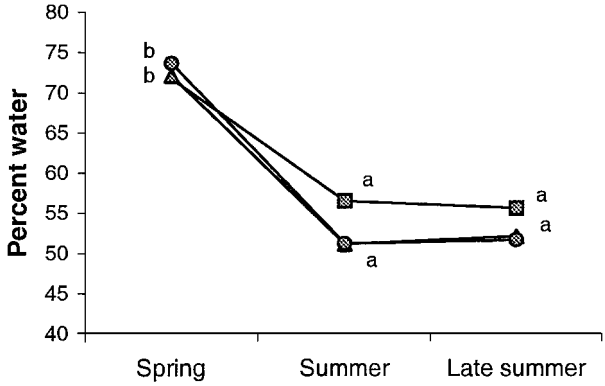

FIG. 1. Percent water in the leaves of *Populus tremuloides* (triangle), *Quercus rubra* (circle), and *Acer saccharum* (square). Different letters denote significant differences between means throughout the entire figure ($P < 0.05$). Standard error bars are smaller than the symbols.

in which each species had a higher antioxidant concentration was compared to an expected distribution of 12 cases for each species.

RESULTS

Seasonal Changes in Foliar Antioxidant Concentrations. The water content of the leaves of each species changed in a similar pattern between spring and summer (Figure 1), suggesting that differences in antioxidant concentrations between species are not due to differences in FW/DW ratios. Only season produced a significant effect ($P < 0.001$), though tree species and the season $\times$ tree species interaction showed a trend toward significance ($P = 0.066$ and 0.071, respectively).

Ascorbate. Ascorbate levels declined by 51% in *P. tremuloides* from the spring to the late summer ($P < 0.001$) (Figure 2A). In comparison, *Q. rubra* and *A. saccharum* leaves contained their lowest levels of ascorbate in the spring, and showed a trend toward increasing levels during the summer. Levels of ascorbate in *A. saccharum* increased by 117% and in *Q. rubra* by 28% in the late summer (Figure 2A), though these were not statistically significant changes in this study ($P = 0.118$ and 0.432, respectively). Significant effects included tree species ($P < 0.001$) and the season $\times$ species interaction ($P < 0.001$).

Glutathione. Glutathione decreased by 53% from the spring to the late summer in *P. tremuloides* ($P < 0.001$) and decreased by 55% in *Q. rubra* ($P = 0.012$) (Figure 2B). By contrast, glutathione increased by 60% in *A. saccharum* from a relatively low level in the spring ($P = 0.011$). An average of over 65% of the total glutathione measured was in the reduced form [calculated as GSH/(GSH + 2$\times$GSSG) $\times$ 100]. Total glutathione concentrations (GSH + 2$\times$GSSG) reported

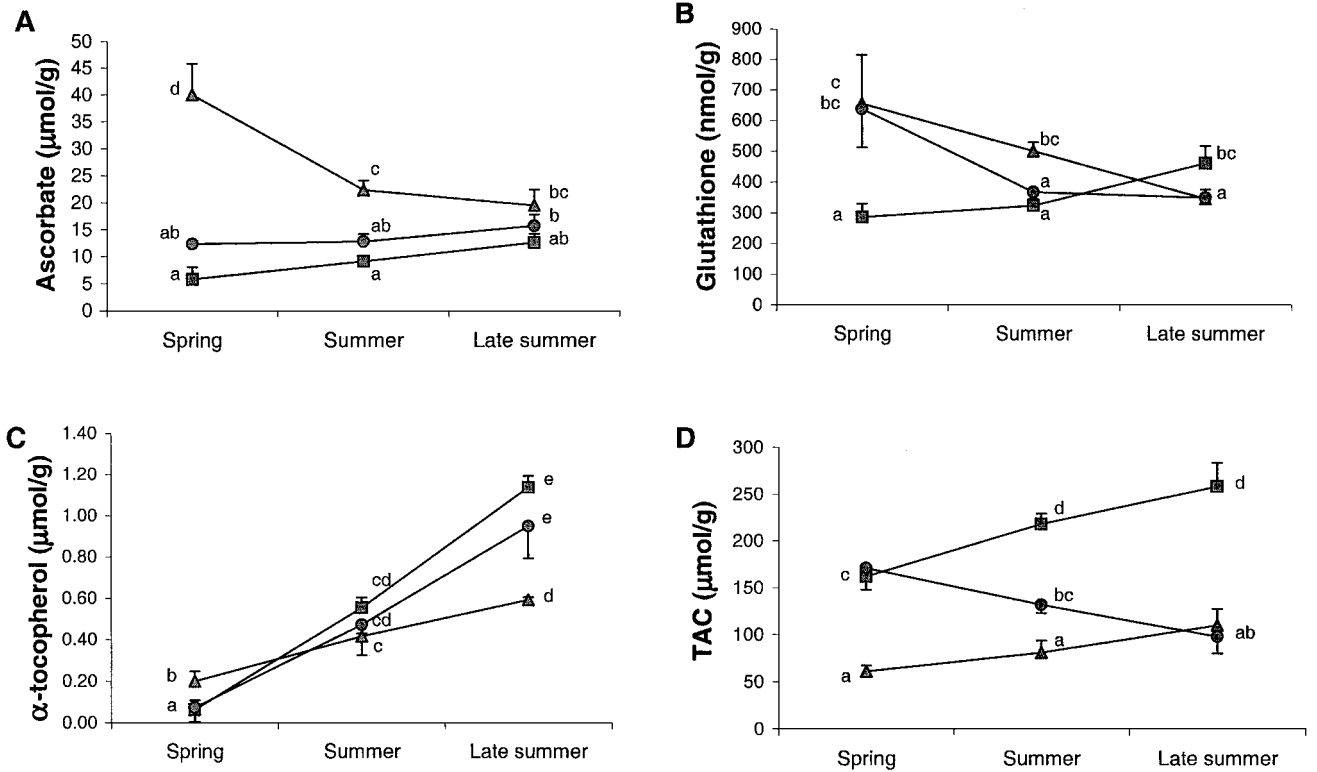

FIG. 2. (A) Ascorbate in the leaves of *Populus tremuloides* (triangle), *Quercus rubra* (circle), and *Acer saccharum* (square). (B) Total glutathione (GSH + 2×GSSG). (C) α-Tocopherol. (D) Total antioxidant capacity (TAC). Concentrations are on a fresh weight basis. Non-overlapping letters denote significant differences between means throughout the entire figure ($P < 0.05$). Standard error bars are presented.

in this study include GSSG that was present in the original samples and that which was formed during sample preparation. Therefore, the fraction of reduced GSH measured by this method is an underestimate. Significant effects included tree species ($P = 0.011$), season ($P = 0.039$), and the interaction between season and species ($P < 0.001$).

α-Tocopherol. α-Tocopherol levels increased substantially in each tree species through the spring and late summer (Figure 2C). Levels increased by 3-fold in *P. tremuloides*, 13-fold in *Q. rubra*, and 19-fold in *A. saccharum* ($P < 0.001$ in each species). *P. tremuloides* foliage contains significantly higher α-tocopherol levels in the spring than the other tree species ($P = 0.004$), but is surpassed by *A. saccharum* and *Q. rubra* by late summer. Significant effects included season ($P < 0.001$), tree ($P = 0.035$), and season × species interaction ($P < 0.001$).

Total Antioxidant Capacity. TAC in the spring foliage of *A. saccharum* and *Q. rubra* was similar ($P = 0.959$), and 166–180% higher than that in *P. tremuloides* ($P < 0.001$) (Figure 2D). In the summer, TAC showed an upward trend in *P. tremuloides* ($P = 0.055$) and increased by 59% in *A. saccharum* ($P = 0.010$), but decreased by 42% in *Q. rubra* by late summer ($P = 0.078$). Significant effects included tree species ($P < 0.001$) and the season × species interaction ($P = 0.002$).

Antioxidant Concentrations in Caterpillars. The water content of midgut fluids was maintained in the range of 88–93% when *O. leucostigma* and *M. disstria* fed on tree leaves of each species, despite differences in the water content of the foliage ingested in the spring and summer. Therefore, seasonal differences in antioxidant concentrations in gut fluids were not affected by changes in their water content.

Ascorbate. O. leucostigma maintained higher ascorbate levels in the summer than did *M. disstria* ($P = 0.002$), but the levels in the two species were not significantly different in the spring ($P = 0.823$) (Table 1). Levels of ascorbate in both species were substantially lower in the summer than in the spring ($P < 0.001$). In several cases, it is evident that foliar ascorbate concentration was positively associated with its concentration in *M. disstria* and *O. leucostigma*. For example, the

TABLE 1. ASCORBATE CONCENTRATIONS (μM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	910 ± 328^{d} (8)	$1{,}589 \pm 632^{d}$ (9)	12 ± 4^{ab} (7)	68 ± 29^{bc} (12)
Acer saccharum	70 ± 16^{c} (8)	66 ± 10^{bc} (7)	27 ± 10^{bc} (6)	178 ± 103^{c} (11)
Quercus rubra	334 ± 55^{d} (8)	555 ± 121^{d} (9)	6 ± 3^{a} (5)	24 ± 6^{bc} (8)

Note: Data are presented as mean ± SE (N). Non-overlapping following SEs letters designate significantly different means across the entire table ($P < 0.05$).

low ascorbate level in *A. saccharum* in the spring is associated with low ascorbate levels in the gut fluids of both caterpillar species, and high ascorbate levels in *P. tremuloides* is associated with high levels in the gut fluids of both caterpillar species (Table 1). It is also evident that foliar ascorbate levels in the summer had little relationship to the concentrations present in the midgut fluids of the insect species at this time (Table 1). Other significant effects included insect species ($P = 0.017$), season ($P < 0.001$), and interactions between insect species and season ($P = 0.037$) and tree species and season ($P < 0.001$).

Glutathione. O. leucostigma midgut fluids contained higher concentrations of glutathione than did those of *M. disstria* in the spring ($P < 0.001$), but *M. disstria* contained higher glutathione concentrations than did *O. leucostigma* in the summer ($P < 0.001$) (Table 2). While concentrations in *O. leucostigma* decreased in the summer to as little as 5.5% of spring levels ($P < 0.001$), glutathione concentrations in *M. disstria* increased in the summer ($P = 0.009$), primarily when feeding on *A. saccharum* (Table 2). Decreases in *O. leucostigma* are partially attributable to decreases in the glutathione levels in the foliage, which dropped to 79% and 58% of spring levels in *P. tremuloides* and *Q. rubra*, respectively (Figure 2b). The total glutathione in midgut fluid contained over 66% and 52% reduced glutathione in *M. disstria* and *O. leucostigma*, respectively. Significant effects included season ($P = 0.015$) and interactions between insect species and season ($P < 0.001$) and tree species and season ($P < 0.001$).

α-Tocopherol. O. leucostigma midgut fluids contained higher concentrations of α-tocopherol than did those of *M. disstria* in the summer ($P < 0.001$). α-Tocopherol concentrations were not significantly different between the insect species in the spring ($P = 0.299$). In both insect species, α-tocopherol increased in the summer when feeding on *P. tremuloides*, and also increased in *O. leucostigma* feeding on *A. saccharum* (Table 3). α-Tocopherol concentrations were lowest in the midgut fluids of both insect species when feeding on *Q. rubra* in the spring and the summer (Table 3). Significant effects included insect species ($P = 0.001$), tree species ($P < 0.001$), season ($P < 0.001$), and interactions between season and insect species ($P = 0.051$), season and tree species ($P = 0.006$), and insect species and tree species ($P = 0.029$).

Total Antioxidant Capacity. TAC was significantly higher in *O. leucostigma* than in *M. disstria* in the summer ($P < 0.001$), but was similar in the spring between the two caterpillar species ($P = 0.294$) (Table 4). Differences were most evident between the insect species when they fed on *A. saccharum*: TAC in *O. leucostigma* remained relatively high in the summer, whereas TAC in *M. disstria* declined in the summer to a lower level than that found in the spring (Table 4). TAC in the midgut fluids of both caterpillar species was consistently low when they were fed *Q. rubra*, lower than when fed on *P. tremuloides* ($P < 0.001$), which in turn was lower than TAC levels noted when they were fed on *A. saccharum* ($P = 0.017$). Significant effects included insect species ($P < 0.001$), tree species ($P < 0.001$),

TABLE 2. GLUTATHIONE CONCENTRATIONS (μM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	81.1 ± 10.4^{cd} (8)	278.6 ± 117.6^{d} (9)	76.9 ± 8.1^{cd} (7)	23.5 ± 3.5^{a} (12)
Acer saccharum	$29.6 + 2.2^{ab}$ (8)	100.5 ± 20.1^{cd} (9)	222.1 ± 59.3^{d} (6)	61.4 ± 11.1^{bc} (11)
Quercus rubra	34.6 ± 7.5^{ab} (8)	397.1 ± 18.1^{d} (9)	49.8 ± 11.4^{abc} (5)	22.4 ± 6.3^{a} (9)

Note: Data are presented as mean $\pm$ SE (N). Non-overlapping letters following SEs.

TABLE 3. α-TOCOPHEROL CONCENTRATIONS (μM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	10.8 ± 1.1^{a} (8)	13.6 ± 1.6^{a} (9)	30.4 ± 3.2^{bc} (7)	61.7 ± 7.8^{d} (12)
Acer saccharum	$9.0 + 1.0^{a}$ (8)	18.5 ± 4.3^{ab} (9)	9.0 ± 1.2^{a} (6)	42.5 ± 7.8^{c} (11)
Quercus rubra	4.2 ± 0.7^{a} (8)	3.4 ± 0.4^{a} (9)	5.2 ± 1.1^{a} (5)	7.6 ± 1.7^{a} (8)

Note: Data are presented as mean $\pm$ SE (N). Non-overlapping letters following SEs designate significantly different means across the entire table ($P < 0.05$).

and interactions between tree species and season ($P < 0.001$), insect species and season ($P = 0.015$), and season $\times$ insect species $\times$ tree species ($P = 0.002$).

Overall Comparison of Insect Species. When antioxidant concentrations in *O. leucostigma* and *M. disstria* were compared pairwise across all tree species and seasons (24 independent comparisons), the midgut fluid of *O. leucostigma* was found to contain significantly greater antioxidant concentrations than the midgut fluid of *M. disstria* ($\chi^2 = 6.0$; $P < 0.025$).

DISCUSSION

Contrary to our expectation, caterpillars that feed in the spring do not generally encounter higher antioxidant concentrations than they would in the summer. Instead, foliar concentrations of antioxidants change in species-specific patterns. While α-tocopherol concentrations increased substantially through time in the examined tree species, seasonal changes in the concentrations of ascorbate and glutathione differed in each tree species: *P. tremuloides* leaves contained higher levels of ascorbate and glutathione in the spring, *Q. rubra* had lower concentrations

TABLE 4. TOTAL ANTIOXIDANT CAPACITY (TAC) (mM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	22.1 ± 1.8^{a} (8)	35.0 ± 3.7^{bc} (13)	43.6 ± 7.5^{cd} (7)	54.1 ± 6.3^{d} (12)
Acer saccharum	$62.1 + 9.4^{d}$ (8)	51.4 ± 4.8^{d} (13)	25.7 ± 6.0^{ab} (6)	58.0 ± 5.6^{d} (10)
Quercus rubra	25.4 ± 2.3^{ab} (8)	26.2 ± 2.0^{ab} (13)	21.0 ± 5.0^{a} (5)	24.6 ± 1.7^{ab} (8)

Note: Data are presented as mean $\pm$ SE (N). Non-overlapping letters following SEs designate significantly different means across the entire table ($P < 0.05$).

of ascorbate but higher concentrations of glutathione in the spring, and *A. saccharum* had lower concentrations of both ascorbate and glutathione in the spring. The trend toward increased ascorbate concentration in the mature leaves of *A. saccharum* and *Q. rubra*, though not significant in this study, fits the pattern of seasonal changes observed previously in *Fagus sylvatica* (Luwe, 1996) and *Pinus strobus* (Anderson et al., 1991), while decreases in glutathione, such as in *P. tremuloides* and *Q. rubra*, have also been observed in *F. sylvatica* and some nondeciduous trees (Dash and Jenness, 1985; Kunert and Ederer, 1985; Schupp and Rennenberg, 1988; Luwe, 1996). On the basis of these examples, the elevated levels of ascorbate in the spring foliage of *P. tremuloides* and the low level of glutathione in the spring foliage of *A. saccharum* appear to be exceptions to the more common patterns of seasonal change in deciduous tree leaves.

In general, previous studies on antioxidants in deciduous tree leaves have found concentrations similiar to those measured in this study, e.g., 6–20 μmol ascorbate/g FW (Luwe, 1996; Schwanz et al., 1996a,b; Marabottini et al., 2001; but see also Roth et al., 1994). In contrast, *P. tremuloides* leaves contain an exceptionally high concentration of ascorbate in the spring (40 μmol/g or 2.5% DW). Previously, concentrations of glutathione and α-tocopherol have been measured in the ranges of approximately 200–700 nmol/g FW and 150–2,400 μmol/g FW, respectively (Kunert and Ederer, 1985; Strohm et al., 1995; Luwe, 1996; Schwanz et al., 1996a,b; Marabottini et al., 2001). The pattern of increasing levels of α-tocopherol during the growing season appears to be typical of developing leaves (Kunert and Ederer, 1985; Hess, 1993).

The results of this study support the hypothesis that *O. leucostigma* maintains higher concentrations of antioxidants in its midgut fluids than does *M. disstria* when they feed on tree leaves, as was the case when they feed on artificial diets (Barbehenn et al., 2001). Significantly greater concentrations of ascorbate and α-tocopherol and higher TAC values were present in the midgut fluids of *O. leucostigma* compared to *M. disstria* when they fed on mature tree leaves. However, glutathione showed the opposite pattern in the summer, and we are unable to explain this or other cases in which the relationship between foliar and larval antioxidant concentrations appears to be complex. The concentration of an antioxidant in the midgut fluid would depend on the efficiency of its extraction from leaf tissues and the relative rates of its metabolism, absorption, and/or secretion in the midgut lumen. Several clear cases were apparent, however, which support the idea that caterpillars could increase the antioxidant concentrations in their midgut fluids by feeding on the foliage of particular tree species, especially in the spring. Most notably, *M. disstria* and *O. leucostigma* contain very high concentrations of ascorbate and glutathione when they feed on the spring foliage of *P. tremuloides*.

Although we have not made measurements that demonstrate whether differences in antioxidant concentration affect insect fitness, our observation of browning in midgut fluids is potentially important in this regard. Browning was common in

M. disstria when it fed on each tree species in the summer, but no browning was observed in *O. leucostigma* feeding in the summer, nor in any case when either species fed on spring foliage. Browning is a well-known result of extensive phenol oxidation (Cilliers and Singleton, 1989) and has been observed previously in the gut lumens of *M. disstria* on phenol-containing diets and in some foliage-feeding insects (Barbehenn and Martin, 1994; Appel and Maines, 1995; Barbehenn et al., 1996; Johnson and Felton, 1996). Decreased TAC in the midgut fluids of *M. disstria* in the summer (primarily on *A. saccharum*) is consistent with the possibility that ingested phenols were oxidized in this species (Barbehenn and Martin, 1994; Barbehenn et al., 2001, 2003). Potential consequences of extensive phenol oxidation in the midgut lumen include decreased essential nutrient availability and oxidative stress.

In an earlier study, it was concluded that the presence of a complete ascorbate recycling system (ascorbate, dehydroascorbate reductase, and glutathione) in the midgut lumen can explain the tolerance of *O. leucostigma* to tannins ingested in an artificial diet (Barbehenn et al., 2001). Efficient ascorbate recycling would maintain sufficiently high levels of ascorbate to prevent tannin oxidation, and the low concentration of glutathione in the midgut fluid of *M. disstria* (6.5 μM) would preclude the recycling of ascorbate in this species. However, it is clear that *M. disstria* obtains much larger amounts of glutathione from tree leaves (Table 2). Yet, even in these conditions its midgut fluids contain extremely low levels of ascorbate and exhibit signs of phenol oxidation when feeding on mature foliage. Although levels of glutathione appear relatively low in the summer in *O. leucostigma*, they are strikingly similar to levels that adequately protect them when they feed on an artificial diet (23 μM): 22 μM on *Q. rubra* and 23 μM on *P. tremuloides*. Given the broad range of glutathione concentrations in these foods (10–1000 μM), this apparent homeostasis suggests that *O. leucostigma* actively maintains a minimum glutathione level in the midgut lumen, possibly as a result of glutathione secretion (Barbehenn et al., 2001).

TAC was initially measured in this study as a means of estimating the overall concentration of antioxidants present in the sample extracts. However, the variety of antioxidants that were not directly measured, such as carotenoids and cysteine, appear to make a relatively minor contribution to foliar TAC. The concentrations of other commonly recognized antioxidants in leaves are quite small compared with the TAC of 60–260 μmol/g measured in this study. For example, β-carotene and other carotenoids are found at between 0.5 and 0.8 μmol/g in tree leaves (Schwanz et al., 1996a,b), and cysteine and γ-glutamylcysteine are at only 0.055 and 0.020 μmol/g, respectively (Schwanz et al., 1996b) (our calculations). When the higher Trolox equivalent antioxidant capacity (TEAC) of carotenoids (2.5- to 3-fold greater than Trolox) (Re et al., 1999) is taken into account, the above antioxidants would contribute no more than 0.6–4.1% of the TAC in tree leaves. In comparison, the contribution of ascorbate and α-tocopherol to the TAC of foliar extracts in this study together ranged from a minimum of 5.4% (*A. saccharum*

in the late summer) to a maximum of 65.9% (*P. tremuloides* in the spring). It is likely that foliar phenols contribute most of the undetermined $ABTS^{\cdot+}$ reducing power measured with the TAC assay: (1) TAC is positively correlated with total phenol concentrations in a wide variety of plant species (Pietta et al., 1998; Prior et al. 1998; Velioglu et al., 1998); (2) phenolic compounds are the most abundant "antioxidants" in leaves (Pratt, 1992), reaching high levels (10–25% DW) in the tree species in this study (Ricklefs and Matthew, 1982; Schultz et al., 1982; Lindroth et al., 1987); (3) phenolic compounds have high TEAC values, e.g., up to 5-fold greater than Trolox for low molecular weight phenols (Re et al., 1999), and 15- and 28-fold greater than Trolox for hydrolyzable and condensed tannins, respectively (Hagerman et al., 1998); and (4) seasonal changes in TAC match the patterns observed previously for total phenols, e.g., concentrations of phenolic compounds in *A. saccharum* and *P. tremuloides* increase seasonally (Lindroth et al., 1987; Hagerman, 1988), and concentrations of total phenolic compounds in *Q. rubra* decrease seasonally (Ricklefs and Matthew, 1982; Rossiter et al., 1988; Appel et al., 2001) (Figure 2d). Unless we are unaware of a major source of $ABTS^{\cdot+}$-reducing antioxidants in leaves, the above results suggest that TAC in our study was commonly dominated by phenols.

Phenolic compounds could function either as antioxidants or prooxidants in the caterpillar midgut fluids (Barbehenn et al., 2001; Johnson and Felton, 2001). The in vitro conditions used in the measurement of TAC do not necessarily discriminate between these possibilities. Measurement of the activity of these compounds in the physicochemical conditions present in vivo is necessary to determine their biological function (Strube et al., 1997). For example, midgut fluids containing a combination of high phenol and low ascorbate concentrations would be expected to produce high TAC. However, phenols in the midgut fluids of caterpillars in these conditions would likely act as prooxidants (i.e., form semiquinone radicals). Recent measurements of semiquinone radical intensities are consistent with this interpretation. Although *M. disstria* has high TAC values when feeding on *A. saccharum* in the spring (Table 4), it also contains a relatively low concentration of ascorbate (Table 1), and high levels of semiquinone radicals are generated in its midgut fluids (Barbehenn et al., 2003). In comparison, when *M. disstria* feeds on *Q. rubra* in the spring, low semiquinone radical intensities are generated (Barbehenn et al., 2003), consistent with lower TAC (i.e., phenol concentration) and a higher ascorbate concentration (Tables 1 and 4). An alternative hypothesis, that ingested phenols function as antioxidants (Johnson and Felton, 2001), appears to be most plausible in cases in which phenols are present at low concentrations relative to ascorbate and/or in which phenols may be relatively stable, such as in acidic biological fluids.

Future research on the antioxidant defenses of *M. disstria* and *O. leucostigma* needs to address three issues, including their oviposition and foraging preferences, the effectiveness of specific antioxidants, such as α-tocopherol, in suppressing

phenol oxidation in the midgut, and the effect of variation in antioxidant concentrations on larval performance. The fact that midgut antioxidant concentrations are affected by the species of tree foliage consumed (primarily in the spring) suggests that adult oviposition preference and/or larval foraging could influence their midgut antioxidant systems, e.g., restricting oviposition or consumption to tree species, tree genotypes, or individual leaves containing high concentrations of the most effective antioxidants.

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SEASONAL AND SPATIAL VARIATIONS OF ALKALOIDS IN *Merendera montana* IN RELATION TO CHEMICAL DEFENSE AND PHENOLOGY

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Abstract—*Merendera montana* (Liliaceae) is an endemic plant broadly, but exclusively, occurring in the Iberian Peninsula where it is one of the most abundant species in rangelands grazed by sheep and on the routes used by migrating sheep herds. In spite of showing a peculiar phenology adapted to Mediterranean regions (leaves remain green from fall to spring and are absent in summer), this species has also successfully colonized high altitude environments (up to 2300 m), and abundant populations can be found in montane and subalpine pastures. Chemical analysis has shown a high forage quality as well as the presence of tropolone alkaloids (similar to other species of the genus *Merendera*), mainly colchicine, 3-demethylcolchicine, and colchicoside. In general terms, the alkaloid content was significantly lower in the subterranean corms than in the leaves. No remarkable seasonal variations in total alkaloids was found, although an increase of colchicoside in the corms and a decrease of colchicine was noted in summer. Both chemical deterrents and phenology could be the cause of the widespread ecological adaptation of *Merendera montana*. The alkaloid content would prevent intake by wild herbivores during winter, while the absence of aerial organs in summer, coinciding exactly with the highest domestic herbivore pressure, would permit this species to colonize rich soil habitats where there is low plant competition.

Key Words—*Merendera montana*, Liliaceae, tropolone alkaloids, colchicine, colchicoside, 3-demethylcolchicine, phenology, chemical defense.

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INTRODUCTION

The genus *Merendera* Ramond (Liliaceae) is closely related to the genus *Colchicum*, and includes about 10 species occurring in Europe, Africa, and Asia. *Merendera montana* (L.) Lange is found in the NW part of this area, exclusively in the Iberian Peninsula and just reaching the Pyrenees. This plant shows a broad ecological extension and it is perhaps the only species among the Iberian flora (around 7000 species) that occurs in grasslands from sea level to the subalpine belt (2300 m) in all kinds of soils, but especially on limestone. *M. montana* is one of the most abundant and common species in pastures, mainly in montane rangelands grazed by sheep. Like other geophytes, its density increases in disturbed areas (vole mounds, wild boar rooting, livestock resting sites, etc.) with low plant cover (Gómez-García et al., 1995). On the routes used by migrating sheep herds and montane road tracks, *M. montana* can attain a high density, and often occurs as the only plant species (pers. obs.). This abundance is remarkable when considering that highly toxic plants in some genera of the family Liliaceae, like *Colchicum* (with the exception of *C. autumnale*), *Bulbocodium*, and *Androcymbium*, normally live in restricted habitats and, at least in the Mediterranean regions, occur in small populations.

M. montana is a small perennial acaulescent geophyte with a subterranean stock-corm and three or four narrow leaves forming a basal rosette. The blooms appear in August–September; the subterranean ovary spends 9 or 10 months in ripening, and then (May–June) a septicidal capsule emerges as a result of elongation of the pedicel. With respect to the leaf phenology, *M. montana* bears leaves from the beginning of fall to the end of spring and, therefore, no aerial part remains green in summer unlike other mountain vegetation. This phenological strategy is adapted to Mediterranean climates with drought and hot summers (Dafni et al., 1981). Nevertheless, *M. montana* has also successfully colonized montane and subalpine pastures that have long and hard winters and a short vegetative period. In these high altitudinal vegetation belts, *M. montana* is the only species showing such a phenology. This plant seems to be rarely consumed by herbivores, although sporadic consumption by chamois (*Rupicapra rupicapra pyrenaica* Bonaparte) at the end of spring has been reported in the Pyrenees (García-González and Montserrat, 1986). In general terms, Pyrenean montane pastures are grazed from June to October by cattle and sheep, while the rest of the year only wild herbivores, mainly chamois, remain in the area.

Although the chemical composition of *M. montana* has not been previously studied, the alkaloid content of different species of the genus have been analysed. Thus, several colchicine type alkaloids are known, for instance, in *M. persica* (Sarg et al., 1993), *M. robusta* (Yusupov, 1996), and in *M. kurdica*, *M. manissadjiani*, and *M. sobolifera* (Husek et al., 1989; Šimánek et al., 1990). Apart from its application in medicine, colchicine is well known for its cytotoxic effects and causes vomiting,

diarrhea, and miscarriage in vertebrates (Paris and Moyse, 1967; Farnsworth et al., 1975). Colchicoside and 3-demethylcolchicine are also toxic compounds, although 100 and 8 fold less so than colchicine, respectively (Jequier and Peterfalvi, 1953).

In trying to understand the ecological success of *M. montana* in terms of spatial occurrence and abundance, we studied some chemical properties related to forage value, as well as the seasonal variation of the alkaloids colchicine, colchicoside, and 3-demethylcolchicine, which appear to be the main deterrents of this species, in different natural populations of Spain throughout a 1-year period. The concentration of alkaloids was analyzed by HPLC in both corms and leaves.

METHODS AND MATERIALS

Plant Material and Sampling. To determine forage quality and temporal and spatial patterns in alkaloid content, *M. montana* was sampled in six locations between 800 and 2000 m in the Spanish Pyrenees. The characteristics of the different locations and the sampling dates are shown in Table 1. Plants were collected on a bimonthly basis from October 1996 to August 1997, except at the end of the vegetative period when some samplings were done monthly in order to obtain more detailed information in relation to leaf senescence. Since there are no leaves in summer, only corms were collected in August. In July, the corm samples of the Aisa and Cubilar de las Vacas locations were accidentally damaged; therefore, only the leaves of these two natural populations were analyzed. Sampling time was always around noon to avoid daily variations in alkaloid concentration. The abundance

TABLE 1. SAMPLING DATES AND SOME CHARACTERISTICS OF THE *M. montana* NATURAL POPULATIONS

Location	Elevation (m)	Vegetation type	Plant cover (%)[a]	Sampling dates
Cuelloarenas	1950	Subalpine	12.4	Oct, Dec, Feb, Apr, Jun, Jul, Aug[b]
Cubilar de las Vacas	1750	Montane	9.4	Oct, Dec, Feb, Apr, Jun, Jul,[c] Aug[b]
Aisa	1550	Montane	7.3	Oct, Dec, Feb, Apr, Jun, Jul,[c] Aug[b]
Guasillo	860	Submediterranean	12.6	Oct, Dec, Feb, Apr, Jun,[b] Jul,[b] Aug[b]
Salto de Roldán	800	Mediterranean	18.3	Oct, Dec, Feb, Apr, May, Jun, Aug[b]
Montmesa	440	Mediterranean	5.2	Oct, Dec, Feb, Apr, May, Jun, Aug[b]

[a] Percentage of plant cover of *Merendera montana* in the different sampling sites.
[b] Only corms were collected.
[c] Only leaves were collected.

of the plant in the sampling areas (Table 1) was estimated by means of the point quadrat method. Ten 2-m transects were randomly sampled in each study area (20 × 20 m) and a contact at 5-cm intervals was gathered to gain knowledge of the plant intercepted; a total of 400 contacts were gathered in each location. The plant cover was estimated as the percentage of contacts with the plant (t' Mannetje, 1978).

A sampling made up of 25 shoots of *M. montana* (around 100 g of fresh weight) was collected at random in each population, dried at 40°C, and then powdered. After that, three subsamples from leaves and corms were separated for chemical analysis.

Forage Quality Determination. Two subsamples of leaves from each location were collected in October, dried, and milled through a 1-mm mesh. This month was chosen because it coincided with the optimal phenological appearence of different populations of *M. montana* and minimum green-vegetation (forage) availability. Nitrogen was determined by the Kjeldahl method (Association of Official Agricultural Chemists) [AOAC], 1964), and other parameters [NDF (neutrodetergent fiber), ADF (acid detergent fiber), ADL (acid detergent lignin), and ADA (acid detergent ashes)] by the sequential method of Van Soest (1994). Enzymatic digestibility was calculated by the pepsin-celullase method (Aufrere and Michalet-Doreau, 1988). All values are expressed as percentage dry weight, and represent the average of the two subsamples that have a percentage of error $<5\%$.

Alkaloid Extraction. Five hundred milligrams of dried and powdered plant material were macerated with 25 ml of MeOH at room temperature for 24 h and sonicated for 45 min in an ultrasonic bath. The extract was filtered and adjusted to a final volume of 25 ml with MeOH. An aliquot of the extract was filtered through 0.22 μm filter (Nylon Acrodisc 4427) before HPLC analysis. Analyses were carried out in triplicate.

HPLC Analysis. Quantitative determination of the alkaloids was carried out by RP-HPLC using a C18 column (150 × 4 mm) and a C18 precolumn (20 × 3.9 mm) packed with Kromasil (5 μm) as previously described (Ellington et al., in press). The mobile phase consisted of water and acetonitrile, and a linear gradient program was used (Table 2). A Hewlett-Packard series 1050 liquid chromatograph,

TABLE 2. THE LINEAR GRADIENT PROGRAM FOR HPLC ANALYSIS

Time (min)	Acetonitrile (%)	Water (%)
0	11	89
1	12	88
12	30	70
16	15	85
18	12	88
20	11	89

equipped with a quaternary pump system, a diode array detector operating at 350 nm, an automatic injector, and an integration system HP 3396 Chemstation for control and data processing were used for the analyses. Pure colchicine, colchicoside, and 3-demethylcolchicine supplied by the French company Roussel Uclaf, were used as reference substances. Two HPLC chromatograms of samples belonging to leaves and corms are shown in Figure 1.

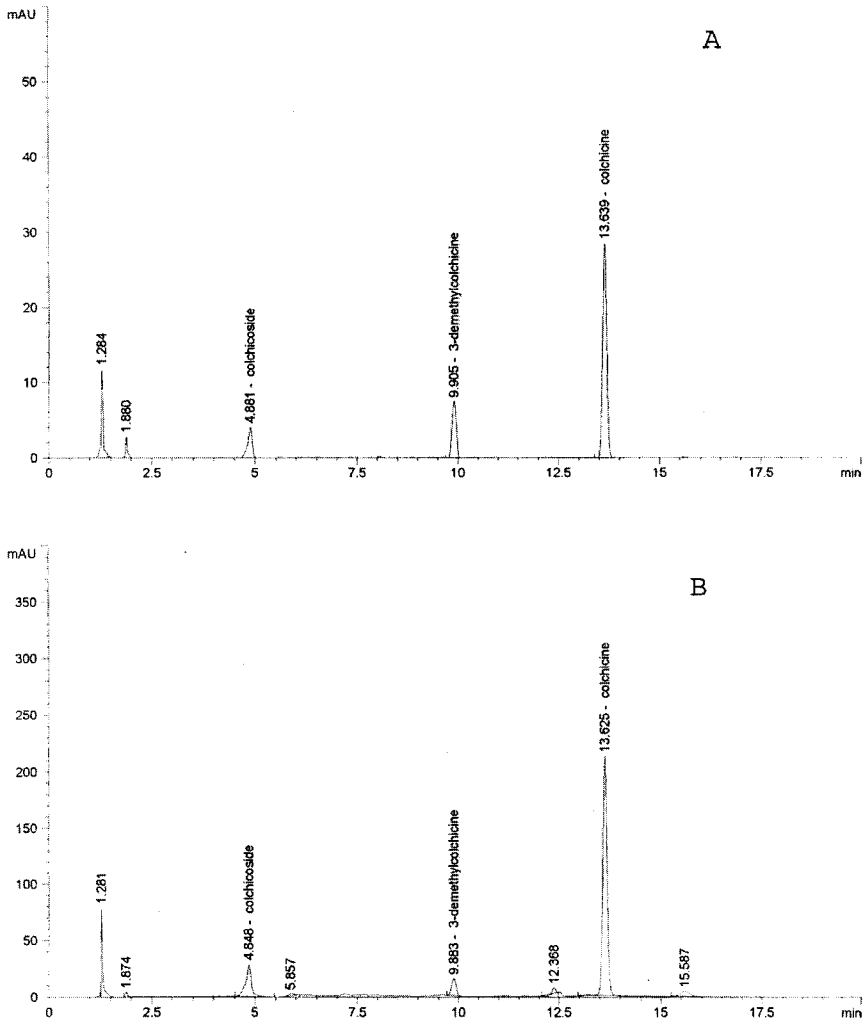

FIG. 1. HPLC chromatograms of two alkaloid extracts from corms (A) and leaves (B) of *Merendera montana*.

Statistical Analysis. The results were subjected to the two-way analysis of variance (ANOVA) with a SPSS/PC package (version 10.0), and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

M. montana has a high nitrogen content (i.e., protein), which is similar to the leguminous plants of the Pyrenees, and higher than that of the graminoids (Table 3; Marinas et al., 2001). It is also highly digestible and has low fiber content when compared with most common grassland species. Thus, *M. montana* is a high quality forage plant. Because it also lacks physical defenses and has a succulent appearance, it should be attractive to herbivores.

The distribution of alkaloids in leaves and corms of *M. montana* throughout the ontogenic cycle is shown in Figure 2. In general, leaves showed a higher amount of alkaloids than corms, with the exception of the two lowest natural sites (Montmesa and Salto del Roldán) in June, and Cubilar de las Vacas in December. The differences between the alkaloid content of leaves and corms in all the locations were statistically significant ($P < 0.05$) for both colchicine and its derivative 3-demethylcolchicine, as well as for the total alkaloids, but not for colchicoside. The exceptions were the Aisa location (1550 m), where no differences were observed in colchicine, and at Guasillo (860 m) and Cubilar de las Vacas (1750 m) where the levels of the alkaloid 3-demethylcolchicine were not significantly different.

Colchicine was found to be the major alkaloid in both leaves and corms. In the three lowest locations (Montmesa, Salto de Roldán, and Guasillo), however, the average of colchicoside content in the corms throughout the ontogenic cycle was higher than that of colchicine (Table 4).

In general, the highest levels of alkaloids in both leaves and corms of *M. montana* were found in plants in the subalpine location. The lowest alkaloid

TABLE 3. DIFFERENT ATTRIBUTES OF FORAGE QUALITY IN LEAVES OF *M. montana*

Location	N	NDF	ADF	ADL	ADA	Enzymatic digestibility
Cuelloarenas	3.26	22,05	14,45	4,32	0,83	90.21
Cubilar de las Vacas	3.47	24.80	18.94	13.48	0.03	85.06
Aisa	3.05	23.05	17.86	14.33	0.09	88.73
Guasillo	2.82	23.52	16.18	4.62	0.06	88.39
Salto de Roldán	2.58	25.71	18.06	6.46	0.88	86.54
Montmesa	3.53	23.50	16.03	6.48	1.03	83.33

Note: N: total nitrogen; NDF: neutrodetergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; ADA: acid detergent ashes. All the values are expressed as % referred to dry weight.

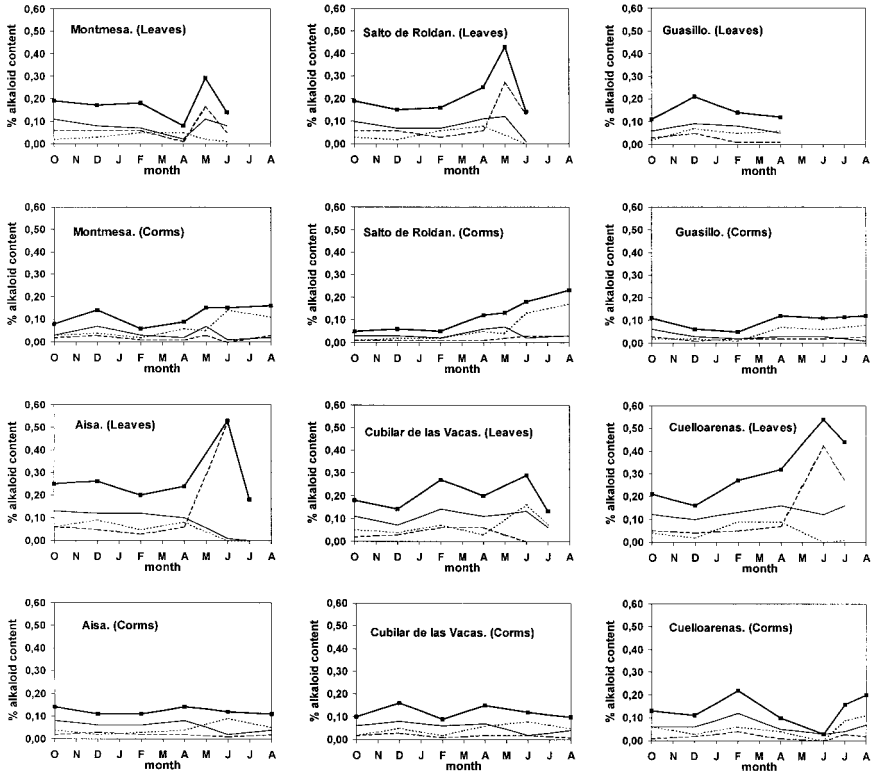

FIG. 2. Seasonal variation of tropolonic alkaloids in leaves and corms of *Merendera montana* in the six sampling locations (colchicine ——, colchicoside . . . . . , 3-demethylcolchicine _ _ _, and total alkaloids ——). Black points correspond to sampling data. Alkaloid content is reported as percentage of dry weight.

content in both leaves and corms was always found in plants in the submediterranean site. Nonetheless, there was no correlation between the alkaloid content and the altitude of the natural populations either in leaves or corms. The alkaloid content of the leaves was found to be location-dependent, as there were statistically significant differences ($P < 0.05$) among the different natural sites for both total and individual alkaloids. The four lowest locations, however, were homogeneous regarding both colchicine and colchicoside in the leaves, so it is the level of 3-demethylcolchicine that determines the differences in the total alkaloid content of leaves at these locations. On the contrary, no statistically significant differences were found among the alkaloid levels in the corms of the different natural sites, with the exception of colchicine.

TABLE 4. AVERAGES OF THE ALKALOID CONTENT (EXPRESSED AS PERCENTAGES REFERRED TO DRY WEIGHT) THROUGHOUT THE 1-YEAR CYCLE IN *M. montana* PLANTS BELONGING TO THE DIFFERENT NATURAL POPULATIONS

Location	Organ	Colchicine	Colchicoside	3-Demethylcolchicine	Total alkaloids
Cuelloarenas	Leaves	0.132	0.041	0.151	0.324
	Corm	0.064	0.056	0.018	0.138
Cubilar de las Vacas	Leaves	0.103	0.069	0.029	0.202
	Corm	0.056	0.046	0.018	0.120
Aisa	Leaves	0.080	0.047	0.150	0.277
	Corm	0.057	0.046	0.020	0.123
Guasillo	Leaves	0.071	0.050	0.025	0.145
	Corm	0.030	0.044	0.021	0.094
Salto de Roldán	Leaves	0.077	0.037	0.103	0.217
	Corm	0.038	0.062	0.017	0.117
Montmesa	Leaves	0.078	0.031	0.065	0.174
	Corm	0.035	0.064	0.022	0.117

The different alkaloid content of leaves and corms of *M. montana* could be due to different defense necessities of the plant. In this way, alkaloid movement within the plant has been seen (Harborne, 1991). A high deterrent content would be neccessary to protect leaves, particularly when the shortage of green grasslands makes *M. montana* especially attractive for herbivores. Since corms are underground, no deterrents would be required to defend them against herbivores, although predation by the subterranean activity of vole-moles with mutual benefit has been noted (Borghi and Giannoni, 1997; Gómez-García et al., 1999).

As occurs in other species (Ralphs et al., 1997), in general no important fluctuations in the leaf alkaloid content were observed during the ontogenic cycle, with the exception of an accumulation of alkaloids in May in the lowest populations (Montmesa and Salto del Roldán) or in June in two of the highest ones (Aisa and Cuelloarenas) (Figure 2). This increase in alkaloid content in leaves is mainly due to the accumulation of 3-demethylcolchicine (reaching 97.73% of the total alkaloids in June in the Aisa site), with the exception of Cubilar de las Vacas, where this alkaloid was not detected during the summer (June–July).

The levels of colchicine were more or less constant throughout the studied period, but the total alkaloid content diminished at the end of the vegetative period coinciding with senescense. This decline, just when chemical defense would be less important for the plant, has also been reported in other toxic species (Ralphs et al., 1997), and can probably be related to nitrogen recycling. In this way, intake by chamois reported by García-González and Montserrat (1986) would also correspond to the period with the lowest leaf alkaloid concentration, just before the start of senescence.

No remarkable fluctuations in the alkaloid levels of the corms were observed during the year (Figure 2). Nonetheless, an increase of the colchicoside content in corms was observed in all locations in the summer season, from June to August (2 months earlier in the Montmesa and Guasillo sites), colchicoside thus becoming the main alkaloid accumulated up to the end of the ontogenic cycle. The glucosylation of colchicine to form colchicoside (via 3-demethylcolchicine) could constitute a detoxification system for the plant in the period when leaves are not present and the new corm is preparing to bloom, when an excess of colchicine could become toxic. At the same time, this process could also represent a system of chemical defense for the plant, as colchicoside could be easily hydrolized to 3-demethylcolchicine after ingestion by animals, thus increasing its toxicity.

After considering both chemical content and phenological behavior of *Merendera montana*, it can be deduced that the ecological success of the plant may be due to the combination of these two factors. The chemical arsenal would allow the plant to avoid herbivore consumption, while the principal forage material remains dormant. On the other hand, the absence of leaves in summer, exactly when intake pressure and trampling by livestock is at its maximum, would facilitate the colonization of extensive territories, particularly pasturelands and livestock routes, optimal habitats owing to the low plant competition and high soil fertility.

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PROFILING OF FLAVONOID CONJUGATES IN *Lupinus albus* AND *Lupinus angustifolius* RESPONDING TO BIOTIC AND ABIOTIC STIMULI

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Abstract—Qualitative and quantitative composition of flavonoid and isoflavonoid glycosides as well as free aglycones in lupin seedlings (roots and aerial parts) grown under different light conditions or responding to infection with *Pleiochaeta setosa*, a fungus causing brown leaf spot, were monitored by liquid chromatography with UV and/or mass spectrometric detection. Both physical and biotic factors affected flavonoid and isoflavonoid levels in lupin tissues. Fungal infection evoked significant increase in the amounts of genistein, 2′-hydroxygenistein, and their prenylated derivatives that are thought to function as lupin phytoalexins. Effect on quantitative changes of glycosylated flavonoids and isoflavonoids in the roots and aerial parts was less significant. Moreover, different light conditions applied during seedling growth caused relative changes of flavonoid and isoflavonoid conjugates composition, especially in the leaves of white lupin plants. The chemical structures of flavonoid and isoflavonoid conjugates present in *Lupinus angustifolius* were elucidated. In addition to genistein and 2′-hydroxygenistein glycosides, flavonol conjugates were identified in leaves,

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while the composition of root isoflavonoids was similar to that of *L. albus* reported earlier.

Key Words—Lupin, metabolic profiling, defense response, flavonoid and isoflavonoid glycosides, liquid chromatography–mass spectrometry, gas chromatography–mass spectrometry, electrospray.

INTRODUCTION

Phenolic compounds constitute an important group of natural products involved in many plant processes, particularly those responding to environmental biotic and abiotic stimuli. Some products of phenylpropanoid metabolic pathways (e.g., isoflavonoids and their conjugates) are involved in defense reactions and act as phytoalexins during plant responses to microbial pathogenic infections, while others are signaling molecules in symbiotic interactions with nitrogen-fixing bacteria or mycorrhizal fungi (Dixon and Paiva, 1995; Spaink, 1995; Shirley, 1996; Paiva, 2000). Phenolic acids and flavonoid derivatives provide a shield protecting plants from high light and UV radiation, and it is thought that the evolutionary acquisition of enzymes biosynthesizing phenolic compounds was among key factors that enabled plants to conquer land environments (Cooper-Driver and Bhattacharya, 1998). Flavonoids are relatively powerful antioxidants that can function, together with respective peroxidases, in vacuoles and cell walls to detoxify hydrogen peroxide (Yamasaki et al., 1997).

Profiling of qualitative and quantitative changes of natural products at various stages of plant development or in stress have been of limited interest to biologists. However, recently the necessity of monitoring changes in the composition of low molecular weight compounds has become obvious with the advent of genome sequencing and functional genomics and/or proteomics (Trethewey et al., 1999; Glassbrock et al., 2000; Glassbrock and Ryals, 2001; Bell et al., 2001; Dixon, 2001). It has been realized that the information elaborated from nucleic acid sequences does not define the function and the physiological status of systems under investigation.

Most flavonoid and isoflavonoid conjugates present in plant tissues are polar compounds and are not amenable to direct profiling by GC-MS. However, in the last decade, new efficient, low energy ionization methods at atmospheric pressure have been introduced, such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI) (Niessen, 1999). Mass spectrometric systems apply these techniques with excellent results for the analysis of proteins, peptides, and low molecular weight secondary metabolites, where liquid chromatographs are interfaced with mass spectrometers (LC-MS).

Liquid chromatography with ultraviolet and electrospray mass spectrometric detection (LC/UV/ESI/MS) has also been used for monitoring changes in the

qualitative and quantitative composition of more polar plant secondary metabolites: flavonoids and isoflavonoids and their conjugates in different plant families (Justesen et al., 1998; Lin et al., 2000; Bednarek et al., 2001; de Rijke et al., 2001; Gu and Gu, 2001; Klejdus et al., 2001). These compounds have also been monitored in alfalfa (*Medicago sativa*) and chick pea (*Cicer arientinum*) with liquid chromatography and continuous-flow liquid secondary-ions mass spectrometry ionization systems LC/CF-LSIMS/MS (Sumner et al., 1996). In other plant species, different glycosidic conjugates of phenolic metabolites have been earlier analyzed using thermospray ionization in LC/MS systems (Wolfender et al., 1997).

In our studies, we used LC/UV and LC/ESI/MS for monitoring changes in the composition of flavonoids and isoflavonoids or their conjugates in lupin seedlings grown under different light conditions or infected with a pathogenic fungus *Pleiochaeta setosa*. The results demonstrate the usefulness of LC-MS for identification of compounds and monitoring changes in the levels of polar phenolic secondary metabolites isolated from plants grown in different environmental conditions. This kind of analysis permits us to draw some conclusions about the role of these natural products in plant biochemistry and physiology. The further comparison of these data with those obtained at the DNA transcription and protein expression levels will help to elucidate the mechanisms of plant defense against, and plant adaptation to, different environmental factors.

METHODS AND MATERIALS

Plant Material. Seeds of white lupin (*Lupinus albus* L. cv. Bac) or narrow-leaf lupin (*L. angustifolius* L. cv. Mirela) were purchased from the Plant Breeding Station Przebędowo, Poland. They were sterilized with 1.5% KOCl for 20 min, washed several times with sterile deionized water, and sown in pots with perlite. Plants (5 seedlings per pot) were grown in a greenhouse for 6 wk.

Growth of Fungus. Pleiochaeta setosa was isolated from infected white lupin leaves. Leaves were surface-sterilized with 70% ethanol and washed with sterile water. Leaf pieces were placed in Petri dishes on a solid glucose–potato nutrient. Growing fungal mycelia were transferred onto fresh medium every 3–4 wk. After 2 wk of growth, *P. setosa* generated conidial spores. The spore suspension was prepared by washing the mycelium with sterile water. Number of spores was determined microscopically by using a Bürker chamber.

Inoculation of Fungi. Seeds of *L. albus* and *L. angustifolius* were sterilized, and sown in pots with steam sterilized garden soil. Seedlings were grown in the open air, and after 6 wk were inoculated by spraying the leaves with the spore suspension (concentration 10^5 spores/ml). Control plants were sprayed with water. To maintain proper humidity during infection, plants were covered with plastic bags for 24 hr. Plants were harvested at the defined time intervals (12, 24, 48, 72 h), divided into roots, stems, and leaves, and the phenolic compounds were extracted.

S*eedlings Grown in Different Light Conditions.* Lupin seeds planted in pots with perlite were grown in the growth chamber under the following light conditions: (1) 14 d in dark; (2) first 7 d in dark, followed by 7 d under artificial light (16/8-h photoperiod, 400 W sodium lamps); (3) first 7 d under artificial light, followed by 7 d in darkness; (4) 14 d under artificial light; (5) 14 d in the greenhouse without additional illumination (the second half of March).

Isolation of Phenolic Compounds. For structural elucidation of flavonoid and isoflavonoid glycosides, plant material was homogenized in 80% methanol (20 ml per g fresh weight) and placed in an ultrasonic bath for 30 min. The suspension was filtered through a Büchner funnel and concentrated under vacuum at 40°C. Compounds of interest were isolated from the extract by liquid extraction with organic solvents, further purified by column chromatography (silica gel, LH-20), and characterized by mass spectrometry according to Frański et al. (1999). Isolated and identified glycosides were used as standards for LC analyses.

For metabolic profiling, phenolic compounds from plants subjected to various treatments were extracted with methanol. Samples of plant extracts for LC analyses were prepared from 2 g of fresh weight lupin tissue. They were purified and concentrated by solid phase extraction (SPE) on cartridges containing a cation exchanger and RP C-18 silica gel (Alltech, Carnforth, England) combined in tandem, according to Stobiecki et al. (1997). Samples for GC analysis were methylated, methanolyzed, and again methylated or acetylated, as described previously (Frański et al., 1999).

Liquid Secondary-Ion Mass Spectrometry (LSIMS). Mass spectral analyses were performed on an AMD Intectra (Germany) double-focusing, reversed-geometry B–E mass spectrometer, which was fitted with a casium ion gun operating at an ion energy of 12 keV and an accelerating voltage of 8 kV. Samples were dissolved in different matrices (glycerol, thioglycerol, nitrobenzyl alcohol, triethanolamine) and analyzed in positive and negative ion modes. Collision-activated dissociation (CAD) in the linked-scan B/E mode was performed with helium as the collision gas in the cell of the first field-free region in such a way that the intensity of the ion beam was reduced by 50% of its value. CAD mass spectra were recorded at a scan rate of 20 decade per sec.

Liquid Chromatography (LC/UV) and Liquid Chromatography–Mass Spectrometry (LC/ESI/MS) Analyses. Quantitative analyses were performed on a Merck Hitachi HPLC pump Model L-7100, equipped with a diode array detector Model L-7450 (Darmstadt, Germany) and Superspher 100 RP-18 column (250 $\times$ 2 mm; Merck). To each analyzed sample, 125-μM *p*-hydroxybenzoic acid were added as an internal standard (LC retention time and UV spectral data did not interfere with those of studied compounds). Quantification of total isoflavones was achieved by integration of UV chromatograms at 259 nm, normalization to the peak of a standard, and comparison with a respective calibration curve for genistein 7-*O*-β-D-glucoside prepared in the range 10–500 μM. The concentrations of lupin

isoflavonoid and flavonoid glycosides were expressed with reference to genistein 7-*O*-glycosides.

LC/ESI/MS analyses were performed on a Nermag model R 10-10 (Quad Service, Argenteuil, France) quadrupole mass spectrometer equipped with an electrospray ion source (Analytica, Brandford, CT, USA). The instrument was coupled with a Varian model 9012 HPLC pump (Harbour City, CA, USA). Samples were injected through a 2-μl injection loop onto a MB2-SP-C18 RP column (200 $\times$ 2 mm id.; Varian) with a 0.2 ml/min flow rate. At the outlet, the effluent was split 3:1 so that 50 μl were delivered to the electrospray ion source and 150 μl passed through a variable-wavelength UV detector (model 481; Waters, Bedford, MA, USA) operating at a wavelength of 259 nm. Mass spectra were recorded in the positive ion mode. Nitrogen was used both as a drying and as an auxiliary gas. Acquisition of the spectra was performed at 1.3 scan/s using an HP Chemstation data system.

During LC/UV and LC/ESI/MS analyses, the elution protocol was carried out with two solvent mixtures: A (95% acetonitrile, 4.5% H_2O, 0.5% acetic acid; v/v/v) and B (95% H_2O, 4.5% acetonitrile, 0.5% acetic acid; v/v/v). Elution steps were as follows: 0–5 min isocratic at 10% A, 5–40 min linear gradient from 10 to 30% of A, 40–48 min linear gradient up to 100% of A, 48–60 min isocratic at 100% of A. Flavonoids were identified by their MS spectra and/or by their retention times compared with data obtained for standards.

Gas Chromatography–Mass Spectrometry (GC/MS) Analysis. Samples were subjected to GC/MS analyses on an Hewlett-Packard (Palo Alto, CA, USA) gas chromatograph Model 5890/II with a mass-selective detector Model 5971A. The instrument was equipped with a XTI-5 fused-silica capillary column (30 m $\times$ 0.25 mm i.d.) (Restek, Bellefonte, PA, USA). The carrier gas was helium at a flow-rate 1 ml/min . The column temperature was programed from 140°C (held for 2 min) at 5°C $\cdot$ min^{-1} to 300°C, which was maintained for 5 min. The injector temperature was 250°C. Electron impact (EI, 70 eV) mass spectra were recorded over the range 50–650 amu.

RESULTS

Flavonoid and Isoflavonoid Glycosides. Structures of flavonoid and isoflavonoid glycosides present in roots and green parts of *L. albus* (white lupin) plants were characterized earlier (Frański et al., 1999; Bednarek et al., 2001). The same mass spectrometric methodological approach was applied to the identification of phenolic compounds isolated as pure compounds from of *L. angustifolius* (narrow-leaf lupin). Liquid secondary-ion mass spectrometry was used for recording the mass spectra of unmodified and methylated flavonoid glycosides isolated from roots and green parts of the narrow-leaf lupin seedlings, and metastable ion MS spectra were also recorded. In the next step of the structural analysis, further chemical

TABLE 1. FLAVONOIDS AND THEIR GLYCOSIDES IDENTIFIED IN NARROW-LEAF LUPIN (*L. angustifolius*)

No	Compound	MW	Root	Leaf
1	2′-hydroxygenistein 4′,7-*O*-diglucoside[a]	610	+	−
2	genistein 4′,7-*O*-diglucoside[a]	594	+	+
3	2′-hydroxygenistein 7-*O*-glucoside[a]	448	+	+
4	genistein 6 or 8-*C*-glucoside[a]	432	+	+
5	genistein 4′-*O*-glucoside-7-*O*-(6″-*O*-malonyl) glucoside[b]	680	+	−
6	genistein 7-*O*-glucoside[a]	432	+	+
7	apigenin 7-*O*-xylosylglucoside[a]	432	−	+
8	2′-hydroxygenistein 7-*O*-(6″-*O*-malonyl) glucoside[b]	534	+	−
9	3′ or 4′-methylluteolin 7-*O*-xylosylglucoside[a]	594	−	+
10	genistein 4′-*O*-glucoside[a]	432	+	−
11	genistein 6 or 8-*C*-(6″-*O*-malonyl) glucoside[b]	518	+	+
12	genistein 4′-*O*-(6″-*O*-malonyl) glucoside[b]	518	+	−
13	3′ or 4′-methylluteolin 7-*O*-glucoside[a]	462	−	+
14	genistein 7-*O*-(6″-*O*-malonyl) glucoside[b]	518	+	+
15	3′ or 4′-methylluteolin 7-*O*-xylosylglucoside malonylated[b]	680	−	+
16	3′ or 4′-methylluteolin 7-*O*-(6″-*O*-malonyl) glucoside[b]	548	−	+
17	2′-hydroxygenistein[a]	286	+	+
18	genistein[a]	270	+	+
19	3′ or 4′-methylluteolin[b]	300	−	+
20	luteone[a]	354	+	+
21	wighteone[b]	338	+	+

[a] Isolated as pure compounds from *L. albus* and/or *L. angustifolius* and identified with mass spectrometric techniques according Frański et al. (1999).
[b] Compounds identified exclusively after LC/ESI/MS analysis.

modification (methanolysis of permethylated glycosides followed by methylation or acetylation) and GC/MS of products were applied. This permitted the identification of the aglycone, substitution pattern of the sugars, and the glycosidic linkage between sugars. Twelve flavone and isoflavone glycosides or free aglycones were identified in *L. angustifolius* (Table 1). These compounds and those described earlier in *L. albus* were used as standards during LC analyses. However, the presence of seven malonylated glycosides in lupin tissue was established after LC/UV/ESI/MS analyses of the methanolic extracts from lupin tissue.

Only isoflavone glycosides were identified in the roots, with some of them malonylated at the sugar moiety. The only difference in the composition of root isoflavone aglycones was the absence of orobole and its methyl derivative in *L. angustifolius*. The most abundant compounds were glycosides of genistein, while 2′-hydroxygenistein conjugates were present in smaller amounts. However, substantial differences were observed in the composition of aglycones present in the glycosides isolated from the green parts of *L. albus* (Frański et al., 1999; Bednarek et al., 2001) and *L. angustifolius* (Figure 1; Table 1). In white lupin,

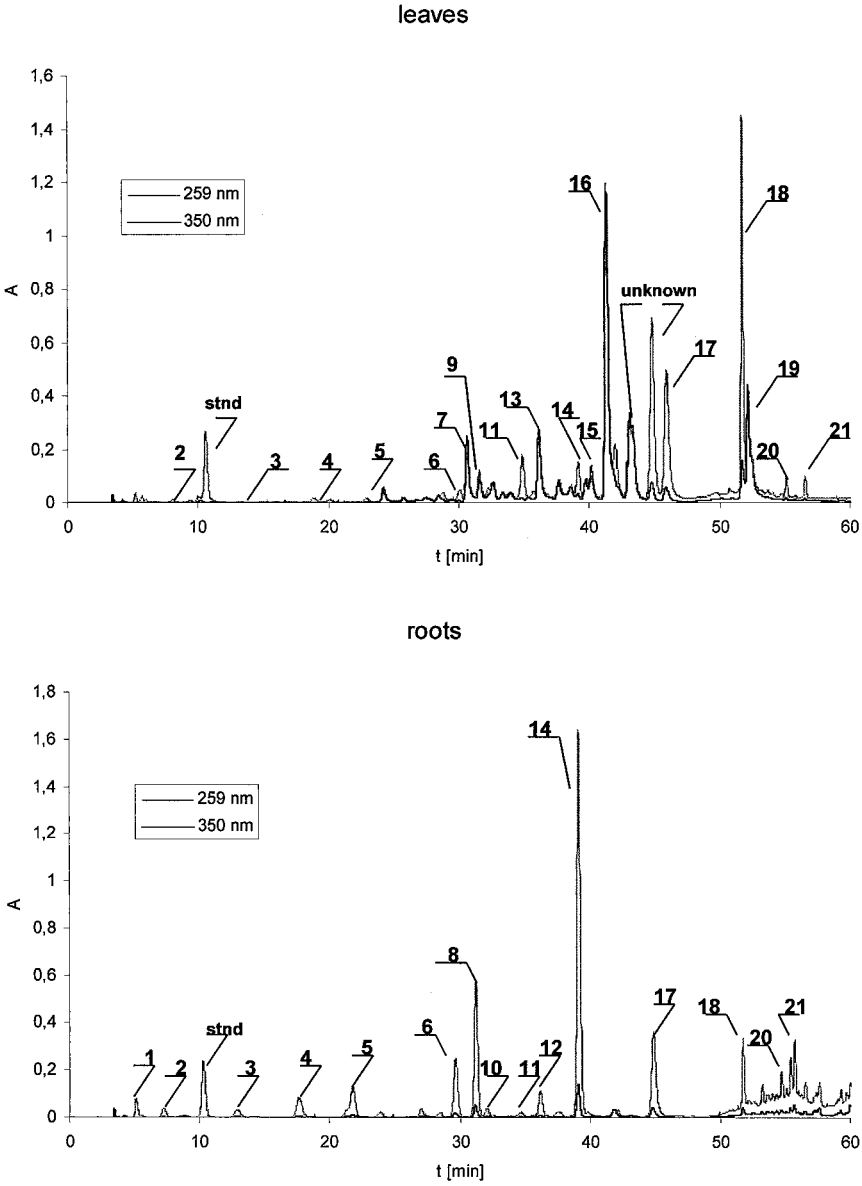

FIG. 1. LC chromatogram of phenolic compounds from narrow-leaf lupin (*L. angustifolius*) leaves and roots. Peak numbers correspond to the flavonoid conjugates listed in Table 1.

glycosides of kaempferol and isorhamnetin were found, while glycosides of apigenin and mainly methylluteolin were isolated from narrow-leaf lupin. Following LC/UV and LC/ESI/MS analyses of fresh extracts from *L. angustifolius* leaves, we also found two *O*-methylluteolin glycosides **15** and **16** (Table 1) that were acylated with malonic acid. This type of modification was not observed earlier in the flavonol (kaempferol and isorhamnetin) glycosides from green parts of *L. albus*. Moreover, the total amount of flavone glycosides present in the leaves was much higher than that of isoflavone glycosides. Thus, the quantitative ratio between flavonoid and isoflavonoid glycosides, found earlier in leaves of *L. albus* plants (Bednarek et al., 2001), was reversed in narrow-leaf lupin.

Infection. It was found earlier that, under field conditions, *L. albus* var. Bac was more susceptible to infection with *P. setosa* than *L. angustifolius* var. Mirela (Rybus-Zając and Kozłowska, 1996). Profiles of flavonoid and isoflavonoid glycosides isolated from leaves of control, healthy lupin plants demonstrated higher concentrations of free isoflavonoid aglycones in roots and leaves of *L. albus*. Throughout the experiments, a constant increase of 2′-hydroxygenistein and luteone concentrations in the leaves of both infected lupin species was observed between 0 and 72 hr after inoculation (Figures 2 and 3). However, concentrations of two other isoflavone aglycones, genistein and wighteon, in leaves of *L. angustifolius* decreased between 48 and 72 hr of the experiment (Figure 3).

The relative increase in the amounts of 2′-hydroxygenistein and luteone in the infected leaves of *L. angustifolius* seedlings 72 hr after infection was higher than that of genistein and wighteone. After 72 hr, the final concentrations of 2′-hydroxygenistein in leaves were also different in *L. albus* and *L. angustifolius*, reaching 190 and 340 μg per gram fresh weight, respectively. Also, the final concentration of genistein was higher in narrow-leaf lupin. In the stems of both lupin species, the accumulation of free isoflavones was also observed, but the concentrations were lower (data not presented). Analysis of LC profiles of flavonoid conjugates in the leaves of control and infected lupin seedlings demonstrated no substantial difference in the amounts of glycosylated isoflavonoids and their acylated conjugates. The only exception was 2′-hydroxygenistein 7-*O*-glucoside in *L. albus* leaves, which increased slightly, reaching maximal concentration after 48 hr (Figure 2).

Influence of Light on Flavonoid Glycosides Profiles. Analysis of results demonstrated that during lupin plant development, illumination conditions influenced qualitative and quantitative composition of free isoflavonoids or flavonoids, as well as their glycosidic conjugates. Total amounts of phenolic compounds in all plant organs were the lowest in etiolated seedlings (1) and those grown in the greenhouse without additional illumination (5) (Figure 4). Levels of all flavonoid and isoflavonoid glycosides in the roots and stems were less affected by different light conditions. Periods of darkness in the growth chamber (1–3) or low intensity of light with shorter photoperiod in the greenhouse (5) did not cause substantial

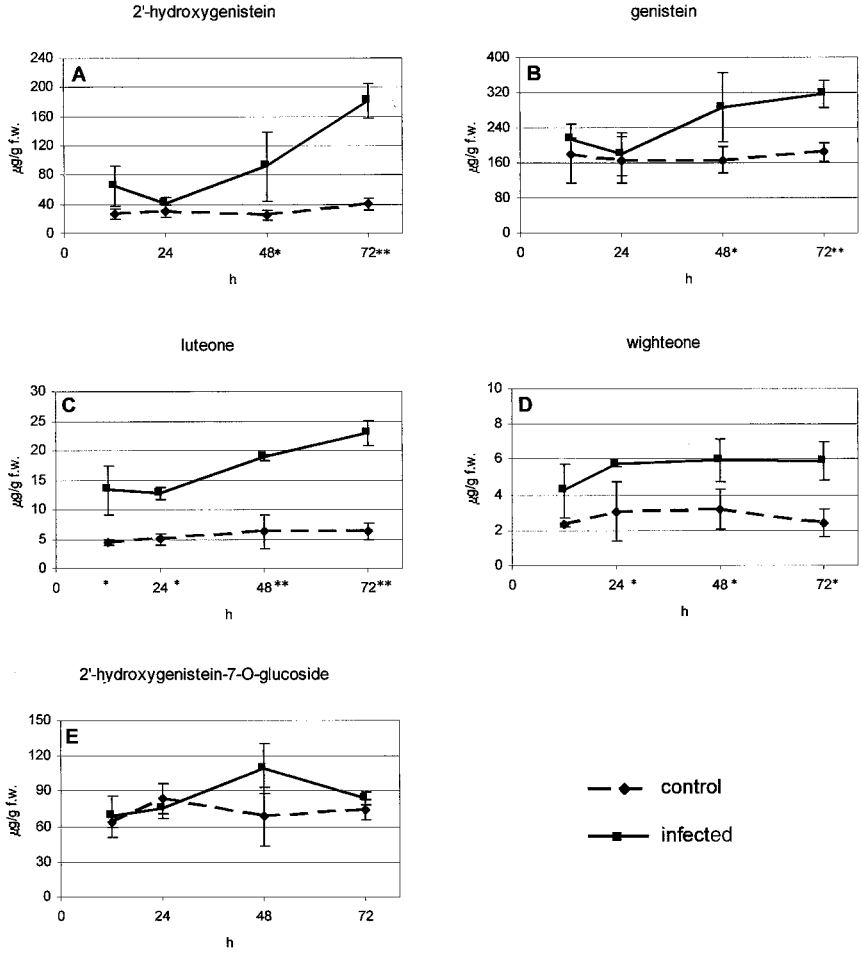

FIG. 2. Changes in the concentrations of compounds in the leaves of control and infected white lupin (*L. albus*) plants: (A) 2′-hydroxygenistein; (B) genistein; (C) luteone; (D) wighteone; (E) 2′-hydroxygenistein 7-*O*-glucoside. The amounts of compounds were calculated from integrated UV chromatograms by comparison with calibration curve prepared for genistein 7-*O*-glucoside. The data were obtained from two experiments, presented as means (±SD) of four analyses, asterisks denote significant differences (*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$).

changes in the amounts of the conjugates extracted from these organs. The highest amounts of phenolic glycosides were observed only in roots and stems of plants cultivated in the growth chamber and illuminated with the artificial light for 14 d (4) (Figure 4).

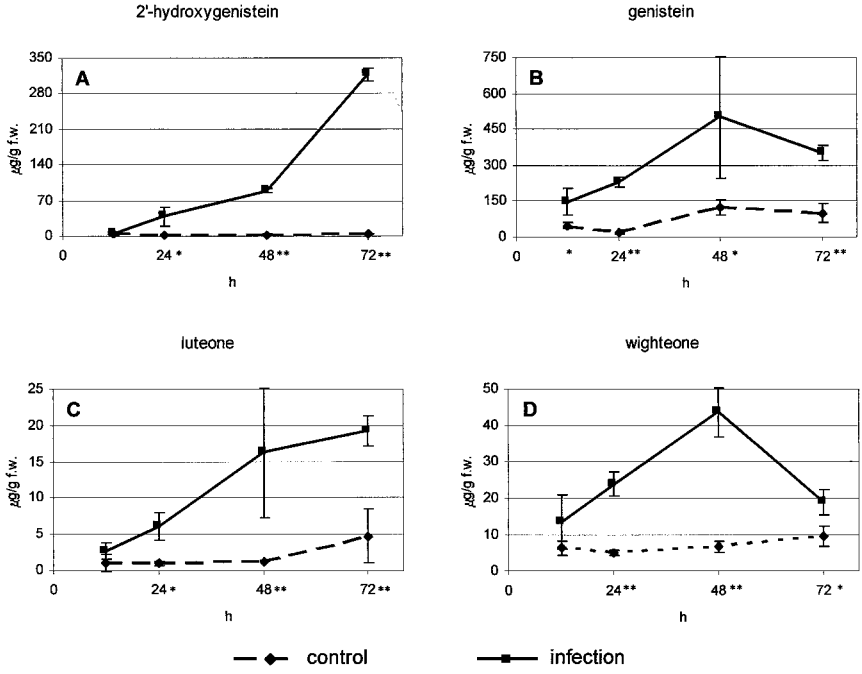

FIG. 3. Changes in the concentrations of aglycones in the leaves of control and infected narrow-leaf lupin (*L. angustifolius*) plants: (A) 2′-hydroxygenistein; (B) genistein; (C) luteone; (D) wighteone. The amounts of compounds were calculated from integrated UV chromatograms by comparison with calibration curve prepared for genistein 7-*O*-glucoside. The data were obtained from two experiments, presented as means (±SD) of four analyses, asterisks denote significant differences (*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$).

The period, the timing, and the intensity of illumination also influenced the relative content of flavonol and isoflavone aglycones and their glycosides in lupin leaves (Figure 5). Substantially higher relative amounts of flavonols were found in the leaves of seedlings cultivated in the growth chamber with different times of illumination (2–4) than in leaves of etiolated seedlings or those grown in the greenhouse. More intense irradiation of seedlings in the first stage of development evoked a relative increase of flavonol glycosides in the total pool of flavonoid conjugates. However, a period of darkness during the second part of the experiment (3) affected the process of deglycosylation of flavonoids to free aglycones and deacylation of isoflavonoid glycosides. This treatment (3) also increased the relative amount of free aglycones, especially isoflavones. In the experimental variants (1) and (3), in which the seedlings were grown in the dark directly before harvest, low light intensity had a negative effect on the relative quantities of malonylated isoflavone

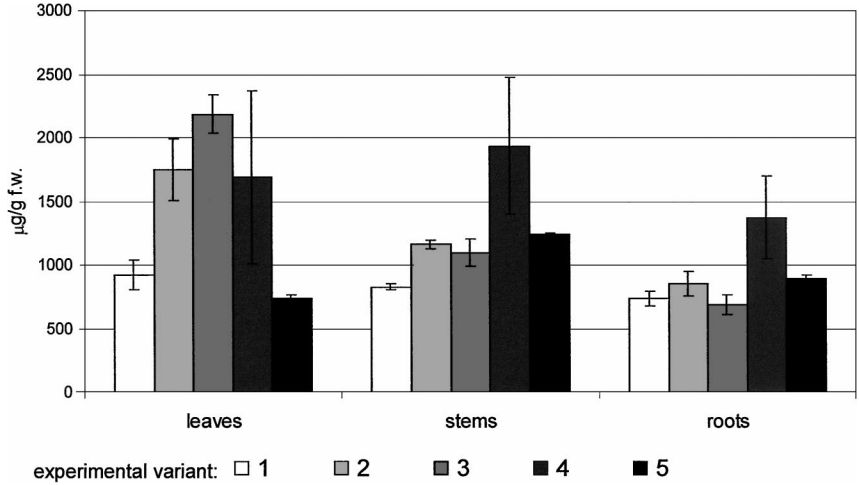

FIG. 4. Total amounts of flavonoid and isoflavonoid glycosides in root, stem, and leaf of white lupin (*L. albus*) seedlings cultivated in different light conditions. The experimental variants are described as follows: (1) etiolated; (2) 7 days in the dark and 7 days with photoperiod; (3) 7 days with photoperiod and 7 days in the dark; (4) 14 days with photoperiod; (5) 14 days in the greenhouse without additional illumination in March 2000, results are mean values of two parallel, independent experiments.

conjugates in leaves. The relative amounts of malonylated isoflavone glycosides varied from 6–25% of relative contribution to the total pool of flavonoid and isoflavonoid conjugates. In experiment (2), where a period of darkness was followed by a period of illumination, dramatic changes in the relative amounts of flavonol glycosides were noted when compared with experiments (1) and (5). However, the qualitative composition was similar to that registered in the experiment (4), where constant illumination during 14 d of growth was applied.

DISCUSSION

Plants have to cope with various challenges coming from the environment, and many classes of biological molecules are involved in their reactions. To precisely evaluate the participation of different low molecular weight compounds in plants' reactions to stress, one has to develop methodological approaches enabling accurate analyses of complicated mixtures of molecules differing in their chemical and biological properties. For example, flavonoid and isoflavonoid glycosides are polar and thermally labile compounds that can be separated efficiently only with liquid chromatographic methods. However, because of various structural

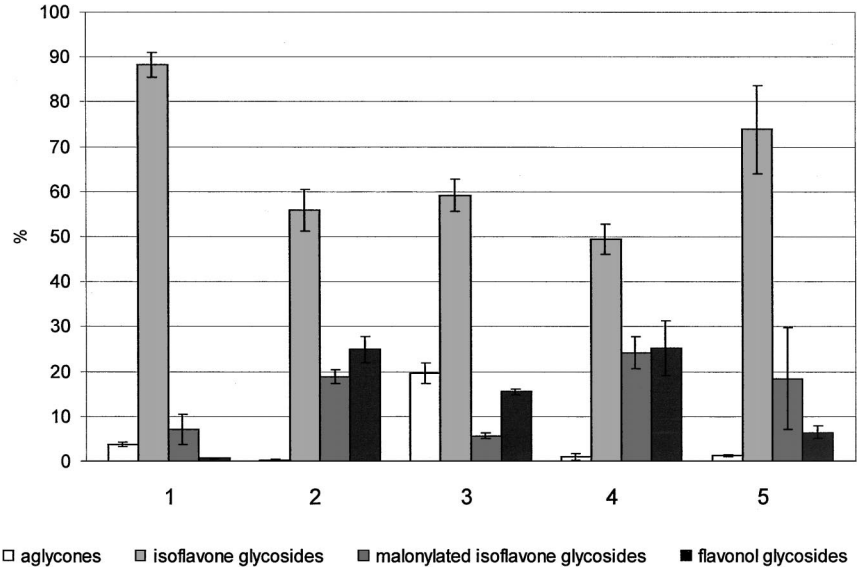

FIG. 5. Relative amounts of flavonoid and isoflavonoid glycosides and free aglycones in the leaves of white lupin (*L. albus*) cultivated in different light conditions. The experimental variants are described as follows: (1) etiolated; (2) 7 days in the dark and 7 days with photoperiod; (3) 7 days with photoperiod and 7 days in the dark; (4) 14 days with photoperiod; (5) 14 days in the greenhouse without additional illumination in March 2000.

modifications of the basic phenolic skeleton (hydroxylation, methylation, and/or prenylation), they reveal a significant structural diversity, and some of them may occur in different isomeric forms. The same problem concerns sugars (pentoses and hexoses, D- or L- forms, α and β configuration on the C-1 carbon atom, placement of interglycosidic linkage). Thus, the choice of detection technique appears to be crucial for successful identification of above mentioned classes of natural products.

Parallel utilization of the UV and mass spectrometric detectors in a LC system facilitates identification of natural products of interest on the basis of UV spectra, *m/z* values of protonated molecules $[M+H]^+$, and fragment ions created after cleavage of glycosidic bonds. However, in the mass spectra of malonylated glycosides of isoflavones and flavones, only fragment ions created after cleavage of the glycosidic bonds were observed, and elimination of malonic acid was not registered during fragmentation of $[M+H]^+$ ions. Selective analysis of differences in UV absorption at 259 nm and 350 nm allowed us to distinguish between isomeric isoflavone and flavone or flavonol structures of aglycones. On the basis of *m/z* values of $[M+H]^+$ ions in the mass spectra, it was possible to detect not only

phenolic glycosides, but also their malonylated conjugates. In a liquid chromatography system that is interfaced with a mass spectrometer equipped with an electrospray source, the amount of accessible structural information is low. Only protonated molecular ions $[M+H]^+$ and fragments created after glycosidic bond cleavage are observed. To obtain more structural information about an aglycone structure, detection with collision-induced dissociation and tandem mass spectrometry capabilities are necessary in the LC/MS system. In many cases, it is possible to use GC/MS with electron impact ionization for identification of the aglycones under investigation, but in such cases, chemical modifications of analyzed compounds are necessary (Bednarek et al., 2001).

The structural diversity of plant flavonoids and isoflavonoids and their conjugates constrains the application of identification methods to those with the highest sensitivity and objectivity. LC/MS systems fulfill these requirements. Thus, liquid chromatography–mass spectrometry may be applied during research in different fields of plant sciences, especially supporting molecular biology techniques used in functional genomic research.

The antimicrobial activity of several prenylated isoflavones from lupin, especially luteone—a derivative of 2′-hydroxygenistein—has been demonstrated *in vitro* (Harborne et al., 1976; Tahara et al., 1984). On one hand, these compounds are secreted constitutively on the surface of healthy plants, and this location may suggest that they function as phytoanticipins (Van Etten et al., 1994). On the other hand, evidence has accumulated that lupin isoflavones may also act as phytoalexins (Shibuya et al., 1992; Wojtaszek and Stobiecki, 1997; Bednarek et al., 2001). The results obtained during infection of lupin plants with *P. setosa* do not allow us to discriminate whether the isoflavone aglycones with antibiotic activity are synthesized *de novo* or if isoflavone glycosides function as substrates providing free aglycones (genistein and 2′-hydroxygenistein) that are utilized further for prenylation. Because flavonoids and their conjugates from all leaves of the infected and control seedlings were extracted, it was not possible to determine if there were differences in the concentrations of isoflavonoid glycosides between infected and uninfected leaves in the localized area. However, in earlier research, we found that PAL (phenylalanine ammonia-lyase) activity in *L. albus* leaves did not increase substantially after elicitation with a yeast cell wall elicitor preparation. Only higher activity of β-glycosidase in elicited lupin plants was observed (Piślewska et al., 2002).

Differences in the intensities of infection symptoms between seedlings of both lupin species were not observed. However, earlier field observation indicated lower susceptibility of *L. angustifolius* to fungal infection (Rybus-Zając and Kozłowska, 1996). These results are consistent with the information obtained from LC/UV/MS profiling of flavonoid conjugates. Comparison of isoflavone aglycone profiles in white and narrow-leaf lupin shows that concentrations of the antibiotic compounds in uninfected seedlings were higher in less resistant species of white

lupin. However, 72 hr after infection, increase of 2′-hydroxygenistein and luteone amounts was more pronounced in the more resistant seedlings of narrow-leaf lupin (Figures 3 and 4).

Two physical parameters characterizing the quality of light appear to be important for the functioning of the phenylpropanoid pathway in plants, namely light spectral characteristics (frequency range) and light intensity. On one hand, UV radiation and high light intensity induce biosynthetic enzymes producing phenolic compounds; also irradiation of green plants with infrared light influences susceptibility to UV damage (Dixon et al., 2001). On the other hand, the composition of phenolic natural products depends on the evolutionary equipment of a given plant species (Rozema et al., 1997). It is usually thought that these pathways have evolved towards generation of compounds with more efficient shielding properties, e.g., phenylpropanoid acids and flavonols (Li et al., 1993; Laundry et al., 1995). Lupins are usually considered to be among the oldest leguminous plants. They appear to lack enzymes of pterocarpan-skeleton biosynthesis, while the isoflavonoid biosynthetic pathway is elaborate and complicated (Gagnon et al., 1992; Shibuya et al., 1992; Wojtaszek and Stobiecki, 1997). Thus, lupins seem to be a good model system to test at least some of these evolutionary implications.

We demonstrated that the concentration and composition of flavonoid and isoflavonoid glycosides in different organs was strongly affected by changes in light intensity (Figures 4 and 5). Kaempferol and isorhamnetin glycosides, which play an important role in the protection of plants against UV-B irradiation (Li et al., 1993; Laundry et al., 1995), had a higher contribution in the relative amount of flavonoid conjugates in the leaves of white lupin grown under more intense light, particularly in the experimental variant (4) (Figure 5). Relative ratios of other phenolic compounds (e.g. malonylated isoflavone glycosides and free aglycones) in the plant tissue were also affected in various experiments. Two hypotheses may explain the decrease in concentration of the isoflavone conjugates and their malonylated derivatives in lupin seedlings cultivated under low light intensity. The first one focuses on the energetic needs of plants as an explanation for the decrease: the secondary metabolites were decomposed for energetic reasons because of the high free energy of hydrolysis. The second hypothesis explains the lower levels of the iosflavone conjugate concentrations by a lesser need of a plant for photoprotection, therefore, synthesis of these compounds is down regulated. As alternative explanations exist, further studies may be necessary. A lower level of malonylation of quercetin and kaempferol glycosides has also been observed in lettuce and endive plants cultivated under different light conditions (DuPont et al., 2000). These data collectively seem to indicate that differences in light intensity during growth and development of white lupin seedlings affects the composition of flavonoid and isoflavonoid conjugates, and this may alter plant resistance to different environmental stresses. Such changes in the relative amounts of glycosides and free aglycones synthesized and/or accumulated in leaves under various

light conditions may also manifest different roles of particular phenolic conjugates in the physiology and biochemistry of plants during interactions with microbes, animals, and other plants (Paiva, 2000).

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INHERITANCE PATTERNS OF PHENOLICS IN F1, F2, AND BACK-CROSS HYBRIDS OF WILLOWS: IMPLICATIONS FOR HERBIVORE RESPONSES TO HYBRID PLANTS

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Abstract—The aim of this study was to determine the inheritance pattern of phenolic secondary compounds in pure and hybrid willows and its consequences for plant resistance to leaf-feeding insects. F1, F2, and back-cross hybrids along with pure species were produced by hand pollination of pure, naturally-growing *Salix caprea* (L., Salicaceae) and *S. repens* (L.) plants. Leaf concentrations of condensed tannins and seven different phenolic glucosides were determined by using butanol-HCl and HPLC analyses. Insect herbivore leaf damage was measured on the same leaves as used for chemical analyses. We found hybrids to be approximately intermediate between the parental species: *S. caprea* with high levels of condensed tannins and no phenolic glucosides, and *S. repens* with low levels of condensed tannins and high levels of phenolic glucosides. We also found a negative correlation between concentrations of condensed tannins and phenolic glucosides, suggesting a trade-off in production of these two substances. F2 hybrids and the hybrid back-crossed to *S. caprea* were significantly more damaged by insect herbivores than the parental species and the F1 hybrid, indicating reduced resistance and possibly a selective disadvantage for these hybrid categories.

Key Words—Phenolic glucosides, condensed tannins, *Salix caprea*, *S. repens*, willows, herbivore resistance, Chrysomelidae.

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INTRODUCTION

Interspecific hybridization in plants may be important for the evolution and speciation of both plants and their associated herbivores (Strauss, 1994). Whitham (1989) suggested that hybridization may cause a breakdown in plant resistance against herbivores. Some later studies have provided at least partial support for Whitham's hypothesis (e.g., Whitham, 1989; Fritz et al., 1994, 1996; Strauss, 1994; Fritz, 1999), but several studies have shown different patterns than those predicted by the hybrid susceptibility hypothesis (Boecklen and Spellenberg, 1990; Strauss, 1994; Hjältén, 1997, 1998; Fritz et al., 1998; Fritz, 1999). In addition, there is considerable interspecific and temporal variation in herbivore response to hybrid plants (Fritz et al., 1994, 1996, 1998; Whitham et al., 1994; Orians et al., 1997).

In most experimental studies of hybrid plant–herbivore interactions, only parental species and F1 hybrids are studied, while data from more complex hybrids, hybrid × hybrid crosses (F2 hybrids), and hybrids back-crossed to the parental species, are lacking (Strauss, 1994; Fritz, 1999). To understand the effect of hybridization in an ecological and evolutionary perspective, these hybrid categories are important to consider (Arnold, 1997; Arnold et al., 1999). Furthermore, as only a small proportion of the herbivore fauna is considered in most hybrid herbivore studies, they provide little information of the total effect of herbivory on plant fitness (Roche and Fritz, 1997). From an evolutionary perspective, it may be more interesting to consider the damage from the whole insect herbivore community. An important step to understand how plant hybrids are affected by herbivory is to study how characters important to herbivores (e.g., secondary chemical compounds) are inherited from the parental species to the hybrids (Orians et al., 2000). By considering both the inheritance of resistance characters and the total effect of herbivores on plants, it may be possible to make some conclusions about how the system might be affected by hybrid–herbivore interactions.

The distribution of leaf-eating insects on willows (Salicaceae) is thought to be strongly influenced by phenolic secondary metabolites (Tahvanainen et al., 1985; Denno et al. 1990; Soetens et al., 1991; Kolehmainen et al., 1994, 1995; Kendall et al., 1996; Roininen et al., 1999). With respect to leaf secondary chemistry, willows can roughly be divided into two different groups. One group contains high concentrations of condensed tannins in the leaves, but low levels of phenolic glucosides, while the other contains phenolic glucosides in high concentrations, but low concentrations of condensed tannins (Julkunen-Tiitto, 1986; Kelly and Curry, 1991). Condensed tannins are phenolic polymers generally known to reduce protein uptake, while phenolic glucosides are phenolic monomers some of which are known to have toxic effects on animals (Lindroth, 1991). It has been suggested that there is a trade-off between the production of phenolic glucosides and condensed tannins, either because of resource allocation in biosynthetic pathways or because of evolutionary genetic factors (Julkunen-Tiitto, 1989; Orians et al., 2000).

Analyses of secondary chemistry in back-cross hybrids and especially F2 hybrids could provide information if such trade-offs exist and if it is due to the genetic arrangement, or as a consequence of resource allocation in phenolic biosynthetic pathways (Orians et al., 2000).

In most cases, phenolic glucosides act as repellents for generalist herbivores (Tahvanainen et al. 1985; Kelly and Curry, 1991). However, for some specialist insect herbivores, they can act as positive cues that trigger oviposition and feeding (Kolehmainen et al., 1994, 1995; Soetens et al., 1998; Roininen et al. 1999). Willows with low levels of phenolic glucosides are usually considered palatable to both generalist and specialist herbivores, while willows with high concentrations of phenolic glucosides are mainly utilized by herbivores that are adapted to these substances (Tahvanainen et al., 1985; Kelly and Curry, 1991; Kolehmainen et al., 1995; Roininen et al., 1999).

Orians and Fritz (1995) reported an additive inheritance pattern of condensed tannins and two phenolic glucosides in the F1 hybrids between *Salix sericea* and *S. eriocephala*. However, in a later study, Orians et al. (2000) found variation between F1 hybrid families and different parent individuals. F1 hybrid families were intermediate between their respective pure siblings, but varied between above midpoint to below midpoint of parental species siblings.

In this study, we determined the concentration of secondary compounds, phenolic glucosides and condensed tannins, in leaves of two willow species, their F1 hybrids, F2 hybrids, and back-cross hybrids. We also determined leaf damage by phytophagous insects on the experimental plants. The following questions were addressed: (1) Are condensed tannins and phenolic glucosides dominantly or additively inherited in F1, F2, and backcross hybrids? (2) Is there a negative correlation between the concentration of condensed tannins and phenolic glucosides in the different plant categories? (3) Do hybrid plants experience more damage by phytophagous insects than the pure parental species?

METHODS AND MATERIAL

Plant Material. Willow hybrids are difficult to identify on the basis of morphological characters (Lid, 1979; Mossberg et al., 1992). To control the origin of the plant material used, seeds of hybrids and parental species were produced during 1996 and 1997 by hand-pollinating willows either growing in the field, or in the case of F2 and back-cross hybrids, potted willows of known origin. Only willow individuals showing morphological characters considered distinctive for *S. caprea* and *S. repens*, were considered as pure parents. The crossing scheme and total replication is shown in Table 1. Each individual plant is regarded as an independent replicate. For further description of methods, see Hjältén (1997).

TABLE 1. HYBRID CROSSES, AND THE NUMBER OF INDIVIDUALS USED FROM EACH PARENTAL CROSS

	Male parent								
Female parent	c6	c1	c10	c2	h1	r1	r2	r5	Sum
C1	4	—	—	—	10	—	—	—	14
C2	10	—	—	10	—	9	—	—	29
C3	—	10	—	—	—	—	—	—	10
C5	—	—	—	10	—	—	—	—	10
C6	—	—	—	—	—	10	—	—	10
H1	—	—	10	—	—	10	—	—	20
H2	—	—	—	9	9	10	—	—	28
H3	—	—	—	—	10	—	—	—	10
R1	—	2	—	—	—	4	8	—	14
R2	—	—	—	—	—	8	—	—	8
R3	—	—	—	—	10	10	—	10	30
R4	—	—	—	—	10				10
Sum	14	12	10	29	49	61	8	10	193

[a] *S. caprea* are represented by C (female) or c (male), *S. repens* by R or, r, and F1 hybrid parents by H or h.

Plants from 1997 (136 plants) were planted one plant to a pot (3 dm^3) and grown in a randomized block design with two plants from each plant category in each block. Pots were placed close to each other (0.01 m between plants). Plants from 1996 were either planted one plant to a pot (3 dm^3) and grown close to each other in a randomized block design with one plant from each of the plant categories-*S. caprea*, F1 hybrid, and *S. repens*-in each block (27 plants) or were planted one plant from each category,-*S. caprea*, F1 hybrid, and *S. repens*-to a pot (5 dm^3, 30 plants). All plants were grown on the same experimental field. The plants received water when needed and were fertilized twice a week (standard liquid fertilizer "SuperbaS"; 10 ml/1; NPK 6.5-1.0-4.7). Winter mortality reduced the sample size and resulted in an unbalanced design (Table 1). The plants were protected from mammalian herbivores. It should be noted that several willow species grow in the neighborhood of the field.

Herbivores. Willows serve as hosts for numerous generalist and specialist leaf beetles (Koch, 1992; Jolivet and Hawkeswood, 1995). The most abundant herbivores on the experimental plants were different species of leaf beetles (Coleoptera: Chrysomelidae), but we also observed lepidopteran and hymenoptera larvae. The leaf beetle species that we observed feeding were *Lochmaea caprea* (L. 1758, all insect nomenclature from Gustavsson, 2001), *Crepidodera fulvicornis* (Fabr. 1792), *Phratora vulgatissima*, *P. polaris* (Suff. 1851), and *P. vitellinae* (L. 1758). These species are commonly observed on wild willows in this area. *P. vitellinae* is known to be abundant on *S. myrsinifolia*, which has a high concentration of

phenolic glucosides in the leaves. Other leaf beetle species are known to be abundant on *S. caprea*, *S. aurita*, and *S. phylicifolia* with low concentrations of phenolic glucosides (see Tahvanainen et al., 1985; Kelly and Curry, 1991; Koch 1992).

Analysis of Leaf Chemistry. Between July 7 and July 11 in 1999, we randomly collected between 20 (*S. caprea*) and 150 (*S. repens*) leaves from each plant. A random sample of 20 of the leaves was photocopied immediately and then all leaves were air-dried at room temperature (18–22°C) in cardboard boxes (see Julkunen-Tiitto and Sorsa, 2001). All leaves in each leaf sample were ground to fine powder in a plant mill. Subsequently, we extracted leaf phenolics for 15 min at +4°C in 20 ml of 100% methanol from a weighed subsample (about 150 mg) of each sample. After the extraction period, the sample was homogenized twice for 3 min with an Ultra-Turrax homogenizer. Solvent phases from both homogenizations were filtered into one collection flask. Finally, solvent was removed by rotary evaporation. Low molecular weight phenolics were analyzed using Hewlett-Packard HPLC. The compounds were separated on a 60 mm × 4.6 mm i.d. HP Hypersil ODS II (3 μm) column. The elution solvents were aqueous 1.5% tetrahydrofuran plus 0.25% orthophosphric acid (A) and methanol (B). Details of the gradient used are in Julkunen-Tiitto et al. (1996). The flow rate was 2 ml/min and the injection volume 20 μl. From each sample, we quantified all detected phenolic glucosides. Individual compounds were identified by comparing their UV–visible spectra and retention times to those of known compounds. We also detected two unknown compounds whose spectra closely resemble known phenolic glucosides and are considered as unknown structural phenolic glucosides. Polymeric condensed tannins were measured with the butanol–HCl assay (Waterman and Mole, 1994).

Measurements of Leaf Damage. To determine the mean leaf area and the mean leaf are removed by herbivores on each plant we measured the photocopied leaves (see Analyses of Plant Chemistry) from each plant using a grid (1 × 1-mm^2 grids) on a transparent scale paper. We were able to discriminate between feeding tracks from Chrysomelidae beetle larvae and other insect herbivores, since the leaf beetles are the only herbivores that skeletonize the leaves when feeding that we have observed. We observed that the lepidopteran and hymenopteran larvae on these plants consume even the vascular system of the leaves. Thus, we report plant damage from leaf beetles separately, as well as the damage from all leaf-chewing insect herbivores together.

Statistical Analyses. Differences in the concentrations of phenolic glucosides between plant categories were first tested in a MANOVA (Hair et al., 1995), and then in individual ANOVAs for each of the studied phenolic compounds (Venables and Ripley, 1999). For variables with a significant difference explained by the plant category factor, a multiple comparison *t*-test with sequential Bonferroni correction (Rice, 1989) was performed to evaluate how concentration varied between different plant categories (Zar, 1996). To study if there is a trade-off between the production of condensed tannins and phenolic glucosides, we performed separate Spearman

rank correlation analyses for each plant category and tested if the correlation coefficient was less than zero.

To test for differences in herbivore susceptibility, we performed two different indicator variable regressions on the two variables estimating the consumed leaf area (leaf area consumed by all herbivores and leaf area consumed by leaf beetles, respectively) to be explained by plant category with leaf area as continuous covariate variable (see results in Tables 2 and 3; Venables and Ripley, 1999). Because the variables in the models were not normally distributed and variation was heteroscedastic, data were log+1 transformed (Zar, 1996; Krebs, 1999; Venables and Ripley, 1999). For all statistical analyses, we used R version 1.2 (Venables et al. 2002).

RESULTS

We detected and measured the concentration of five known phenolic glucosides, salicin, salicortin, diglucose salicin, tremulacin, tremuloidin, and two structurally unidentified phenolic glucosides in the plant material. A significant part of the overall variation in concentration of these phenolic glucosides was explained by plant category (MANOVA; Pillai's trace $= 1.0558$, $P < 0.001$). Plant category also explained a significant part of the variation in the concentration

TABLE 2. STATISTICS FOR INDICATOR VARIABLE REGRESSION OF HERBIVORE LEAF DAMAGE

	df	*F* value	*P*
Intercept, plant category	5	39.51	<0.001
Slope, leaf area	1	50.11	<0.001
Residuals	186		
Model variables	Coefficient estimate	*t*	*P*
Slope, leaf area	1.08	7.08	<0.001
Intercept, *S. caprea*	−4.44	−3.9	<0.001
Additional intercept, *S. repens*	0.05	0.14	0.89
Additional intercept, F1 hybrid	−0.25	−0.98	0.33
Additional intercept, F2 hybrid	0.79	2.77	0.01
Additional intercept, F1 × *S. caprea*	0.94	4.19	<0.001
Additional intercept, F1 × *S. repens*	0.32	1.1	0.27

[a] The table shows the statistics for the regression model (first three rows) and statistical test if coefficents in the model are different from 0. From the table we can see that there is a positive relationship between the plant's average leaf size and the average amount of leaf damage. There is no difference between *S. caprea*, *S. repens*, F1 hybrid, and the hybrid back-crossed to *S. repens* but the F2 hybrid and the hybrid back-crossed to *S. caprea* were significantly more damaged than *S. caprea* (Figure 5).

TABLE 3. STATISTICS FOR INDICATOR VARIABLE REGRESSION OF LEAF BEETLE DAMAGE

	df	*F* value	*P*
Intercept, plant category	5	4.15	0.001
Slope, leaf area	1	15.02	<0.001
Residuals	186		
Model coefficients	Coefficient estimate	*t*	*P*
Slope, leaf area	0.67	3.88	<0.001
Intercept, *S. caprea*	−3.49	−2.72	0.01
Additional intercept, *S. repens*	0.9	2.29	0.02
Additional intercept, F1 hybrid	0.15	0.52	0.60
Additional intercept, F2 hybrid	1.07	3.32	<0.001
Additional intercept, F1 × *S. caprea*	0.88	3.46	<0.001
Additional intercept, F1 × *S. repens*	1.2	3.56	<0.0001

[a] See Table 2 for further explanations (Figure 6). The results are similar to Table 2 but there is also a difference between *S. caprea* and *S. repens* and the hybrid back-crossed to *S. repens*.

of total phenolic glucosides and the seven individual phenolic glucosides. (See Figure 1 for *F* and *P* values for each separate ANOVA). The distribution of these substances in the parents and the different hybrid categories were similar for all the different phenolic glucosides except for diglucose salicin and suggested additive inheritance of phenolics. The concentration of all phenolic glucosides, except diglucose salicin, were significantly higher in *S. repens* than in *S. caprea* and the F1 hybrids. The F2 (F1 × F1) hybrids were approximately intermediate between the two parental species. The hybrids back-crossed against *S. repens* were approximately intermediate between the F1 hybrid and *S. repens*. The hybrids back-crossed against *S. caprea* were not significantly different from *S. caprea*, but in several cases there was an indication of being intermediate between the F1 hybrid and *S. caprea*. For diglucose salicin, the concentration was lower in *S. repens* than in the other plant categories, while the hybrid back-crossed against *S. repens* was intermediate between the F1 hybrid and *S. repens*, but did not differ from either *S. caprea*, the F2 hybrid, or the hybrid back-crossed against the *S. caprea*.

Plant category also explained a significant amount of the variation in condensed tannins (ANOVA $F_{5,187} = 32.6$; $P < 0.001$; Figure 2). The pattern for condensed tannins was opposite to the pattern in most of the phenolic glucosides. *S. caprea* and the hybrid back-crossed against *S. caprea* had significantly higher concentrations of condensed tannins than either of the other plant categories. There was no difference between the F1 and F2 hybrids. *S. repens* and the hybrid back-crossed against *S. repens* had significantly lower concentrations of

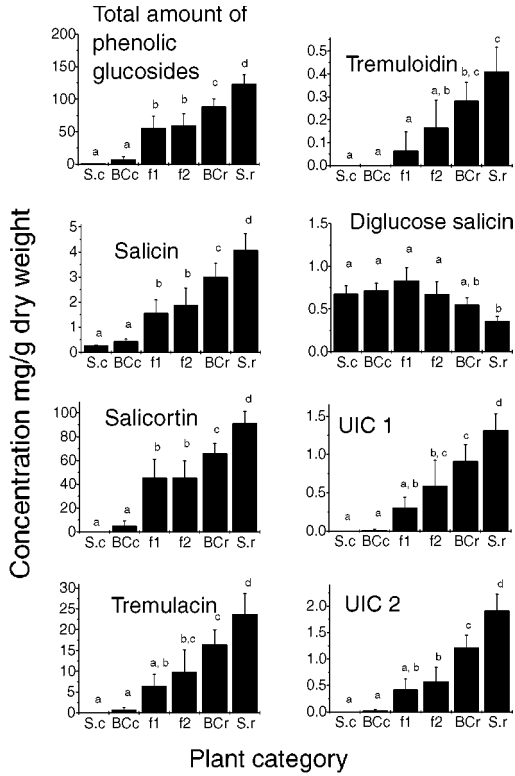

FIG. 1. Mean concentration of 7 different phenolic glucosides and the total amount of phenolic glucosides with 95% confidence limits for the 6 different plant categories. Plant category is shown in a short format *S. caprea* = S.c, hybrid back-crossed to *S. caprea* = BCc, F1 hybrid = f1, F2 hybrid = f2, hybrid back-crossed to *S. repens* = BCr and *S. repens* = S.r. Plant categories with no letter in common differ significantly ($P < 0.05$). Observe that each individual diagram has different scales of leaf concentration. ANOVA, for salicin $F_{5,187} = 40.5$, $P < 0.001$; ANOVA, for salicortin $F_{5,187} = 74.4$, $P < 0.001$; ANOVA, for tremulacin $F_{5,187} = 32.8$, $P < 0.001$; ANOVA, for tremuloidin $F_{5,187} = 20.5$, $P < 0.001$; ANOVA, for diglucose salicin $F_{5,187} = 10.5$, $P < 0.001$; ANOVA, for unidentified compound 1 (UIC 1) $F_{5,187} = 36.8$, $P < 0.001$; ANOVA, for UIC 2 $F_{5,187} = 52$, $P < 0.001$.

condensed tannins than the F1 and F2 hybrids, thus making the F1 and F2 hybrids intermediate between the two parental species and respective back-crosses.

There was a negative correlation between the concentration of condensed tannins and total concentration of phenolic glucosides for the four plant categories that had several individuals that contained phenolic glucosides (Figure 3). For

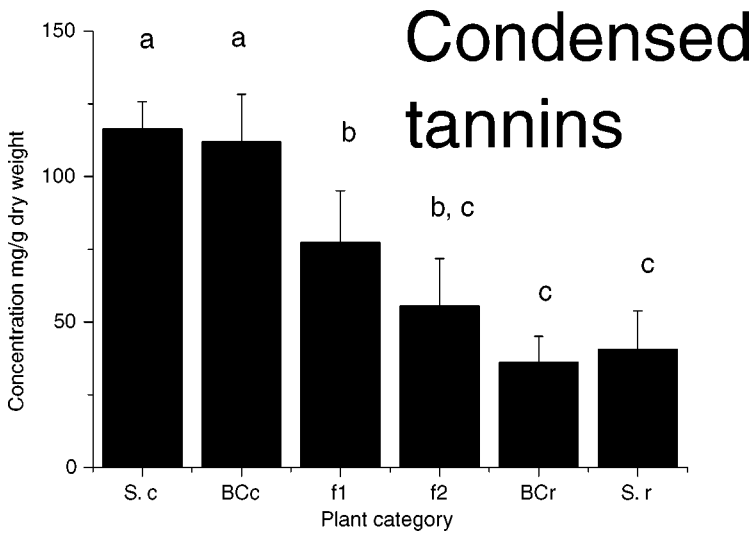

FIG. 2. Mean concentration of condensed tannins with 95% confidence limits for the six different plant categories. Symbols and abbreviations as in Figure 1.

S. caprea and the hybrid back-crossed to *S. caprea*, there was no correlation between the substances, but there were also very few individuals that contained any phenolic glucosides.

Morphologically, leaves from the different plant categories are different (Figure 4). The results from the two indicator variable regressions show that mean leaf area explained a significant amount of the variation in the mean leaf area consumed by leaf beetles and other herbivorous insects (Tables 2 and 3). We compared the results from indicator variable regression models with independent slopes and intercepts for each plant category with more simple models using the same slope but independent intercepts for plant categories (parallel linear models), but there was no improvement by using the more complex model (with independent slope and intercept for each plant category, ANOVA $F_5 = 1.83$, $P = 0.11$ for leaf damage from all herbivores and ANOVA $F_5 = 2.08$, $P = 0.07$ for leaf damage from leaf beetles). Thus, we base our results on a simple indicator variable regression model with the same slope, but independent intercepts for plant categories. There were no differences in leaf damage from all leaf-feeding insect herbivores between the two parental species and the F1 hybrids. However, the F2 hybrid and the hybrids back-crossed against *S. caprea* were more utilized than the parental species and the F1 hybrids. Hybrids back-crossed against *S. repens* were intermediate between the pure species and the F2 hybrid in damage (Figure 5). The pattern for leaf consumption by leaf beetles was similar to all leaf-eating insects

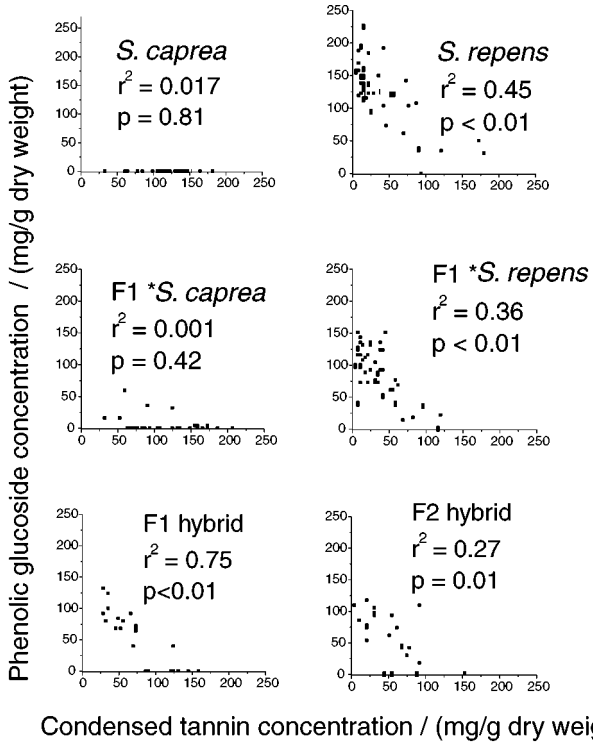

FIG. 3. Correlation between condensed tannins and phenolic glucosides. Condensed tannins plotted against phenolic glucosides for all plant categories. For each graph, the coefficient of determination (R^2) and the one-tail probability that R is not different from zero is shown.

except that *S. repens* was slightly more damaged than *S. caprea* and that the hybrids back-crossed against *S. repens* were damaged as much as the other back-crossed hybrid and the F2 hybrid (Figure 6).

DISCUSSION

Orians (2000) found that the most common pattern is that hybrids are intermediate compared to parental species with regard to concentration of secondary compounds. However, there are also hybrid studies that show other patterns (Rieseberg and Ellstrand, 1993; Orians, 2000). The concentration of specific substances in hybrids may also be similar to one parental species (Court et al., 1992), higher than any of the parental species (Spring and Schilling, 1989; Court et al., 1992; Häggström, 1997), or lower than any of the parental species. In addition, hybrids

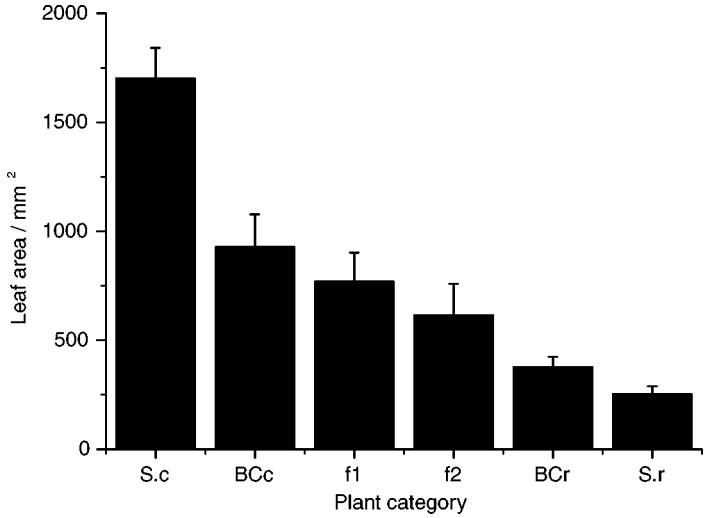

FIG. 4. Mean and 95% confidence limits of the plants, mean leaf area (mm^2) for the different plant categories. Symbols and abbreviations as in Figure 1.

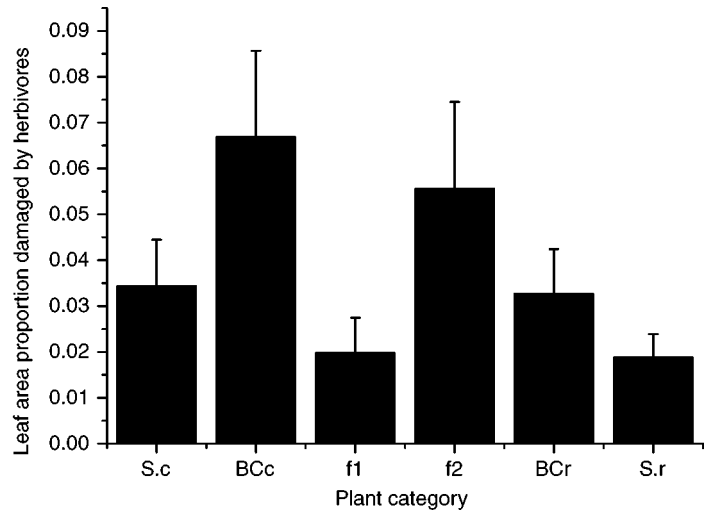

FIG. 5. Mean and 95% confidence interval (error bars) of the proportion of leaf area damaged by herbivores for each plant category. Symbols and abbreviations as in Figure 1.

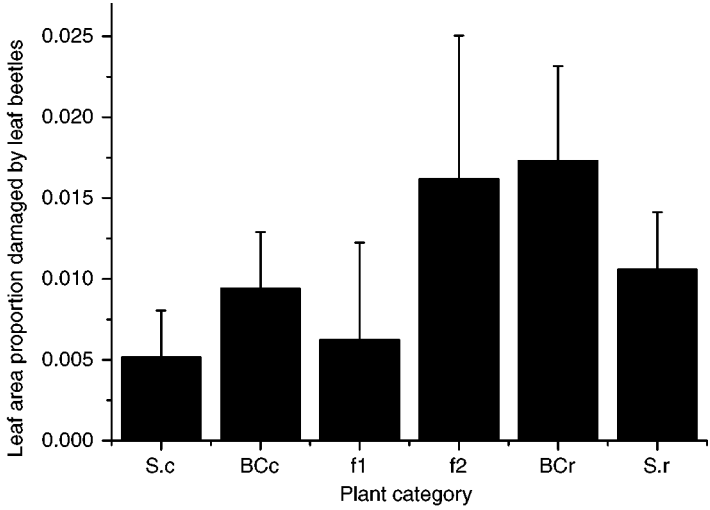

FIG. 6. Mean and 95% confidence interval (error bars) of the proportion of leaf area that was damaged by leaf beetles for each plant category. Symbols and abbreviations as in Figure 1.

may also lack chemicals that are produced by both parental species or express chemicals that are not produced in any of the parental species (Levy and Levin, 1974; Rieseberg and Ellstrand, 1993; Buschmann and Spring, 1995; for more references see Orians, 2000). However, only a few studies have measured inheritance patterns of secondary substances in woody plants.

Our results strongly suggest an additive inheritance of most phenolic compounds. One possible explanation for the only exception, diglucose salicin, is that diglucose salicin contains two glucose molecules, while the other substances only contain one. Thus, there may be a trade-off for the plant between producing a high concentration of total phenolic glucoside and producing diglucose salicin when the amount of available glucose is limited. However, the concentration of diglucose salicin was low compared to the other phenolic glucosides and could indicate its minor biological significance. The condensed tannins differ slightly from the additive pattern in that neither of the back-crosses differ from the respective parental species. This pattern could be explained if condensed tannin concentrations depend on epistatic genes, i.e., their concentration depends on genes that influence each others' phenotypic expression. This is a common pattern for resistance characters and other complex characters (R. S. Fritz, personal communication).

It has been suggested that both the quantitative and qualitative variation in secondary chemicals should be higher in later-generation hybrids than in F1 hybrids (reviewed by Orians, 2000). F2 recombinants and back-crosses could either contain a complete set of alleles responsible for production of secondary chemicals from

both parents or no such alleles at all. These would then produce high concentrations of the secondary chemicals found in both parents or none at all. However, our data on F2 hybrids and back-crosses show no such pattern. We found no individuals that produced high levels of both phenolic glucosides and condensed tannins, or low levels of both these groups. Instead, we found a negative correlation between concentrations of phenolic glucoside and condensed tannins in four plant categories, in which a large fraction of the individual plants produced phenolic glucosides in detectable amounts. This may indicate a trade-off in resource allocation. Both condensed tannins and phenolic glucosides are produced by the shikimate pathway, and they share a common precursor, cinnamate. Thus, production of condensed tannins or phenolic glucosides in high amounts may limit the production of another group of phenolics. It should be noted, however, that the relatively low number of crosses for some of the plant categories could potentially bias our result if nonrepresentative parent individuals have been included by chance.

The data on insect feeding damage does not fit well with any of the suggested patterns of herbivore response to hybrids with additive inheritance in resistance traits (see Fritz et al., 1998). The most interesting pattern is that the F2 hybrids were more utilized than the parental species and the F1 hybrids. That is consistent with theories that recombination in F2 could disrupt coadapted resistance mechanisms (Messina et al., 1996; Fritz, 1999). Similar observations have been made on mouse resistance against nematode parasites that show that while F1 hybrids are resistant against the pinworm *Aspicularis tetraptera*, the more advanced hybrids (F2's and back-crosses) show less resistance (Moulia, 1999). Our result suggests that in this hybrid system, herbivory may cause a decrease in the fitness of F2 and back-cross hybrids compared to parental species and F1 hybrids and, thus, herbivory may cause selection against hybrids.

A breakdown in hybrid resistance could occur by several mechanisms (Fritz, 1999). However, when resistance seems to be additively inherited, as in our case hybrid plants could express a particular character to such a low extent that it would not confer much resistance. This would then cause a break down in the resistance of hybrids. In that case, we should also find a reduced resistance in the F1 hybrids. Alternatively or additionally, herbivores may have responded to other resistant characters than the ones that we studied. Moreover, we have clumped all leaf-eating herbivores together, even though they probably do respond in different ways to the different plant characters. For example, earlier studies of the current system show that the leaf beetle *L. caprea* shows additive responses to hybrids whereas *C. fulvicornis* shows dominance toward the susceptible parent (Hjältén et al., 2002). Orians et al. (1997) have shown that leaf-eating insects with specific adaptations to phenolic glucosides select differently than leaf-eating insects that are not adapted to phenolic glucosides when selecting between parental species and hybrids, and between high and low phenolic glucoside willows. Both insect species that feed on willows with and without phenolic glucosides are present

in our system (Hallgren, personal observation). Overall, the susceptibility of the plant categories in our study reflects the total effect of several insect species that may differ in food and oviposition selection. From an evolutionary perspective, it may be even more important to study resistance to herbivory as one process rather than considering individual herbivore species. At the moment, few studies have discussed or measured the total effect of herbivory on hybrid plants (see Fritz, 1999).

To conclude, this study shows that both phenolic glucosides and condensed tannins were expressed in concentrations that support an additive inheritance between hybrids and parental species. Furthermore, the more complex hybrids, hybrid $\times$ hybrid crosses (F2), and back-crosses, were more utilized by phytophagous insects than parental species and F1 hybrids. This may indicate that in this case there is a fitness disadvantage of complex hybridization that may result in a hybridization barrier. Despite previous results showing that hybrids are not preferred by herbivores in foraging selection studies, the increased susceptibility of hybrids in this study may be explained because even though hybrids are not the most preferred plants by any single herbivore species, they may be rather susceptible to several different herbivores, and, thus, the total herbivore pressure on the plants may be greater than on parental species. It is also possible that more resistant characters have to be considered to understand the reasons for the breakdown in resistance.

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EFFECT OF GREEN MANURE ON THE INCIDENCE OF CYANOGENIC *Pseudomonas* STRAINS IN HOP GARDEN SOILS

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Abstract—Incidence of cyanogenic *Pseudomonas* strains in hop garden soils in relation to the kind of fertilization was studied. Incidence differed with respect to the fertilization treatment and the age of the plantation. Amendment of soil with rye and with white mustard as green manures limited the number of cyanogenic *Pseudomonas* strains relative to farmyard manures and NPK fertilization. Among all fertilization treatments, cyanogenic *Pseudomonas* spp. strains had lowest populations in soils amended with white mustard.

Key Words—Cyanogenic *Pseudomonas*, hop, green manure, rye, white mustard.

INTRODUCTION

One of the main problems in agriculture is that successive cropping (monoculture and short rotation) tends to increase in soil the incidence and longevity of pathogens and deleterious microorganisms such as cyanogenic *Pseudomonas*. Hydrocyanic acid produced from glycine and methionine, mainly by fluorescent pseudomonads, is a highly toxic compound both to prokaryotic and eukaryotic organisms (Dubey and Holmes, 1995). Numerous studies have focused on the harmful effects of cyanogenic *Pseudomonas* spp. on the growth and development of plants and microorganisms (Alström and Burns, 1989; Åström, 1991; Schippers et al., 1991; Piotrowska-Seget, 1995; Paszkowski et al., 1996; Piotrowska-Seget and

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Sroka, 1996; Kremer and Souissi, 2001; Owen and Zdor, 2001). Root-colonizing *Pseudomonas* spp. capable of producing HCN have been implicated in growth inhibition and reduction of potato yields when plants were grown in short rotation (Bakker and Schippers, 1987). In previous studies (Dwornikiewicz, 1994; Solarska, 1996), incidence of disease in plants and yield reductions of cones were recorded as a consequence of long-term (up to 20 years) conventional tillage of hops. Addition of organic matter to soil can increase the number of specific groups of microorganisms, e.g., pathogen antagonists, and decrease the population of pathogens and deleterious rhizosphere microorganisms (DRMO) (Sikora, 1992). Amendment of soil with green manure reduced the incidence of *Verticillium*-wilt of hops and increased the yields of cones (Szewczuk, 1995; Solarska et al., 1996; Dwornikiewicz et al., 2001). This paper reports results from field studies, the aim of which was to determine the effect of green manure on the incidence of cyanogenic *Pseudomonas* spp. strains in hop garden soils at different ages, 5- and 20-year-old plants.

METHODS AND MATERIALS

A 2-year study was carried out on young (5-year old) and old (20-year old) hop plantations at the Jastkow Experimental Station – IUNG, Pulawy, Poland. Hop (*Humulus lupulus* L. cv. "Lubelski") was grown in rows about 3 m apart on a brown soil developed from loess. Experiments were conducted on three plots of 1112 m^2 area. Soil samples for microbiological analysis were taken twice each year (3 weeks after green manures were plowed under) from the soil interrows of the following treatments: (1) NPK (control), (2) farmyard manure (3) white mustard cv. "Borowska" (*Sinapis alba* L. var. "Borowska"), (4) rye cv. "Dankowskie Zlote" (*Secale cereale* L. var. "Dankowskie Zlote"). Mineral fertilizers were applied every year at the rates (kg/ha): 140 N, 140 P, 180 K. Farmyard manure from cows was applied every 2 years (last application in autumn before the 1st year of study) at the rate of 30 tons wet weight per hectare. The white mustard and rye for green manure were sown in August and September and then incorporated into the soil in the 1st week of September or May, respectively.

Soil samples for microbiological analyses were taken (10-cm depth) from four treatments of the experimental field with a 2.5-cm-wide soil core twice each year in spring and autumn. Five cores per plot were randomly collected, sieved (2 mm), and composited into a single sample. Ten grams of soil were placed into an Erlenmeyer flask containing 90 ml 0.1 M sterile $MgSO_4$ solution and shaken for 15 min at 200 rpm on a rotary shaker. Soil was allowed to settle from solution for a period of 5 min. At this time, 10-fold serial dilutions of the suspensions made with 0.1 M $MgSO_4$ were spread on Sands–Rovira agar (Sands and Rovira, 1970). Plates were incubated at 28°C for 3 days, at which time colonies were counted. Numbers were expressed as cfu/g dry soil. After counting the total number of

fluorescent *Pseudomonas* spp., representative fluorescent colonies were isolated, purified, and stored at 4°C until identification to generic level using an API-20NE diagnostic kit (Bio Merieux). About 20 strains of *Pseudomonas* spp. selected at random from each treatment were tested for the ability to produce HCN in tubes. Production of HCN was tested by strains inoculated into a tube of semisolid King's B supplemented with glycine (4.4 g/l) and 10 μM $FeCl_3$. An indicator paper (prepared by dipping filter paper into saturated aqueous picric acid, drying, dipping into 10% aqueous sodium carbonate, and drying again) was placed between the tube and the plug. Production of HCN was indicated by the paper turning from yellow to light brown, brown, or reddish brown and recorded as weak, moderate, or strong cyanogenic potential, respectively, 48 hr after inoculation at 25°C (Paszkowski et al., 1996).

All data were subjected to analysis of variance, and means were compared by using Tukey's protected least significant differences (LSD) test at the 5% level of probability.

RESULTS AND DISCUSSION

Marked differences were found in the number of cyanogenic *Pseudomonas* spp. strains isolated from hop garden soils in both young and old plantations. As shown in Tables 1 and 2, fertilization treatment was the major factor that influenced the incidence of cyanogenic *Pseudomonas* spp. strains. Higher numbers were noted in soils receiving NPK and farmyard manures. Independent of the soil fertilization treatment, the age of the plantation also affected the population size of these bacteria, with the mean number of strains isolated from the old garden

TABLE 1. INCIDENCE OF CYANOGENIC *Pseudomonas* STRAINS IN COMMUNITIES OF FLUORESCENT *Pseudomonas* ISOLATED FROM YOUNG HOP GARDEN SOILS

	Number of cyanogenic strains[a]				
	1st year		2nd year		
Treatment	Spring	Autumn	Spring	Autumn	Mean number (%)
NPK	7	8	8	2	6.25 a (31.25)
Farmyard manure	4	3	2	3	3.00 ab (15.00)
Rye	4	3	2	2	2.75 b (13.75)
White mustard	3	1	1	1	1.50 b (7.50)
LSD (0.05)					3.45

Note: Means followed by the same letter in a column are not significantly different at 5% level.

[a] Based on evaluation of 20 *Pseudomonas* strains collected from each treatment during 2 years of the field experiments.

TABLE 2. INCIDENCE OF CYANOGENIC *Pseudomonas* STRAINS IN COMMUNITIES OF FLUORESCENT *Pseudomonas* ISOLATED FROM OLD HOP GARDEN SOILS

Treatment	Number of cyanogenic strains[a]				Mean number (%)
	1st year		2nd year		
	Spring	Autumn	Spring	Autumn	
NPK	4	9	9	6	7.0 a (35.00)
Farmyard manure	4	9	9	6	7.0 a (35.00)
Rye	3	5	4	4	4.0 ab (20.00)
White mustard	3	1	3	3	2.5 b (12.50)
LSD (0.05)					3.8

Note: Means followed by the same letter in a column are not significantly different at 5% level.

[a] Based on evaluation of 20 *Pseudomonas* strains collected from each treatment during 2 years of the field experiments.

soils higher than that from the young. Addition of farmyard manure to soil slightly decreased, compared with NPK treatment, the number of cyanogenic *Pseudomonas* spp. only in the young plantation. Amendment of soil with green manures reduced the incidence of cyanogenic *Pseudomonas* spp. in both trials. Incorporation of rye reduced strains by factors of 2.3 and 1.8 in young and old hop garden soil, respectively, compared to NPK treatment. Among all fertilization treatments examined, numbers of cyanogenic *Pseudomonas* spp. strains were lowest in soils amended with white mustard. The mean numbers of cyanogenic *Pseudomonas* spp. strains isolated from such soils were 1.5 and 2.5 for young and old plantations, respectively. The results from young hop garden soils summarized in Table 1 show that the influence of farmyard manure, rye, and mustard are not significantly different. Table 2 shows that rye does not significantly reduce *Pseudomonas* in comparison to farmyard manure in old hop garden soils.

Manipulation and modification of soil microbial community structure and species composition by soils amended with organic material is commonly known. Soil microorganisms are sensitive to environmental change, and significant changes in the microbial community occur following stress conditions, both in terms of total numbers and species composition (Turco et al., 1994). In young hop garden soils, no significant differences among treatments (farmyard manure, rye and mustard) occurred, suggesting that the same or similar plant organic stress factor(s) affected the bacterial species compositions. An increase in cyanogenic *Pseudomonas* spp. strains in young hop garden soils occurred only with addition of NPK. The lack of a significant difference between rye and mustard in the amount of cyanogenic *Pseudomonas* spp. strains in the old hop garden soils can be linked to the chemical composition of root exudates and rhizodeposits or to the level of compounds

released during decomposition of these green manures. Modification of microbial community structures as a consequence of modification of substrate availability in the rhizosphere have been previously reported (Grayston et al., 1998).

Presence of available substrates, e.g., glycine, methionine, iron, and phosphorus, in the rhizosphere and/or soil solution have been shown to affect the incidence and the degree to which cyanogenic pseudomonad strains produce hydrogen cyanide (Bakker and Schippers, 1987; Alström and Burns, 1989). Another factor is the species of plant cultivated (Grayston et al., 1998). Paszkowski and Wróblewska (2001) examined the incidence of cyanogenic strains from fluorescent pseudomonads colonizing the rhizosphere of barley, maize, oat, winter wheat, and rye at three successive plant growth stages and showed that the frequency of cyanogenic strains in the bacterial communities varied depending on the growth stage and plant species. The highest percentage (80%) of isolates capable of HCN production was found among strains originating from the rhizosphere of maize. However, only 9% of the strains isolated from the rhizosphere of rye were able to produce HCN. Kloepper et al. (1991) found similar results. As shown here, amendment of soil with rye green manure reduced the number of cyanogenic pseudomonads in soils. Competition for iron between phytosiderophores released in the rye rhizosphere and cyanogenic bacteria may be a possible mechanism. The roots of some *Poaceae* plants excrete into the soil iron-complexing compounds termed phytosiderophores (phytochelates). Roots of rye produce mugineic acid and their hydroxy-derived compounds, which have a binding constant for ferric ion of about 10^{18} (Marschner et al., 1989). This suggests that phytosiderophores of rye may inhibit, through deprivation of iron—an important element in the regulation of the synthesis of HCN by cyanogenic *Pseudomonas* spp.—the growth and incidence of these bacteria in soil.

Results presented here also show that cyanogenic pseudomonad strains occurred at the lowest numbers in soils amended with white mustard. It is difficult to explain the mode of action. White mustard and other species of the *Brassicaceae* produce glucosinolates that are antimicrobial and phytotoxic (Koide and Schreiner, 1992; Oleszek et al., 1996). Compounds derived from hydrolysis products of glucosinolates, the mustard oils (isothiocyanates) may reduce cyanogenic pseudomonads in soils. More information is needed, however, on how cyanogenic *Pseudomonas* interact with these substances, and further work is needed to determine how isothiocyanates inhibit cyanogenic bacteria.

Based on these results and on earlier findings (Dwornikiewicz, 1994; Szewczuk, 1995; Solarska, 1996; Solarska et al., 1996; Dwornikiewicz et al., 2001) we recommend the use of these green manures in practice for creating beneficial conditions in hop garden soils. This is not only an inexpensive and nonhazardous strategy closely connected with sustainable agriculture, but also seems to be the best and simplest method to reduce the use of high doses of mineral fertilizers and agrochemicals in hop plantations.

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SYSTEMIC EFFECTS OF *Heterobasidion annosum* ON FERULIC ACID GLUCOSIDE AND LIGNIN OF PRESYMPTOMATIC PONDEROSA PINE PHLOEM, AND POTENTIAL EFFECTS ON BARK-BEETLE-ASSOCIATED FUNGI

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Abstract—Concentrations of soluble phenolics and lignin in the phloem of ponderosa pines inoculated with the pathogen *Heterobasidion annosum* were assessed over a period of 2 years in a 35-year-old plantation in northern California, USA. The major effect of the pathogen on phloem-soluble phenolics consisted of a significant accumulation of ferulic acid glucoside: 503 ± 27 μg/g fresh weight (FW), compared with 366 ± 26 μg/g FW for mock-treated and 386 ± 27 μg/g FW for control trees. Lignin content was negatively correlated with ferulic acid glucoside concentration, and there was an indication of lignin reduction in the

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cell walls of inoculated trees. Lignin had a negative effect on the *in vitro* growth of two common bark beetle fungal associates, *Ceratocystiopsis brevicomi* and *Ophiostoma minus*. For this reason it, is hypothesized that lower lignification may facilitate the growth of beetle-associated fungi, resulting in greater susceptibility of the presymptomatic host to bark beetle colonization.

Key Words—Pine root rot, induction, systemic accumulation, phenolics, lignin depletion.

INTRODUCTION

A considerable body of evidence points to predisposition of root-diseased conifers to colonization by tree-killing bark beetles (Cobb et al., 1974; Goheen and Cobb, 1980; Livingston et al., 1983; Goheen and Hansen, 1993). This appears to be particularly important in the maintenance of endemic beetle population densities (summarized in Paine and Baker, 1993), presumably because the availability of disease-weakened trees may shorten the time required by colonizing beetles to find a suitable substrate and, thus, reduce the risks of losses due to predation or exposure (Wood, 1982). However, root disease centers are also often foci of bark beetle outbreaks, because once diseased trees are colonized, pheromone production is induced and amplification of bark beetle attack occurs on adjacent healthy trees (Wood, 1982). Death of these healthy trees apparently results from the overwhelming of tree defenses brought about by the dynamic combination of insect attack and infection by bark-beetle-associated fungi (Paine et al., 1997). Thus, understanding how diseased trees become susceptible to bark beetle attack will provide insight into mechanisms by which bark beetle outbreaks occur. In particular, an understanding of systemic physiological changes in the phloem of root-diseased-trees and potential effects of those changes on the fungi associated with bark beetles, is lacking (Paine et al., 1997; Wood, 1982).

Prelanding olfactory cues may or may not aid in the location of a host tree (Moeck et al., 1981; Nebeker et al., 1995; Brattli et al., 1998; Macias-Samano et al., 1998; Bonello et al., 2001b). In the postlanding environment, close range olfactory and/or gustatory cues (Elkington and Wood, 1980; Wood et al., 1986; Raffa et al., 1993), as well as constitutive and induced plant defenses against insects and associated fungi (Harrington, 1993), such as resinosis (summarized in Lorio, 1993) and lignification, are important in the host-selection process. In several reports, secondary metabolites, notably phenylpropanoid derivatives, terpenoids, and host cell wall lignin have been demonstrated to have significant effects on bark beetles infesting conifers (Wainhouse et al., 1990; Klepzig et al., 1996; Storer and Speight, 1996; McNee, 2000). Because an attack can occur away from the direct influence of the root pathogen, interaction between host tree and bark beetles may be affected by systemic chemical changes induced by the pathogen in the phloem of the host tree; similar systemic chemical changes may explain the induced

resistance phenomenon recently observed in Monterey and Austrian pine (Blodgett and Bonello, 2001; Bonello et al., 2001a).

Systemic effects of disease on plant metabolism are well known, particularly in the case of secondary metabolism associated with systemic acquired resistance (SAR) (Heil, 1999). Secondary metabolites in the phenolic and isoprenoid pathways have been studied in great detail in many plant species, including herbaceous and arboreal angiosperms, and gymnosperms (Strack, 1997). Many of these pathways and compounds can be profoundly affected by disease. A phenolic derivative, salicylic acid, has been implicated in signaling in the SAR phenomenon (Metraux, 2001).

There are many reports of secondary metabolite changes induced locally in conifers by microbial infection. For example, accumulation of soluble and cell-wall-bound phenolics has been shown in mycorrhizal roots of Douglas fir (Sylvia and Sinclair, 1983; Strobel and Sinclair, 1988), while in European larch the phenomenon is more variable, with accumulation (Weiss et al., 1997) or decreased concentration (Muenzenberger et al., 1995) of phenolics in mycorrhizal roots, depending on the particular host–mycobiont combination. Localized pathogenic induction of changes in secondary metabolism are also well known. Stilbenes, a class of phenylpropanoid derivatives (Strack, 1997) with antimicrobial properties (Hart, 1981; Kemp and Burden, 1986), are present constitutively in spruce secondary phloem (Woodward and Pearce, 1988) and in pine heartwood (Hart, 1981; Kemp and Burden, 1986), but are synthesized *de novo* in pine sapwood and juvenile tissues following microbial challenge (Prior, 1976; Bonello et al., 1993; Lieutier et al., 1996). Some of these phenomena may be related to localized (Christiansen et al., 1999; Krokene et al., 1999; Franceschi et al., 2000) or SAR (Bonello et al., 2001a) phenotypes. Studies specifically designed to induce systemic effects of disease on the secondary metabolism of large coniferous trees in controlled experiments are unknown, although in one case systemic effects of natural infection were demonstrated (Klepzig et al., 1995a).

In plants, localized lignification of host cell walls is another documented constitutive and inducible defense mechanism against pathogens (Vance et al., 1980; Aist, 1983; Ride and Barber, 1987). At the tissue level, lignification can occur as an early response in the periderm-restoration process in woody plants (Woodward, 1992). Little is known about the systemic effects of disease on lignification, with most information deriving from studies of cell wall appositions in SAR induced in crop plant species (Hammerschmidt and Kuc, 1982). No information is available on the systemic effects of disease on lignification in coniferous trees and, more generally, woody plants.

To contribute to a better understanding of these interactions, we tested the hypothesis that root disease of ponderosa pine (*Pinus ponderosa* Dougl. ex Laws), caused by *Heterobasidion annosum* (Fr.) Bref (an important root rot of conifers, see Woodward et al., 1998), induces systemic changes in the phenolic constituents

of the phloem, which may directly or indirectly affect bark beetle behavior. The relevance of this model system follows from the important role played by *H. annosum* as a predisposing factor to bark beetle outbreaks (summarized in Paine and Baker, 1993). In particular, we report results highlighting systemic effects induced by *H. annosum* on soluble low-molecular-weight phenolics; as well as on the lignin content of the phloem. Because enhanced lignification is a known defense response against fungi, we also investigated the effect of lignin on the *in vitro* growth rates of two bark beetle-associated fungi: *Ceratocystiopsis brevicomi* Hsiau et Harrington and *Ophiostoma minus* (Hedgcock) Sydow et Sydow.

METHODS AND MATERIALS

Field Site and Inoculation. Two adjacent stands of 35-year-old ponderosa pines (east and west plots) at the University of California's Blodgett Forest Research Station (BFRS) (El Dorado County, California, 38°55′ N, 120°39′ W) were selected in the spring of 1997. Diameter at breast height was determined for a random sample of 30 trees in each plot.

In each of the two plots (i.e., among a total of 60 trees), 10 trees were assigned at random to each of three treatments: inoculation with *H. annosum* (strain AWR-282: intersterility group P, isolated from ponderosa pine in the Modoc National Forest, California), mock inoculation, and unwounded control. Inoculation with the root pathogen was carried out by insertion of colonized wood dowels at four orthogonal positions around the base of the trunk by using a modification of a previously reported method (Garbelotto et al., 1997). Dead outer bark was pared away using a hatchet to produce a flat area, and inoculation holes 0.9 cm in diameter, 6–7 cm deep, were made with a cordless drill at approximately 5 cm above litter line. One wood dowel, either colonized by the pathogen or autoclaved, but not colonized (mock), was inserted into each hole. Autoclaved cork stoppers were inserted into each hole, and the infection court was coated with pruning seal (Ace Hardware, USA). Inoculations and mock inoculations were carried out on June 14–19, 1997.

Five inoculated trees from each plot ($N = 10$) were sampled on May 5, 1998, approximately 11 months after inoculation, to test for *H. annosum* activity. Cores from 3 cm above and 3 cm below each inoculation site were taken to the lab, where they were surface-sterilized by immersion in 1% sodium hypochlorite for 2 min. The cores were then incubated in moist chambers and determined to support active growth of *H. annosum* when the characteristic conidiophores were formed (Woodward et al., 1998).

A separate cohort of 40 trees in the same plantation, located at least 30 m away from any of our test trees, was similarly inoculated in October 1997, and felled at the inoculation level in October 1999 (McNee et al., 2003). Extent of fungal

invasion was estimated visually based on resin soaking and/or discoloration/drying of the host sapwood and phloem, and considered representative of the trees in the main experiment.

Sampling. Trees were sampled for analysis of phenolics five times over 2 years, on October 28, 1997, July 21 and 30 October, 1998, and July 12 and September 24, 1999. One core per tree, comprising outer bark, phloem, and 3–5 cm of sapwood, was taken with an increment borer at breast height on the first three dates. Phloem plugs were taken with a 2 cm diameter cork borer on the last two dates from the same relative positions. On each occasion, the distance around the tree circumference at breast height between new and previous samples was maximized to avoid the effects of localized host responses to the wounds. Additionally, in September 1999, phloem samples were taken from the base of the trees, next to one of the inoculation sites selected at random. The cores and plugs were immediately frozen in liquid nitrogen and taken to the laboratory for analysis of phenolics. In the laboratory, phloem was separated from the sapwood of each core and homogenized at liquid nitrogen temperature by using a Mikrodismembrator S (BBraun Biotech, Allentown, PA). Celite (Sigma, St. Louis, MO) was used as a carrier for phloem homogenization (Rosemann et al., 1991) when this came from cores, because of the low amounts of fresh material. Phloem from plugs was homogenized without celite.

Analysis of Methanol-Soluble Phenolics. The homogenized biomass ($\sim$50 mg (FW)) was extracted twice in 0.5 ml of HPLC grade methanol overnight at 4°C; the supernatants were combined, centrifuged, and the extracts analyzed directly by HPLC or stored at -20°C (or -80°C) until analysis. Compounds were separated on a Beckman Ultrasphere 5 μm, ODS C-18 reverse phase, 4.6 $\times$ 250 mm column, using the water/methanol-based gradient system described by Rosemann et al. (1991) at a flow rate of 1 ml/min.

Analysis of Cell Wall-Bound Phenolics. Pellets left over from the analysis of the October 1998, and July and September 1999 soluble phenolics were processed and extracted for determination of cell wall-bound phenolics by using either a modification of the method of Strack et al. (1988), as described in Bonello et al. (1993), or the following. Briefly, the pellets were washed three times with methanol and once with water, 100 μl of 1 N NaOH containing 100 mM ascorbic acid and 0.2% sodium borohydride ($NaBH_4$) (to limit oxidation of the phenolic metabolites) were added, and the samples were hydrolyzed overnight on a shaker. Formic acid (1.5 m; 100 μl) was added to the mixture to stop the reaction, followed by 200 μl of methanol. The mixture was centrifuged for 10 min at 11,000 rpm, and the supernatant was analyzed by HPLC, or stored at -80°C until analysis.

Analysis of Lignin-Like Material. Phloem collected from the stem in October 1998 and July and September 1999, and from the tree base next to inoculation sites in September 1999 was processed and extracted for quantitative determination of lignin-like material complexed to the cell wall by using a modification

of the method of Bruce and West (1989), as described by Bonello et al. (1993). This method involves derivatization of lignin polymers with thioglycolic acid (Sigma–Aldrich, St. Louis, MO) and solubilization with alkali. Lignin concentration was determined spectrophotometrically against a standard of pure spruce lignin (Sigma–Aldrich) using a Shimadzu UV-260 spectrophotometer.

Effects of Lignin on in Vitro Growth Rates of C. brevicomi and O. minus. *C. brevicomi* and *O. minus* used in this study were isolated from western pine beetles, *Dendroctonus brevicomis* LeConte, collected from ponderosa pines at BFRS by Tom Harrington (Iowa State University), who donated them to us. Cultures were maintained on 2% malt extract agar (MEA) at room temperature.

Pure spruce lignin (Sigma–Aldrich) was incorporated into 2% MEA at the following rates: 0, 2, 4, 8, 16, and 20 mg/ml. This range includes the *in vivo* lignin concentrations described in the results, if one assumes correspondence between 1 ml of culture medium and 1 g FW phloem. The two fungal species were tested on the lignin-amended medium by placing 5-mm mycelial plugs (mycelium side down), taken from the margins of actively growing cultures, at the edge of 60-mm diameter Petri dishes. The cultures were maintained at room temperature in the dark for the duration of the experiment. Cumulative radial growth was measured at 6, 12, and 21 days for *C. brevicomi* (a slow growing species), and at 6 and 9 days for *O. minus* (a fast growing species).

Identification and Synthesis of Ferulic Acid Glucoside. Ferulic acid glucoside was identified by diode array spectroscopy before and after co-chromatography with prepared reference material. The compound eluted at 20.93 min and showed two maxima at 290 and 313 nm.

Ferulic acid glucoside was prepared by using a slight modification of the methods of Pearl and Beyer (1951) and Schuster et al. (1986). A solution of 157 mg vanillin glucoside (0.5 mmol; Apin, UK) and 115 mg malonic acid (1.1 mmol) in 1 ml dry pyridine and 20 μl piperidine (Fluka, Germany) was kept at 45°C for 8 days, when only one product, and no vanillin glucoside, was detected by HPLC (Turunen et al., 1999). The solvents were removed in a rotary evaporator at 40°C and the pale yellow residue was partly dissolved by vigorously vortexing in 10 ml dry ethanol–acetic acid (95:5, v/v) for 1 min. It was not possible to dissolve 100% of the residue. The dissolved product was then precipitated from the supernatant by dropwise addition of 10 ml dry diethyl ether–acetic acid, (95:5, v/v). The supernatant was carefully removed and the residue dried in a stream of nitrogen. The washing procedure was repeated twice with abs. ethanol and diethyl ether. The dry powder (150 mg, 0.42 mmol, yield 84%) was identified as ferulic acid glucoside by ^{1}H NMR spectroscopy (DMSO-d_6, 500 MHz, DMX-500, Bruker, Germany).

Data Analysis. Data were analyzed using SPSS 10.0 for Windows. Differences in mean diameter of trees between the two plots (east and west) were tested using analysis of variance (ANOVA) (Sokal and Rohlf, 1981). Differences in the

mean amounts of metabolites in the phloem samples were tested using ANOVA, following appropriate data transformation (Sokal and Rohlf, 1981). Where correlation coefficients between data for each compound extracted from phloem collected on different dates were significant, repeated measurements ANOVA was used (SPSS 10.0 for Windows). For each compound extracted from phloem samples, orthogonal contrasts (Sokal and Rohlf, 1981) compared the inoculated treatment to the mock-inoculated and control treatments combined, followed by a comparison between the mock-inoculated and the control trees. Relationships between the compounds found in the phloem were tested by calculating correlation coefficients (Sokal and Rohlf, 1981).

The relationships between the radial growth of *C. brevicomi* and *O. minus* and the concentration of lignin in MEA were tested by using linear and exponential regressions.

RESULTS

Tree Size and Pathogen Activity. When the experiment was set up in 1997, the difference in the mean diameters of trees in the east plot (29.2 ± 1.1 cm) and in the west plot (34.3 ± 1.3 cm) was significant ($F = 9.08$; df: 1, 59; $P = 0.004$). Active mycelium of the pathogen was recovered from at least one inoculation site in three trees from the west plot and four from the east plot (from five sampled in each plot). Thus, $70 \pm 14.5\%$ (SE) of tested inoculated trees were actively infected 11 months after inoculation. Visual assessment of the infection sites on the stumps of the separate trees felled in October of 1999, showed that limited development of the infection occurred over the 2 years of the study, with sapwood discoloration evident only just around the inserted inoculation dowels (Figure 1).

FIG. 1. Extent of colonization of ponderosa pine stems by *Heterobasidion annosum* 2 years after artificial inoculation. Note the limited sapwood area occupied by discolored/resin-soaked tissue (arrows).

Oct. '97	r = 0.432 P = 0.001 N = 60	r = 0.661 P < 0.001 N = 55	r = 0.742 P < 0.001 N = 59	r = 0.508 P < 0.001 N = 59
	July '98	r = 0.312 P = 0.020 N = 55	r = 0.508 P < 0.001 N = 59	r = 0.423 P = 0.002 N = 53
		Oct. '98	r = 0.548 P < 0.001 N = 54	r = 0.471 P = 0.001 N = 49
			July '99	r = 0.588 P < 0.001 N = 53
				Sept. '99

FIG. 2. Bivariate correlations between ferulic acid glucoside concentrations in the phloem of ponderosa pines at breast height at different combinations of dates. All correlations between dates were significant and positive ($P \leq 0.028$).

Phloem Samples from the Stem at Breast Height. No qualitative changes were detected throughout the study. However, the concentration of the constitutive compound ferulic acid glucoside was affected by treatment. Bivariate Pearson correlations between ferulic acid glucoside concentrations in all trees at different dates were all positive and significant (Figure 2, $P \leq 0.028$), indicating that trees producing higher amounts of this compound did so consistently over time. Thus, repeated measurements ANOVA was performed. The difference in ferulic acid glucoside concentration between trees in the east and west plots was not significant, and there were no significant interaction between plot and treatment. However, differences in the mean concentrations of ferulic acid glucoside among treatments were not significant. Ferulic acid level in all inoculated trees pooled over plots was 503 ± 27 μg/g FW, compared with 366 ± 26 μg/g FW for mock-treated and 386 ± 27 μg/g FW for control trees ($F = 3.68$; df : 2, 43; $P = 0.033$. *Contrasts*: inoculated vs. (mocks + controls): $P = 0.010$; mocks vs. control: $P = 0.784$). Other constitutive compounds, e.g., taxifolin-3′-glucoside and an unidentified

cinnamic acid derivative, also showed some changes, but not consistently and not in the same magnitude, and were not considered further in the analysis. No significant qualitative or quantitative differences in cell wall-bound phenolics were observed among treatments throughout the study. Both of the methods used worked well and gave comparable results.

Correlations between lignin contents at different dates were not significant, so the data were analyzed at individual dates. Differences among treatments were not statistically significant, either in one-way or two-way ANOVAs, except for the east plot in October 1998, when the phloem of inoculated trees contained less lignin than the other treatments: inoculated, 3.87 ± 1.59 mg/g FW; mock-inoculated, 18.93 ± 5.58 mg/g FW; controls, 7.0 ± 3.09 mg/g FW ($F = 4.25$; df: 2, 25; $P = 0.027$; inoculated vs. (mocks + controls): $P = 0.041$; mocks vs. control: $P = 0.065$). The mean concentrations of lignin from all trees pooled over plots and all three dates were inoculated, 7.92 ± 1.29 mg/g FW; mock-inoculated, 9.66 ± 1.39 mg/g FW; controls, 10.49 ± 2.37 mg/g FW (differences not significant).

There was an apparent negative association between lignin content and ferulic acid glucoside concentration up the stem, so a partial correlation controlling for plot and treatment was carried out between these two variables averaged for each tree over the last three dates. The correlation between lignin and ferulic acid glucoside was negative and significant ($r = -0.30$, $P = 0.021$, $N = 56$).

Phloem Samples from Base of Trees (September 1999). Differences between treatments in ferulic acid glucoside at the base were not significant. The mean concentrations of ferulic acid glucoside from all trees pooled over plots were inoculated, 372 ± 55 μg/g FW; mock-inoculated, 253 ± 49 μg/g FW; controls, 303 ± 58 μg/g FW. Similarly, effects of treatment on lignin content at the base were not significant.

Effects of Lignin on in Vitro Growth Rates of C. brevicomi and O. minus. Soluble lignin had a significant negative effect on the growth rates of the two bark beetle-associated fungi (Figure 3, shows growth rates at 6 days). Assuming that 1 ml of lignin-amended MEA is equivalent to 1 g phloem FW, and based on the regression functions, it was found that a reduction in lignin concentration from 10.07 to 7.92 mg/g FW [corresponding to the observed decrease in the phloem of inoculated trees compared with that in control trees (mean of mock-inoculated and control trees)], results in 12% and 25.9% increases (at 6 days), in *in vitro* growth rates of *C. brevicomi* and *O. minus*, respectively. Similar growth rate changes were also observed for the other test dates (data not shown).

DISCUSSION

The activity of a pathogen can result in increased susceptibility to other pathogenic and nonpathogenic microbes, presumably because of weakened host

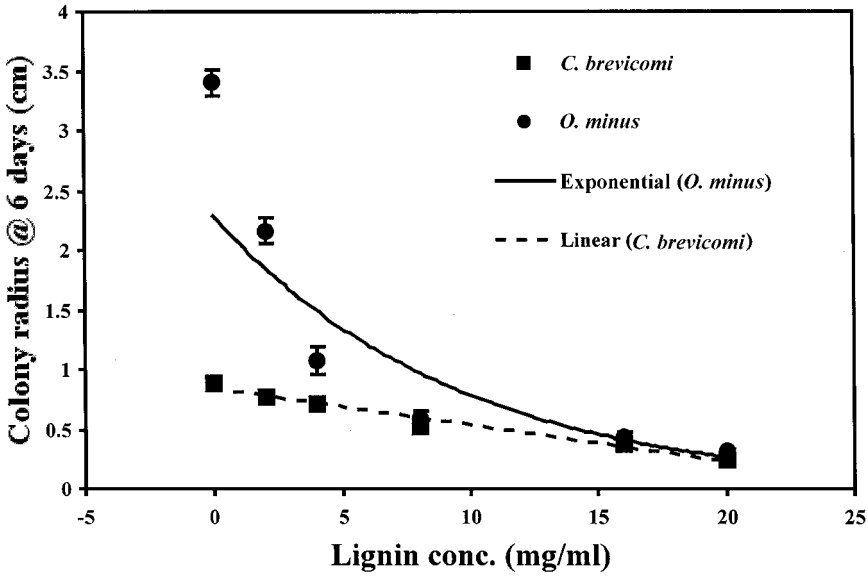

FIG. 3. Negative effect of lignin on *in vitro* growth rates of *Ceratocystiopsis brevicomi* and *Ophiostoma minus* at 6 days. Bars are SE and are not visible when smaller than the symbol. Linear and exponential regression equations fitting growth rates at 6 days for *C. brevicomi* and *O. minus* were $y = -0.03x + 0.84 (R^2 = 0.97, P < 0.001)$ and $y = 2.29\ e^{-0.107x} (R^2 = 0.87, P < 0.01)$, respectively (where "$x$" is lignin concentration and "y" is colony radius).

defenses. For example, establishment of the root pathogen *Armillaria* spp. on various conifers often follows attacks by other root, foliar, or stem pathogens (Wargo and Harrington, 1991). Successful attacks by most bark beetle species appear mediated, in part, by certain fungi that these insects vector (Whitney, 1982), such as species in the ophiostomatoid ascomycete genera *Ceratocystis*, *Ceratocystiopsis*, and *Ophiostoma*, and at least one species from the genus *Leptographium* (i.e., *L. terebrantis* Barras & Perris) (Owen et al., 1987; Klepzig et al., 1995b). Some of these fungi are capable of killing their hosts independently of their vectors (Owen et al., 1987; Parmeter et al., 1989; Harrington, 1993; Krokene and Solheim, 1998), while others are mycangial fungi that appear to confer a direct fitness advantage to the beetles without being injurious to the host tree (Barras, 1973; Bridges, 1983; Six and Paine, 1998). Consequently, one mechanism by which root disease affects bark beetle populations may be through increased susceptibility of the host to invasion by bark beetle-vectored fungal symbionts and plant pathogens.

Despite this evidence, the physiological and biochemical mechanisms underlying these associations in coniferous hosts are imperfectly understood, particularly

with respect to systemic effects. We now have evidence, for the first time in a conifer, of sustained systemic induction over a period of 2 years of ferulic acid glucoside accumulation in the phloem of presymptomatic trees inoculated with the root pathogen *H. annosum*. The concentrations of ferulic acid glucoside proved to be elevated despite the fact that inoculated trees appeared to have effectively contained the pathogen (Figure 1).

Systemic ferulic acid glucoside accumulation in the phloem was mirrored by an overall average decrease in lignin content in the same trees. Lignin content of inoculated trees pooled over all dates was 21.4% lower than in the mock-inoculated and control trees combined. Correlative analysis showed an inverse relationship between ferulic acid glucoside and lignin concentrations. The mean reduction in stem lignin content was not significant, in part due to high variability within and between dates and treatments. However, this result, when coupled with the negative correlations between the concentrations of ferulic acid glucoside and lignin, may be biologically meaningful because ferulic acid glucoside is an indirect precursor in lignin biosynthesis (Strack, 1997). Glucosides are thought to be storable and transportable forms of otherwise reactive molecules, and ferulic acid glucoside is such an example. Following a hydrolytic reaction, the aglycone, ferulic acid, is available for further processing in the lignin biosynthetic pathway, which proceeds through enzymatic transformation of ferulic acid to coniferyl alcohol and eventual incorporation into the lignin polymer via condensation reactions with other precursors (Strack, 1997). Ferulic acid also functions as a major bridge in anchoring the lignin polymers to the carbohydrate constituents of the cell walls (Strack, 1997). We hypothesize that accumulation of ferulic acid glucoside in the phloem of inoculated trees indicates that this lignin precursor is diverted from lignin synthesis in noninfected areas, i.e., it is stored and transported to the infection courts to contribute to the localized, pathogen-induced lignification response. This hypothesis aims to explain the apparent contradiction between accumulation of this compound and concurrent reduction of lignin content in the same tissues, but would require tracer studies to be confirmed. Interestingly, systemic stresses like air pollution (e.g., ozone exposure) have also been shown to bring about a reduction in lignification rates and a concurrent increase in susceptibility to disease and bark beetle attacks in conifers (Stark et al., 1968; Bonello et al., 1993; Pearce, 1996), perhaps pointing to a generalized mechanism of stress-induced weakening of tree defenses.

Pathogen-induced systemic accumulation of ferulic acid glucoside and concurrent reduction in lignification may directly affect the success of bark beetle attacks on diseased trees. Preliminary evidence suggests that *Ips paraconfusus*, an important tree-killing bark beetle of pines, is not affected in its laboratory feeding behavior when fed a wide range of ferulic acid glucoside concentrations in an artificial diet (McNee, 2000). However, similar information on the effects of reduced lignification on *I. paraconfusus* feeding behavior is not presently available.

In addition to a possible effect of decreased lignification on adult beetle feeding, other indirect mechanisms may also render infected trees more susceptible to successful bark beetle establishment. Lignification of cell walls is one of the main disease resistance mechanisms in plants (Vance et al., 1980; Pearce, 1996b). However, the effect of systemic reduction in lignin content of inoculated ponderosa pine phloem on bark beetle—associated fungi remains untested. Our *in vitro* growth studies suggest that such reduction may contribute to faster growth of these fungi in root- and butt-infected trees (particularly *O. minus*, whose growth rate was 26% higher at the lower end of mean lignin concentration observed in the inoculated trees). However, this result has to be interpreted cautiously, because (1) the difference in lignification rates was not significant and (2) it does not provide information about the localized induced lignification response that would result from inoculation of pine stems with the ophiostomatoid fungi in the presence of root and butt infections with *H. annosum*. Indeed, it is possible that infection of pine phloem in the presence of elevated precursor concentrations observed in root- and butt-infected trees could result in higher lignification rates in response to infection by fungi such as *C. brevicomi* and *O. minus*. Furthermore, our tests with solubilized lignin may not accurately reflect the role of cell wall-bound lignin. Thus, the effect of root and butt rot fungi on the success of bark beetle colonization via a reduction in the host lignin deposition response remains to be clarified.

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FEEDING RESPONSE OF *Ips paraconfusus* TO PHLOEM AND PHLOEM METABOLITES OF *Heterobasidion annosum*–INOCULATED PONDEROSA PINE, *Pinus ponderosa*

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Abstract—In studies of feeding by the bark beetle, *Ips paraconfusus*, two pine stilbenes (pinosylvin and pinosylvin methyl ether), ferulic acid glucoside, and enantiomers of the four most common sugars present in ponderosa pine phloem (sucrose, glucose, fructose, and raffinose) did not stimulate or reduce male feeding when assayed on wet α-cellulose with or without stimulatory phloem extractives present. When allowed to feed on wet α-cellulose containing sequential extracts (hexane, methanol, and water) of ponderosa pine phloem, methanol and water extractives stimulated feeding, but hexane extractives did not. Males confined in wet α-cellulose containing aqueous or organic extracts of culture broths derived from phloem tissue and containing the root pathogen, *Heterobasidion annosum*, ingested less substrate than beetles confined to control preparations. In an assay using logs from uninoculated ponderosa pines, the mean lengths of phloem in the digestive tracts increased as time spent feeding increased. Males confined to the phloem of basal logs cut from ponderosa pines artificially

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inoculated with *H. annosum* ingested significantly less phloem than beetles in logs cut from trees that were (combined) mock-inoculated or uninoculated and did not contain the pathogen. However, individual pathogen-containing treatments were not significantly different from uninoculated controls. It was concluded that altered feeding rates are not a major factor which may explain why diseased ponderosa pines are colonized by *I. paraconfusus*.

Key Words—*Ips paraconfusus, Heterobasidion annosum, Pinus ponderosa*, bark beetle, feeding, extractives, stilbene, pinosylvin, pinosylvin methyl ether, ferulic acid glucoside, phloem broth.

INTRODUCTION

The California five-spined ips, *Ips paraconfusus* Lanier (Coleoptera: Scolytidae), is a major bark beetle pest of second-growth pines in California and southern Oregon (Struble and Hall, 1955; Bright and Stark, 1973). It attacks and kills saplings and the tops of mature trees, and also breeds in logging debris and fallen trees. Its principal host is ponderosa pine, *Pinus ponderosa* Dougl. ex Laws. but it colonizes any pine species within its range (Struble and Hall, 1955; Bright and Stark, 1973). Bark beetles must discriminate between host and nonhost species in their geographic range (Wood, 1982), and previous research has shown that male *I. paraconfusus* recognize ponderosa pine after entering the phloem (Elkinton and Wood, 1980). Elkinton et al. (1981) demonstrated that there are extractable compounds in ponderosa pine phloem that elicit feeding behavior by *I. paraconfusus*. Other researchers have described a stimulation of feeding behaviors in the southern pine beetle, *Dendroctonus frontalis* Zimmermann, and the mountain pine beetle, *D. ponderosae* Hopkins, by outer bark extracts of their host pine species (Thomas et al., 1981; Raffa and Berryman, 1982).

Feeding stimulants or extracts stimulating feeding behaviors have been identified for a number of species from other beetle families, including Chrysomelidae (e.g., Matsuda and Matsumoto, 1975; Lin et al., 1998), and Curculionidae (e.g., Maxwell et al., 1963). Among the Scolytidae, feeding stimulants have been identified for the European elm bark beetle, *Scolytus multistriatus* (Marsham) (Doskotch et al., 1970), and the fruit tree bark beetle, *S. mediterraneus* (Egger) (Levy et al., 1974). Several simple sugars present in the phloem of American elm, *Ulmus americana* L, did not stimulate feeding by *S. multistriatus* (Norris and Baker, 1967). The feeding of larval and adult bark beetles has been reported to be greatly enhanced by the addition of ground phloem to artificial diets, suggesting that unidentified phloem metabolites are primarily responsible for feeding stimulation in the species tested (Bedard, 1966; Byers, 1995).

At low population densities, aggressive bark beetles such as *I. paraconfusus* colonize diseased trees more frequently than healthy trees (e.g., Cobb et al., 1974;

Hertert et al., 1975; Ferrell and Smith, 1976; Goheen and Cobb, 1980; Klepzig et al., 1991). Moeck et al. (1981) reported no evidence of long range nonpheromone attraction of the western pine beetle, *D. brevicomis* Leconte, and *D. ponderosae*, to ponderosa pines naturally infected with *Leptographium wageneri* (Kendrick) Wingfield, the pathogen that causes blackstain root disease. Thus, it was hypothesized that diseased trees may produce specific compounds that facilitate this selective colonization behavior following beetle landing on the tree.

Localized, defense-associated physiological changes such as the accumulation of resins, resin acids, stilbenes, and ferulic acid glucoside occur in pines following infection by a root pathogen such as *Heterobasidion annosum* (Fries) Brefeld (e.g., Jorgensen, 1961; Gibbs, 1968; Rishbeth, 1972; Prior, 1976; Hart, 1981; Bonello and Pearce, 1993; Bonello et al., 2003). Resins are known to be antagonistic to bark beetles and microorganisms (e.g., Smith, 1961; Vité, 1961; Russell and Berryman, 1976; Wong and Berryman, 1977). Two pine stilbenes, pinosylvin and pinosylvin methyl ether, have antifungal (e.g., Erdtman and Rennerfelt, 1944; Rennerfelt and Nacht, 1955; Gottstein and Gross, 1992; Schultz et al., 1992), antinematode (Suga et al., 1993), and antiherbivore properties (e.g., Bryant et al., 1983; Clausen et al., 1986; Sullivan et al., 1992; Russell et al., 2000). Ferulic acid glucoside was the first host compound observed to systemically accumulate in the phloem following inoculation of ponderosa pines with *H. annosum* in a field study (Bonello et al., 2003). Ferulic acid has been implicated in plant resistance to insects and fungal pathogens (e.g., Bergvinson et al., 1994, 1997; Classen et al., 1990; Cabrera et al., 1995; Dowd and Vega, 1996), and it was hypothesized that ferulic acid glucoside may play a role in the host-mediated interactions between bark beetles and root pathogens.

The aim of this study was to determine whether the alteration of phloem metabolite concentrations following root pathogen infection results in increased bark beetle feeding rates that may partially account for the differential colonization of root-diseased trees (e.g., Person, 1931; Cobb et al., 1974; Goheen and Cobb, 1980; Moeck et al., 1981; Bonello et al., 2003). The study was conducted using *I. paraconfusus* because of prior feeding studies using this species (Elkinton and Wood, 1980; Elkinton et al., 1980, 1981) and because it is a less destructive bark beetle than *D. brevicomis* (e.g., Bright and Stark, 1973; Wood, 1982). It was believed that altered feeding rates would be more easily detected with a less aggressive species (e.g., Wood, 1972, 1982; Borden, 1974). In order to test the hypothesis of differential feeding behaviors, studies were conducted between 1997 and 1999 to characterize feeding behavior in ponderosa pine in response to (1) intact phloem and phloem extracts; (2) the most common simple sugars in the phloem; (3) pathogen-induced metabolites present in the phloem of *H. annosum*-inoculated trees; (4) extracts of an aqueous culture of *H. annosum*; and (5) the intact phloem of *H. annosum*-inoculated and-uninoculated trees.

METHODS AND MATERIALS

Collection of Beetles. *Ips paraconfusus* were collected from ponderosa pine logging debris or from aggregation pheromone-baited trees at the University of California Blodgett Forest Research Station (BFRS), El Dorado County (38° 55′ N, 120° 39′W), California, and reared at the University of California, Berkeley. Emerged beetles were incubated at 5°C on moist filter paper for up to 10 days prior to use in experiments, and all beetles had intact legs and antennae. Following sex determination (Wood, 1982), male beetles were randomly assigned to treatments and controls in all experiments.

Feeding in Intact Phloem. An 8-year-old, 12-cm-diam. ponderosa pine with 3–4-mm-thick phloem at breast height was felled at BFRS in July 1999, and a basal section of the main stem with little taper was cut into five logs approximately 30 cm long. The ends of each log were sealed with paraffin wax to reduce desiccation, and each log was randomly assigned a feeding period of 2, 4, 8, 24, or 48 hr. Three-mm-diam. holes were drilled through the outer bark to the xylem–phloem interface at an approximate 45° angle to the longitudinal axis of each log, and 30 male and 30 female *I. paraconfusus* were individually inserted into alternating longitudinal rows of 4–5 holes around each log. Holes were spaced approximately 2.5 cm apart, and rows were 8–10 cm apart. Beetles were confined by wire mesh stapled to the outer bark of the logs, which were vertically oriented and maintained at 22–24°C under constant light. At the end of each feeding period, all beetles in the corresponding log were removed and killed by freezing. Beetles were dissected, and the lengths of phloem in their digestive tracts were measured using a calibrated ocular micrometer under 30× magnification. The hindguts of many beetles contained small amounts of older food material that was different in color and texture, and this was excluded from any analyses.

Preparation of Extracts. A healthy, 30-cm-diameter ponderosa pine was felled at BFRS in August 1998, and the basal 1-m section was refrigerated at 5°C for 24 hr prior to phloem collection. Phloem was removed from the log, immediately frozen in liquid nitrogen, and then stored at −50°C until needed. The phloem was freeze-dried for 48 hr, ground into particles less than 1 mm^3 in size, and sequentially extracted for 48 hr each in hexane, methanol, and water, at a ratio of 10 ml/g dry weight. Extracts were filtered using a 0.45-μm filter (Micron Separations, Inc., Westboro, MA) and then concentrated up to 10-fold using a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) or speedvac (Savant Instruments, Holbrook, NY). The methanol extract was used as a positive control and to elicit feeding in subsequent experiments at 1× nominal concentration unless otherwise noted. At this concentration, the extractives removed from 1 g wet phloem were added to 1 g wet substrate (α-cellulose). The methanol extract was used as a positive control owing to methanol's ability to extract a wider polarity range of metabolites than water.

Assay Methodology. Powdered α-cellulose (Sigma-Aldrich, St. Louis, MO) containing 5% orange powdered dye by weight (Day-Glo Color Corp., Cleveland, OH) was used as an artificial feeding substrate. Hexane and methanol extracts were applied in concentrated form so that approximately 1 ml of solvent was applied to each gram of dry α-cellulose. Substrates and extracts were mixed with a mortar and pestle and then spread on petri dishes in a fume hood to allow for solvent evaporation. Hexane was allowed 30 min to evaporate and methanol was evaporated overnight. α-Cellulose samples containing hexane or methanol extractives were mixed with distilled water at room temperature to a water content similar to that of the phloem from which the extracts were taken (66% by weight), and were packed into 5-cm lengths of plastic drinking straws (Wood et al., 1986) at a density of approximately 0.25 g/cm^3. Concentrated water extracts were warmed to room temperature and mixed with α-cellulose prior to use in assays. For controls, pure solvents were mixed with α-cellulose and prepared in the same manner as samples containing phloem extracts. pH paper (Micro Essential Laboratory, Brooklyn, NY) was used to confirm that assays were conducted at a pH of approximately 4.5, the pH of ponderosa pine phloem measured in several trees and also reported by Elkinton et al. (1981).

Beetles were introduced into preformed tunnels extending 2–3 mm into the substrate, as in Wood et al. (1986). Straws were 3–4 cm longer than the substrate to prevent beetles from escaping, and were vertically oriented in microcentrifuge tube holders. After a feeding period of 4 h, beetles were removed from the straws, killed by freezing, and dissected to measure the length of α-cellulose in their digestive tracts. All experiments were conducted at 21–23°C under constant light between August 1998 and December 1999.

Feeding in α-Cellulose Containing Combined Phloem Extractives. In order to determine the baseline feeding response in α-cellulose containing phloem extractives, all extracts were applied to one sample of α-cellulose at 1$\times$ nominal conc. in the order of methanol, hexane, and water using the methods described above. For each time period (2, 4, 8, 24, or 48 hr), 30 male *I. paraconfusus* were inserted into drinking straws containing either treated or blank α-cellulose, and were assayed using the standard methodology. Few beetles tunneled to the end of the α-cellulose and escaped the straw segments, and those that did were excluded from data analyses.

Hexane, methanol, and water extracts were assayed individually at 1$\times$, 0.5$\times$, and 0.1$\times$ nominal conc. One treatment consisting of 1$\times$ nominal conc. methanol plus water extractives and one treatment consisting of all three extracts at 1$\times$ nominal conc. in one sample of α-cellulose were also tested to determine whether the observed feeding activity could be explained solely by the polar extractives. The standard assay was used, with 25 males in each treatment.

Sugars. In order to determine whether the most abundant soluble sugars in ponderosa pine phloem reported by Smith and Zavarin (1960) stimulated *I.*

paraconfusus feeding, sucrose, D(+)-glucose, D(−)-fructose, and D(+)-raffinose (pentahydrate) (Sigma-Aldrich, St. Louis, MO) were tested using the α-cellulose assay. In each assay, α-cellulose containing 0.25$\times$ nominal conc. methanol extractives was used as a positive control, and blank α-cellulose was used as a negative control. Preliminary studies determined that the mean lengths of ingested substrate were similar with 0.25$\times$ and 1$\times$ nominal conc. extracts. The total soluble sugar content of dry phloem was previously reported to be approximately 5% by weight (Smith and Zavarin, 1960). In the first assay, the four sugars were combined in the relative proportions reported by Smith and Zavarin (1960) (glucose 1.5%, fructose 1.8%, sucrose 1.5%, and raffinose 0.1% dry weight), and were tested at total concentrations of 1.7 and 3.3% wet weight (5 and 10% dry weight, respectively) using 25 male *I. paraconfusus* per treatment. In the second assay, each sugar was tested individually at 3.3% wet weight using 25 males per treatment. A third assay was conducted to determine whether sugars interacted synergistically with methanol extractives to increase feeding. The combined sugar mixture used in the first assay was tested at 3.3% wet weight in combination with 0.25$\times$ nominal conc. methanol extractives, using 30 male *I. paraconfusus* per treatment.

Stilbenes and Ferulic Acid Glucoside. In order to determine whether the two pine stilbenes, pinosylvin and pinosylvin methyl ether, stimulated feeding by male *I. paraconfusus*, the compounds (Apin Chemicals Ltd., Abingdon, England) were added to α-cellulose individually at concentrations of 12, 120, and 1200 nmol/g wet weight, and together in equal molar proportions at total concentrations of 24, 240, and 2400 nmol/g wet weight. The mean (combined) pinosylvin and pinosylvin methyl ether concentration found in potted ponderosa pine seedlings inoculated with *H. annosum* was approximately 120 nmol/g wet weight (Bonello et al., unpublished). Blank α-cellulose and α-cellulose containing 1$\times$ nominal conc. methanol extractives were used as negative and positive controls, respectively. The stilbenes were dissolved in several ml of methanol, and were added to α-cellulose the evening prior to the experiment in order to evaporate the solvent. Twenty beetles were tested in each treatment, using the standard assay.

In order to determine whether the stilbenes inhibited feeding or synergistically enhanced feeding in combination with stimulatory methanol extractives, 30 males per treatment were added to negative and positive controls, and to α-cellulose containing stilbenes plus 1$\times$ nominal conc. methanol extractives. Stilbenes were tested using the standard assay methodology in combination at total concentrations of 24, 240, and 2400 nmol/g wet weight, and individually at concentrations of 12, 120, and 1200 nmol/g wet weight.

Ferulic acid glucoside was synthesized as described by Bonello et al. (2003) and dissolved in several ml of a 50:50 mixture of methanol and distilled water prior to addition to α-cellulose. The solvent was allowed to evaporate overnight. In order to determine whether ferulic acid glucoside stimulated *I. paraconfusus* feeding, 30 males per treatment were added to negative and positive controls and to

α-cellulose containing 300, 1200, or 3000 ng/mg wet weight ferulic acid glucoside using the standard assay methodology. HPLC analysis demonstrated that the $1\times$ nominal conc. crude methanol extract also contained ferulic acid glucoside, at a concentration of 660 ng/μl wet weight.

In order to determine whether ferulic acid glucoside reduced feeding or acted synergistically as a feeding stimulant in combination with stimulatory methanol extractives, the compound was assayed in two experiments at 30, 300, and 1350 ng/mg wet weight. 1350 ng/mg was the highest individual concentration observed in *H. annosum*-inoculated seedlings in a study by Bonello et al. (unpublished). Twenty-five males were allowed to feed in negative controls, 50 males were confined to positive controls, and 50 males were fed in substrate containing $1\times$ nominal conc. methanol extractives plus 1350 ng/mg wet weight ferulic acid glucoside.

Phloem Broth. Several cultures of *H. annosum* were grown for 2.5 months in a filter-sterilized, aqueous extract of ponderosa pine phloem using 20 ml water per g wet phloem. Cultures were combined, and 100 ml of broth were filtered using a 0.45-μm filter and partitioned into aqueous and organic phases (water and chloroform, respectively). As a control, sterile broth without the pathogen was prepared and incubated using the same methods. The four extracts (aqueous and organic phases from broth with and without the pathogen) were concentrated to dryness using a rotary evaporator and redissolved in several milliliters of methanol. Extracts were added to α-cellulose at a ratio of 10 ml of original broth per gram of dry substrate and, after allowing methanol to evaporate overnight, were assayed using the standard methodology, with 30 male *I. paraconfusus* in each treatment.

Feeding in H. annosum-Inoculated and Uninoculated Trees. In order to determine whether *H. annosum* infection resulted in altered rates of phloem feeding by male *I. paraconfusus*, 40 ponderosa pines, 20–40 cm in diam., were selected from a 30-year-old plantation located at BFRS in October 1997. No trees showed any signs of bark beetle attack, stem resinosis, or mechanical damage, and all trees were at least one row (6 m) from the edge of the stand. Ten trees were randomly assigned to each of four categories: uninoculated controls, basal mock-inoculation with 4 sterile wooden dowels [6 mm (diam.) $\times$ 25 mm (length)] equally spaced around the tree, or basal inoculation with either 4 or 8 wooden dowels colonized by *H. annosum* (Bonello et al., 2003).

In October 1999, those trees in each category without any observable signs of bark beetle attack (3 mock-inoculated, 4 with 4 inoculations, 4 with 8 inoculations, and 4 uninoculated controls) were felled. Logs were cut at or below the inoculations to approximately 60 cm above the inoculation sites. They were transported to the University of California, Berkeley, and the cut ends were sealed with paraffin wax prior to assays in order to reduce desiccation. These logs were maintained at 5°C overnight and allowed to warm to room temperature for several hours before feeding assays were conducted at 22–23°C. Each log was divided into four longitudinal quadrants centered around the inoculation sites, or randomly oriented

for uninoculated trees. In each quadrant, a total of six male *I. paraconfusus* were inserted into two vertical rows of 3-mm diam. holes extending to the xylem–phloem interface at an approximate 45° angle to the longitudinal axis of the log, beginning at 5 cm above the inoculations. Six males were also inserted in the same manner 45 cm above each lower beetle cohort. Beetles were confined to the tunnels by wire screen stapled to the outer bark, and logs were vertically oriented during the assay. After 4 hr, beetles were removed from the logs, killed by freezing, and dissected to measure the lengths of phloem in their digestive tracts.

Portions of the xylem adjacent to all inoculations, and samples from the middle of each quadrant of uninoculated trees, were removed and analyzed for the presence of *H. annosum*. Samples were surface-sterilized in 1% sodium hypochlorite for 2 min and then incubated on sterile, wet filter paper sealed inside petri dishes. Characteristic conidiophores were produced within 2 wk on samples from which the pathogen was successfully recovered.

Data Analysis. Data were analyzed using SPSS for Windows 6.1 (SPSS, 1994). Differences in the mean lengths of α-cellulose in the digestive tracts were tested using analysis of variance with orthogonal contrasts between individual and combined treatments (Sokal and Rohlf, 1981). In both assays where feeding was followed over time, treatments were compared at each time period. Differences between treatments in the amount of feeding by *I. paraconfusus* in all other assays were tested using analysis of variance, followed by multiple comparisons using the least significant difference test (SPSS, 1994). In the assay of *H. annosum*-infected trees, phloem thickness was included in the analysis as a covariate, and analysis of variance was used to test whether the mean lengths of phloem in the digestive tracts were dependent upon inoculation treatment, reisolation of the pathogen in each quadrant, or the spatial placement of beetles in relation to the inoculation sites. Orthogonal contrasts were made between individual and combined treatment categories (Sokal and Rohlf, 1981).

RESULTS

Feeding in Intact Phloem. Both sexes of *I. paraconfusus* were observed to contain greater mean lengths of phloem in their digestive tracts as feeding times increased (Table 1, Expt. 1a). The mean lengths of ingested phloem differed between the sexes only at 24 hr, where males contained more phloem than females ($P = 0.02$).

Feeding in α-Cellulose Containing Phloem Extractives. At each time period, beetles in treatments containing all three phloem extracts had greater mean lengths of α-cellulose in their digestive tracts than those feeding in blank controls (Table 1, Expt. 1b). In the assay of individual and combined extractives, most treatments that included methanol and/or water extracts resulted in greater feeding than the control treatment (Table 2). Hexane extractives did not stimulate

TABLE 1. MEAN LENGTHS OF SUBSTRATE (mm ± *SE*) IN THE DIGESTIVE TRACTS OF *Ips paraconfusus* ADULTS CONFINED TO THE INTACT PHLOEM OF PONDEROSA PINE LOGS (EXPERIMENT 1A) AND WET α-CELLULOSE CONTAINING COMBINED HEXANE, METHANOL, AND WATER EXTRACTS OF PONDEROSA PINE PHLOEM (EXPERIMENT 1B)

	Feeding period (hr)				
	2	4	8	24	48
Experiment 1a: Intact phloem					
Males	3.71 ± 0.39	4.11 ± 0.34	5.60 ± 0.23	6.47 ± 0.20	7.12 ± 0.28
Females	4.32 ± 0.33	4.82 ± 0.25	5.27 ± 0.29	5.51 ± 0.28	6.35 ± 0.25
Significance[a]	ns	ns	ns	$P < 0.05$	ns
Experiment 1b: α-Cellulose					
Extract	3.72 ± 0.44	3.98 ± 0.52	5.59 ± 0.48	4.43 ± 0.51	4.53 ± 0.53
Control	0.30 ± 0.19	0.29 ± 0.19	0.14 ± 0.07	0.08 ± 0.04	0.13 ± 0.11
Significance[b]	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

[a] Test of difference between sexes for that feeding period. ns = not significant.
[b] Test of difference between extract and control for that feeding period.

feeding at any of the concentrations tested, and the addition of hexane extractives to α-cellulose containing methanol and water extractives did not significantly affect the amount of feeding. None of the 0.5× and 1× nominal conc. treatments containing methanol extractives were significantly different, while at 0.1× nominal conc., only treatments containing methanol extractives induced more feeding than the negative control. Water extractives alone induced feeding only at 1× nominal conc.

TABLE 2. MEAN LENGTHS (mm ± *SE*) OF α-CELLULOSE IN THE DIGESTIVE TRACTS OF MALE *I. paraconfusus* CONFINED TO WET α-CELLULOSE CONTAINING INDIVIDUAL AND COMBINED EXTRACTS OF PONDEROSA PINE PHLOEM AT THREE CONCENTRATIONS (0.1×, 0.5×, AND 1× NOMINAL CONC.)[a]

	Concentration		
Extractives	0.1×	0.5×	1.0×
Negative control	0.11 ± 0.07a	—	—
Hexane	0.08 ± 0.05a	0.33 ± 0.25a	0.09 ± 0.06a
Methanol	1.63 ± 0.34bc	4.48 ± 0.55d	4.35 ± 0.43d
Water	0.06 ± 0.04a	0.78 ± 0.25ab	2.21 ± 0.44c
Combined	1.74 ± 0.50bc	4.04 ± 0.54d	4.15 ± 0.52d
Methanol + water	—	—	3.89 ± 0.43d

[a] Means followed by different letters differ significantly across all rows and columns ($P < 0.05$, LSD test).

Sugars. The mean lengths of α-cellulose in the digestive tracts of beetles allowed to feed in substrate containing a total 3.3% wet weight (10% dry weight) sugar mixture [0.44 ± 0.15 (*SE*) mm] and a negative control (0.09 ± 0.07 mm) were not significantly different, while the methanol extractives elicited more feeding (4.27 ± 0.47 mm) than the sugar mixture ($P < 0.05$). At 3.3% wet weight, differences in the amount of feeding between individual sugars and the negative control were not significant. Likewise, differences in the amount of feeding on α-cellulose containing methanol extractives from ponderosa pine phloem (2.06 ± 0.39 mm) and α-cellulose containing methanol extractives plus a 3.3% wet weight mixture of the sugars (1.77 ± 0.35 mm) were not significant.

Stilbenes and Ferulic Acid Glucoside. On wet α-cellulose, none of the treatments containing individual or combined stilbenes differed from the negative control (Table 3, Expt. 1). When assayed in combination with $1\times$ nominal conc. methanol extractives, none of the individual or combined stilbene treatments (Table 3, Expts. 2a and 2b, respectively) differed from the positive controls.

Treatments containing ferulic acid glucoside added to wet α-cellulose were not significantly different from the negative control (Table 4, Expt. 1). In two assays containing $1\times$ nominal conc. methanol extractives plus 30, 300, and 1350 ng/mg

TABLE 3. MEAN LENGTHS OF α-CELLULOSE (mm $\pm$ *SE*) IN THE DIGESTIVE TRACTS OF MALE *I. paraconfusus* CONFINED TO WET α-CELLULOSE CONTAINING PINOSYLVIN AND PINOSYLVIN METHYL ETHER (nmol/g WET WEIGHT)[a]

Treatment		Pinosylvin	Pinosylvin methyl ether	Pinosylvins, combined
Methanol extractives absent (Experiment 1)				
Negative control	0.19 ± 0.17a	—	—	—
Positive control	4.46 ± 0.60b	—	—	—
12 nmol/g	—	0.12 ± 0.12a	0.00 ± 0.00a	0.11 ± 0.11a
120 nmol/g	—	0.04 ± 0.04a	0.00 ± 0.00a	0.05 ± 0.05a
1200 nmol/g	—	0.06 ± 0.06a	0.00 ± 0.00a	0.14 ± 0.14a
Methanol extractives present (Experiment 2)		← Experiment 2a →		Experiment 2b
Negative control	0.06 ± 0.06a	—	—	0.00 ± 0.00a
Positive control	5.47 ± 0.52b	—	—	4.10 ± 0.46b
12 nmol/g	—	5.45 ± 0.42b	4.77 ± 0.52b	4.17 ± 0.35b
120 nmol/g	—	5.59 ± 0.61b	4.79 ± 0.51b	4.12 ± 0.40b
1200 nmol/g	—	4.35 ± 0.55b	4.83 ± 0.58b	4.52 ± 0.37b

[a] Stilbenes were added to either wet α-cellulose (Experiment 1) or wet α-cellulose containing $1\times$ nominal conc. methanol extractives from ponderosa pine phloem (Experiment 2). Within each experiment (1, 2a, and 2b), means followed by different letters differ significantly ($P < 0.05$, LSD test).

TABLE 4. MEAN LENGTHS OF α-CELLULOSE (mm $\pm$ *SE*) IN THE DIGESTIVE TRACTS OF MALE *I. paraconfusus* CONFINED TO WET α-CELLULOSE CONTAINING FERULIC ACID GLUCOSIDE (ng/mg WET WEIGHT)[a]

Extractives	Glucoside added (ng/mg wet weight)	Length of food in gut (mm)	
Methanol extractives absent (Experiment 1)			
Absent	0	0.00 ± 0.00a	
Absent	300	0.07 ± 0.07a	
Absent	1200	0.00 ± 0.00a	
Absent	3000	0.05 ± 0.05a	
Present	0	3.72 ± 0.62b	
Methanol extractives present (Experiment 2)		Experiment 2a	Experiment 2b
Absent	0	0.41 ± 0.23a	0.00 ± 0.00a
Present	0	6.47 ± 0.39b	3.67 ± 0.38b
Present	30	6.26 ± 0.38b	—
Present	300	6.24 ± 0.40b	—
Present	1350	—	3.33 ± 0.38b

[a] Within each experiment (1, 2a, and 2b), means followed by different letters differ significantly ($P < 0.05$).

wet weight ferulic acid glucoside (Table 4, Expts. 2a and 2b), the mean lengths of α-cellulose in the digestive tracts of beetles in glucoside-containing treatments were not significantly different from positive controls.

Phloem Broth. Beetles feeding in aqueous and organic preparations from broths without *H. annosum* ingested more α-cellulose than those confined to preparations from broths containing *H. annosum* ($P < 0.05$) (Fig. 1). Feeding in the organic and aqueous controls was not different, and feeding in both preparations from broths with *H. annosum* was also not significantly different.

Feeding in H. annosum-Inoculated and Uninoculated Trees. The mean lengths of phloem in the digestive tracts (Fig. 2) varied by treatment ($P = 0.04$), but did not vary by beetle position on the log (5 and 45 cm above the inoculation sites) or reisolation of the pathogen at the inoculation sites. The mean length of phloem in the guts of beetles confined to all inoculated logs combined (5.62 ± 0.09 mm) was lower than in all uninoculated and mock-inoculated logs combined (5.95 ± 0.09 mm) ($P = 0.03$). There was no difference between treatments with 4 and 8 *H. annosum* inoculations, or between mock-inoculated and uninoculated treatments. The mean $\pm$ *SE* proportion of quadrants from which the pathogen was reisolated was, uninoculated $0 \pm 0\%$, mock-inoculated $17 \pm 11\%$, inoculated with 4 dowels $69 \pm 13\%$, and inoculated with 8 dowels $50 \pm 13\%$. The two positive samples from the mock-inoculated trees were likely due to contamination during

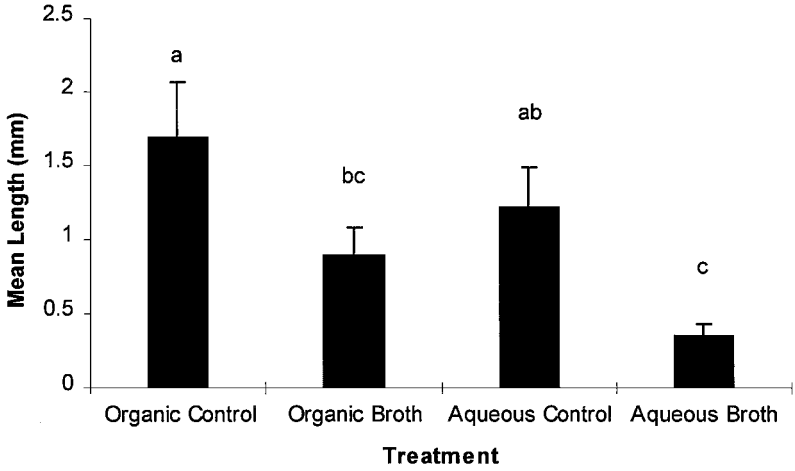

FIG. 1. Mean lengths of α-cellulose (mm $\pm$ *SE*) in the digestive tracts of male *I. paraconfusus* confined to wet substrate containing extractives of *H. annosum* liquid cultures. Different letters indicate significant differences among treatments ($P < 0.05$).

incubation. Visible disease symptoms consisted of phloem resinosis and a dry zone in the xylem of inoculated trees extending from the pathogen-colonized dowels towards the center of the tree (reviewed in Coutts, 1976). There was no sign of insect attack immediately around the inoculation sites, although many of the trees

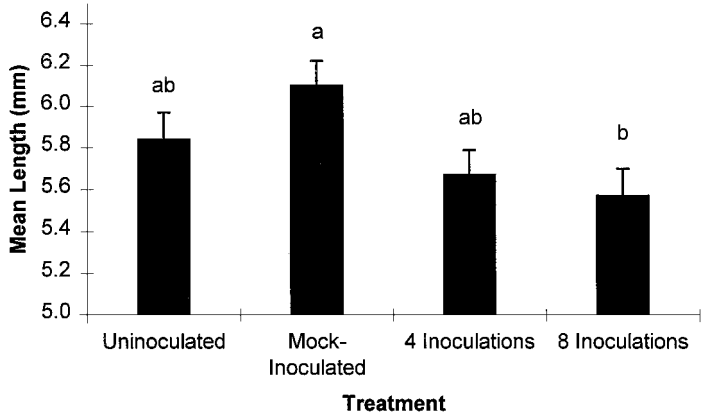

FIG. 2. Mean lengths of phloem (mm $\pm$ *SE*) in the digestive tracts of male *I. paraconfusus* confined to the phloem of uninoculated, mock-inoculated, and *H. annosum*–inoculated ponderosa pine logs. Different letters indicate significant differences among treatments ($P < 0.05$).

that were inoculated in 1997 but not used in 1999 had been unsuccessfully attacked by bark beetles.

DISCUSSION

Feeding in *H. annosum*-inoculated logs was lower than in logs that did not contain the pathogen. However, no effect on feeding was observed in any of the stilbene or ferulic acid glucoside assays. Therefore, the reduced feeding in inoculated logs was not due to *H. annosum*-induced increases in these compounds. Localized defensive reactions near an infection site have been reported for infections of *H. annosum* and other conifer pathogens (e.g., Lieutier and Berryman, 1988; Brignolas et al., 1995; Klepzig et al., 1996; Bonello et al., 2003). For example, stilbene concentrations dramatically increased only around the inoculation sites of most trees in greenhouse studies of *H. annosum*-inoculated ponderosa pines, and no systemic induction was observed in the field (Bonello et al., 2003). Thus, beetles colonizing most of the stem of root-diseased trees are unlikely to encounter greatly increased stilbene concentrations. Stilbenes are known to have antimicrobial activity at very low concentrations (e.g., Rennerfelt, 1945; Shain, 1967; Prior, 1976), and may reduce the likelihood of oviposition by some bark beetles (Storer and Speight, 1996). However, their underlying role appears to be as antimicrobial agents rather than insect antifeedants (Woodward and Pearce, 1988; Storer and Speight, 1996).

Ferulic acid glucoside was not stimulatory to male *I. paraconfusus*, and when assayed in combination with stimulatory methanol extractives, did not increase or reduce feeding at the highest concentration observed in a field study of *H. annosum*-inoculated ponderosa pines (Bonello et al., 2003). In that study, considerable variation in ferulic acid glucoside concentration was observed in both *H. annosum*-inoculated trees (range = 5–1350 ng/mg wet weight) and uninoculated trees (range = 18–777 ng/mg wet weight), yet feeding rates in numerous logs in this study and in other studies were relatively similar (McNee, 2000). Ferulic acid glucoside may have a role in insect and/or disease resistance that is unrelated to bark beetle feeding (Bonello et al., 2003), and it is possible that increased ferulic acid glucoside concentrations may be a secondary effect of other biochemical changes that were not examined here.

The reduced feeding in *H. annosum*-inoculated logs, compared with logs that did not contain the pathogen, was contrary to the hypothesis that infection by a root pathogen would increase feeding by bark beetles. The lack of differential feeding between the upper and lower regions of the logs, and between quadrants from which the pathogen was and was not recovered, may indicate the induction of a systemic response that reduces the overall suitability of the tree for bark beetle colonization. Likewise, increased amounts of liquid and crystalline resin were

present in the phloem of pathogen-inoculated trees, compared with uninoculated or mock-inoculated trees (McNee, 2000). It is possible that basal inoculation with *H. annosum* induces localized and systemic formation of traumatic resin ducts (e.g., Nagy et al., 2000; Francheschi et al., 2002). In pines, resin production in response to pathogen infection has been described for other pathosystems (e.g., Reid et al., 1967; Owen et al., 1987; Lieutier and Berryman, 1988), and resins are known to be antagonistic to bark beetles and their associated, pathogenic fungi (e.g., Smith, 1961; Vité, 1961; Russell and Berryman, 1976; Wong and Berryman, 1977). It is not known whether the reduced feeding observed in this study is due to a reduced concentration of stimulatory metabolites, the production of antifeedant compounds, increased lignification or other physical barriers, changes in the chemical and physical properties of resins, other metabolic changes, or some combination of these factors.

The difference between individual inoculated and uninoculated treatments may have become statistically significant if the infections had been given more time to progress. Root-diseased trees that are killed by bark beetles are typically infected longer than the 2-year incubation period used in this study (e.g., Shaw and Kile, 1991, and references therein; Smith, 1993). No external disease symptoms were visible 2 years after inoculation, but the amount of inoculum initially introduced to the tree was probably greater than the initial stage of a natural infection. Even if feeding rates, and possibly pheromone production rates, were significantly reduced in trees with advanced infections, this probably has no ultimate effect on tree survival, since these trees are eventually colonized by bark beetles.

The consumption of phloem metabolites by fungi (e.g., Hodges et al., 1968; Barras and Hodges, 1969; Barras, 1970), or the production of antagonistic secondary metabolites (e.g., Claydon et al., 1985), may be responsible for the reduced feeding observed in broth preparations originally containing *H. annosum*. Similar results were obtained by Klepzig et al. (1996), who demonstrated that male *I. pini* spent less time tunneling in artificial media containing extracts of phloem from *Leptographium*-inoculated red pine, *P. resinosa* Ait., than in media containing extracts of uninfected phloem.

Some compounds in the phloem of healthy ponderosa pines stimulated feeding while other compounds did not. Methanol and water extractives induced significant feeding, in contrast to the stilbenes, ferulic acid glucoside, soluble sugars, and hexane extractives that did not. Female *I. paraconfusus* were stimulated to feed by the same 1× nominal conc. methanol and water extracts as males (McNee, 2000). The compounds responsible for the feeding stimulation of *I. paraconfusus* are extractable and polar, as was first demonstrated by Elkinton et al. (1981) in a study that recorded substrate preference without dissecting beetles. Feeding and biting stimulants for *Scolytus multistriatus* and *S. mediterraneus* have also been reported to be polar (Doskotch et al., 1970; Levy et al., 1974). The finding that the most common sugars in ponderosa pine phloem did not stimulate

feeding is consistent with earlier research on other bark beetle species (Bedard, 1966; Norris and Baker, 1967). These sugars are ubiquitous throughout the higher plants (e.g., Salisbury and Ross, 1991) and are common in nonhost conifers present within the geographic range of *I. paraconfusus* (Zimmerman, 1957; Smith and Zavarin, 1960). However, it is possible that other structural forms may be stimulatory.

Beetles allowed to feed in α-cellulose containing combined hexane, methanol, and water extractives contained similar amounts of substrate at all tested feeding periods, whereas beetles confined to blank α-cellulose contained little ingested material at any of the observation periods. It has been hypothesized that a need for water would stimulate beetles to ingest a neutral substrate after a period of starvation (Elkinton et al., 1980), but this period may be greater than the time intervals used in this study. Elkinton et al. (1980) concluded that nonhost white fir, *Abies concolor* (Gordo & Glendl.) Lindl. ex Heloleb, lacks both feeding stimulants and deterrents for *I. paraconfusus*, yet 30 min of confinement in the phloem of white fir resulted in the ingestion of amounts of phloem comparable to those confined to ponderosa pine phloem (McNee, 2000). These findings are consistent with Raffa (1988), who suggested that once the preliminary stages of host orientation have been satisfied by *D. ponderosae*, attacks are abandoned in the presence of repellants and not in the absence of chemical stimulants.

Both sexes of *I. paraconfusus* ingested significant amounts of food material (phloem or α-cellulose) within 2 hr of confinement, and the guts became 80–90% filled after 48 hr of confinement. The relatively similar feeding pattern in males and females may be due to a common requirement for water and nutrients rather than for sex-specific behaviors such as nuptial chamber construction and aggregation pheromone production by male *I. paraconfusus* (e.g., Struble and Hall, 1955; Wood and Vité, 1961; Wood, 1962; Wood and Stark, 1968). This study demonstrated that detection of aggregation pheromone is not required for the nonpioneering female *I. paraconfusus* to feed, supporting the hypothesis that bark beetles tunnel into uncolonized trees during dispersal to obtain water and nutrients before a pheromone plume is detected and/or before producing pheromone (reviewed in Borden, 1974; D.L. Wood, 1982). Male *I. pini* (Say) feeding has also been demonstrated to stimulate *de novo* pheromone biosynthesis (Tillman et al., 1998).

In summary, there was no evidence that increased feeding rates are a major factor in explaining why root-diseased trees are preferentially killed by bark beetles. Although it is possible that factors such as decreased resistance to colonization by beetle-vectored fungi may play a significant role in the preferential colonization of root-diseased trees, published research generally supports the hypothesis that factors such as osmotic pressure, resin composition, pressure, crystallization, and flow rates are mainly responsible for the resistance of pines to bark beetle colonization (e.g., Vité, 1961; Rudinsky, 1962; Cobb et al., 1968; Berryman, 1972; Wood, 1972; Cook and Hain, 1988; Byers, 1995; Phillips and Croteau, 1999).

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DIFFERENT FOOD SOURCES AFFECT THE GUSTATORY RESPONSE OF *Anaphes iole*, AN EGG PARASITOID OF *Lygus* SPP.

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Abstract—The gustatory response of female *Anaphes iole* wasps to naturally occurring carbohydrates, a commercial food source, and host (*Lygus lineolaris*) frass was determined. Wasps responded to all 14 of the sugars at the highest concentration tested (2 M). At this concentration, sucrose, glucose, maltose, melezitose, fructose, and erlose all elicited >90% acceptance. The lowest concentration that evoked a response (=acceptance threshold) for these sugars was <1/256 M, with the exception of glucose, which was 1/16 M. Raffinose, trehalose, mannose, galactose, melibiose, rhamnose, stachyose, and lactose led to <50% gustatory response by the wasps at 2 M, and were categorized as "moderately stimulatory sugars." The acceptance threshold for these sugars was >1/4 M, with the exception of raffinose, which was 1/256 M. In trials with moderately stimulatory sugars combined with either sucrose or maltose, only the rhamnose+maltose mixture significantly inhibited the gustatory response of *A. iole*. Food and water deprived parasitoids readily accepted the moderately stimulatory sugars. Eliminade™, a commercial food supplement, was readily accepted (92%) by *A. iole*. Conversely, the wasps did not feed on host frass. Chemical analysis of *L. lineolaris* frass demonstrated the presence of glucose, sucrose, fructose, trehalulose, and melezitose, apparently at concentrations below those perceived by *A. iole*. The latter two compounds were previously known only from homopteran honeydew (trehalulose and melezitose) and bacteria (trehalulose). With respect

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to gustatory response to nectar and honeydew sugars, *A. iole* differs markedly from other hymenopterans that have been studied in that this parasitoid accepted all the naturally occurring sugars with which it was tested. Moreover, this parasitoid had lower acceptance thresholds than other hymenopterans for many of the sugars. This broad and sensitive range of gustatory perception might be helpful in the development of a food source for the wasp that is not exploited by the host.

Key Words—*Anaphes iole*, Mymaridae, carbohydrate, nectar, honeydew, artificial food supplement, feeding behavior, conservation biological control, *Lygus lineolaris*, frass.

INTRODUCTION

Many adult parasitic Hymenoptera require food to satisfy metabolic energy and reproductive needs (Quicke, 1997). Energy and maintenance requirements are generally met by sugar-foraging, while host-feeding provides wasps with protein nutrients necessary for successful reproduction. Floral and extrafloral nectar and honeydew excreted by homopteran insects provide sugar-foraging wasps with rich sources of carbohydrates (Rogers, 1985; Jervis et al., 1993; Longley and Jepson, 1996; Jacob and Evans, 1998). Provisioning parasitoids with carbohydrates generally increases longevity and subsequent rates of parasitism (Stapel et al., 1997; Baggen and Gurr, 1998; Damon et al., 1999; Olson et al., 2000; Siekmann et al., 2001; Wäckers, 2001). Consequently, the availability of carbohydrates for foraging parasitoids is an important factor of habitat management strategies aimed at enhancing the effectiveness of biological control agents (Gurr and Wratten, 1999; Landis et al., 2000).

Successful sugar foraging depends on the availability of suitable nectar at the time of foraging. Most parasitoids readily accept sucrose, fructose, and glucose (Jervis et al., 1993, 1996), the most common components of nectar and honeydew (Baker and Baker, 1983a). However, other carbohydrates occur in nectar and honeydew as well (Baker and Baker, 1983b; Davidson et al., 1994; Hendrix and Wei, 1994; Koptur, 1994). The carbohydrate composition of nectar can also vary within and between plant species (Baker and Baker, 1983a; Mohr and Jay, 1990). The resulting spatial and temporal variability in the sugar composition, i.e., suitability of nectar available to parasitoids, can result in unprofitable food encounters. With the exception of the predominant carbohydrates, little is known about the suitability of most sugars present in nectar and honeydew for parasitic Hymenoptera. The gustatory response of *Cotesia glomerata* (L.) wasps exposed to individual nectar and honeydew sugars varied greatly (Wäckers, 1999), and was positively correlated with longevity (Wäckers, 2001). Romeis and Wäckers (2000) demonstrated differences in sugar utilization patterns between *C. glomerata* and its host, *Pieris brassicae* L. The parasitoid utilized several sugars to which the host did not respond. These findings have practical relevance for pest control, because

they suggest that natural or artificial food sources might be designed to benefit natural enemies to a greater extent than the pest. Food sprays and other artificial supplements increase attraction and retention of natural enemies (Hagen et al., 1971; Evans and Swallow, 1993; Evans and Richards, 1997; Mensah, 1997), and can lead to a reduction in oviposition by the pest (Mensah, 1996; Mensah et al., 2000).

Lygus spp. (Heteroptera: Miridae) are important pests of many crops in North America (Wheeler, 2001). In cotton, *Gossypium hirsutum* L., annual losses due to *Lygus lineolaris* (Palisot de Beauvois) damage can exceed $40 million in the southcentral United States (Williams, 1999). Control of *L. lineolaris* populations in cotton is achieved mostly by broad-spectrum insecticides aimed at several pests, including *L. lineolaris*, lepidopterous larvae, and the boll weevil, *Anthonomus grandis* Boheman. However, the total insecticide load in this region is expected to decrease as a result of the use of transgenic cotton, the success of the boll weevil eradication program (Hardee et al., 2001), and concerns about insecticide resistance (Snodgrass, 1996). Although reduced insecticide use might lead to an increase in *L. lineolaris* densities, it will also create a scenario in which biological control has potential for controlling this pest (Ruberson and Williams, 2000).

Anaphes iole Girault (Hymenoptera: Mymaridae) is an egg parasitoid that attacks *L. lineolaris* and other mirids in North America (Jackson and Graham, 1983; Huber and Rajakulendran, 1988; Udayagiri et al., 2000). *Anaphes iole* is pro-ovigenic and does not require a food source in order to mature eggs (Jervis et al., 2001). Nevertheless, foraging by adult *A. iole* is important because increased longevity would allow the wasp more time to search for and parasitize hosts, thus leading to a possible increase in realized fecundity. An increase in longevity afforded by sugar foraging is especially important when host densities are low and parasitoids must spend considerable time searching. Adult *A. iole* does not host-feed, and apparently relies on food sources such as nectar and honeydew, although field observations of *A. iole* feeding are lacking. *Lygus lineolaris* excretes liquid frass in the form of small (<2 mm diam.) droplets. Although this frass is not as sugar-rich as honeydew, it contains plant-derived carbohydrates that might be used as a food source by *A. iole*. Under laboratory conditions, longevity of *A. iole* wasps is limited to <3 days in the absence of food, but can exceed 10 days when honey is provided (Jones and Jackson, 1990). However, nothing is known about the suitability of individual sugars for *A. iole*. A better understanding of nutritional ecology of *A. iole* may facilitate the development of natural or artificial food sources that confer greater benefit to this parasitoid than to *L. lineolaris*.

Our objectives were to describe the gustatory discrimination of *A. iole* to 14 carbohydrates, a commercial food source, and host (*L. lineolaris*) frass. Carbohydrate components of the frass were identified and quantified on a relative basis, and related to gustatory response by *A. iole*.

METHODS AND MATERIALS

Insects. *Anaphes iole* were obtained from a laboratory colony maintained on *Lygus hesperus* Knight eggs at the USDA-ARS Biological Control and Mass Rearing Research Unit, Mississippi State, MS. Parasitized host eggs were held in Plexiglass cages (26 × 26 × 20 cm) at 27 ± 1°C, 65–85% RH, and 14:10 L:D photoperiod. Depending upon the study emerging wasps were either water-satiated (provided with distilled water *ad lib* via saturated absorbent matting and frequent misting), or were food and water deprived. Wasps used in the experiments were <3 day old females that appeared healthy, were assumed mated (*A. iole* exhibits protandry, and male wasps copulate with females soon after the females emerge), and had not engaged in oviposition. In all the experiments described herein, each wasp was subjected to only one treatment, after which it was killed. Under the existing laboratory conditions, the longevity of food and water deprived *A. iole* is about 3 days (Williams, unpublished). All experiments were conducted between 0800 and 1700 h central standard time, USA.

Gustatory Response and Acceptance Threshold. Acceptance thresholds for the 14 sugars in Table 1 were determined for *A. iole*. Where appropriate, effective concentrations (the concentration at which 50% of the wasps were scored positive, i.e., the EC_{50}) were also estimated. With the exception of lactose, all the sugars tested are known to be associated with plants (e.g., nectar) or insects (e.g., honeydew or bee honey). Lactose was included as a control sugar that *A. iole* is unlikely to encounter in nature. A 2 M concentration of each sugar was prepared

TABLE 1. LIST OF SACCHARIDES, COMMERCIAL SOURCE, FORMULA WEIGHT, PURITY, AND NATURAL SOURCE

Sugar	Source	F. W.	Purity (%)	Major sources in nature
D(+)-Sucrose	Fluka	342.3	>99.5	Bee honey, nectar, and phloem sap
D(+)-Glucose	Fluka	180.16	>99	Bee honey, nectar, and honeydew
D(+)-Maltose monohydrate	Fluka	360.32	>99	Bee honey
D(+)-Melezitose monohydrate	Fluka	522.48	>99	Some honeydews and bee honey
D(−)-Fructose	Fluka	180.16	>99	Bee honey, nectar, and honeydew
Erlose	Senn	504.4	98.90	Bee honey and some honeydews
D(+)-Raffinose pentahydrate	Fluka	594.53	>99	Bee honey, some honeydews, and sap of some plants
α,α−D(+)-Trehalose	Fluka	342.31	>99.5	Bee honey, honeydews, and fungi
D-Mannose	Aldrich	180.16	>99	Bee honey and mannosides
D(+)-Galactose	Fluka	180.16	>99	Seeds and galactosides
D(+)-Melibiose monohydrate	Fluka	360.32	>99	Bee honey and some honeydews
L(+)-Rhamnose monohydrate	Fluka	182.18	>99	Glycosides
Stachyose tetrahydrate	Aldrich	738.64	98	Seeds, some honeydews, and sap of some plants
D(+)-Lactose monohydrate	Fluka	360.32	>99	Milk

with distilled water. This concentration approximates that found in nectar and honeydew (Baker and Baker, 1983a). Because of the limited solubility of lactose, the highest concentration for this sugar was 1 M. Preliminary observations of gustatory discrimination were used to help choose the appropriate low concentration for each sugar. Serial dilutions were then made from the stock solution in a geometric progression (i.e., 1 M, 1/2 M, 1/4 M, etc.) for each sugar. When estimation of an EC_{50} was justified, gustatory response data were subjected to probit analysis, including tests of parallelism (equal slopes) and equality (equal slopes and intercepts) (LeOra Software, 1987). Comparisons of EC_{50}s and slopes were then made (Robertson and Preisler, 1992). Significant differences for EC_{50} values were indicated when the 95% confidence interval for an effective concentration ratio did not bracket 1.0.

Gustatory response by *A. iole* was also assessed for a commercial food supplement, Eliminade™ (Entopath, Easton, PA), and *L. lineolaris* frass. Eliminade™ was developed as part of a conservation biological control program to enhance the effectiveness of parasitoids of the southern pine beetle, *Dendroctonus frontalis* Zimmerman (Hanano, 1996; Mathews and Stephen, 1999). For this study, Eliminade™ was prepared as recommended by the manufacturer. *Lygus lineolaris* frass was collected from adult bugs provided with cotton, *G. hirsutum* L. (var. DPL NuCOTN 33B), seedlings at the three true leaf stage. Bugs, field-collected from alfalfa, *Medicago sativa* L., were caged individually in sleeve cages (10 × 15 cm), each cage enclosing three cotton seedlings, and held at 27 ± 1°C, 65–85% RH, and 14:10 L:D photoperiod. After 2 days, bugs were transferred to sleeve cages enclosing three undamaged cotton seedlings and a piece of aluminum foil (10 × 20 cm), and held under the same conditions given above. The foil was removed after 24 hr, and a cork borer was used to cut disks (5 mm diam.), each disk with a single spot of frass (ca. 1 mm diam.) in the center. The disks of foil were either used immediately in gustatory response bioassays, or were dehydrated at 70°C for 2 hr then frozen at −80°C until chemical analysis.

Bioassay methodology was as follows. Water-satiated wasps were placed individually into a 0.5 dram glass shell vial containing a 5 μl drop of the treatment (sugar solution or Eliminade™) into the bottom of the vial. For assays using host frass, a disc of foil with frass was placed in the vial. Wasps were observed at 50 × with a Nikon SMZ-2T dissecting scope for 5 min. "Acceptance" was recorded if the wasp fed for more than 5 sec upon contact with the treatment, or if the total time spent feeding surpassed 5 sec. Otherwise, the encounter was scored as "rejection." Each sugar-concentration combination was presented to 25 wasps in a completely randomized design. Sugar treatment combinations were replicated 3–8 times. Fresh sugar solutions were prepared for each replicate. Bioassays using Eliminade™ or frass were set up as were those with sugars, with the following exceptions. Fifty wasps were presented with Eliminade™ in each of two replicates. For bioassays using frass, 5–12 parasitoids were tested in each of five replicates. Wasp behavior

(e.g., walking speed, turning frequency, and antennation) was also noted in the frass bioassays. Laboratory conditions during the study were $24 \pm 3^{\circ}$C and 17–52% RH.

Feeding Deterrence of Sugar Mixtures. Of the 14 sugars tested in the acceptance threshold study, seven produced <50% gustatory acceptance at the 2 M concentration. Two types of studies were set up to determine whether these "moderately stimulatory" sugars (mannose, galactose, melibiose, stachyose, raffinose, trehalose, and rhamnose) acted as feeding deterrents. The first was a comparison of moderately stimulatory+highly stimulatory sugar mixtures versus a highly stimulatory sugar. The moderately stimulatory sugars were 2 M concentrations of mannose, galactose, melibiose, stachyose, raffinose, trehalose, and rhamnose. The highly stimulatory sugars were $\frac{1}{2}$ M concentrations of sucrose and maltose. The following six treatments were tested for each moderately stimulatory sugar. A control consisting of the 2 M moderately stimulatory sugar only, a control consisting of distilled water only, a control consisting of $\frac{1}{2}$ M sucrose, a 1:1 mixture of $\frac{1}{2}$ M sucrose + 2 M moderately stimulatory sugar, a control consisting of $\frac{1}{2}$ M maltose, and a 1:1 mixture of $\frac{1}{2}$ M maltose + 2 M moderately stimulatory sugar. Thus, there were 42 treatment combinations. The bioassay methodology was identical to that used in the acceptance threshold study. Briefly, a 5 μl drop of treatment solution was placed on the bottom of a 0.5 dram glass shell vial and a single female wasp (<3 days old, presumed mated, and water-satiated) was inserted and observed with a dissecting scope at 50x. If the wasp fed for more than 5 sec after contact with the solution, the response was considered "acceptance," otherwise the response was scored as "rejection." Twenty-five wasps were used in each treatment combination. Treatment combinations were set up in a completely randomized design, and were replicated 3–4 times. Fresh sugar solutions were prepared for each replicate. Conditions in the laboratory during the study were $24 \pm 2^{\circ}$C and 19–50% RH. Percentage data were arcsine transformed prior to comparison of moderately stimulatory+highly stimulatory sugar mixtures versus highly stimulatory sugar only controls (Student's t-test for unequal variances, $\alpha < 0.05$, with one-tailed hypotheses for feeding deterrence in moderately stimulatory+highly stimulatory sugar mixtures).

Feeding Deterrence of Individual Sugars. This study was designed to ascertain wether individual moderately stimulatory sugars inhibited feeding by *A. iole*. In this deterrence study, food and water deprived (<2 days old, presumably mated) female wasps were presented with a treatment solution, and the duration of feeding was recorded. The 10 treatments consisted of 1 M concentrations of each of the 7 moderately stimulatory sugars tested in the previous deterrence study, and controls consisting of 1 M sucrose, 1 M lactose, and distilled water. The bioassay methodology was identical to that used in the previous studies. After a wasp was placed into a vial, it was observed at 50x for 5 min, and the time spent feeding was recorded to the nearest second. Fifteen wasps were used in each treatment combination. Treatment combinations were set up in a completely randomized

design, and were replicated three times. Fresh sugar solutions were prepared for each replicate. Environmental conditions in the laboratory during the study were $24 \pm 3^{\circ}$C and 13–44% RH. Data for moderately stimulatory sugars for which the duration of wasp feeding was numerically less than in the distilled water control (mannose and rhamnose) were normalized by $\log_{10}$ transformation and analyzed by single factor analysis of variance (Zar, 1996).

Voucher Specimens. Voucher specimens of *A. iole* and *L. lineolaris* are deposited in the National Entomological Collection, National Museum of Natural History, Smithsonian Institution, Washington, DC, USA.

Chemical Analysis of L. lineolaris Frass. Identification and quantification of the major carbohydrates present in frass produced from bugs fed cotton was done by high-performance anion-exchange chromatography (HPAEC) analysis (Davidson et al., 1994; Hendrix and Wei, 1994). The quantity of glucose was determined for frass in the following manner. One hundred foil disks with frass were randomly sorted into 10 samples each with 10 pieces of foil. For each sample, the dry weight of frass was determined by weighing foil disks on a microbalance, after which the frass was washed off the foil in ca. 5 ml hot (80°C) water. This suspension was lyophilized. Washed foil disks were reweighed to give the dry mass of frass in each sample. The lyophilized samples were then suspended in 0.5 ml deionized water filtered through a 0.45 μM membrane (Amicon Ultrafree-MC Centrifugal Filters), and the clarified solutions were dried in a SpeedVac sample concentrator. We added 0.11 ml deionized water to each dried sample. One hundred microliters of each sample were analyzed by HPAEC. Since detector response varies among individual sugars (Larew and Johnson, 1988), peaks were identified and quantified by comparing the time of retention and peak areas with known sugar standards. Peak areas were determined using Dionex PeakNet software.

Five additional samples of frass were obtained in order to estimate the amount of the other frass carbohydrates in relation to glucose. These samples were obtained as described for the foil disks except that frass samples were collected from ca. 30 bugs caged together on 10 cotton seedlings. Samples were analyzed by HPAEC, and the relative amount of frass carbohydrates was then determined by multiplying the previously determined mean glucose content by the proportion of carbohydrate determined from peak areas relative to the peak area created by a known amount of glucose, correcting for the differences in detector response to various sugars (Larew and Johnson, 1988).

RESULTS

Gustatory Response and Acceptance Threshold. All 14 sugars elicited a response by *Anaphes iole*, although marked differences were observed (Figure 1). At the 2 M concentration, gustatory responses grouped into two categories. Sucrose, glucose, maltose, melezitose, fructose, and erlose evoked $>90\%$ acceptance at

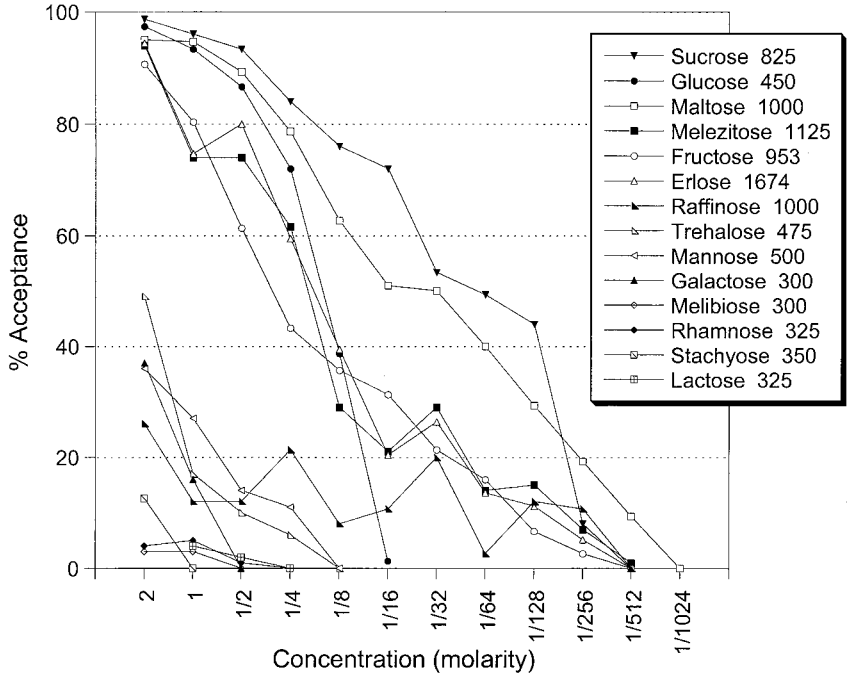

FIG. 1. Gustatory responses of food deprived female *Anaphes iole* to 14 sugars at 12 concentrations. Numbers of wasps tested with each carbohydrate are given in legend.

this concentration. These sugars were considered to be "highly stimulatory" sugars. The acceptance threshold was 1/256 M, with the exceptions of glucose and maltose, which were 1/16 and 1/512 M, respectively. Response curves declined in a relatively linear manner. Probit analysis revealed that the EC_{50} values ranged from 0.026 M for sucrose to 0.28 M for fructose (Table 2). EC_{50}s for sucrose and maltose did not differ significantly from each other ($P > 0.05$), but were lower than for the other sugars ($P < 0.05$). Slopes ranged from 0.99 for maltose to 2.48 for glucose (Table 2). Slopes for maltose, melezitose, erlose, and fructose were not different ($P > 0.05$). Slopes for sucrose, melezitose, and erlose were not different ($P > 0.05$). The slope for glucose was different from all others ($P < 0.05$).

The remaining sugars (raffinose, trehalose, mannose, galactose, melibiose, rhamnose, stachyose, and lactose) led to <50% gustatory response by the wasps (Figure 1). The acceptance thresholds did not exceed 1/4 M, except for raffinose, which was 1/256 M. Response curves were also relatively linear, with the exception of raffinose, which displayed an irregular response and never exceeded 30% gustatory acceptance between 2 and 1/512 M.

TABLE 2. REGRESSION ANALYSIS OF GUSTATORY RESPONSE OF FEMALE *Anaphes iole* WASPS TO HIGHLY STIMULATORY SUGARS

Sugar	EC_{50} moles (95% CI)[a]	Slope ± SE[b]	No. wasps	χ^2	df
Sucrose	0.026 (0.019–0.035) a	1.19 ± 0.07 B	825	63.8	31
Maltose	0.038 (0.026–0.054) a	0.99 ± 0.05 A	1000	92.5	36
Melezitose	0.152 (0.102–0.235) bc	1.06 ± 0.06 AB	1125	127	31
Erlose	0.166 (0.117–0.242) bd	1.12 ± 0.05 AB	1674	349	65
Glucose	0.192 (0.150–0.241) cd	2.48 ± 0.20 C	450	34.5	16
Fructose	0.280 (0.181–0.466) e	1.00 ± 0.06 B	953	137	37

[a] Lowercase letters denote EC_{50} values that are not significantly different as determined by estimation of 95% confidence limits for each ratio (Robertson and Preisler, 1992).
[b] Slopes that are not significantly different are followed by the same uppercase letters.

Ninety-two percent of the wasps accepted Eliminade™ ($N = 100$, SD = 5.66). Conversely, none of the wasps presented with *L. lineolaris* frass fed on it ($N = 34$). Our observations during the bioassays indicated that *A. iole* was neither repelled by nor attracted to the frass of its host. The behavior of *A. iole* did not appear to be affected after wasps encountered frass.

Feeding Deterrence of Sugar Mixtures. Results presented in Table 3 indicate that gustatory responses to the moderately stimulatory controls were similar to those observed in the acceptance threshold study. Response to the distilled water control was negligible (<1% for the study), which confirmed that wasps were satiated prior to experimentation and that gustatory response was not attributable to thirst. Only the rhamnose+maltose mixture significantly inhibited ($P < 0.05$) the feeding response by *A. iole* in comparison to maltose only.

Feeding Deterrence of Individual Sugars. All of the moderately stimulatory sugars were accepted by food and water deprived *A. iole* (Figure 2). In fact, with the exception of mannose and rhamnose, all sugars tested led to longer duration of feeding than did the distilled water control. The durations of feeding for mannose and rhamnose were not shorter than for the distilled water control ($P > 0.05$).

Chemical Analysis of L. lineolaris Frass. Results of HPAEC analysis indicated that the predominant carbohydrates in *L. lineolaris* frass were inositol, glucose, fructose, trehalulose, sucrose, and melezitose (Table 4). The glucose content (mean ± SD) in *L. lineolaris* frass was 0.59 ± 0.40 μg/mg dry weight frass. Trace amounts of other carbohydrates were also present (Figure 3). Concentrations of glucose and fructose were relatively consistent across the five trials, while concentrations of the other carbohydrates were variable (Table 4). Several compounds, inositol, trehalulose, and melezitose, were not detected in every trial. In four of the five trials, sucrose was the most abundant carbohydrate detected.

TABLE 3. TEST FOR FEEDING DETERRENCE OF MODERATELY STIMULATORY SUGARS MIXED WITH HIGHLY STIMULATORY SUGARS ON THE FEEDING RESPONSE OF *Anaphes iole* FEMALES[a]

Moderately stimulatory sugar (2 M)	1:1 Mixture with 1/2 M sucrose	1/2 M sucrose control	*t*-test[b]	1:1 Mixture with 1/2 M maltose	1/2 M maltose control	*t*-test[b]	Moderately stimulatory sugar (2 M) control	Distilled water control
Mannose	100	98.67	NS	100	100	NS	57.33	1.33
Galactose	97.33	100	NS	98.67	97.33	NS	34.67	0
Melibiose	97.33	100	NS	100	98.67	NS	13.33	1.33
Stachyose	93.33	92	NS	80	82.67	NS	13.33	0
Raffinose	98.67	98.67	NS	98.67	97.33	NS	44	1.33
Trehalose	97	88	NS	97	99	NS	41	0
Rhamnose	94	99	NS	74	99	<0.05	8	2

[a] Figures represent the percentage of wasps exhibiting a feeding response for each treatment averaged over all replicates. Tests for feeding deterrence of moderately stimulatory+highly stimulatory sugar mixtures were done using Student's *t*-test on arcsin transformed data.
[b] NS = Not significant ($t > 0.05$).

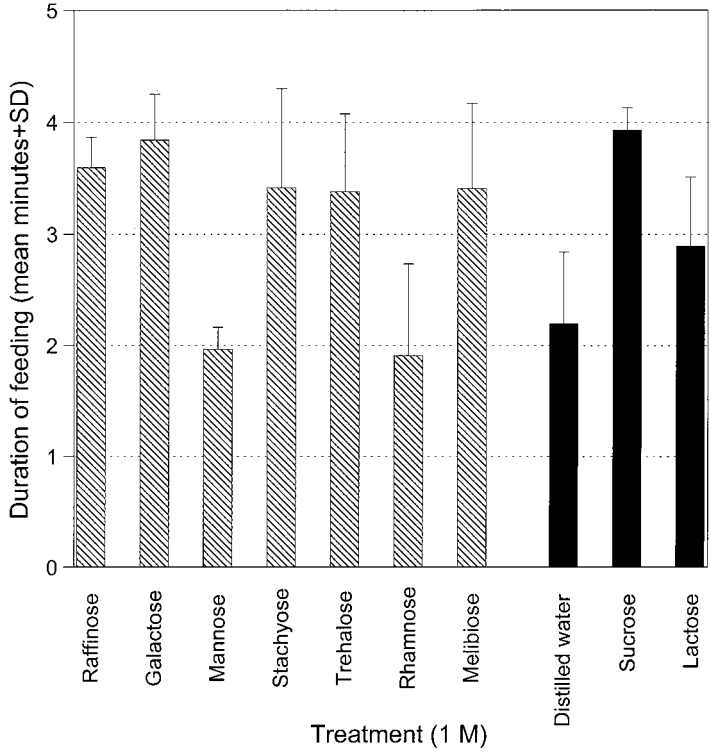

FIG. 2. Duration of feeding by food and water deprived *Anaphes iole* in response to moderately stimulatory sugars (1 M), and control treatments of distilled water, sucrose (1 M), and lactose (1 M) ($N = 45$ female wasps in each treatment).

TABLE 4. CARBOHYDRATE COMPOSITION OF *Lygus lineolaris* FRASS[a]

Trial	μg/mg dry weight frass[b]				
	Inositol	Fructose	Trehalulose	Sucrose	Melezitose
1	0.28	0.37	0.00	5.49	0.67
2	0.59	1.19	1.75	4.80	1.76
3	0.18	0.36	0.32	0.87	0.00
4	0.17	0.28	2.19	0.73	0.10
5	0.00	0.21	0.03	0.49	0.00

[a] Bugs were fed cotton, *G. hirsutum* L. (var. DPL NuCOTN 33B), seedlings prior to frass collection and analysis with high-performance anion-exchange chromatography.
[b] Amount of carbohydrate relative to glucose, based on 0.59 μg glucose/mg dry weight frass.

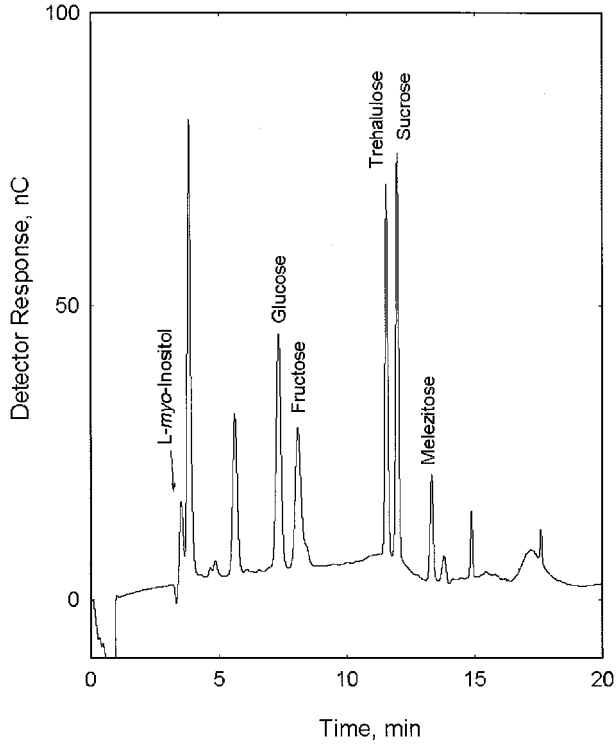

FIG. 3. Typical HPAEC chromatogram of *Lygus lineolaris* frass sugars. Analysis conditions as in Hendrix and Wei (1994).

DISCUSSION

Female *A. iole* wasps responded to all carbohydrates tested. Parasitoids were most sensitive to six sugars present in nectar (sucrose, glucose, and fructose), honeydew (glucose, melezitose, erlose, and fructose), and bee honey (maltose and erlose). We observed a greater than 10-fold range between EC_{50} values for these highly stimulatory sugars. *Anaphes iole* was most sensitive to sucrose, and it is interesting that this parasitoid was significantly more sensitive to sucrose than to its components, glucose and fructose. These sugars are usually the primary components of nectar (Baker and Baker, 1983a), an important food source for parasitoids (Jervis et al., 1993). Sucrose is the most widely found disaccharide in nature, and is the primary form in which fixed carbon and energy are translocated in plants. The acceptance thresholds for sucrose and other highly stimulatory sugars were much lower than the concentrations (10–50% w/v) at which they naturally occur (Baker and Baker, 1983a). High sensitivity to these carbohydrates by

A. iole would enable this wasp to exploit sources with low concentrations of these sugars. The remaining sugars were moderately stimulatory (<50% acceptance at 2 M). These sugars are found in bee honey (mannose, trehalose, raffinose, and melibiose), honeydew (trehalose, raffinose, and stachyose), plant seeds (raffinose, galactose, stachyose, and rhamnose), and phloem sap (raffinose, melibiose, and stachyose) (Baker and Baker, 1983a,b; Kuo et al., 1988; Nakajima et al., 1990; Donner, 1991; Hendrix et al., 1992; Wei et al., 1996). Collectively, these results suggest that *A. iole* can detect a wide range of potential food sources, some of them at low concentrations. In particular, it appears that nectar and honeydew are natural sources of sugars that *A. iole* can perceive, even if the sources have been diluted by precipitation or dew.

Similar feeding studies conducted with other hymenopterans allow us to put our results with *A. iole* into perspective (Table 5). For half of the sugars tested, *A. iole* exhibited lower acceptance thresholds than for any hymenopteran tested to date. Moreover, *A. iole* was the only hymenopteran tested that accepted all the sugars. Differences in sensitivity and range of carbohydrates detected by parasitoids may be a function of the insect's reproductive physiology. Like many egg parasitoids, *A. iole* is pro-ovigenic and produces eggs that rely on the host egg's protein for nourishment (Quicke, 1997). Conversely, the parasitoid studied by Wäckers (1999), *C. glomerata*, is synovigenic, meaning that female wasps emerge with a limited number of nutrient-rich eggs (Quicke, 1997). When provided with a protein-rich diet, synovigenic wasps can mature additional eggs. In the absence of food, these wasps sometimes resorb their eggs and utilize the resources for self-preservation. Therefore, protein may not be an important component in the diet of a pro-ovigenic wasp that emerges with a full complement of matured eggs. Carbohydrates, which provide a source of quick energy for locomotion, may play a more important role in the nutritional ecology of *A. iole* than proteins. Reliance on carbohydrates may explain the high sensitivity and range of perception of *A. iole* to sugars. Future studies with other pro-ovigenic and synovigenic parasitoids are necessary before the relationship between gustatory response and reproductive physiology for *A. iole* and *C. glomerata* can be generalized to other parasitoids.

Gustatory response may also be influenced by nectar accessibility. Wäckers (1999) proposed that responses by *Apis mellifera* L. and *C. glomerata* to sucrose, glucose, and fructose were related to morphological differences of mouthparts. *Apis mellifera*, strongly responsive to sucrose, but less so to glucose and fructose (von Frisch, 1934), is a long-tongued bee and is able to exploit floral nectaries rich in sucrose. *Cotesia glomerata* exhibited a similar response to sucrose as did *A. mellifera*, but was considerably more sensitive to glucose and fructose (Wäckers, 1999). *Cotesia glomerata* has unspecialized mouthparts, and may be unable to access floral nectaries of many species. Thus, this wasp may be forced to feed at exposed structures, like extrafloral nectaries, that produce nectar with greater proportions of glucose and fructose than do floral nectaries (Baker and

TABLE 5. SUGAR ACCEPTANCE THRESHOLDS IN *Anaphes iole* AND OTHER HYMENOPTERA

Sugar	*Anaphes iole*	*Cotesia glomerata*	*Apis mellifera*	*Myrmica rubra*	*Myrmica rubida*	*Lasius niger*
Glucose	0.0625	0.0625	0.25	0.02	0.0313	0.125
Fructose	0.0039	0.0156	0.25	0.02	0.0313	0.0156
Galactose	1	NR	2	0.5	NR	NR
Mannose	0.25	NR	NR	0.25	NR	NR
Rhamnose	0.5	NR	NR	1	NR	NR
Sucrose	0.0039	0.0625	0.0625	0.0067	0.0025	0.005
Trehalose	0.25	0.5	0.25	-	-	-
Maltose	0.0020	0.5	0.125	0.01	0.01	0.01
Melibiose	1	NR	NR	NR	NR	-
Lactose	0.5	NR	NR	NR	NR	NR
Raffinose	0.0039	NR	NR	0.0025	0.01	0.0025
Melezitose	0.0039	0.25	0.125	0.005	0.0156	0.01
Erlose	0.0039	0.0313	-	-	-	-
Stachyose	2	1	-	-	-	-
Reference	Current study	Wäckers (1999)	von Frisch (1934)	Schmidt (1938)	Schmidt (1938)	Schmidt (1938)

Note: NR = no response; - = not tested.

Baker, 1983a; Rogers, 1985). Patt et al. (1997) demonstrated that floral architecture and odor influenced the selection of floral food sources by two eulophid wasps. *Pediobius foveolatus* Crawford can utilize flowers with either exposed or partially exposed nectaries, while *Edovum puttleri* Grissell is limited to flowers with exposed nectaries. These results corresponded with comparisons between head width of these wasps and the gaps between petals and styles or stamens of different floral resources. In the present study, *Anaphes iole* exhibited the same acceptance threshold for sucrose and fructose (1/256 M), but the threshold for glucose was higher (1/16 M). Estimates of EC_{50}s indicated the order of sensitivity to be sucrose>glucose>fructose, i.e., that *A. iole* is most responsive to sucrose. Although *A. iole* has unspecialized mouthparts, its minute size (<1 mm in length) probably allows it access to floral resources, including flowers with long corollas that would exclude larger wasps with similar mouthparts. Thus, despite its unspecialized mouthparts, *A. iole* might exploit the same sucrose-rich flowers as larger insects with specialized mouthparts, such as *A. mellifera* and Lepidoptera. Our data support the idea that differential responsiveness to sugars is related to nectar accessibility (Wäckers, 1999).

Anaphes iole readily accepted Eliminade™, a protein-rich commercial food supplement developed for synovigenic parasitoids. Its acceptance by *A. iole* suggests that it is capable of utilizing complex food sources and that proteins and other ingredients in Eliminade™ that are not required for its diet are not necessarily deleterious to this parasitoid. However, further studies are necessary to elucidate the effects of Eliminade™ on longevity and other life history traits.

High sensitivity to a broad range of carbohydrates might allow *A. iole* to detect and exploit a variety of potential food sources, such as host frass. However, exposure of females to *L. lineolaris* frass did not induce feeding or other marked changes in behavior. Our gustatory response bioassays included several sugars (glucose, fructose, sucrose, and melezitose) that were detected in *L. lineolaris* frass. Although chemical analysis of frass did not permit direct comparison with results from gustatory response bioassays, the our results suggest that these sugars were present in concentrations below the detectable limits for *A. iole*. Parasitoids used in these studies were reared from host eggs embedded in an artificial media, Gelcarin® (FMC, Food Ingredients Division, Rockland, ME), and it is possible that these rearing conditions adversely affected the ability of *A. iole* to respond to frass excreted by hosts fed cotton. However, we believe that this is unlikely because other studies using *L. lineolaris* eggs oviposited in plant hosts have demonstrated that the host-searching behavior of *A. iole* reared from eggs embedded in Gelcarin® is not compromised (Williams, unpublished). Host frass apparently does not play a significant role in the nutritional ecology of *A. iole*.

Low to moderate acceptance of some of the sugars suggests that these compounds may act as mild feeding deterrents to *A. iole*. However, results from the feeding deterrence trials indicate that the moderate gustatory responses of wasps are

probably not based on deterrency. In the study with sugar mixtures, only rhamnose mixed with maltose inhibited the acceptance of maltose. Wäckers (1999) found that sucrose mixed with mannose or raffinose inhibited feeding by *C. glomerata* wasps. Reduced gustatory acceptance by *A. iole* to the moderately stimulatory sugars may be due to a paucity of sense receptors for these sugars.

Sensory receptors in insects occur on a wide range of structures (Chapman, 1998). In parasitic wasps, gustatory receptors are poorly understood, but may occur on the tarsi, antennae, and mouthparts (Quicke, 1997). In the honeybee, *A. mellifera*, proboscis extension is elicited by stimulation of any of these receptors (Bitterman et al., 1983). However, in the present study, we regularly observed antennal and tarsal contact of sugar solutions by *A. iole* wasps that did not elicit a feeding response. Acceptance or rejection of a solution was typically preceded by contact of the sugar solution with the mouthparts. These observations were also reported for *C. glomerata* (Wäckers, 1999), and suggest a role of mouthparts for sugar perception in parasitic Hymenoptera.

Understanding the possible chemosensory role of *A. iole* mouthparts is difficult in the absence of physiological and anatomical data. However, the molecular configuration of sugars and the type of receptor protein determine if a particular sugar is perceived (Chapman, 1998). Thus, correlation of *A. iole* gustatory response and sugar configuration might lead to a better understanding of the molecular basis of receptor sensitivity. *Anaphes iole* exhibited a higher sensitivity (more than 7× for EC_{50}) to sucrose than to its components, glucose and fructose. This sensitivity exceeds the 2× molecular weight difference between sucrose and its components, indicating that the sensitivity to sucrose apparently is not a result of weight concentration effects. The basis for this stimulatory synergy between glucose and fructose may lie in the linkage between the molecules, and will be the topic of future work. Several chemosensitive neurons (the sugar, amino acid, salt, and deterrent cells) comprise the contact chemoreceptor sensillae of insects (Chapman, 1998). In the calypterate dipterans, the structural requirements for two sugar receptor types have been described (Shimada et al., 1974; Shimada and Isono, 1978). The pyranose site is believed to bind with sugar molecules containing equatorial hydroxyl groups on C-2, C-3, and C-4. Compounds with such structures include sucrose, maltose, glucose, and trehalose. Mannose and rhamnose, with axial hydroxyl groups on C-2 and C-4, are believed to inhibit the pyranose site. Our results are consistent with this framework of pyranose receptor sensitivity and suggest that *A. iole* possess pyranose-type receptors. However, trehalose was only moderately accepted by the wasps (ca. 50% acceptance at 2 M). The reduced acceptance of rhamnose mixed with maltose suggests that rhamnose may interfere with the pyranose site. The furanose site is thought to bind with fructose, galactose, and mannose (Shimada et al., 1974; Shimada and Isono, 1978). *Anaphes iole* readily accepted fructose, but mannose and galactose were only moderately accepted. This insect would be regularly exposed to fructose but rarely, if ever, have the opportunity to feed upon

mannose or galactose. *Cotesia glomerata* likewise accepted fructose, but did not respond to mannose or galactose (Wäckers, 1999). The reason for this is unclear, but it is possible that sugar cells other than the pyranose or furanose-types occur on *A. iole* and *C. glomerata*. Widely differing results from experimental studies, with regard to the range of sugars accepted and their acceptance thresholds, suggest that receptor configurations differ between parasitic Hymenoptera and calyptrate Diptera.

The ability of *A. iole* to detect and utilize a broad range of food sources has practical implications for the development of a food supplement for this parasitoid. However, it must be remembered that *L. lineolaris* might also utilize a food supplement intended for *A. iole*. The presence of nectar can benefit pest herbivores (Schuster et al., 1976; Adjei-Maafo and Wilson, 1983; Belcher et al., 1984) as well as their natural enemies (Bugg et al., 1989; Stapel et al., 1997; Damon et al., 1999). Therefore, knowledge of gustatory discrimination in *L. lineolaris*, as well as *A. iole*, is critical for the development of a selective food supplement. Hatfield et al. (1982) identified carbohydrates, amino acids, and allelochemicals that act as feeding deterrents to *L. lineolaris* at certain concentrations. For example, 1 M sucrose inhibited feeding by *L. lineolaris* females, as did leucine concentrations $\geq$0.01 M. Our current investigations addressing the effects of potential food sources on perception, gustatory acceptance, and longevity of *L. lineolaris* and *A. iole* will guide the development of selective food supplements allowing successful integration of conservation biological control with inoculative releases of *A. iole* in noncrop habitats. Use of food supplements that benefit the biological control agent to a greater extent than the pest herbivore may have broad potential in other biological control programs. Ultimately, it may be possible to breed crops that produce nectar selectively benefiting natural enemies of pests (Rogers, 1985; Lewis et al., 1997; Cortesero et al., 2000).

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FLAVONOID INCREASE IN SOYBEAN AS A RESPONSE TO *Nezara viridula* INJURY AND ITS EFFECT ON INSECT-FEEDING PREFERENCE

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Abstract—The ability of the stink bug (*Nezara viridula*) to induce and/or increase production of chemical defenses, i.e., flavonoids, in immature seeds of five genotypes of soybean (BR-16, IAC-100, PI 227687, PI 229358, and PI 274454) was investigated under greenhouse and laboratory conditions. Samples from pods of each genotype damaged by stink bug were analyzed for flavonoid content with high performance liquid chromatography. A dual-choice test was conducted to evaluate the feeding preference of *N. viridula* comparing BR-16 pods treated with extracts of PI 227687 seeds (with and without stink-bug injury), with water-treated pods. Seeds of PI 227687 damaged by *N. viridula* presented the highest concentration (352 μg/g) of daidzin (4′-hydroxyisoflavone-7-glucoside). The same trend was observed with genistin (4′,5,7-trihydroxyisoflavone-7-glucoside): PI 227687 contained 142.4 μg/g, PI 274454, 31.6 μg/g, and PI 229358, 38.9 μg/g. Seeds damaged by stink bugs had higher isoflavone contents (daidzin and genistin), compared to controls. However, after being damaged, PI 274454 and PI 229358 produced less genistin than the other genotypes and no differences in concentration between damaged and nondamaged plants of this genotypes were observed. The numbers of observations of the insect feeding and the numbers of stylet sheaths left in water-treated BR-16 pods were greater than in those treated with PI 227687 extracts. The insects fed for longer periods on BR-16 pods treated with extract of PI 227687 without injury compared to those that were treated with extract of PI 227687

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previously injured by stink bugs. Extracts of PI 227687 pods (damaged or not) were deterrent to adults of *N. viridula*, and insect injury increased concentrations of daidzin and genistin in PI 227687 seeds. The deterrence seemed to be more pronounced after pods had suffered stink-bug injury.

Key Words—Resistance, green stink bug, Heteroptera, isoflavones, daidzin, genistin, deterrence.

INTRODUCTION

The majority of wild and cultivated plant species possess some degree of resistance to insects (Kogan, 1986) resulting from chemical factors that, in general, are secondary metabolites (Kubo and Hanke, 1986). Compared with other plant families (such as Cruciferae and Solanaceae) soybeans (*Glycine max* (L.) Merr.), and grain legumes in general, lack dominant secondary metabolites with remarkable allelochemic potential (Kogan, 1986). However, flavonoids have been identified in various soybean organs (Hoffmann-Campo, 1995). These natural products, synthesized from phenylpropanoid and acetate-derived precursors, play important defensive roles against microorganisms and insect pests (Dixon and Steele, 1999). Some of them have shown antifeedant and/or antibiotic effects against soybean pests (Neupane and Norris, 1990; Sharma and Norris, 1991; Hoffmann-Campo et al., 2001).

Depending on the compounds involved, soybean resistance can be constitutive (Smith, 1985) or inducible (Kogan and Fischer, 1991). Isoflavones are flavonoids regularly found in the Papilionoideae (Harborne, 1989) a subfamily of Leguminosae, to which soybean belongs. The most common soybean isoflavonoids are conjugates of daidzin (4′-hydroxyisoflavone-7-glucoside) and genistin (4′,5,7-trihydroxyisoflavone-7-glucoside). The isoflavonoid concentrations in soybean vary considerably among cultivars, due to both genetic and environmental factors (Carrão-Panizzi and Kitamura, 1995). Daidzin and genistin are mainly reported as constitutive, while the hydrolyzed aglycone daidzein has been considered an immediate precursor of the glyceollins (Graham et al., 1990). These inducible pterocarpan phytoalexins are primarily fungitoxic (Harborne, 1986; Graham et al., 1990), but they can also negatively affect the biology and feeding preferences of insects (Fischer et al., 1990; Kogan and Fischer, 1991).

Flavonoids were previously identified from soybean leaves (Hoffmann-Campo, 1995). A foliage-fraction of PI 227687, containing the flavonol rutin (quercitin 3-*O*-glucosyl rhamnoside) and the isoflavone genistin, negatively affects the behavior and physiology of *Heliothis zea* and *Trichoplusia ni* (Hoffmann-Campo, 1995; Hoffmann-Campo et al., 2001). Rutin also negatively affects *Anticarsia gemmatalis*, the most important defoliating pest of soybean in Brazil (Hoffmann-Campo, unpublished data). Nevertheless, the role of flavonoids in the

interaction between soybean seeds and stink bugs remains unclear. Seed-sucking stink bugs (*N. viridula, Euschistus heros*, and *Piezodorus guildinii*) are important pests of soybean in Brazil that reduce yield, seed quality, oil content, and delay crop maturity (Villas Bôas et al., 1990). We conducted this study to investigate, (i) the role of *N. viridula* damage in inducing flavonoid production in immature seeds of five soybean genotypes, and (ii) the effects of extracts obtained from injured or noninjured PI 227687 seeds on feeding preferences of *N. viridula*.

METHODS AND MATERIALS

Plant Material and Insects. Pods of resistant soybean plant introductions PI 227687, PI 229358, and PI 274454 alongside the cultivars IAC-100 (resistant) and BR-16 (susceptible) were produced under greenhouse conditions (mean temperature $23 \pm 2^{\circ}$C, mean relative humidity 78%). At reproductive stages R5 and/or R6 (Fehr and Caviness, 1977), four field-collected adult males of *N. viridula* were maintained for 18 hr in cages made from Petri dishes bottoms, containing two soybean pods. Pods were also kept in stink bug-free cages for use as controls. To perform the experiments, pods were collected and opened 72 hr after the removal of stink bugs from the cages.

Extraction and HPLC Analysis. A 0.10 g sample from the injured part of seeds (identified under a stereomicroscope) of each genotype was excised, macerated, and ground with a pestle adapted for 5-ml microcentrifuge tubes, and homogenized with 0.5 ml of 80% methanol (MeOH). After centrifugation (14,000 rpm, 10 min), the supernatant was dried under flowing N_2 and redissolved in 100 μl 80% MeOH. Aliquots of 20 μl were subjected to high-performance liquid chromatograph (HPLC, Shimadzu, model SPD-M10A VP) for isoflavonoid analysis.

The analyses were performed by using a reverse phase octadesyl group column (CLS-ODS-C18-M, 4.6 mm ID $\times$ 250 mm). The isoflavonoids were eluted in a linear gradient system (Graham, 1991) of 0–55% acetonitrile (CH_3CN, Merck, HPLC grade) water followed by a step increase to 100% acetonitrile for 2 min, returning to initial condition (water) at 30 min. The ultra-pure water was adjusted to pH 3 with acetic acid (CH_2H_4O, Vetec, UV/spectrometry grade). The flow rate of the solvent was 1.0 ml/min, and absorption was measured at 260 nm.

Isoflavonoid concentrations in the five genotypes were estimated by comparing the concentrations of daidzin and genistin in the plant samples with those obtained from pure compounds (standards). Equations were constructed by injecting concentrations of 0.1, 0.2, 0.4, and 0.8 mg/ml of daidzin (Fujicco), and 0.1, 0.2, and 0.4 mg/ml of genistin (Sigma). After sample injection, scannings of photo-diode arrays were performed to compare the UV spectra and retention times of each HPLC sample trace with those of the standards. The area corresponding

to each isoflavonoid trace was calculated and applied to the equation (standards) to estimate the concentration of daidzin and genistin in each genotype.

A randomized design was used in the experiments, and treatments were arranged in a factorial design (five genotypes, two treatments, and four replicates). Data were analyzed by ANOVA, and the isoflavone content averages of each genotype in the treatment (injured) and control (noninjured) were compared by the Tukey test ($P < 0.05$), using the SAS statistical (SAS Institute, 1996) and SANEST (Zonta et al., 1982).

Preparation of PI 227687 Extracts for the Feeding Experiment. Extracts of injured and noninjured PI 227687 seeds were prepared as described earlier (*Plant Material and Insects*). To prepare sufficient amounts of extract for the feeding experiment, whole seeds (instead of the damaged part only) were ground, centrifuged (4,000 rpm, 3 min), and the supernatant was rotoevaporated to dryness. Samples were kept at 4°C, until the beginning of the test, when they were redissolved in 100 ml of 80% MeOH. Distilled water (control) or PI 227687 extracts (injured or noninjured) were applied with a camel brush onto pods of BR-16 and dried with a hair dryer.

Dual-Choice Test. The tests were conducted in Petri-dish (9.1 cm diam. × 1.8 cm high) arenas lined with a moistened filter paper. Four soybean pods were placed in each, two of which had been treated with plain water (check) and two treated with injured or noninjured PI 227687 extracts, disposed alternately, as close as possible to the arena borders. There were 10 replicates of each treatment.

Four field-collected adult males of *N. viridula* were prestarved for 24 hr, and then placed in the center of each arena; one stink bug was marked with white ink (not toxic) and observed at 2-min intervals for 12 hr, using an adaptation of the method developed by Chambers et al. (1995). The activity of these marked insects, during the observation period, was entered manually into a computer spreadsheet. The behavior categories were, in motion, quiescent on a pod or in the arena, feeding on the extract-pods (injured or noninjured), or feeding on the water-pods. The data were analyzed to determine the time spent on each pod. Observations of behavior lasting only one interval were added to the precedent behavior observation, with duration longer than 2 min. At the end of the test, the numbers of stylet sheaths left in the pods by the stink bugs were evaluated by immersing seeds in fuchsin for 25 min, then washing and observing them under the stereomicroscope (Bowling, 1979).

The Wilcoxon matched-pairs test was used to analyze the number of observation and the average time spent in each behavior category, as well as the number of the stylet sheaths left by *N. viridula*. The following comparisons were made among the pod samples: (i) injured extract vs. noninjured extract; (ii) injured extract vs. water; (iii) noninjured extract vs. water; and (iv) water vs. water. The statistical tests were performed in SAS (SAS Institute, 1996) and SANEST (Zonta et al., 1982).

TABLE 1. DAIDZIN CONCENTRATION IN EXTRACTS PREPARED WITH SEEDS OF FIVE SOYBEAN GENOTYPES INJURED OR NONINJURED BY *Nezara viridula* ($N = 4$)

Genotypes	Daidzin concentration (μg/g) (mean $\pm$ SEM)	
	Injured	Noninjured
PI 227687	352.1 $\pm$ 49.0 a A[a]	75.0 $\pm$ 17.0 a B
IAC-100	268.4 $\pm$ 58.5 b A	0.0 $\pm$ 0.0 a B
BR-16	204.4 $\pm$ 24.9 bc A	25.8 $\pm$ 25.8 a B
PI 274454	128.1 $\pm$ 19.7 c A	0.0 $\pm$ 0.0 a B
PI 229358	28.5 $\pm$ 4.3 d A	12.3 $\pm$ 2.6 a B
F value – Genotype	12.5***	
F value – Treatment	90.7***	
F value – Genotype $\times$ Treat	6.64***	

[a] Means followed by the same lower-case letter in the column and upper-case letter in the row do not differ by Tukey's test ($P < 0.05$).
*** $P < 0.001$.

RESULTS AND DISCUSSION

Constitutive concentrations of daidzin of soybean did not significantly differ in the five genotypes we tested (Table 1). PI 227687 and PI 274454 had the highest and the lowest concentrations of genistin, respectively (Table 2). Seeds from pods damaged by *N. viridula*, in general, had higher isoflavone glycoside contents (daidzin and genistin), than those without damage (Tables 1 and 2; Figure 1). In field trials, Gilmar et al. (1982) reported that PI 227687 was moderately resistant to *N. viridula* damage; in our experiment, both before and

TABLE 2. GENISTIN CONCENTRATION IN EXTRACTS PREPARED WITH SEEDS OF SOYBEAN GENOTYPE INJURED OR NONINJURED BY THE STINK-BUG *Nezara viridula* ($N = 4$)

Genotypes	Genistin concentration (μg/g) (mean $\pm$ SEM)	
	Injured	Noninjured
PI 227687	142.4 $\pm$ 18.0 a A[a]	41.7 $\pm$ 11.5 a B
IAC-100	89.1 $\pm$ 9.0 b A	38.0 $\pm$ 7.1 ab B
BR-16	58.8 $\pm$ 5.2 c A	20.4 $\pm$ 3.5 bc B
PI 229358	38.9 $\pm$ 2.6 cd A	36.0 $\pm$ 6.3 ab A
PI 274454	31.6 $\pm$ 3.0 d A	15.4 $\pm$ 3.0 c A
F value – Genotype	20.61***	
F value – Treatment	64.58***	
F value – Genotype $\times$ Treat	8.79***	

[a] Means followed by the same lower-case letter in the column and upper-case letter in the row do not differ by Tukey's test ($P < 0.05$).
*** $P < 0.001$.

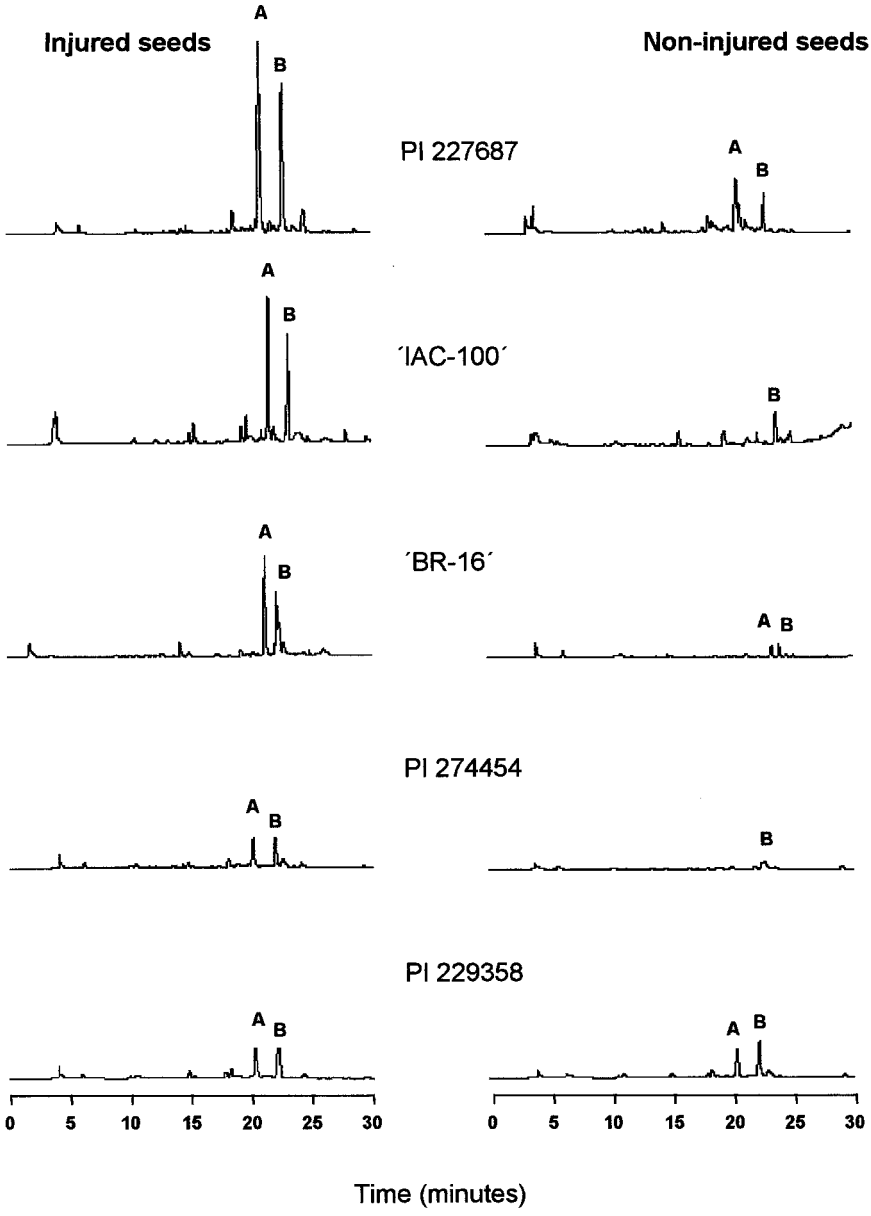

FIG. 1. HPLC traces of extracts prepared with injured or noninjured seeds of soybean genotypes. A = Daidzin; B = Genistin.

after insect damage, PI 227687 had the highest concentrations of daidzin and genistin.

After *N. viridula* injury, the concentration of daidzin increased, at least to some extent, in the seeds of all the genotypes. PI 274454 and PI 229358 produced less genistin than the other genotypes even after being damaged by stink bugs, and showed no differences in isoflavone concentration between damaged and non-damaged pods. In fact, substantial genetic diversity among soybean genotypes has been observed in regard to isoflavone content (Carrão-Panizzi and Kitamura, 1995) and capacity to respond to factors of induced resistance (Kogan and Fischer, 1991).

The phytoalexin glyceollin was not detected in any of the genotypes. However, isoflavone concentrations increased in most cases, following stink bug injury. It is known that the aglycone daidzein (hydrolyzed daidzin) can be a precursor of glyceollin and that concentrations of aglycone and glyceollins increase rapidly at the same time that daidzin conjugates, including glycoside, decrease to nearly non-detectable levels 48 hr after fungal (*Phytophthora megasperma*) infection (Graham et al., 1990). The type and timing of reaction to phytoalexin turnover may differ depending on the elicitor; we found that daizin and genistin (glycosides) greatly increased in concentration 72 hr after stink-bug injury, therefore, our results are not comparable to those of Graham et al. 1990.

Preference Test. The average numbers of observations of insect-feeding on BR-16 water-treated were about four and three times higher when compared to pods treated with extracts of injured and noninjured PI 227687, respectively (Figure 2). Insects were more frequently observed to be quiescent on water-treated pods than on extract-treated pods (injured and noninjured), suggesting that PI 227687 extract, even when prepared from seeds without *N. viridula* injury, was deterrent to adults of *N. viridula*.

Fewer stylet sheathes were observed on BR-16 pods treated with PI 227687 extracts (injured and noninjured) than on water-treated pods (Table 3). There were no differences in the numbers of stylet sheaths among the treatments with PI 227687 injured and noninjured pods and water-treated pods. However, the average time of stink-bug feeding on pods where noninjured seed extract was applied was significantly greater than on pods with injured seed extract (Table 4). This suggests that after *N. viridula* injury, the deterrent effect of the PI 227687 increased, since the insect remained feeding on noninjured seed extract for longer period of time. According to Harborne (1986), plants can respond rapidly to herbivory by becoming increasingly unpalatable. Additionally, it is known that a cascade of enzyme reactions occurs in leaves in response to insect attack, altering flavonoid profiles and, thus, host selection (Simmonds, 2001), likely by decreasing host palatability. Probably this phenomenon occurs not only for *de novo* production of compounds (phytoalexins), but also to increase the amounts of constitutive compounds already present in plants.

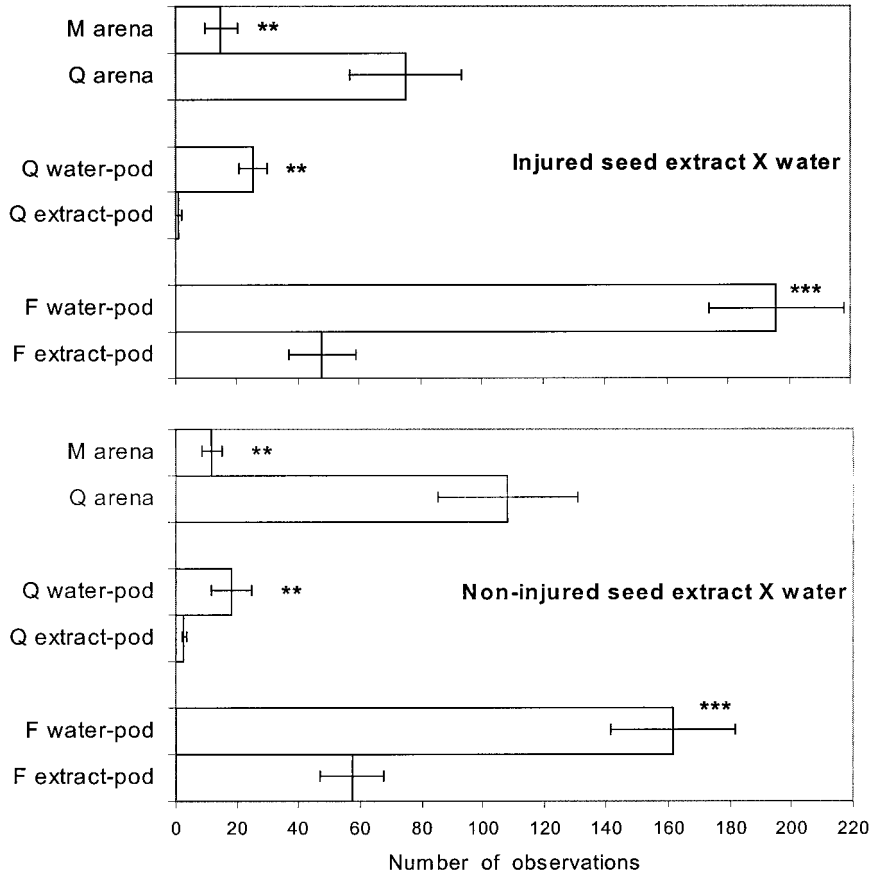

FIG. 2. Number (mean ± SEM) of 2-min-interval observations of *Nezara viridula* behavior categories (M = in motion; Q = in quiescence; F = feeding), giving them dual-choice for pods of BR-16 treated with extract of PI 227687 injured seeds × water or extract of PI 227687 noninjured seeds × water ($N = 10$). Wilcoxon matched-pairs test: $^{**}P < 0.01$; $^{***}P < 0.001$.

The isoflavonoids are a subclass of flavonoid that exhibit a wide range of biological proprieties (Williams and Harborne, 1989). They are the largest group of inducible phytoalexins, and, although fungitoxicity is their most characteristic biological activity (Harborne, 1986; Graham et al., 1990), phytoalexins of legumes can also be toxic (Harborne, 1986) or deterrent (Hart et al., 1983) to insects.

The biology of *N. viridula* was affected by PI 227687. Higher mortality, lower initial and final weights, less growth, smaller weight gain, and less lipid accumulation were all associated with this genotype (Piubelli, 2000). This may

TABLE 3. NUMBERS OF *Nezara viridula* STYLET SHEATHS LEFT IN PODS OF BR-16 WITH OR WITHOUT PI 227687 EXTRACT PREPARED WITH INJURED OR NONINJURED SEEDS ($N = 10$)

Pods	Stylet Sheaths (mean ± SEM)[a]		
	Injured	Noninjured	*P*
BR-16 + PI 227687 extract	7.8 ± 1.9	6.6 ± 1.2	NS
BR-16 + Water	14.1 ± 1.0	14.1 ± 2.9	NS
P	*	*	

[a] Comparison between treatments on the column and on the row, Wilcoxon matched-pairs test.
* $P < 0.05$, NS = not significantly different.

have been due to the presence of isoflavones. Daidzin and genistin are known to be toxic to mosquito larvae (Rao et al., 1990), and the aglycone daidzein is known to have antibiotic and antifeeding effects on soybean pests, such as *Trichoplusia ni* (Sharma and Norris, 1991).

Methanolic extracts of PI 227687 seeds affected stink-bug behavior. After being injured, this genotype increased its isoflavone contents, thus enhancing deterrence of *N. viridula*. Wild relatives of cultivated species generally contain a greater diversity of constitutive defense compounds. It is also known that some primitive soybean lines can maintain detectable levels of induced resistance 30 days after elicitation (Kogan, 1999). It is likely that soybean and other crops are losing mechanisms of defense against pests as a result of breeding for higher yield and other agronomic traits (Kogan, 1999). Thus, the identification of genotypes with rapid, intense, and persistent responses to stresses should be seen as an important component of breeding programs to protect plants during their most sensitive stages of development (Kogan and Fischer, 1991).

Cultivars with at least moderate levels of resistance, combined with other integrated pest-management techniques, can diminish or even eliminate pesticide applications, thus contributing to agronomic sustainability. Regarding isoflavones and many other flavonoids, there are additional advantages: they possesses

TABLE 4. FEEDING-TIME AVERAGES FOR *Nezara viridula* ON PODS OF BR-16 WITH OR WITHOUT PI227687 EXTRACT PREPARED WITH INJURED OR NONINJURED SEEDS ($N = 10$)

Pods	Feeding Time (min) (mean ± SEM)[a]		
	Injured	Noninjured	*P*
BR-16 + PI 227687 extract	29.2 ± 5.3	65.8 ± 15.1	*
BR-16 + Water	51.2 ± 8.7	70.4 ± 25.1	NS
P	NS	NS	

[a] Comparison between treatments on the column and on the row, Wilcoxon matched-pairs test.
* $P < 0.05$, NS = not significantly different.

antioxidant properties and health-promoting "nutraceutical" qualities (Dixon and Steele, 1999), augmenting considerably the interest in their potential for metabolic engineering. Consequently, soybean cultivars with high-isoflavone content have various potential advantages and should, therefore, be maintained in germplasm collections and used in breeding programs.

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CHEMICAL ANALYSIS OF VOLATILES EMITTED BY *Pinus sylvestris* AFTER INDUCTION BY INSECT OVIPOSITION

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Abstract—Gas chromatography – mass spectrometry analyses of the headspace volatiles of Scots pine (*Pinus sylvestris*) induced by egg deposition of the sawfly *Diprion pini* were conducted. The odor blend of systemically oviposition-induced pine twigs, attractive for the eulophid egg parasitoid *Chrysonotomyia ruforum*, was compared to volatiles released by damaged pine twigs (control) that are not attractive for the parasitoid. The mechanical damage inflicted to the control twigs mimicked the damage by a sawfly female prior to egg deposition. The odor blend released by oviposition-induced pine twigs consisted of numerous mono- and sesquiterpenes, which all were also present in the headspace of the artificially damaged control twigs. A quantitative comparison of the volatiles from oviposition-induced twigs and controls revealed that only the amounts of (*E*)-β-farnesene were significantly higher in the volatile blend of the oviposition-induced twigs. Volatiles from pine twigs treated with jasmonic acid (JA) also attract the egg parasitoid. No qualitative differences were detected when comparing the composition of the headspace of JA-treated pine twigs with the volatile blend of untreated control twigs. JA-treated pine twigs released significantly higher amounts of (*E*)-β-farnesene. However, the JA treatment induced a significant increase of the amount of further terpenoid components. The release of terpenoids by pine after wounding, egg deposition, and JA treatment is discussed with special respect to (*E*)-β-farnesene.

Key Words—Egg deposition, terpenoids, monoterpenes, sesquiterpenes, induction, jasmonic acid, systemic effect, *Pinus sylvestris*, parasitoids, sawfly, Diprionidae, Eulophidae.

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INTRODUCTION

Plants are able to respond to herbivore damage with direct or indirect defenses (see overviews in Karban and Baldwin, 1997; Agrawal et al., 1999; Dicke, 1999; Dicke and van Loon, 2000; Walling, 2000; Kessler and Baldwin, 2002). While direct defenses act directly against the herbivore by e.g., production of toxins or repellents (Edwards and Wratten, 1987; Baldwin, 1994; Duffey and Stout, 1996), indirect plant defenses employ the third trophic level, carnivores (Price et al., 1980, Price, 1986). Numerous studies show that a plant can attract predators and parasitoids of their herbivores by release of volatiles in response to herbivore attack (e.g., Dicke et al., 1990; Turlings et al., 1990; De Moraes et al., 1998; Dicke and Vet, 1999). This induction of volatiles results in a change of plant odor that may be a quantitative alteration (i.e., the volatiles released by the undamaged and damaged plants are the same, but are released in differing amounts) or a qualitative one (i.e., the damaged plant produces components that are not emitted by the undamaged plant) (e.g., Boland et al., 1999; Dicke, 1999; Paré et al., 1999). The herbivore-induced change of a plant's odor can be specific both for the plant and herbivore species, for the plant's age, and for the attacking herbivore stage (e.g., Takabayashi et al., 1994; De Moraes et al., 1998; Stout and Bostock, 1999). Release of induced volatiles is often not restricted to the area of feeding damage, but the signal may be transported systemically to undamaged plant parts (e.g., Turlings et al., 1993; Dicke, 1994; Karban and Baldwin, 1997).

Several wound signals are involved in induced plant defenses (Aducci, 1997; Staswick and Lehman, 1999, and references therein). Jasmonates have intensively been studied in the context of plant defense against herbivores (Sembdner and Parthier, 1993; Creelman and Mullet, 1997; Beale and Ward, 1998). Exogenous application of jasmonic acid (JA) or methyl jasmonate to undamaged plants was found to induce a quantitative and qualitative change of the plant's odor that is similar to the change of volatiles induced by infestation with herbivores (Hopke et al., 1994; Boland et al., 1995, 1999; Koch et al., 1999; Ozawa et al., 2000). Even though JA-induced plant volatiles were found to be attractive to predators and parasitoids (Dicke et al., 1999; Gols et al., 1999; Thaler, 1999), some carnivores are able to distinguish between odor from herbivore- and that from JA-induced plants (Dicke et al., 1999).

Apart from feeding, egg deposition by herbivores can also induce plants to emit volatiles that attract specialized egg parasitoids. This was shown in two different tritrophic systems:

(1) Meiners and Hilker (1997) demonstrated that the eulophid egg parasitoid *Oomyzus gallerucae* is attracted by volatiles of the field elm (*Ulmus minor*) induced locally and systemically by oviposition of the elm leaf beetle *Xanthogaleruca luteola*. Emission of volatiles attractive to the egg

parasitoid is also inducible by application of JA (Meiners and Hilker, 2000). Analyses of volatiles emitted by elm leaves revealed that egg deposition induces the emission of a complex odor blend qualitatively and quantitatively different from the odor of undamaged elm leaves. Treatment of elm leaves with JA also results in a qualitative and quantitative change of the plant's odor, which almost exclusively consists of terpenoids (Wegener et al., 2001).

(2) In the second tritrophic system, oviposition by the herbivorous sawfly *Diprion pini* (Hymenoptera, Diprionidae) induces Scots pine (*Pinus sylvestris*) to produce volatiles that attract the egg parasitoid *Chrysonotomyia ruforum* (Hymenoptera, Eulophidae). Prior to oviposition, a female of *D. pini* slits the pine needle with its ovipositor. Mimicking this mechanical damage with a razor blade does not induce pine twigs to emit attractive volatiles. The elicitor is located in the oviduct secretion that envelopes the sawfly eggs, since application of oviduct secretion (without eggs) elicits the release of attractive volatiles. The oviposition-induced plant response is systemic, i.e., volatiles emitted from needles adjacent to the oviposition sites are attractive towards the egg parasitoids 72 hr after egg deposition. As has been shown in the elm system, exogenous application of JA results in an induction of attractive volatiles (Hilker et al., 2002a,b; Hilker and Meiners, 2002).

In this study, we conducted GC-MS (gas chromatography – mass spectrometry) analyses of the headspace of *P. sylvestris* induced systemically by egg deposition of *D. pini*. To detect the volatiles that attract the egg parasitoid, the attractive odor blend of systemically oviposition-induced pine was compared to the nonattractive odor released by artificially damaged pine twigs mimicking the slitting conducted by a *D. pini* female prior to oviposition. In addition, the odor blend of attractive JA-treated pine twigs was analyzed.

METHODS AND MATERIALS

Plants and Insects. All branches of *P. sylvestris* used for analyses were detached from crowns of 35- to 45-year-old trees in the forests near Berlin. The lower part of the stems was cleaned and sterilized according to Moore and Clark (1968). *D. pini* was reared in the laboratory as described by Bombosch and Ramakers (1976) and Eichhorn (1976) at 25°C, L/D = 18:6, and 70% relative humidity.

Plant Treatments, General. Small pine twigs with about 90–120 needles were cut, placed into water, and treated for a period of 72 hr at 25°C, L/D = 18:6, and approximately 2000 lux. All treatments were conducted as described by Hilker et al. (2002a). Headspace samples were taken from the twigs immediately after treatment (see below).

Systemically Oviposition-Induced Pine Twig. Females of *D. pini* were offered the lower half of a pine twig for egg deposition, while the upper part was wrapped in a polyethylene terephthalate (PET) foil to avoid egg deposition and adsorption of volatiles from other parts of the twig or sawflies. This ensured that collected volatiles originated from plants only. The bag was ventilated with purified air through an in- and outlet. When *D. pini* females had laid at least eight egg clusters after 72 hr on the lower part of the pine twig, the foil wrapping the upper half without eggs was removed. The upper half of the twig was cut off, and volatiles from this systemically oviposition-induced half of the pine twig were collected.

Artificially Damaged Pine Twig. This treatment served as control for the systemically oviposition-induced twigs. Because terpenoid concentrations in pine species are known to show a high genotypic variability (e.g., Petrakis et al., 2001) and even vary within an individual tree (e.g., Gershenzon and Croteau, 1991; Manninen et al., 2002), control twigs were taken from the same large pine branch as the twigs for the oviposition-induction treatment and cut next to them. Control twigs were kept under the same conditions as the oviposition-induced ones, but 10 needles of the lower half of a pine twig were slit with a scalpel to mimic the mechanical damage that a *D. pini* female conducts with her ovipositor prior to oviposition. Headspace samples were taken from the upper, undamaged half of this pine twig mechanically damaged in its lower half.

Treatment of Pine Twigs with Jasmonic Acid. Pine twigs were supplied with 0.3 μmol/ml JA dissolved in an aqueous Tween 20 solution (0.05%). This concentration of JA induces the emission of attractive volatiles for the parasitoid *C. ruforum* (Hilker et al., 2002a). Control twigs were provided with Tween 20 solution only and taken from the same branch next to the ones used for the JA treatment.

Collection of Headspace Volatiles and Internal Standard. Volatile compounds emitted by pine twigs were collected using a dynamic headspace sampling method. A control twig that was compared with an oviposition-induced pine twig was cut from the same large branch as the twig for induction by oviposition. A control that was compared with a JA-treated pine twig was taken from the same large branch as the twig for JA treatment. Thus, test and control twigs may be considered as paired samples (see below statistical analyses). The cut end of a twig was carefully and tightly wrapped with Parafilm® during the sampling period of 5 hr. To collect volatiles from test and control twigs, the air was initially purified by passing over activated charcoal, then split before entering the flasks (250 ml) containing either the test or the control samples. Volatiles of each sample were collected separately on 5-mg charcoal filters and eluted with 50-μl dichloromethane containing 125 ng/μl methyl octanoate as internal standard. Two flowmeters (Supelco, Germany) ensured a constant airflow of 110 ml/min. All components of the sampling setup were connected with teflon tubing. The conditions during sampling were the same as those used for the treatments (see above).

Chemical Analysis of Headspace Samples. Headspace samples were analyzed by coupled GC-MS on a Fisons GC model 8060 and a Fisons MD 800 quadrupole MS using a J&W 30-m DB5-ms capillary column (0.32-mm I.D., film thickness 0.25 μm). Samples (1 μl) were injected in splitless mode (injector temperature 240°C) with helium as the carrier gas (inlet pressure 10 kPa). The temperature program started at 40°C (4-min hold) and rose 3°C/min to 280°C. The column effluent was ionized by electron impact (EI) ionization at 70 eV. Mass range was from 35 to 350 m/z with a scan time of 0.9 sec and an interscan delay of 0.1 sec. Eluted compounds were identified by comparing mass spectra and linear retention indices with those of authentic reference compounds or with NIST library spectra. Retention indices were calculated for each compound according to van den Dool and Kratz (1963) and compared to critically evaluated tabulated data (Adams, 1995; Joulain and König, 1998).

Enantiomer Separation. Enantiomer separations of several chiral monoterpenes were performed on an Hewlett Packard GC 6890 coupled to a MSD 5973, equipped with a 35-m capillary column (0.25-mm I.D.) coated with hydrodex-6-TBDMS (Macherey-Nagel, Düren, Germany). Helium was used as the carrier gas at a flow rate of 1 ml/min. The temperature of the gas chromatograph was set at 50°C. The injection port temperature was 200°C. Samples were injected in splitless mode. The mass spectrometer was operated in EI ionization mode (70 eV).

The enantiomers of limonene, α-pinene, and β-pinene were obtained from Fluka (Taufkirchen, Germany). (+)-Sabinene, which contained minor amounts of (−)-sabinene, was purchased from Carl Roth GmbH & Co., KG (Karlsruhe, Germany), while racemic and (−)-camphene were obtained from Sigma-Aldrich (Taufkirchen, Germany) as was (+)-3-carene. The enantiomer (−)-3-carene, which is a major component of the essential oil of black pepper (König et al., 1992), was obtained by steam distillation of a commercially available sample (coarse variety, ALDI, Germany). While (−)-β-phellandrene occurs in *P. sylvestris* (Borg-Karlson et al., 1993; Sjödin et al., 1996), (+)-β-phellandrene was obtained by SPME sampling of leaves of lovage (*Levisticum officinale* Koch.), in which it was the major component (König et al., 1992). 2-Carene is known to occur naturally only as the (+)-enantiomer (Fugmann et al., 1997). All the mentioned enantiomers separated on the hydrodex column (see above). The only problem occurred with (+)-limonene, which eluted in the beginning of the (−)-β-phellandrene peak. However, careful analysis of the ion traces of the characteristic ions of these compounds [(+)-limonene $m/z = 68$, (−)-β-phellandrene $m/z = 93$] allowed determination of their relative amounts. The retention times (in minute) of the compounds were as follows: (−)-α-pinene (21.0), (+)-α-pinene (22.3), (−)-camphene (24.6), (+)-camphene (26.8), (+)-sabinene (27.3), (+)-β-pinene (29.9), (−)-sabinene (30.3), (−)-β-pinene (31.8), (+)-3-carene (34.0), (−)-3-carene (35.3), (−)-limonene (40.9), (+)-limonene (44.4), (−)-β-phellandrene (44.5), (+)-β-phellandrene (45.7).

Statistical Analysis. Headspace samples were taken from 10 oviposition-induced twigs and 10 respective controls (artificial damage), as well as from 10 JA-treated twigs and 10 respective controls. A calibration with a set of pure standards and the internal standard had been performed to calculate the response factors. Peak areas of the components compared to the peak areas of the internal standard were quantified with respect to the response factors. Limonene and β-phellandrene were not separated on the DB-5 column. Analogous to the enantiomeric separation of these two components, careful analysis of the area of the ion traces ($m/z = 68$ for limonene and $m/z = 93$ for β-phellandrene) with respect to the portion of these ions of the total ion current allowed the determination of their relative quantities (compare formula given in Table 1 for details of calculation). The relative peak areas of test and control twigs were statistically compared as paired samples by the Wilcoxon matched pairs test using the software program SPSS (SPSS, Inc.). Ratios of enantiomers in test and control twigs were also compared by the Wilcoxon matched pairs test. Numbers of samples analyzed quantitatively differ from the number of samples in which enantiomer separation was performed, because the quantitative analyses were conducted first, followed by enantiomer separation of the remaining samples by use of different equipment.

RESULTS

Compounds detected in at least 5 of 10 samples of the oviposition-induced pine twigs are listed in Table 1. A characteristic total ion current chromatogram of the headspace of an oviposition-induced pine twig is shown in Figure 1. All components detected in the headspace of oviposition-induced pine twigs and the JA-treated ones were also found in the respective controls, i.e., no qualitative change in the headspace of pine twigs occurred because of oviposition or JA treatment. The pine twigs emitted predominantly mono- and sesquiterpenes regardless of their treatment. The major compounds in the headspace of oviposition-induced pine twigs were myrcene, 3-carene, and β-phellandrene (at least 10% of total peak area, Table 1, Figure 1). These three compounds were also dominant in the headspace of JA-treated pine twigs, but here additionally α-pinene and β-pinene occurred as major components.

The volatile pattern of JA-treated pine twigs was not as consistent as oviposition-induced twigs. Only 9 out of 31 compounds could be detected in the headspace of JA-treated pine twigs as often as in oviposition-induced pine twigs (Figure 2; see numbers written in columns for components no. 2, 3, 6, 21, 24, 26, 27, 30, 31). Only one component, α-humulene (no. 25), was detected more often in JA-treated samples than in oviposition-induced ones. All other components of the headspace of JA-treated pine twigs were detected less frequently than in the volatile blend of oviposition-induced pine twigs.

TABLE 1. LIST OF VOLATILES COLLECTED FROM *P. sylvestris* TWIGS AFTER DIFFERENT TREATMENTS[a]

No.	Compound	Oviposition induced	Control	JA induced	JA control
1	Thujene	2 ± 0.4	1 ± 0.3	2 ± 0.3	2 ± 0.5
2	α-Pinene	46 ± 10.8	60 ± 14.4	100 ± 15.3	154 ± 34.5
3	Camphene	3 ± 0.8	5 ± 1.8	6 ± 1.0	5 ± 0.6
4	Sabinene	22 ± 5.3	21 ± 6.6	55 ± 15.7	43 ± 13.3
5	β-Pinene	29 ± 12.2	22 ± 4.4	79 ± 42.6	94 ± 31.7
6	Myrcene	170 ± 65.4	131 ± 27.6	142 ± 35.5	138 ± 35.0
7	3-Carene	432 ± 76.3	423 ± 104.4	360 ± 130.5	359 ± 145.9
8	2-Carene	4 ± 0.9	5 ± 1.9	10 ± 2.8	7 ± 2.5
9	*p*-Cymene	10 ± 1.6	12 ± 4.4	20 ± 7.1	16 ± 6.1
10	Limonene	12 ± 2.3	13 ± 3.3	26 ± 7.8	18 ± 8.6
11	β-Phellandrene	107 ± 37.0	70 ± 15.6	68 ± 34.1	65 ± 47.2
12	(*E*)-β-Ocimene	2 ± 0.4	2 ± 0.6	7 ± 2.6	1 ± 0.6
13	γ-Terpinene	5 ± 1.0	7 ± 2.7	13 ± 3.3	9 ± 3.1
14	Unknown monoterpene, B93, M136	3 ± 0.6	4 ± 1.9	8 ± 2.5	6 ± 2.2
15	Terpinolene	26 ± 5.7	36 ± 15.0	48 ± 18.1	34 ± 14.8
16	*p*-Cymenene	4 ± 0.8	5 ± 1.9	11 ± 4.1	9 ± 3.2
17	1,3,8-*p*-Menthatriene	7 ± 1.5	10 ± 4.1	28 ± 9.6	21 ± 8.4
18	Unknown monoterpene, B119, M152	5 ± 1.1	8 ± 3.7	6 ± 2.3	4 ± 2.6
19	(*E*,*E*)-2,6-dimethyl-3,5, 7-octatrien-2-ol	7 ± 1.6	10 ± 5.2	8 ± 2.8	5 ± 3.4
20	Thymyl methyl ether	2 ± 0.7	1 ± 0.2	2 ± 0.6	2 ± 1.7
21	Bornyl acetate	1 ± 0.3	1 ± 0.5	1 ± 0.3	1 ± 0.3
22	α-Copaene	1 ± 0.5	1 ± 0.4	1 ± 0.3	1 ± 0.2
23	β-Elemene	2 ± 0.7	3 ± 1.0	3 ± 0.7	3 ± 1.3
24	β-Caryophyllene	15 ± 5.0	19 ± 5.2	19 ± 5.4	17 ± 6.3
25	α-Humulene	3 ± 0.7	3 ± 0.7	2 ± 0.7	2 ± 0.9
26	(*E*)-β-Farnesene	7 ± 2.7	5 ± 2.8	43 ± 19.8	12 ± 7.3
27	Germacrene D	6 ± 1.1	9 ± 2.0	3 ± 0.9	3 ± 1.0
28	Bicyclogermacrene	6 ± 1.8	7 ± 2.0	4 ± 1.1	3 ± 1.1
29	α-Muurolene	2 ± 0.6	2 ± 0.4	1 ± 0.2	1 ± 0.3
30	γ-Cadinene	7 ± 2.7	8 ± 2.5	4 ± 1.4	2 ± 0.9
31	δ-Cadinene	11 ± 3.8	10 ± 2.7	5 ± 1.4	3 ± 0.8

[a] Only compounds that were identified in at least 5 out of 10 samples of oviposition-induced pine twigs are given. Values are given in ng/μl/sampling unit $\pm$ standard error. Numbers of replicates for each component are given within the columns of Figure 2. Limonene and β-phellandrene were not separated from each other on DB-5 column. The formula given below was used to calculate the relative quantities of limonene and β-phellandrene. X: portion of limonene in TIC-peak of β-phellandrene + limonene, Y: portion of β-phellandrene in TIC-peak of β-phellandrene + limonene, A_{68}: peak area of ion trace 68; A_{93}: peak area of ion trace 93; B_{68}: portion of mass 68 of the total ion yield of a limonene pure sample (%); B_{93}: portion of mass 93 of the total ion yield of a limonene pure sample (%); C_{93}: portion of mass 93 of the total ion yield of a β-phellandrene pure sample (%).

$$X = \frac{A_{68} \cdot \frac{C_{93}}{B_{68}}}{A_{68} \cdot \frac{C_{93}}{B_{93}} + \left(A_{93} - A_{68} \cdot \frac{C_{93}}{B_{68}} \cdot \frac{B_{93}}{B_{68}}\right)} \qquad Y = 1 - X$$

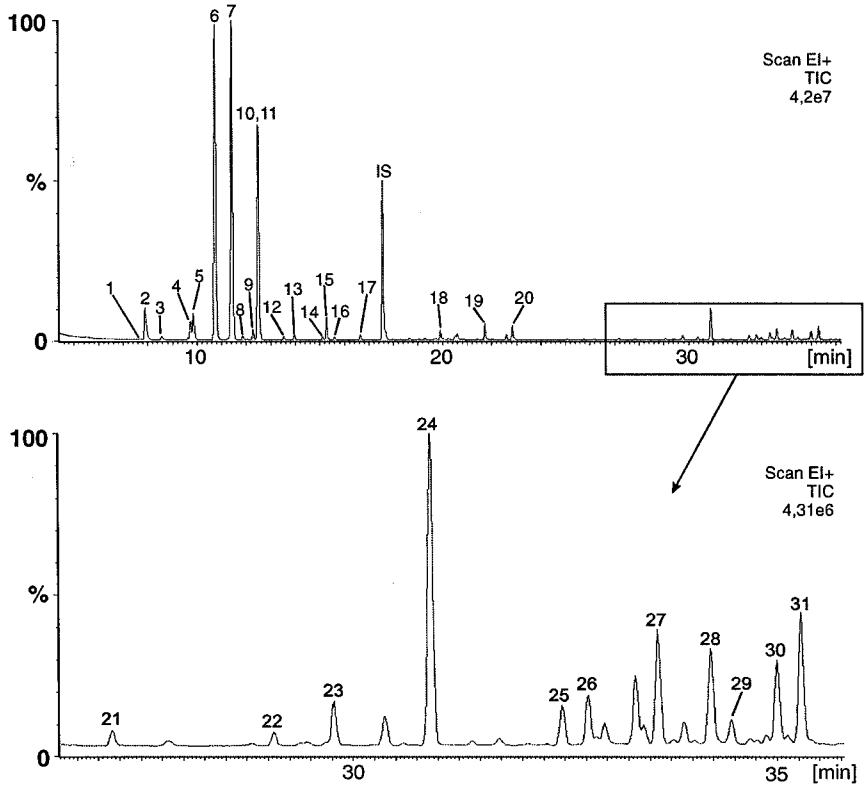

FIG. 1. Total ion current chromatogram of the headspace volatiles from pine twigs after egg deposition of *D. pini*. Numbers of peaks refer to compounds listed in Table 1. IS: internal standard (125 ng/μl methyl octanoate). Peaks without numbers denote compounds not present in more than five samples.

Separation of enantiomeric monoterpenes revealed that α-pinene, camphene, β-pinene, and limonene were present in both enantiomeric forms whereas sabinene and β-phellandrene were found only as (−)-enantiomers in all samples. 3-Carene was detected only as (+)-enantiomer. None of the treatments resulted in a significant change of the enantiomeric composition of the chiral monoterpenes analyzed (Table 2).

Since neither oviposition nor JA-treatment induced a qualitative change of pine volatiles, samples were analyzed statistically for quantitative differences. As characteristic for terpenoid contents in conifers (Latta et al., 2000, and references therein), the absolute quantities of emitted compounds varied a great deal among samples (Table 1). However, we wanted to elucidate whether quantities of volatiles

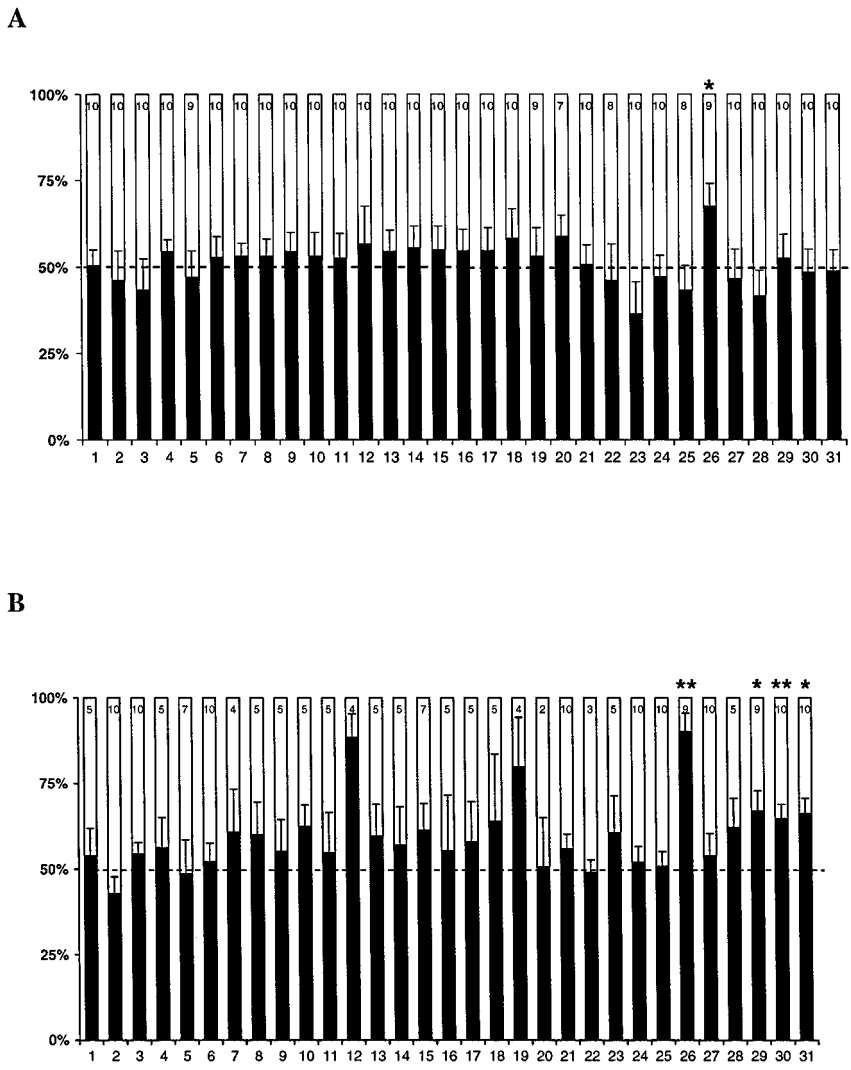

FIG. 2. Percentages of the relative amounts of compounds from treated twigs (dark columns) and control twigs (white columns) are given. The emitted amounts of a volatile component emitted by the test twigs and control twigs were considered 100%. (A): Oviposition-induced twigs (dark columns) compared with artificially damaged twigs (white columns); (B): Twigs treated with JA (dark columns) compared with untreated twigs (white columns). Mean ± standard error, columns refer to the detected compounds given in Table 1, numbers inside the columns indicate number of paired samples in which the compounds have been identified. Statistical analysis: Wilcoxon matched pairs test, $^{*}P < 0.05$; $^{**}P < 0.01$.

TABLE 2. ENANTIOMERIC COMPOSITION (%) OF CHIRAL MONOTERPENES COLLECTED FROM DIFFERENTLY TREATED PINE TWIGS[a]

Component	Oviposition induced	Control	N^b	JA-induced	JA-control	N^b
(−)-α-Pinene	51.5	49.3	7	31.1	30.6	10
(+)-α-Pinene	48.5	50.7		68.9	69.4	
(−)-Camphene	95.8	93.4	6	81.2	85.6	6
(+)-Camphene	4.2	6.6		18.8	14.4	
(−)-Sabinene	100.0	100.0	7	100.0	100.0	5
(+)-Sabinene	0.0	0.0		0.0	0.0	
(−)-β-Pinene	100.0	98.5	7	99.2	98.3	9
(+)-β-Pinene	0.0	1.5		0.8	1.7	
(−)-3-Carene	0.0	0.0	7	0.0	0.0	7
(+)-3-Carene	100.0	100.0		100.0	100.0	
(−)-Limonene	95.8	95.1	5	92.0	96.7	8
(+)-Limonene	4.2	4.9		8.0	3.3	
(−)-β-Phellandrene	100.0	100.0	7	100.0	100.0	8
(+)-β-Phellandrene	0.0	0.0		0.0	0.0	

[a] Since a test and control twig were always taken from the same branch, they were considered as paired samples. There were no significant differences between the ratios of enantiomers detected in test and control twigs (Wilcoxon matched pairs test).
[b] N = number of paired samples.

from a test twig change relative to the respective control. Therefore, we calculated the relative amount of each volatile component emitted by a test and its control twig on the basis of the sum of the respective volatile component emitted by both twigs. Figure 2A shows that relative amounts of nearly all the components detected in the headspace of oviposition-induced pine twigs were about the same as those detected in the headspace of the respective control twigs. However, one sesquiterpene, (*E*)-β-farnesene (no. 26), occurred in higher relative quantities than in the controls, although the absolute amounts were not highly different (Table 1). Interestingly, relative amounts of (*E*)-β-farnesene were also significantly higher in JA-treated pine twigs than in the control (Figure 2B). Additionally, the headspace of JA-treated pine twigs contained α-muurolene (no. 29), γ-cadinene (no. 30), and δ-cadinene (no. 31) in higher relative amounts than the controls. The relative amounts of several other components seem to be higher in JA-treated samples than in the controls; however, the number of samples in which these components were detected ($N = 5$ as defined minimum for statistical analysis) was too low for a valid statistical analysis.

DISCUSSION

Headspace analyses of oviposition-induced pine twigs, JA-treated ones, and their respective controls showed numerous terpenoid components that are typical

constituents of conifer resin. Most have been reported before from *P. sylvestris* (see Manninen et al., 2002, and references therein), with the exception of *p*-cymenene (16), 1,3,8-*p*-menthatriene (17), thymyl methyl ether (20), (*E*)-β-farnesene (26), and bicyclogermacrene (28). Also (*E*,*E*)-2,6-dimethyl-3,5,7-octatrien-2-ol has not been identified in pine oleoresin before, but is known from *Ulmus minor* (Wegener et al., 2001), *Narcissus sp.* (van Dort et al., 1993), and several plant essential oils (e.g., Baser et al., 2002). When compared to artificially damaged pine twigs, induction by sawfly egg deposition did not result in a qualitative change of the composition of the pine's volatile blend, but only a significant relative increase in a single sesquiterpene, (*E*)-β-farnesene.

Since wounding of conifers is known to affect resin flow and formation of resin canals (e.g., Nebeker et al., 1995; Oven and Torelli, 1999; Tomlin et al., 1998), the mere artificial damage of pine needles mimicking the wounding conducted by a sawfly female prior to egg deposition was expected to cause an odor change when compared to undamaged pine. However, the artificially damaged pine here served as control since odor from these twigs did not attract the egg parasitoid (Hilker et al., 2002a). The impact of wounding and insect attack on the formation of oleoresin mono-, sesqui-, and diterpenes of conifers has been investigated in several studies (see Gershenzon and Croteau, 1991; Trapp and Croteau, 2001, and references therein). With respect to the storage capacity of constitutively produced oleoresin, two wound responses of conifers may be differentiated (Gijzen et al., 1993):

(1) Conifers, like pines with well-developed resin ducts and high storage capacity for large amounts of constitutively produced oleoresin, are known to translocate resin in response to damage to the site of wounding (e.g., Gijzen et al., 1993, and references therein). Nevertheless, increased activity of monoterpene cyclase induced by damage was found in needles of Ponderosa pine (*Pinus ponderosa*). Interestingly, feeding-induced enzyme activity was much higher than artificially wounding induced activity (Litvak and Monson, 1998). However, when considering woody pine tissue, as e.g., *Pinus taeda*, wound-induced resin flow is increased compared to constitutive flow (Lombadero et al., 2000), while no wound inducibility of monoterpene synthase activity is detectable in stem tissue of this pine species (Phillips et al., 1999).
(2) In contrast, conifers, like grand fir (*Abies grandis*), with low constitutive terpene biosynthetic activity, show increased terpene biosynthesis in response to stem wounding due to the inducibility of mono-, sesqui-, and diterpene synthases at the site of damage (Bohlmann et al., 1998; Steele et al., 1998, and references therein).

A comparison of terpene patterns of wounded conifer tissue and of undamaged tissue revealed mainly quantitative differences rather than qualitative ones (Popp et al., 1995; Raffa and Smalley, 1995). Particularly, woody tissue has been

studied when analyzing the effect of wounding on terpenoid synthesis in conifers (Gershenzon and Croteau, 1991; Trapp and Croteau, 2001, and references therein). For example, the relative amounts of monoterpene enantiomers isolated from trunk xylem of *Pinus caribaea* changed after insect attack, whereas no obvious change in relative amounts of monoterpenes was detected in another pine species, *P. yunnanensis* (Fäldt et al., 2001). Similarly, defoliation did not induce a significant change of monoterpene concentrations in needles of several other pine species (Watt et al., 1991; Barnola et al., 1994) and clones of Douglas fir *Pseudotsuga menziesii* (Chen et al., 2002). When focusing on studies of needles from *P. sylvestris*, again no significant changes in the composition of monoterpenes, sesquiterpenes, and resin acids were found after defoliation (Honkanen et al., 1999). Also, Sadof and Grant (1997) did not find differences in monoterpene composition and ratio of enantiomers between volatile samples of wounded and unwounded *P. sylvestris*.

With respect to known results from other studies and the ones obtained in our analyses, we conclude that neither wounding nor induction by egg deposition, nor induction by JA, leads to a qualitative change of the mono- and sesquiterpene pattern in *P. sylvestris*. Also, no effect on the enantiomer composition was detected in response to these treatments. When comparing the inducibility of *P. sylvestris* by egg deposition and JA, the latter treatment seems to have a stronger effect on the quantities of mono- and sesquiterpenes than does oviposition (Figure 2). Thus, terpenoid-based responses of *P. sylvestris* were shown to be inducible by JA, as is known for numerous angiosperms (Boland et al., 1995, 1999; Karban and Baldwin, 1997; Gols et al., 1999; Rodriguez-Saona et al., 2001). Recently, Martin et al. (2002) and Fäldt et al. (2003) showed that methyl jasmonate induces transient transcript accumulation of terpene synthases resulting in an increased terpenoid resin biosynthesis and terpenoid accumulation in *Picea abies*. Furthermore, other than terpenoid-based responses towards JA have been shown in gymnosperms (e.g., Kaukinen et al., 1996; Richard et al., 1999, 2000; Franceschi et al., 2002). In some angiosperm species, JA treatment results in only a quantitative shift within the volatile blend, while in others *de novo* volatile induction was shown (e.g., Hopke et al., 1994; Boland et al., 1995). In *P. sylvestris*, we could not detect any qualitative changes after JA treatment, only quantitative shifts in the volatile blend.

Terpenoids seem to play a major role in the attraction of egg parasitoids to oviposition-induced plants (see also: ecological role of terpenoids; Langenheim, 1994). Not only the odor of oviposition-induced pine twigs but also the volatile blend of elm leaves induced by egg deposition of the elm leaf beetle is dominated by terpenoids (Wegener et al., 2001). The odor of JA-treated elm leaves, which also attracts the elm leaf beetle's egg parasitoid, was shown to consist almost exclusively of terpenoids. However, (*E*)-β-farnesene was not detected in oviposition-induced and JA-treated elm leaves (Wegener et al., 2001). In contrast to oviposition-induced pine, elm leaves induced by egg deposition showed both quantitative and qualitative changes in their volatile blends.

(*E*)-β-Farnesene, the only component that was emitted in significantly higher amounts in both oviposition-induced pine twigs and JA-treated ones, is a common terpenoid component constitutively present in several plants (Teuscher and Lindequist, 1994). Numerous studies have shown increased amounts or *de novo* production of this sesquiterpene in plants induced by feeding herbivores (e.g., Turlings et al., 1991, 1993; Takabayashi et al., 1995; Bolter et al., 1997; Paré and Tumlinson, 1997; De Moraes et al., 1998; Gols et al., 1999; Paré et al., 1999), by mechanical damage (e.g., McAuslane and Alborn, 1998), and by treatment with regurgitate (Turlings et al., 1993), volicitin, or JA (e.g., Rodriguez-Saona et al., 2001; Schmelz et al., 2001). The increased amount of (*E*)-β-farnesene emitted is not restricted to the leaves under attack, but also adjacent, nondamaged leaves of a plant under attack by feeding herbivores may release higher amounts of this sesquiterpene (e.g., Röse et al., 1998). Thus, both systemically oviposition-induced and feeding-induced plants may respond by increased emission of (*E*)-β-farnesene.

Numerous insect species are able to respond to (*E*)-β-farnesene. For example, this sesquiterpene is known as an alarm pheromone in aphids (Nault et al., 1973). Both herbivorous insects (e.g., Bengtsson et al., 2001) and predatory insects show EAG responses towards (*E*)-β-farnesene (e.g., Zhu et al., 1999; Al Abassi et al., 2000; Weissbecker et al., 2000). Also, aphid parasitoids have been shown to respond electrophysiologically as well as behaviorally towards (*E*)-β-farnesene (Micha and Wyss, 1996; Du et al., 1998). To elucidate the role of (*E*)-β-farnesene within the tritrophic system of *P. sylvestris*, the sawfly *D. pini*, and its egg parasitoid *C. ruforum*, it will be necessary to study the behavioral and electrophysiological responses of the egg parasitoid towards synthetic (*E*)-β-farnesene. Dicke and Vet (1999) discussed the informational value of a herbivore-induced quantitative change in the volatile blend of a plant for carnivores. Up to now, we do not know how carnivores are attracted to a quantitatively changed, herbivore-induced plant odor blend. Future studies need to investigate whether carnivores rely on a specific plant volatile that is released in specific quantities, or whether they respond finely tuned to the pattern of ratios of the volatiles within a blend emitted by a herbivore-induced plant.

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ATTRACTIVENESS OF FRUIT AND FLOWER ODORANTS DETECTED BY OLFACTORY RECEPTOR NEURONS IN THE FRUIT CHAFER *Pachnoda marginata*

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Abstract—We studied the attraction of the African fruit chafer *Pachnoda marginata* Drury (Coleoptera: Scarabaeidae) to banana and 34 synthetic plant compounds previously shown to be detected by *P. marginata* olfactory receptor neurons. The behavioral studies were carried out in a two-choice olfactometer, where the attraction of beetles to lures and controls was monitored in 30-min intervals during whole days. Monitoring of the attraction over time gave additional information when comparing relative attractiveness of different compounds. Seventeen of the test compounds, primarily phenylic compounds, fruit esters, isovaleric acid, acetoin, and some floral or fruit terpenes, were attractive to *P. marginata.* Compounds showing no attractiveness included green leaf volatiles, lactones, and several alcohols, but also phenylic compounds and esters. One case of blend synergism was demonstrated, as well as some examples of sexual dimorphism in attraction. The significance of certain compounds and receptor neurons for olfactory-guided behavior of phytophagous scarabs is discussed.

Key Words—Coleoptera, Scarabaeidae, Cetoniinae, olfaction, Odor, attraction, olfactometer, food, fruit, flower, plant, bioassay.

INTRODUCTION

Phytophagous scarabs are important agricultural or horticultural pests in many parts of the world, which has led to the identification of scarab semiochemicals for use in integrated pest management (reviewed in Leal, 1998). The primary target

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for plant kairomones in scarab field trapping has been the Japanese beetle *Popillia japonica*, an introduced pest in North America since the early twentieth century (reviewed in Metcalf and Metcalf, 1992; Leal, 1998). Investigations of plant attractants for other scarabs have, to a large extent, been modelled on studies of the Japanese beetle using test compounds attractive for this species (e.g., Williams and Miller, 1982; Yokomizo and Nagata, 1984; Donaldson et al., 1986, 1990; Klein and Edwards, 1989; Ben-Yakir et al., 1995). Decades of empirical testing have yielded some compounds that are potent attractants for numerous species of phytophagous scarabs.

Even if the search for scarab attractants through reiteration of previously used stimuli has often been successful, a potential problem is that the conformity among studies may have led investigators to overlook potential attractants. Although there is a pronounced conservatism in the olfactory cues utilized in host plant searches, marked differences exist between species. The rose chafer *Macrodactylus subspinosus* is not attracted to any of the common floral lures, despite being a notorious destroyer of flowers, instead preferring short-chain carboxylic acids, esters, and alcohols (Williams and Miller, 1982; Williams et al., 1990). Other *Macrodactylus* species follow the same strategy (Arredondo-Bernal et al., 1995). Some investigators have aimed for a fresh start by going back to natural odor sources to find attractants, through comparative studies of attractiveness of different substrates (Domek and Johnson, 1988; Harari et al., 1994; Loughrin et al., 1995, 1996a,b, 1997a,b, 1998) and/or direct testing of volatile constituents from preferred food sources (Leal et al., 1994; Loughrin et al., 1998). While these investigations have not always yielded more efficient lures, they have often shed some light on the mechanisms behind olfactory-mediated attraction.

Two helpful tools in the study of semiochemicals are the use of insect antennae as chemical detectors to screen biological extracts for active compounds, and laboratory bioassays to select among potential attractants before proceeding to field tests. For scarabs, electrophysiological screening of plant odors is rare and has largely limited to synthetic compounds (e.g., Ruther et al., 2000), while GC-electrophysiological studies of biological extracts have been used almost exclusively for identification of pheromone components (e.g., Leal, 1991; Ruther et al., 2001). To our knowledge, the first combined GC-electrophysiological study of actual plant extracts in a scarab beetle was performed with the African fruit chafer *Pachnoda marginata* Drury (Coleoptera: Scarabaeidae) as a model species, with the main objective to investigate how the olfactory system of a polyphagous insect handles the multitude of volatile compounds released from potential food sources (Stensmyr et al., 2001). Using GC-single cell screening (Wadhams et al., 1982; Van der Pers and Löfstedt, 1983) of fruit extracts supplemented with screening of additional synthetic compounds, Stensmyr et al. (2001) identified more than 50 compounds eliciting responses from olfactory receptor neurons on the antennae of *P. marginata*. Most neurons exhibited narrowly tuned responses to single

or a few structurally similar compounds, which could be grouped into categories: 1) typical "flowery" or "fruity" compounds like aromatics, esters, or lactones; 2) certain simple terpenes commonly found in flower or fruit aromas but also in other plant tissues; 3) simple alcohols and carboxylic acids commonly found as microbial degradation or fermentation products; 4) certain six-carbon alcohols, aldehydes, and their derivatives commonly referred to as "green leaf volatiles" (GLVs), which are common by-products of plant metabolism released from all plant tissues.

In this paper, we show results from behavioral bioassay experiments with *P. marginata* that test the attractiveness of selected compounds from these four categories. Although olfactory screening is helpful during the initial stages of identification of semiochemicals, it has generally proven difficult to design robust and efficient behavioral bioassays for scarab beetles (Heath et al., 2001). We developed a two-choice olfactometer for *P. marginata*, using banana as a test lure, and assessed the attractiveness of 34 selected synthetic compounds and three blends of compounds to *P. marginata* males and females.

METHODS AND MATERIALS

Experimental Animals. *Pachnoda marginata* (Drury) is a scarab with several subspecies native to equatorial Africa (Rigout, 1989). Beetles used for behavioral experiments, *P. m. peregrina* (Kolbe), were reared in our laboratory. The origin of the culture and the method of rearing have been described (Stensmyr et al., 2001). Animals were collected immediately after emergence from pupal cocoons, and sorted by sex and date of emergence. They were kept on 12:12 h L/D periods, consistent with approximate equatorial light conditions. Beetles were used between 1 and 7 days after emergence and were not fed prior to the experiments. Newly emerged beetles generally survive for at least 2 weeks without food. During this period, we have observed no change in unfed beetles, either in their motivation to forage or in their preference for food odors. The number of beetles tested (= put in the central test chamber), and the numbers "choosing" (= entering either of the collection jars) are shown for each compound in Table 1.

Behavioral Evaluations. The olfactometer was developed specifically for assessing behavioral responses of *P. marginata*, through a series of trial-and-error testing of different prototypes. The final version used is shown (Figure 1). The olfactometer had a central test chamber consisting of a transparent plastic box (30 $\times$ 22 $\times$ 12 cm) covered with a transparent lid. Through each short side, an exit tube made of nontransparent plastic material (inner diameter 6 cm, length 17 cm) entered 4 cm into the box. Exit tubes connected the central chamber with transparent plastic collection jars containing either an odor lure or a control. Collection jars were screwed onto the plastic box and easily removed. A 2-cm-thick styrofoam sheet covered the bottom of the box and raised the floor to the level of the exit

TABLE 1. LIST OF OLFACTORY STIMULI TESTED IN THE BEHAVIORAL ASSAY

Compound/blend	Source/purity(%)	#Tes	#Choos	Dispenser
(−)-Beta-pinene	Fluka/ >98, e.e unknown	30	23	Wick+Tube
2,3-Butanediol	Fluka/ >99	30	27	Wick+Tube
Acetoin	Aldrich/>99	45	32	Wick+Tube
Anethole	Fluka/ >98	48	37	Wick
Benzyl alcohol	Aldrich/ >99	39	34	Wick
Beta-ionone	Aldrich/ >96	43	37	Wick
Butyl butyrate	Aldrich/ >98	45	42	Wick+Tube
Cinnamic aldehyde	Aldrich/ >98	40	30	Wick
Citronellol	Aldrich/ >95	59	53	Wick
Dimethyl malonate	Aldrich/ >98	56	47	Wick+Tube
Eugenol (Eug)	Sigma/ >99	51	46	Wick
4-Ethyl phenol	Aldrich/ >99	30	26	Wick
Gamma-decalactone (G-decal)	Aldrich/ >99	60	49	Wick
Gamma-hexalactone	Aldrich/ >98	40	29	Wick
Gamma-undecalactone	Aldrich/ >99	45	41	Wick
Geraniol (Ger)	Sigma/ >98	57	45	Wick
(*E*)-2-Hexenal (E2HxAL)	Aldrich/ >98	60	49	Wick+Tube
(*E*)-2-Hexenol	Aldrich/ >96	30	28	Wick+Tube
(*E*)-3-Hexenol	Aldrich/ >98	42	34	Wick+Tube
(*Z*)-3-Hexenol	Aldrich/ >98	45	35	Wick+Tube
Isoamyl acetate	Aldrich/ >98	45	38	Wick+Tube
Isoamyl alcohol (IsoamOH)	Aldrich/ >99	41	38	Wick+Tube
Isobutanol	Riedel-de Haen/ >99	42	32	Wick+Tube
Isovaleric acid	ICN/ >97	46	32	Wick
Linalool (LinOH)	ICN/ >98	91	77	Wick
Linalool oxide mix isomers	Fluka/ >97	59	50	Wick
Methyl anthranilate	Aldrich/ >99	39	36	Wick
Methyl benzoate	Aldrich/ >99	40	29	Wick
Methyl cinnamate	Fluka/ >99	31	26	Wick
Methyl hexanoate	Aldrich/ >99	45	39	Wick+Tube
Methyl salicylate	Sigma/ >99	53	48	Wick
Phenethyl propionate (PEP)	Sigma/ >98	51	43	Wick
2-Phenyl ethanol	Sigma/ >98	38	27	Wick
Phenylacetaldehyde (PAA)	Aldrich/ >90	42	31	Wick
Phenylacetaldehyde 1 mg	Aldrich/ >90	52	46	Wick
PEP+ Ger+Eug	Blend (10+10+10 mg)	46	45	Wick
LinOH+Acet+E2HxAL	Blend (30+10+10 mg)	30	29	Wick+Tube
G-decal+E2hxAl+IsoamOH	Blend (30+10+10 mg)	119	100	Wick+Tube
Banana		115	99	Fruit pulp
Total		1920	1609	

Note: The source and purity, numbers of beetles tested (= put in the central test chamber), and the numbers "choosing" (= entering either of the collection jars) are shown for each compound. *N* values for males and females have been pooled.

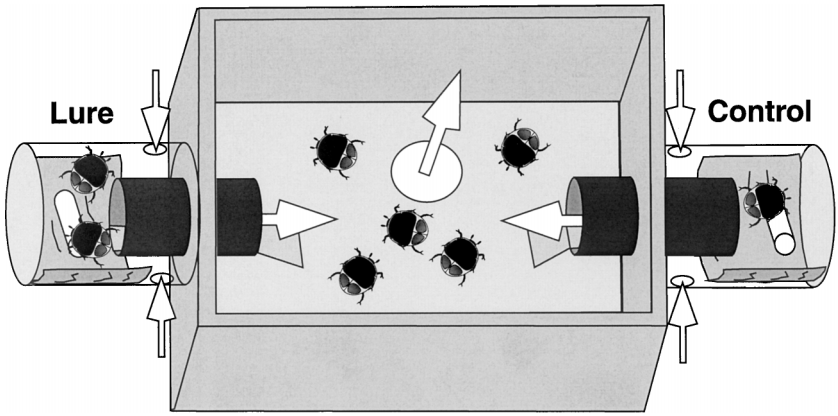

FIG. 1. Schematic drawing of the main elements in the olfactometer used for the behavioral tests (not drawn to scale) (see text for details). Beetles were placed in a central chamber and could exit through either of two tubes leading to collection jars. Air was evacuated through the lid of the central chamber, creating air streams through the collection jars and into the chamber. Lure and control dispensers were placed in the collection jars. The number of beetles entering each jar was monitored every 30 min, and the position of the collection jars switched every hour to avoid position effects.

tubes. The styrofoam was covered with protective paper for work surfaces, lined with plastic foil on the side facing down, and held in place by adhesive tape. A ramp of adhesive tape with a rough surface went from the floor through the smooth exit tubes to facilitate walking. The protective paper and the tape in the exit tubes were frequently changed to avoid contamination.

Through an exhaust at the center of the lid, air was evacuated from the central chamber, creating two gentle air streams through holes in the collection jars and into the central test chamber. To moisten the air entering the olfactometer, each collection jar contained sheets of folded wet paper towels. The behavioral tests were conducted at 30°C, with the olfactometer illuminated by two strong greenhouse lamps. A combination of high temperature and high light intensity was essential for performance of the beetles in the bioassay experiments. Each test started 2–3 hr into the photophase, when 10–15 beetles were put in the central test chamber and allowed to acclimatize for several minutes, before the lure and the control were placed in the collection jars. Beetles were allowed to walk freely in the central chamber and could exit through any of the two tubes during a test period that went on for a maximum of 7 hr or until no beetles were left in the central chamber. Collection jars were emptied every 30 min, and the position of the jars switched every 60 min to avoid position effects. Using several beetles simultaneously was necessary because of time constraints imposed by the great number of compounds

tested and to the long duration of each trial. This method is potentially subject to error caused by the animals being attracted to early arriving conspecifics rather than to the test compounds, but in young animals, such as those used in the experiments, we have observed neither any display of sexual behavior nor any attraction to conspecifics when tested separately in the bioassay (unpublished data). The continuous removal of beetles from the collection chambers during each trial should also minimize any interactions among individuals during the experiments.

Two-to-eight tests were conducted for each treatment, and the total numbers choosing the lure and control sides were combined for χ^2 analysis. Male and female beetles were tested separately to assess any sexual dimorphism in odor preferences. Single compounds were tested in more or less random order. Tests with the blends were interspersed towards the end of the study, after the attractiveness of each single component had been established.

Olfactory Stimuli. Initial evaluation of the bioassay was performed using pieces of ripe banana, weighing approximately 30 g each, as lures. Each piece was mashed into a pulp and placed in one of the plastic jars, out of sight from the central chamber, while the control jar was left without banana. During the following tests, a total of 34 single synthetic compounds and 3 blends of compounds were tested. The name, source, and purity of each test stimulus are shown in Table 1. Single compounds were chosen to represent most of the classes of receptor neurons previously identified (Stensmyr et al., 2001), and subsequent dose–response studies of single receptor neurons (unpublished data). Some compounds (e.g., acetoin and 2,3-butanediol) were chosen specifically to evaluate behavioral effects of different compounds apparently stimulating the same receptor neuron. Of the three odor blends tested, the first (phenethyl propionate, geraniol, eugenol) was chosen because they are part of the most attractive kairomone lure for the Japanese beetle (Ladd and McGovern, 1980). The constituents of the other two blends were chosen simply to represent a diverse spectrum of compounds.

Dental cotton wicks loaded with defined amounts of test compounds were used as dispensers. This procedure worked well with most compounds, but with some the evaporation rate was too high, causing complete evaporation before the test was completed (unpublished data). To create a more homogenous evaporation rate, these compounds were, therefore, tested with the cotton wick placed in an open glass tube. Test compounds were applied in 100-μl solvent portions, which were absorbed into the cotton material, followed by evaporation of the solvent. To control dispensers, we applied 100 μl of pure solvent. The odor solutions were prepared by dissolving known amounts of test compounds, determined by weight, in hexane or acetone depending on their solubility. All single compounds were tested with 10-mg loads in each lure (100 μl of 100 μg/μl solutions). Exhaustive dose–response experiments were beyond the scope of this study because of the great number of stimuli tested, but for comparison, one moderately attractive compound, phenylacetaldehyde, was also tested at 1-mg loads (100 μl

of 10 μg/μl solution). In the blends, some compounds were added at 30-mg loads to compensate for their lower volatility when the wick was placed in a glass tube.

RESULTS

We used a total of 1920 beetles. Of these, 1609 entered either of the collection chambers, and were used for statistical comparisons. The numbers of beetles tested for each treatment are shown in Table 1. Results from the olfactometer show that the standard lure (banana) and 17 of the 34 synthetic compounds were significantly attractive to *P. marginata* (Figure 2). None of the compounds appeared to elicit avoidance behavior, based on the choice between the lure and the control (Figure 2(B)). Most of the attractive synthetic compounds were phenylic compounds, including several esters, plus some aliphatic fruit esters and terpenes. The only exceptions were isovaleric acid and acetoin. None of the green leaf volatiles or other aliphatic alcohols were attractive by themselves; neither were any of the lactones. There were also examples of phenylic compounds, terpenes, and aliphatic esters that were not attractive, indicating that the attraction of beetles was specifically directed to certain compounds.

Without taking different release rates into account, there are apparently great differences in attractiveness among stimuli (Figure 2), when comparing the proportions of beetles choosing the lure. These differences could sometimes be directly observed from behavior of beetles in the test chamber. Some of the attractive compounds, especially methyl salicylate, methyl anthranilate, and cinnamic aldehyde, induced visible directed search behavior resulting in a fast attraction to the lure. This is illustrated in Figure 3, showing the attraction to the lure during the first 2 hr for a selection of compounds. With methyl salicylate and cinnamic aldehyde, most beetles exiting the central test chamber had already chosen the lure after 0.5–1 hr. Attractiveness may be a function of dose, as shown by tests with two different concentrations of phenylacetaldehyde, where only the highest elicited significant attraction (Figure 4(A)). The banana odor was less attractive than any of the single compound attractants, including its constituent isoamyl acetate, likely due to higher release rates from the dispensers than from the fruit. Most single compounds were equally attractive to males and females (data not shown). Linalool was the only exception, eliciting significant attraction only in male beetles (Figure 4(B)).

Tests of three blends showed mixed results (Figure 5). Two blends, including compounds that were attractive by themselves, did not exhibit any additive or synergistic effects, compared with the most attractive single constituent. A blend of three compounds, individually neutral to beetles, resulted in a dramatic increase in attractiveness to males. Females, however, were not attracted to this blend.

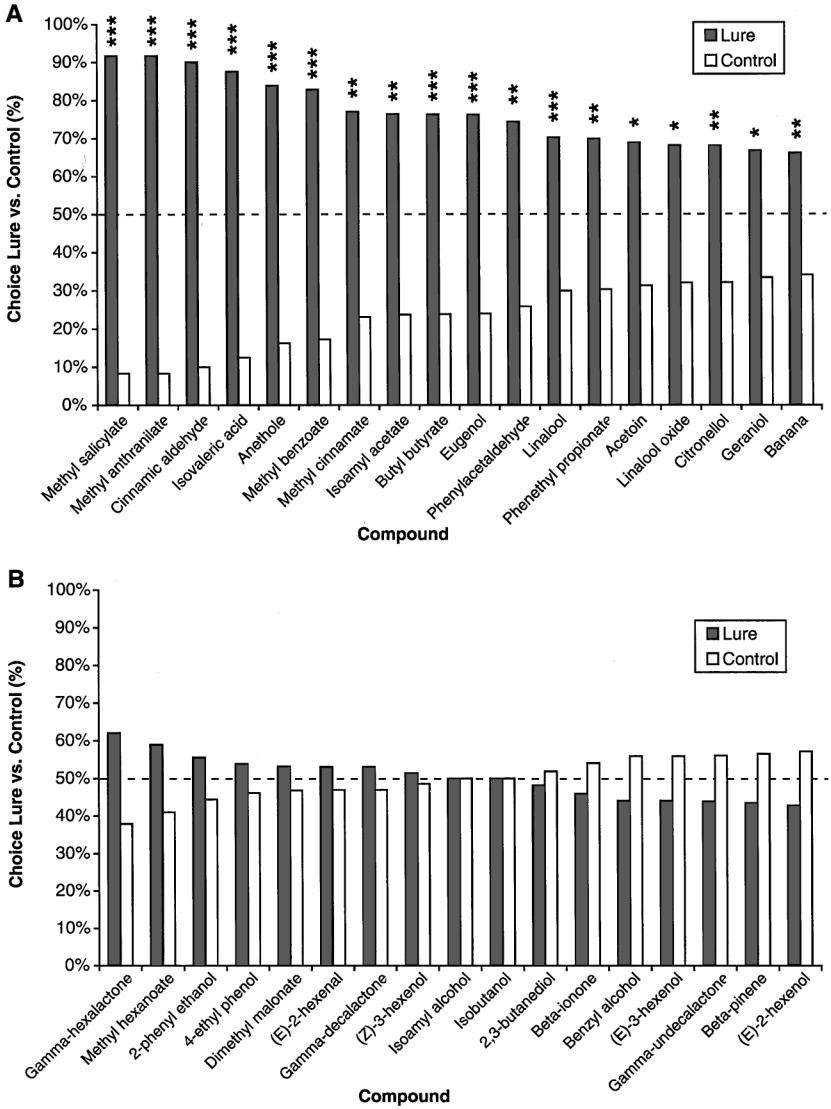

FIG. 2. Results for banana and each single compound tested at the 10-mg dose in the olfactometer, showing the relative proportions of beetles entering the chamber containing the lure and the control, respectively. Results from males and females have been pooled. The dashed line indicates the expected random distribution (50/50). N values are given in Table 1. (A) Stimuli that were significantly attractive to *P. marginata* according to χ^2 tests (1 degree of freedom) against a random distribution ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (B) Compounds showing no attractiveness, i.e., not deviating from a random distribution.

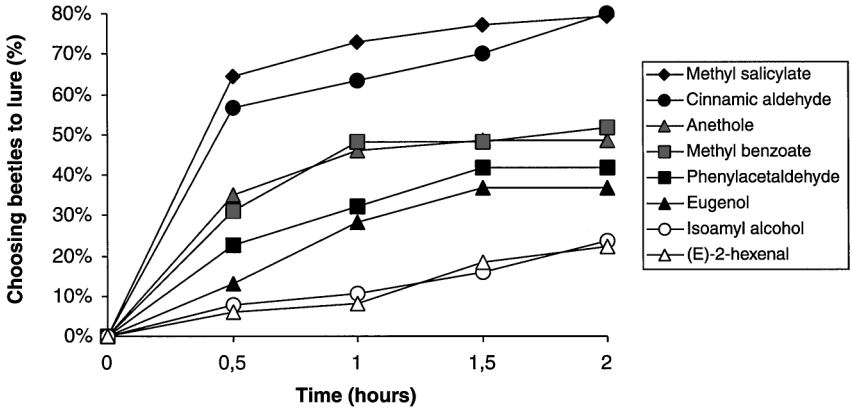

FIG. 3. Cumulative proportion of all "choosing" beetles entering the chamber containing the lure during the first 2 hr. Figure shows results from a selection of compounds for which complete data were available. All compounds except isoamyl alcohol and (E)-2-hexenal were considered attractive (compare Figure 2), but the beetles were attracted to methyl salicylate and cinnamic aldehyde much faster than to any of the other compounds. Results from males and females have been pooled.

DISCUSSION

The results show that many of the electrophysiologically active compounds previously identified by Stensmyr et al. (2001) were attractive to *P. marginata* in the olfactory bioassay. When obtaining behavioral data from *P. marginata*, the major problem has been that the beetles are sensitive to the inevitable disturbances that occur during handling, and that their behavior is affected by constraints imposed by the laboratory environment. In a laboratory choice setting, much behavior manifests itself as erratic and random escape behavior, leading to relatively high responses to the control. In our olfactometer, we circumvented this by exploiting the strongly phototactic behavior of the beetles, forcing them to enter and walk through a tube receiving lower illumination than the central chamber or the collection jars before a choice was recorded. As beetles appear reluctant to enter the exit tubes unless drawn by some attractive stimulus, most of the random control encounters are suppressed.

Combinations of gas chromatography and single-cell recordings can direct the search towards minor constituents of plant extracts, which would otherwise be overlooked or regarded as insignificant in chemical analyses (e.g., Blight et al., 1995; Wibe et al., 1997; Stensmyr et al., 2001; Barata et al., 2002). The limiting factor in electrophysiological or behavioral screening for semiochemicals is the choice of test stimuli, regardless whether these are biological extracts or

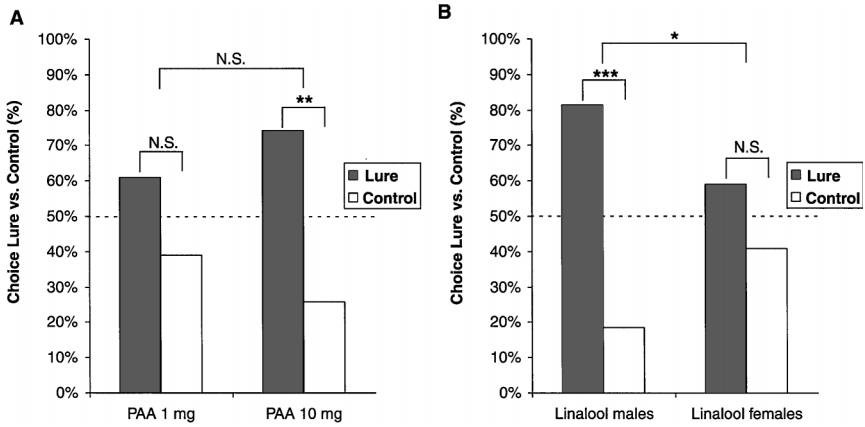

FIG. 4. (A) Comparison of responses to 1 and 10 mg of phenylacetaldehyde (PPA). Diagram shows relative proportions of beetles entering the chamber containing the lure and the control, respectively. N values are given in Table 1. Results from males and females have been pooled. Beetles were attracted to the lure containing 10 mg of PAA (data same as Figure 2), while there was no attraction to the 1-mg lure, according to a χ^2 test (1 degree of freedom). The two distributions were not different from each other. Dashed line indicates the expected random distribution (50/50) for each treatment. (B) Comparison of the responses of males ($N = 38$) and females ($N = 39$) to linalool. Diagram shows relative proportions of beetles entering the chamber containing the lure and the control, respectively. Males were attracted to linalool, according to a χ^2 test (1 degree of freedom) ($^{***}P < 0.001$), while females show no or little attraction. Responses of males and females were different from each other ($^{*}P < 0.05$). Dashed line indicates the expected random distribution (50/50) for each treatment.

synthetic compounds. Polyphagous species like *P. marginata* could be expected to feed opportunistically on whatever fruits or flowers are available, utilizing odors commonly found in many food substrates (Loughrin et al., 1996a). Under this assumption, Stensmyr et al. (2001) chose a wide selection of locally available fruits. All elicited search behavior and feeding when presented to the beetles, but only banana has now been tested in an olfactometer (this paper). Indeed, fruit extracts turned out to be the source of 11 of the 17 single compounds eliciting attraction. Although several *P. marginata* attractants are also common scarab attractants (e.g., geraniol and eugenol), their presence in fruit extracts is new information. While there is overlap in the attractants demonstrated for *P. marginata* and for other scarabs, the rank order of attractiveness is different among species. Methyl salicylate, a potent attractant for *P. marginata*, exhibits low attractiveness to the Japanese beetle (Langford et al., 1943). Perhaps for this reason it has not been included in studies of other species. Acetoin and linalool oxides are novel scarab kairomones, although acetoin (3-hydroxy-2-butanone) is a component of male-

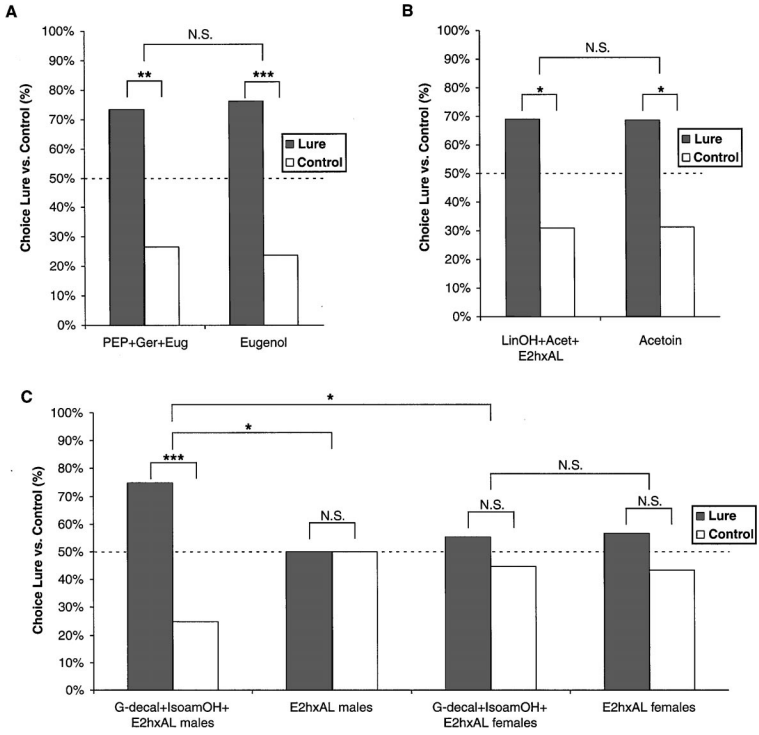

FIG. 5. Comparisons of responses to blends of compounds and responses to the most attractive constituents. Diagrams show relative proportions of beetles entering the chamber containing the lure and the control, respectively. Dashed lines indicate expected random distribution (50/50) for each treatment. N.S. = no significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, according to χ^2 tests (1 degree of freedom). (A) Comparison of the responses to a blend of phenethyl propionate, geraniol, and eugenol (10 + 10 + 10 mg in the lure) and to 10-mg lure of eugenol, the most attractive component. *N* values are given in Table 1. Results from males and females have been pooled. The two distributions were virtually identical. (B) Comparison of the responses to a blend of linalool, acetoin, and (*E*)-2-hexenal (30 + 10 + 10-mg in the lure) and to 10-mg lure of acetoin, the most attractive component. Diagram shows the relative proportions of beetles entering the chamber containing the lure and the control, respectively. *N* values are given in Table 1. Results from males and females have been pooled. The two distributions were virtually identical. (C) Comparison of the responses to a blend of gamma-decalactone, isoamyl alcohol, and (*E*)-2-hexenal (30 + 10 + 10 mg in the lure) and to 10-mg lure of (*E*)-2-hexenal. Responses of males and females are shown separately. *N* (males, blend) = 44, *N* (males, (*E*)-2-hexenal) = 26, *N* (females, blend) = 56, *N* (females, (*E*)-2-hexenal) = 23. Females exhibited no attraction to the blend, nor to (*E*)-2-hexenal. Males exhibited strong attraction to the blend, but not to (*E*)-2-hexenal. Responses of males and females to the blend were significantly different from each other.

released aggregation pheromones of two dynastine scarab beetles (Rochat et al., 2000a,b).

The majority of potential odorants tested were largely ignored by the olfactory system of *P. marginata*, which appears to be selectively tuned to a small range of compounds that form representative cues to its food substrates. Using a limited number of characteristic compounds as cues could suffice for identifying a large spectrum of preferred food sources against a noisy background of irrelevant odors. Investigations of flowers (Knudsen et al., 1993) and fruit (Stensmyr et al., 2001) show that certain compounds form specific signals for these high-quality food sources. Other compounds with more widespread distribution, such as acetoin and isovaleric acid, convey a selective message of fermenting, sugar-rich substrates despite their ubiquity (Phelan and Lin, 1991; Nout and Bartelt, 1998). Even among scarabs attacking green plant tissue, attraction often appears to be mediated primarily by odor mixtures containing floral-like or fruit-like compounds released in response to insect feeding (Loughrin et al., 1995, 1996b, 1997a,b, 1998). Thus, polyphagy of phytophagous scarabs may be mediated by a relatively small number of compounds serving as informational cues regardless of the specific source. This probably applies to coprophagous scarabs as well, although we do not know of any specific attractants identified from dung odor.

Using specific key compounds to locate food or oviposition sites appears to be a strategy used by monophagous or oligophagous insect herbivores where such characteristic compounds can be found (Renwick, 1989). Scarab beetles are an example of polyphagous insects that use a similar strategy applicable to a wide variety of unrelated hosts. Comparisons between *P. marginata* and other insects attracted to decaying or fermenting plant materials, including distantly related species such as beetles, butterflies, and *Drosophila*, show great overlap between behaviorally and/or electrophysiologically active compounds (Lin and Phelan, 1991; Phelan and Lin, 1991; Nout and Bartelt, 1998; de Bruyne et al., 1999, 2001; Omura et al., 2000; Rochat et al., 2000b). A prerequisite for this strategy may be the evolution of signals that indicate the desired resource with a high degree of certainty, which seems to be the case with flowers and fruits that produce a limited set of stereotyped odors (including secondary production by colonizing microbes) to advertise nutrient-rich rewards in return for pollen or seed dispersal. Polyphagous insects ovipositing on noncooperative plants may have a harder time selecting specific compounds providing sufficient information. An indication of the cooperative scenario may be that phytophagous scarabs often display strong attraction to single plant volatiles that presumably have high informational value, and that they appear to have a "trusting nature," with host choice based on attractive signals and with no antagonistic effects reported from specific volatiles. One study demonstrates that Japanese beetles are unable to resist toxic plants with an attractive smell, despite repeated paralysis as a result of feeding on these plants (Potter and Held, 1999). In this, scarabs are different from, for example, bark beetles that use

the presence of specific chemicals as cues for avoidance of nonhosts or unsuitable hosts (Byers, 1995; Schlyter and Birgersson, 1999). In the system of rewards for services provided, many scarabs are actually cheaters, destroying flowers without providing pollen or seed dispersal in return.

Half of the tested compounds previously shown to be electrophysiologically active failed to elicit attraction in *P. marginata* beetles. In some cases, e.g., 2,3-butanediol, which stimulates the same receptor neurons as acetoin, the electrophysiological responses could have resulted from a high stimulus dose from GC, while under natural conditions *the compound* may be poorly detected. Another likely explanation is that some compounds primarily provide information in combination with others or may be exploited in another context. GLUs are an interesting case, as all studies of receptor neurons in phytophagous scarabs to date have demonstrated high abundance of specific neurons for GLVs (Hansson et al., 1999; Larsson et al., 2001; Stensmyr et al., 2001; Nikonov et al., 2002), while these compounds generally are not attractive to scarab beetles as noted in this paper as well as by others (Loughrin et al., 1998; Larsson et al., 2001). Recent studies of *Melolontha* cockchafers have, however, demonstrated that males are attracted to specific GLV compounds that work as "sex kairomones" released from leaves through mechanical damage by feeding females (Reinecke et al., 2002 a,b; Ruther et al., 2002). The GLVs are even more attractive than the female sex pheromone itself, which rather works as a synergist to the GLVs, indicating that even these ubiquitous compounds can relay specific information in the right context.

Most investigations of olfactory-guided search behavior in scarabs have demonstrated strong synergism among compounds. Blends are generally more attractive than single constituents (Langford et al., 1943; Ladd and McGovern, 1980; Williams et al., 1990; Loughrin et al., 1998). In monophagous insects, specific ratios of GLVs or other compounds may be one factor that mediates attraction to the host plant (Visser and Avé, 1978; Peacock et al., 2001), while polyphagous insects like scarab beetles could be expected to utilize nonspecific blends of compounds as olfactory cues (Loughrin et al., 1996a). In our investigation, combining attractive compounds neither increased nor decreased their attractiveness. This may be due to experimental design; a better approach when using a two-choice bioassay might be to determine differences in threshold amounts needed for attraction, which would necessarily require a more precise control of release rates. One blend of nonattractive compounds induced attraction, but in male beetles only. Male-specific attraction was demonstrated for linalool as well, indicating that there could be some differences in search strategy between the sexes. Many scarabs are primarily attracted to substrates that have already been attacked by conspecifics (Domek and Johnson, 1988; Harari et al., 1994; Loughrin et al., 1995, 1998); an adaptive response, as the beetles choose already favored substrates. In some scarab species, like *P. japonica* (Fleming, 1972) and *Maladera matrida* (Harari et al., 1994), males are often the pioneers initially attacking the host plants, while females appear later

and presumably utilize the olfactory cues generated by feeding males. In this system, females could afford to be more choosy in their responses to olfactory cues.

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ALLELOPATHIC POTENTIAL OF *Macaranga tanarius* (L.) MUELL.–ARG.

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Abstract—*Macaranga tanarius* is widely distributed in the abandoned lowlands of Taiwan where substantial amounts of leaves accumulate on the ground. A unique pattern of weed exclusion underneath trees is often found and thought to result from allelopathic interactions. Density-dependent phytotoxicity analysis of *Lactuca sativa* L. (lettuce) growing in soil mixed with the powder of *M. tanarius* leaves showed a significant deviation from the expected yield–density relationship. Lettuce growth was most suppressed in the low seed density experiment suggesting that the phytotoxins produced during leaf decomposition inhibit the growth of lettuce seedlings. *Bidens pilosa* and *Leucaena leucocephala*, growing in soil mixed with the leaf powder of *M. tanarius* were also suppressed. Aqueous leaf extracts were bioassayed against lettuce and *B. pilosa*, and exhibited a significant suppression in radicle growth. Compounds identified from leaves included nymphaeol-A (**1**), nymphaeol-B (**2**), nymphaeol-C (**3**), quercetin (**4**), abscisic acid (ABA) (**5**), blumenol A (**6**), blumenol B (**7**),

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roseoside II (**8**), tanariflavanone A (**9**), and tanariflavanone B (**10**). ABA was the major growth inhibitor. At concentrations of 20 ppm, ABA suppressed lettuce germination, while at 120 ppm it inhibited the growth of *Miscanthus floridulus*, *Chloris barbata*, and *Bidens pilosa*. At 600 ppm, quercetin, blumenol A, and blumenol B, caused 20–25% inhibition of radicle and shoot growth of *M. floridulus*. The amount of ABA in *M. tanarius* leaves was approximately 3–5 μg g^{-1} dry weight, significantly higher than previously reported. We conclude that the pattern of weed exclusion underneath stands of *M. tanarius* and its invasion into its adjacent grassland vegetation results from allelopathic interactions.

Key Words—Phytotoxicity, allelopathy, allelochemicals, *Macaranga tanarius*, abscisic acid, fallen leaves, prenylflavanones, quercetin, blumenols, nymphaeols.

INTRODUCTION

Allelopathic compounds are released from plants that can be beneficial or detrimental to the growth of receptor plants. The source of allelopathic compounds includes decomposition and leaching of plant debris, volatilization, leaf leachate, or root exudates (Putnam and Tang, 1986; Chou and Leu, 1992; Chou et al., 1998; Mallik and Pellissier, 2000; Chaves et al., 2001). In habitats with limiting environmental conditions, dominant species can suppress the growth of neighboring plants and compete for more resources by releasing allelochemicals into the environment. Allelopathy has increasingly been recognized as playing an appreciable role in plant dominance, succession, formation of plant communities and climax vegetation, and crop productivity (Muller, 1969; Rice, 1984; Chou, 1999a).

Macaranga tanarius, an endemic species, is commonly distributed in abandoned areas throughout Taiwan. It is an evergreen species, but leaf-fall may take place at any time of year, resulting in large quantities of fallen leaves underneath tree stands. *M. tanarius* is an early succession tree and often spreads into adjacent grasslands, resulting in a secondary forest with few other species growing in the understory. *Alocasia macrorrhiza* is common beneath *M. tanarius* stands. On the basis of field observation, it was hypothesized that fallen *M. tanarius* leaves might release allelopathic compounds that inhibit the growth of weeds adjacent to *M. tanarius* stands. Experiments based on bioassay and the yield–density relationship for lettuce and *A. macrorrhiza* were conducted to evaluate the allelopathic potential of *M. tanarius*. Bioassay of the responsible phytotoxic compounds released from the plant was conducted in order to confirm the allelopathic effect.

METHODS AND MATERIALS

Study Sites. Four study sites were selected for field experiments. Data were obtained from two sites, namely Yuansan and Chauchou, which were previously

abandoned by the Taipei Water Department and by local farmers, and representing different climate types. The Yuansan site is located in Taipei City and belongs to the northeast humid zone, while the Chauchou site is located in Pingtung County in southern Taiwan and has a long drought season in winter. Precipitation in southern Taiwan is usually concentrated in the summer. Typhoons frequently hit Taiwan from August to October. Northeastern Taiwan receives a substantial amount of rainfall in winter in addition to summer rain; however, in the south it is often dry in autumn and winter.

Field Observation. M. tanarius. is widely distributed over the island and commonly found in thickets forming pure secondary forests in abandoned fields at low elevations. *M. tanarius* is a fast-growing, light-demanding species with the characteristics of early seral trees. A unique pattern, with relatively few species growing in the understory, is found in the Yuansan site (Figure 1A), and the pattern is particularly pronounced in the Chauchou site in southern Taiwan during the winter season (Figure 1B). Light intensity, relative humidity, and temperature underneath *M. tanarius* stands and its adjacent grassland were measured by a light intensity logger (StowAway), and a relative humidity and temperature logger (HOBO H8 Pro Series). Light intensity ranged from 20 to 110 μmol $m^{-2}s^{-1}$ at noon in summer under the canopy of *M. tanarius* stand, while it was 220–240 μmol $m^{-2}s^{-1}$ in its adjacent grassland. The temperature underneath the *M. tanarius* stand was 29–32°C, and 36–38°C outside of the stand. Relative humidity ranged from 47 to 52% under *M. tanarius*, higher than in that grassland (40–42%), indicating that soil moisture was not a limiting factor in regulating the understory species (Tseng, 2001).

Sampling and Field Measurement. In order to understand the influences of the invasion of *M. tanarius* into grassland, the vegetative compositions beneath the canopy and the grasslands were compared. In each site, 20 plots (1×1 m^2 each) were randomly selected in *M. tanarius* stands and 20 plots in the vicinity adjacent to the *M. tanarius* stand. Plant species in each plot were identified, and the coverage of each species per plot was measured.

Greenhouse Pot Experiments. Seedlings of *Bidens pilosa* and *Leucaena leucocephala* were planted in pots containing a mixture of *M. tanarius* fallen leaf powder and treated soil (1 g leaves/30 g soil), or untreated soil (vermiculite-perlite-peat moss, 3:1:1, v/v/v) as control. Each set of experiments consisted of three replicates. Pots were placed into a greenhouse of Academia Sinica, Taipei, Taiwan.

Density Dependent Phytotoxic Effects. Treated soil mixed with or without the powder of fallen leaves was transferred into $30 \times 36 \times 10$ cm^3 plastic seed trays. Lettuce and *Alocasia macrorrhiza* seeds were sown in the trays and the emerging seedlings were thinned to allow three levels of density, namely low (4500 seedlings m^{-2}), medium (8500 seedlings m^{-2}), and high (17,500 seedlings m^{-2}) for lettuce, respectively. Each treatment was set with three replicate trays and placed in the greenhouse. Approximately 5 weeks after treatment, plants were

FIG. 1. The understory of *M. tanarius* at the Yaunsan site (A) and the Chauchou site (B), showing the relatively few species present, except *Alocasia macrorrhiza*.

harvested and soil was carefully washed out of roots. Roots and shoots were separated, oven-dried, and weighed.

Preparation of Aqueous Extracts. Fallen leaves were allowed to air dry, then ground to powder for water extraction. A series of aqueous extracts, 0.5 (0.5 g plant sample + 99.5 ml distilled water), 1.0, 1.5, and 2.0% were prepared as described by Chou and Muller (1972) and Chou (1999b). The ionic concentration of the aqueous extract was determined by using a Fiske Osmometer (model G-66). The osmolarity of the 0.5, 1.0, 1.5, and 2.0% aqueous extracts of *M. tanarius* fallen leaves was 20, 24, 26, and 32 milliosmols, respectively.

Bioassays. To determine the allelopathic potential of *M. tanarius*, aqueous extracts of fallen leaves were bioassayed using lettuce and *Bidens pilosa* as test plants. Extracts were bioassayed separately by a modification of Muller's standard sponge bioassay as described by Chou (1999b). In addition, a chromatographic bioassay described by Chou (1999b) was employed to determine the presence of phytotoxic compounds in the ether fraction of the aqueous extract. Washed paper strips spotted with a methanol solution of the ether fraction or an unspotted control strip were developed simultaneously with 2.0% acetic acid. Phytotoxicity was evaluated by bioassaying relevant R_f segments of the paper chromatogram. The pure or crude compounds were dissolved in spectroscopic grade methanol to prepare variable concentrations, and spread onto silica gel TLC sheets (1.5×5 cm^2). The methanol was allowed to evaporate completely in a laminar flow hood. Prior to bioassay, the TLC sheets were placed into Petri dishes and moistened with distilled water, then surrounded by wet sponges without contact. The test seeds were soaked in distilled water for 2 hr, and then placed onto the TLC sheets containing the pure compound or onto untreated control sheets. Petri dishes were sealed with parafilm "M" (American National Can^{TM}), and placed in an incubator at 25°C for 48 hr in the dark.

Extraction and Isolation. Four kilogram of crushed *M. tanarius* fallen leaves were twice extracted with 100 l MeOH at room temperature for 7 days. The extract was evaporated *in vacuo*, the residue was suspended in 1 l H_2O, and partitioned with hexane, ethyl ether, and finally with butanol. Extracts were chromatographed on silica gel (Merk 70-230, 230-400 mesh, ASTM). Crude compounds from the ether fraction were eluted with 25–100% ethyl acetate in dichloromethane. Further purification was accomplished with Sephadex LH 20, TLC, HPTLC, and HPLC [Merk Lichro CART 250-10 Cat. 1.50179 Lichrosorb Si 60 (7 μm)]. For each separation, the fractions obtained were concentrated to dryness *in vacuo* at 40 ± 2°C, then bioassayed against lettuce. The isolation procedures of the phytotoxic fraction are schematically presented in Figure 2.

Identification. All purified compounds were subjected to spectroscopic identification using IR (Perkin-Elmer 983G Spectrophotometer), ^{1}H and ^{13}C NMR (Varian Unity Plus 400 spectrometer), MS (JEOL JMS-HX 300 mass spectrometer), and specific rotations (JASCO DIP-1000 digital polarimeter), respectively.

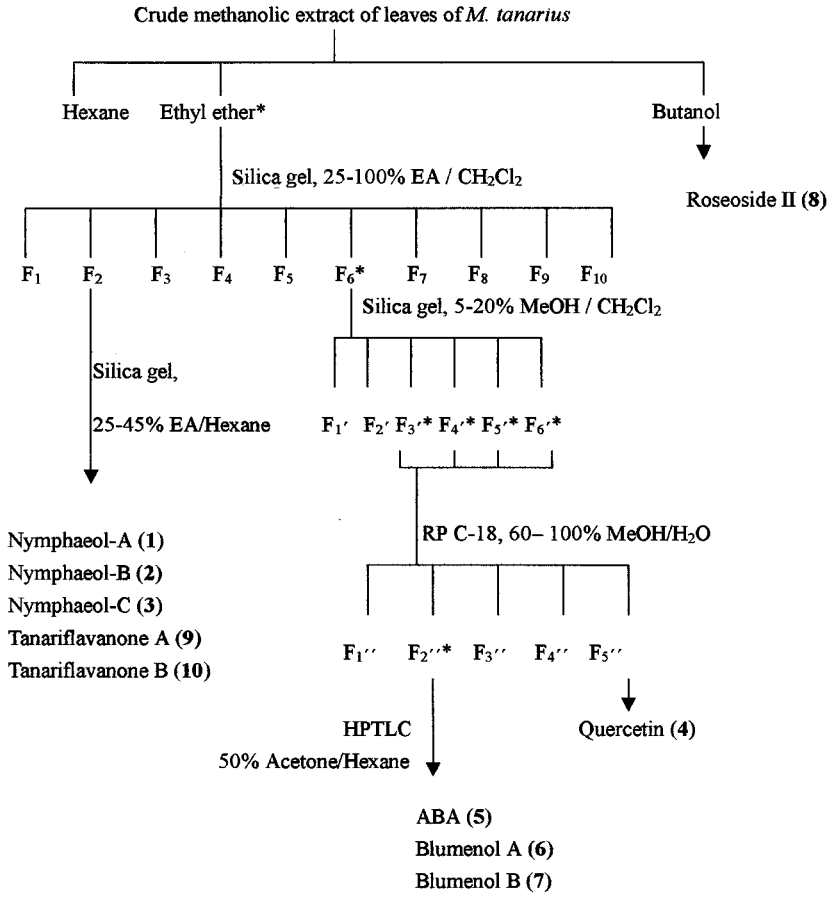

FIG. 2. Scheme for isolating phytotoxic active components from crude methanolic extract of *M. tanarius* leaves. *High phytotoxic active fraction.

Statistics. Data were analyzed by the Duncan's multiple range test (Gomez and Gomez, 1976), *t* test, and SAS for Windows v. 6.12.

RESULTS

Comparison of Vegetation Composition. The floristic composition and absolute coverage of each species per plot in *M. tanarius* stands and the adjacent grassland areas are given in Table 1. The vegetation composition on the *M. tanarius* floor at the two sites was similar, but the grasslands at the two sites were different.

TABLE 1. FLORISTIC COMPOSITION AND COVERAGE OF SPECIES PRESENT IN CHAUCHOU AND YAUNSAN STUDY SITES

Species name	Actual coverage (%)/m^2			
	Chauchou		Yaunsan	
	Grassland	*M. tanarius*	Grassland	*M. tatarius*
Aeschynomene indica	20.0	-	-	-
Alocasia macrorrhiza	-	43.0	6.0	61.1
Alpinia formosana	-	-	-	6.3
Areca catechu	-	-	-	0.3
Bidens pilosa	70.0	-	-	-
Broussonetia papyifera	-	1.0	-	-
Callicarpa remotiserrulata	-	-	-	5.0
Cassia mimosoides	2.0	-	-	-
Cenchrus echinatus	0.3	-	-	-
Chloris barbata	5.0	-	-	-
Christella acuminata	-	0.7	-	7.0
Cinnamomum camphora	-	0.07	-	-
Crassocephalum rabens	-	-	10.0	-
Cyperus rotundus	-	0.4	-	-
Elaeocarpus serratus	-	-	-	11.5
Eragrostis amabilis	0.3	-	-	-
Euphoria longana	-	-	-	1.5
Ficus pumila	-	-	-	1.5
Ipomoea batatas	-	-	1.0	-
Ipomoea digitata	-	-	4.5	0.4
Ipomoea obscura	7.0	0.2	5.0	-
Lygodium japonicum	-	-	-	1.1
Macaranga tanarius	-	-	4.5	-
Machilus kusanoi	-	-	4.0	5.7
Mallotus paniculatus	-	-	-	2.3
Malvastrum coromandelianum	5.0	-	-	-
Melanolepis multiglandulosa	3.3	6.0	-	-
Mimosa invisa	35.0	-	-	-
Mimosa pudica	3.3	-	-	-
Momordica charantia	1.0	-	-	-
Miscanthus floridulus	7.0	-	50.0	
Murraya paniculata	-	-	-	2.1
Musa sapientum	-	-	30.0	-
Oplismenus compositus	-	2.0	50.0	-
Panicum repens	4.0	-	-	-
Paspalum conjugatum	-	0.7	81.0	-
Passiflora foetida	3.0	-	-	-
Pennisetum alopecuroides	5.0	-	-	-
Peristrophe japonica	-	-	20.0	-
Polygonum chinense	-	-	10.0	-
Psychotria rubra	-	-	-	5.3
Pueraria lobata	5.0	0.48	-	-

TABLE 1. CONTINUED

Species name	Actual coverage (%)/m^2			
	Chauchou		Yaunsan	
	Grassland	*M. tanarius*	Grassland	*M. tatarius*
Rhaphidophora aurea	-	6.6	-	40.0
Rhynchelytrum repens	5.0	-	-	-
Schefflera octophylla	-	-	-	8.6
Solanum capsicastrum	-	0.4	-	-
Typhonium divaricatum	-	0.004	-	-
Wedelia chinensis	-	0.07	-	-
Total coverage (%)	196.2	62.0	276.0	159.7
% of grassland control	100.0	32.0	100.0	58.0
Number of species	18.0	14.0	12.0	16.0

Note: "-" indicates that plant was not found.

Most plants under *M. tanarius* canopy were shade-tolerant plants; for example, the mean coverage of dominant species *Alocasia macrorrhiza* was above 40%, and *Christella acuminata* and *Rhaphidophora aurea* were also dispersed on the floor of *M. tanarius* stands at the two sites. In the grassland plots at the Chauchou site, the coverage of *B. pilosa* and *Mimosa invisa* reached 70 and 35%, respectively. *Aeschynomen indica*, *Chloris barbata*, *Malvastrum coromandelianum*, *Pennisetum alopecuroides*, *Rhaphidophora aurea*, and *Rhynchelytrum repens* were found. Coverage of *Miscanthus floridulus* and *Oplismenus compositus* was significantly higher than 50%, and *Peristrophe japonica*, *Polygonum chinense*, *Musa sapientum*, and *Crassocephalum rabens* were also found at the Yaunsan site. Some seedlings of *Machilus kusanoi* were dispersed under the *M. tanarius* stand at the Yaunsan site, but were absent in the Chauchou site. Differences could be due to different weather conditions at the two sites. It is concluded that total coverage of understory species on the *M. tanarius* floor was significantly lower than that of the grassland area at the two sites (Table 1).

Density-Dependent Phytotoxicity. Lettuce growth was reduced in soil containing *M. tanarius* leaf powder (Table 2). The highest reduction of lettuce growth was observed in the low-density treatment. Total dry weight per plant was reduced by 68% in soil containing leaf powder compared to the control (Table 2). Growth was reduced by 55% in the medium-density treatment, and only by 23% in the high-density treatment. The results of this experiment may be better understood by studying the slope of the log yield–log density relationship. Harvesting at the 5th week, the slope was reduced in the soil mixed with *M. tanarius* leaves as compared to the soil alone control (0.004 vs. -0.53, Figure 3A), showing significance below the 5% level ($F = 44.86$). In contrast, there was no reduction of slope in the growth of *A. macrorrhiza* in soil mixed with *M. tanarius* leaf powder (-0.46

TABLE 2. GROWTH PERFORMANCE OF LETTUCE SEEDLINGS AT THREE DENSITIES IN SOIL CONTAINING *Macaranga tanarius* LEAF POWDER OR SOIL ALONE AS CONTROL

Seedling density (no./m^2)	Dry weight per plant (mg)				% reduction of control
	Soil	Shoot	Root	Total ± SE	
Low (4500)	Soil (control)	8.00 a	2.70 a	10.65 ± 0.07	
	Soil + leaf powder	2.20 d	1.20 b	3.45 ± 0.07	68 a
Medium (8500)	Soil (control)	4.75 b	2.40 a	7.10 ± 0.14	
	Soil + leaf Powder	2.05 d	1.15 b	3.20 ± 0.28	55 a
High (17,500)	Soil (control)	3.05 c	1.45 b	4.55 ± 0.21	
	Soil + leaf Powder	2.35 d	1.20 b	3.50 ± 0.71	23 b

Note: Means in the column followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple range test. The measurements were taken 5 weeks after planting.

vs. −0.44, Figure 3B), indicating that the growth of this plant was not affected by phytotoxins released by *M. tanarius*.

Phytotoxicity of Aqueous Extracts. Aqueous extracts (0.5, 1.0, 1.5, and 2.0%) of *M. tanarius* leaves were bioassayed by using lettuce and *B. pilosa* seeds as test material. In general, radicle growth of lettuce was suppressed by the extract at as low as 0.5%, and the inhibition increased with greater concentrations (Figure 4). The same inhibition was observed when *B. pilosa* grew in the aqueous extract of *M. tanarius* leaves (Figure 4). Radicle growth of both lettuce and *B. pilosa* was suppressed by increased concentrations of the aqueous leaf extract. The 2.0% aqueous extract of *M. tanarius* leaves caused 95 and 70% inhibition of the radicle growth of lettuce and *B. pilosa*, respectively. The aqueous extract was not active at the 0.5% concentration for *B. pilosa*, and more nutrients were in the extract than in the pure water control. Therefore, response of the low concentration of extract promoted the growth of *B. pilosa*. One might question whether the inhibition might be due to the effect of osmolarity and pH of the solutions. The osmolarity of the 0.1% extracts of *M. tanarius* leaves was 18 milliosmols, while that of 2.0% extracts was 32 milliosmols. The pH of the 0.1–2.0% aqueous extracts ranged mostly above 4.0 (Tseng, 2001). Chou and Young (1974) indicated that osmolarity of extracts below 30 milliosmols would not cause a significant osmotic inhibition, but a pH below 4.0 or above 8.0 did cause significant inhibition of lettuce radicle growth. They also indicated that when the osmolarity of extracts exceeds 50 milliosmols, it might cause 20–30% inhibition. In the present study, even at 2.0%, the osmolarity of extracts was below 35 milliosmols, and the pH ranged from 4.0 to 4.5. The 2.0% (or above) extract caused both osmotic inhibition and phytotoxic effects; however, the osmotic inhibition was about 20% while the total inhibition was up to 70%. Thus, phytotoxic inhibition was about 50% (70–20%). In addition to bioassays of the aqueous extract, soil containing *M. tanarius* leaf powder also suppressed the growth of *B. pilosa* and *L. leucocephala* (Figure 5).

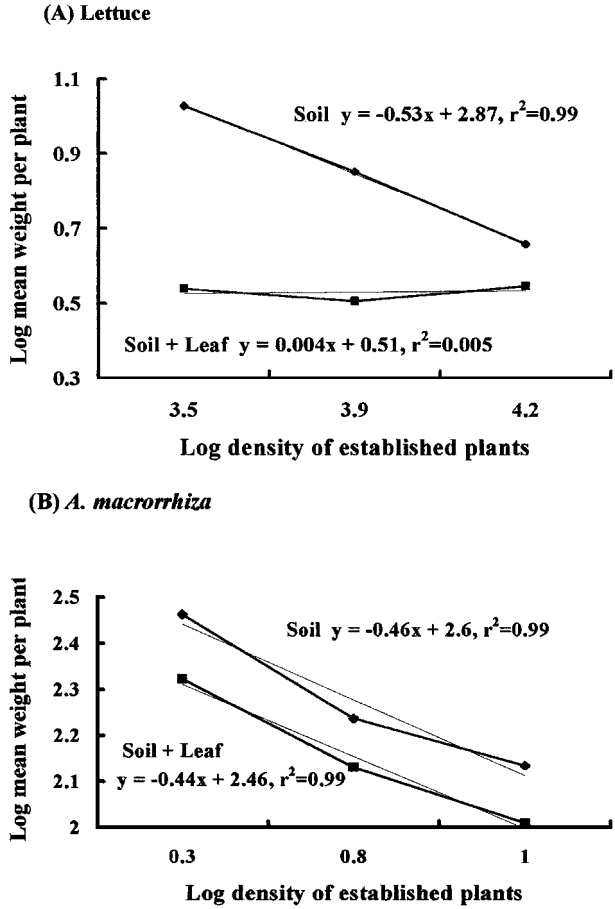

FIG. 3. Relationship between log mean weight per plant and log plant density for lettuce and *A. macrorrhiza* grown in soil containing leaf powder and soil alone: (A) lettuce, (B) *A. macrorrhiza.* Solid dark lines indicate the observational data while the light lines indicate the statistical linear regression on the basis of experimental data.

Phytotoxicity of Ether Extracts. The result of chromatographic bioassay of the ether fraction of an aqueous extract of *M. tanarius* leaves showed significant suppression of radicle growth at R_f 0.42–0.49 and R_f 0.76–0.84 (Figure 6). These findings led us to isolate phytotoxins from the ethyl ether fraction (Figure 2). Silica gel filtration of the ethyl ether fraction (80 g) gave 10 fractions (F_1–F_{10}). Fraction F_6 showed the most inhibition of lettuce germination. Further purification of F_6 using silica gel resulted in six fractions ($F_{1'}$–$F_{6'}$). At 500 ppm, fractions $F_{3'}$–$F_{6'}$ inhibited lettuce seed germination (Tseng, 2001). The active fractions ($F_{3'}$–$F_{6'}$)

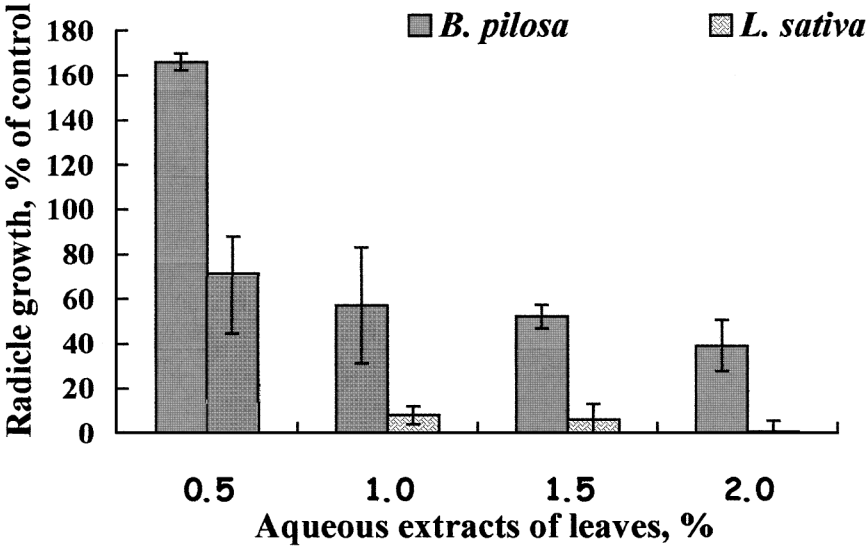

FIG. 4. Effect of aqueous extracts (0.5–2.0%) of *M. tanarius* leaves on radicle growth of lettuce and *B. pilosa*.

were combined and further fractionated by reverse-phase chromatography into 5 fractions ($F_{1''}$–$F_{5''}$). Fraction $F_{2''}$, at 500 ppm, exhibited the most inhibitory effect on germination. Fraction $F_{2''}$ was further purified by HPTLC, and three bands were visualized. The pure compound from the first band (R_f value 0.3) was eluted and bioassayed against lettuce. Lettuce germination was remarkably suppressed by the isolated compound at 20 ppm (Tseng, 2001).

Identification. The spectroscopic data were checked with those of Yakushijin et al. (1980) and confirmed the structure of compound **1** as nymphaeol-A, compound **2** as nymphaeol-B, and compound **3** as nymphaeol-C (see Figure 7). The spectroscopic data for compound **4** were in agreement with Lin et al. (2000) and confirmed the structure as quercetin (Figure 7). The spectroscopic data of compound **5** agree with Ohkuma et al. (1965) and Constantino et al. (1989) and confirm the structure of the first band (R_f value 0.3) as abscisic acid (ABA) (Figure 7). The spectroscopic data obtained for compound **6**, and **7** agree with Galbraith and Horn (1972) and confirm the structures as blumenol A and blumenol B, respectively (Figure 7). The spectroscopic data of compound **8** agree with Otsuka et al. (1995) and confirms the structure as roseoside II (Figure 7).

Phytotoxicity of Pure Compounds. Pure compounds isolated from *M. tanarius* leaves, namely quercetin (**4**), abscisic acid (**5**), blumenol A (**6**), blumenol B (**7**), and roseoside II (**8**) were prepared separately at various concentrations and bioassayed against *Miscanthus floridulus*, *Bidens pilosa*, and *Chloris barbata.* Results

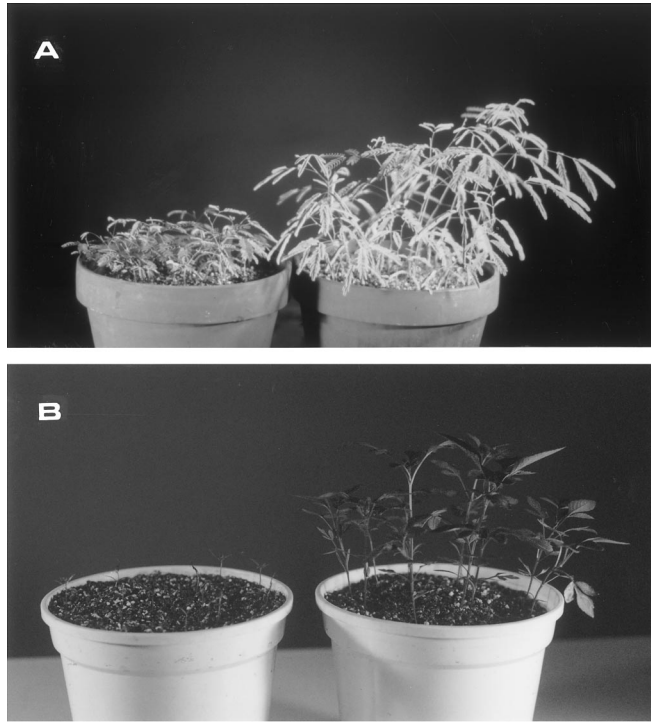

FIG. 5. Comparison of inhibitory effect of soil mixed with leaf powder (left) and soil alone as control (right) on the seedling growth of *L. leucocephala* (A) and *B. pilosa* (B). The measurement was taken 1 month after planting.

varied with different compounds (Table 3). ABA (**5**) exhibited the most inhibitory effect. At a concentration of 120 ppm, ABA caused about 75% inhibition of shoot growth of *M. floridulus* and *C. barbata*, and about 55% inhibition of radicle growth of both weeds. At 40 ppm, the radicle growth of *B. pilosa* was inhibited up to 45% compared to the distilled water control. The other compounds, including quercetin, blumenol A, and blumenol B, at 600 ppm showed 20–25% inhibition of radicle and shoot growth of *M. floridulus* (Table 3). Nymphaeol-A (**1**), nymphaeol-B (**2**), nymphaeol-C (**3**), and roseoside II (**8**) did not inhibit lettuce or weed germination (data not shown).

DISCUSSION

Analysis of yield–density relationships can be used as a tool to understand the resource competition and allelopathic interference between plants prior to a

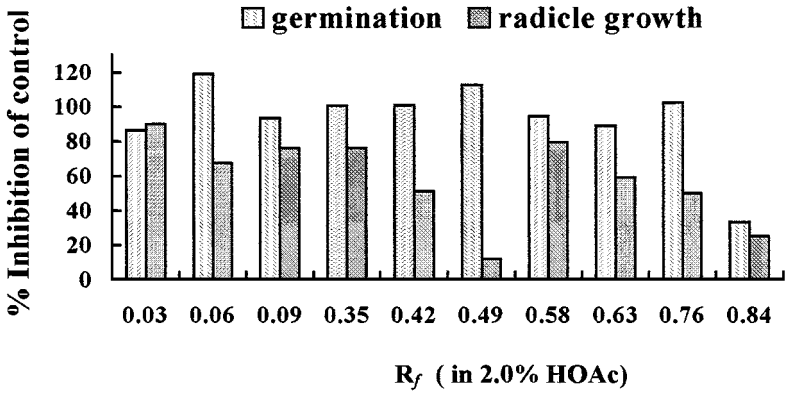

FIG. 6. Chromatographic bioassay of compounds isolated from the ether fraction of aqueous extract of *M. tanarius* leaves. The corresponding R_f segments of chromatogram with or without spotting extract were used as test and control, respectively. The paper strip was developed with 2.0% acetic acids.

prolonged and expensive phytochemical investigation of a suspected toxic species (Weidenhamer et al., 1989). As plant density increases, total yield increases linearly. Beyond a certain density, reductions of individual plant growth caused by density-dependent mortality and resource competition result in a constant total yield per unit area (Kira et al., 1953). Studies of both allelochemicals and herbicides have shown that phytotoxic effects are density-dependent (Skipper, 1966; Hoffman and Lavy, 1978; Winkle et al., 1981; Weidenhamer et al., 1987). These studies suggest that the presence of high phytotoxin concentrations may cause a reversal in the slope of predicted log yield–log density relationships at low plant densities, in contrast to the expected consequences of increased density and resource competition (Weidenhamer et al., 1989). The relationship of log mean weight per plant and log plant density for tomatoes exhibited a reversal in slope of the predicted log yield–log density relationship, indicating the presence of a toxic substance in walnut soil (Weidenhamer et al., 1989). The retrogressive slope of the log weight–log density relationship in lettuce indicated a positive response of plant growth to phytotoxins from the leaves of *M. tanarius* (Figure 3A). In a given soil volume containing a finite amount of leaf powder, each lettuce plant growing at low density might uptake a higher amount of available phytotoxins than at high density. In other words, lettuce growing at low density would be exposed to higher levels of phytotoxins, leading to a greater inhibition of growth. Total dry weight per plant was most reduced at low density in soil containing leaf powder (Table 2). The presence of phytotoxins in leaves was further confirmed by the standard sponge bioassay against seed germination of lettuce and *B. pilosa*. *Alocasia macrorrhiza* found in the understory of Australian tropical forests and

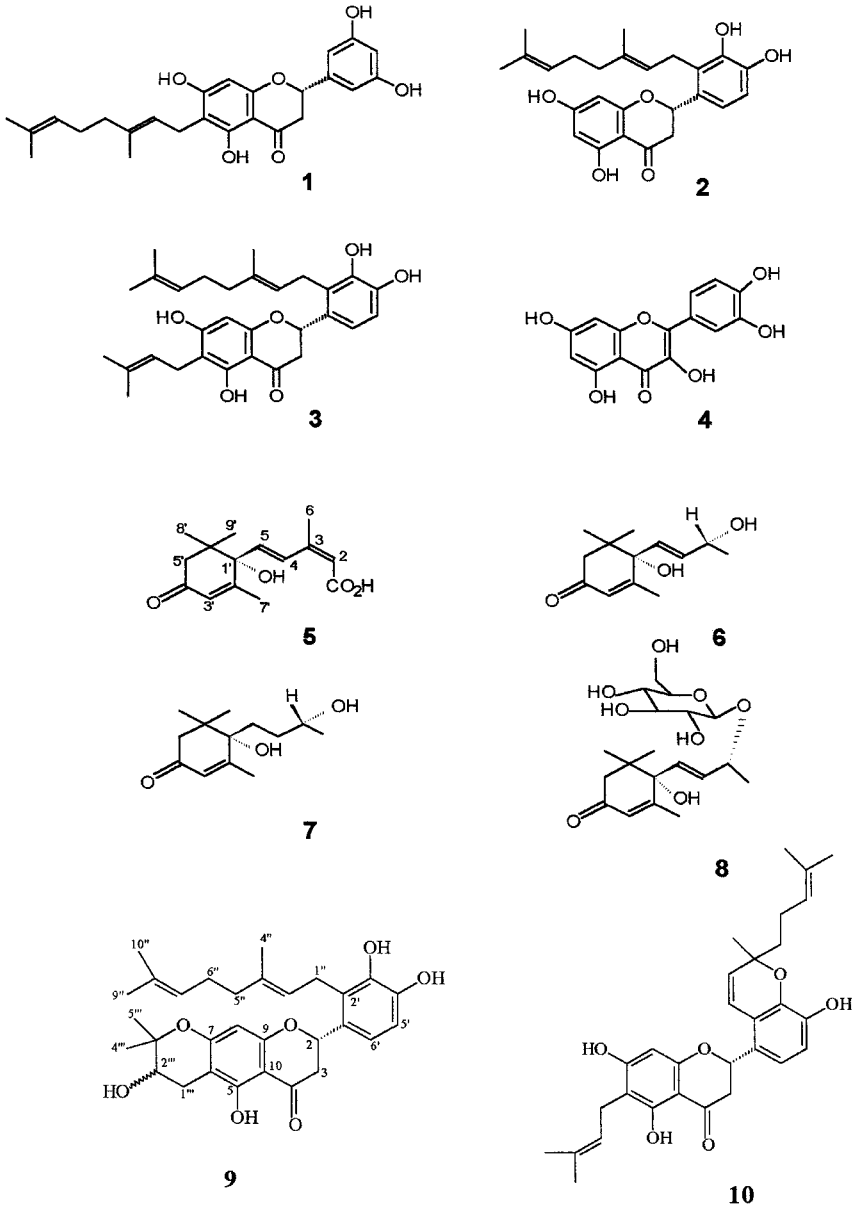

FIG. 7. Chemical structures of the 10 identified compounds from *M. tanarius* leaves. (**1**) nymphaeol-A; (**2**) nymphaeol-B; (**3**) nymphaeol-C; (**4**) quercetin; (**5**) abscisic aicd; (**6**) blumenol A; (**7**) blumenol B; (**8**) roseoside II; (**9**) tanariflavanone A; (**10**) tanariflavanone B. The compounds are structurally confirmed by ^{1}H NMR, ^{13}C NMR, and MS spectra.

TABLE 3. EFFECTS OF DIFFERENT CONCENTRATIONS OF COMPOUNDS ISOLATED FROM *M. tanarius* LEAVES ON RADICLE AND SHOOT GROWTH OF TESTED PLANTS

Compound	Conc. (ppm)	Radicle growth (% of control)	Shoot growth (% of control)	Tested species
Abscisic acid	10	80*	-	*Biden pilosa*
	20	82*	-	
	30	67**	-	
	40	55**	-	
Abscisic acid	40	71**	57**	*Chloris barbata*
	80	65**	62**	
	120	41**	25**	
Abscisic acid	40	142**	68*	*Miscanthus floridulus*
	80	86	49**	
	120	44**	23**	
Quercetin	200	106	82	*M. floridulus*
	400	98	77	
	600	72*	80	
Blumenol A	200	90	96	*M. floridulus*
	400	75*	81	
	600	71*	70	
Blumenol B	200	98	86	*M. floridulus*
	400	82	89	
	600	81*	87	

Note: "-" indicates that the shoot length of test plants was not visualized.
* Values are significant from control ($P < 0.05$) according to t test.
** significant at $P < 0.01$.

subtropical forests has been extensively studied in terms of adaptation to extreme shade conditions (Boardman, 1977). On the basis of our field observation, the canopy of *M. tanarius* seems to provide a moderate habitat for *A. macrorrhiza*. In the greenhouse, *A. macrorrhiza* was apparently not sensitive to the phytotoxins released from the leaves of *M. tanarius*.

Compounds identified from *M. tanarius* leaves, namely quercetin (**4**), ABA (**5**), blumenol A (**6**), blumenol B (**7**), tanariflavanone A (**9**), and tanariflavaone B (**10**) (Tseng et al., 2001), exhibited inhibitory effects on radicle growth of lettuce and weeds. The major growth inhibitor, however, was ABA, which is well recognized as a plant growth regulator (Zeevaart and Creelman, 1988; Leung and Giraudat, 1998). Wheat straw collected from the soil surface after harvest also contains ABA (Hall et al., 1986). The existence of ABA in straw residues from preceding crops was the cause for poor growth of successive crops (Hall et al., 1986). Leachates of tall fescue grass (*Festuca arundinacea*) contained three principal allelochemicals including ABA, caffeic acid, and *p*-coumaric acid. After quantitative analysis, ABA was found to be a predominant inhibitor (Buta and Spaulding, 1989). The concentrations of ABA in grass leachates of desiccated tall fescue were

about 390 ng g^{-1} fw and approximately the same as from straw leachates (Buta and Spaulding, 1989). The concentration of ABA in leaves of *M. tanarius* was much higher. Since ABA inhibits seed germination and influences stomatal control (Zeevaart and Creelman, 1988; Leung and Giraudat, 1998), nanogram quantities of ABA found in dried grass leachates were sufficient to cause inhibition (Buta and Spaulding, 1989). Likely, a certain quantity of ABA would be leached from senescent or dehydrated leaves after the extended desiccation of 30 days (Buta and Spaulding, 1989). ABA is a potent phytotoxin and its concentration was higher in *M. tanarius* leaves than that of previous reports, suggesting that ABA is involved in the allelopathy of *M. tanarius*. Although it is probable that blumenol A (**6**), blumenol B (**7**), and roseoside II (**8**) are biosynthetically derived from abscisic acid (Bhakuni et al., 1974), they did not inhibit radicle growth of lettuce like ABA (**5**). The different biological activities are probably due to structural differences, suggesting that C-1, C-2, C-3, and C-6 of ABA are important for biological activity. Tanariflavanone B (**10**) inhibited lettuce germination more effectively than nymphaeol-A (**1**), nymphaeol-B (**2**), nymphaeol-C (**3**), and tanariflavanone A (**9**), suggesting the importance of the pyran ring for biological activity, but this remains to be confirmed.

When *M. tanarius* invades grasslands, its fallen leaves accumulate on the ground over time and can inhibit the growth of the nearby weeds allowing the plant to compete for more resources. One or two years after invasion of the grassland, development of a *M. tanarius* canopy leads to a gradual reduction in light and increases the concentration of allelopathic compounds, resulting in a change in the floral composition. *M. tanarius* has larger leaves with a dense canopy that would lead to shading, resulting in lower temperature and higher humidity in the understory. The dense shade might provide moderate habitat for shade-adapted plants, such as *A. macrorrhiza*, that could resist the *M. tanarius* phytotoxins. *M. tanarius*, therefore, becomes a dominant in the community after invasion into grasslands.

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ANNOUNCEMENT

The International Allelopathy Foundation is organizing the IV International Congress on Allelopathy–Allelopathy in Sustainable Terrestrial and Aquatic Ecosystems–to be held August 23–25, 2004 at CCS Haryana Agricultural University, Hisar, India. There will be both oral and poster sessions. Fifteen symposia are being organized. The deadline for abstract submission is July 31, 2003.

The conference aims to bring together active allelopathy scientists working worldwide to review progress, identify constraints, and project future goals for basic and applied allelopathy research in Terrestrial an Aquatic Ecosystems. Previous conferences were attended by approximately 200 scientists representing 17 countries. Accommodations are included with the registration fee and will be in the Faculty House at the University. Hisar is a small ancient city about 165 km west of New Delhi.

For more information contact:

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EFFECTS OF SAMPLE DRYING AND STORAGE, AND CHOICE OF EXTRACTION SOLVENT AND ANALYSIS METHOD ON THE YIELD OF BIRCH LEAF HYDROLYZABLE TANNINS

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Abstract—In this study, I investigated the effects of different methods of sample drying and storage, and the choice of extraction solvent and analysis method on the concentrations of 14 individual hydrolyzable tannins (HTs), and insoluble ellagitannins in birch (*Betula pubescens*) leaves. Freeze- and vacuum-drying of birch leaves were found to provide more reliable results than air- or oven-drying. Storage of leaves at −20°C for 3 months before freeze-drying did not cause major changes in tannin content, although levels of 1,2,3,4,6-penta-*O*-galloylglucose and isostrictinin were altered. Storage of dried leaf material at −20°C is preferred because 1 year storage of freeze-dried leaves at 4°C and at room temperature decreased the concentration of the pedunculagin derivative, one of the main ellagitannins of birch. Furthermore, storage at room temperature increased the levels of isostrictinin and 2,3-(*S*)-HHDP-glucose, indicating possible HT catabolism. Of the extraction solvents tested, aqueous acetone was superior to pure acetone, or aqueous or pure methanol. The addition of 0.1% ascorbic acid into 70% acetone significantly increased the yield of ellagitannins, presumably by preventing their oxidation. By comparing the conventional rhodanine assay and the HPLC–ESI-MS assay for quantification of leaf galloylglucoses, the former tends to underestimate total concentrations of galloylglucoses in birch leaf extract. On the basis of the outcomes of all the method and solvent comparisons, their suitability for qualitative and quantitative analysis of plant HTs is discussed, emphasizing that each plant species, with its presumably unique HT composition, is likely to have a unique combination of ideal conditions for tissue preservation and extraction.

Key Words—Sample drying, storage, extraction, tannin quantification, HPLC, ESI-MS, hydrolyzable tannins, leaves, birch, *Betula pubescens*.

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INTRODUCTION

Hydrolyzable tannins (HTs) form a large group of polyphenolic compounds that are hypothesized to play an important role in plant–herbivore interactions. Despite the wide occurrence of HTs in the plant kingdom, their structural diversity has seldom been taken into account in their quantification. Accordingly, the simplest and, therefore, widely used quantitative methods, like the radial diffusion assay for total tannins (Hagerman, 1987), the rhodanine assay for gallotannins (GTs) (Thies and Fischer, 1973; Inoue and Hagerman, 1988), and the $NaNO_2$ assay for ellagitannins (ETs) (Bate-Smith, 1972; Wilson and Hagerman, 1990) are based on the analysis of total protein-precipitable tannins or HT subgroups only. Although these methods are only partially GT- or ET-specific (i.e., galloyl groups often present in ETs are quantified by the rhodanine assay for GTs, but not with the $NaNO_2$ assay for ETs), the preparation of analyzable extracts from plant material can be an even more crucial point than the choice of analysis method itself, in measuring the natural content of tannins within a living plant. Once harvested, the metabolic state of the organism may change, and with it the quantity and quality of tannins (Waterman and Mole, 1994). Immediate extraction of freshly collected plant tissue or rapid lyophilization after collecting into liquid nitrogen would presumably minimize alterations in tannin concentrations. However, in the presence of a large number of samples, the usual case in ecological studies, and in the absence of sophisticated equipment, as is usual in field experiments, sample preparation cannot always be performed with the fastest and most optimal methods. Nevertheless, all plant extracts within a comparative set of experiments should be prepared from similarly pretreated plant material. It is necessary to know which methods can be used in different situations for sample drying, storage, and extraction, and how these methods affect the yields of individual HTs.

Although there have been studies on the effects of sample drying, storage, or extraction on the yield of plant phenolic compounds (Hagerman, 1988; Raynal et al., 1989; Peng and Jay-Allemand, 1991; Orians, 1995; Torti et al., 1995; Keinänen and Julkunen-Tiitto, 1998; Julkunen-Tiitto and Sorsa, 2001; Zafrilla et al., 2001), only a few have dealt with HTs, focusing on total protein-precipitable tannins measurable with the radial diffusion assay (Hagerman, 1987, 1988; Peng and Jay-Allemand, 1991). The aim of the present study was to investigate the effects of different methods of sample drying and storage, and the choice of extraction solvent on the concentrations of 14 individual birch (*Betula pubescens*) leaf HTs measured with a high-performance liquid chromatographic—electrospray ionization mass spectrometric (HPLC–ESI-MS) method (cf. Salminen et al., 1999, 2001, 2002; Salminen and Lempa, 2002). Also, the effects on the total concentrations of insoluble, cell-wall-bound ETs were measured, since insoluble ETs presumably contribute to leaf toughness and, therefore, to herbivore performance as well

(Ossipov et al., 2001; Salminen et al., 2002). In addition, the rhodanine assay for total GTs was compared with the HPLC–ESI-MS method in the quantitative analysis of birch leaf galloylglucoses (GGs, precursors for more complex GTs) by analyzing birch leaf extracts with both of the methods. On the basis of the results, suitability of these methods for the qualitative and quantitative analysis of plant HTs is discussed.

METHODS AND MATERIALS

Samples for Comparisons of Drying and Storage Methods. Short shoot leaves of white birch (*Betula pubescens* Erhr.) were collected in May 1999 between 9 and 10 A.M. from lower branches of a single tree growing in the Botanical garden of the University of Turku, SW Finland. Young, approximately 2-wk-old leaves were clipped from the petioles and placed randomly in 40 sealed plastic bags (10–15 leaves per bag), which were enclosed in an insulated box filled with wet ice and transported immediately to a laboratory.

Sets of five bags were treated as follows: (1) The first was vacuum-dried (vacuum drying = freeze-drying of fresh, nonfrozen leaf material) immediately for 48 hr. (2) The second was oven-dried at 60°C for 12 hr. (3) The third was air-dried in a fume hood at room temperature for 4 d. (4) The fourth set containing 20 bags (15 bags for storage method comparisons) was kept in a freezer (−20°C) for 2 d and subsequently freeze-dried for 48 hr. (5) The fifth set was kept in a freezer (−20°C) for 3 months and subsequently freeze-dried for 48 hr. Samples were ground into fine powder each time the drying was completed, stored at −20°C in sealed plastic bags, and extracted with 70% acetone within a week, except that the 15 samples used in the storage method comparison were extracted after 1 year of storage. The three 5-bag sets were stored at (1) room temperature, (2) 4°C, and (3) −20°C. All storage comparison bags were sealed after removal of excess air and wrapped in aluminium foil to keep material in the dark.

Samples for Comparison of Extraction Solvents. Samples for the extraction solvent comparisons were collected as described above but a week later and from a different white birch tree growing in the Botanical garden. Samples were placed randomly in 28 sealed plastic bags, which were enclosed in an insulated box filled with wet ice and transported immediately to a laboratory freezer. After 2 d of freezing, the frozen material was freeze-dried for 48 hr. Lyophilized samples were ground into fine powder and extracted with the following solvents: (1) 50% methanol, (2) 70% methanol, (3) 100% methanol, (4) 50% acetone, (5) 70% acetone, (6) 70% acetone containing 0.1% (m/v) ascorbic acid, and (7) 100% acetone.

Samples for Comparison of Analysis Methods. Samples for the analysis method (rhodanine assay vs. HPLC–ESI-MS) comparisons were collected near the

Kevo Subarctic Research Station of the University of Turku, in northern Finland. Leaves of 24 mountain birch (*B. pubescens* ssp. *czerepanovii*) trees were collected five times (June 3, 8, 16, 21, and 28) during summer 2000 using the same procedure as above. Since three of the study trees did not yet have leaves at the point of first sampling, the total number of mountain birch samples was 117. Leaves of 30 dwarf birches (*B. nana* L.) were collected twice (June 21 and July 2) during summer 2001. Samples were freeze-dried and extracted with 70% acetone containing 0.1% ascorbic acid (117 mountain birch samples) or with 70% acetone only (60 dwarf birch samples). Voucher specimens of all birch species have been deposited at TUR under the following accession codes: TUR 360093–TUR 360103 and TUR 564208–TUR 564212.

Extraction. The dried and ground leaves (300 mg dry wt) were extracted three times (3×1 hr) with corresponding solvent (3×10 ml) in a shaker (homogenizer). The insoluble residue was extracted once again (5 sec by hand) with the same solvent (5 ml) to remove all extracted compounds. After evaporation of organic solvents at room temperature, the aqueous extract was freeze-dried and dissolved in water (3×2 ml). After centrifugation (10 min at 2500*g*), the supernatant was filtered through a 0.45 μm PTFE filter and kept frozen at -20°C until analyzed with HPLC–ESI-MS. The insoluble residue of leaves that was not extractable with the solvents was lyophilized, weighed, and stored at -20°C until used in the analysis of insoluble ETs.

Analysis of Hydrolyzable Tannins with HPLC–ESI-MS. HPLC–ESI-MS analysis was performed with a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ion-spray (pneumatically assisted electrospray) interface. The HPLC system consisted of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT, USA) connected to a Series 200 autosampler. The eluate passed through a Merck Superspher 100 RP-18 column (75×4 mm i.d., 4 μm), and the UV trace was recorded with a 785A UV/VIS detector at 280 nm. After UV detection, part of the eluate was split off and introduced into the ESI-MS system. The elution gradient of reversed phase HPLC consisted of water and acetonitrile, both including 0.4% formic acid as a modifier; other chromatographic and ESI-MS conditions were as described in a previous paper (Salminen et al., 1999). Leaf extracts were qualitatively analyzed with negative ion HPLC–ESI-MS, and HTs were identified on the basis of their $[M—H]^-$ values and retention times as in Salminen et al. (1999, 2001). The identified HTs were quantified in mg/g dry wt using ion trace analysis of deprotonated molecules ($[M—H]^-$) of individual tannins and calibration curves of the appropriate HTs reported previously (Salminen et al., 1999, 2001, 2002). Differences in the ESI-MS performance among individual runs were standardized by using 6-bromo-2-naphthyl-β-D-glucopyranoside as an internal standard. Following the established practice in the ecological literature, we use the term

"concentration" to refer to the mass of a particular compound per unit of leaf mass (mg/g drywt).

Analysis of Total Contents of Galloylglucoses with the Rhodanine Assay. Quantitative analysis of gallic acid in the hydrolyzate extracts was used for the determination of total gallotannins (Ossipov et al., 1997; a modification of the method by Inoue and Hagerman, 1988). One milliliter of extract was placed in a 2-ml Teflon-lined screw-cap glass tube, freeze-dried, and dissolved in 1 ml of 1 M H_2SO_4. The tube was capped, and hydrolysis was run for 4 hr at 100°C. A 0.1-ml aliquot of the hydrolyzate was diluted to 1 ml with distilled water; 0.2 ml of this sample was mixed with 0.3 ml of 0.667% methanolic rhodanine solution. After exactly 5 min, 0.2 ml of 0.5 M KOH solution was added. After 2.5 min, the mixture was diluted to 5 ml with distilled water. Five to 10 min later the absorbance at 520 nm was read. The rhodanine assay was standardized with gallic acid.

Analysis of Insoluble Ellagitannins. Insoluble ETs were quantified with a modification of the method described by Peng et al. (1991). A total of 50 mg of lyophilized insoluble residue of birch leaves was hydrolyzed for 3 hr with 5 ml MeOH/6 M HCl (9:1) at 120°C to liberate the ellagic acid that was bound to insoluble ETs as hexahydroxydiphenoyl (HHDP) residues. With the given conditions, hydrolysis time of 3 hr was enough to liberate all ellagic acid from the insoluble residue (cf. Salminen, 2002). After cooling to room temperature, the hydrolyzate was filtered through 0.45 μm PTFE filters and analyzed directly with HPLC at 258 nm. The HPLC system (Merck-Hitachi, Tokyo, Japan) consisted of a pump L-7100, a UV detector L-7400, a programmable autosampler L-7200, and an interface D-7000. The column was Superspher 100 RP-18 (75 $\times$ 4 mm i.d., 4 μm, Merck, Germany), and eluents were 0.05 M H_3PO_4 (A) and CH_3CN (B). The elution profile was 0–3 min, 98% A (isocratic); 3–22 min, 2–20% B in A (linear gradient); 22–30 min, 20–30% B in A (linear gradient) at a flow rate 1 ml/min. Ellagic acid was detected based on its retention time compared to an authentic standard. The identity of the ellagic acid peak was confirmed with its UV and mass spectra. The content of insoluble ETs was quantified as mg ellagic acid/g dry wt of birch leaves.

Statistical Methods. The effects of different methods for sample drying and storage, and of extraction solvents on the yields of birch leaf HTs were analyzed with one-way analysis of variance. The Student–Newman–Keuls multiple range test was performed on all treatment means to determine statistically significant differences among individual treatments (proc GLM, SAS Inc. 1996). Analysis of the Pearson product moment correlation coefficient (proc CORR, SAS Inc. 1996) was performed to analyze relationships between the values obtained with the two methods for the quantitative analysis of HTs.

RESULTS AND DISCUSSION

Every time a new plant species is investigated, a survey for the optimal sample preparation protocols (e.g., drying, storage, and extraction) should be undertaken. As the ultimate goal is to obtain an accurate estimate of the quantity and quality of chemical components present in a living plant tissue, and not only in a plant extract, one has to be aware of possible chemical transformations occurring to the compounds of interest. Furthermore, it is necessary to be able to translate the quantitative results of, e.g., drying method comparisons, in order to determine the best protocols for sample preparation. The only way to achieve this goal is to know the chemical structures of individual compounds and the relationships among them, i.e., their order in the biosynthetic pathway and their possible catabolic products. These factors are well-known for hydrolyzable tannins of birch (*B. pubescens*) leaves; only the full chemical structure of the pedunculagin derivative, and its position in the biosynthetic pathway of HTs is unclear (cf. Salminen et al., 1999, 2001, 2002). However, because of the complexity of interactions among individual HTs in the biosynthetic pathway (see Figure 1), even their detailed quantification at a specific time (as the plant extract is ready to be analyzed) may not reveal all transformations that have occurred after the sample was collected. Nevertheless, as the traditional methods that quantify only total contents of GTs or ETs cannot separate individual tannins from each other, it is evident that by using those methods only a minor portion of the transformations can be revealed.

By comparing several methods for leaf sample drying and storage, and different solvent compositions for sample extraction, we found that the yields of HTs vary significantly depending on the method or solvent chosen (Tables 1–3). Of the sample drying methods tested, vacuum- and freeze-drying of newly collected ($\leq$2 d before drying) leaves were more reliable than air- or oven-drying. The two latter methods, without lowering temperatures at beginning of the drying process, resulted in decreased yields of 1-*O*-galloylglucose, the first metabolite and the main galloyl donor in GG biosynthesis (see Table 1 and Figure 1; cf. Gross, 1999a,b). Presumably, the enzymes responsible for the galloylations of other GGs were able to function during the first steps of air- and oven-drying and utilized the galloyl group of 1-*O*-galloylglucose, thus depleting its content in the foliage. Additionally, oven-drying degraded the pedunculagin derivative almost totally, and changed the balance of individual ETs towards the end products of the pathway (cf. Figure 1; Salminen et al., 2001, 2002). This is best seen as accumulated levels of isostrictinin, 2,3-(*S*)-HHDP-glucose, and insoluble ETs. Apart from the metabolic processes presumably occurring at 60°C, the increased concentrations of many ETs could be explained by their enhanced extractability from the heat-treated plant tissue. Nevertheless, the majority of the increase in the levels of 2,3-(*S*)-HHDP-glucose and insoluble ETs could be simply attributed to the degradation of the pedunculagin derivative that was shown to produce

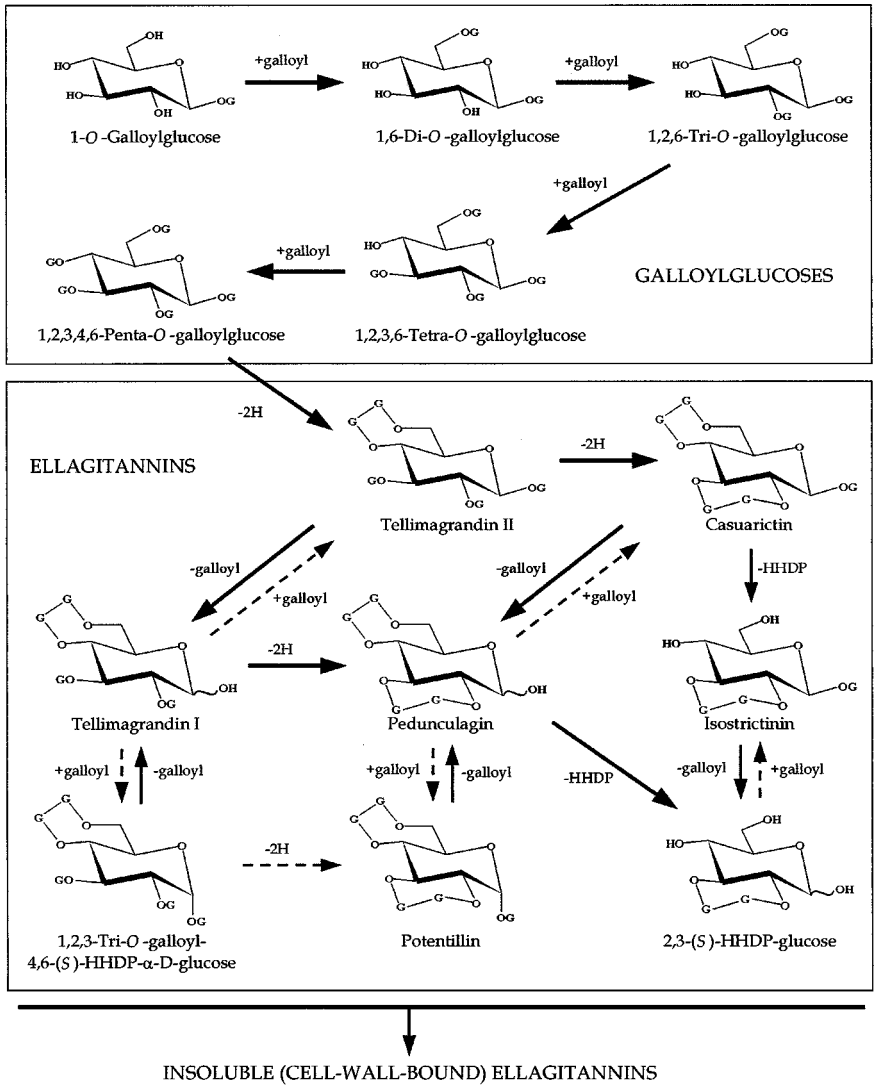

FIG. 1. The biosynthetic pathway of birch (*Betula pubescens*) leaf hydrolyzable tannins, divided into three consecutive biosynthetic groups, i.e., galloylglucoses, ellagitannins, and insoluble ellagitannins according to Salminen et al. (2001, 2002). The letter G is used to describe a galloyl unit, and G-G to describe an HHDP unit. The text besides the arrows indicates whether a galloyl group is added to the precursor, or whether a galloyl or an HHDP group is hydrolyzed from it during that reaction step.

TABLE 1. EFFECTS OF DRYING METHOD[a] ON THE CONCENTRATION (mg/g dry wt) OF BIRCH LEAF HYDROLYZABLE TANNINS[b]

Compound	Freeze drying	Freeze drying (after 3 months storage)	Vacuum drying	Air drying	Oven drying
1-*O*-Galloylglucose	0.52 ± 0.03 a	0.54 ± 0.03 a	0.57 ± 0.03 a	0.37 ± 0.02 b	0.38 ± 0.03 b
1,6-Di-*O*-galloylglucose	0.51 ± 0.04 a	0.46 ± 0.03 a	0.51 ± 0.04 a	0.54 ± 0.03 a	0.52 ± 0.03 a
1,2,6-Tri-*O*-galloylglucose	0.81 ± 0.03 ab	0.72 ± 0.03 a	0.87 ± 0.02 b	0.74 ± 0.02 ab	0.54 ± 0.03 c
1,2,3,6-Tetra-*O*-galloylglucose	0.48 ± 0.02 a	0.44 ± 0.03 a	0.51 ± 0.02 a	0.43 ± 0.02 a	0.30 ± 0.02 b
1,2,3,4,6-Penta-*O*-galloylglucose	2.18 ± 0.18 a	1.69 ± 0.10 bc	2.14 ± 0.11 ab	1.68 ± 0.05 bc	1.46 ± 0.07 c
Total galloylglucoses	4.50 ± 0.25 a	3.85 ± 0.16 b	4.59 ± 0.21 a	3.77 ± 0.07 b	3.22 ± 0.11 c
Pedunculagin derivative	5.23 ± 0.10 a	5.50 ± 0.05 a	5.34 ± 0.33 a	5.71 ± 0.23 a	0.09 ± 0.02 b
Pedunculagin	0.83 ± 0.04 a	0.88 ± 0.03 a	0.87 ± 0.08 a	0.86 ± 0.03 a	1.15 ± 0.06 b
Tellimagrandin I	1.23 ± 0.06 a	1.18 ± 0.11 a	1.17 ± 0.06 a	1.26 ± 0.04 a	1.61 ± 0.09 b
Casuarictin	0.95 ± 0.04 a	0.90 ± 0.05 a	1.13 ± 0.04 b	0.83 ± 0.03 a	1.24 ± 0.03 c
Potentillin	1.02 ± 0.06 ab	1.10 ± 0.07 a	0.86 ± 0.03 b	0.81 ± 0.04 b	2.43 ± 0.09 c
Tellimagrandin II	0.82 ± 0.05 a	0.79 ± 0.06 a	0.77 ± 0.03 ab	0.71 ± 0.02 a	0.62 ± 0.0 bb
1,2,3-Tri-*O*-galloyl-4,6-(*S*)-HHDP-α-D-glucose	0.55 ± 0.04 a	0.49 ± 0.06 a	0.58 ± 0.04 a	0.45 ± 0.02 a	0.92 ± 0.02 b
Isostrictinin	0.17 ± 0.01 a	0.21 ± 0.01 b	0.15 ± 0.01 a	0.09 ± 0.01 c	0.30 ± 0.02*d*
2,3-(*S*)-HHDP-glucose	0.05 ± 0.01 a	0.02 ± 0.01 a	0.03 ± 0.01 a	0.04 ± 0.00 a	0.47 ± 0.04 b
Total ellagitannins	10.86 ± 0.22 a	11.06 ± 0.36 a	10.87 ± 0.43 a	10.73 ± 0.17 a	8.37 ± 0.32 b
Total hydrolyzable tannins	15.35 ± 0.41 a	14.90 ± 0.46 a	15.46 ± 0.61 a	14.50 ± 0.16 a	11.58 ± 0.42 b
Insoluble ellagitannins	0.82 ± 0.01 a	0.77 ± 0.02 ab	0.73 ± 0.02 b	0.72 ± 0.01 b	2.38 ± 0.03 c

Note: Concentrations of tannins with same letters between the methods are not significantly different.
[a] The choice of the drying method had a significant effect on the total yield of birch leaf GGs, ETs, and insoluble ETs (for all $P < 0.001$).
[b] Data are means ± SE, $N = 5$.

2,3-(*S*)-HHDP-glucose and ellagic acid upon its hydrolysis (Salminen et al., 2002). Since the ellagic acid released from the pedunculagin derivative has a limited solubility in 70% acetone, it remained in the insoluble residue of birch leaves, just to be detected by the method of insoluble ETs.

If leaf samples cannot be freeze- or vacuum-dried soon after collection, they may be stored at −20°C for some time before drying without major changes in the content of individual tannins; 3 months of storage altered only the concentrations of 1,2,3,4,6-penta-*O*-galloylglucose and isostrictinin (see Table 1). Similarly, after freeze- or vacuum-drying, the dried leaf material should be stored preferably at −20°C, since storage at 4°C and at room temperature decreased the content of the pedunculagin derivative (see Table 2), one of the main ETs of birch. Just like oven-drying (see above) and the opposite of sample storage at 4°C or −20°C, storage at room temperature elevated levels of isostrictinin and 2,3-(*S*)-HHDP-glucose, indicating possible catabolism of HTs. Elevated yields of ETs (other than isostrictinin and 2,3-(*S*)-HHDP-glucose) after sample storage at −20°C are difficult to explain other than by the possible increase in their extractability due to a partial loss of interaction between leaf material and ETs during the 1 year storage. This assumption, however, was not supported by the subsequently increased yields of insoluble cell-wall-bound ETs as well. Although possible controversial, these results suggest that if quantitative results of plant samples are to be compared with each other, all samples should be extracted after being stored at the same temperature for similar periods of time.

After drying, plant material should be ground into as fine a powder as possible to maximize the efficiency of the subsequent extraction process. Although both aqueous methanol and aqueous acetone are commonly used to extract tannins from plant samples (Hagerman, 1988; Scalbert et al., 1989), the latter was found superior over the former for the extraction of birch leaf HTs (Table 3). In contrast, pure methanol is better than pure acetone, the weakest solvent. The percentage of water in acetone also had an effect on the yield of HT subgroups; 50% acetone extracted ETs better than 70% and produced higher yields of GGs. The addition of 0.1% ascorbic acid into the 70% acetone increased the yield of ETs significantly, especially casuarictin (see Table 3). This cannot be explained by enhanced extractability, since there were no differences in the contents of insoluble ETs between the two 70% acetone extractions (with or without ascorbic acid). With the other solvents, it was shown that if a portion of ETs remained unextracted in the insoluble residue, the content of insoluble ETs subsequently increased (see Table 3), suggesting that part of the amounts of insoluble tannins found in plants may be due to insufficient extraction. This was illustrated by the negative correlation ($r = -0.962$) between the combined content of pedunculagin and its derivative (the major sources of ellagic acid in birch leaves), and the content of insoluble ETs. Therefore, if, for example, the approx. 13 mg/g difference in casuarictin and pedunculagin content between the two 70% acetone extractions were due to

TABLE 2. EFFECTS OF 1 YEAR STORAGE[a] OF BIRCH LEAF MATERIAL[b] IN DIFFERENT TEMPERATURES ON THE CONCENTRATION (mg/g dry wt) OF BIRCH LEAF HYDROLYZABLE TANNINS[c]

Compound	Control	Room temperature (~22°C)	Fridge (4°C)	Freezer (−20°C)
1-*O*-Galloylglucose	0.52 ± 0.03 a	0.52 ± 0.03 a	0.45 ± 0.01 a	0.49 ± 0.01 a
1,6-Di-*O*-galloylglucose	0.51 ± 0.04 a	0.59 ± 0.04 a	0.46 ± 0.03 a	0.45 ± 0.03 a
1,2,6-Tri-*O*-galloylglucose	0.81 ± 0.03 a	0.99 ± 0.04 b	0.81 ± 0.02 a	0.90 ± 0.03 ab
1,2,3,6-Tetra-*O*-galloylglucose	0.48 ± 0.02 a	0.77 ± 0.02 b	0.64 ± 0.03 c	0.73 ± 0.02 b
1,2,3,4,6-Penta-*O*-galloylglucose	2.18 ± 0.18 a	2.32 ± 0.10 a	1.84 ± 0.10 a	2.31 ± 0.11 a
Total galloylglucoses	4.50 ± 0.25 ab	5.19 ± 0.20 a	4.19 ± 0.14 b	4.90 ± 0.09 ab
Pedunculagin derivative	5.23 ± 0.10 a	4.00 ± 0.20 b	4.40 ± 0.08 c	5.20 ± 0.01 a
Pedunculagin	0.83 ± 0.04 a	1.08 ± 0.10 ab	0.82 ± 0.05 a	1.53 ± 0.30 b
Tellimagrandin I	1.23 ± 0.06 a	1.59 ± 0.09 b	1.39 ± 0.04 ab	1.50 ± 0.03 b
Casuarictin	0.95 ± 0.04 a	1.69 ± 0.04 b	1.31 ± 0.05 c	1.46 ± 0.04 c
Potentillin	1.02 ± 0.06 a	1.35 ± 0.11 b	1.52 ± 0.11 b	1.95 ± 0.03 c
Tellimagrandin II	0.82 ± 0.05 a	1.23 ± 0.04 b	1.03 ± 0.06 c	1.24 ± 0.04 b
1,2,3-Tri-*O*-galloyl-4,6-(S)-HHDP-α-D-glucose	0.55 ± 0.04 a	0.70 ± 0.01 b	0.81 ± 0.02 c	1.05 ± 0.03 c
Isostrictinin	0.17 ± 0.02 a	0.48 ± 0.05 b	0.17 ± 0.04 a	0.17 ± 0.01 a
2,3-(*S*)-HHDP-glucose	0.05 ± 0.01 a	0.09 ± 0.01 b	0.05 ± 0.01 a	0.05 ± 0.01 a
Total ellagitannins	10.86 ± 0.22 a	12.22 ± 0.38 b	11.49 ± 0.24 a*b*	14.15 ± 0.19 c
Total hydrolyzable tannins	15.35 ± 0.41 a	17.41 ± 0.55 b	15.68 ± 0.22 a	19.05 ± 0.24 c
Insoluble ellagitannins	0.82 ± 0.01 a	0.98 ± 0.05 b	1.06 ± 0.03 b	1.03 ± 0.03 b

Note: Concentrations of tannins with same letters between the methods are not significantly different.

[a] The storage of the freeze-dried birch leaf powder for 1 year in different temperatures affected the yield of total GGs, total ETs, and insoluble ETs ($P = 0.0157$, $P < 0.001$, and $P < 0.001$, respectively).

[b] The dried and ground samples were sealed in plastic bags and wrapped in aluminium foil to keep the samples dry and in the dark.

[c] Data are means ± SE, $N = 4$ (except for control $N = 5$).

TABLE 3. EFFECTS OF EXTRACTION SOLVENT[a] ON THE CONCENTRATION (mg/g dry wt) OF BIRCH LEAF HYDROLYZABLE TANNINS[b]

Compound	50% Methanol	70% Methanol	100% Methanol	50% Acetone	70% Acetone	70% Acetone + 0.1% ascorbic acid	100% Acetone
1-*O*-Galloylglucose	0.89 ± 0.02 a	0.85 ± 0.02 a	0.84 ± 0.03 a	0.95 ± 0.05 a	0.97 ± 0.02 a	0.96 ± 0.05 a	0.07 ± 0.01 b
1,6-Di-*O*-galloylglucose	1.56 ± 0.09 a	1.53 ± 0.03 a	1.44 ± 0.06 a	1.47 ± 0.15 a	1.53 ± 0.03 a	1.64 ± 0.06 a	0.17 ± 0.01 b
1,2,6-Tri-*O*-galloylglucose	1.64 ± 0.03 a	1.71 ± 0.03 ab	1.38 ± 0.04 c	2.04 ± 0.15 d	1.94 ± 0.01 bd	2.05 ± 0.02 d	0.31 ± 0.01 e
1,2,3,6-Tetra-*O*-galloylglucose	0.81 ± 0.01 a	0.95 ± 0.03 b	0.83 ± 0.04 a	1.40 ± 0.04 c	1.30 ± 0.03 c	1.26 ± 0.05 c	0.49 ± 0.05 d
1,2,3,4,6-Penta-*O*-galloylglucose	0.91 ± 0.02 a	1.87 ± 0.07 b	1.62 ± 0.13 b	1.77 ± 0.16 b	2.85 ± 0.13 c	3.04 ± 0.18 c	0.74 ± 0.09 a
Total galloylglucoses	5.81 ± 0.16 a	6.91 ± 0.07 b	6.11 ± 0.18 a	7.63 ± 0.26 b	8.59 ± 0.17 c	8.95 ± 0.30 c	1.77 ± 0.11 d
Pedunculagin derivative	11.68 ± 0.37 a	11.52 ± 0.05 a	8.79 ± 0.25 b	16.39 ± 0.36 c	15.87 ± 0.23 c	15.31 ± 0.55 c	1.52 ± 0.14 d
Pedunculagin	3.37 ± 0.13 a	3.09 ± 0.11 a	2.22 ± 0.11 b	3.98 ± 0.08 c	3.84 ± 0.07 c	8.05 ± 0.22 d	1.52 ± 0.14 e
Tellimagrandin I	4.73 ± 0.17 a	4.73 ± 0.10 a	4.50 ± 0.27 a	6.12 ± 0.19 b	5.86 ± 0.09 b	6.53 ± 0.18 b	3.22 ± 0.17 c
Casuarictin	3.56 ± 0.16 a	1.89 ± 0.08 b	2.12 ± 0.03 b	3.25 ± 0.10 a	3.11 ± 0.17 a	11.91 ± 0.10 c	1.29 ± 0.11 d
Potentillin	0.41 ± 0.04 a	3.01 ± 0.02 b	2.23 ± 0.05 c	5.97 ± 0.16 d	4.24 ± 0.18 e	3.18 ± 0.23 b	1.14 ± 0.15 f
Tellimagrandin II	0.94 ± 0.07 ab	1.32 ± 0.06 c	1.17 ± 0.07 bc	1.91 ± 0.13 d	1.79 ± 0.15 d	2.37 ± 0.08 e	0.70 ± 0.05 a
1,2,3-Tri-*O*-galloyl-4,6-(*S*)-HHDP-α-D-glucose	1.08 ± 0.07 ab	1.20 ± 0.04 a	0.84 ± 0.03 b	2.34 ± 0.06 c	1.93 ± 0.03 d	2.66 ± 0.17 e	0.34 ± 0.03 f
Isostrictinin	1.20 ± 0.03 a	1.13 ± 0.04 a	0.96 ± 0.05 b	1.67 ± 0.12 c	1.48 ± 0.03 c	0.92 ± 0.07 b	0.53 ± 0.04 d
2,3-(S)-HHDP-glucose	0.51 ± 0.03 a	0.51 ± 0.05 a	0.23 ± 0.01 b	0.56 ± 0.05 a	0.52 ± 0.04 a	0.79 ± 0.06 c	0.06 ± 0.01 d
Total ellagitannins	27.48 ± 0.81 a	28.39 ± 0.30 a	23.06 ± 0.70 b	42.18 ± 0.91 c	38.63 ± 0.73 d	51.72 ± 1.26 e	10.31 ± 0.75 f
Total hydrolyzable tannins	33.29 ± 0.95 a	35.30 ± 0.36 b	29.17 ± 0.85 c	49.82 ± 1.07 d	47.22 ± 0.82 d	60.67 ± 1.54 e	12.08 ± 0.75 f
Insoluble ellagitannins	1.63 ± 0.05 a	1.63 ± 0.05 a	3.31 ± 0.07 b	0.80 ± 0.02 c	0.74 ± 0.01 c	0.72 ± 0.01 c	5.92 ± 0.07 d

Note: Concentrations of tannins with same letters between the methods are not significantly different.

[a] The choice of the extraction solvent had a significant effect on the yields of both individual HTs and HT groups ($P < 0.001$ for each compound and group).

[b] Data are means ±SE, $N = 4$.

differences in ET extractability, this should have been demonstrated by differences in the insoluble ET content as well. Rather than affecting the extractability of ETs, presumably ascorbic acid prevented the oxidative processes that reduced the contents of many ETs while using only 70% acetone (or other solvent compositions not having antioxidants added) for the extraction. Since these findings are consistent with those of Peng and Jay-Allemand (1991), the use of antioxidants in the extraction of plant HTs is recommended. As a precaution, however, ascorbic acid will give a peak on HPLC, where it may interfere with the detection of the α- and β-isomers of 2,3-(*S*)–HHDP-glucose. This is a problem only with UV detection, since HPLC-ESI-MS can differentiate between ascorbic acid and 2,3-(*S*)-HHDP-glucose on the basis of different molecular weights.

Comparison of the HPLC–ESI-MS and the rhodanine assay for quantification of birch leaf galloylglucoses demonstrated that results obtained with the two methods correlate with each other, but that the absolute values are quite different (for mountain birch samples see Figure 2a, and for dwarf birch samples Figure 2b). Especially above the level of 4 mg/g of total GGs, the rhodanine assay tended to underestimate results compared to HPLC–ESI-MS (see Figure 2a,b). This is not surprising since the rhodanine method is best used with samples containing so-called true gallotannins (i.e., hexa- and higher-substituted GGs), and not with samples containing only simple GGs (i.e., penta- and lower-substituted GGs). This is because the method calculates only the amount of galloyl residues cleaved from GGs after acid hydrolysis (Inoue and Hagerman, 1988). For instance, in 1-*O*-galloylglucose, the single galloyl group comprises only 45.7% of the molecular weight of 1-*O*-galloylglucose, while in hexagalloylglucose the six galloyl groups comprise 83.5% of the molecular weight of hexagalloylglucose. With 1-*O*-galloylglucose, the rhodanine assay makes a 54.3% underestimation, and in the case of hexagalloylglucose, the underestimation is only 16.5%.

By such compound-specific calculations, it is possible to convert quantitative results of individual and identified GGs into a galloyl load corresponding to the load that would have been obtained from the same GGs with the rhodanine assay. In theory, such conversion of the natural levels of individual GGs in an extract would result in a total GG level matching exactly that obtained by the rhodanine assay. However, since birch leaves also contain ETs, some of which carry galloyl moieties in their structures, the amounts of these ET-bound galloyl groups have to be taken into account as well. Accordingly, here conversions were made to the contents of individual GGs and ETs quantified with HPLC–ESI-MS from 117 mountain birch (Figure 3a) and 60 dwarf birch samples (Figure 3b). As shown in Figure 3, these converted results of HPLC–ESI-MS match much better with the absolute results of the rhodanine assay than before the conversion (Figure 2a,b). This suggests that the quantitative results of individual HTs obtained with HPLC–ESI-MS have to be close to the natural levels of HTs actually present in the plant extract analyzed (cf. theoretical calculations above).

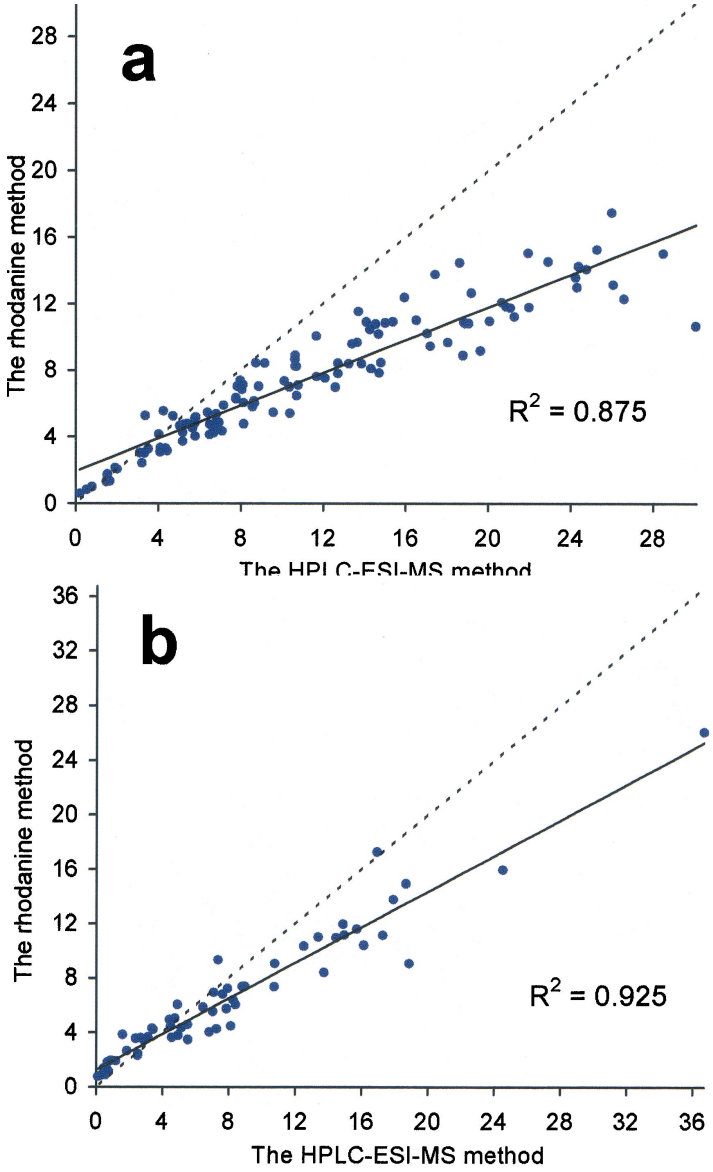

FIG. 2. Contents of total galloylglucoses from 117 mountain birch samples (a), and 60 dwarf birch samples (b), measured both with the rhodanine (gallic acid equivalents mg/g dry wt) and the HPLC–ESI-MS (sum of individual galloylglucoses mg/g dry wt) methods. The solid line shows linear regression between the results; dashed line represents the situation where both methods would give equal results.

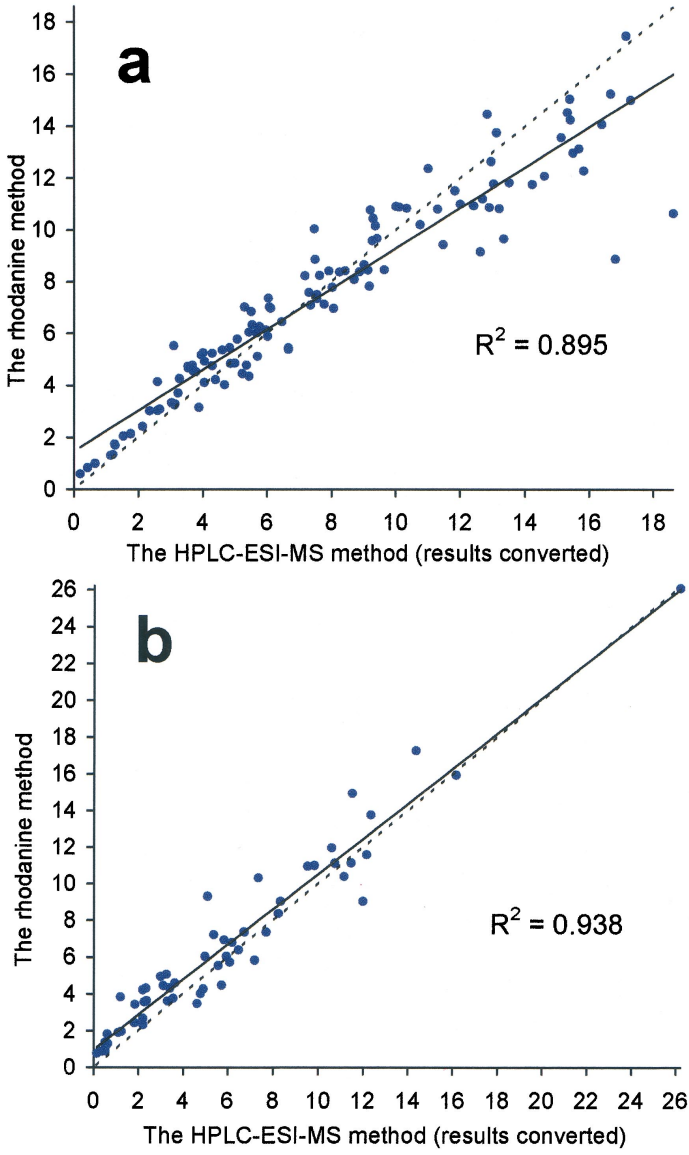

FIG. 3. Contents of total galloylglucoses from 117 mountain birch samples (a), and 60 dwarf birch samples (b). The HPLC–ESI-MS results on the contents of individual galloylglucoses and ellagitannins containing galloyl residues have been converted into gallic acid equivalents mg/g dry wt, after simulation of galloyl residue hydrolysis by the rhodanine assay. The solid line shows linear regression between the results; dashed line represents the situation where both methods would give equal results.

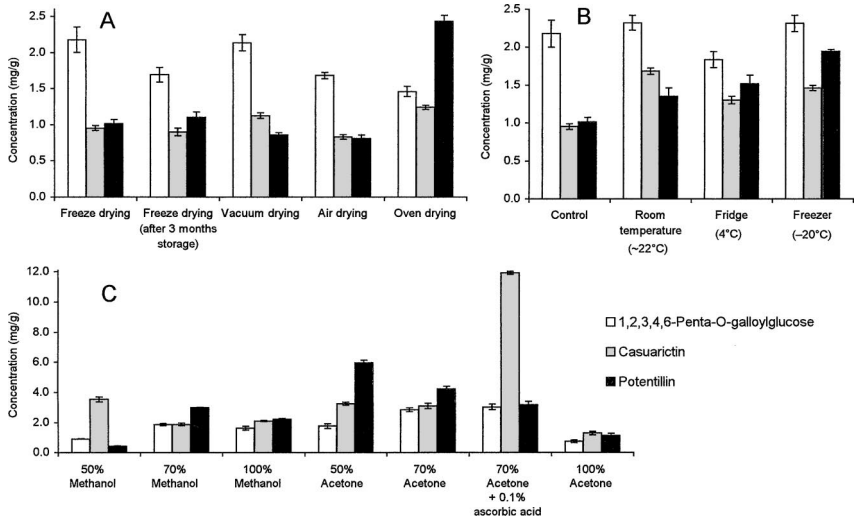

FIG. 4. Summary figure emphasizing that individual HTs (1,2,3,4,6-penta-*O*-galloylglucose, casuarictin, and potentillin as examples) respond uniquely to different conditions of sample drying (A), storage (B), and extraction (C). Statistically significant differences in the contents of tannins are those shown in Tables 1–3.

In addition to the rhodanine assay underestimating total GG levels in birch leaves, its quantitative results are a combination of galloyl containing GGs and ETs, some of which have relatively high protein precipitation capacities and some none at all (Ozawa et al., 1987; Spencer et al., 1988; Haslam 1992; Kilkowski and Gross, 1999). For these reasons, the rationale for quantifying total contents of GGs—especially in plant–herbivore studies—could be questioned. However, since methods such as HPLC are not available in every laboratory, it should be emphasized that the rhodanine assay provides at least a useful relative GG content of a plant extract (highly correlated with HPLC–ESI-MS data in Figure 2a,b) even though it does not provide absolute values for specific compounds.

Figure 4 shows using 1,2,3,4,6-penta-*O*-galloylglucose, casuarictin, and potentillin as examples—that individual HTs respond uniquely to different conditions of sample drying, storage, and extraction. By using different combinations of methods for plant tissue preservation together with the subsequent extraction, it is possible to end up with highly variable yields of individual plant HTs, even though their quantification would meet the highest standards. If the studies of plant–herbivore interactions aim to obtain a close estimate of hydrolyzable tannins present in a living plant being consumed by a herbivore, they should use only the methods of ecological chemistry that enable this goal. On the basis of this study, samples should be either vacuum- or freeze-dried, stored preferably at -20°C, extracted

with 70% acetone containing antioxidants like 0.1% ascorbic acid, and quantified with a method such as HPLC that does not confuse the subsequent correlative studies by mixing individual tannins of varying biological activities with each other. However, whether these methods are optimal for a particular plant species, should be tested before running any large-scale field experiment.

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QUANTITATIVE ASSESSMENT OF NATURAL TOXICITY IN SPONGES: TOXICITY BIOASSAY VERSUS COMPOUND QUANTIFICATION

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Abstract—Microtox® assay was used to assess the natural toxicity of two sponges, *Dysidea avara* and *Ircinia variabilis*. The activity of crude extracts and major metabolites were compared. Methanol extract of *D. avara* was more toxic than that of acetone and was as toxic as pure avarol, thus suggesting that the toxicity of the sponge was mainly due to this metabolite. We also quantified palinurin, the major metabolite of *I. variabilis*, in specimens from several habitats. With the same methanol extracts used for palinurin quantification, we ran the Microtox® assay and found a positive significant regression between toxicity and concentration of this metabolite. Pure palinurin was tested at the same concentration present in the extract, and the toxicity recorded was higher than that of the methanol extract. As with avarol from *D. avara*, palinurin is the main secondary metabolite that confers toxicity to *I. variabilis*. The results confirm that the standardized Microtox® assay is an accurate and reproducible tool for assessing the toxicity of crude extracts and pure metabolites of marine organisms. These results also suggest that methanol is more suitable than acetone for the detection of species toxicity by Microtox®. The method is faster and easier to perform than chemical quantification even when the sponge chemistry is known, and is appropriate for studies on variation in natural toxicity over a range of environmental conditions.

Key Words—Natural toxicity, sesterpene, avarol, palinurin, secondary metabolites, crude extracts, bioactivity, bioassays, Microtox® , chemical quantification, sponges, *Dysidea avara*, *Ircinia variabilis*.

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INTRODUCTION

Intraspecies variation in the production of bioactive metabolites is a key topic in chemical ecology studies because (a) this is the kind of variation on which evolution acts (Hay, 1996), and (b) most of the biological functions associated with secondary metabolites, such as deterrence and competition for substrate, are dependent on concentration (e.g., Hay et al., 1987; Schmitt et al., 1995; Becerro et al., 1998). Consequently, quantification of these metabolites and the assessment of their activities are basic considerations needed to understand the chemical ecology of marine assemblages (e.g., Paul and van Alstyne, 1988; Van Alstyne and Paul, 1990; Harvell et al., 1993; Maida et al., 1993; Becerro et al., 1995a, 1998; Steinberg, 1995; De Nys et al., 1996; Jayatilake and Baker, 1996; Turon et al., 1996; Betancourt-Lozano et al.,1998).

Disappointingly, there is no agreement on how to address studies within the scientific community. Chemists consider the isolation and identification of bioactive metabolites a necessary first step before approaching biochemical, biological, and ecological aspects (Scheuer, 1996; Faulkner, 2001; Tringali, 2001). Biologists claim the need for "biologically or ecologically relevant" experiments (e.g., Hay, 1996). This implicitly requires the assessment of the crude extract toxicity as a whole, since the different metabolites present in a species may interact and produce different bioactivities when assayed altogether or separately. In fact, other chemical products (including salts) occurring in the organism might also be biologically relevant or interact with a major metabolite by changing, inhibiting, or increasing (synergetic effect) its activity (e.g., Hay et al., 1994; Lumbang and Paul, 1996). Ecologists also prefer standardized, reproducible assays to evaluate the patterns of variation in chemical defense over spatial or temporal scales, such as latitudinal or environmental gradients (e.g., Bolser and Hay, 1996; Puglisi et al., 2000). However, when trying to assess the ecological role of pure metabolites, only major metabolites can be considered, because they are the only ones that can be isolated in sufficient amounts for the assays.

All these approaches with their pros and cons provide only partial knowledge on the biological and ecological functions of secondary metabolits. Thus, it would be desirable to compare results obtained from different methodologies, but this has been seldom done on the same species (Uriz et al., 1996a). When secondary chemistry of a given species is poorly known, general bioassays of the crude extract are a suitable first approach to verify whether the species is bioactive. The results of these bioassays integrate the response of all the metabolites present in the organisms (Karban and Myers, 1989; Martín and Uriz, 1993; Turon et al., 1996; Becerro et al., 1998) and give a more holistic view of the chemical ecology of the species than bioassays using pure metabolites (Harper et al., 2001).

The aim of this study was to assess the relationship between the chemical content and the toxicity in two common Mediterranean sponges, *Dysidea avara*

Schmidt, 1862 and *Ircinia variabilis* Schmidt, 1862. We compared the activity of the main metabolite(s) versus crude extracts of the two sponges. If a correlation between both variables exists, then Microtox® assay could also be used to assess intraspecies chemical variation. Further, similar studies on additional sponge species, will allow one to establish the extent of the relationship between toxicity of the crude extract and the amount of major metabolites.

METHODS AND MATERIALS

Source Organisms and Sampling Sites. D. avara and *I. variabilis* were collected by SCUBA diving in Tossa de Mar (NE Iberian Peninsula) and the Cabrera Archipelago (Balearic Islands) in August 1998 and October 1999, respectively. *D. avara* was sampled to compare the toxicity of the crude extract with that of its main secondary metabolite. Nine specimens of *I. variabilis* were collected from the entrance to the innermost part of a cave to study possible differences in toxicity or chemistry among individuals from distinct habitats. Samples were immediately frozen after collection and lyophilized. They were then weighed and stored at -30°C in the dark until chemical extraction.

Chemical Analyses. For *D. avara*, we compared the toxicity of crude extracts obtained from two different extraction solvents. An individual of *D. avara* was divided into two equal portions of 0.25 g each (dry weight), and these were independently extracted with methanol and acetone. Hydroquinone avarol ($C_{21}H_{30}O_2$), the major metabolite in this species (Figure 1), together with minor amounts of its quinone, avarone, account for approximately 6% of the sponge dry weight (Minale et al., 1974). Crystallized avarol (mixed with avarone) was isolated as described in Uriz et al. (1996b).

With respect to *I. variabilis*, the purpose was to compare the variations of toxicity of the crude extracts with the amount of the main metabolite present in the samples (palinurin) and the toxicity of the pure metabolite. Extraction was performed by soaking 1-g dry weight sponge samples in 25 ml of methanol, three successive times for 5, 10, and 15 min in an ultrasonic bath. The solvents from each extraction were combined and evaporated to dryness under reduced pressure.

To quantify the main metabolites, known amounts of dry crude extracts of *I. variabilis* were dissolved in CD_3OD and analyzed by NMR to obtain the ^{1}H NMR spectrum. A known amount of methyl fumarate, which is chemically stable, nontoxic, and nonreactive, was added as an internal standard. The major secondary metabolite was quantified by comparing the integrated area corresponding to the metabolite in the spectrum with that of the amount of fumaric acid. Results were expressed as milligram of metabolite per dry weight unit of sponge. Pure palinurin for the toxicity assays was isolated from *I. variabilis* following the protocol described in Alfano et al. (1979).

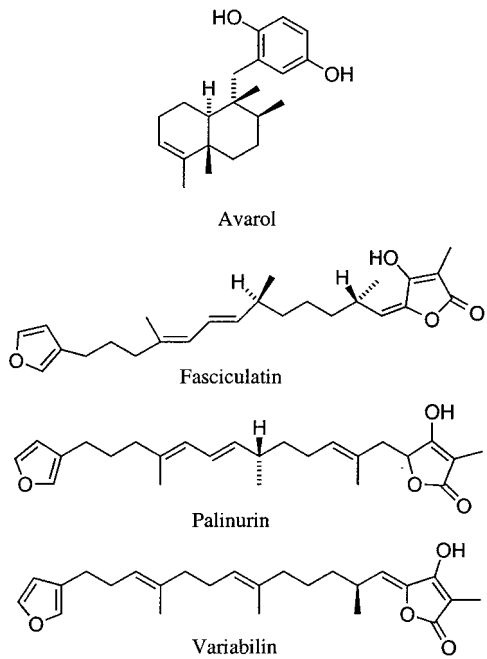

FIG. 1. Structure of avarol, the major metabolite of *D. avara*. Structures of the furano sesterterpenoids reported in the literature for *I. variabilis*, fasciculatin, palinurin, and variabilin. (Only palinurin was found in our study)

Toxicity Bioassay. We used Microtox® to assess toxicity. This bioassay was selected because of its high sensitivity, precision, and repeatability (Vasseur et al., 1984; Ribo and Kaiser, 1987; Kaiser and Ribo, 1988; Ribo and Rogers, 1990; Becerro et al., 1995b; Martí, 2002). Dry crude extracts of acetone and methanol of *D. avara*, as well as pure avarol, were assayed. Crude extracts were dissolved in artificial seawater at 34% salinity (Subov, 1931). Solutions were tested in four diluted concentrations, with a dilution factor of 2. Microtox® quantifies the toxicity of the samples (detrimental effects on the bacterium physiology) by measuring decreases in the light produced by the abyssal marine photobacterium, *Vibrio fischeri* (previously *Photobacterium phosphoreum*). By assaying a range of concentrations, a regression equation between concentration and decrease in light can be outlined. Toxicity is measured in conventional "gamma" units. The *EC*50 value indicated in the regression equation (Figures 2 and 4) is the concentration that reduces bacteria light to a half the initial light, and corresponds to a gamma unit of 1. This *EC*50 represents a percentage relative to the initial concentration tested; therefore, to find the real EC50, the previous value (*EC*50) must be multiplied by the initial concentration tested.

In both cases, the initial concentration tested was 1000 μg of crude extract per milliliter of artificial seawater. A small amount of acetone (up to 0.4% of the solution) was added to promote dissolution of the extracts. An acetone trial at the same concentration as in the treatments was performed to test the possible toxicity of acetone. Avarol dissolved in artificial seawater with 0.4% acetone was also assayed at the same concentration present in the crude extract (i.e., 6% of the sponge dry weight).

For *I. variabilis*, the same crude extracts were used to quantify metabolites and to assess toxicity. The highest concentration tested was 1500 μg of crude extract per milliliter of artificial seawater. In this case, we compared the real EC50 of the crude extract with the amount of palinurin (the major metabolite) in the samples to address whether the toxicity of the crude extract was positively correlated with the abundance of this metabolite. Palinurin dissolved in artificial seawater with 0.4% of acetone was also assayed for toxicity at the same concentration as in the extract.

Methanol dissolves part of the seawater salt in the samples. To test whether the salt in the extracts contributed to the toxicity, salt obtained from seawater evaporation was assayed at the same concentration as the extracts. We were conservative with this estimate because we considered the extreme case (i.e., as if the whole crude extracts were salt).

Statistical Analyses. Regression analyses were performed to assess the relationships between (1) crude concentration and toxicity of the methanol and acetone crude extracts of *D. avara*, (2) pure avarol concentration and toxicity, (3) crude concentration and toxicity of the methanol extract of *I. variabilis*, (4) pure palinurin concentration and toxicity, and (5) palinurin concentration in the crude extracts and toxicity of these extracts.

RESULTS

Dysidea avara. None of the salt concentrations assayed (always higher than those in the methanol extract) was toxic (gamma units always lower than 0.005). *D. avara* extracted with methanol was more toxic, with an EC50 value of 159.6 μg/ml ($EC50 = 15.96\%$ of the initial concentration assayed), than the acetone extract (EC50 = 935 μg/ml; $EC50 = 93.5\%$) (Figure 2A and B). The $EC50$ for pure avarol (Figure 2C) was closer (EC50 = 44.7 μg/ml; $EC50 = 14.23\%$) to that found for the methanol extract than for acetone.

Significant negative regressions were detected between concentration of the methanol and acetone extracts of *D. avara* and that of pure avarol, and bacteria bioluminescence ($P < 0.01$, for the three cases). The slopes of the regression lines for the methanol and acetone extracts were similar, and in both cases were lower than that for pure avarol. Higher slopes are expected for pure toxic compounds,

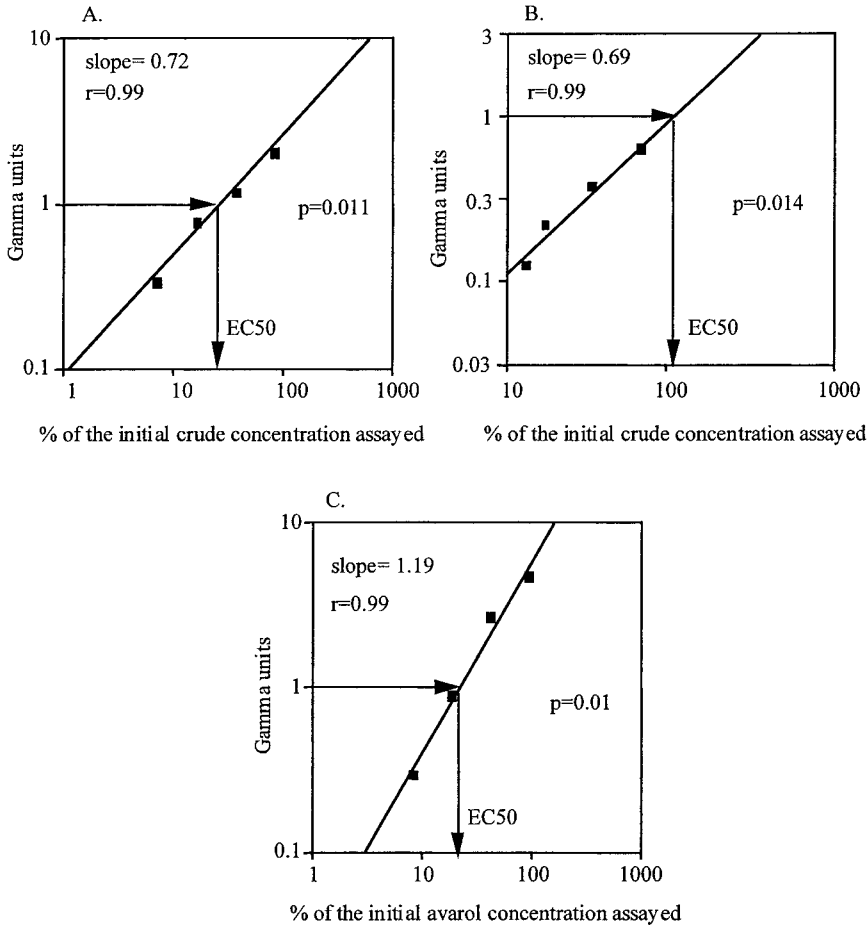

FIG. 2. Microtox® results for methanol (A) and acetone (B) extracts of *D. avara*, and pure avarol (C). Regression equations were log(gamma) = 0.724 log(conc.) − 0.870, log(gamma) = 0.689 log(conc.) − 1.358, and log(gamma) = 1.194 log(conc.) − 1.377 for methanol extract, acetone extract, and pure avarol, respectively.

since the slope value indicates the strength of the toxic response to a given increase in the concentration of the substance analyzed (Turon et al., 1996).

Ircinia variabilis. Palinurin was the major metabolite detected in the ^{1}H NMR spectra of the specimens of *I. variabilis* (Figure 1). The regression between the concentration of palinurin and the corresponding values of toxicity was significant ($r = 0.87$; $P < 0.005$), indicating a positive correlation between the amount of this metabolite and toxicity of the crude extract (Figure 3). The concentration of

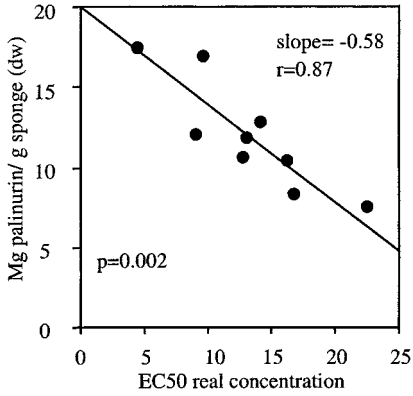

FIG. 3. Regression analysis between toxicity (real EC50) and concentration of the major metabolite in samples of *I. variabilis*. EC50 concentration is expressed in milligrams of sponge dry weight per milliliter. Notice that the slope of the regression line is negative (i.e., the higher the real EC50, the less toxic sample). Regression equation was palinurin concentration = 0.577 EC50 + 19.570.

palinurin found among individuals ranged from 0.75 to 1.75% (Table 1), approximately a twofold variation of the sponge dry weight, while EC50 values ranged from 4.4 to 22.4 mg sponge dry weight per milliliter (ca fivefold variation). Chemical analyses and toxicity assays showed an intraspecific variation in palinurin content and toxicity, respectively, but the latter was more sensitive at detecting this variation.

Figure 4 shows the Microtox® results for the methanol extract of a specimen of *I. variabilis*, which contained 1.2% of palinurin per sponge dry weight (Figure 4A), as well as for pure palinurin (Figure 4B). Pure palinurin was more toxic (EC50 = 99.5 μg/ml; *EC*50 = 38.28%) than methanol extract (EC50 =

TABLE 1. QUANTIFICATION OF PALINURIN IN SAMPLES OF *I. variabilis* FROM DIFFERENT HABITATS

Specimen	Habitat	% Palinurin per sponge dry weight
1		0.825
2	Seaweed community	1.209
3		1.060
4		1.290
5	Semi-dark-cave community	0.750
6		1.039
7		1.182
8	Dark-cave community	1.752
9		1.709

990 μg of crude extract per milliliter, $EC50 = 66\%$). The slope of the pure palinurin toxicity regression line was greater than that of the methanol extract, as occurred with pure avarol with respect to *D. avara.*

DISCUSSION

The sponges *Dysidea avara* Schmidt, 1862 and *Ircinia variabilis* Schmidt, 1862 were selected to test the relationship between compound concentration and toxicity because their toxic activity and secondary chemistry are known (e.g., Faulkner, 1973; Minale et al., 1974; De Rosa et al., 1976; Müller et al., 1985 for *D. avara,* and Cafieri et al., 1972; Alfano et al., 1979; Ishibashi et al., 1993 for *I. variabilis*). Moreover, these species are among the most abundant littoral sponges in the western Mediterranean (Uriz et al., 1992a; Carballo et al., 1997).

Secondary metabolites of *D. avara* are active against a wide array of organisms. They show toxicity against embryos of sea urchins and larvae of bryozoans (Cariello et al., 1980; Martín and Uriz, 1993). This species also shows antibacterial, antimitotic, and cytostatic activities against pathogenic microbes and tumor cells (Seibert et al., 1985; Müller et al., 1985; Uriz et al., 1992a). Feeding deterrence during distinct stages of the life history of this sponge has also been reported (Uriz et al., 1996a). Furthermore, avarol (Figure 1), the major secondary metabolite of *D. avara,* is an effective antioxidant that inhibits some enzymatic activities (Belisario et al., 1990, 1992).

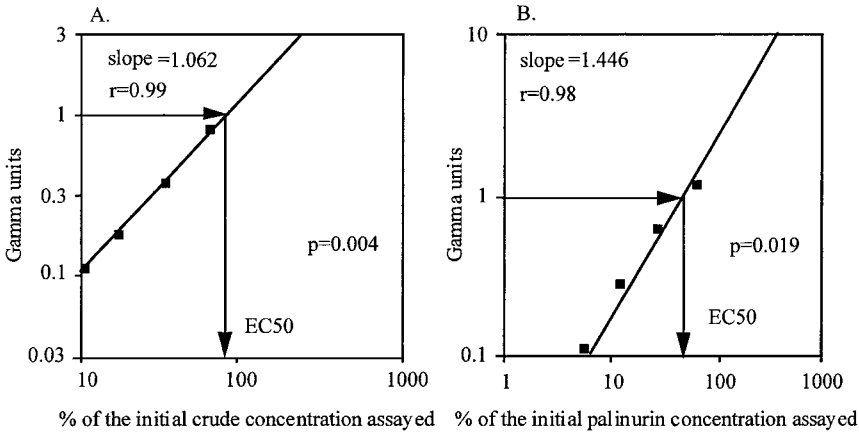

FIG. 4. Microtox® results for the methanol extract of *I. variabilis* (A), and pure palinurin (B). Regression equations were log(gamma) = 1.062 log(conc.) − 1.933, log(gamma) = 1.446 log(conc.) − 2.307 for the methanol extract and pure palinurin, respectively.

Metabolites of *I. variabilis* are also toxic against sea-urchin embryos, bryozoan (*Bugula neritina*) larvae, and several classes of bacteria and fungi (Amade et al., 1987; Uriz et al., 1992a; Martín and Uriz, 1993). A chemical polymorphism has been reported for this species, with the presence of furanosesterterpenes fasciculatin, palinurin, or variabilin (Figure 1) or combinations of these (Marin et al., 1997), although the species *I. fasciculata* may have been misidentified for *I. variabilis* in some of these studies (authors' interpretation).

The chemical characteristics of the organic solvents used in extraction procedures roughly determine the kind of molecules extracted. The distinct affinity of methanol and acetone for apolar substances produces extractions that vary both quantitatively and/or qualitatively, which may explain the variations in toxicity found in the two crude extracts of *D. avara*. These two extracts differed in appearance, in solubility in artificial seawater, and in toxicity. The higher toxicity of the methanol extract may be due both to a more efficient extraction or a better dissolution in artificial seawater, because a uniform solution or suspension is required to obtain a reliable result in the Microtox® bioassay (Becerro et al., 1995b).

We knew from the preliminary bioassays that the marine salt in the methanol extract did not contribute to toxicity. However, since the methanol crude extract contained salts, the true concentration of metabolites in the extract assayed was probably underestimated and the toxicity of the metabolites extracted with methanol even higher than reported here (i.e., lower EC50).

For both *D. avara* and *I. variabilis*, the main metabolite chiefly accounted for toxicity. For the former, the results of pure avarol were similar to those of the whole methanol crude extract, indicating that avarol is mainly responsible for the toxicity of the sponge. The higher slope of the regression between concentration and toxicity for avarol than for the crude extract is probably due to the presence of other nontoxic molecules in the crude. Also, for *I. variabilis*, after quantification of the major metabolite, we found a significant regression between concentration of palinurin and toxicity. Furthermore, pure palinurin was more toxic than the methanol extract, and the slope was also higher for the pure metabolite than for the extract. Consequently, as in the case of avarol, species toxicity can be mainly ascribed to palinurin.

Intraspecies variation in the secondary chemistry has been reported in a number of groups of benthic animals, such as gorgonians (Harvell et al., 1993), soft corals (Maida et al., 1993), and sponges (e.g., Thompson et al., 1987; Bobzin and Faulkner, 1992; Turon et al., 1996; Betancourt-Lozano et al., 1998). These variations may be due to genetic, physiological, and a wide range of environmental factors. A high intraspecies variation in the production of secondary metabolites was found in this study for *I. variabilis* specimens living in the same cave but under different light and trophic conditions (i.e., different assemblages, Uriz et al., 1992b).

To summarize, the standardized Microtox® assay proved to be an accurate and repeatable tool to assess the toxicity of crude extracts and pure metabolites of

marine species, when toxic activity can be associated with a specific metabolite. The procedure may be even better (equally accurate, and less time-consuming) than chemical quantification, since there is a good correlation between Microtox® results and major toxic metabolites.

Microtox® analysis seems to be particularly suitable to study variation in natural toxicity in a range of environmental conditions along geographic or ecological gradients. Although the bioactive responses of the main metabolites were similar to those of the methanol extracts in the species studied, crude extract analyses are, in general, preferable, in order to avoid minor toxic metabolites being disregarded (Harper et al., 2001). Furthermore, highly toxic species exist in which there is not a single major metabolite. In these cases, Microtox® can be used as a bioassay-guided screening to identify the metabolites responsible of the toxicity observed.

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DOES MOWING HEIGHT INFLUENCE ALKALOID PRODUCTION IN ENDOPHYTIC TALL FESCUE AND PERENNIAL RYEGRASS?

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Abstract—The mutualistic symbiosis following infection of tall fescue, *Festuca arundinacea*, and perennial ryegrass, *Lolium perenne*, by fungal endophyte (*Neotyphodium* spp.) results in the production of alkaloids that are feeding deterrents or toxic to insects and livestock. If the levels of the alkaloids can be manipulated by cultural practices in the grasses that are used for home lawns and golf courses, this could alleviate the need for pesticide applications in urban environments. We evaluated the influence of mowing height on the levels of some alkaloids in a greenhouse experiment for two consecutive months. In tall fescue, levels of four of the nine alkaloids, including one presumptive alkaloid, showed increased levels with increasing the mowing height from 2.5 to 7.5 cm. The alkaloids were ergonovine, ergocryptine, perloline methyl ether, and an unidentified alkaloid designated as unknown C. In perennial ryegrass, three out of six alkaloids, perloline methyl ether, chanoclavine, and unknown A, showed similar increases. The alkaloid levels in perennial ryegrass showed more variability than those in tall fescue between the two sampling dates. It was clear in both grasses that the relative levels of the alkaloids varied with mowing height, as well as over time.

Key Words—Fungal endophytes, *Neotyphodium coenophialum*, *Neotyphodium lolii*, alkaloids, tall fescue, *Festuca arundinacea*, perennial ryegrass, *Lolium perenne*, moving height, resource allocation.

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INTRODUCTION

Tall fescue, *Festuca arundinacea* Schreb, often harbors a fungal endophyte *Neotyphodium coenophialum* Morgan-Jones & Gams, and perennial ryegrass, *Lolium perenne* L. harbors *Neotyphodium lolii* Latch, Christensen & Samuels. These mutualistic symbioses lead to the production of several alkaloids that provide infected plants with chemical defenses against herbivory (Prestidge et al., 1982). The impact of these alkaloids has been shown on livestock (Porter et al., 1974; Hoveland, 1993), nematodes (West et al., 1988), fungi (White and Cole, 1985), and at least 15 species of insects (Siegel et al., 1990; Rowan et al., 1990; Breen, 1993).

Tall fescue and perennial ryegrass are important turf grasses that are commonly used in residential lawns, golf course fairways, roughs, driving ranges, and in institutional and public parks. The resistance to insect herbivory provided by endophytic alkaloids could offer an alternative to the use of chemical pesticides, or at least decrease the need for them (Clay, 1989). Efforts to control insects in the field have been plagued by inconsistent results (Clay et al., 1985). If levels of the alkaloids could be increased by cultural practices (fertilization, irrigation, mowing), this could decrease the need for pesticide use, resulting in both economic and health benefits to home owners and managers of golf courses and public parks.

A reduction in alkaloid content by repeated clipping has been demonstrated in tall fescue (Belesky and Hill, 1997). This reduction in the alkaloid production was attributed to the diversion of nonstructural carbohydrates towards the synthesis of leaf matter at the expense of alkaloid production. Salminen and Grewal (2002) demonstrated a trend towards increases in some alkaloids in tall fescue and perennial ryegrass when mowing frequency was reduced from weekly to biweekly. Here, we report results from another greenhouse study on the effects of mowing height on the levels of major alkaloids in the endophytic tall fescue and perennial ryegrass. We hypothesize that increased mowing height will lead to increased alkaloid production in the two grasses.

METHODS AND MATERIALS

Grass Management and Mowing Frequency. Nine 15-cm cores of established stands of endophytic tall fescue, *F. arundinacea*, and nine of endophytic perennial ryegrass, *L. perenne*, were taken in fall 2000 from field plots established in 1999 at the Ohio Agricultural Research and Development Center in Wooster, Ohio. Cores were placed in 15-cm diam pots and transferred to a greenhouse. Percentage of tillers infected by the endophyte was in the range 30–100%, with an average of 58%, for the tall fescue, and 50–90%, with an average of 77%, for the perennial ryegrass (Grewal, unpublished data). A temperature regime 21°C day and 16°C

night was maintained. The day/night cycle consisted of 16 hr light and 8 hr night. Daylight was supplemented with artificial lighting as required. Plants were watered as needed, and weekly applications of Peters Professional fertilizer (20-20-20 N-P-K) (O.M. Scott Co., Maysville, OH) were provided. Pots were maintained in the greenhouse for four wk, the plants were cut weekly to 5 cm above the soil surface, before the mowing height treatments were imposed. Three pots were selected for each of the three plant heights, and the grass was cut weekly to a height of 2.5, 5.0, or 7.5 cm above the soil surface.

Sampling. Triplicate samples were collected, one per pot, at 4 and 8 wk (two months). To collect the samples, grass was cut at the soil level, about a fourth of a pot. Samples were immediately frozen in liquid nitrogen and lyophilized. Dried samples were ground to a powder in a Cyclotech 1093 mill and stored in closed glass vials in a freezer until analysis.

Alkaloid Extraction. A method described by Hill et al. (1993) with some modifications was used. Briefly, 200 mg of dried sample were weighed and transferred to a liquid scintillation vial. Two hundred nanograms of ergotamine were added as an internal standard. Nine ml of $CHCl_3$ and 1 ml of 0.01 N NaOH were added, and the sample was agitated by wrist action for 30 min. Sample was filtered through Whatman 1 PS filter paper and loaded onto 5-ml solid phase extraction column prepared as described by Hill et al. (1993) containing 0.5 g of Ergosil at the bottom and 1 g of Na_2SO_4 on top, and separated by Whatman # 41 filter paper disks. Pigments were eluted in an aspirated manifold with 5 ml of acetone/chloroform (75:25), and the residual acetone/chloroform was removed with 1 ml of diethyl ether. A detachable Nylon 66 filter (22 μm) was attached to the syringe and the alkaloids eluted by slowly plunging 2 ml of methanol through. The sample was evaporated to dryness under N_2 and stored in the freezer until analysis.

Data Collection. The sample was suspended into 100 μl of methanol, and analysis was carried out with a Waters LC/MS system using an Xterra MS C18, 3.5-μm, 2.1 $\times$ 150 mm column. The flow rate was 0.27 ml/min. The mobile phase was H_2O/methanol/acetonitrile plus 0.03% NH_4OH (60:10:20:10) in a linear gradient for 48 min, changed to 10:10:70:10 for 4 min, and at 52 min returned to the original composition until 60 min. Peaks were detected with a Waters 996 Photodiode Array Detector and Waters 474 Scanning Fluorescence Detector with excitation wavelength at 316 nm and emission wavelength at 413 nm. Positive ion electrospray spectra were recorded on a Waters ZQ instrument.

Data Analysis. Quantification was based on the ergotamine internal standard. Data for each alkaloid were analyzed using repeated measures ANOVA where the level of each alkaloid, e.g., $\text{chanoclavine}_{\text{mo 1}}$ vs. $\text{chanoclavine}_{\text{mo 2}}$, was compared for the two months. A comparison of the effect of mowing height for each alkaloid was done separately for each month by one-way ANOVA followed by Tukey HSD test (Statistica, StatSoft, Inc., Tulsa, Oklahoma, USA).

RESULTS

Tall Fescue. All effects of the repeated measures ANOVA are shown in Table 1. Chanoclavine levels were not affected by mowing height, but an effect by month was significant (Table 1, Figure 1A). Ergovaline levels showed a Month × Height interaction (Table 1). There was a decline in ergovaline levels at month 2 where Tukey HSD test indicated that 5-cm-high grass was significantly different from 2.5-cm grass (Figure 1B). Ergonovine levels were not affected by grass height for the first month, but Tukey HSD showed a significant effect at month 2, where the level in the 7.5-cm-high grass was greater than in the 5 cm ($P = 0.018$) or 2.5 cm ($P = 0.010$) high grass (Figure 1C). No interaction was seen (Table 1). Ergocristine levels were influenced by both height and month, but not by Month × Height interaction (Table 1). Ergocristine increased from month 1 to month 2 at all mowing heights. Tukey HSD for month 1 showed that 7.5-cm-high grass was significantly different from 2.5 cm ($P = 0.008$) and 5 cm ($P = 0.033$) high grasses. At month 2, the 5-cm grass had a lower level than the 7.5-cm grass ($P = 0.034$) (Figure 1D). Ergocryptine levels showed Month × Height interaction (Table 1). Levels increased with increasing mowing height at both months (Figure 1E). Tukey HSD test for month 1 indicated that the 7.5-cm-high grass had significantly higher ergocryptine level than the 2.5 cm ($P = 0.001$) or 5 cm ($P = 0.002$) high grass. At month 2, the 7.5-cm-high grass had a greater level than either 2.5 cm ($P = 0.038$) or 5 cm ($P = 0.006$) high grass. Perloline methyl ether also conformed to this pattern (Table 1, Figure 1F). At month 1, the perloline methyl ether levels were significantly lower in the 2.5-cm-high grass than either 5 cm ($P = 0.003$) or 7.5 cm ($P = 0.002$) high grass. At month 2, Tukey HSD test showed that 7.5-cm-high grass contained greater amounts of perloline methyl ether than either 5 cm ($P = 0.001$) or 2.5 cm ($P < 0.001$) high grass. Three unidentified, presumptive alkaloids, designated as unknowns A, B, and C, were present. An effect of mowing height was seen on unknown alkaloid A with a pseudomolecular ion ($(M + H)^+ = 537$) (Table 1, Figure 1G) at month 2 where Tukey HSD test indicated that the 7.5-cm-high grass had more unknown A than the 5-cm-high grass ($P = 0.047$). Mowing height had an effect on unknown B ($(M + H)^+ = 571$) at month 1 where the amount in the 7.5-cm-high grass was less than in 2.5 cm ($P = 0.011$) or 5 cm ($P = 0.006$) high grass. (Figure 1H). This pattern was repeated at month 2 where the level in the 7.5- cm-high grass was less than in 2.5 cm ($P = 0.008$) or 5 cm ($P = 0.010$) high grass. Unknown C, ($(M+H)^+ = 574$), showed an increasing trend both with increased mowing height and month (Table 1, Figure 1I). At month 1, Tukey HSD test indicated that the 7.5-cm grass had a higher level of unknown C than the 2.5 cm ($P = 0.002$) or 5 cm ($P = 0.003$) high grass. This pattern repeated at month 2 where the 7.5-cm-high grass had a higher level than 2.5 cm ($P = 0.003$) or 5 cm ($P = 0.002$) high grass.

TABLE 1. REPEATED MEASURES ANALYSIS OF THE ALKALOID LEVELS IN TALL FESCUE DETERMINED ONCE A MONTH FOR TWO MONTHS

Effect	df[a]	Chanoclavine	Ergovaline	Ergonovine	Ergocristine	Ergocryptine	Perloline methylether	Unk.[b] A	Unk.[b] B	Unk.[b] C
Height	2	NS	24.53***	15.84***	10.43***	24.96***	100.23***	5.57**	39.43***	374.91***
Error	6									
Month	1	10.37**	36.90***	68.62***	14.31**	991.66***	8.48**	NS	NS	366.23***
Month × Height	2	NS	6.59**	NS	NS	6.38**	NS	NS	NS	11.71***
Error	6									

Note: F values and their significance levels are given.
[a] df = degrees of freedom.
[b] Unk. = unknown. * $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$.

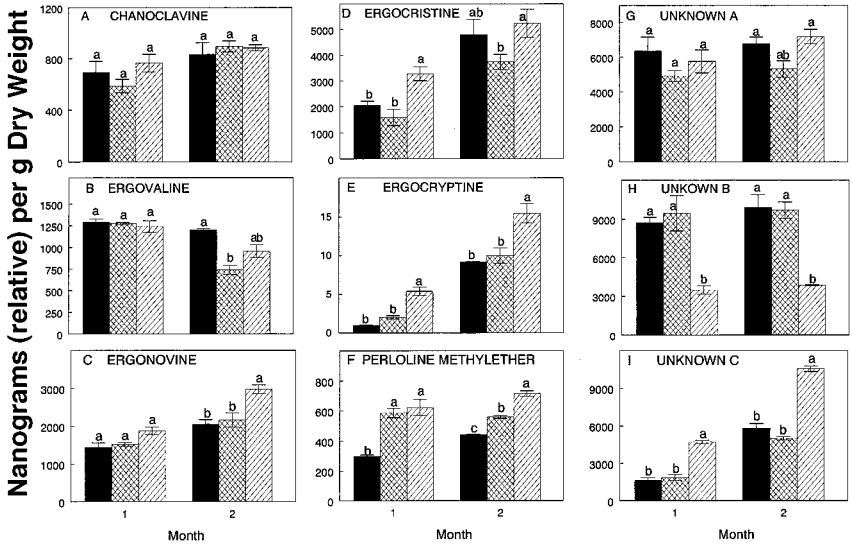

FIG. 1. Mean (±SE) levels of chanoclavine (A), ergovaline (B), ergonovine (C), ergocristine (D), ergocryptine (E), perloline methyl ether (F), unknown alkaloid A (G), unknown alkaloid B (H), and unknown alkaloid C (I) in above-ground tall fescue grown in the greenhouse. Mowed weekly to 2.5 cm (■), 5.0 cm (⊠), and 7.5 cm (⊿) height. Figures 1 E and F are in micrograms per gram dry weight.

Perennial Ryegrass. The relationship between alkaloid levels and mowing height in perennial ryegrass was variable. All six alkaloids showed Month × Height interaction (Table 2). Perloline methyl ether showed a similar increase with mowing height as in tall fescue (Figure 2D). At month 1, Tukey HSD test indicated that a significant increase in the perloline methyl ether level was associated with

TABLE 2. REPEATED MEASURES ANALYSIS OF THE ALKALOID LEVELS IN PERENNIAL RYEGRASS DETERMINED ONCE A MONTH FOR TWO MONTHS

Effect	df[a]	Chanoclavine	Ergovaline	Ergocristine	Perloline methylether	Unk.[b] A	Unk.[b] B
Height	2	187.78***	23.32***	15.39***	21.04***	163.04***	39.06***
Error	6						
Month	1	12.84***	60.307***	127.54***	24.93***	NS	11.08**
Month × Height	2	38.48***	79.32***	23.15***	6.12**	139.67***	40.10***
Error	6						

Note: F values and their significance levels are given.
[a] df = degrees of freedom.
[b] Unk. = unknown. $^{*}P < 0.1$; $^{**}P < 0.05$; $^{***}P < 0.01$.

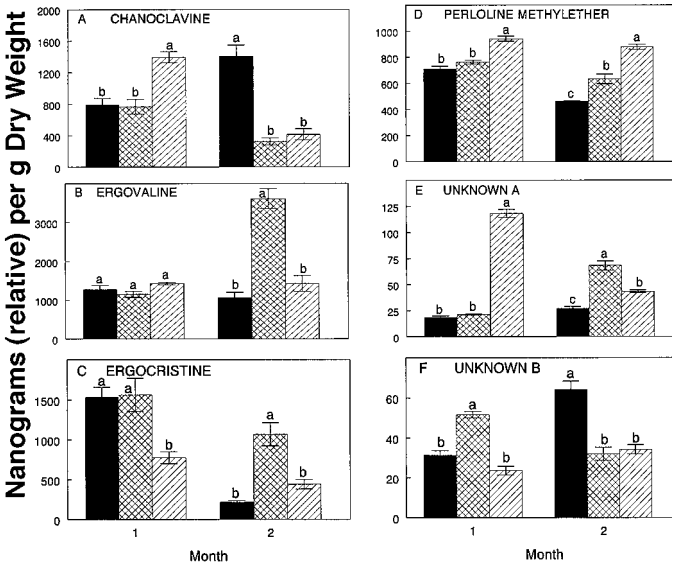

FIG. 2. Mean (±SE) levels of chanoclavine (A), ergovaline (B), ergocristine (C), perloline methyl ether (D), unknown alkaloid A (E), and unknown alkaloid B (F) in above-ground perennial ryegrass grown in the greenhouse. Mowed weekly to 2.5 cm (■), 5.0 cm (⊠), and 7.5 cm (◪) height. Figures 1 D, E, and F are in micrograms per gram dry weight.

increasing mowing height. Thus, 2.5-cm-high grass had a lower level than 5 cm ($P = 0.003$) or 7.5 cm ($P < 0.001$) high grass, and the 5-cm grass was different from the 7.5 cm ($P = 0.001$) high grass. Results for unknown A ($(M + H)^+ = 537$) indicated Month × Height interaction (Table 2, Figure 2E). At month 1, the amount of unknown A in the 7.5-cm-high grass was more than in the 2.5 ($P < 0.001$) or 5 cm ($P < 0.001$) high grass (Tukey HSD). At month 2, levels in the 2.5- and 5-cm grasses had increased, whereas in the 7.5-cm grass unknown A had decreased. The level now found in the 5-cm-high grass was higher than in 2.5 cm ($P = 0.001$) or 7.5 cm ($P = 0.002$) high grass. The 7.5-cm-high grass still had higher levels ($P = 0.050$) than the 2.5-cm-high grass. Chanoclavine also showed Month × Height interaction (Table 2). The 2.5-cm-high grass had increased chanoclavine level from month 1, whereas the opposite was true with the other mowing heights (Figure 2A). Tukey HSD test indicated that for month 1 the 7.5-cm grass had higher levels than either 2.5 cm ($P = 0.002$) or 5 cm ($P = 0.002$) high grass. This pattern was reversed at month 2, where the 2.5-cm grass had higher chanoclavine content than 5 cm ($P < 0.001$) or 7.5 cm ($P < 0.001$) high grass. The Month × Height interaction seen with unknown B ($(M + H)^+ = 571$) (Table 2, Figure 2F) showed that the level of unknown B in 2.5- and 7.5-cm-high grasses increased from month 1 to month 2 and decreased in the 5-cm- high grass. Tukey HSD test

for month 1 showed that unknown B content in the 5-cm-high grass was higher than either 2.5 cm ($P = 0.001$) or 7.5 cm ($P < 0.001$) high grass. At month 2, the 2.5-cm-high grass had a higher level than either 5 cm ($P = 0.001$) or 7.5 cm ($P = 0.001$) high grass. Ergovaline exhibited Month × Height interaction, where in 2.5-cm grass it declined slightly, and in 5-cm grass it showed a large increase (Table 2, Figure 2B). There were no significant differences in ergovaline levels at month 1, but at month 2, Tukey HSD test indicated that the 5-cm-high grass had higher ergovaline levels than either 2.5 cm ($P < 0.001$) or 7.5 cm ($P = 0.001$) high grass. A Month × Height interaction was also seen in ergocristine levels, where the levels declined at all mowing heights from month 1 to month 2 (Table 2, Figure 2C). At month 1, the 7.5-cm-high grass had a lower ergocristine content than 2.5 cm ($P = 0.010$) or 5 cm ($P = 0.008$) high grass. At month 2, Tukey HSD indicated that the 5-cm-high grass had a higher ergocristine content than 2.5 cm ($P = 0.002$) or 7.5 cm ($P = 0.007$) high grass.

DISCUSSION

The alkaloids we report are included among those described by other workers in endophytic tall fescue and perennial ryegrass (Porter et al., 1974; Lyons et al., 1986; Siegel et al., 1987, 1990; Salminen and Grewal, 2002). The levels we found here, however, are much higher than in our previous study (Salminen and Grewal, 2002). We attribute the differences to a new column, as well as to improved extraction. The Xterra HPLC column is more tolerant of the alkaline pH of the solvent system and minimizes the bleeding off of material that we experienced with a previously used Nova-Pak C18 column. It should be emphasized that the amounts are relative values based on the ergotamine used as an internal standard.

In tall fescue, we found that the amount of four out of nine alkaloids increased significantly with height when the grass was mowed weekly to 2.5-cm, 5-cm, or 7.5-cm height. This pattern would seem to be consistent with our previous study on mowing frequency where the data suggested that mowing every 14 days would result in higher alkaloid levels than would weekly mowing (Salminen and Grewal, 2002). Allocation of resources in the 2.5-cm- high grass would be directed toward building more photosynthetic leaf area, whereas in the 7.5-cm-high grass more photosynthate may be available to allow synthesis of secondary metabolites, such as the alkaloids. This would imply that the allocation of resources is mainly controlled by the plant host rather than by the endophytic symbiont. Belesky and Hill (1997) reported a decline in alkaloids as a result of repeated (6) defoliation in tall fescue. We did not see evidence for this in tall fescue samples, where alkaloid levels generally increased or were similar when samples from months 1 and 2 were compared. This could reflect the specific endophyte-grass genotype used.

The response to mowing height in perennial ryegrass showed increases in alkaloid levels in three out of six cases. Only perloline methyl ether showed

increasing levels with increasing mowing height for both months. Chanoclavine and the unknown alkaloid A appeared to show some dependence on the mowing height, but this was actually reversed by month 2. We saw no clear-cut effects of mowing on the other alkaloids. Alkaloid production in endophytic perennial ryegrass in all cases showed Month × Height interaction, whereas in two cases there was interaction present in the tall fescue. The response to mowing height was more variable in perennial ryegrass than in tall fescue. Specific host–endophyte combinations could account for this. It also is possible that the difference reflects different evolutionary stages of transition between mutualism and parasitism of the two host and fungi combinations, as discussed by Clay (1988).

Perloline and its methyl ether have been found in both infected and uninfected tall fescue (Hovin and Buckner, 1983). Both were identified in both grasses in the present study, but only the data for the methyl ether are given, as perloline comes off the column very close to the solvent peak where interfering compounds made identification and quantification questionable. However, it is interesting to note that the pattern of perloline methyl ether, increasing levels with increasing mowing height, followed that of the other endophyte-associated alkaloids most affected by mowing height.

Ergovaline levels are higher in the leaf sheaths than in the leaves (Rottinghaus et al., 1991). We harvested the samples at the soil level, so a 2.5-cm-tall grass would have had a higher sheath to shoot ratio than 5- or 7.5-cm-tall grass and, thus, be expected to have a higher alkaloid content. Finding the opposite patterns (Figures 1, 2) would suggest that these data reflect an actual increase rather than a change in the sheath-to-shoot ratio.

It was clear that some alkaloids showed a definite accumulation with increasing mowing height, in both tall fescue and perennial ryegrass. Furthermore, it was clear that the levels of alkaloids could change rapidly. This would result in changes in their ratios. Yates et al. (1989) reported a synergistic interaction between perloline and ergocryptine on mortality of *Oncopeltus faciatus* (Dallas). Thus, the changes in the ratios of alkaloids could have a significant impact on the feeding behavior and survival of insects. Fluctuations in levels of alkaloids and their changing ratios could also account for the inconsistent control of insects with the use of endophytes sometimes observed in the field (Clay et al., 1985). Our data clearly indicate that it is possible to influence the levels of endophytic alkaloids by mowing practices in tall fescue and perennial ryegrass. Taller mowing height appears to enhance alkaloid production in both grass species and might have important implications for pest management.

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PHYTOTOXICITY AND FATE OF 1,1,2-TRICHLOROETHYLENE: A LABORATORY STUDY

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Abstract—1,1,2-Trichloroethylene (TCE), a chlorinated organic contaminant, poses serious environmental concerns. A study was conducted to evaluate the phytotoxicity of TCE to a crop species and its fate in vermiculite. Growth bioassays were carried out using carrot (*Daucus carota* L.) as the test species. Three different concentrations, 0.25, 0.50, and 1 ppm were used to evaluate phytotoxicity of TCE. When added to petri plates with cotton pads, TCE did not have any effect on carrot seedling growth. However, when added to vermiculite, it significantly suppressed growth. Shoot growth was inhibited only at the 1 ppm concentration. Recovery experiments were carried out to study the fate of TCE in vermiculite. A significant decline in the percent recovery was observed with time. Interestingly, TCE additional peaks (unknown organic molecules) were detected with declining concentrations. The available chloride ion concentration in vermiculite containing 1 ppm of TCE for 24 hr was significantly higher compared to control.

Key Words—*Daucus carota* L., degradation, organic pollutant, phytoremediation, trichloroethylene.

INTRODUCTION

1,1,2-Trichloroethylene (TCE) is one of the common organic pollutants found at hazardous waste sites (Cunningham et al., 1996). TCE has been known to influence structure and function of the soil community, enzyme activities, and nitrogen cycling (Kanazawa and Filip, 1986; Fuller et al., 1997; Fuller and Scow, 1997). The bioactive concentration of TCE in the substratum depends upon microbial

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degradation and sorption onto soil particles. Microbial degradation of TCE is well documented (Walton and Anderson, 1990, 1992; Cunningham et al., 1996). Soil sorption of organic volatiles may influence the degradation process and, thus, their availability in the medium (Walton et al., 1992).

The uptake of organic pollutants by selected plant species has been suggested, and they have been employed in phytoremediation of contaminated soil (Cunningham et al., 1996; Anderson et al., 1997). Under one patented scheme, carrots are being used to specifically absorb DDT (1,1,1-trichloro-2,2′-bis(*p*-chlorophenyl)ethane) (Cunningham and Ow, 1996). The contaminated sites, however, may have adverse effects on the growth and establishment of plant species used for phytoremediation. It is, therefore, important to study the growth response of plant species in the contaminated environment. The present research was conducted to understand the phytotoxicity and bioavailability of TCE when added to the growing medium. The objectives of the present study were to (i) examine phytotoxic effects of TCE on seedling growth of carrot, (ii) determine TCE recovery in the growth medium after different time intervals, and (iii) assess the changes, if any, in the available chloride ion concentration in TCE amended medium incubated for different time intervals.

METHODS AND MATERIAL

TCE was dissolved in a few drops of methanol followed by addition of appropriate amount of distilled water to obtain final concentrations of 0.25, 0.50, and 1 ppm.

Growth Experiments. In the present research, carrot (*Daucus carota* L.) was selected as the bioassay species. Three separate growth experiments were performed. In the first, 30 carrot seeds were placed on filter paper moistened with 50 ml distilled water (control) or 0.25, 0.50, and 1 ppm TCE (treatments) and placed in 15-cm Petri plates. To maintain uniform moisture in the Petri plates in this experiment, a cotton pad soaked in water or different concentrations of TCE was placed below the filter paper. In the second, 30 carrot seeds were placed on filter paper moistened with 50 ml water (control) or 1 ppm TCE treatment and placed in 15-cm Petri plates that were placed in a desiccator to minimize the loss of TCE into the environment. In the third experiment 50 g of vermiculite was placed in each 15-cm Petri plate and irrigated with 190 ml of distilled water (control) or 190 ml of 0.25, 0.50, and 1 ppm TCE (treatment), respectively. Thirty carrot seeds were placed on the vermiculite. Petri plates in each growth experiment were maintained at 22°C under light levels of 131 μE/m^2/sec. Root and shoot lengths were measured on day 8 of each experiment ($N = 3$). Data were analyzed using one way analysis of variance ($P < 0.05$).

TCE Recovery. Five gram of vermiculite were treated with 5 ml of 1 ppm (1000 μg/l) TCE and extracted with 20 ml hexane after incubation periods of

0, 16, 24, 48, and 72 hr. The extracts were filtered through Whatman No. 44 and subjected to GC-MS (GC:HP 5890 series II, MS:HP 5971, Hewlet Packard, Wilmington). The initial oven temperature was 40°C for 10 min followed by a 10°C increase per minute, and hold at 210°C for 4 min. The helium flow was 19.1 cm/sec. The column used was a Ttx-Volatile 30 m, 0.25 mm i.d., 2 μ mdf. TCE and other unknown peaks were differentiated on the basis of their retention times. All recovery experiments were replicated three times.

Soil Chloride Concentration. Five grams of vermiculite were soaked with 5 ml 1 ppm TCE for 0, 16, 24, 48, and 72 hr followed by extraction with 25 ml water. This soil suspension was used to determine available chloride concentration using an Ion Analyzer (IA-100, TOA Electronics, Tokyo). The mobile phase was a mixture of 3 mM phthalic acid, 2.74 mM tris(hydroxymethyl) amino methane, and 200 mM boric acid solution. The flow rate was 1.3 ml/min. The volume of vermiculite suspension injected was 20 μl. The experiment was replicated three times.

RESULTS AND DISCUSSION

Seedling growth of carrot was only affected at the 1 ppm TCE concentration in filter paper bioassays measuring shoot growth (Table 1). However, when carrot was grown on filter paper moistened with 1 ppm TCE placed in a desiccator, root growth was significantly suppressed (−19.3%) but shoot growth was not affected. This differential growth response of carrot could be explained by the volatile nature of TCE. In desiccator experiments, carrot seedlings experienced additional TCE vapors, which were absent in the filter paper bioassay carried out in an open environment. In contrast, root growth of carrot was significantly suppressed in vermiculite treated with 0.25 (−17.6%), 0.50 (−20.1%), and 1 (−17.8%) ppm of TCE (Table 1). The shoot growth was inhibited in vermiculite treated with 1 ppm TCE (Table 1). In the present study, data on seed germination were not given, since seed germination may not be a good parameter to check the phytotoxicity of the chemical (Inderjit, 1996).

Recovery studies show that TCE concentration declined with time after its addition to the vermiculite (Figure 1). TCE concentrations in vermiculite incubated with 1 ppm TCE for 0, 16, 24, 48, and 72 hr were 89.9 (±1.7), 19.2 (±11.5), 9.19 (±1.7), 0.69 (±0.8), and 0.047 (±0.25) μg/l, respectively (Figure 1). The lower levels of TCE in vermiculite, with time, could be due to degradation, sorption, and/or evaporation (Madhun et al., 1986; Anderson and Walton, 1995a,b; Perkovich et al., 1996). Walton and Anderson (1990) reported TCE degradation in slurries of rhizosphere soil. Data given in Table 2 could explain the possible degradation of TCE. Total number of unidentified peaks in vermiculite incubated with TCE for 0, 16, 24, 48, and 72 hr are 0, 2, 2, 6, and 7, respectively (Table 2). This suggests that TCE has been degraded into other organic compounds

TABLE 1. EFFECT OF 0.25, 0.50, AND 1 PPM 1,1,2-TRICHLOROETHYLENE ON CARROT SEEDLING GROWTH[a]

	Bioassay			
	Filter paper		Vermiculite	
TCE (ppm)	Root (cm)	Shoot (cm)	Root (cm)	Shoot (cm)
0	2.73 ± 1.33	2.19 ± 0.59	4.93 ± 1.6	3.52 ± 0.66
0.25	2.51 ± 1.22 (−8.1)[b]	2.06 ± 0.74 (−5.93)[b]	4.06 ± 1.3* (−17.64*)[b]	3.29 ± 0.79 (−6.5)[b]
0.50	2.61 ± 1.4 (−4.4)[b]	2.22 ± 0.44 (+1.4)[b]	3.94 ± 1.2*(−20.1*)[b]	3.29 ± 0.73 (−6.53)[b]
1	2.30 ± 1.4 (−15.75)[b]	1.83 ± 0.64* (−16.43*)[b]	4.05 ± 1.3* (−17.84*)[b]	3.14 ± 0.79* (−10.8*)[b]

[a] Values indicate mean ± standard deviation, and are significantly different from control (0 ppm) at the level of $P < 0.05$.
[b] Values in parentheses indicate percent decrease in length compared to control.

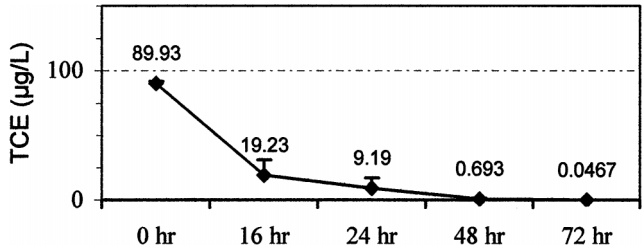

FIG. 1. TCE concentration (μg/l) in vermiculite treated with 1 ppm TCE for 0, 16, 24, 48, and 72 hr.

with time. Under aerobic conditions, TCE has been reported to degrade to phenol, toluene, propylene, methane, and ammonia (Ensley, 1991; Ensign et al., 1992; Fuller et al., 1997). Barker et al. (1987) reported that toluene degraded into catechol, cresol, benzoic acid, and benzaldehyde. These phenolic compounds are reported to interfere with growth and establishment of plant species (Rice, 1984). Previous literature shows that TCE degrades to ethylene (Friedman and Gossett, 1989), which is known to possess allelopathic effects (Rice, 1984; Butler, 1995). It is important to identify the degradation products of particular pollutants, and understood how they influence the growth and establishment of plant species being used for phytoremediation. Research is needed to identify TCE degradation products and to study their possible allelopathic activities.

TABLE 2. PRESENCE (+) OR ABSENCE (−) OF UNIDENTIFIED PEAKS AFTER ADDITION OF 1,1,2-TRICHLOROETHYLENE INTO VERMICULITE[a]

Retention time (min)	Time after incubation (hr)				
	0	16	24	48	72
1.5	−	−		−	+
2.3	−	−	−	+	+
2.5	−	+	+	+	+
4.9	−	+	+	+	−
5.05	−	−	−	+	+
5.9	−	−	−	−	+
6.4	−	−	−	+	+
11.8	−	−	−	−	+
18.2	−	−	−	+	−
Total number of peaks	0	2	2	6	7

[a] Five gram vermiculite were treated with 5 ml of 1 ppm TCE for 0, 16, 24, 48, and 72 hr, and extracted with 20-ml hexane. The hexane extracts were analyzed using GC-MS spectroscopy.

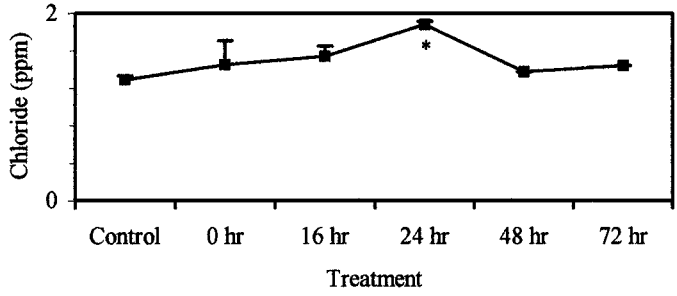

FIG. 2. Chloride ion concentration (ppm) in vermiculite treated with 1 ppm TCE for 0, 16, 24, 48, and 72 hr.

When a chlorinated hydrocarbon degrades, chemically or microbially, there may be release of chloride ions. Our preliminary study shows a significant increase in Cl^- when vermiculite was treated with TCE for 24 hr compared to control (Figure 2). The higher Cl^- values may make soil unsuitable for plant growth (Richards, 1968; Black, 1973). The degradation of TCE to 1,2-dichloro and then to vinyl chloride has been documented (Barrio-Lage et al., 1986; Wilson et al., 1986). Friedman and Gossett (1989) reported the conversion of TCE to ethylene, and proposed the following pathway for reductive dechlorination: polychloroethylene → trichloroethylene → dichloroethylene → vinyl chloride → ethylene. These studies support the hypothesis of higher Cl^- concentration in TCE contaminated soils.

In summary, the present study shows the phytotoxic effects of TCE on carrot seedling growth, its degradation in vermiculite, and trends toward higher Cl^- values in TCE treated vermiculite over time. When inspecting TCE contaminated sites, it is not only important to determine TCE concentration, but equally important to find out (1) degradation byproducts, and (2) available chloride ion concentration in the contaminated soil. Even if TCE degrades to nonphytotoxic levels, its degraded products and/or the resulted higher Cl^- concentrations may cause adverse effects on the plant species being used for the phytoremediation program.

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GROWTH STIMULATION OF ECTOMYCORRHIZAL FUNGI BY ROOT EXUDATES OF BRASSICACEAE PLANTS: ROLE OF DEGRADED COMPOUNDS OF INDOLE GLUCOSINOLATES

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Abstract—Brassicaceae plants are nonmycorrhizal. They were found to inhibit VA mycorrhizal infection in their host plants. We tested if they can influence growth of ectomycorrhizal (ECM) fungi. When roots and leaves of Brassicaceae plants and ECM fungi were cultured together in the same petri dishes, the root exudates of turnip (*Brassica rapa*), swede (*B. napobrassica*), cabbage (*B. oleracea*, var. *capitata*), broccoli (*B. oleracea*, var. *italica* Plenck), kohlrobi (*B. caulorapa* Pasq.), mustard (*B. juncea*), radish (*Raphanus sativus*), and choy (*B. napus*) significantly stimulated hyphal growth of the ectomycorrhizal fungus *Paxillus involutus*. Root exudates of turnip and cabbage stimulated hyphal growth of *Pisolithus tinctorius* and two isolates of *P. involutus.* Colony area of *P. involutus* was increased by 452 and 414%, respectively, in the presence of turnip and cabbage germinants. Root exudates of turnip increased the biomass of *P. involutus* and *P. tinctorius* by 256 and 122% and cabbage by 220 and 82%, respectively. The stimulatory effect was not affected by autoclaving the root exudates. Root exudates had chemical reactions with glutathione and lysine, which resulted in a reduction of the growth stimulation of ECM fungi. Myrosinase enhanced further the stimulatory effects of turnip on the ECM colony diameter growth by 23%. Autoclaved roots and leaves of turnip did not stimulate fungal growth, but mechanically ground roots and leaves of turnip stimulated growth of *P. involutus* by 147 and 135%, respectively. After desulfuration with aryl sulphatase, the glucosinolates (GLSs) in turnip roots and leaves were identified by HPLC. The major ones were indole GLSs. Prominent compounds identified were 1-methoxy-3-indolymethyl GLS and 4-methoxy-3-indolymethyl GLS. The

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finding provides an opportunity to field test the use of Brassicaceae plants in enhancing ectomycorrhizal formation in conifers by interplanting conifers with Brassicaceae plants in forest tree nursery and agroforestry systems.

Key Words—Ectomycorrhizal fungi, *Paxillus involutus*, *Pisolithus tinctorius,* growth stimulation, Brassicaceae, M-factor, indole glucosinolate, indole isothiocyanate.

INTRODUCTION

Ectomycorrhizal (ECM) fungi improve the absorption of mineral nutrition of host trees and increase the tolerance of trees to stress environments. Most ECM fungi are slow growing in pure culture (Satyanarayana et al., 1996). Melin (1954) found root exudates of trees increased the growth rates of ECM fungi *in vitro*. The effective compound(s) in the exudates was called "M-factor." Its chemical identity was not determined. He used 10 water soluble vitamins and 19 amino acids to see whether the activity of the root exudates belonged to vitamins or amino acids, but found that the growth stimulation was caused by unknown compounds. Few M-factors have been identified (Sun and Fries, 1992). Chemicals responsible for the so-called M-factor could be used to enhance multiplication of ECM fungi for use in tree seedling inoculation. If more M-factors are identified, they can be used to culture ECM fungi for commercial seedling inoculation.

In developing countries, agroforestry has become an important approach for growing multiple crops in a sustainable way. In agroforestry systems, trees often grow in soils with low fertility and in a stressed environment. Use of mycorrhizae enhanced tree seedling growth (Haselwandter and Bowen, 1996; Brunner, 2001). Since the principal mode of action of mycorrhizas is mediated through the growth of mycelia into soils, factors affecting mycelial growth are important to the symbiosis (Haselwandter and Bowen, 1996). A large amount of mycelia of ECM fungi was found to enhance plant growth (Jones et al., 1990). A combination of trees and crops in agroforestry should be beneficial for mycorrhizal formation and fungal mycelial growth. However, before selecting the combinations, it is important to determine which crop trees will influence mycorrhizal formation and mycelial growth.

Brassicaceae are important crops. A functional mycorrhizal association has never been found in plants of this family (Vierheilig et al., 2000). Glucosinoaltes (GLSs), a group of β-D-thioglucosides that are characteristic of all species in the family, are relatively nontoxic (Fahey et al., 2001). The hydrolytic products (isothiocyanates, ITCs) of these compounds, however, are bioactive by the coexisting enzyme myrosinase (thioglucoside glucohydrolase (EC 3.2.3.1)). Isothiocyanates have long been known for their fungicidal, bactericidal, nematocidal, and allelopathic properties, and have recently attracted intense research interest because of their cancer chemoprotective attributes (Fahey et al., 2001; Petersen et al.,

2001). The presence of glucosinolates in Brassicaceae plants has been implicated in their resistance to invasion of arbuscular mycorrhizal (AM) fungi (Tester et al., 1987; Schreiner and Koide, 1993). In Southern China, farmers often plant cabbage, choy, and radish underneath trees, especially when trees are young. It was reported that plants became less mycorrhizal when they grew together with Brassicaceous plants (Tester et al., 1987). We asked the question whether the Brassicaceae plants influence ECM fungal growth and mycorrhizal formation in higher plants. The presence of glucosinolates in plants is believed to be linked with the absence of mycorrhization.

The specific objectives of our study were: (1) to investigate the M-factor effects of Brassicaceous plants on ectomycorrhiza, and (2) to determine the role of glucosinoaltes in the M-factor.

METHODS AND MATERIALS

Plant Materials. Seeds of turnip (*Brassica rapa* L), swede (*B. napobrassica* L), cabbage (*B. oleracea* L., var. *capitata*), broccoli (*B. oleracea* L, var. *italica* Plenck), kohlrobi (*B. caulorapa* Pasq.), mustard (*B. juncea* L.), radish (*Raphanus sativus* L), choy (*B. napus* L), cucumber (*Cucumis sativus*), and oat (*Avena sativa*) were purchased from Mckenzie seed Co. (Brandon, MB, Canada). Seeds of jack pine ((*Pinus banksiana*) and black spruce (*Picea mariana*) were obtained from Hills Nursery, Thunder Bay, Canada.

Ectomycorrhizal Fungi. Paxillus involutus (Batsch. Ex Fr.) Fr. Strains, 211804 and 196554 were provided by the Canadian Collection of Fungal Culture, Ottawa, and *Pisolithus tinctorius* Coker and Couch (strain 99132) from Prof Gong, Research Institute of Tropical Forestry, Guangzhou, P.R. China.

Chemicals and Enzymes. Lysine, glutathione, sinigrin, DEAE-Sephadex A-25, myrosinase (thioglucosidase, EC 3.2.3.1), sulfatase type H-1 (EC 3.1.6.1) from *Helix pomatia* were purchased from Sigma Chemical Co. Malt extract and granulated agar were obtained from Becton Dickinson Co. All solvents used were of analytic or chromatographic grades. Mustard oil was purchased from a local market in Thunder Bay, Canada.

Seed Surface Sterilization . Plant seeds, except black spruce and Jack pine, were surface-sterilized with 1% $NaClO_3$ for 10 min and 75% ethanol for 10 sec, and then rinsed with sterile distilled water three times. Seeds of black spruce and Jack pine were surface-sterilized only with 1% $NaClO_3$ for 10 min and then rinsed with sterile water three times.

Fungal Culture. Modified Melin-Norkrans' nutrient solution (hereafter MMN) was used for growing ectomycorrhizal fungi (Marx, 1969). Because plants grow poorly in full strength MMN medium, the $1/4 - 1/2$ strength was used. The petri dish with the MMN media was inoculated with one agar plug

(4 mm diameter) cut from the edge of 2- to 3-week old colonies of ectomycorrhizal fungi. Three surface sterilized seeds were sowed around the inoculum. The distance between the seeds and inocula was approximately 1.5 cm. Inoculated plates were incubated at room temperature (20–25°C). Diameters of fungal colonies were measured every 5 d. Each treatment of each experiment had at least three petri dishes as replicates.

Effects of Brassicaceae Plants on ECM Fungi

Plant and Fungal Species. Turnip, swede, turnip, cabbage, broccoli, kohlrabi, radish, mustard, and choy were used. The non-Brassicaceae plants, mung bean, oat, and cucumber, representing three different families, were used for comparison. Since pines have been reported to stimulate the growth ECM fungi (Melin, 1954), Jack pine and black spruce, host plants of ECM fungi, were also used for comparison. A plug of inoculum of *P. involutus* (211804) and three seeds of one of the above 12 species were added in each plate. Seeds of Brassicaceae plants, mung bean, and cucumber germinated at room temperature (22–25°C) within 24 hr, Jack pine within 48 hr, and black spruce within 4 d. Fungal colony diameters were measured 16 d following inoculation.

Two isolates (211804, 196554) of *P. involutus* and one (99132) of *P. tinctorius* were tested with turnip and cabbage as plant materials. The same culture method mentioned above was used.

Biomass Determination. Dry mycelial biomass of the fungi was determined after growing them in 2/5 MMN agar medium. A piece of 0.45 μm millipore filter (46 mm diam) was used to separate the media and mycelia in each petri dish. A plug of fungal mycelia was inoculated in the center of the filters. Three surface-sterilized seeds of turnip and cabbage were planted around the filter. A control was made with the same filter but without seeds. When the mycelia of turnip treatment grew to the edge of the filter, all mycelia on top was collected, dried at 60°C for 24 hr, and the dry biomass was weighed with a 0.1 mg balance.

Nutrient Effects. To test if stimulatory effects of fungal growth were caused by the addition of carbon and nitrogen, we used three nutrient levels, full, 1/2, and 1/4 MMN agar media. A plug of inoculum was inoculated in the center of a petri dish, and three seeds of turnip or cabbage were added in each plate. The distance between the seeds and fungal inocula was approximately 1.5 cm. After 20 d, diameters of the colonies were measured.

Nonvolatile Compounds. High CO_2 concentration may stimulate ECM fungal growth (Straatsma *et al.*, 1986), and Brassicaceae plants can produce volatile mustard oil. To test whether the growth stimulation of ECM fungi was caused by increasing concentration of CO_2 or other volatile compounds from the aerial parts of the seedling, after the 1/3 MMN agar media had solidified in a 10 mm petri dish, a 5 mm ditch was aseptically dug with an inoculating needle in the middle of

a plate. On one side of the ditch, *P. involutus* 211804 was inoculated, and on the other side three seeds of turnip were added. This ensured the exposure of volatile compounds from turnip to ECM fungi, but the nonvolatile root exudates could not reach the opposite side. Two controls were used: (1) no ditch cutting with turnip seeds, and (2) ditch cutting without turnip seeds. Diameters of the fungal colonies were measured after 16 and 25 d.

Isothiocyanates in Mustard Oil Mustard oil contained volatile ITCs that were degraded from GLSs in mustard seeds. We tested whether the fungal growth stimulation was caused by these volatile ITCs. Mustard oil was sterilized by passing it through two layers of millipore filter (46 mm diam). Sterile mustard oil was then added to autoclaved 1/3 MMN agar medium when it cooled to 40–50°C. The media with 1, 10, 25, and 100 mg/l of mustard oil were poured into petri dishes and inoculated with *P. involutus* 211804. Plates were incubated at room temperature (20–25°C). Diameters of the colonies were measured after 20 d.

Root Exudates. We tested whether the fungal growth stimulation was caused by the root exudates released into the growth medium. Five germinated turnip seeds were grown in 20 ml of 1/3 MMN agar medium for 7 d in a petri dish (90 mm diam). Whole seedlings including roots were removed and prepared for the following three treatments: (1) *Nonautoclaved root exudates* – four dishes from which the turnip seedlings had been removed were inoculated directly with *P. involutus* 211804. (2) *Autoclaved root exudates* – the medium collected from another four petri dishes from which the turnip seedlings had been removed was autoclaved at 121°C for 15 min. We collected the media with root exudates from four dishes, autoclaved the media, and then adjusted the volume with sterile water to 80 ml, which was equal to the original volume for four dishes. The media were then added to four dishes. Thus, the total amounts of root exudates in autoclaved and nonautoclaved systems were almost the same. Dishes were then inoculated. (3) *Root pieces* – turnip roots collected from one of the above petri dishes were aseptically transferred to another dish containing 1/3 MMN agar medium and lined around the inoculated plug. When taken from the culture medium, most roots were broken into pieces. The control was 1/3 MMN medium without turnip germinants and was prepared in the same way. Petri dishes were inoculated with *P. involutus* 211804. To compare stimulatory effects with living turnip germinants, a fungal plug and three turnip seeds were added onto 1/3 MMN medium in a petri dish. Colony diameters were measured after 14 d.

Another experiment was conducted to compare the effects of root exudates and extracts of turnip shoots and roots. Turnip seedlings were prepared as described above. There were five seeds in each petri dish. Roots and shoots collected from three dishes (15 seedlings) were added onto 60 ml 1/3 strength MMN agar medium. Media with roots and shoots and root exudates in medium were autoclaved at 121°C for 15 min, followed by cooling and inoculation with *P. involutus* 211804. Diameters of the colonies were measured after 17 d.

Glucosinolate Hydrolysis

Induced GLS Hydrolysis. We hypothesized that GLSs in leaves and roots of Brassicaceae plants cannot stimulate ECM fungal growth, but ITCs from GLS hydrolysis resulting from mechanical damage can. Hydrolysis of GLSs was induced by mechanically blending roots and leaves. Roots and leaves were harvested from 4-month old greenhouse grown turnip plants. Plant materials (2.5 g) were ground in 100 ml sterile water in a Waring commercial blender at low speed for 2 min. Ground materials were sterilized by passing through Whatman filter paper (No. 42), followed by a 0.45-μm millipore filter. Forty ml of the filtrate were mixed with 40 ml autoclaved MMN agar media cooled to 50–60$\circ$C. The resulting medium contained 2/5 MMN medium and 12.5 g/l plant material. It was poured into four petri dishes.

Indigenous myrosinase in leaves and roots was inactivated by autoclaving. One gram of root or leaf material was added in 80 ml 2/5 MMN agar medium, followed by autoclaving at 121° for 15 min. The control consisted of 2/5 MMN agar medium without root or leaf material. *P. involutus* 211804 was inoculated. Diameters of colonies were measured after 20 d.

Enzymatic Hydrolysis. We hypothesized that roots of turnip could also release glucosinolates into growth medium. Addition of myrosinase to growth medium could produce a hydrolytic reaction with GLSs to produce ITCs, which could increase growth stimulation of turnip. The same nutrient level (2/5 MMN agar medium) was used to culture *P. involutus* 211804 in all treatments. Four dishes were prepared containing myrosinase (0.1 mg/ml) and turnip seeds (three seeds for each dish). Four dishes contained only turnip seeds. The two controls included only nutrient medium and nutrient medium with the same concentration of myrosinase. After 20 d, the diameters of the colonies were measured.

Isothiocyanate Reduction. We hypothesized that growth stimulation of ectomycorrhizal fungi by Brassicaceae plants could be due to ITCs released from roots. Isothiocyanates are reactive with lysine and glutathione and convert to thiourea derivatives (Kawakishi and Kaneko, 1985). If ITCs stimulate fungal growth, stimulation would be decreased by the addition of amines. Two concentrations (10, 100 mg/l) of lysine and glutathione were added in 1/3 MMN media to react with any ITCs released from the turnip roots. The amines were dissolved in sterile water and passed through a 0.45-μm millipore filter. When the temperature of autoclaved MMN agar medium dropped to 50–60°C, nutrient medium and amine solution were mixed and poured into four petri dishes. After the media solidified, a plug of 4 mm inoculum and three surface-sterilized turnip seeds were added. Another treatment was prepared with no amines added. The same concentrations of amines without turnip seeds were used as controls. There were four replicates for each treatment.

Glucosinolate Analysis. We identified GLSs in turnip roots and leaves with reversed phase HPLC, which is the most widely used technique for GLS analysis (Fahey et al., 2001). Roots and leaves were collected from plants grown in vermiculite and peat-moss (1:1) mixture in a greenhouse for 4 months. Roots were washed carefully with tap water and dried with a paper blotter. Desulphoglucosinolates of turnip roots and leaves were prepared as described by Minchinton et al. (1982) with some modification. Forty grams of fresh roots and 80 g fresh leaves were extracted separately in 400 ml boiling methanol for 15 min. Each was re-extracted three times with boiling methanol (2 × 200 ml). Extracts were combined and filtered through Whatman 42 filter paper. Methanol in the filtrate was removed by rotary evaporation at 35°C. The aqueous solution was transferred to a 50-ml volumetric flask. Five ml of 0.5 M lead and barium acetate were added, and the volume made up to 50 ml with water. The solution was centrifuged (10000 × g, 15 min) at 0°C. Five milliliters of the supernatant were applied to 200 mg DEAE-Sephadex A-25 and desulphated with 6 mg aryl sulphatase. Two mg of sinigrin were added onto the column as an internal standard before desulphation. After overnight reaction at room temperature, the desulphoglucosinolates were eluted with 5 × 1 ml water. The volume was adjusted to 7 ml, and this sample was used for HPLC. Analyses were carried out by using a Varian Prostar Chromatograph equipped with an automatic injector, a Chrompak column (250 × 4.6 mm). The chromatograph was operated at a constant flow rate at 1.0 ml/min, with an oven temperature of 32°C. Ultraviolet detection was carried out with a PDA detector (Model 330) at a wavelength of 227.5 nm. The solvent program was 100% water for 10 min; 0–12% CH_3CN over the next 30 min; then constant 12% CH_3CN. Individual GLSs of turnip roots and leaves were identified by comparing retention times with the internal standard and on the basis of available literature data (Sang et al., 1984).

Statistical Analysis. The data were subjected to one-way analysis of variance (ANOVA), and significant treatment differences were tested at 0.01 level by using Duncan's multiple range test.

RESULTS

Effects of Brassicaceae Plants on ECM Fungi. Colony diameter of *P. involutus* increased significantly in the presence of all eight Brassicaceae plants tested, while cucumber and oat did not (Table 1). Hyphal density in the medium with Brassicaceae germinants was much higher than in the control. Hyphal density with cucumber and oat was sparser than the control. Mung bean also stimulated fungal growth, but significantly less than Brassicaceae plants. Jack pine, the host plant of *P. involutus*, also stimulated the fungal growth (Table 2) although it was not as strong and fast as turnip. Although black spruce germinants did not increase

TABLE 1. EFFECTS OF GERMINANTS OF 11 BRASSICACEAE AND NON-BRASSICACEAE PLANTS ON COLONY DIAMETRIC GROWTH OF *P. involutus* 211804

Plant species	Colony diameter (mm (mean ± SE))	Plant species	Colony diameter (mm (mean ± SE))
Control	29.5 ± 2.1 e	Radish	55.0 ± 1.0 bc
Broccoli	67.3 ± 6.7 a	Kohlrobi	50.3 ± 2.5 c
Swede turnip	64.5 ± 1.5 ab	Choy	54.7 ± 2.5 bc
Turnip	64.3 ± 1.5 ab	Mung bean	36.3 ± 1.5 d
Cabbage	59.7 ± 3.2 b	Cucumber	32.0 ± 2.0 df
Mustard	65.0 ± 4.3 ab	Oat	27.7 ± 2.0 e

Note: Values are mean ± standard error ($N = 4$), means in the table not followed by the same letter are significantly different ($P < 0.01$) ANOVA with Duncan's multiple range test.

colony diameters of the fungus significantly (Table 2), the fungus produced more aerial mycelium. When the fungus was cultured longer, black spruce also stimulated growth. Oat inhibited growth. Cucumber stimulated diametric growth, but hyphal density was even sparser than that of control.

Colony diameters of *P. involutus* 211804 were increased by 135 and 127% in the presence of turnip and cabbage germinants, respectively (Table 3). Because colonies were almost round, total area increased by 452 and 414%, respectively. Compared to *P. involutus*, growth stimulation in *P. tinctorius* was less dramatic; colony diameter increased by 42 and 41%, respectively, in the presence of turnip and cabbage (Table 3). There was more mycelia growing in the medium with turnip and cabbage germinants than in the control.

Within 15 d, the biomass of *P. involutus* 211804 and *P. tinctorius* cultured with turnip germinants increased by 256 and 122%, respectively (Figure 1). The biomass of *P. involutus* 211804 and *P. tinctorius* cultured with cabbage germinants increased by 220 and 82%, respectively, during the same time (Table 1).

TABLE 2. STIMULATORY EFFECTS OF TURNIP, JACK PINE, AND BLACK SPRUCE ON COLONY DIAMETRIC GROWTH OF *P. involutus* 211804

Plant species	Colony diameter (mm (mean ± SE))
Control	3.8 ± 0.12 c
Turnip	7.1 ± 0.10 a
Jack pine	5.3 ± 0.15 b
Black spruce	4.2 ± 0.98 bc

Note: Values are mean ± standard error ($N = 4$), means in the same column not followed by the same letter are significantly different ($P < 0.01$) ANOVA with Duncan's multiple range test.

TABLE 3. STIMULATORY EFFECTS OF CABBAGE AND TURNIP GERMINANTS ON COLONY DIAMETRIC GROWTH OF TWO ISOLATES OF *P. involutus* AND ONE ISOLATE OF *P. tinctorius*

Treatments	Colony diameters (mm (mean ± SE))		
	P. involutus 211804	*P. involutus* 196554	*P. tinctorius* 99132
Control	31.0 ± 2.6 b	22.7 ± 1.5 b	30.3 ± 1.5 b
Turnip	73.0 ± 6.1 a	43.7 ± 2.1 a	43.0 ± 1.0 a
Cabbage	70.3 ± 2.1 a	41.0 ± 1.0 a	42.7 ± 1.2 a

Note: Values are mean ± standard error ($N = 4$), means in the same column not followed by the same letter are significantly different ($P < 0.01$) ANOVA with Duncan's multiple range test.

Nutrient Effects. Turnip and cabbage significantly stimulated the growth of *P. involutus* at all nutrient levels (Figure 2). For the control, 1/2 MMN agar medium provided the best growth. The colony diameter of the fungus in the 1/4 MMN medium with turnip and cabbage was significantly higher (67 and 115%, respectively) than the 1/2 MMN control. With nutrient increase, fungal growth stimulation by turnip and cabbage also increased. The hyphal density of *P. involutus* in the media with turnip and cabbage germinants was higher than in the media without them. These results indicate that the fungal growth stimulation was not caused by the enhanced carbon and nitrogen supply in the growing media.

Volatile Compounds. When fungal and turnip media were separated physically by a ditch, no significant stimulation of fungal growth was found (Table 4).

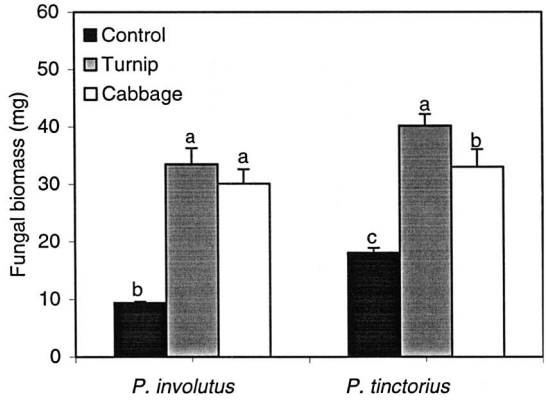

FIG. 1. Stimulatory effects of turnip and cabbage germinants on biomass of *P. involutus* 211804 and *P. tinctorius* 99132. Values are mean + standard error ($N = 4$). Significant difference ($P < 0.01$) among treatments in each group are indicated by different letters above bars.

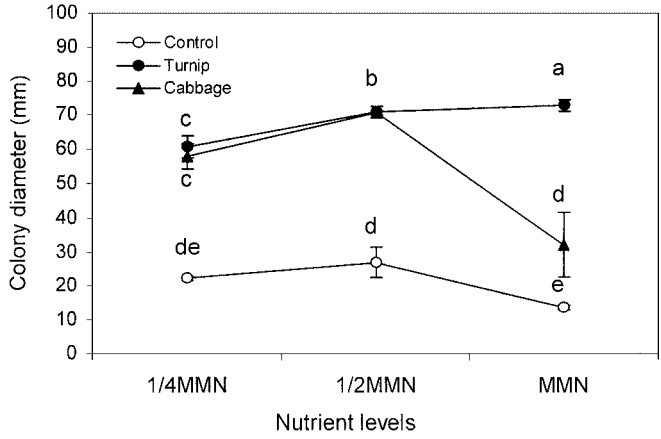

FIG. 2. Effects of germinants of turnip and cabbage on colony radial growth of *P. involutus* 211804 at three nutrient levels of MMN cultural media. Values are means ± standard error ($N = 3$). Significant differences ($P < 0.01$) among treatments are indicated by different letters.

Hyphal density was the same as control. Strong stimulation was observed when there was no separation. Diameter was 66% longer in no separation dishes than in separation dishes, and hyphal density was much higher. When cultured for more than 25 d, some roots in two petri dishes penetrated the ditches, and the growth rate of *P. involutus* there was faster than in the other two replicates. We, therefore, concluded that growth stimulation was caused by root exudates rather than volatile compounds released from aerial parts of turnip seedlings.

Isothiocyanates in Mustard Oil. No stimulatory effects of *P. involutus* was found at concentrations of 1, 10, 25 mg/l. Mustard oil significantly inhibited the

TABLE 4. INFLUENCE OF DITCH SEPARATION ON STIMULATORY EFFECTS OF TURNIP GERMINANTS ON COLONY DIAMETRIC GROWTH OF *P. involutus* 211804

Treatments	Colony diameter (mm (mean ± SE))	
	16 d	25 d
Control	20.3 ± 0.6 b	34.3 ± 0.6 b
Ditch separation	22.3 ± 0.6 b	39.7 ± 3.1 b
No ditch separation	30.3 ± 2.1 a	66.0 ± 2.6 a

Note: Values are mean ± standard error ($N = 4$), means in the same column not followed by the same letter are significantly different ($P < 0.01$) ANOVA with Duncan's multiple range test.

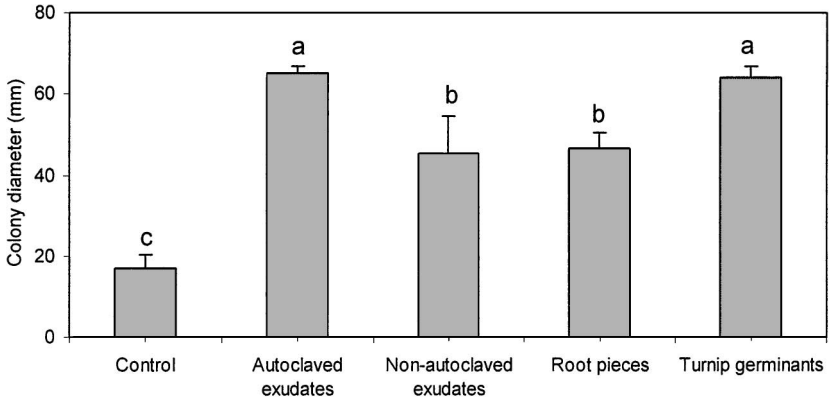

FIG. 3. Effects of autoclaved and nonautoclaved root exudates, root pieces, and living germinants of turnip on colony diametric growth of *P. involutus* 211804. Values are mean + standard error ($N = 4$). Treatments not followed by the same letter on the top of bars are significantly different ($P < 0.01$).

fungal growth at 100 mg/l. Inhibition was 18% compared to control. Hyphal density of the fungus treated with all concentrations of mustard oil was as sparse as that of control.

Root Exudates. Since the entire seedling including shoot and roots had been removed, the only thing left in the medium was turnip root exudates. When the medium (non-autoclaved) with exudates was directly inoculated with *P. involutus*, it significantly stimulated the fungal growth (Figure 3). The fungal colony diameter was increased by 165% compared to control. When the medium with exudates was autoclaved, it stimulated the fungal colony diameter by 280%. The growth stimulatory effect of autoclaved root exudates of turnip was significantly higher than that of the fresh turnip germinants. Roots (root pieces) without shoots also produced chemicals that stimulated the fungal growth, but the autoclaved roots and shoots did not (Figure 4). Hyphal density was higher in the media containing root exudates than that of control and autoclaved roots (Figure 5). These results indicate that only chemicals released from roots had the fungal growth stimulatory effects.

Glucosinolate Hydrolysis

Induced GLS Hydrolysis. Ground roots and leaves of turnip significantly stimulated the colony diameters of *P. involutus* by 147, and 135%, respectively (Figure 6). Hyphal density in both treatments was higher than control. Autoclaved roots stimulated the colony diameter, but hyphal density was as sparse as control. Autoclaved leaves did not stimulate fungal growth.

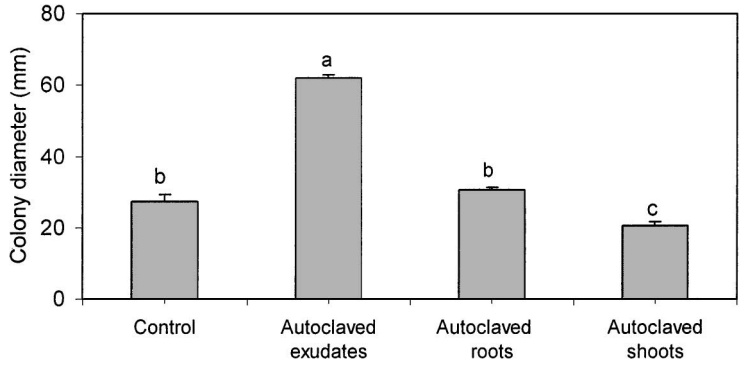

FIG. 4. Effects of autoclaved roots, shoots, and root exudates of turnip on colony diametric growth of *P. involutus* 211804. Values are mean + standard error ($N = 4$). Significant differences ($P < 0.01$) among treatments are indicated by different letters above bars.

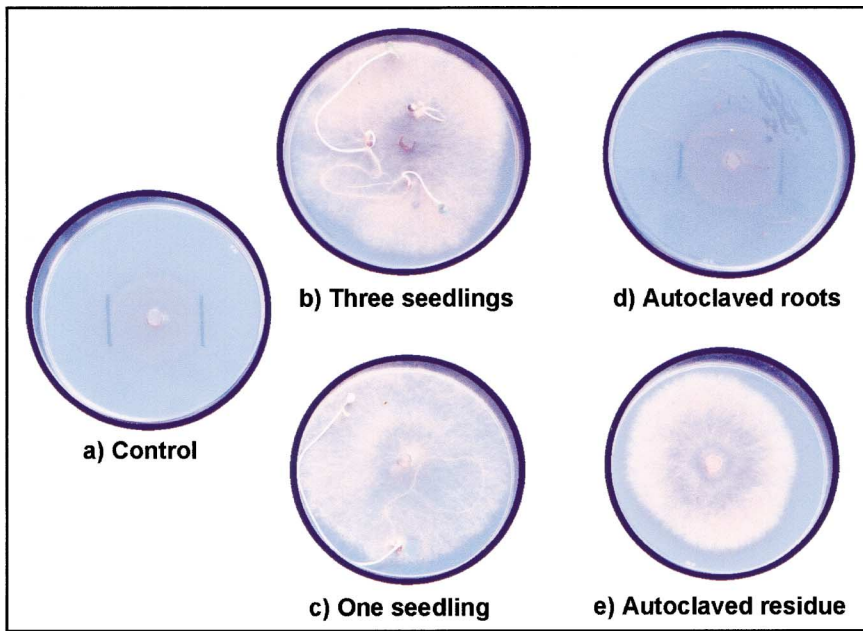

FIG. 5. Colony growth of *P. involutus* 211804 (a) in control, 1/3 MMN agar medium, (b) in presence of three turnip germinants, (c) in presence of one turnip seed, (d) in presence of autoclaved cabbage roots, and (e) in autoclaved turnip root exudates in the growing medium.

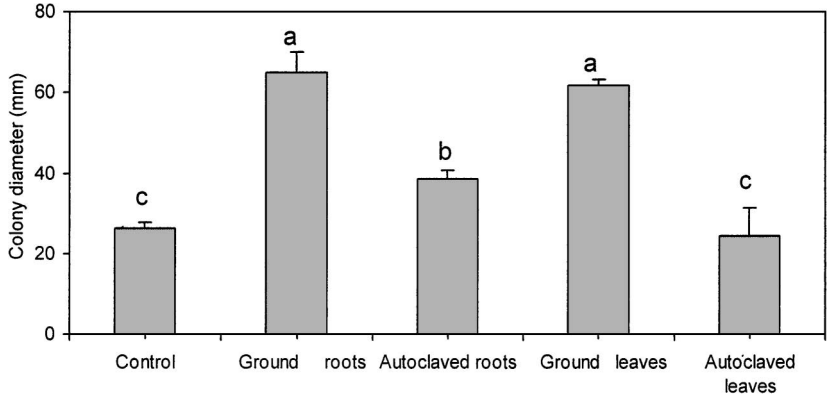

FIG. 6. Effects of ground and autoclaved roots, and ground and autoclaved leaves on colony diametric growth of *P. involutus* 211804. Values are mean + standard error of four replicates. Treatments not followed by the same letter on the top of bars are significantly different ($P < 0.01$).

Enzymatic Hydrolysis. When turnip and *P. involutus* grew together in the medium with 0.1 mg/ml myrosinase, the growth stimulatory effect increased significantly (Figure 7). Fungal colony diameter increased by 23% compared to control. Myrosinase itself had no effect on fungal growth at this concentration.

Isothiocyanate Reduction. When lysine and glutathione were added to growth media, the stimulatory effects of turnip significantly decreased. Glutathione alone stimulated growth at a concentration of 100 mg/l (Figure 8). When turnip root exudates contacted glutathione, the growth stimulation decreased drastically. Lysine alone did not have any significant effect at 10 mg/l concentration (Figure 8). However, this concentration of lysine reduced the stimulatory effect of turnip. Lysine

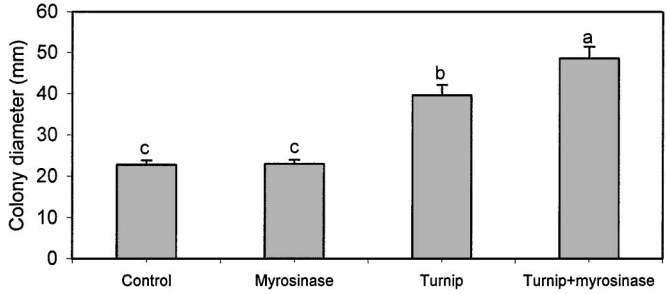

FIG. 7. Effects of myrosinase on the stimulation of turnip on colony diametric growth of *P. involutus* 211804. Values are mean + standard error ($N = 4$). Significant differences ($P < 0.01$) among treatments are indicated by different letters above bars.

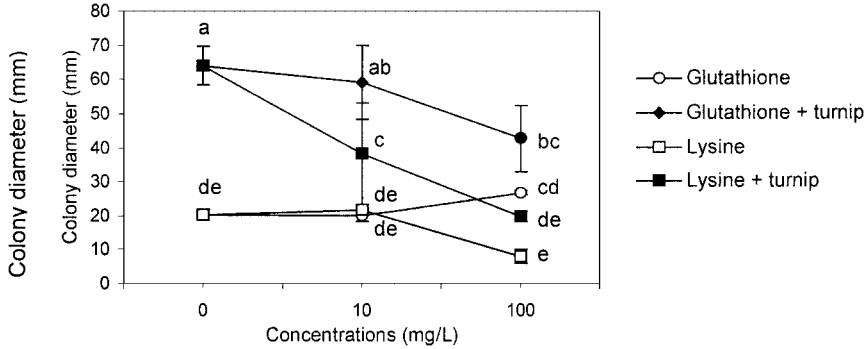

FIG. 8. Effects of two concentrations of glutathione and lysine on colony radial growth of *P. involutus* 211804 in 1/3 MMN medium with and without turnip germinants. Values are means ± standard error ($N = 3$). Significant differences ($P < 0.01$) among treatments are indicated by different letters.

inhibited fungal growth at a concentration of 100 mg/l. On the other hand, turnip in the presence of 100 mg/l lysine had no effects on colony diameter of *P. involutus* compared to control.

Glucosinolate Analysis. The HPLC profiles of desulphoglucosinolates in roots and leaves of turnip are presented in Figure 9a and b. The GLSs were identified by comparing retention times with the internal standard as well as with literature

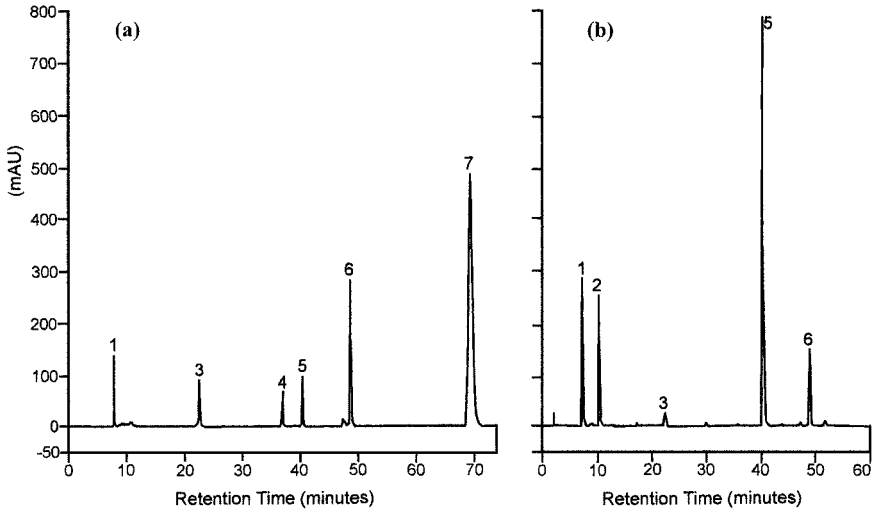

FIG. 9. HPLC profiles of desulphoglucosinolates in roots (a) and leaves (b) of turnip.

TABLE 5. RETENTION TIME OF DESULPHOGLUCOSINOLATES FROM HPLC AND CORRESPONDING GLUCOSINOLATES IN TURNIP ROOTS AND LEAVES

Compound no.	Retention time (min)	Name of corresponding glucosinolate (GLS)	Trivial name
1	7.3	2-Hydroxybut-3-enyl-GLS	Progoitrin
2	10.2	Allyl-GLS	Sinigrin (internal standard)
3	22.2	2-Hydroxypent-4-enyl-GLS	Napoleiferin
4	37.4	4-Methylthiobutyl-GLS	Glucoerucin
5	40.2	3-Indolylmethyl-GLS	Glucobrassicin
6	48.7	4-Methoxy-3-indolylmethyl-GLS	4-Methoxy glucobrassicin
7	71.8	1-Methoxy-3-indolylmethyl-GLS	Neoglucobrassicin

data (Sang et al., 1984). The most prominent GLSs in the roots were 1-methoxy-3-indolymethyl GLS and 4-methoxy-3-indolymethyl GLS (Table 5, Figure 9a). Turnip roots also contained 3-indolymethyl GLS, 2-hydroxybut-3-enyl-GLS, 2-hydroxypent-4-enyl-GLS, and 4-methylthiobutyl-GLS. The most prominent GLS in the leaves was 3-indolymethyl GLS. The leaves also contained large amounts of 4-methoxy-3-indolymethyl GLS and 2-hydroxybut-3-enyl-GLS (Table 5, Figure 9b).

DISCUSSION

All Brassicaceae plants tested stimulated the growth of *P. involutus*, while cucumber and oat did not. The colony diameter of the fungus *P. tinctorius* was also increased significantly in the presence of turnip and cabbage germinants compared to control. We conclude that Brassicaceae plants produce one or more chemicals that stimulate the growth of the ectomycorrhizal fungi. When *P. involutus* and turnip were cultured in the same dish but separated by a ditch in the agar the stimulation disappeared. Straatsma et al. (1986) reported that hyphal growth stimulation of ECM fungus *Cantharellus cibarius* in living roots of tomato was caused by increased CO_2 concentration. Our experiment conducted in Parafilm sealed petri dishes showed that CO_2 and other volatile compounds could not be responsible for the observed growth stimulation. When only the volatile compounds reached the medium, no stimulation was found. On the other hand, when turnip roots reached the medium of the fungus, significant growth stimulation occurred.

Autoclaving may cause a change in pH and chemical composition of the medium. Our experiment, however, showed that the stimulatory effect of the exudates was not affected by autoclaving. It showed instead that root exudates in the culture media stimulated the fungal growth with or without autoclaving. The growth stimulation of autoclaved root exudates of three turnip germinants was

nearly as strong as that of the three living germinants. Thus, autoclaving did not have any significant effect on the stimuli released from the cabbage roots. In fact, the stimulation of autoclaved root exudates was higher than that of nonautoclaved root exudates. One explanation for this additional growth stimulation may be that when the turnip roots were mechanically removed from the agar medium before autoclaving, the medium was broken, which may have caused additional growth enhancement. Autoclaved leaves and roots of turnip seedlings and adult plants did not stimulate fungal growth, but autoclaved root did stimulate colony radial growth of the fungus but did not affect hyphal density. This is probably because some roots were broken when they were harvested from the greenhouse plants, and some exudates were released during the process. We conclude from these results that the fungal growth stimulation was caused by the root exudates and that the growth stimulatory compounds are heat resistant.

Glucosinolates are typically present in Brassicaceae. Usually by themselves they are not active in plants but their hydrolytic products (ITCs) formed by the catalytic action of myrosinase are Glucosinolates are compartmented in the vacuole, while myrosinase appears to be associated with various membranes in the cytoplasm (Koide and Schreiner, 1992). Hydrolysis of glucosinolates in living plants is believed to occur primarily as a result of tissue damage, as a result of the mixing of glucosinolates with myrosinase, and thus releasing ITCs and other potentially toxic compounds, including nitriles and thiocyanates (Luthy and Matile, 1984). When fresh roots and leaves of cabbage were autoclaved, the myrosinase was likely inactivated by heating and the autoclaved medium did not stimulate fungal growth. When the roots and leaves were mechanically ground, they stimulated fungal growth. It is likely that during grinding the myrosinase and GLSs were mixed and a hydrolytic reaction resulted in the production of ITCs and associated metabolites.

Our results indicate that GLSs themselves did not stimulate fungal growth unless they were hydrolyzed. The results also showed that when myrosinase was added to the growth medium, the stimulatory effect of turnip on ECM fungi increased significantly. This indicates that turnip roots produce GLSs. Coming in contact with the added myrosinase in the medium, GLSs were changed into ITCs and ITC-related compounds causing growth stimulation of the fungi.

Roots of Brassicaceae plants are reported to produce ITCs (Tang and Takenaka, 1983; Schreiner, 1992). Isothiocyanates react with lysine and glutathione and convert ITCs to thiourea derivatives (Kawakishi and Kaneko, 1985). Since our experiment showed that the stimulatory effects of turnip on ECM fungal growth decreased significantly when lysine or glutathione were added in the culture medium, the amines added apparently reacted with ITCs released from turnip roots, and resulted in the reduction of the fungal growth. This experiment suggests that the root exudates of turnip contain ITCs that are responsible for the growth stimulation.

The most common GLSs are the aliphatic, aromatic, and indole GLSs (Bennett and Wallsgrove, 1994). Indole GLSs represent a significant proportion of the total GLS content in Brassicaceae. 3-Indolyl-methyl GLS is the predominant indole GLS in cabbage, swede, turnip, broccoli, Brussels sprouts, and cauliflower (Sang et al., 1984; Mithen et al., 1987). Vierheilig et al. (2000) showed that the aromatic and indole GLSs may be involved in preventing AM formation in some Brassicaceae, and aliphatic GLSs are not responsible for incompatibility between Brassicaceae plants and AM fungi. We found that mustard oil had no stimulatory effect on ECM fungal growth. Mustard oil contained mainly volatile aliphatic ITCs. Our experiment showing the absence of fungal growth stimulation with mustard oil implies that the growth stimulation was not caused by the aliphatic GLSs. The HPLC analysis showed rather that the main GLSs in turnip roots were indole GLSs. This is consistent with the results of Sang et al. (1984). No aromatic GLSs were detected. The ITCs formed from the hydrolysis of indole GLS are unstable, and decompose spontaneously to indole-3-carbinol, indole-acetonitrile, thiocyanate ions, and 3,3-diindolymethane (Chevolleau et al., 1997). The compounds degraded from indole GLSs are not volatile. This leads us to believe that ITCs and other degraded compounds from indole GLSs in turnip are responsible for the growth stimulation of ECM fungi.

Our study shows that Brassicaceae plants have obvious M-factor effects on ECM fungi, and the ECM fungi not only use the root exudates from the host plants (in this case jack pine and black spruce) as a nutrient source and as signal compounds, but also the root exudates from other nonhost plants. In this way, the ECM fungi may obtain additional nutrients from the forest ecosystem. However, not all root exudates are used as stimuli for ECM fungal growth. Although ITCs have antagonistic activity against many soil-borne fungal and bacterial plant pathogens (Rosa and Rodrigues, 1999), they can also be used as M-factors by some ECM fungi. In an agroforestry system, root exudates of certain nonmycorrhizal crops underneath trees may become an important stimulus for M-factors for the ECM fungi associated with tree species. An appropriate mix of tree species and understory crops with stimulus for M-factors may help ECM formation and mycelial development in the tree species resulting an increase in growth and yield.

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DIFFERENTIAL ACTIVITY AND DEGRADATION OF PLANT VOLATILE ELICITORS IN REGURGITANT OF TOBACCO HORNWORM (*Manduca sexta*) LARVAE

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Abstract—Plants respond to insect herbivory by emitting volatile compounds that attract natural enemies of the herbivores. Biosynthesis of many of these volatiles in plants is induced by herbivore-produced compounds. Components of tobacco hornworm (THW) regurgitant were investigated for their efficacy as elicitors of corn seedling volatiles. Two components that elicited the strongest release of volatiles were isolated and identified as *N*-linolenoyl-L-glutamine (18:3-GLN) and *N*-linolenoyl-L-glutamic acid (18:3-GLU). The approximately 10 times more active 18:3-GLN, which also is found in the regurgitant of several other Lepidopteran larvae, was rapidly degraded when THW regurgitant was left at room temperature, while 18:3-GLU degraded at a much slower rate. Different dietary sources of THW and tobacco bud worm larvae, including both host and nonhost plants, did not affect the amino acid composition of the fatty acid–amino acid conjugates in the regurgitant.

Key Words—Plant volatiles, elicitors, insect herbivory, regurgitant, *Manduca sexta*, tobacco hornworm, *Nicotiana tobaccum*.

INTRODUCTION

Injury by insect herbivores induces plants to release a blend of volatile terpenoids and indole that attracts natural enemies of the herbivores (Turlings et al., 1990, 1991). This intriguing defensive reaction is triggered when substances produced by insect herbivores contact damaged plant tissue. Application of a compound, named

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volicitin (*N* [17-hydroxylinolenoyl]-L-glutamine), isolated from beet armyworm (BAW), *Spodoptera exigua*, oral secretion, to damaged leaves of corn seedlings induced the same release of volatiles as feeding by the larvae (Alborn et al., 1997). Further analyses of BAW regurgitant showed the presence of several related fatty acid–glutamine conjugates (Pare et al., 1998; Alborn et al., 2000), among which *N*-linolenoyl-L-glutamine is the only conjugate that has been shown to have activity comparable to that of volicitin (Alborn et al., 2000). These fatty acid–amino acid (FAA) conjugates are also present in regurgitant of other lepidopteran larvae (Pohnert et al., 1999; Halitschke et al., 2001; Mori et al., 2001), and regurgitant from some Lepidopteran larvae has been shown to contain the corresponding glutamic acid conjugates (Pohnert et al., 1999). Halitschke et al. (2001) showed that tobacco hornworm (THW), *Manduca sexta*, regurgitant contains fatty acid–glutamine conjugates as well as the corresponding glutamic acid conjugates, while volicitin was not detected. They also showed that THW regurgitant induced herbivore specific release of volatiles in *Nicotiana attenuata* and, by eliminating the FAA conjugates, that collectively these conjugates were necessary and sufficient for this induction.

We chose THW as a candidate for a study of the biosynthesis of these conjugates and of their function in the insect. A preliminary investigation indicated that regurgitant obtained from wild THW collected near Gainesville, FL contained the glutamic acid FAA conjugates but only trace amounts of the glutamine FAA conjugates. Therefore, we investigated whether the proportions of glutamine/glutamic acid conjugates were population dependent, or if they were related to the diet (host plant). Furthermore, Mori et al. (2001) showed that digestive enzymes originating in the midgut lumen of tobacco budworm (TBW), *Heliothis virescens*, and corn ear worm, *Helicoverpa zea*, could alter the proportions of the various FAA conjugates found in the insects regurgitant. This made it necessary to investigate the presence of digestive enzymes in the regurgitant and their time-dependent effect on the FAA conjugate composition. A further objective of this study was to determine which of the FAA conjugates in THW regurgitant are active in inducing corn seedlings to release volatiles, and to compare the relative activity of the FAA conjugates.

METHODS AND MATERIAL

Plants. All plants were grown in 1 gal containers (Miracle Grow #92695 potting soil, fertilized with 1 tsp. of Osmocote 14-14-14, Scotts-Sierra Horticulture Marysville, OH[3]).

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Sweet corn, *Zea mays L.*, var. Delprim, was planted 10 seeds per pot, and plants were maintained on a 12 hr L/D cycle. Light intensity was 15,900 lx at the top of the plant canopy, with day/night temperatures at 25/20°C, respectively. Relative (RH) humidity ranged from 60 to 70%.

All other plants, tobacco (*Nicotiana tobaccum*, strain K326), eggplant (*Solanum melongena, var esculentum*), bell pepper (*Capsicum annum* crispy hybrid), tomato (*Lycopersicon esculentum* cv Castlemart), devil's claw (*Proboscidea parviflora var.* hohokamiana), and Knuckle Purple Hull cowpea (*Vigna unguiculata*) were planted one seed per pot and grown in an air conditioned greenhouse. Temperature was 29 ± 5°C, RH ranged from 95 ± 5% in the morning hours to 40 ± 5% in the late afternoon.

Larvae. Wild tobacco hornworm larvae of all ages and instars were collected from tobacco fields in northwest Alachua County, Florida, and regurgitant collected the same day. THW larvae were also reared from eggs (North Carolina State University – Department of Entomology, Raleigh, NC) on THW artificial diet (Southland Products Incorporated, Lake Village, AR), at 28°C, 74% RH with a 14:10 L/D cycle until late third instar and then allowed to feed on the different host plants 2 d prior to sampling. BAW and tobacco budworm eggs were received from USDA (Tifton, GA) and transferred to pinto bean–based artificial diet (Burton, 1970) under the same rearing conditions as THW larvae. Third instars were placed on host plants and allowed to feed for 48 hr before regurgitant was collected.

Collection and Treatment of Regurgitant. THW regurgitant was obtained as described in Turlings et al. (1993). However, because of the discovery that the composition of larval regurgitant changes over time at room temperature (Mori et al., 2001), the regurgitant was collected directly into a container packed in ice, or dry ice/acetone, and then stored at −70°C. For isolation of active components, collections from a large number of larvae were pooled. Before use, the vials with regurgitant were directly transferred from the freezer to an oven and immediately heated at 95°C for 30 min to inactivate hydrolytic enzymes, and then centrifuged at 11,000 g for 10 min (Eppendorf Centrifuge 5415) to remove the solids. For HPLC analyses, 20 μl of the supernatant were combined with 30 μl 4:1 acetonitrile/water and 5 μl of a 1 μg/μl solution of the internal standard *N*-arachidonoyl-L-glutamine. The samples were centrifuged again at 11,000 g for 10 min to remove precipitate, and the supernatant transferred to micro vials for HPLC analyses.

To test the effect of starvation on regurgitant composition, lab strain THW larvae were reared on tobacco plants for 4 d. Half of the larvae were removed, placed in a plastic container, and starved for 5 hr, while the other larvae continued to feed. Fresh oral secretion was collected on ice from larvae of both groups, heated, centrifuged, and analyzed by HPLC as described below.

HPLC Analyses. Regurgitants were analyzed, and fractions were collected by injecting 5–25 μl of sample onto a C18 reverse phase column (ODS-AMQ, S-5um, 200A, 250 × 4.6 mm id, YMC Co. Kyoto, Japan). Column temperature was

maintained at 60°C with a flow of 1.0 ml/min of a gradient programmed from 20/80 to 95/5, 0.08% acetic acid in acetonitrile/0.05% acetic acid in water, over 40 min, and then returned to the initial solvent ratio at 45 min (acetic acid, Aldrich, Milwaukee, WI, acetonitrile UV, B&J Brand , High Purity Solvent, Muskegon, MI, and water, Milli-Q UV Plus System, Millipore, Bedford, MA). UV absorption was monitored at 200 nm, and the results were analyzed with Chromquest software (Thermo Separation Products, Riviera Beach, FL). The HPLC system consisted of an AS3500 autosampler, a Constametric 4100 pump, and a Spectromonitor 3200 variable wavelength detector (Thermo Separation Products, Riviera Beach, FL) .

Degradation of Fresh Regurgitant. Fresh regurgitant from laboratory reared THW fed on tobacco was collected on ice from individual larvae and immediately placed in a −70°C freezer. Individual collections were combined to 300 μl total volume. Five 50 μl aliquots were pipetted from each combined sample and treated as follows: immediately heated at 95°C for 30 min (time 0 hr) or left at room temperature for 2, 4, 6, and 8 hr, respectively, and then heat treated. All samples were centrifuged and analyzed by HPLC as described above.

Different Host and Nonhost Plants. To test if the amino acid composition of the FAA conjugates was diet dependent, regurgitant was collected from laboratory strain THW that had been fed tobacco, green pepper, Devil's Claw, tomato, eggplant, and the nonhost and nonsolanaceous cowpea for 48 hr before regurgitant was collected and analyzed by HPLC as described above. To verify that the synthesis of glutamic acid FAA was not specific for insects feeding on solanaceous plants, regurgitant was also collected and analyzed from tobacco budworm (TBW) that had been allowed to feed on tobacco for 48 hr. The influence of diet on the quantitative composition of the FAA conjugates was investigated in a separate experiment by feeding laboratory strain THW both tobacco and cowpea and then collecting and analyzing the regurgitant.

Bioassay Procedure and Statistical Analyses. Approximately 2 hr into the scotophase 11- to 12-d-old corn seedlings were cut off near the base of the stem with a razor blade, and the cut end was immediately immersed in 1200 μl of 50 mM Na_2HPO_4 buffer pH 8 (control) or a test solution diluted in 1200 μl of the same buffer, contained in a 1.5 ml vial. For bioassay concentrations, see below. Plants were incubated in the solutions for approximately 16–17 hr, at the same day/night schedule they were grown in, but with reduced day time light (2000 lx). Plants would routinely take up more than 80% of the test solution. At about 6 hr into the next photophase, the stem of each plant was recut, wrapped in wet cotton, and placed in a glass volatile collection chamber (30 cm long × 4 cm id) under 15,900 lx. A push/pull volatile collection system similar to that described by Turlings et al. (1991) was used. Humidified and purified air (570 ml/min) was passed over the plants through the glass chamber and drawn by vacuum through a trap containing 25 mg of a polymeric adsorbent, Super Q 80/100 (Catalog #2735

Alltech Associates, Deerfield, IL) positioned at the downwind end of the chamber. Volatiles were collected for 2 hr, and the trap was removed and extracted with 170 μl methylene chloride. Five μl of an 80 ng/μl solution of *n*-nonyl-acetate were added as an internal standard, and the samples were injected on a gas chromatograph (HP 6890, Hewlett Packard Palo Alto, CA) by splitless injection at 220°C. The methyl silicone column, (HP1, 15 m × 0.25 mm (id) × 0.1 μm film thickness, Hewlett-Packard) was kept at 40°C for 0.5 min, and then temperature was programmed 12°C/min to 180°C. The He carrier gas flow rate was 40 cm/sec (constant flow), and the detector temperature was 250°C. The response by the plant to each test sample was measured as the combined amount of four induced terpenoids: caryophyllene, bergamotene, alpha-humulene, and beta-farnesene. Although several more volatile components are released by this corn cultivar in response to insect herbivory (Gouinguene et al., 2001), these four components show the strongest induced response. All bioassays also included positive controls (1000 pmol volicitin) and a negative control (buffer only). All samples were replicated 3 times within each bioassay and, unless differently stated, each bioassay was repeated 3 times (giving a total of $N = 9$ per treatment). To reduce the variation in response between bioassay times, the (combined induced terpenoid) response for each test sample was normalized for each test date as the percent of the mean response for the volicitin treatments. Data were analyzed by using a Tukey pairwise multiple comparison test.

Dose–Response Tests. Small samples of THW regurgitant (5–15 μl) were used in preliminary tests for the bioassay procedures. However, once *N*-linolenoyl-L-glutamic acid (18:3-GLU) was identified as the predominant amino acid–fatty acid conjugate, the amount of this component in each regurgitant collection was HPLC analyzed and used to normalize the bioassay doses. To decide an optimal test concentration, crude regurgitant was bioassayed at concentrations containing 100, 300, 1000, and 3000 pmol of 18:3-GLU, which corresponded to 0.4, 1.1, 3.7, and 11 μl of the batch of combined regurgitant that was used for the isolation and purification process . In each case, the total volume was adjusted to 1200 μl with 50 mM phosphate buffer (pH 8.0). Synthetic versions of the most active conjugates were also bioassayed at doses ranging from 10 to 3000 pmol/plant.

Analysis and Identification of Regurgitant Components. Regurgitant collected from THW larvae as described above was fractionated by HPLC, and individual fractions were derivatized by procedures modified after Mee et al. (1977). Samples were placed in 2 ml vials, concentrated to dryness by vacuum, 50 μl of methanol and 10 μl of acetic acid anhydride were added, the vials purged with nitrogen, capped, and heated at 100°C overnight. Samples were cooled, concentrated with nitrogen to dryness, dissolved in 50 μl of methylene chloride, and analyzed by GC/MS (HP 6890/5973 in both EI and CI mode). Samples were introduced by splitless injection at 220°C, and the methyl silicone column (HP1, 30 m × 0.25 mm (id) × 0.1 μm film thickness, Hewlett-Packard) was kept at 40°C for 0.5 min and

then temperature programed 10°C/min to 240°C and held at the final temperature for 15 min. The He carrier gas flow rate was 30 mm/sec (constant flow), and the ion source temperature was 220°C in EI mode and 250°C in CI mode with isobutane as the reaction gas.

A VG Analytical Zab-spec (Fison Instruments, Manchester, England) high resolution mass spectrometer was used for Fast Atom Bombardment (FAB) analysis of the natural products (Alborn et al., 1997). Up to 4 μl of 10 ng/ml solutions in 50% acetonitrile/water were added to a 10-μl glycerol matrix. Samples were protonated by addition of 1 μl trifluoroacetic acid. Sodium adducts were obtained by adding 1 μl of 1 M aqueous sodium chloride.

The stereochemistry of 18:3-GLN and 18:3-GLU was determined by using a chirobiotic T column (250 $\times$ 4.6 mm id, Advanced Separation Technologies, Whippany, NJ) as described in Alborn et al., 1997 and Mori et al., 2001. For the glutamine conjugate, the column was eluted with 10% CH_3CN in 10 mM $AcONH_4$-AcOH buffer, pH 4.5, at a flow rate of 1 ml/min. For the glutamic acid conjugate, the percent CH_3CN was increased to 15%.

Regurgitant Fractionation for Bioassay. HPLC fractionated THW regurgitant samples were concentrated to near dryness on a Speed Vac and redissolved in 50 mM phosphate buffer, pH 8.0. Initially, the regurgitant was separated into three major fractions. Individual peaks were then collected in the area of the HPLC chromatogram where biological activity was observed. The area of activity was further resolved by bioassaying combined peaks and eventually individual peaks were assayed.

Synthesis. The procedures developed for the coupling of fatty acids and glutamine described in Alborn et al., 2000 were modified for the coupling with glutamic acid. Five hundred μg of L-glutamic acid di-*t*-butyl ester were dissolved in 3 ml of dimethyl-formamide (DMF), and then 200 μl of water and 250 μl of triethylamine were added. In a second flask, 400 mg of the linolenic or linoleic acid were dissolved in 3 ml DMF, with the successive addition of 500 mg of 1-hydroxybenzotriazole and 300 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. After stirring for 2 min, the contents of both bottles were combined, and the mixture was stirred overnight at ambient temperature under N_2. The following day, 5 ml of CH_2Cl_2 were added, and the mixture was extracted repeatedly with 100 mM aqueous ammonia, 100 mM acetic acid, and water before the CH_2Cl_2 fraction was concentrated under vacuum to a thick yellow liquid. The *t*-butyl groups were removed by dissolving and stirring the product overnight in 2 ml of 88% formic acid. Five μl of CH_2Cl_2 were then added, and the solution was extracted repeatedly with water. The organic phase was concentrated to dryness under vacuum to give 600 mg of the product ($>$95% pure). Synthetic material to be tested in bioassays was further purified by HPLC to $>$99% purity using the same conditions as for the isolation of the naturally occurring FAA conjugates.

RESULTS

Isolation and Identification. HPLC comparisons between BAW and THW regurgitants indicated that THW regurgitant contained 18:3-GLN and trace amounts of 18:2-GLN, as well as several other components eluting in the proximity of these components (Figure 1). Eleven of the components eluting in this area were collected from the HPLC and derivatized with methanol/acetic anhydride. GC/MS analyses of derivatized peak 4 yielded three dominating GC peaks, identified as pyroglutamate methyl ester, the acetylated dimethyl ester of glutamic acid, and the methyl ester of linolenic acid. These data, in agreement with results published in Mori et al. (2001), and the fact that this component had the same HPLC retention volume as synthetic and natural 18:3-GLN collected from BAW regurgitant, confirmed that peak 4 in Figure 1 was identical to 18:3-GLN. The major component in the HPLC trace, peak 7, gave the same three characteristic GC/MS peaks, which suggested that this component was *N*-linolenoyl-glutamic acid (18:3-GLU).

GC/MS analysis of HPLC peaks 10 and 11 gave the same glutamine/glutamic acid derived GC peaks as above and the methyl ester of linoleic acid, which

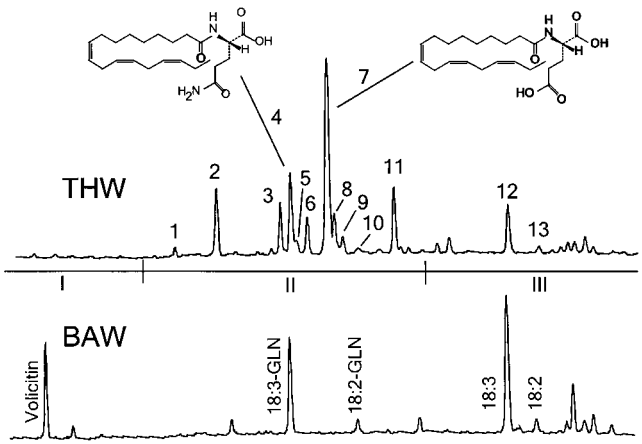

FIG. 1. HPLC comparison of 2 μl regurgitant equivalents collected from THW-fed tobacco and BAW-fed corn. The three THW fractions that were assayed for ability to elicit the release of volatiles in corn seedlings are marked **I** (1–24 min), **II** (25–36 min), and **III** (37–45 min). The peak numbers 1–11 correspond to components that were bioassayed individually. The two most active components in THW regurgitant are peak 4: *N*-linolenoyl-L-glutamine (18:3-GLN), and 7: *N*-linolenoyl-L-glutamic acid (18:3-GLU). Peaks 1 and 2 were tentatively identified as *N*-hexadecatrienoyl-glutamine and, *N*-hexadecatrienoyl-glutamic acid, respectively. Peak 10 is *N*-linoleoyl-glutamine (18:2-GLN), and peak 11 is *N*-linoleoyl-glutamic acid (18:2-GLU). Peak 3, 5, 6, 8, and 9 are unidentified plant components. Peaks 12 and 13 are linolenic acid (18:3) and linoleic acid (18:2).

indicated that peak 10 was 18:2-GLN and that peak 11 was 18:2-GLU. Peak 10 also coeluted with natural and synthetic 18:2-GLN. Interestingly, 1 peaks and 2 also gave the characteristic glutamine/glutamic acid peaks and the methyl ester of a hexadecatrienoic acid, which indicated that these peaks were *N*-hexadecatrienoyl-glutamine (16:3-GLN) and *N*-hexadecatrienoyl-glutamic acid (16:3-GLU), respectively. These small peaks that showed little bioassay activity were not investigated further. However, a comparison with the NIST library tentatively identified the fatty acid as 4,7,10-hexadecatrienoic acid. GC/MS analyses of the remaining peaks gave no indications of amino acid – fatty acid conjugates. Furthermore, these peaks were also present in the HPLC chromatogram of a crude tobacco extract, which indicated that they were plant components (unpublished results).

The identities of peaks 4, 7, 10, and 11 were confirmed by FAB/MS analyses, which gave an M + 1 ion at m/z 407 for peak 4, m/z 408 for peak 7, m/z 409 for peak 10, and at m/z 410 for peak 11. All components also gave characteristic sodium adducts, confirming the molecular weights as 406 D for peak 4, 407 D for peak 7, 408 D for peak 10, and 409 D for peak 11, which strengthened the evidence that peaks 7 and 11 were glutamic acid conjugates of linolenic and linoleic acid. The L-glutamic acid conjugates of linolenic and linoleic acid were synthesized using procedures developed for glutamine–fatty acid conjugates (Alborn et al., 2000). The approximately 95% pure synthetic materials, which had the same retention times as the natural components, were further purified by HPLC with the same methods that were used for the purification of the crude regurgitant. The synthetic conjugates were subjected to the same GC/MS and FAB-MS analyses as above, which gave identical results for the synthetic and natural conjugates.

Stereochemistry of 18:3-GLN and 18:3-GLU. Analyses of HPLC collected 18:3-GLN on the chiral column under the conditions given in Mori et al. (2001) gave a retention time of 6.30 min, which was identical to synthetic *N*-linolenoyl-L-glutamine. It was necessary to increase the acetonitrile content in the mobile phase from 10 to 15% for a chiral analysis of 18:3-GLU. Under these conditions, 18:3-GLU from THW regurgitant as well as synthetic *N*-linolenoyl-L-glutamic acid had a retention time of 7.50 min, while synthetic *N*-linolenoyl-D-glutamic acid had a retention time of 9.70 min. This confirmed that both FAA-conjugates contained the L-form of the amino acids.

Bioassay Results. Preliminary dose–response bioassays of crude regurgitant showed that maximum activity was reached when the test samples contained approximately 1000 pmol of the main component 18:3-GLU, which corresponded to a dose of 3.7 μl of the pooled THW regurgitant that was used in the bioassays. This dose was, therefore, chosen for all the following bioassays. However, the maximum activity for the crude THW regurgitant was only 67% of the 1000 pmol volicitin control (7.7 ± 0.5 μg for THW vs. 11.4 ± 1.5 μg of combined induced terpenoids for the volicitin control).

Bioassay results of crude regurgitant, separated by HPLC into three fractions (Figure 1), indicated all biological activity to be located in one fraction eluting between 25 and 36 min. This fraction, which contained the earlier identified FAA conjugates, was as active as a combination of all the fractions (1.74 ± 0.3 μg for fraction 25–36 vs. 1.78 ± 0.7 μg of combined induced terpenoids for the recombined fraction 1–45, no significant difference, $P > 0.95$) . However, in this bioassay, the volicitin treated control seedlings released almost 3 times more induced volatiles than the most active THW fraction. The two other fractions, 1–24 min (252 ± 75 ng of induced volatiles) and 37–45 min (172 ± 19 ng of induced volatiles), showed no increased activity compared to the buffer control (165 ± 49 ng of induced volatiles, $P = 0.39$ and $P = 0.92$, respectively). Interestingly, the latter fraction contained the free linolenic and linoleic acid. The active fraction was further separated into 11 individual peaks that were bioassayed in combination and individually (Figure 2). The combined fraction $1 + 2$ (containing 16:3-GLN+16:3-GLU) as well as the combined fraction 9–11 (containing 18:2-GLN and 18:2-GLU) showed some activity, which in both cases was significantly different from the buffer control (Figure 2B). However, most of the activity was located in the combined fractions 3–6 and 7–8 (Figure 2B). Fraction 7–8 showed lower mean activity than fraction 3–6, but the difference was not significant due to large variation in the response.

The most active fractions (3–8) were further bioassayed as individual peaks (Figure 2C). Fraction 4, which contained approximately 114 pmol of 18:3-GLN, and 7, which contained 1000 pmol of 18:3-GLU, were equally active. This indicated that 18:3-GLN and 18:3-GLU are both major contributors to the activity of the THW regurgitant.

Synthetic forms of 18:3-GLN, 18:3-GLU, and 18:2-GLU were bioassayed over a range of concentrations. The 18:2-GLU was included in this test because it was the second or third most abundant of the identified glutamine and glutamic conjugates in all THW regurgitant collections, while 18:2-GLN was excluded due to its consistently low abundance. We also established that this component had low activity compared to 18:3-GLN (data not shown). 18:3-GLN was the most active of the tested components (Figure 3). It was significantly more active than the control and 18:3-GLU at 30 pmol, and significantly more active than 18:2-GLU at 10 pmol. 18:3-GLU was significantly more active than the control and 18:2-GLU at 300 pmol. Finally, 18:2-GLU only showed some activity at the highest doses tested (significantly more active than the control at 1000 pmol).

Effect of Diet on FAA Conjugate Composition. Analyses by HPLC of regurgitant from THW fed the normal host plants green pepper, devil's claw, tomato, eggplant, and cowpea showed that all collections contained the same characteristic glutamine and glutamic acid FAA conjugates (Table 1). A large variation in the data combined with a low number of replicates ($N = 3$) made a quantitative comparison impossible. To verify that the synthesis of glutamic acid FAA

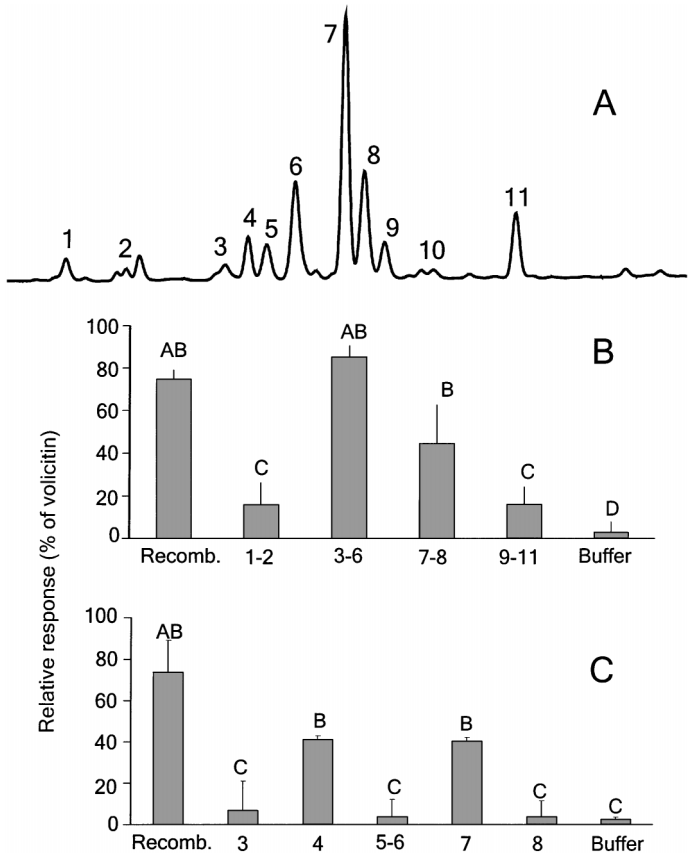

FIG. 2. HPLC fractionation and bioassay of THW regurgitant. (A) The active area, peaks 1–11 in Figure 1. (B) Results of bioassay of different combinations of fractions. (C) Results of bioassay of individual peaks from fractions 3–6 and 7–8. The concentration of each fraction in all bioassays was 3.7 μl equivalents of THW regurgitant. The amount of 18:3-GLU in 3.7 μl of THW regurgitant is about 1000 pmol. The response for each fraction is given relative to the release of induced volatiles by corn seedlings treated with 1000 pmol of volicitin. Different letters indicate significant differences ($P < 0.01$). The letter A indicates no significant difference from the response to the volicitin controls.

was not specific for insects feeding on solanaceous plants, regurgitant was also collected and analyzed from larvae of TBW fed on tobacco. The analyses showed no detectable amounts of any glutamic acid conjugates (Table 1), but the same glutamine conjugates (including volicitin) as earlier reported from TBW feeding on corn (Mori et al., 2001). In a separate experiment, regurgitant from cowpea fed THW ($N = 17$) was compared quantitatively with regurgitant from tobacco fed

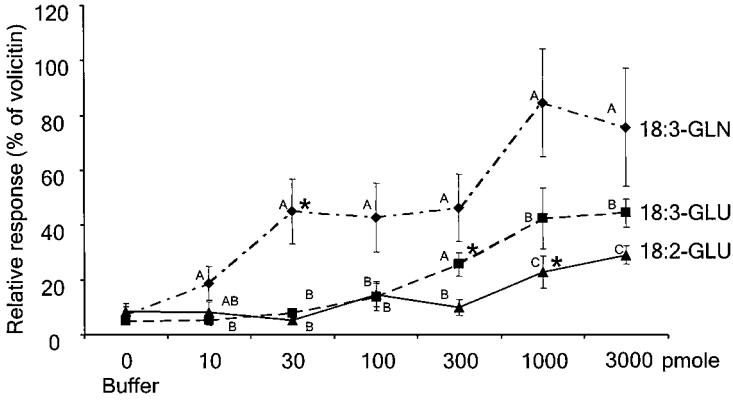

FIG. 3. Dose response bioassay of synthetic 18:3-GLN, 18:3-GLU, and 18:2-GLU. The relative response is calculated as in Figure 2. Different letters indicate significant difference in the activity at that specific dose ($P < 0.05$). *indicates the lowest dose for each component that gave a significantly stronger response than the buffer control.

THW ($N = 15$). The cowpea regurgitants contained lower ($P < 0.05$) amounts of 18:3-GLN than the regurgitant of tobacco-fed THW (for cowpea-fed 86 ± 12 ng 18:3-GLN/μl and 436 ± 40 ng 18:3-GLU/μl, vs. tobacco-fed 126 ± 15 ng 18:3-GLN/μl and 410 ± 15 ng 18:3-GLU/μl).

Degradation of Regurgitant Components. HPLC analysis of regurgitant collected from wild insects at room temperature and without heat treatment showed the amount of 18:3-GLN to be less than 2% of the amount of 18:3-GLU (2.2 ± 1.3 ng/μl vs. 169 ± 38 ng/μl). In contrast, when the regurgitant was collected on ice, frozen immediately, and heat treated before analysis, the 18:3-GLN content

TABLE 1. PRESENCE OF GLUTAMINE AND GLUTAMIC ACID-BASED FAA CONJUGATES AND VOLICITIN IN REGURGITANT OF THW AND TBW FED DIFFERENT HOST AND NONHOST LLANTS

Insect	Plant	Volicitin	Glutamine-FAA	Glutamic acid-FAA
THW	Tobacco	n/d	+	+
	Tomato	n/d	+	+
	Bell pepper	n/d	+	+
	Eggplant	n/d	+	+
	Devil's claw	n/d	+	+
	Cowpea	n/d	+	+
TBW	Corn	+	+	n/d
	Cowpea	+	+	n/d

Note: n/d = No detectable amounts by HPLC analyses; + = Detectable amounts present.

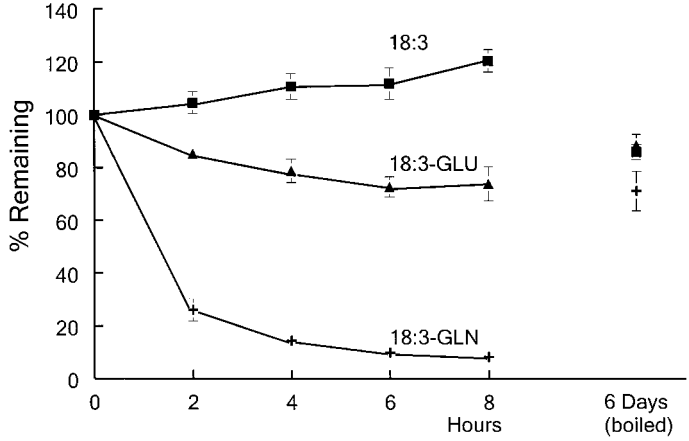

FIG. 4. The amounts of 18:3-GLN, 18:3-GLU, and linoleic acid (18:3) in crude regurgitant left at room temperature for 2, 4, 6, and 8 hr, presented as % of the amounts at the start of the experiment, and the amounts in heat-treated regurgitant 6 d after start of the experiment.

was 11% (37 ± 8 ng/μl vs. 322 ± 15 ng/μl for 18:3-GLU). Furthermore, regurgitant collected from actively feeding THW contained higher concentrations of all known components than regurgitant from starved insects. There was an 85% reduction in 18:3-GLN (from 139 ± 25 ng/μl to 18 ± 11 ng/μl) and a 61% reduction in 18:3-GLU (from 394 ± 16 ng/μl to 158 ± 46 ng/μl) in the oral secretion from starved insects compared to regurgitant from actively feeding insects.

Degradation experiments (Figure 4) using fresh THW oral secretion that was processed immediately vs. secretion that was allowed to sit at room temperature for 2, 4, 6, 8, and 24 hr showed that within the first 2 hr about 72% of 18:3-GLN had disappeared in nonheat treated regurgitant (from 113 ± 24 ng/μl to 31 ± 9 ng/μl) However, after 8 hr, only 26% of 18:3-GLU had been degraded (from 407 ± 41 ng/μl to 299 ± 37 ng/μl). During the same time period, the amount of free linolenic acid increased from 296 ± 18 ng/μl to 356 ± 28 ng/μl. Heat treated regurgitant did not show any significant degradation even after 6 d.

DISCUSSION

Halitschke et al. (2001) showed that a combination of FAA conjugates found in THW regurgitant was necessary and sufficient to induce herbivore-specific release of volatiles in *Nicotiana attenuata*. However, to date the activities of the individual FAA conjugates found in the THW regurgitant have not been tested. We found that regurgitant collected from THW larvae induces corn seedlings to

release the same pattern of volatiles as corn seedlings treated with BAW regurgitant (Turlings et al., 1993). By HPLC and bioassay driven fractionation of THW regurgitant, we isolated one area with activity equal to a combination of all HPLC fractions. Further separation of this fraction showed that several components had activity. However, two fractions (containing 18:3-GLN and 18:3-GLU, respectively) were significantly more active than any of the others. These two HPLC fractions had nearly equal activity despite the fact that the amount of 18:3-GLN was only approximately 10% of 18:3-GLU, which was the most abundant of all conjugates in the THW regurgitant. Bioassays of synthetic FAA conjugates confirmed that 18:3-GLN was significantly more active than 18:3-GLU. The second most abundant conjugate in the regurgitant was 18:2-GLU, which was identified as the main component in one of the less active HPLC fractions. Dose–response bioassays of synthesized 18:2-GLU confirmed that this component also had some activity, but only at 1000 pmol or higher concentrations. This fraction also contained 18:2-GLN but only in trace amounts, and this component was not tested. A second HPLC fraction with some activity contained two peaks that were identified as the glutamine and glutamic acid conjugates of a hexadecatrienoic acid. These components were not reported by Halitschke et al. (2001) in THW regurgitant. However, they reported the presence of glutamine and glutamic acid conjugates with palmitic and oleic acid, which we did not detect. One possible reason for this discrepancy is that Halitschke et al. (2001) used LC/MS to directly separate and identify FAA conjugates in the THW regurgitant. In this study, we used HPLC with UV detection at 200 nm, where the detector response is directly correlated with the number of double bonds and consequently linolenic and hexadecatrienoic acid will give a stronger detector response than the corresponding less unsaturated fatty acids. The use of nondestructive UV detection in combination with fractionation and bioassays enabled us to focus on individual components that induced volatile production. Furthermore, we used THW feeding on a commercial tobacco cultivar, while Halitschke et al. (2001) used insects that were feeding on a wild relative (*Nicotiana attenuata*). It is possible that these plants have a different fatty acid composition. In most plants investigated, there seems to be a correlation between the free fatty acids and the corresponding amino acid conjugate composition (unpublished). In this study, we decided not to further investigate the hexadecatrienoic acid conjugates. However, the fact that these components showed activity could justify future investigation.

The amounts of the two major active components varied greatly among different collections of THW regurgitant. This variation could be due to differences in collection, sample handling, THW population differences, or time between the last feeding and regurgitant collection. Regurgitant from field collected THW, which had been collected at room temperature, contained large amounts of the glutamic acid conjugates but only trace amounts of the glutamine conjugates. However,

analyses of regurgitant from insects from the same field, collected into a container in an ice bath, and then immediately transferred to a −70°C freezer, showed less variation in the FAA conjugate amounts and also contained significantly more 18:3-GLN (the most active component) than the earlier collections. There also appeared to be a difference in the composition of regurgitant from field collected insects compared to lab reared THW. The regurgitant from lab reared insects contained 3.4 times more 18:3-GLN than the wild insects but approximately equal amounts of 18:3-GLU. This might have been due to a natural variation among populations. However, regurgitant from the wild insects was obtained after field collection and a car transport, during which the insects might not have been actively feeding. When we compared the regurgitant collected from insects that had been starved for 5 hr with regurgitant from actively feeding insects, the former showed a significantly reduced amount of all conjugates, particularly the glutamine conjugates. Recently, Mori et al. (2001) showed that for at least some Lepidopteran larvae the fatty acid–glutamine conjugates in the regurgitant are rapidly broken down due to the presence of enzymes originating in the midgut lumen. We found that 18:3-GLN was subject to a rapid degradation when THW regurgitant was left at room temperature. The degradation was biased towards glutamine conjugates because 18:3-GLU degraded at a much slower rate during the same time period. This confirmed that the amounts of FAA conjugates, and especially the glutamine conjugates, are affected by enzymatic degradation of the regurgitant, and highlights the problems with regurgitant collection for identification and quantification of FAA conjugates.

Thus far, only *Epirrita autumnata* in addition to THW has been shown to produce both glutamic acid–and glutamine–linolenic acid conjugates (Pohnert et al., 1999). All other lepidopteran larvae investigated (Paré et al., 1998;. Pohnert et al., 1999; Mori et al., 2001) produce only the glutamine conjugates. However, most of these other species produce volicitin (*N*-[17-hydroxylinolenoyl]-L-glutamine) and analogous hydroxylated conjugates.

The formation of glutamine and/or glutamic acid conjugates does not appear to be directly dependent on the supply of those amino acids in the diet. Instead, the amino acid composition of the FAA's appears to be regulated by the herbivore species. THW fed different solanaceous and nonsolanaceous plants produced the same glutamine and glutamic acid FAA conjugates, although both absolute amounts and the glutamine/glutamic acid ratio appeared to vary. For example, regurgitant collected from cowpea fed THW contained less of both 18:3-GLN and 18:3-GLU than tobacco fed. However, cowpea is not normally a host plant, and the insects were less willing to feed on these plants. The hypothesis that the amino acids of the FAA conjugates are species specific is further strengthened by the fact that tobacco fed TBW produced the same glutamine conjugates (including volicitin) as corn fed, and no detectable amounts of glutamic acid FAA conjugates. Furthermore, Pare et al. (1998) showed with ^{13}C labeling experiments that BAW

rapidly incorporated labeled plant fatty acids into FAA conjugates with unlabeled glutamine that originated in the insect.

The fact that THW does not appear to produce volicitin or any of the hydroxylated analogs, but produces both glutamine and glutamic acid conjugates raises many interesting questions. Are the THW coupling enzymes less specific than in other Lepidoptera, or are different enzymes responsible? Alternatively, are glutamine FAA conjugates converted to glutamic acid FAA conjugates in some insects, and do different plant species respond differently to the different amino acid conjugates? In this investigation, we showed that 18:3-GLU was less active than 18:3-GLN in inducing volatile release in corn seedlings, which suggests that THW might benefit from such a conversion. However, the biological activities of the individual or combined conjugates in THW regurgitant may be different on tobacco than on corn, a nonhost plant. It is not yet clear why insect herbivores produce compounds that trigger plant defensive reactions. The implications are that the elicitors are critically important to the insects in some way. Otherwise, they would have been discarded through evolution. This hypothesis is supported by the results of this study in that the composition of the active compounds may be determined more by the herbivore than by the food it eats. However, the number of insect species from which elicitors have been identified and the species of plants on which these elicitors have been tested is still too small to allow any general conclusions to be drawn. We intend to address these and related questions in future studies.

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HOST NUTRITIVE QUALITY AND HOST PLANT CHOICE IN TWO GRASS MINERS: PRIMARY ROLES FOR PRIMARY COMPOUNDS?

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Abstract—The relationship between host plant choice and plant nutritional quality was investigated in two oligophagous grass miners *Chromatomyia milii* and *C. nigra* (Diptera, Agromyzidae). We tested whether host choice is determined by chemically mediated host suitability for offspring performance and/or adult performance. A second goal was to relate the observed variation among the different fitness parameters to quantitative and qualitative variation in foliar food quality. Choice experiments illustrated that both miners discriminated among grass species, and that *C. milii* has a smaller host range than *C. nigra*, as observed under natural conditions. Oviposition preference was correlated with adult feeding preference and related adult performance (longevity and fecundity) for both miners. Offspring performance measures (survival and pupal size) of at least *C. nigra* were more weakly related to host preference. Nearly all variation in adult performance of both miners was explained by foliar protein content, which had a positive effect on adult longevity and fecundity. Pupal size of both miners was positively related to foliar water and amino acid content and negatively related to lignin content. No clear relationship between host chemistry and offspring survival was observed. These observations show that fitness parameters are differentially related to host chemistry. Secondly, they suggest that chemically mediated host suitability for adult performance is an important determinant of host choice in this species. Finally, the results suggest a primary role for foliar protein content in host choice of the study species in general and in shaping the host range of *C. milii* in particular.

Key Words—Host choice, adult and offspring performance, nutritional quality, primary compounds, miners, Poaceae.

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INTRODUCTION

Disentangling the selective forces that shape host preference patterns and host plant ranges of phytophagous insects is central to our understanding of plant–insect interactions (e.g., Thompson, 1988; Courtney and Kibota, 1989; Mayhew, 2001). The most common approach to examining this subject is investigation of the relations between plant chemistry, offspring performance, and host plant selection because chemically mediated host plant suitability for offspring development is considered to be the major selective force in the evolution of the diet of plant-feeding insects (Courtney and Kibota, 1989), especially in holometabolous insects. However, recent research has shown that selection pressures intervening on adult performance should also be considered when investigating host choice (Scheirs et al., 2000; Mayhew, 2001; Scheirs and De Bruyn, 2002). Although adult performance is affected by host quality in many phytophagous insects (Awmack and Leather, 2002), few studies have addressed the role of chemically mediated host plant suitability for adult performance in host choice (Awmack and Leather, 2002; Scheirs and De Bruyn, 2002).

Chemically mediated host plant suitability for the development of phytophagous insects is determined by the balance of nutrients, chemical defenses, and structural defenses (Bernays and Chapman, 1994). Nevertheless, plant–insect researchers have been concerned less with nutrients and structural defenses than with the role of chemical defenses as mediators of host recognition and resistance (Bernays, 1991; Feeny, 1992). Although it is clear that structural defenses may determine host choice (Bernays, 1991), nutrients or primary compounds are believed to play only a secondary role in maintaining the host range of phytophagous arthropods (Moran and Hamilton, 1980; Feeny, 1992; Berenbaum, 1995). Grasses (Poaceae) are a group of plants wherein secondary metabolites active as chemical defenses occur only sporadically in mature plants. When occurring, as in several crop species, they are of low diversity (Bernays and Barbehenn, 1987; Tscharntke and Greiler, 1995). Instead, grasses have a generally high structural defense and low nutrient content (Bernays and Barbehenn, 1987; Scheirs et al., 2001a,b). Grass–insect interactions are, therefore, a good model system to study the potential role of nutrients and structural defenses in host range evolution.

Closely related herbivorous insects often differ in host plant specificity, and a comparative approach in these species is frequently used to study the determinants of host plant preference patterns (e.g., Rausher, 1979; Roininen and Tahvanainen, 1989; Ohsaki and Sato, 1994). We used this approach in order to study the influence of plant chemistry on host choice of two closely related grass miners, i.e., *Chromatomyia milii* (Kaltenbach) and *C. nigra* (Meigen) (Diptera: Agromyzidae). Both miners are oligophagous, but the host range of *C. nigra* is considerably wider than that of *C. milii*. *C. nigra* is recorded from over 40 genera of C_3 grasses, while *C. milii* is recorded from only four C_3 grass genera (Griffiths, 1980; J. Scheirs,

personal observation). The aim of this study was to determine the nutritional basis for the difference in host use between the species. More precisely, we addressed the following three questions: (1) Are there differences in host range between the miners under controlled conditions? (2) Is there variation in adult and offspring performance among hosts and to what extent can this be related, both quantitatively and qualitatively, by host nutritional quality? (3) Can chemically mediated host plant suitability for adult and/or offspring performance explain the observed host preference patterns?

METHODS AND MATERIALS

Life History of C. milii and C. nigra. Females of both miners insert their eggs directly in the mesophyll layer between two veins of a grass leaf (Scheirs et al., 2001a). Larvae emerge within 1 week and immediately eat their way through the internal leaf tissues. They mainly consume mesophyll cells, but also parts of the sclerenchyma strands and bundle sheath cells when crossing the numerous adjoining veins (Scheirs et al., 2001b). Larvae complete four stadia (three larval and one prepupal) and then pupate within their mines. Adults emerge through the leaf surface. Females feed on leaf sap that exudes from feeding punctures made by their ovipositor in the leaf blades of host grasses (Scheirs et al., 2001a).

Grasses and Miners. Four grass species were used, *Agrostis tenuis* Sibth., *Dactylis glomerata* L., *Lolium perenne* L., and *Poa trivialis* L. Seeds were derived from the firm Barenburg-Maes (Marke, Belgium). Grasses (100 mg) were sown in $10 \times 10 \times 11$ cm pots containing a soil mixture consisting of 50% white sand and 50% vermiculite. Grasses were grown in a greenhouse under controlled conditions (16:8 hr L:D, $20 \pm 1^{\circ}$C, 70% RH in the light – 100% RH in the dark). The same greenhouse conditions were used in all experiments. Plants were watered weekly with 150 ml half-strength Hoagland solution per pot (Hoagland and Arnon, 1950). Grasses were used when they were 12-weeks old. This growth stage has at least four, fully expanded leaves, which corresponds to the size of plants used by *C. milii* and *C. nigra* in natural conditions.

C. milii and *C. nigra* females were collected from a field population living exclusively on *Holcus lanatus* L. at Hoboken, Belgium. *Holcus lanatus* was not included in the experiments to avoid biased female preference due to past experience and to mitigate maternal effects on offspring performance. All four grasses used occur within the geographical range of *C. milii* and *C. nigra*. We used only gravid females of unknown age. Flies and grasses were only used once to avoid pseudoreplication.

Host Preference. Host preference of *C. milii* and *C. nigra* was determined by two types of choice experiments. In four-way choice experiments, one pot of each of the four grass species was offered simultaneously to five females of

C. milii or *C. nigra* during one trial. All grasses in a trial had a comparable amount of biomass. Pots were randomly positioned in a rectangle in a flight cage (50 cm long × 75 cm wide × 75 cm high). A trial lasted 48 hr, after which the number of eggs and feeding punctures were recorded as a measure of adult oviposition and feeding preference, respectively. Feeding punctures and eggs were counted by examining each individual leaf under a microscope with light projecting through the leaf. We conducted six trials for each miner species.

In two-way choice experiments, we offered every combination of two grass species. A female was placed into a small plastic vial (10 cm diam. × 10 cm height) with two slits at opposing sides. Randomly selected green leaves of a particular combination of grasses were pushed through the slits without detaching from the leaf stem or damaging the leaf. By offering only leaves to the flies, we controlled for potential differences in plant architecture (i.e., size differences). A trial lasted 24 hr, after which preference was recorded as above. We conducted 10 trials for most combinations of grasses for each miner species. For the combinations *Lolium-Agrostis* and *Agrostis-Dactylis* with *C. milii*, only five trials could be performed. Females that died during the course of the experiment without having fed or oviposited were excluded from the analysis.

Adult and Offspring Performance. Adult performance was determined by no-choice trials in which a single grass species was offered to individual females. Females were placed into a small plastic vial (10 cm diam. × 10 cm height) with slits in its side through which grass leaves were offered. Leaves were replaced every 24 hr. No extra water or food was offered as in the choice experiments in order to study the direct effect of host quality on female choice or performance. As a measure of adult performance, we recorded adult longevity and fecundity (number of eggs laid) (see also Scheirs et al., 2000; Awmack and Leather, 2002).

Offspring performance was determined by raising the eggs that were laid in the four-way choice experiments. Grasses were checked daily for pupated and dead larvae. Pupated larvae were removed from leaves and put into individual rearing containers and checked daily for emerging adults. The positions of plants and the rearing containers were randomized daily within the greenhouse to avoid position effects. The length of the puparium was assessed to the nearest 0.05 mm. We determined three measures of offspring performance: offspring survival, development time, and pupal size. Only larvae that completed their development in the absence of competition were considered in the analysis, because larval competition decreases offspring performance of Agromyzidae (Parrella, 1983; Quiring and McNeil, 1984a,b; Petitt and Wietlisbach, 1992). We considered intraspecific competition to be present when the mine of a larva was crossed or used by one or more other mining larvae and/or the mine crossed a batch of feeding punctures.

Almost all eggs of *C. milii* were laid on *P. trivialis* in the four-way choice experiments. In order to be able to compare offspring performance among grass

species, we conducted no-choice experiments with *C. milii* for all grass species. This experiment yielded enough eggs for comparison of offspring performance between *P. trivialis* and *L. perenne* only.

Leaf Nutritional Quality. We assessed the nutritional quality of leaves of all four grasses by determining water content, the concentration of nutrients (proteins, amino acids, and soluble carbohydrates), and the lignin content as a measure of the amount of structural defenses. We used whole leaf samples instead of the mesophyll, as suggested for selective feeders (Kimmerer and Potter, 1987). Whole leaf samples are a good estimate of the nutritional quality of larval food because (a) the mesophyll layer of grasses consists of one large homogeneous layer (Vincent, 1982), and (b) the larvae feed on both the mesophyll and the veins (Scheirs et al., 2001b). Samples were taken from pots that were not used in the preference trials or the performance experiments when plants were 12-weeks old. Thirty leaves of each grass species were immediately weighed after collection to measure their fresh mass (FM), dried at 105°C, and reweighed after 48 hr to obtain their dry mass (DM). Water content is expressed as $([FM-DM]/FM) \times 100$. Afterwards, we determined the lignin content of these leaves using the acetyl bromide technique of Morrison (1972a) modified for small quantities (Morrison, 1972b; Lin and Dence, 1992).

For nutrient analyses, we collected 25 leaves of each grass species and immediately froze them at −80°C. Leaves were subsequently lyophilized, ground, and extracted on ice for 30 min with a Tris/EDTA buffer (pH = 7.6). Extracts were centrifuged at 5500 rpm and supernatants collected and stored at −80°C until further analysis. Protein levels were analyzed using the Bradford assay with bovine serum albumin as a standard (Sigma, B6916) (Jones et al., 1989). Soluble carbohydrate content was determined using the anthrone method (Yemm and Willis, 1954), utilizing glucose as a standard. Prior to amino acid analysis, proteins were precipitated using trichloroacetic acid (Bollag et al., 1996), and supernatants were collected after centrifugation at 5500 rpm. Amino acid content was measured using the ninhydrin method (Sigma, N1632) (Moore, 1968; Allen, 1989) with leucine as a standard.

Statistical Analyses. Choice experiments were analyzed with exact Quade tests (Quade, 1979; Conover, 1980) using StatXact (Mehta and Patel, 1995). This nonparametric test takes the lack of independence among the simultaneously offered food types into account (Roa, 1992). For the two-way choice experiments, we applied a Sequential Bonferroni correction to reduce the chance of making a Type I error (Rice, 1989).

Adult longevity was analyzed using the Kaplan–Meier method and the log-rank test in the PROC LIFETEST module of SAS 6.12 (Allison, 1995). Adult fecundity was analyzed with a mixed model regression analysis with log link function and Poisson errors. Grass species and miner species were treated as fixed effects.

Offspring survival was analyzed using mixed model logistic regression with a logit link function and binomial errors. We used choice trial as a random variable in the case of *C. nigra*, but not for *C. milii* because of a different experimental design. Grass species was added to the model as a fixed variable for *C. milii* and *C. nigra*. Development time and pupal size were analyzed with mixed model regression analysis with normally distributed errors. In the case of *C. nigra*, we used pot, nested within choice trial, as random variables. For *C. milii*, only pot was used as a random variable. Grass species and sex were considered as fixed variables, the interaction between grass species and sex was never significant and excluded from the model.

Nutritional quality of the different grasses was compared with analysis of variance (ANOVA) using the PROC GLM module in SAS (SAS Inst., 1990). Protein, amino acid, carbohydrate, and lignin content were square root transformed, while water was arcsine transformed in order to meet the assumptions of ANOVA.

The relation between preference and performance was studied by Pearson's correlation analyses, which were calculated using STATISTICA (Statsoft, 1994). We calculated the correlations between mean preference as observed in the four-way choice experiments and mean performance for both study species (see also Scheirs et al., 2000).

The effect of host nutritional quality on the performance of both miners was analyzed with stepwise multiple regression analysis using the PROC REG module in SAS (SAS Inst., 1990). In the case of adult performance, we analyzed the relationship between host nutritional quality and adult longevity only because adult longevity and adult fecundity were almost perfectly correlated for *C. milii* ($r = 0.995, t = 14.33, P = 0.005$) and *C. nigra* ($r = 0.994, t = 12.63, P = 0.006$). In the case of offspring performance, we only considered the performance measures that differed among grasses (pupal size and offspring survival). Mean performance measures were included in the dependent variable list. Miner species and mean content of all nutritional quality components were included in the independent variable list. For the analyses of the effect of host nutritional quality on pupal size, we also included sex as a class variable in the independent variable list.

All previous mixed model analyses were calculated using the PROC MIXED module in SAS (Littell et al., 1996) in the case of normal errors, and with the GLIMMIX macro in the case of binomial or Poisson errors (Littell et al., 1996). The degrees of freedom of the fixed effects F-test were adjusted for statistical dependence using Satterthwaite formulas, while variance components were estimated by restricted maximum likelihood (Littell et al., 1996).

RESULTS

Host Preference. The results of the four-way choice experiments illustrate that the number of feeding punctures and eggs (Figure 1) differed significantly

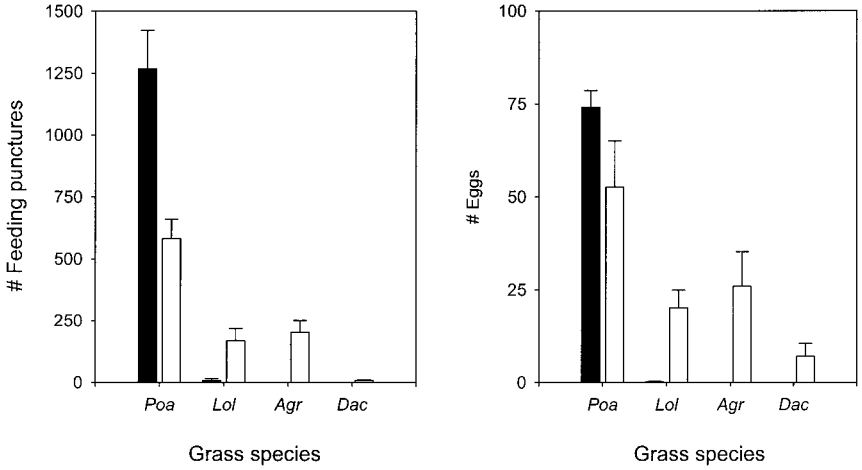

FIG. 1. Feeding and oviposition preference of *C. milii* (black bars) and *C. nigra* (white bars) in the four-way choice experiments. Abbreviations used: *Poa trivialis* (*Poa*), *Lolium perenne* (*Lol*), *Agrostis tenuis* (*Agr*), and *Dactylis glomerata* (*Dac*). Error bars represent ± 1 SE.

among grasses for both *C. milii* (feeding punctures: $Q = 14.02$, $P < 0.001$; eggs: $Q = 20.53$, $P = 0.001$) and *C. nigra* (feeding punctures: $Q = 13.93$, $P < 0.001$; eggs: $Q = 5.62$, $P < 0.01$). The high correlation between feeding and oviposition preference in *C. milii* ($r = 0.999$, $t = 310.88$, $P < 0.001$) and *C. nigra* ($r = 0.996$, $t = 16.00$, $P < 0.01$) illustrated that both preference hierarchies were nearly identical. *C. milii* had a smaller host range than *C. nigra*, which agrees with the pattern observed under natural conditions (Griffiths, 1980). *C. milii* used only *P. trivialis* and, to a far lesser extent, *L. perenne* as hosts. *C. nigra* fed and oviposited on all four grass species in the following preference order: *P. trivialis*, *A. tenuis*, *L. perenne*, and *D. glomerata*.

The two-way choice experiments gave comparable results to the four-way choice trials (Table 1), which indicates that the number of choices had little effect on the results (see also Raffa et al., 2002). *P. trivialis* was preferred over the other grass species for feeding and oviposition by *C. milii*, while differences among the other combinations of grass species were not significant. Host preference of *C. nigra* differed significantly among most combinations, except for feeding and oviposition preference among *P. trivialis–L. perenne* and *L. perenne–A. tenuis*, and for oviposition preference between *A. tenuis–D. glomerata*.

Adult and Offspring Performance. Adult longevity differed among grass species for *C. milii* (Figure 2) ($\chi^2 = 30.02$, df $= 3$, $P < 0.001$) and *C. nigra* ($\chi^2 = 17.69$, df $= 3$, $P < 0.001$). In *C. milii*, longevity was the highest on *P. trivialis*, much lower on *L. perenne*, and the lowest on the two other grasses.

TABLE 1. FEEDING AND OVIPOSITION PREFERENCE OF *C. milii* AND *C. nigra* IN THE TWO-WAY CHOICE EXPERIMENTS

Trial	#Feeding punctures) (mean ± 1 SE)				# Eggs (mean ± 1 SE)			
A – B	A	B	*Q*	*P*	A	B	*Q*	*P*
Results two-way choice trials *C. milii*								
Poa-Lol	169.90 ± 31.98	0.50 ± 0.50	33.00	0.002[a]	5.10 ± 0.87	0 ± 0	34.68	0.002[a]
Poa-Agr	141.60 ± 17.78	2.90 ± 1.10	3.00	0.002[a]	4.70 ± 0.84	0 ± 0	33.61	0.002[a]
Poa-Dac	160.30 ± 17.11	0.30 ± 0.30	33.00	0.002[a]	4.90 ± 1.15	0 ± 0	34.25	0.002[a]
Lol-Agr	2.10 ± 6.30	0.70 ± 1.49	0.01	1.000	0 ± 0	0 ± 0	0	1.000
Lol-Dac	9.60 ± 7.44	3.80 ± 3.10	0.26	0.313	0.50 ± 0.34	0 ± 0	2.24	0.500
Agr-Dac	0.50 ± 0.27	0.20 ± 0.13	3.82	0.250	0 ± 0	0 ± 0	0	1.000
Results two-way choice trials *C. nigra*								
Poa-Lol	122.30 ± 301.93	60.50 ± 21.91	4.20	0.084	2.80 ± 0.59	2.00 ± 0.65	1.19	0.324
Poa-Agr	136.30 ± 22.03	28.10 ± 10.39	33.00	0.002[a]	6.50 ± 0.93	0.20 ± 0.13	29.19	0.004[a]
Poa-Dac	143.40 ± 37.78	0.90 ± 0.90	33.00	0.002[a]	4.20 ± 0.90	0.50 ± 0.34	35.59	0.002[a]
Lol-Agr	168.00 ± 30.00	118.60 ± 25.49	2.10	0.193	5.90 ± 1.13	3.20 ± 1.04	2.78	0.129
Lol-Dac	138.40 ± 33.96	1.20 ± 0.80	33.00	0.002[a]	7.70 ± 1.48	1.10 ± 0.90	24.76	0.004[a]
Agr-Dac	88.10 ± 23.30	1.80 ± 0.84	33.00	0.002[a]	2.00 ± 1.00	1.10 ± 0.67	0.96	0.422

Note: Plant Species names are abbreviated in the following way: *Poa trivialis* (*Poa*), *Lolium perenne* (*Lol*), *Agrostis tenuis* (*Agr*), and *Dactylis glomerata* (*Dac*).
[a] Probabilities significant after sequential Bonferroni correction.

In *C. nigra*, adult performance was the highest on *P. trivialis*, lower on *L. perenne* and *A. tenuis*, and lowest on *D. glomerata*. Adult fecundity differed in a comparable way among grasses, and the fecundity of *C. milii* was consistently lower than that of *C. nigra*. (grass species: $F_{3,127} = 15.95$, $P < 0.001$; miner species: $F_{1,127} = 2.25$, $P < 0.001$).

Offspring survival of *C. milii* was higher on *P. trivialis* compared to *L. perenne* (Table 2, Figure 3). The other indicators of offspring performance did not differ for this miner. In the case of *C. nigra*, offspring survival and pupal size differed among grasses (Table 2, Figure 3). Offspring survival was the highest on *P. trivialis* and decreased from this host over *L. perenne* and *A. tenuis* to *D. glomerata*. The largest pupae were found on *L. perenne* and *P. trivialis*.

Nutritional Quality. All food quality parameters differed among the grass species (Table 3). Water content was highest in *P. trivialis*, lower in *L. perenne* and *D. glomerata*, and lowest in *A. tenuis*. Protein content was higher in *P. trivialis*, intermediate in *L. perenne* and *A. tenuis*, and lowest in *D. glomerata*. The amino acid content was lower in *D. glomerata* compared to the other grasses. Carbohydrate content was high in *P. trivialis* and *A. tenuis* and lower in the other two grasses. The lignin content was low in *D. glomerata*, higher in *L. perenne* and *A. tenuis*, and highest in *P. trivialis*.

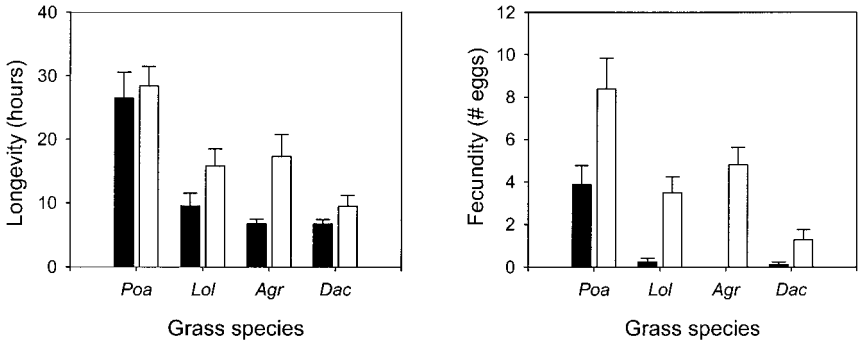

FIG. 2. Adult performance of *C. milii* (black bars) and *C. nigra* (white bars) on the different grass species. See Fig. 1 for abbreviations. Error bars represent ± 1 SE.

Relationship between Preference and Performance. Both oviposition and feeding preference were highly correlated with adult performance characteristics for both miners (Table 4). Only the correlation between feeding preference and adult fecundity of *C. nigra* became insignificant after sequential Bonferroni correction. Correlations between host preference and offspring performance characteristics of *C. nigra* were weaker than those between host preference and adult performance characteristics, and were never significant. The latter correlation analysis could not be performed for *C. milii* because of the lack of offspring performance characteristics on *A. tenuis* and *D. glomerata*.

Relationship between Host Plant Suitability and Nutritional Quality. Stepwise multiple regression analyses (Table 5) of the effects of host nutritional quality on adult performance, yielded two equivalent models. The first model explained a large amount of the variation in adult performance (93%) when miner species

TABLE 2. MIXED MODEL REGRESSION ANALYSES OF OFFSPRING PERFORMANCE OF *C. milii* AND *C. nigra* ON THE DIFFERENT GRASSES

Effect	*C. milii*			*C. nigra*		
	df	*F*	*P*	df	*F*	*P*
Offspring survival						
Species	1, 7	6.23	0.041	3, 10	23.43	<0.001
Total development time						
Species	1, 6.78	1.65	0.241	3, 11.4	1.48	0.272
Sex	1, 22.8	5.52	0.028	1, 175	5.20	0.024
Pupal length						
Species	1, 4.76	0.03	0.868	3, 12.9	3.98	0.033
Sex	1, 22.6	9.58	0.005	1, 176	179.67	<0.001

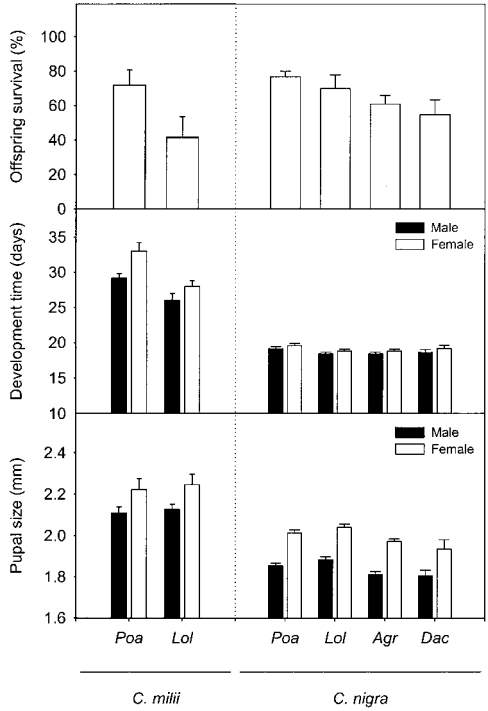

FIG. 3. Offspring performance of *C. milii* and C. *nigra* on the different grass species. See Fig. 1 for abbreviations. Error bars represent ± 1 SE.

and protein content were included. The second model explained an almost equal amount of variation (94%) when miner species and lignin content were included. Protein and lignin content were positively related to adult longevity in both models. The positive relationship of adult performance with foliar protein content is

TABLE 3. NUTRITIONAL QUALITY OF THE DIFFERENT GRASS SPECIES

Grass species	Water (% FM)	Protein (mg/g DM)	Amino acid (mg/g DM)	Carbohydrate (mg/g DM)	Lignin (% DM)
P. trivialis	87.44 ± 0.22	39.86 ± 1.95	11.97 ± 0.76	104.13 ± 5.96	5.51 ± 0.30
L. perenne	85.73 ± 0.38	21.86 ± 1.38	12.07 ± 0.61	65.90 ± 5.75	3.93 ± 0.12
A. tenuis	82.39 ± 0.19	19.10 ± 1.26	12.10 ± 1.06	107.00 ± 7.56	3.73 ± 0.24
D. glomerata	85.55 ± 0.29	11.30 ± 0.60	5.89 ± 0.25	58.43 ± 2.97	3.16 ± 0.14
ANOVA	$F_{3,116} = 26.96$ $P < 0.001$	$F_{3,96} = 82.96$ $P < 0.001$	$F_{3,96} = 21.69$ $P < 0.001$	$F_{3,96} = 20.95$ $P < 0.001$	$F_{3,96} = 21.50$ $P < 0.001$

Note: Data shown are means ± 1 SE.

TABLE 4. CORRELATION MATRIX BETWEEN PREFERENCE AND PERFORMANCE FOR BOTH MINERS

		C. milii		*C. nigra*	
Preference	Performance	*r*	*P*	*r*	*P*
Feeding	Adult longevity	0.991	0.009[a]	0.996	0.004[a]
	Adult fecundity	0.999	0.001[a]	0.984	0.016
	Offspring survival			0.879	0.122
	Pupal size male			0.398	0.603
	Pupal size females			0.527	0.473
Oviposition	Adult longevity	0.991	0.010[a]	0.999	0.001[a]
	Adult fecundity	0.999	0.001[a]	0.996	0.004[a]
	Offspring survival			0.859	0.141
	Pupal size male			0.362	0.638
	Pupal size female			0.514	0.487

[a] Probabilities significant after sequential Bonferroni correction.

probably the only genuine relationship, as lignin content is unlikely to have a positive effect on adult performance as it is a structural defense compound (Bernays and Barbehenn, 1987; Bernays and Chapman, 1994). The artificial relationship of adult performance and lignin content is probably the result of the fact that foliar protein and lignin content covary ($r = 0.998$, $t = 28.82$, $P = 0.001$).

Stepwise multiple regression analyses showed that a significant and large (99%) portion of the variation in pupal size of both miners is explained when miner species, sex, amino acid content, water content, and lignin content were included in the model (Table 6). The latter nutrient components were always positively related to pupal size, while the lignin content negatively influenced pupal size. No significant model could be constructed to explain the variation in offspring survival, although there was a positive relationship with protein content (Table 6).

TABLE 5. RESULTS OF MULTIPLE REGRESSION ANALYSIS OF THE EFFECTS OF HOST NUTRITIONAL QUALITY ON ADULT LONGEVITY OF *C. milii* AND *C. nigra*

Variable	df	Estimate	SE estimate	*F*	*P*
Model A ($F_{2,5} = 37.49$, $P = 0.0010$, Adj-$R^2 = 0.93$)					
Intercept	1	−9.03	3.45	6.85	0.047
Miner species	1	5.37	1.79	9.01	0.030
Protein content	1	0.70	0.09	65.97	<0.001
Model B ($F_{2,5} = 44.87$, $P = 0.0006$, Adj-$R^2 = 0.94$)					
Intercept	1	−27.31	4.65	34.44	0.002
Miner species	1	5.38	1.65	10.67	0.022
Lignin content	1	8.40	0.94	79.06	< 0.001

TABLE 6. RESULTS OF MULTIPLE REGRESSION ANALYSIS OF THE EFFECTS OF HOST NUTRITIONAL QUALITY ON OFFSPRING PERFORMANCE OF *C. milii* AND *C. nigra*

Variable	df	Estimate	SE estimate	*F*	*P*
Offspring survival					
Model ($F_{1,4} = 7.65$, $P = 0.066$, Adj-$R^2 = 0.84$)					
Intercept	1	59.107	9.357	39.91	0.008
Miner species	1	−16.67	6.291	7.02	0.077
Protein content	1	1.001	0.278	13.07	0.036
Pupal size					
Model ($F_{5,6} = 265.04$, $P < 0.001$, Adj-$R^2 = 0.99$)					
Intercept	1	−0.548	0.351	2.44	0.169
Miner species	1	0.227	0.010	545.08	<0.001
Sex	1	0.139	0.008	308.67	<0.001
Amino acid content	1	0.019	0.003	8.17	<0.001
Water content	1	0.023	0.004	18.91	0.002
Lignin content	1	−0.040	0.010	29.72	0.005

DISCUSSION

Our results show that there exists substantial qualitative variation in the relations between host chemistry and the different fitness parameters. First, nearly all variation in adult performance of both miners was explained by foliar protein content. Second, pupal size was positively related to water and amino acid content and always negatively to the lignin content within a leaf. Third, no significant regression model explained the variation in offspring survival, which suggests that one or more unmeasured compounds determine pupal survival. Two mutually nonexclusive reasons may explain the qualitative variation in the relations between host chemistry and the different fitness parameters. First, different life stages may have different nutrient requirements (Simpson and Simpson, 1990; Stockhoff, 1993; Schoonhoven et al., 1998). Adults may for instance require more proteins than larvae as they are essential for egg development, especially in synovigenic insects (Mattson, 1980; Wheeler, 1996; Awmack and Leather, 2002). A second explanation is differences in the food consumed during different life stages. Both adults and larvae of *C. milii* and *C. nigra* feed selectively on the mesophyll of leaves. However, mining larvae cross the heavily lignified veinal tissues in order to reach the mesophyll, and have difficulties doing so (Scheirs et al., 2001b), while adults feed between the veins (Scheirs et al., 2001a). This may explain why lignin content is negatively related to offspring size but not to adult performance. These results suggest that the relationship between plant chemistry and herbivore fitness may be more complex than previously thought. More research on the nutrient requirements of different life stages of phytophagous insects and their role in host choice are clearly needed as this aspect

has largely been neglected in plant–insect research (see Joern and Behmer, 1997, 1998).

Chemically mediated host suitability for adult performance seems to be an important determinant of host choice in *C. milii* and *C. nigra*. This is indicated by the nearly perfect correlations between host choice and host specific variation in adult performance, foliar protein content, and adult performance. The tight link between adult performance and protein content seems logical knowing that proteins are essential for egg development, and concentrations are directly related to adult fecundity in many species, especially in synovigenic insects (Mattson, 1980; Wheeler, 1996; Awmack and Leather, 2002). Moreover, plant nitrogen content has also been shown to correlate with adult performance in another agromyzid species (Minkenberg and Ottenheim, 1990). The latter observations conflict with the common assumption that host plant choice is shaped, both in ecological and evolutionary time, by selection pressures that intervene on offspring performance only (see Jaenike, 1978; Thompson, 1988; Courtney and Kibota, 1989; Mayhew, 1997; Scheirs and De Bruyn, 2002).

Optimality theory predicts that animals will use the strategy or behavior that yields the highest lifetime fitness (Parker and Maynard Smith, 1990). Ideally, females in our study should, therefore, not only optimize adult performance, but also try to optimize offspring performance (Scheirs and De Bruyn, 2002). However, it is not clear to what extent either species optimizes offspring performance. In the case of *C. nigra*, offspring performance is more weakly related to host choice than adult performance, but the trade-off or fitness cost for not optimizing offspring performance seems to be small (see also Scheirs et al., 2000). The latter aspect could not be evaluated for *C. milii* as we were missing offspring performance measures on two grass species. The role of chemically mediated host suitability for offspring performance in host choice of both study species, therefore, remains unclear.

Our results indicate that host nutritional quality may limit the host range of *C. milii*. This is supported by the observation that adult performance is lowered dramatically on nonhost grasses, and this is related to a lower protein content of these plants. We hypothesize that the threshold nutrient concentration, i.e., protein content, that enables exploitation of a grass species becomes limited at a higher concentration in *C. milii* relative to *C. nigra*. Many plant–insect researchers do not consider nutrients or primary plant metabolites as possible determinants of the host range of phytophagous insects (Feeny, 1992). Others are more moderate and believe it is unlikely, but albeit, possible under restricted conditions (Moran and Hamilton, 1980; White, 1993; Berenbaum, 1995). Besides evidence being scarce (but see Auclair et al., 1957; Maltais and Auclair, 1957; Sogowa and Pathak, 1970), the main argument for the small role of nutrients in host range evolution is that most herbivores may compensate for a low nutrient content by increasing consumption rates. However, herbivores cannot compensate by overeating

ad infinitum (Mattson, 1980; White, 1993) or they may not be mobile enough to compensate for nutritional differences by switching host plants (Berenbaum, 1995). Compensatory feeding may also be difficult in our study species because of the low essential nutrient (e.g., proteins) and water content of grasses (Bernays and Barbehenn, 1987) and the time consuming feeding method of adult Agromyzidae (see Bethke and Parrella, 1985). Nevertheless, the chemical basis of host choice by *C. milii* and *C. nigra* needs further study in order to conclude definitely that foliar protein content plays a primary role in host choice of both study species, as our evidence is merely correlative. Second, the seemingly important role of foliar protein content does not exclude a role for secondary substances in host recognition. Finally, the influence of other selection pressures like plant anatomy (Bernays, 1991; Scheirs et al., 2001a) or higher trophic level effects (e.g., Bernays and Graham, 1988; Berdegué et al., 1996) needs to be addressed. We hope that our results will encourage plant–insect researchers to devote more attention to the quantitative and qualitative variation of primary nutrient compounds, as interest in the defensive role of primary constituents has virtually disappeared (Berenbaum, 1995).

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ALTERATION OF KAIROMONE-INDUCED ANTIPREDATOR RESPONSE OF THE FRESHWATER AMPHIPOD *Gammarus roeseli* BY SEDIMENT TYPE

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Abstract—The importance of sediment type and of kairomones released by juvenile burbot (*Lota lota*) on behavioral responses of the freshwater amphipod *Gammarus roeseli* was studied in laboratory experiments. In the absence of kairomones, *G. roeseli* preferred coarse substrates; the highest preference was shown for the most coarse sediment. In the presence of kairomones, the amphipods preferred the less-coarse substrate pebbles over gravel. This difference is an effective antipredator response; *G. roeseli* is able to hide efficiently in the interstices of the pebbles. Juvenile burbot have a low foraging efficiency on pebbles and preferentially forage in the interstices of coarser sediments. *G. roeseli* responded differently to kairmones in different sediment types. The amphipods decreased their activity only in pebbles. In gravel, the rate of exploration behavior increased, and on a bare surface, the area containing fish kairomones was avoided.

Key Words—Predator avoidance, habitat preference, flexible response, chemical communication, burbot, *Lota lota*.

INTRODUCTION

Flexible antipredator responses imply the ability of prey organisms to assess the current predation risk. When predators are nearby or occur in high densities, predation risk increases. In addition to predator proximity and density, other information, such as the availability of refuges for the prey organisms or the occurrence of conspecifics, may be important in risk decision making. Since predator avoidance

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is usually associated with costs related to other behavioral traits, e.g., foraging, mating, or reproduction, antipredator responses can be the result of a complex risk assessment and a trade-off between different needs (e.g., Lima and Dill, 1990; Mathis and Hoback, 1997; Wellborn, 2002).

In freshwater habitats, chemical cues are frequently used to gather information about predators before encounter (see reviews by Dodson et al., 1994; Kats and Dill, 1998). The behavioral responses of the prey organisms, such as escape, refuge use, lowered activity, and shoaling, lead to differences in their distribution, thereby modifying the impact of the predators on local prey density and the prey population (e.g., Sih, 1987; Cooper et al., 1990; Sih and Wooster, 1994).

Predicting impacts of predators on prey is a central issue in ecology, and the mechanisms behind variable behavioral responses of prey organisms are of particular interest. In benthic environments, activity and habitat choice of invertebrates are strongly affected by spatial heterogeneity and habitat complexity, making it likely that sediment characteristics can also influence predator–prey interactions (Gilinsky, 1984; Mayer et al., 2001). For freshwater amphipods, either decreased activity and reduced drift rates (Williams and Moore, 1982; Andersson et al., 1986; Holomuzki and Hoyle, 1990; Andersen et al., 1993; Wudkevich et al., 1997; Wooster, 1998) or active avoidance of microhabitats with predators and increased dispersal rates have been described (Dahl, 1998; Dahl et al., 1998; Baumgärtner et al., 2002). Whether the amphipods decrease or increase their activity strongly determines their local abundance. While active escape and higher dispersal rates lead to lower prey density, lowered activity causes an accumulation of prey organisms in the vicinity of the predator (e.g., Cooper et al., 1990). These antagonistic responses are probably evoked by different feeding modes of the predators (Dahl and Greenberg, 1996; Dahl, 1998; Wooster, 1998). For amphipods, a protective mechanism would be to respond to a water column feeding predator by reducing activity and moving toward the sediment (Wudkevich et al., 1997; Starry et al., 1998), whereas a benthic feeder would generally be better avoided by active escape or increased drift (Sih and Wooster, 1994). Considering the spatial heterogeneity in the benthos, however, a more flexible response that takes into account habitat quality features for the assessment of the current predation risk could increase the probability of survival of the amphipods.

It is well known that amphipods can perceive sediment characteristics by the presence and size of substrate interstices (Olyslager and Williams, 1993, and citations therein) and that these substrate characteristics play an important role for activity (Starry et al., 1998) and habitat selection of *Gammarus* spp. (Pringle, 1982; Williams and Moore, 1986; Dahl and Greenberg, 1996). The ultimate factors for choosing a particular sediment include protection from predators, potential availability of food, and protection from stressful physical conditions. As these benefits cannot be achieved equally in every substrate type, there should be a trade-off between different factors affecting the sediment choice of the amphipods, e.g.,

a trade-off between foraging behavior and predator avoidance. To investigate the influence of such a trade-off, we studied the combined effect of sediment type and predator chemical cues (fish-conditioned water) on the microhabitat choice and the antipredator response of *Gammarus roeseli.*

METHODS AND MATERIALS

Origin and Maintenance of the Animals. The freshwater amphipod *G. roeseli*, a common, widespread species in central and southeastern Europe, was used as a model organism. Like other amphipods, *G. roeseli* is an important prey for many fish species (MacNeil et al., 1999), including juvenile burbot *Lota lota* (Ryder and Pesendorfer, 1992), which we used as the benthivorous predator.

G. roeseli amphipods were caught once a week in the littoral zone of Lake Constance, Germany, where they occur in high abundance. In the laboratory, the amphipods were kept in 5 l plastic tanks filled with water from Lake Constance; the lake water was supplied by a pipe at 15 m depth and filtered through a 100-μm mesh. The gammarids were fed with leaves and provided with sediments of different grain sizes as shelter.

Juvenile burbot (age class 0) were caught once by electrofishing in the littoral zone of Lake Constance, maintained in lake water in 40 l glass tanks during the experimental time from September to December 2001, and fed frozen chironomids.

Experimental Set-Up. All experiments were carried out in a y-shaped flow-through system (y-maze) in an 18°C climate chamber, according to the protocol in Baumgärtner et al. (2002). The y-maze (root length $\times$ width: 36 $\times$ 13 cm; y-arm: 30 $\times$ 9 cm) consisted of grey PVC and was filled with 7 l of lake water. The inflow was adjusted to 0.5 l/min for each of the y-arms. Mixing and flow of the water were checked using colored water.

Two factors of the microhabitat characteristics were altered: sediment grain size and the presence or absence of fish-conditioned water, containing fish kairomones. The sediments were obtained commercially and autoclaved before use. Fish-conditioned water was produced by incubating groups of 12 juvenile burbot in tanks with 24 l of lake water. After 24 hr, the tank water was removed and replaced with fresh lake water. The fish-conditioned water was then filtered through 100-μm mesh and used for the experiments. The experimental set-up was illuminated evenly by fluorescent lights (Osram Biolux 15 W and Sylvania Standard 36 W). The day–night cycle followed the natural cycle. All experiments were carried out during daytime.

Treatments. To test the influence of different sediment grain sizes on the choice of microhabitat by *G. roeseli* and how this choice is affected by fish-conditioned water, a 4 $\times$ 2 factorial design with four levels of sediment grain size [bare surface, sand, pebbles (grain size 6–20 mm), and gravel (grain

size > 63 mm)] and two levels of kairomone concentration (water from Lake Constance and burbot-conditioned lake water) was used. Although burbot occur in Lake Constance, the concentration of burbot kairomones in lake water is too low to induce predator-avoidance behavior by *G. roeseli* (Baumgärtner et al., 2002).

Habitat preference in the absence of fish-conditioned water was tested with sediment added to only one of the branches of the y-maze. In each treatment, the preference of the amphipods for the sediment in the treatment branch and the bare surface in the reference branch was compared. Effects of fish-conditioned water were tested with the same set-up; only the treatment arm was exposed to fish kairomones. The reference arm always consisted of a bare surface and was flushed with unconditioned lake water. With this experimental design, a potential preference for a certain sediment grain size and the effects of fish-conditioned water on this preference compared to a single reference were evaluated.

Each experimental trial lasted 30 min. After a lead time of 1 min, when the inflowing water had reached the point of confluence, 20 adult *G. roeseli* (length: 1–1.7 cm; mixed sexes) were released at the end of the mixing zone (starting area). Every 2 min, the number of individuals in each of the branches and the mixing zone was counted. As it proved impossible to count the amphipods correctly when they were hiding in the sediment interstices, their number was determined indirectly by counting the amphipods in the reference arm and the mixing zone and subtracting this value from the initial value of 20.

Each treatment was repeated eight times, except for the pebbles with the fish-conditioned water treatment, which was repeated six times. New *G. roeseli* individuals were used for every replicate. After each replicate, the treatment side of the y-maze was changed, and the y-maze was cleaned carefully by rinsing it several times with hot and cold tap water.

Statistical Analysis. Data were analyzed with the statistical package Statistica Vers. 5.5 (StatSoft Inc., 1999) and a repeated measures ANOVA (RMANOVA). The difference in the number of amphipods in the branches of the y-maze, i.e., the number in the treated branch minus the number in the reference branch, was the dependent variable. Negative differences indicate discrimination against the treatment conditions; positive differences indicate preference for the treatment. Independent factors were sediment size (four levels) and the presence or absence of fish-conditioned water.

During the first 10 min of the experiment, before the water in the root of the y-maze was fully mixed, differences in the number of *G. roeseli* specimens between the branches of the y-maze should increase (dependent on the treatment), reaching a dynamic equilibrium thereafter. In the RMANOVA, this would lead to a significant Time $\times$ Treatment interaction effect for the first 10 min of the experiment and a significant treatment main effect thereafter. To avoid confounding effects, only the values from minute 10 on were included in the calculations.

Amphipod responses to different substrate types in lake water and burbot-conditioned water were checked separately using Tukey's test. The influence of the kairomone in the burbot-conditioned water on each substrate was tested by linear contrasts between the results for lake water and fish-conditioned water. The test-wise significance level of 0.05 was maintained by a sequential Bonferroni adjustment (Rice, 1989). Variance homogeneity was checked using Bartlett's test.

RESULTS

G. roeseli responded differently to the various sediment types, and these responses were altered by the presence of chemical cues from burbot in the fish-conditioned water (Table 1; RMANOVA, Substrate × Kairomone, $P < 0.001$). Variances were homogenous at all observation times (Bartlett's test, $P > 0.05$). Although only data from minute 10 on were included in the calculations, there was a significant Time × Substrate interaction in the partial model without kairomone (Table 1, $P < 0.001$). This was caused by amphipod numbers increasing over time in the pebbles treatment, where a steady state of the differences was not reached

TABLE 1. RESULTS OF RMANOVA ON SEDIMENT PREFERENCE OF *Gammarus roeseli*[a]

	Selection condition	df	MS	*F*	*P*
Kairomone		1	107.16	0.61	0.44
Substrate		3	4682.53	26.82	<0.001
Kairomone × Substrate		3	1270.70	7.28	<0.001
Error		54	7.51		
Time		10	16.72	2.23	0.015
Time × Kairomone		10	3.86	0.51	0.88
Time × Substrate		30	10.34	1.38	0.090
Time × Kairomone × Substrate		30	12.91	1.72	0.011
Error		540	174.58		
Substrate	No kairomone	3	2164.41	12.07	<0.001
Error	No kairomone	28	179.36		
Time	No kairomone	10	12.77	1.44	0.16
Time × Substrate	No kairomone	30	18.95	2.13	<0.001
Error	No kairomone	280	8.88		
Substrate	With kairomone	3	3576.97	21.11	<0.001
Error	With kairomone	26	169.42		
Time	With kairomone	10	8.00	1.33	0.21
Time × Substrate	With kairomone	30	4.94	0.82	0.74
Error	With kairomone	260	6.02		

[a] Independent factors were sediment grain size (four levels: bare surface, sand, pebbles, gravel) and kairomone concentration (two levels: burbot-conditioned lake water and lake water). Results for the entire model and for the partial models of different kairomone treatments are shown.

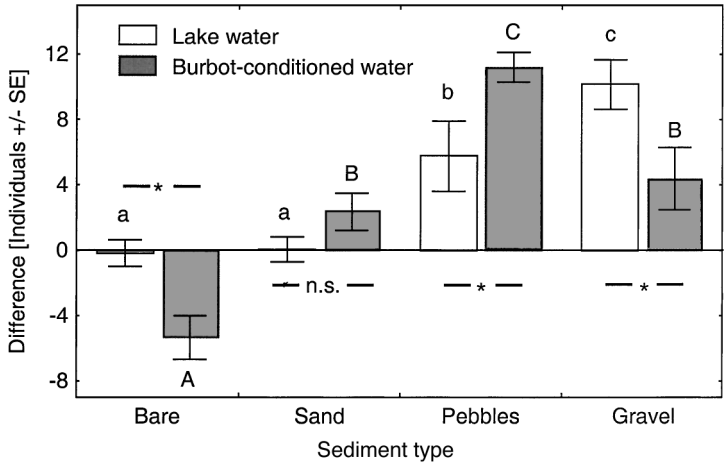

FIG. 1. Sediment preference of *Gammarus roeseli* in lake water and burbot-conditioned lake water. Values are differences in numbers of animals between the two arms of the y-maze, averaged from time 10 min to 30 min. Significant differences in the preference of a sediment type are indicated by different letters. Lowercase letters indicate differences within the lake water treatment, and capital letters indicate differences within the burbot-conditioned water treatment (Tukey's test, $P < 0.05$). Significant differences in the preference of a certain sediment type in lake water and burbot-conditioned water are marked with an asterisk (- * -) (linear contrast, $P < 0.05$ after sequential Bonferroni adjustment). n.s. = not significant.

until 26 min (data not shown). When the model without the pebbles treatment was calculated, the Time × Substrate interaction effect disappeared ($P = 0.17$). Apart from this, most variation could be explained as expected with the substrate main effect (Table 1).

In lake water, the amphipods had a marked preference for the coarser substrates in ascending order, i.e., preference for gravel > preference for pebbles > preference for sand (Tukey's test, $P < 0.05$; Figure 1). There was no difference between sand and bare surface (Tukey's test, $P > 0.05$). The preferences for the substrates compared to the bare surface arose mainly from a longer duration of stay by the gammarids in the arm with sediment, while they left the reference arm after a shorter period.

With burbot-conditioned water, the response of the amphipods changed. On the bare surface, they avoided the kairomone-containing water and stayed preferably in the reference branch of the y-maze. However, on the other sediment types, this escape behavior was not observed. The amphipods showed no avoidance of the sand habitat with burbot-conditioned water, and on the coarser substrates, *G. roeseli* even preferred a microhabitat with pebbles or gravel in kairomone-containing water to the kairomone-free reference. The amphipods preferred pebbles to gravel in

fish-conditioned water (Tukey's test, $P < 0.05$). Their response to sand and gravel was nearly similar (Tukey's test, $P > 0.05$).

When the amphipods entered the branch with fish-conditioned water and pebbles, most of them ceased swimming and moved toward the sediment where they lay inactively or crawled around slowly until the experiment was terminated. Over the course of the experiment, sometimes all 20 specimens accumulated in the pebbles substrate. In the other treatments with fish-conditioned water, the amphipods frequently left the treatment arm and entered it again.

A comparison of the responses on the different substrates with respect to the influence of the fish-conditioned water revealed significant differences of means for all sediment types except sand (linear contrasts, $P < 0.05$ after sequential Bonferroni adjustment). The positive response to pebbles was increased twofold by burbot-conditioned water, while the positive response to gravel was reduced to less than half, but was still evident. This indicated a shift in the preferred habitat caused by an effect of kairomones on sediment preference.

DISCUSSION

The results of our study show that predation risk affects the sediment preference of the freshwater amphipod *G. roeseli* and that the behavioral antipredator response depends on sediment grain size. In lake water, *G. roeseli* preferred the coarse sediments pebbles and gravel. This corresponds with field observations in streams, in which amphipods settled preferably in places where large substrate particles were offered (Williams and Moore, 1986; Dahl and Greenberg, 1996). Whether it is more important that in running water, large sediment sizes constitute a low-current microhabitat and provide a velocity refuge where the amphipods can save energy, or whether the tendency to colonize coarse substrates is due to general positive thigmotaxis (Starry et al., 1998) is not quite clear. In our experiments, *G. roeseli* distinguished between the two coarse sediment sizes and preferred the larger gravel substrate to a higher degree than pebbles. This means that the amphipods stayed longer in the coarser substrate and reduced the rate at which they left the microhabitat to explore a new environment.

When fish-conditioned water flowed through the sediments, the gammarids changed their habitat preference: on the bare surface, they markedly avoided the branch with fish-conditioned water, as described by Baumgärtner et al. (2002). Even though the amphipods did not discriminate between the sand and the coarse substrates, there were important changes in habitat use: pebbles were more strongly preferred in kairomone-containing water, while the preference for gravel diminished to a comparable extent. This change is meaningful since the preference for a certain habitat is measured as the difference in the number of individuals in the habitat compared to the number in the reference branch (with a bare surface and

lake water in all treatments) and hence is determined mainly by the level of activity in the respective habitat or by exploration behavior away from it.

In terms of response to enhanced predation risk, we observed that the switch in preference for pebbles and gravel resulted from a changed behavior: the amphipods decreased activity in the smaller pebble substrate and left the substrate less often to inspect the environment. They undergo a trade-off within the habitat in decreasing their foraging activity and potential energy intake on one hand, and reducing predation risk on the other hand. A decrease of activity has been shown in many studies with amphipods as a response to predatory fish and fish-conditioned water (e.g., Williams and Moore, 1982; Andersson et al., 1986; Holomuzki and Hoyle, 1990; Friberg et al., 1994; Wooster, 1998).

On the gravel substrate, in contrast, the emigration rate of *G. roeseli* was higher when fish kairomones were present, connecting a minor preference for this sediment type with higher activity. Here, the amphipods accepted a higher predation risk when leaving their habitat in search of better shelter.

Remarkably, *G. roeseli* neither preferred sand substrate to the bare surface in the lake water treatment nor avoided it in the presence of fish-conditioned water. This behavior seems hardly adaptive since the amphipods are more prone to fish predation on sand. Perhaps the sand substrate offers some sort of refuge, i.e., when kairomones are present, the amphipods look for additional substrate complexity. One could argue that thigmotaxis in the form of contact to sand particles is more important for microhabitat choice than the presence of kairomones, but in this case, *G. roeseli* should also have preferred sand to the bare surface in the lake water treatment.

It is still controversial as to whether amphipods are able to distinguish kairomones of different predators and consequentially can assess the actual predation risk on the basis of different chemical cues. In favor of a universal response to kairomones from different fish species is the finding that *Gammarus pseudolimnaeus* responds with a significant decrease of activity to eight fish species, including both predators and nonpredators of *Gammarus* and one cichlid species from the Niger Delta in West Africa, where freshwater amphipods are absent (Williams and Moore, 1985). In contrast, the observations that latency times for precopulatory mate guarding in *G. pseudolimnaeus* differ depending on the kairomones from predatory and nonpredatory fish (Mathis and Hoback, 1997), and microhabitat choice of *G. roeseli* differed in water conditioned with predatory and nonpredatory fish (Baumgärtner et al., 2002), argue for the possibility of selective responses.

Assuming that *G. roeseli* can respond selectively, the potential effectiveness of the responses can be related to the foraging strategy of the predator. The juvenile burbot used in our experiment coexists with *G. roeseli* in the littoral zone of Lake Constance. Burbot is a benthivorous predator, and *G. roeseli* is one of the main prey organisms (unpublished data). Juvenile burbot preferentially live in coarse sediments larger than 20 mm in diameter; pebbles and smaller sediments are rarely

used as a habitat (Fischer, 2000). Also, the foraging efficiency of the burbot, evaluated with chironomids, is highest on substrates with large interstices (grain size > 110 mm) and lowest on sediments with 30–50 mm grain size (Fischer, 2000). Considering the sediment preference of burbot, the response of *G. roeseli* is highly adaptive. The amphipods change their microhabitat preference toward a sediment grain size on which they are better protected from the predator. They reduce their activity and thereby accumulate in the pebble substrate, where the burbot usually do not occur, and they increase their exploration behavior on gravel, where they are likely to be caught by the predator.

A comparable habitat preference of *Gammarus fasciatus* has been observed, although in these studies, the factors of fish presence and habitat complexity were not combined. The amphipods preferred complex habitats with attached zebra mussels, where the capture rate of their predator, yellow perch (*Perca flavescens*), was markedly reduced, over low-complexity habitats (Gonzalez and Downing, 1999; Mayer et al., 2001).

In contrast to the findings of a field study by Holomuzki and Hoyle (1990), in which microhabitat use by *Gammarus minus* was not affected by the presence of fish, the amphipods in our study changed their sediment preference in the presence of fish-conditioned water. The influence of the size of substrate interstices on microhabitat choice as shown by Olyslager and Williams (1993), can be superimposed by the influence of fish kairomones. These contrary results are probably due to differences in concentrations of kairomones in the fish-conditioned water used in the experiment *vs.* those concentrations in the field. Different responses to fish-conditioned water with different predator densities have been found, for example, for mayfly drift (McIntosh et al., 1999) and *G. roeseli* microhabitat choice (Baumgärtner et al., 2002). Obviously, the fish density for the production of fish-conditioned water in our experiment was higher than the average field abundance of burbot (Fischer and Eckmann, 1997). Since burbot and amphipods live in the same macrohabitat, a low background concentration of fish infochemicals should be present at all times. A high concentration of kairomones represents situations when a predator approaches the prey or when the amphipods swim or drift toward places where burbot have settled under stones. The response of the amphipods then indicates a small-scale response to acute predation risk (e.g., Dahl et al., 1998).

In conclusion, there are different modes of predator avoidance for the same predator on different substrates. On a bare surface, the amphipods avoided the microhabitat with fish-conditioned water; on pebbles, the activity of *G. roeseli* was reduced; and on gravel, exploration behavior and dispersal increased. These results indicate a trade-off between the use of optimal sediment grain sizes and predator avoidance. *G. roeseli* amphipods use predator chemical cues as well as sediment characteristics for the evaluation of the predation risk and, consequently, for a flexible, effective antipredator response.

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A ROLE FOR ISOTHIOCYANATES IN PLANT RESISTANCE AGAINST THE SPECIALIST HERBIVORE *Pieris rapae*

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Abstract—We experimentally reanalyzed the classic interaction between *Pieris rapae*, a specialist lepidopteran herbivore, and isothiocyanates (mustard oils) that are characteristic phytochemicals of the Brassicaceae. Previous investigations have suggested that *P. rapae* is unaffected by isothiocyanates. Using whole plants, root extracts, and a microencapsulated formulation of allyl isothiocyanate, we now show that isothiocyanates reduce herbivore survival and growth, and increase development time, each in a dose-dependent manner. Neither the substrate allyl glucosinolate, nor myrosinase, the enzyme that results in the breakdown of glucosinolates, negatively affected *P. rapae.* Thus, we present strong evidence for a role for isothiocyanates in plant resistance against the specialist herbivore *P. rapae.*

Key Words—*Arabidopsis lyrata*, *Brassica* spp., Brassicaceae, glucosinolates, herbivory, horseradish, host range evolution, induced plant resistance, microencapsulation, myrosinase, plant–insect interactions.

INTRODUCTION

Organisms specialize on the consumption of particular foods because of trade-offs in fitness when eating alternate resources (Futuyma and Moreno, 1988; Agrawal, 2000a). For herbivorous macrolepidoptera in particular, approximately 25% of species feed on species in one plant family (Schoonhoven et al., 1998). Specialist herbivores restrict their counterdefensive measures to the small range of defensive tactics of the plants on which they specialize, while generalists invest in broad detoxification strategies (Krieger et al., 1971; Schoonhoven et al., 1998). Thus, specialist herbivores have long been predicted to be less affected by a

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given chemical defense in plants compared to generalists (the specialist herbivore paradigm) (Whittaker and Feeny, 1971; Feeny, 1976; Blau et al., 1978; Rhoades, 1979; Van Dam et al., 1993; Dyer, 1995; Giamoustaris and Mithen, 1995; Van Der Meijden, 1996). While the specialist herbivore paradigm must be true to some extent, several studies have challenged this notion by demonstrating strong effects of plant chemicals on specialists (Berenbaum et al., 1989; Adler et al., 1995; Malcolm and Zalucki, 1996; Agrawal, 2000b; Van Dam et al., 2000; Kliebenstein et al., 2002; Traw and Dawson, 2002).

White butterflies in the genus *Pieris* are largely restricted to feeding on plants in the Brassicaceae and have been a prominent focus in the study of the ecology and evolution of host specialization (Verschaffelt, 1910; Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991; Ohsaki and Sato, 1994; Giamoustaris and Mithen, 1995; Renwick, 2001). Although plants in the Brassicaceae contain diverse phytochemicals thought to serve defensively, *Pieris rapae*, a herbivore that widely feeds on plants in the Brassicaceae, is purportedly unaffected by either glucosinolates (and their breakdown products) (Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991) or proteinase inhibitors (Broadway, 1995). In particular, glucosinolates are sulfur- and nitrogen-containing secondary compounds characteristic of the Brassicaceae. Myrosinase is an enzyme stored separately from glucosinolates in plants, and does not react with glucosinolates until tissues are macerated, as occurs when lepidopteran larvae feed on leaves. The resulting hydrolysis products, isothiocyanates or mustard oils, give mustards their characteristic pungent odor and are toxic to a broad range of organisms (Chew, 1988; Louda and Mole, 1991).

The groundbreaking studies on the response of *P. rapae* to plant defense involved feeding larvae diets that were variable in glucosinolate concentration and assumed that hydrolysis to isothiocyanates occurred in the herbivore gut (Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991). These studies confirmed that glucosinolates *per se* were not effective agents of plant resistance against *P. rapae*, and that the compounds serve as both feeding and oviposition stimulants (Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991; Giamoustaris and Mithen, 1995; Renwick and Lopez, 1999). Although putative plant defenses are known to have manifold effects in communities (Linhart, 1991), stimulatory effects on specialists have reinforced the specialist herbivore paradigm. Nonetheless, the defensive role of the breakdown products of glucosinolates (isothiocyanates) against *P. rapae* has not been rigorously addressed.

We were motivated to reinvestigate the role of isothiocyanates as a defense against *P. rapae* because no previous experiments had successfully evaluated the role of isothiocyanates on larval performance. We consistently observe a reduction in the performance of *P. rapae* on plants that have been previously damaged by herbivores, challenging the application of the specialist herbivore paradigm to *P. rapae* (Agrawal, 1998, 2000b; Agrawal et al., 1999b). To demonstrate unambiguously

the effects of the glucosinolate–myrosinase system and their hydrolysis products on *P. rapae*, we took a three-pronged approach employing whole plants, root extracts, and synthetic diets incorporating volatile isothiocyanates in microcapsules. In particular, we first show that phenotypic and genetic manipulations of the glucosinolate–myrosinase system in plants affect *P. rapae*'s growth and development. We then isolate the specific effects of allyl isothiocyanate on *P. rapae* by employing active and deactivated pastes of horseradish root extract and a microencapsulated formulation of isothiocyanate in synthetic diets. Although previous studies have directly attempted to examine the effects of allyl isothiocyanate on herbivores, they have failed because allyl isothiocyanate is volatile; when added to synthetic diets, these isothiocyanates are released and fumigate the larvae (Mcloskey and Isman, 1993). Our final approach involving microencapsulated single molecules of allyl isothiocyanate in β-cyclodextrin surmounts previous difficulties of dosing diets with volatile defense compounds (Usher et al., 1989; Szente et al., 1990).

METHODS AND MATERIALS

Whole Plant Experiments. Induced plant resistance to *P. rapae* was assessed with *Arabidopsis lyrata* and *Brassica oleracea*, two host plants commonly used by *P. rapae* in the field. *A. lyrata* ($N = 52$) were grown in a growth chamber in 500-ml pots. At the 6–10 leaf stage, a newly hatched *P. rapae* larva was introduced to half of the plants. *P. rapae* larvae are sluggish and do not move from plant to plant as long as leaves are not touching. Thus, larvae were not caged or restricted. After 5 days of feeding, less than 20% of the leaf tissue was damaged and the caterpillars were removed. A newly hatched *P. rapae* larva was then added to each plant and weighed to the microgram after 5 days. *Brassica oleracea* var. Marathon ($N = 76$) were grown in a glasshouse. All procedures were the same as above except that damaging *P. rapae* larvae were introduced when the plants had one fully expanded true leaf. Chemical analyses were conducted on 15 replicates of each treatment of *B. oleracea*. The analytical procedure for determining glucosinolates was modified from published protocols, and glucosinolates were identified using predetermined HPLC retention times (Brown and Morra, 1995).

To assess the performance of *P. rapae* on whole plants, we worked with four lines of *Brassica* with genetically determined differences in glucosinolate concentrations. A double haploid line was derived from *B. oleracea* var. Green Duke, and three additional lines were produced from its progeny with three wild *Brassica* species (*B. drepanensis, B. villosa*, and *B. atlantica*) (Faulkner et al., 1998). Methods and glucosinolate inheritance are reported in Faulkner et al. (1998). Seeds from these crosses were grown in a glasshouse as described earlier. At the four-leaf stage, a single, newly hatched *P. rapae* larva was introduced to each

plant. The plants were not caged, and caterpillars were not restricted. When the caterpillars entered the wandering phase, approximately 4 days before pupation, we confined the larvae to deli cups and provided them with cut foliage from the plants on which they were feeding.

Root Extract Experiment. To examine the specific effects of allyl isothiocyanate on *P. rapae*, we employed diets that vary in their allyl isothiocyanate levels. We used Japanese Horseradish Powder (JFC International, Inc., San Francisco, CA), which contains only horseradish, mustard seed, cornstarch, and coloring. Allyl isothiocyanate is the primary hydrolysis product released by damage to horseradish (Yu et al., 2001). The powder was obtained by freeze-drying plant tissues, to remove water without letting the reaction between glucosinolates and myrosinase take place. When this powder is wetted, the reaction takes place in a few minutes, as evidenced by the pungent odor and burning taste of horseradish. Our hypothesis was that higher doses of active horseradish extracts would result in larger negative effects on the herbivore. We prepared three types of insect diet: (1) active horseradish paste (1:1 mixture of dry powder and tap water based on mass), (2) deactivated horseradish paste (dry powder wetted to release volatile isothiocyanates, then dried and ground using a mortar and pestle); and subsequently rewetted at the same (1:1) powder-to-water ratio, and (3) 10% active horseradish paste (with a 1:9 ratio of active to deactivated powder). The deactivated paste had the same appearance and texture as the active paste, but none of the spicy, vaporous emanations indicative of isothiocyanate production. Sixty, newly hatched *P. rapae* larvae were initially introduced singly to 30-ml plastic cups with approximately 0.5 g of paste ($N = 20$ on active paste and 40 on deactivated paste). The horseradish paste was refreshed each day of the experiment. After 3 days, the surviving caterpillars on the deactivated paste were split into two equal groups, one maintained on deactivated paste and one on 10% active paste.

Synthetic Diet Experiments. We prepared synthetic diets suitable for *P. rapae* (Webb and Shelton, 1988) and assessed the consequences of dosing these diets with microencapsulated allyl isothiocyanate, allyl glucosinolate, or myrosinase on *P. rapae*'s growth and development. Natural host plants of *P. rapae* range in the concentrations of allyl isothiocyanate up to 1.7 μmol/g fresh mass (Crucifer Genetics Cooperative, University of Wisconsin – Madison). Our synthetic diets had 0, 0.282, 0.565, 1.129, and 1.693 μmol/g fresh mass of allyl isothiocyanate (Sigma Chemical Co.) microencapsulated in β-cyclodextrin ($N = 18$ of each concentration). β-Cyclodextrin is a cycloamylose molecule containing 7 glucose units obtained by the action of *Bacillus macerans* on starch. The cyclodextrin cavity is slightly hydrophobic and large enough to envelop allyl isothiocyanate in a 1:1 ratio. An additional sham control was prepared with an equal amount of empty β-cyclodextrin as in the highest allyl isothiocyanate treatment ($N = 18$). The microencapsulated allyl isothiocyanate was mixed with prepared diet (when cooled $< 50^{\circ}$C) made with agar that does not gel until 28°C. The diet was prepared in a

single large batch (to avoid differences between batches) and then split into equal groups to which the microcapsules were added and thoroughly mixed. Larvae were individually reared in 30-ml plastic cups from freshly hatched eggs.

Experiments with the substrate (allyl glucosinolate) and enzyme (myrosinase) that ultimately result in the production of allyl isothiocyanate were conducted as above, employing synthetic diets and singly reared larvae. Diets containing allyl glucosinolate (Sigma Chemical Co.) were prepared at six incremental concentrations ranging from 0 to 30 μmol/g fresh mass, $N = 15$. The myrosinase experiment compared control diets to diets containing 0.156 mg/g myrosinase (Sigma Chemical Co.); myrosinase was added when the diet cooled below 45°C because temperatures above 60°C denature myrosinase. The amount we added would produce 4.8 μmol glucose per minute when reacting with allyl glucosinolate at pH 6 at 25°C (Bones et al., 1994).

Statistical Analyses. For analyses of continuous data, we employed *t* tests where there were two treatment groups, and ANOVA where there were more treatment groups (or additional variables included in the model such as sex of the caterpillar). Where our treatments involved increasing doses of compounds, we assessed the effect of dose on caterpillars by employing response-curve analyses using polynomial contrasts following ANOVA (Dawkins, 1981, 1983). Response-curve analyses are superior to discrete ANOVAs followed by contrasts because the hypothesis tested is a prediction about an ordered set of treatments. However, response-curve analyses do not assume that the predictor variable is continuous as in regression (Dawkins, 1981, 1983). The polynomial contrast procedure in Systat version 9 (SPSS, Inc., Chicago, IL) was employed. In all response-curve analyses, results represent the first-order polynomial (linear) analysis; no higher order polynomial analyses were significant. *G* tests were employed to test for differences in mortality of the caterpillars.

RESULTS AND DISCUSSION

P. rapae larvae grew 32 and 12% more slowly when feeding on previously damaged plants of *Arabidopsis lyrata* and *Brassica oleracea* than when feeding on the respective undamaged plants (Figure 1, *A. lyrata*, $t = 3.21$, df $= 50$, $P = 0.002$; *B. oleracea*, $t = 2.09$, df $= 44$, $P = 0.042$). This increased resistance in *B. oleracea* was correlated with a 176% and a 94% increase in isothiocyanate-producing indolyl ($t = 5.55$, df $= 28$, $P < 0.001$) and allyl ($t = 2.55$, df $= 28$, $P = 0.017$) glucosinolates, respectively (Figure 1). These results challenge the specialist herbivore paradigm because *P. rapae* is negatively affected by induced responses of plants, and this effect is correlated with induction of compounds previously believed to be ineffective against *P. rapae.* Other recent studies have reported negative effects of induced plant responses on performance of *P. rapae* (Agrawal et al., 1999a; Agrawal, 2000b; Traw and Dawson, 2002).

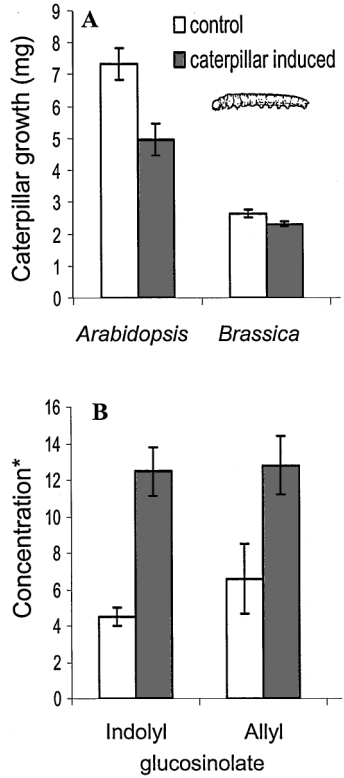

FIG. 1. (A) Induced resistance of *Arabidopsis lyrata* and *Brassica oleracea* to *Pieris rapae*. Low growth of caterpillars on *B. oleracea* was correlated with significant increases in (B) isothiocyanate-producing indolyl and allyl glucosinolate (sinigrin). *Units for indolyl glucosinolate are μmol/g dry mass while units for allyl glucosinolate are 1×10^{-2} μmol/g dry mass. Shown are means $\pm$ SE.

Our experiments with four plant lines with a range of isothiocyanate-producing glucosinolates from 7 to 154 μmol/g dry mass (Faulkner et al., 1998) also showed negative effects on *P. rapae*. Glucosinolate concentration in the *Brassica* lines was negatively correlated with *P. rapae* pupal mass, a strong indicator of butterfly fitness (Figure 2, response-curve ANOVA, $F_{1,44} = 17.354$, $P < 0.001$, sex: $F_{1,44} = 8.059$, $P = 0.007$). Other recent evidence, using lines of *B. rapa* artificially selected for high and low concentrations of glucosinolates, also supported a role for these secondary compounds in defense against *P. rapae* (Stowe, 1998). Neither approach of using induced plant responses nor bred lines of plants, however, unambiguously addresses the role of isothiocyanates in plant resistance.

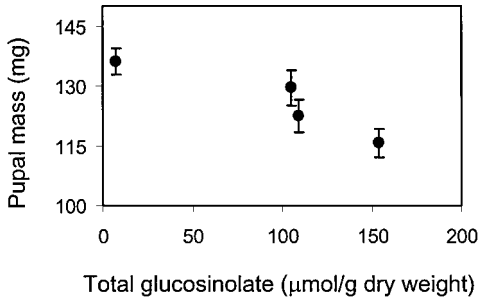

FIG. 2. Pupal mass of *Pieris rapae* as affected by four lines of *Brassica* that varied in their glucosinolate content. From left to right, the varieties are *B. oleracea* var. Green Duke (GD), GD X *B. drepanensis*, GD X *B. villosa*, and, GD X *B. atlantica*) (Faulkner et al., 1998). Shown are least squares means ± SE.

To test more directly for the effects of isothiocyanates on *P. rapae,* we fed larvae diets of active and deactivated horseradish root extracts. After 3 days, mortality was 100% (20 out of 20) on the active paste and 12.5% (5 out of 40) on the deactivated paste (G test, $G = 13.78$, $P < 0.001$). On the 4th day, the surviving caterpillars on the deactivated paste were randomly split into two equal groups, one maintained on deactivated paste and one on 10% active paste. After three more days, no additional mortality occurred, but caterpillars on 10% active paste grew 45% slower than caterpillars on deactivated paste (Figure 3, $t = 3.03$, df $= 33$, $P = 0.005$). Although this approach directly implicates isothiocyanates in plant resistance to *P. rapae*, it has the drawback of volatile loss (and possible fumigation) as in previous studies (Mccloskey and Isman, 1993).

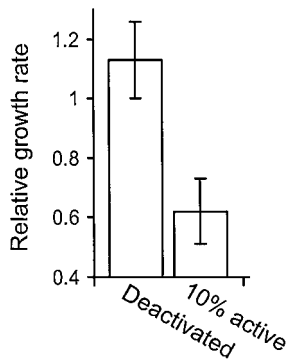

FIG. 3. The relative growth rate of *Pieris rapae* larvae growing on pastes of deactivated and 10% active horseradish root extract. Shown are means ± SE.

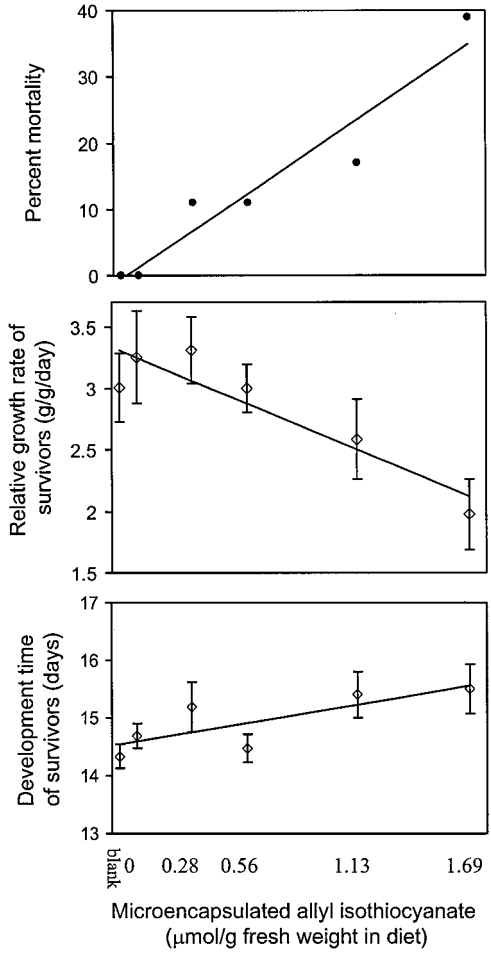

FIG. 4. Effects of microencapsulated allyl isothiocyanate on performance of *Pieris rapae* larvae. (A) percent mortality, (B) relative growth rate, and (C) development time. Shown are means ± SE. The left most data points above "blank" represent larvae on diet with empty β-cyclodextrin microcapsules.

Our final approach to examine the effects of isothiocyanates on *P. rapae* was to compare performance of *P. rapae* on synthetic diets containing microencapsulated allyl isothiocyanate. Microencapsulation prevented the volatilization of the isothiocyanate. Mortality of larvae was dependent on treatment, with no mortality on control diets, but strongly increasing mortality thereafter (Figure 4A, G test, $G = 17.419$, $P < 0.001$). For the survivors, the dose of allyl isothiocyanate negatively

affected the relative growth rate of larvae (Figure 4B, response-curve ANOVA, $F_{1,96} = 10.885$, $P = 0.001$), positively affected development time (Figure 4C, $F_{1,83} = 6.863$, $P = 0.010$), but did not affect pupal mass ($F_{1,89} = 0.079$, $P = 0.779$, sex: $F_{1,89} = 5.720$, $P = 0.019$). These data unequivocally point to isothiocyanates as effective agents of resistance to *P. rapae* via effects on mortality, growth, and development.

Some studies have suggested that specialist herbivores may prevent the defensive enzymatic reactions from occurring upon plant damage, thereby disarming the plant's defense (Engler et al., 2000; Ratzka et al., 2002). For example, a recent study has identified enzymes released by a *Brassica* specialist (*Plutella xylostella*) that prevent isothiocyanates from being formed following ingestion. Sulfatase activity of the enzyme produced by *P. xylostella* competes with myrosinase for attachment to glucosinolates, resulting in very little exposure to isothiocyanates in the herbivore gut. Two lines of evidence suggest that *P. rapae* is not preventing the glucosinolate–myrosinase reaction. First, Ratzka et al. (2002) report that *P. rapae* does not produce glucosinolate sulfatases like *P. xylostella*. Second, the frass of *P. rapae* feeding on *Brassica* contains allyl isothiocyanate, indicating that hydrolysis of glucosinolates does indeed occur during digestion (Agelopoulos et al., 1995). Thus, *P. rapae* does not "disarm the mustard oil bomb" (Ratzka et al., 2002).

There was no negative relationship between phytochemicals and *P. rapae* performance when only the substrate, allyl glucosinolate, was added to synthetic diet (effect on mortality: $G = 4.763$, $P = 0.445$; time to pupation: $F_{1,13} = 0.478$, $P = 0.502$; pupal mass: $F_{1,13} = 0.589$, $P = 0.457$). Similarly, when we added myrosinase to synthetic diets at realistic concentrations found in fresh *Brassica* leaves, *P. rapae* was not affected in growth rate ($t = 0.826$, df $= 38$, $P = 0.414$), development time ($t = 0.056$, df $= 37$, $P = 0.955$), or pupal mass ($t = 0.246$, df $= 37$, $P = 0.870$) (Table 1). Thus, as has been suspected for decades, neither glucosinolate nor myrosinase that together result in the production of isothiocyanate has an independent negative impact on *P. rapae.*

Even if specialists are, to some extent, able to cope with their hosts' phytochemicals, we suggest that a coevolutionary interaction where specialist herbivores reduce plant fitness must impose natural selection for more effective plant defenses against specialists (Berenbaum and Zangerl, 1998; Thompson and Cunningham, 2002). Both glucosinolate and myrosinase are strongly inducible

TABLE 1. EFFECTS OF DIETS CONTAINING 0.156 MG/G MYROSINASE ON THE MEANS ± 1 SE GROWTH AND DEVELOPMENT OF *Pieris rapae*

	Mass day 8 (mg)	Development time (days)	Pupal mass (mg)
Control diet	29.9 ± 5.1	14.3 ± 0.4	167.4 ± 5.4
Myrosinase diet	25.7 ± 3.8	14.1 ± 0.4	164.2 ± 5.4

following herbivory by specialists (Bodnaryk, 1992; Siemens and Mitchell-Olds, 1998; Agrawal et al., 1999b; Bartlet et al., 1999). Some studies find that specialist herbivores of the Brassicaceae feed less on previously damaged plants than controls (Bodnaryk, 1992; Agrawal, 1998, 2000b; Agrawal et al., 1999a; Bartlet et al., 1999). Thus, although many of these same studies find that glucosinolates are attractive to adults of the specialists (e.g., Giamoustaris and Mithen, 1995), differential effects on attraction of adults versus larval performance may explain the paradoxical induction of these compounds that are seemingly beneficial to herbivores. Similar results have been reported for specialist Buckeye butterflies (*Junonia coenia*) on *Plantago lanceolata*, where iridoid glycosides are attractive to adults (Bowers, 1991), but negatively affect larvae (Adler et al., 1995). Ultimately, the net effects of the putative defense compounds will determine the outcome of the plant–herbivore interaction. Indeed, correlations between the myrosinase-glucosinolate system and damage by specialist herbivores in the laboratory and field are often negative (Louda and Mole, 1991; Siemens and Mitchell-Olds, 1998; Stowe, 1998; Li et al., 2000).

Our experiments with microencapsulated isothiocyanates allowed for a realistic evaluation of the effects of these compounds against *P. rapae*. Our results resurrect a long-standing debate on plant–herbivore chemical coevolution and point to multiple positive and negative ecological roles for secondary plant metabolites. While certain aspects of the glucosinolate–myrosinase system may be essential for recognition, oviposition, and feeding by specialist herbivores, these same chemicals reduce larval performance of *P. rapae* and are likely to serve as a plant defense. More generally, differential effects of phytochemicals on different life stages of herbivores may resolve the paradox of why plants appear to be producing compounds seemingly beneficial to specialist herbivores (Malcolm and Zalucki, 1996; Agrawal, 2001).

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A SAPONIN CORRELATED WITH VARIABLE RESISTANCE OF *Barbarea vulgaris* TO THE DIAMONDBACK MOTH *Plutella xylostella*

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Abstract—Two types of *Barbarea vulgaris* var. *arcuata*, the G-type and the P-type, differed in resistance to larvae of the diamondback moth (DBM) *Plutella xylostella*. Rosette plants of the G-type were fully resistant to the DBM when grown in a greenhouse or collected in the summer season, but leaves collected during the late fall were less resistant, as previously found for flea beetle resistance. The P-type was always susceptible. Extracts of resistant leaflets inhibited larval growth in a bioassay, and a growth-inhibiting fraction was isolated by activity-guided fractionation. A triterpenoid saponin **(1)** was isolated from this fraction and identified as 3-*O*-β-cellobiosyloleanolic acid from spectroscopic data and analysis of hydrolysis products. The decrease in resistance of the G-type in the fall was correlated with a decrease in the level of **1**, from 0.6–0.9 to <0.2 μmol/g dry wt. Compound **1** was not detected in the susceptible P-type. We conclude that **1** is correlated with the variable resistance of *B. vulgaris* foliage to the DBM.

Key Words—*Barbarea vulgaris*, Brassicaceae, diamondback moth, 3-*O*-β-cellobiosyloleanolic acid, 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-oleanolic acid, G-type, oleanolic acid, *Plutella xylostella*, P-type, resistance, saponin, seasonal variation.

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INTRODUCTION

The wild cruciferous plant *Barbarea vulgaris* R. Br. (Brassicaceae) is accepted for oviposition by gravid females of the diamondback moth (DBM) *Plutella xylostella* (Lepidoptera: Plutellidae) (Idris and Grafius, 1996; Hughes et al., 1997). However, this behavior represents an oviposition mistake, as DBM larvae do not survive on *B. vulgaris* (Idris and Grafius, 1996). The DBM is the world's most destructive insect pest of cruciferous crops such as *Brassica oleracea* (cabbage) and *Brassica napus* (oilseed rape), probably because of its notorious ability to develop resistance against synthetic insecticides (Talekar and Shelton, 1993).

Several varieties of *B. vulgaris* have been described (Bush, 1939, 1970; Ball, 1993). A common variety of *B. vulgaris* in Canada (MacDonald and Cavers, 1991), the former Soviet Union (Bush, 1939/1970), and Denmark (Nielsen, 1997; Agerbirk et al., 2001a) is var. *arcuata* (Opiz.) Fries (syn. ssp. *arcuata* (Opiz.) Simkovics). Two types of *B. vulgaris* var. *arcuata* can be recognized in Denmark—the **g**labrous G-type and the **p**ubescent P-type—on the basis of differences in leaf pubescence, flea beetle resistance, and glucosinolate content (Agerbirk et al., 2001a). The classification of both types within the same variety of *B. vulgaris* has been questioned elsewhere (Agerbirk et al., 2003); it is likely that future classifications will regard the P-type as a separate variety or subspecies. In this paper, the two types will simply be referred to as the G-type and the P-type. The G-type is resistant to the flea beetle *Phyllotreta nemorum*, while the P-type is susceptible to *P. nemorum*.

As indicated by the common name of *B. vulgaris*—Wintercress—rosette plants do not wilt in the fall, but stay green throughout the winter. The flea beetle resistance of the G-type varies during the growth season: Rosette plants are resistant (lethal) in the spring and summer, but gradually lose resistance during the fall (Nielsen, 1997). We recently reported that levels of glucosinolates in the leaves may vary significantly during the transition from resistance to susceptibility, but there was no correlation with flea beetle resistance (Agerbirk et al., 2001a).

In this paper, we report that the P- and G-types also differ in resistance to the DBM, and that there is a seasonal variation in DBM resistance, similar to the previously reported seasonal variation in flea beetle resistance. A triterpenoid saponin **(1)** was isolated from the G-type by activity-guided fractionation. The structure of **1** was determined to be 3-*O*-β-cellobiosyloleanolic acid. The saponin was not present in the susceptiple P-type. Finally, it was found that DBM resistance of one Danish and one New York accession of the G-type was strongly correlated to the levels of **1** in the fall. While our work was in progress, resistance to DBM was reported to be due to the presence of a different triterpenoid saponin, 3-*O*-β-cellobiosylhederagenin (**2**), in *B. vulgaris* var. *variegata*, a horticultural cultivar with variegated leaves (Shinoda et al., 2002). The latter report is discussed and compared to our findings.

METHODS AND MATERIALS

Plants. Seeds of *B. napus* cv. Polo were obtained from Danisco Seed, Holeby, Denmark. Seeds of P-type *B. vulgaris* var. *arcuata* (accession B4) were obtained as previously described (Agerbirk et al., 2001b, 2003). Three accessions of the G-type were used: B1 (Denmark), B15 (New York), and B44 (Denmark), with the country or state of origin indicated. Details about the origin of B1 and B15 are described elsewhere (Agerbirk et al., 2001b, 2003). Accession B44 was collected as seeds by J.K.N. at the same locality where B1 was collected, but in a later year (1999). Plants for initial studies (Figures 1 and 2) were grown in the Boyce Thompson Institute greenhouse with supplemental light as previously described (Renwick et al., 1992). Plants (accession B1) for purification of **1** were grown in a growth chamber as previously described (Nielsen, 1999). The *Barbarea* plants used were first year rosette plants up to 4 months old, and the *B. napus* plants used were preflowering or early flowering plants, about 1 month old. Voucher specimens of the G-type (B1) and the P-type (B4) have previously been deposited at the Herbarium of Danish Vascular Plants, Botanical Museum, University of Copenhagen, Denmark (Agerbirk et al., 2001b).

Insects. DBM eggs were from a colony kept by Dr Patrick Hughes, Boyce Thompson Institute; the colony was originally obtained from the NYS Agricultural Experiment Station in Geneva, New York, in 1994. Adult rearing and collection of eggs on filter paper discs with cabbage surface extracts was carried out as described by Hughes et al. (1997). For maintaining the colony, larvae were reared on high

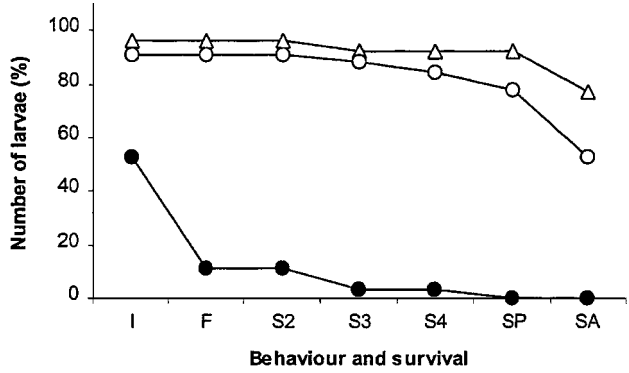

FIG. 1. Behavior and survival of DBM neonates offered leaves of either *Brassica napus* (triangles), or the P-type (open circles) or the G-type (closed circles) of *B. vulgaris* var. *arcuata*. I: Initiation of feeding; F: Continued feeding; S2: Survival to 2nd instar; S3: Survival to 3rd instar; S4: Survival to 4th instar; SP: Survival to the pupal stage; SA: Survival to the adult stage. The numbers of tested leaves and larvae were 26 for *B. napus*, 38 for the G-type, and 32 for the P-type. For statistical significance, see text.

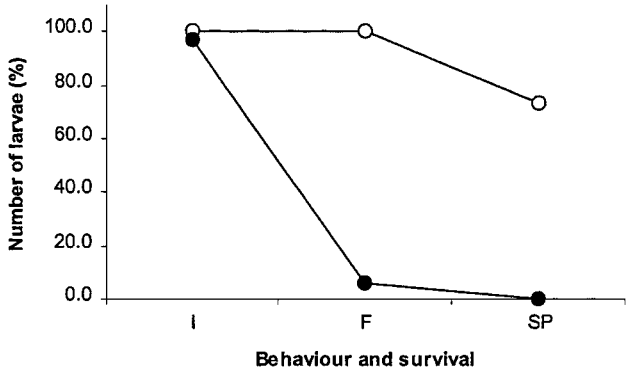

FIG. 2. Feeding behavior and survival of later DBM instars reared on *Brassica napus*, on leaves of either the P-type (open circles) or the G-type (closed circles). Results for second and third instars were pooled, since they were not significantly different. I: Initiation of feeding; F: Continued feeding; SP Survival to the pupal stage. The numbers of tested leaves and larvae were 30 for the P-type and 35 for the G-type. For statistical significance, see text.

wheat germ diet (Bell et al., 1981). Larvae for use in experiments were either inexperienced newly hatched from filter paper, or older reared on *B. napus*.

Chemicals and Chromatography Equipment. Oleanolic acid was from ICN Biomedicals, Costa Mesa, CA. Benzylglucosinolate was from Merck, Darmstadt, Germany. Vanillin was from Sigma, St. Louis, MO. Ingredients for the high wheat germ diet were from Bioserv, Frenchtown, NY. DEAE Sephadex A-25 was from Pharmacia, Uppsala, Sweden. C18 column material was "Poligoprep 60-50 C18" from Macherey-Nagel, Düren, Germany. Isolute C18 SPE columns (500 mg) were from International Sorbent Technology, Mid Glamorgan, UK. Solvents were of HPLC quality, except CD_3OD, which was of NMR quality. The HPLC apparatus was as described elsewhere (Agerbirk et al., 2001a), equipped with an analytical C18 column (Phenomenex Prodigy ODS3 100 A, 250 $\times$ 4.6 mm, particle size 5 μm) and a homemade column oven set at 30°C. TLC aluminium sheets with silica gel 60 F254 were from Merck, Darmstadt, Germany. GC-MS was performed in a system consisting of an HP5890 Series II gas chromatograph coupled to a Jeol JMS-AX505W mass spectrometer (head pressure 100 kPa, splitless injection). An SGE column (BPX5, 25 $\times$ 0.25 mm, 0.25 mm film thickness) was used.

Behavioral Experiments with DBM Larvae on Leaves. Fresh, randomly picked leaves from 2- to 6-week-old greenhouse-grown plants of accession B1 and B4 were used. Leaves were placed in transparent or semitransparent plastic containers of suitable size (30–100 ml, according to the size of the leaf tested), with a piece of moist filter paper. Neonates, or older larvae reared on *B. napus*, were gently placed on the filter paper with a moist paint brush on day 0. Containers were kept at 22°C,

L16:D8. Each container contained one larva and one leaf. Experiments were left undisturbed on days 0 and 1 (and sometimes 2). On day 2 or 3, and every day thereafter, the filter papers were moistened if needed, wilted leaves were replaced with fresh ones, and the instar or life stage, feeding status, and survival or death of the larvae were noted. Leaves were inspected for signs of initiation of feeding or continued feeding under a dissection microscope. Initiation of feeding (I) was defined as the presence of any sign of feeding, including the presence of tiny biting marks of a size approximate to the larval head capsule. Continued feeding was defined as signs of substantial feeding, either mines or surface-feeding, where the largest fed area was at least 10 times larger than the larval head capsule, but typically much larger. Larval instars (abbreviated 1, 2, 3, or 4) were determined from the width and color of the head capsule: (1) 0.15 mm, black; (2) 0.2 mm, black; (3) 0.4 mm, brown; (4) 0.6 mm, spotted brown.

Bioassay of Crude Extract and Fractions. The bioassay diet was similar to the standard high wheat germ diet. Extracts or fractions were mixed with 6.0 g wheat germ in a beaker, and the solvent was allowed to evaporate. Fifty ml of bioassay diet was prepared as follows. A total of 0.75 g of agar was dissolved in 42 ml of boiling water. The hot agar solution was transferred to the beaker containing 6.0 g wheat germ with or without added extracts or fractions, followed by addition of 1.25 g casein, 0.40 g Wesson Salt Mix, 0.10 g sorbic acid, and 0.050 g methylparaben (total volume 50 ml). After boiling for 1 min, with thorough stirring to ensure homogeneity, the mixture was allowed to cool to 60°C–65°C, and 0.50 g USDA Vitamin Mix and 0.025 g aureomycin were added. The warm mixture was stirred carefully again, and poured into a petri dish and allowed to cool. The diet was kept at 5°C until use. The diet was divided in 18 lumps (1–1.5 g), each of which was placed in a 20 ml plastic beaker with a tight plastic lid (Nunc, Denmark). A piece of moist filter paper was placed on top of the diet. A healthy looking, 4-day-old, second instar DBM reared on *B. napus* was gently placed on the filter paper with a moist paint brush on day 0 of the assay period. The cup was closed with a lid, and kept at 24°C, L18:D6. On day 5, each larva was inspected for survival, and, if alive, the developmental stage was noted. The start of the pupal stage was defined as the time when the larva had left the diet and started constructing a cocoon, although strictly speaking this is the prepupal stage (Talekar and Shelton, 1993). The distribution of surviving larvae at the various stages was reported (Table 1). The dry weight content of leaves was close to 10%, and for calculations of gle (gram leaf equivalent), it was assumed throughout that the ratio of dry weight to fresh weight was 1:10.

*Isolation of **1**.* Leaves of the G-type (accession B1) were air-dried, and the petioles were discarded. Dry leaflets (50 g = 500 gle) were blended to a fine powder, and 250 ml of boiling 70% aq. MeOH were added. After 1 min of heating, the mixture was poured through filter paper, and the residue was extracted with an additional 500 ml of warm 70% MeOH. The extract was centrifuged for 20 min

TABLE 1. BIOASSAYS FROM THE ACTIVITY-GUIDED ISOLATION OF A GROWTH-INHIBITING FRACTION

	Survival (%)	Number of larvae at various instars after 6 days				Sign.[a]
		II	III	IV	P	
A: Extraction and initial separation steps[b] (1 gle/ml[c])						
Blind	100	0	0	4	14	
Crude extract	100	0	15	3	0	***
Post DEAE supernatant	89	0	2	6	8	—
Post DEAE precipitate (*2 gle/ml*[c]):	94	0	3	11	3	n.s.
Post DEAE precipitate	100	0	9	9	0	*
B: Open column fractionation of redissolved post DEAE precipitate[d] (2.8 gle/ml[c])						
f 0–9	89	0	0	3	13	
f 10–13[e]	83	0	9	6	0	**
f 14–20	100	0	1	14	3	**
f 21–22	100	0	0	6	12	n.s.
f 23–31	100	0	0	5	13	n.s.
f 32–40	89	0	0	10	6	*

[a] For each fraction or extract, 18 larva were tested. Statistical significance of differences in the distribution of instars II to IV was analyzed as pairwise comparisons by Fishers exact test. Significance levels (sign.) are indicated with asteric as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s.: not significant;—not calculated.

[b] Statistical significance relates to the data in the preceeding line.

[c] Gle indicates gram leaf equivalents, on a fresh weight basis, and gle/ml indicates the amount of plant material or fraction per milliliter of diet.

[d] *f* indicates fraction number. Statistical significance relates to *f* 0–9. **1** was mainly present in *f* 10–13.

[e] Instar distribution significantly different** from f14–20.

at 20°C, 5000 g, to provide the crude extract, which was quickly passed through a DEAE Sephadex column in the acetate form (13 g dry wt. DEAE Sephadex A-25 equilibrated with 0.5 M AcOH–NaOH, pH 5.0, washed with water, and finally equilibrated with 70% aq. MeOH). Use of a column allowing a high flow rate seemed essential to avoid precipitation on the column. The column was washed with two column volumes of solvent, and the eluant was left in a crystallization beaker for evaporation of MeOH. When MeOH had evaporated, the precipitate (ppt.) was obtained by centrifugation for 20 min at 5°C and 10,000 g and freeze dried. The ppt. was dissolved in a minimum of warm MeOH (75 ml), and reprecipitated by addition of 300 ml of cold water and incubation at −5°C. The final ppt. was dissolved in 450 ml of 70% aq. MeOH with moderate heating. The solution was applied to a C18 open column equilibrated in 70% aq. MeOH. For the purification, which was followed by bioassays, 150 gle of redissolved ppt. was applied to a 2.5 × 38 cm column, which was washed with 100 ml 70% MeOH. The run-through and wash were collected as fraction 0. The column was eluted with a 300 ml linear gradient from 70% to 100% MeOH, then a 400 ml linear

gradient from 100% MeOH to 100% ethylacetate, and finally 100 ml of ethyl acetate. Fractions of 20 ml were collected, and 1/15 of each fraction was taken for TLC analysis. A suitable volume was applied to the TLC plate, which was eluted in EtAc–MeOH–H_2O (100:13.5:10). The dry plate was developed with freshly mixed 1% vanillin in EtOH-10% sulfuric acid in EtOH (1:1) and heated at 100°C for 5–7 min. Compound **1** appeared as a bright violet spot with an R_f of ca. 0.3. Fractions (remaining 14/15) were pooled guided by the TLC results, and each pool (140 gle) was used for bioassay (Table 1, part B). For later preparative purification of **1**, about 500 gle of redissolved ppt. were applied to a 2.5 × 100 cm C18 column, which was eluted first with 150 ml of 70% MeOH, then a 1200 ml linear gradient from 70% to 100% MeOH, and finally 3000 ml of MeOH. Fractions with **1** were pooled, and the solvent was evaporated to yield a pale yellow material. Final purification was obtained by repeated preparative HPLC with an analytical C18 column, with the gradient described below for the HPLC analysis of **1**. Fractions were collected, guided by the absorption at 200 nm; TLC showed that **1** coeluted with a peak at ca. 15 min. Fractions pure in TLC and analytical HPLC were pooled and submitted to NMR, MS, and LC-MS.

NMR Spectroscopy and MS. NMR spectra were recorded on a Bruker Avance 400 instrument as previously described (Agerbirk et al., 2001b), except that the solvent was CD_3OD and the chemical shifts were based on TMS as the internal reference (0 ppm). Abbreviations used for 2D experiments were explained previously (Agerbirk et al., 2001b), except the J-resolved ^{1}H NMR spectrum, abbreviated JRES. Mass spectra were obtained as previously described (Agerbirk et al., 2001b).

*Hydrolysis of **1**, and Analysis by GC-MS.* Ten μl of the solution used for NMR were evaporated to dryness. The substance was then hydrolyzed by addition of 20 μl 1 M aq. trifluoroacetic acid and 10 μl MeOH, followed by heating in a 110°C heating block for 1 hr. After evaporation to dryness and trimethylsilylation in 30 μl pyridine/*N*,*N*-bis-trimethylsilyl-trifluoroacetamide 1:1, the sample was subjected to GC-MS. Oleanolic acid and glucose were trimethylsilylated similarly and run separately for comparison. The GC oven temperature program was as follows: 80°C for 2 min, 80°C to 300°C at 20°C/min, 300°C for 20 min. The ion source was run in EI mode (70 eV) at 200°C. Retention times for the trimethylsilyl derivatives of glucose were 11.6 min and 12.5 min and 39 min for oleanolic acid.

*Analysis of **1** in Leaf Material.* A crude leaflet extract of 0.1 g dry wt. in boiling 70% MeOH was prepared as previously described for glucosinolate extraction (Agerbirk et al., 2001a). Eighty percent of the crude extract in 70% aq. MeOH was loaded onto a C18 SPE column, and the column was washed with 3 × 3 ml of 70% aq. MeOH. The wash was discarded, since it did not contain **1** in control experiments. The column was then eluted with 5 × 1 ml of 90% MeOH, which was collected and evaporated to dryness under an airstream. The eluate was redissolved

in 300 μl of MeOH, and 10 μl of this solution were injected into the HPLC. The following HPLC program was used (1 ml/min): 40 min linear gradient from 40% to 85% B, 8 min of 85% B, 5 min gradient from 85% to 40% B, 5 min of 40% B, where A is water and B is MeCN. Detection was at 200 nm, and diode array spectra from 190 to 400 nm were recorded. The UV spectrum of **1** showed only a shoulder at 200 nm, and no significant absorption above 225 nm. Quantification of **1** in the samples was based on comparison with a standard of **1**, which was quantified by NMR as follows.

An amount of **1** was dissolved in 5 ml of MeOH, and 200 μl of the solution was transferred to a small-size HPLC vial for use as the HPLC standard. The remaining 4.8 ml of the solution of **1** were mixed with 1.0 μmol of benzyl glucosinolate. The solvent was evaporated, and the residue was dissolved in CD_3OD and analyzed by ^{1}H NMR. The peak area of the two anomeric saponin hydrogens at 4.4 ppm divided by the peak area of the two methylene hydrogens in benzylglucosinolate at 4.1 and 4.2 ppm was 2.7, indicating that 2.7 μmol of the saponin were present. The concetraction of **1** in the HPLC standard was, thus, 2.7 μmol/4.8 ml = 0.56 μmol/ml. In the calculation of concentrations of **1** in leaf material, it was assumed that the analytical recovery of **1** was 100%. The method's limit of detection was estimated from integration of small baseline variations in P-type chromatograms, assumed to be due to noise, at and near the expected retention time of **1**.

*Seasonal Variation in DBM Resistance, and Correlation with **1**.* Three accessions, B4 (P-type), B15 (G-type), and B44 (G-type), were grown in a common garden in Lyngby, Copenhagen, Denmark. Leaves were picked on August 30 and October 28, 2001, and randomly divided into two batches. One batch was freeze-dried for later analysis. From the other batch, 8 leaves were tested for resistance to DBM second instars reared on oilseed rape: Each leaf was placed in a plastic container, a piece of moist filter paper was placed above the leaf, and five larvae were gently placed on the filter paper. Surviving larvae were counted after a 4-day incubation at 24°C, L18:D6. The resulting data are reported in Table 2. Leaflets from each freeze dried sample were divided into fine pieces, and the resulting material was analyzed for **1** in duplicate. Chemical data for the samples in Table 2 are reported in Figure 4.

Statistical Analysis of Correlation. Correlation between DBM survival and the level of **1** was tested statistically with a logistic regression model, with the concentration of saponin as a covariate, accession as a factor, and leaf as a random effect (see, for instance, Collett, 1991, section 6.7). Saponin concentrations less than the detection limit (0.005 μmol/g dry wt.) were assumed to be zero in the statistical calculations. The equation of the general correlation curve was $\log\left(P_i/(1-P_i)\right) = a\ (\text{accession}_i) + b\ (\text{accession}_i)x_i + \varepsilon(\text{leaf}_i)$, where P_i is the survival probability for the i'th larva and accession_i, x_i, leaf_i are the accession, saponin concentration, and the leaf corresponding to the ith larva. Here, we assume that the εs are independent and $N(0,\sigma^2)$ -distributed.

TABLE 2. SURVIVAL OF DBM LARVAE WHEN OFFERED LEAVES OF *B. vulgaris* VAR. *arcuata* COLLECTED IN LATE SUMMER OR MID FALL

			August 30		October 28	
Accession	Type	Origin	*S* (%)	*N*	*S* (%)	*N*
B4	P	Denmark	95.0	40	87.5$^{n.s.}$	40
B15	G	New York	12.5	40	65.0**	40
B44	G	Denmark	5.0	40	82.5***	40

Note: Survival (S) of second instars on *Barbarea* leaves, collected on either August 30 or October 28, 2001, was examined after a 4-day incubation. Accession, type, and geographical origin of the seeds are indicated. N = number of larvae tested, at 5 larvae per leaf. For each accession a logistic regression model with random effect of leaf and fixed effect of date was used. P-values of the approximate F-test are indicated as follows: n.s. = not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Performance and Behavior of DBM Larvae on Resistant and Susceptible B. vulgaris Types. Leaves of greenhouse-grown G-type plants were resistant to neonate DBM larvae, while P-type leaves were susceptible (Figure 1). Indeed, growth and survival on the P-type leaves was comparable to the performance on the favorable host plant *B. napus* (survival to pupation and to the adult stage on

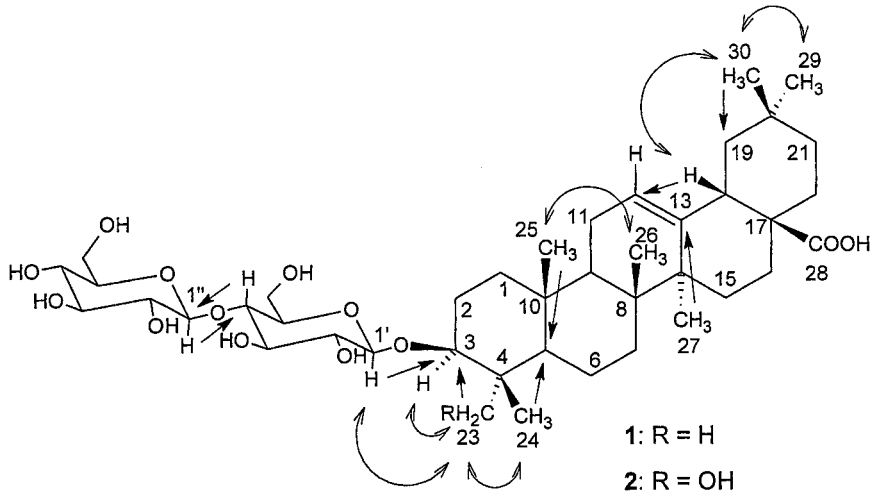

FIG. 3. The structures of saponins **1** and **2**. The numbering system used for reporting the NMR data is indicated. Selected NOE (curved arrows) and HMBC (straight arrows) long-range interactions that confirm the structure of **1** are indicated.

P-type and *B. napus* not significantly different by chi-square test). In contrast, no larvae survived to pupation on G-type leaves, and the lower survival on the G-type compared to the P-type was statistically significant (chi-square test, $P < 0.001$). Biting marks indicating initiation of feeding were observed for 53% of the neonate larvae offered G-type leaves. However, only 11% of the larvae offered G-type leaves continued feeding. The lower rate of continued feeding on the G-type compared to the P-type was statistically significant (chi-square test, $P < 0.001$). G-type leaves were likewise resistant to older DBM larvae transferred from *B. napus*, while P-type leaves were fully accepted as food plant (Figure 2). For the older larvae, initiation of feeding could be determined with higher certainty, and it was clear that most or all larvae took at least one or a few bites before rejection took place. The frequencies of initiation of feeding by older larvae on the P- and G-types were obviously not statistically different, since only one larva (on the G-type) did not initiate feeding (Figure 2). The frequency of continued feeding, however, was significantly different for the two types (chi-square test, $P < 0.001$). The mechanism of resistance seemed to be an inhibition of continued feeding, which is referred to as deterrency. When other food sources were not available, DBM larvae eventually died without substantial feeding from the resistant G-type leaves, although repeated initiation of feeding sometimes took place.

Activity-Guided Isolation of a Deterrent Fraction. Addition of the crude 70% methanolic extract to the artificial diet resulted in a significant reduction of larval growth rate, compared to that on the pure wheat germ diet (Table 1, part A). Subsequent fractionation was designed to isolate compounds responsible for this growth-inhibiting activity. A partially purified active subfraction free from the abundant glucosinolates and flavonoids was obtained in the following way. By passage through a DEAE Sephadex anion exchange column, acetate form, glucosinolates were exchanged with acetate. Evaporation of MeOH from the post DEAE extract resulted in a pale brown precipitate and a syrupy aqueous supernatant. The supernatant contained a complex mixture that included flavonoid glycosides (Brown et al., unpublished). When bioassayed, the precipitate resulted in a numerically, but statistically not significant ($P = 0.17$), lower growth rate than the supernatant. The precipitate resulted in a dose-dependent lowered growth rate, and the dose-dependency was statistically significant ($P < 0.05$, Table 1, part A), showing that the precipitate had growth-inhibiting activity.

The active precipitate could be redissolved in MeOH, and reprecipitated by addition of water, without significant loss of activity (data not shown). The redissolved precipitate was subjected to TLC and developed with vanillin-sulfuric acid, resulting in several yellow and grey spots, as well as two distinct violet spots with R_f values of 0.3 (later identified as **1**) and 0.6, as expected for saponins or other terpenoid glycosides (Wagner and Bladt, 1996). The faster moving of these TLC bands with R_f 0.6 was not identified, and is named compound B in the rest of this paper. Separation of **1** and compound B from other compounds was

achieved by open column chromatography on a C18 column, and fractions yielding the violet TLC bands were pooled as indicated in Table 1, part B. When the resulting pooled fractions were subjected to bioassays, the main growth-inhibiting activity occurred when **1** was present (Table 1, part B), while fractions containing compound B (Fractions 21 and 22) and other fractions showed lower or no activity.

*Final Isolation of **1***. HPLC of the open column fractions that contained **1** showed a complex mixture of UV absorbing compounds. Final purification of **1** required repeated preparative HPLC, which could not be activity-guided, because of the low capacity of the analytical HPLC, and yielded about 5 mg of **1**.

*Structural Elucidation of **1***. A combination of one- and two-dimensional NMR techniques and comparison with the reported structure and NMR data of **2** led to the structure 3-*O*-β-cellobiosyloleanolic acid (Table 3, Figure 3). MS showed the molecular weight of **1** to be 780 (m/z for $[M+Na]^+ = 803$, m/z for $[M+K]^+ = 819$). ^{1}H NMR showed signals from two anomeric sugar protons (δ 4.35 and δ 4.41), indicating that **1** was a glycoside. The β-cellobiosyl moiety was identified as follows: Starting from 4.35 ppm, COSY identified the protons of the first glucoside moiety, and a ^{1}H-^{13}C-correlated spectrum (HSQC) identified the corresponding carbon signals. Starting from 4.41 ppm, signals for the second glucoside moiety were similary identified. HMBC and NOESY confirmed the assignments of the glucoside signals. HMBC proved that the two glucosides were linked by a $4 \rightarrow 1$ bond, as ^{1}H-^{13}C long-range coupling from position $1''$ to $4'$ and vice versa was detected (Figure 3). The coupling constants of all sugar protons except $5'$, $3''$, and $5''$ were resolved by JRES, and the coupling constants of the three unresolved signals could be deduced from the coupling constants of the neighboring protons. Since the coupling constants among the protons $1'$–$5'$ and $1''$–$5''$ were all large, about 8 Hz, the disaccharide moiety was composed of glucose units, and the configuration of both anomeric centers was β. The connectivity of the β-cellobiosyl moiety to C3 of the aglycone (δC 91.0) was shown by HMBC (Figure 3). Finally, a combination of HMBC, HSQC, and NOESY proved the structure of the fragment C3—C4—(C5; C23; C24) and corresponding protons (Figure 3), but further structural elucidation was difficult because of the crowded signals. At this point, the structure and NMR data of **2** were published (Shinoda et al., 2002), and we used these data as a model for the further structural elucidation. Compared to **2**, the molecular weight of **1** (780) was 16 lower. The elucidated fragment contained one less oxygen than the corresponding part of **2**, suggesting that the remaining parts of **2** and **1** were identical. The NMR data were in agreement with this (the chemical shifts of the carbon atoms agreed well, even though our spectrum was measured in CD_3OD, while the spectrum of **2** was measured in deutero pyridine). The assignments in Table 2 were based on analogy to the ^{13}C spectrum of **2**, supported by HMBC and NOESY to detect long-range interactions (Figure 3), DEPT to classify carbon signals as quaternary, secondary, or primary/tertiary, and HSQC to identify corresponding carbon and

TABLE 3. ^{1}H AND ^{13}C NMR DATA FOR SAPONIN **1** AND ^{13}C NMR DATA FOR AUTHENTIC OLEANOLIC ACID

	Oleanolic acid	Saponin **1**	
Assignment	δ C (ppm)	δ C (ppm)	δ H (ppm)
1	39.9	39.8	ca. 0.97[c]; ca. 1.62[c]
2	27.9	27.1	N.D.
3	79.8	91.0	3.49 (tr., 8 Hz)
4	39.9[a]	40.2[a]	—
5	56.8	57.1	0.78[c]
6	19.5	19.4	N.D.
7	33.9	34.1	N.D.
8	40.6[a]	40.6[a]	—
9	49.1[b]	ca. 49.0[c]	ca. 1.58[c]
10	38.2	37.9	—
11	24.6	24.6[aa]	N.D.
12	123.7	123.4	5.24 (tr., 3 Hz)
13	145.2	145.6	—
14	42.9	43.0	—
15	28.9	29.0	N.D.
16	24.1	24.2[aa]	N.D.
17	47.7	47.5	—
18	42.8	43.0	2.87 (br. d., 11 Hz)
19	47.3	ca. 47.7[c]	ca. 1.1[c]; ca. 1.67[c]
20	31.7	31.7	—
21	34.9	35.1	N.D.
22	34.1	34.1	N.D.
23	28.8	28.6	1.05 (s.)
24	16.4	17.0	0.85[c] (s.)
25	15.9	16.0	0.95[c] (s.)
26	17.8	17.9	0.83[c] (s.)
27	26.4	26.4	1.15 (s.)
28	181.9	183.1[d]	—
29	33.6	33.7	0.89 (s.)
30	24.0	24.1	0.94[c] (s.)
1′	—	106.7	4.35 (d., 8 Hz)
2′	—	75.4	3.24[e] (tr., 8 Hz)
3′	—	76.3	3.49[e] (tr., 8 Hz)
4′	—	80.7	3.56[e] (tr., 8 Hz)
5′	—	76.7	3.37[f]
6′	—	62.0	3.85[e] (d., 3 Hz)
1″	—	104.6	4.41 (d., 8 Hz)
2″	—	75.0	3.21[e] (tr., 8 Hz)
3″	—	77.9	3.37[f]
4″	—	71.4	3.29[e] (tr., 8 Hz)
5″	—	78.2	3.33[f]
6″	—	62.5	a: 3.65[e] (dd., 12 Hz; 5 Hz) b: 3.87[e] (dd., 12Hz; 2 Hz)

Note: [a] & [aa] Assignments can be interchanged. N.D.: Not determined.
[b] Chemical shift from DEPT spectrum.
[c] Chemical shifts extracted from HSQC spectrum.
[d] Very weak signal.
[e] Chemical shifts extracted from JRES.
[f] Chemical shifts extracted from COSY, coupling constants could not be measured because of overlap with neighboring signal or solvent signal at 3.3 ppm.

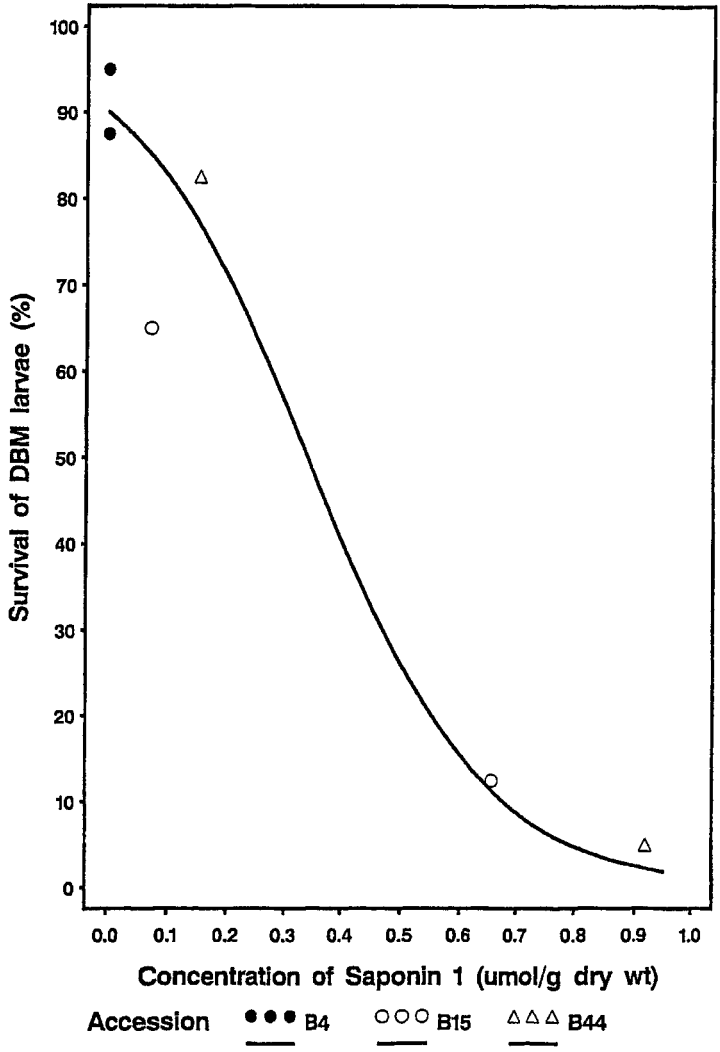

FIG. 4. Correlation between levels of saponin **1** and DBM larval survival in bioassays with leaves from three accessions. The estimates (with approximate standard errors) used to draw the durve were $a = 2.2008$ (0.3328); $b = -6.4853$ (0.8547).

proton signals. As expected, the chemical shifts of C3, C4, C5, C23, and C24 differed significantly between **1** and **2** because of the difference in hydroxylation at C23. The weak signal in our ^{13}C NMR spectrum at 183.1, which was assigned to C28 (carboxylate C), differed by 3 ppm from the carboxylate signal in **2**.

To further test the proposed structure, we examined authentic oleanolic acid by NMR using the same conditions and solvent as for **1**. The signal of the carboxylic acid C of oleanolic acid was almost as intense as the remaining signals, and was found at 181.9, which is only 1.2 ppm lower than the corresponding signal in the spectrum of **1**. The signal of C3 of oleanolic acid was detected at 79.8 ppm, which is 11.2 ppm lower than for **1**. This large difference was expected for the glycoside compared to the aglycone. Chemical shifts of the remaining carbons of oleanolic acid differed between 0 and 0.8 ppm from the corresponding chemical shifts found for **1**, to further support the proposed structure. Finally, a sample of **1** was hydrolyzed and the products trimethylsilylated. GC-MS of the resulting mixture showed the presence of trimethylsilylated oleanolic acid and glucose, since the retention times as well as EI mass spectra agreed with those of authentic references.

*Analysis of G-Type and P-Type Leaves for **1**.* We tested whether variations in the level of **1** could explain the biological phenomenon described in the first part of this paper, i.e., the difference in DBM resistance between the G-type and the P-type. Indeed, high levels of **1** were found in leaves of the G-type (mean 1.5 μmol/g dry wt., $SD = 0.2$, $N = 5$), while **1** was not detected in the susceptible leaves of the P-type. Variation of the amount of G-type leaf material extracted, from 10 to 200 mg dry wt., showed that the HPLC peak area of **1** was proportional to the amount of leaf material, confirming the linearity of the entire analytical procedure.

Seasonal Variation in B. vulgaris Resistance to DBM. Since we had previously found a seasonal variation in flea beetle resistance for the G-type (Agerbirk et al., 2001a), we carried out a similar experiment where DBM larvae were offered G-type or P-type leaves collected in either the late summer or the late fall from plants grown in a common garden (Table 2). G-type leaves collected in August 2001 were almost completely resistant to second instar DBM larvae. However, survival of larvae was significantly higher on those G-type leaves collected in October 2001, for both the Danish accession B44 and the New York accession B15. In contrast, P-type leaves were equally susceptible to DBM larvae in August and October.

*Seasonal Correlation of DBM Resistance and **1**.* Saponin **1** was not detected in the P-type; the estimated limit of detection was 0.005 μmol/g dry wt. In the G-type, levels of **1** were higher in August than in October. The hypothesis that the level of **1** was correlated with DBM resistance was tested statistically (Figure 4). In our experiments, larval survival rather than mortality was actually measured. For this reason, we tested statistically whether *survival* and levels of **1** were (negatively) correlated. There was no effect of the *Barbarea* accession (G-types: B15, and B44, P-type: B4) on the slope ($P = 0.38$). Subsequently, there was no effect of accession on the intercept ($P = 0.12$). The slope parameter was significantly different from zero ($P < 0.001$), indicating a strong correlation between the concentration of saponin **1** and survival of DBM larvae.

DISCUSSION

Variation in levels of **1** explained both the difference between the two plant types, and the seasonal variation in DBM resistance. This result suggests that **1**—possibly in combination with additional, related saponins—constitutes the chemical basis of the resistance of the G-type to DBM larvae. Correlation of levels of a 3-*O*-β-cellobiosyl triterpenoid saponin and insect resistance in *B. vulgaris* supports the results reported by Shinoda et al. (2002). However, the fact that the saponin isolated by us is structurally different from that isolated by Shinoda et al. (2002) may indicate that the insect resistance of *B. vulgaris* could be due to several structurally related saponins, or that *B. vulgaris* var. *arcuata* (G-type) and *B. vulgaris* var. *variegata* contain different saponins. The finding that the P- and G-types differ in saponin content may prove useful in attempts to identify the genes and enzymes resposible for saponin biosynthesis in *B. vulgaris*, and hybridization experiments with the two plant types have been initiated.

The levels of **1** reported here, around 1.5 μmol/g dry wt. in fully resistant leaves of the G-type, are lower than the content of **2** in *B. vulgaris* var. *variegata* (1.1 mg/g fresh wt.) reported by Shinoda et al. (2002), which can be recalculated to about 13 μmol/g dry wt. Whether **2** was present in our *B. vulgaris* (G-type) foliage is not known. The unidentified, minor compound B detected by TLC in this work was apparently less hydrophilic than **1**, and so was probably not identical to **2**.

The isolation of **1** included passage through an anion exchange column, to remove glucosinolates, and so it may seem surprising that a carboxylic acid passed through this column at the near-neutral pH of a crude extract. However, this may be explained by the tendency of saponins to form micelles. The carboxylate group could be shielded by the micelle, or the pK_a of the carboxylic acid group could be unusually high because of the apolar conditions inside the micelles. It is likely that the anion exchange step can be omitted from the isolation procedure, but this has not been tried. It is possible that our inability to detect **2** in the precipitate after the anion exchange step was due to binding of this saponin to the anion exchange column. We did not try elution and test of bound material from the column, since salts in such eluates were likely to interfere with the bioassay. The recovery of growth-inhibiting activity in the anion exchange step cannot be calculated from our data, because of the separate bioassay of the precipitate and supernatant from the post-DEAE fraction. Thus, further experiments are needed to test whether **2** co-occurs with **1** in the G-type.

It seems likely that **1** is a DBM deterrent in G-type foliage, although the evidence presented here is mainly correlative. Structure–activity studies of saponins as DBM deterrents would, therefore, be useful. Since the P- and G-types differ in resistance to DBM and the flea beetle *P. nemorum* in the same way (Agerbirk et al., 2001a), it seems likely that saponins are also responsible for resistance to the flea beetle, but this hypothesis remains untested. We have recently presented evidence

for a common mechanism of flea beetle resistance in *B. vulgaris* (G-type), *Barbara verna*, and *Barbara intermedia* (Agerbirk et al., 2003), and it would be relevant to investigate whether these species contain **1**, **2**, or additional saponins.

Toxic effects of saponins to various organisms appear to be linked to their interaction with biological membranes, and may be related to their soap-like properties (Osbourn, 1996; Osbourn et al., 1998). Thus, detoxification of saponins is likely to involve enzymatic hydrolysis of the glycosidic bonds, as already shown for fungi (e.g., Bowyer et al., 1995; reviewed by Osbourn, 1996; Osbourn et al., 1998). Several crucifer specialist insects, such as *Pieris brassicae* and *P. rapae* (Chew, 1981; Agerbirk, unpublished) and *P. nemorum* with R-genes (Nielsen, 1999; de Jong et al., 2000), are insensitive to the defenses of *B. vulgaris*. The elucidation of the structures of saponins in *B. vulgaris* (Shinoda et al., 2002; this work) should allow for investigations into the mechanism by which these insensitive insects can cope with the potentially toxic saponins.

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PYRROLIZIDINE ALKALOIDS AS OVIPOSITION STIMULANTS FOR THE CINNABAR MOTH, *Tyria jacobaeae*

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Abstract—In choice experiments with artificial leaves, we tested related pyrrolizidine alkaloids (PAs) for their stimulatory effects on the oviposition of the cinnabar moth, a specialist on the PA-containing plant *Senecio jacobaea*. The PAs from *S. jacobaea* that we tested stimulated oviposition. Monocrotaline also stimulated oviposition although this PA is not found in plants of the genus *Senecio*. The moths preferred ovipositing on filter paper with a PA mixture extracted from *S. jacobaea* to ovipositing on filter paper with single PAs. Senkirkine, heliotrine, and retrorsine did not stimulate oviposition. The nonactive retrorsine differs only in one OH group to the active senecionine, indicating that small structural differences alter the stimulatory activity of PAs. However, a PA mixture extracted from a nonhost plant, *Senecio inaequidens*, that consisted of 81% of the nonactive retrorsine did stimulate oviposition. Oviposition preferences between *Senecio* species seem to be determined by chemical compounds other than PAs.

Key Words—Arms-race hypothesis, plant–insect interactions, chemical diversity, oviposition, stimulants, *Senecio jacobaea*, pyrrolizidine alkaloids.

INTRODUCTION

The oviposition behavior of Lepidoptera is determined by a complex set of visual, olfactory, and chemical cues (Papaj and Rausher, 1983). After locating the potential host plant through visual and olfactory cues, acceptance of the host plant often depends on chemotactile stimuli (e.g., Myers, 1969; Miller and Stickler, 1984). Leaf allelochemicals can be important oviposition stimulants for specialist butterfly and moth species (Schoonhoven, 1972; Feeny et al., 1983; Honda, 1995). For example, glucosinolates are known to stimulate oviposition for *Pieris* species (van

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Loon et al., 1992; Renwick et al., 1992; Huang and Renwick, 1994); glycosides and flavonoids in *Citrus* species are oviposition stimulants for some *Papilio* species (Ohsugi et al., 1985; Honda, 1986; Honda, 1995 and references therein); and cnicin, a sesquiterpene lactone, stimulates oviposition for the specialist moth *Pterolonche inspersa* (Landau et al., 1994).

The stimulatory response of specialist lepidopteran species to allelochemicals in their host plants presents an important dilemma for the plant. Plant secondary metabolites may deter generalist herbivores while attracting specialist herbivores (Linhart, 1991; van der Meijden, 1996). Many plant species display a diversity of secondary metabolites, and often a variety of structurally related compounds can be found within a plant. The evolution of this staggering diversity of compounds is still poorly understood. One possible explanation for the diversity of structurally related secondary metabolites is that new compounds evolve in a continuous evolutionary arms race between a plant and its specialist insect herbivores. In such an arms race, a plant that synthesizes new compounds is able to escape herbivory and the insect herbivores, in turn, adapt to these compounds (Ehrlich and Raven, 1964). This coevolutionary model implies that structurally related compounds differ in their effects on specialist herbivores. Most emphasis has been on the toxicity of related compounds when discussing the arms-race hypothesis. However, for specialists, for which related compounds are mostly not toxic, as well as for generalist herbivores, for which related compounds are mostly all toxic, there is little evidence indicating differences in toxicity of structurally related compounds. Alternatively, related compounds may differ in their stimulatory effect on specialists. Thus, within the evolutionary arms race, plants may be able to (temporarily) overcome the dilemma of contrasting selection pressures of specialists and generalists if a new compound evolves that masks the plant for specialists and still deters generalist herbivores. Under this model, it is expected that structurally related compounds differ in the stimulation of oviposition of specialist lepidopteran species. The response to plant secondary metabolites by specialist lepidopteran species can be highly specific, acting only on specific compounds or combinations of compounds, or can be more general, acting on a certain group of compounds (Honda, 1995). Huang and Renwick (1994) found that all the aromatic glucosinolates they tested, except for glucosinalbin, stimulated oviposition by *Pieris rapae*, while the tested aliphatic glucosinolates did not stimulate oviposition. For another *Pieris* species, *P. napi oleracea*, structurally related glucosinolates differed in their stimulatory activity and there was no clear-cut relationship between activity and structure.

An excellent system for studying the evolution of related secondary metabolites are the various pyrrolizidine alkaloids (PAs) in *Senecio jacobaea*. This species can contain more than 10 structurally related pyrrolizidine alkaloids (Witte et al., 1992), and variation in PA composition in *S. jacobaea* is at least partly genetically determined (Vrieling et al., 1993). PAs can be deterrent to generalist herbivores (Van Dam et al., 1995; Hägele and Rowell-Rahier, 2000). In contrast, the specialist *Tyria jacobaeae* (cinnabar moth) sequesters PAs from its host plant *S. jacobaea*

(Aplin and Rotschild, 1972; Rotschild et al., 1979), and all life-stages of *T. jacobaeae* contain pyrrolizidine alkaloids (van Zoelen and van der Meijden, 1991). If specialist herbivores, like the cinnabar moth, have been selective forces in the evolution of different PAs, we expect that structurally related PAs differ in their effects on the cinnabar moth. Larval performance of *T. jacobaeae* did not differentiate between *S. jacobaea* plants with different PA composition (Macel et al., 2002). *T. jacobaeae* can detoxify PAs from *S. jacobaea* through N-oxidation by an enzyme that must have been recruited during the coevolutionary adaptation of *T. jacobaeae* to PAs (Lindigkeit et al., 1997; Naumann et al., 2002). Little is known about the importance of PAs as feeding or oviposition stimulants for the cinnabar moth. Adding PAs to the leaves of *S. jacobaea* did not increase oviposition (van der Meijden et al., 1989), suggesting that PA concentration on the leaf surface is not important as a proximate factor in the selection of plants for oviposition. It is not clear if PAs in themselves can stimulate oviposition.

In this study, we addressed the following questions: (1) Do PAs stimulate oviposition for the cinnabar moth? (2) If they do, is there a relationship between stimulatory activity and structure of the PAs? (3) Are specific (combinations of) PAs preferred over others? (4) Do PAs stimulate oviposition at a range of concentrations? We tested whether or not a PA mixture from *S. jacobaea* and a PA mixture extracted from a nonhost plant species (*Senecio inaequidens*) stimulated oviposition by the cinnabar moth. We also tested several single PAs from *S. jacobaea* as well as related and nonrelated plant species for stimulating effects on oviposition. To determine whether other chemical compounds can also play a role in the oviposition preference of *T. jacobaeae*, we tested whether leafsap from the preferred host plant, *S. jacobaea*, and from the nonhost plant, *S. inaequidens*, stimulated oviposition.

METHODS AND MATERIALS

Tyria jacobaeae. Female moths of *T. jacobaeae* used in the experiments were either captured in the field or reared in the lab. Female moths were captured in the dunes of Meijendel, The Netherlands, in the summer of 2000 and 2001. Moths reared in the lab were collected from Meijendel as fifth (last) instar caterpillars in the summer of 1999 and 2000. Each caterpillar was put into a glass tube without food until pupation. Pupae were stored for 10 months in a cold growth chamber (photoperiod L8:D16, 4°C, RH 70%) for hibernation. Following hibernation, pupae were placed into another growth chamber (L16:D8, 20:15°C, RH 70%) to emerge. Prior to the oviposition experiments, male and female moths were kept together for at least 1 week to mate. Lab-reared moths used for the experiments were 1–3 weeks old. The age of the moths collected in the field was not known.

Pyrrolizidine Alkaloids. Most of the PAs, we used belong to the structural group of senecionine type PAs, except for heliotrine, which is a lycopsamine type

PA, and monocrotaline, which is a monocrotaline type PA (Hartmann and Witte, 1995). Senecionine-type PAs are mostly 12-membered, macrocyclic diesters with a retronecine or otonecine base. Lycopsamine-type PAs are monoesters or diesters containing a hydroxylated 2-isopropylbutyric acid as a necic acid. Monocrotaline-type PAs are 11-membered, macrocyclic diesters with a retronecine base (Hartmann and Witte, 1995). The structures of the pyrrolizidine alkaloids we used are given in Table 1. The pyrrolizidine alkaloid mixtures were extracted from flowering plants of *S. jacobaea* and *S. inaequidens* using a modified version of the method developed by Koekemoer and Warren (1951) as described by Hol and van Veen (2002). The composition of the PA mixtures (Table 2) was determined by GC (HP 6890, 30 m $\times$0.25 μm, HP-1). GC conditions were, injector 250°C, temperature program 0-22-5-250, split mode 1–30, carrier gas N_2 at 0.9 ml/min, pressure 56 kPa, detector FID. All observerd peaks coincided with known PAs. The PA mixtures were 99% pure as determined by a color reaction modified after Mattocks (1967). Monocrotaline and retrorsine were obtained from Sigma; senecionine, seneciphylline, and senkirkine were obtained from Roth; and heliotrine was obtained from LATOXAN, France. In the plant, PAs mainly occur as N-oxides (Hartmann and Toppel, 1987). It is not known whether PAs on the leaf surface are free-base PAs or PA N-oxides. We tested free-base PAs to determine whether they can serve as oviposition stimuli.

Oviposition Choice Experiments. All tests were performed in a growth chamber (L16:D8, 20:15°C, RH 70%). In two-choice experiments, we tested the preference of *T. jacobaeae* for PAs using artificial "leaves" composed of filter paper. Two white filter paper leaves (Whatmann 91, 10 cm long and 2 cm wide) were attached to the bottom of a glass vial (2.5 cm diam.) with a small piece of tape (Figure 1). The vials were partly filled with sand to secure stability and covered with a white lid.

PAs were dissolved in methanol (5 mg PA per 1 ml methanol). We added 100 μl of test solution, PAs (25 μg/cm^2) or methanol, to the artificial leaves. Single PAs were all tested against the control (methanol). PA mixtures were tested against the control and against each other. We also tested the preference of *T. jacobaeae* for the sap of *S. jacobaea* versus the sap of *S. inaequidens*. Saps were made by squeezing leaves of the two *Senecio* species and collecting 100 μl of the sap that was added to the artificial leaves. Further tests were performed with diluted pure saps, by adding 50% of demi-water to each sap. A 1:1 mixture of both undiluted saps was also made. Again, 100 μl of diluted sap or mixture of saps were added to the artificial leaves.

One vial with test solutions and one female *T. jacobaeae* were placed in to a transparent plastic cylinder (30-cm diam. 50-cm height) with top and bottom covered with gauze. The vials were placed in such a way that direction of the filter paper leaves was arranged randomly. Every 24 hr, the number of eggs on each filter paper leaf as well as the number of eggs elsewhere in the cage (i.e.,

TABLE 1. STRUCTURES OF PYRROLIZIDINE ALKALOIDS USED IN THIS STUDY

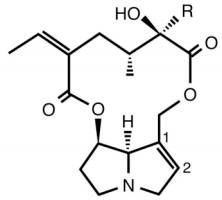
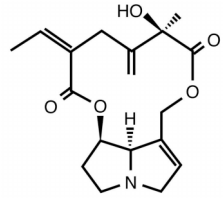
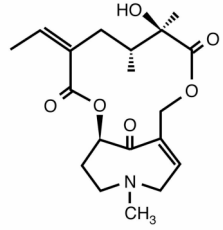
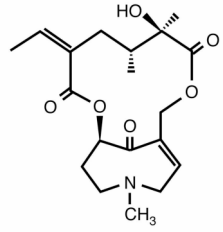
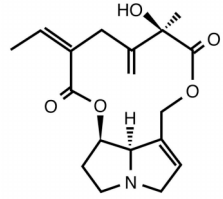
Name	Structure	Plant species
Senecionine: R=CH_3 Retrorsine: R=CH_2OH		*Senecio* sp., e.g., *S. jacobaea* (senecionine) *S. inaequidens* (senecionine + retrorsine)
Seneciphylline		*Senecio* sp., e.g., *S. jacobaea*
Senkirkine		*Senecio* sp., e.g., *S. vernalis*
Monocrotaline		*Crotalaria* sp.
Heliotrine		*Heliotropum* sp.

TABLE 2. PA COMPOSITION (% OF TOTAL PAS) OF MIXTURES EXTRACTED FROM *S. jacobaea* AND *S. inaequidens*

PA	S. jacobaea	S. inaequidens
Senecivernine		5.4
Senecionine	10.5	6.7
Integerrimine	4.6	3.6
Seneciphylline	17.4	
Jacobine	30	
Jacozine	3.6	
Jacoline	3.6	
Jaconine	25.2	
Retrorsine		81.3
Erucifoline	5.1	
Usaramine		1.9
Otosenine		1.1

other than on the paper leaves) was recorded. The cage covers an area of 50 × 70 cm, far greater than the surface area of the artificial leaves. Egg batches laid on the cage were considered neither a negative nor a positive response to our PA treatment since such egg batches could indicate that the moths had not encountered the artificial leaves. Each female was used only once. Experiments were replicated 15–21 times, depending on the number of female moths available.

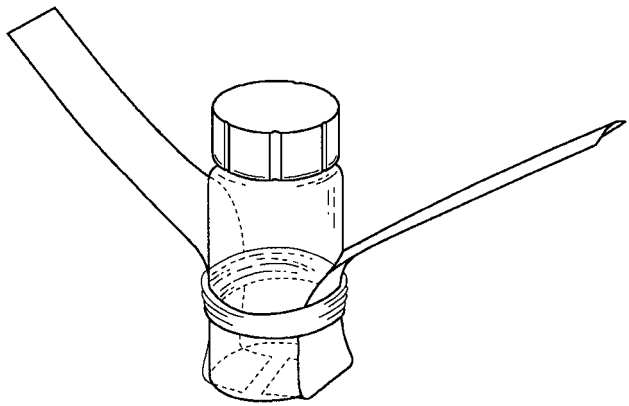


FIG. 1. Experimental design with artificial leaves. The glass vial is 6 cm in height and 2.5 cm in diameter, the filter paper leaves are Whatmann 91, 10 × 2 cm. The vial was placed in a transparent plastic cylinder with top and bottom of gauze. Test solutions were added to the artificial leaves.

RESULTS

On average (over all experiments), the moths laid 1.7 egg batches per female, and 23.9% of the moths did not oviposit at all. The percentage of females that did not lay any eggs did not differ among the experiments ($\chi^2 = 10.80$, $df = 14$, $P = 0.73$). Although egg batches were also found elsewhere in the cage, in most experiments more than 50% of the eggs were laid on the artificial leaves.

Oviposition Stimulation of PAs. Table 3 shows the results of the oviposition choice experiments using the different PAs against the control treatment. *T. jacobaeae* females strongly preferred to oviposit on filter paper with the PA

TABLE 3. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN PAS AND CONTROL

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
PAs *S. jacobaea*	1370	16	<0.001	18	Field
Control (methanol)	146	1			
Cage	160	8			
PAs *S. inaequidens*	374	13	0.002	17	Lab
Control	56	3			
Cage	164	10			
Senecionine	515	10	0.011	14	Field
Control	60	2			
Cage	111	6			
Retrorsine	438	7	0.705	11	Lab
Control	158	6			
Cage	189	8			
Seneciphylline[a]	363	13	0.024	11	Lab
Control	87	4			
Cage	175	6			
Senkirkine	356	10	0.102	9	Field
Control	64	2			
Cage	554	10			
Monocrotaline	939	11	0.009	10	Field
Control	0	0			
Cage	33	3			
Heliotrine	266	5	0.180	11	Lab
Control	117	2			
Cage	290	10			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on control and on PAs. The last column indicates if field or lab reared females were used. *N* = number of ovipositing females.

[a] 7.5 μg PA/cm^2 was applied instead of 25 μg/cm^2.

TABLE 4. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN PA MIXTURES FROM *S. jacobaea*, *S. inaequidens* AND SINGLE PAS

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
PAs *S. jacobaea*	351	11	0.180	14	Lab
PAs *S. inaequidens*	325	8			
Cage	143	8			
PAs *S. jacobaea*	654	17	0.020	12	Lab
Senecionine	162	7			
Cage	117	6			
PAs *S. jacobaea*	747	14	0.001	16	Lab
Monocrotaline	30	3			
Cage	38	6			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on PAs. The last column indicates if field or lab reared females were used. N = number of ovipositing females.

mixture from *S. jacobaea*, and also preferred the PA mixture from *S. inaequidens* to the control. In the experiments with single PAs, senecionine and seneciphylline had a stimulatory effect on oviposition. Both senecionine and seneciphylline are found in *S. jacobaea*. Monocrotaline was also preferred to the control. Interestingly, this PA is not found in *Senecio* species. Retrorsine, a PA found in *S. inaequidens* and other *Senecio* species but not found in *S. jacobaea*,did not stimulate oviposition of the cinnabar moth. Senkirkine and heliotrine also did not have a stimulatory effect on oviposition.

Preference for Specific PAs. Females did not distinguish between the two PA mixtures from *S. jacobaea* and *S. inaequidens* (Table 4). When females were given a choice between senecionine and the PA mixture of *S. jacobaea*, they preferred the mixture to the single senecionine. In addition, this PA mixture was also preferred to monocrotaline (Table 4).

Different Concentrations of PAs. The PA mixture from *S. jacobaea* stimulated oviposition at all three concentrations (Table 5).

Other Leaf Chemicals. Significantly more eggs and egg batches were laid on the filter paper with the sap of *S. jacobaea* than on the filter paper with sap of *S. inaequidens* (Table 6). The PA concentration in the *S. jacobaea* sap (223 μg/ml) was twofold higher than the PA concentration in the *S. inaequidens* sap (108 μg/ml), which might explain the oviposition difference. However, moths equally preferred the pure sap of *S. jacobaea* and a mixture of 50% *S. jacobaea* sap and 50% *S. inaequidens* sap, and preferred this mixture to the pure sap of *S. inaequidens* (Table 6). This is a clear indication that besides PAs other leaf chemicals in *S. jacobaea* also stimulate oviposition in the cinnabar moth.

TABLE 5. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN THREE CONCENTRATIONS OF THE PA MIXTURE FROM *S. jacobaea* AND CONTROL

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
PAs *S. jacobaea* 5 μg/cm^2	338	11	0.002	11	Lab
Control	0	0			
Cage	96	5			
PAs *S.jacobaea* 0.5 μg/cm^2	225	9	0.038	12	Lab
Control	22	2			
Cage	342	12			
PAs *S. jacobaea* 0.05 μg/cm^2	361	8	0.034	11	Lab
Control	6	1			
Cage	414	16			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on control and on PAs. The last column indicates if field or lab reared females were used. N = number of ovipositing females.

DISCUSSION

The attraction of Lepidoptera to PAs in food sources has been studied extensively (Pliske, 1975; Boppré, 1984, 1986; Schneider, 1987). To our knowledge, this is the second study that shows that PAs can also be oviposition stimuli for specialist lepidopteran species. Honda et al. (1997) showed that PAs from *Parsonia laevigata* stimulated oviposition by the danaid butterfly *Idea leuconoe*. In our study, the PAs from *S. jacobaea* stimulated oviposition by the cinnabar moth. Monocrotaline also stimulated oviposition even though this PA is not found in *S. jacobaea* or its relatives. Furthermore, the PA mixture from *S. inaequidens*, a nonhost plant, also stimulated oviposition. Our results show that the response of *T. jacobaeae* to PAs

TABLE 6. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN SAPS OF *S. jacobaea* AND *S. inaequidens*

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
S. jacobaea sap	1464	14	<0.001	14	Lab
S. inaquidens sap	2	1			
Cage	10	2			
50% *S. jacobaea* sap[a]	420	20	0.196	15	Field
Sap mixture	214	19			
50% *S. inaequidens* sap[a]	140	5	0.013	15	Field
Sap mixture	738	24			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on saps. The last column indicates if field or lab reared females were used. N = number of ovipositing females.
[a] In tests with sap mixtures no measurements were made of eggs on the cage.

is not specific, since nonhost PAs stimulated oviposition. Senkirkine, heliotrine, and retrorsine did not stimulate oviposition. Retrorsine differs only in one OH group at C-12 from senecionine, a PA that did stimulate oviposition. Remarkably, the PA mixture of *S. inaequidens*, which consists of 81% retrorsine (Table 1), did stimulate oviposition.The preference of *T. jacobaeae* for the *S. jacobaea* PA mixture over the single PAs senecionine and monocrotaline, and equal preference for both *S. jacobaea* and *S. inaequidens* PA mixtures, suggests that *T. jacobaeae* is most stimulated by (*Senecio*) PA mixtures irrespective of the composition of these mixtures.

It is unclear whether females of *T. jacobaeae* can detect PAs inside the leaf. The PA concentration we used in most of our experiments was comparable to the average concentration of 0.5% dry weight that is found inside the leaves of *S. jacobaea* plants (Vrieling et al., 1993). PAs are present on the leaf surface of *S. jacobaea* at an average concentration of 0.04 μg/cm^2 (Derridj and Vrieling, unpublished data). This concentration is comparable to the 1 μg (0.05 μg/cm^2) we used with different concentrations of PA mixture from *S. jacobaea* and which stimulated oviposition. It is not clear whether there is a threshold value for PA concentration or whether the stimulatory activity of PAs are concentration-dependent. The results of Van der Meijden et al. (1989) suggest that there is a threshold value for PA concentration since adding PAs to leaves of *S. jacobaea* did not increase oviposition. If there is a threshold, it must be at a concentration that is lower than 0.05 μg/cm^2 based on our results with different concentrations of the PA mixture of*S. jacobaea*.

In previous oviposition experiments with plants, we have shown that the cinnabar moth strongly preferred *S. jacobaea* to *S. inaequidens* (Macel et al., 2002). The cinnabar moth does not distinguish between the PA mixtures of *S. jacobaea* and *S. inaequidens* and, therefore, the oviposition preference of the cinnabar moth for *S. jacobaea* is not based on the PA composition of these plant species. The sap experiments suggest that the oviposition preference between these two *Senecio* species is determined by other chemical factors. A whole set of chemical cues likely determines the oviposition behavior of *T. jacobaeae*, of which PAs are only a part.

Although the attraction of the cinnabar moth to PAs in its host plant certainly presents an evolutionary dilemma for the plant, it is doubtful whether the cinnabar moth is a selective force in the evolution of the diversity of PAs in *S. jacobaea*. On one hand, small differences in structure can influence the stimulatory activity of PAs, as is shown by the active senecionine and non-active retrorsine. On the other hand, a nonhost plant PA of a different structure (monocrotaline) stimulated oviposition. Furthermore, the PA mixture of *S. inaequidens* with 81% of the non-active retrorsine still stimulated oviposition. By producing a new PA within its existing set of PAs, *S. jacobaea* might not be able to escape oviposition by the cinnabar moth.

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EFFECTS OF TWO PLANT SECONDARY METABOLITES, CINEOLE AND GALLIC ACID, ON NIGHTLY FEEDING PATTERNS OF THE COMMON BRUSHTAIL POSSUM

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Abstract—We investigated effects of two plant secondary metabolites (PSMs), cineole and gallic acid, on the nightly feeding behavior of the common brushtail possum (*Trichosurus vulpecula*), a generalist folivore. We tested whether possums altered their feeding behavior in response to increasing levels of cineole, a dietary terpene. Possums were fed artificial diets containing three levels of cineole; zero (basal diet), medium (6.8% of total dry matter, DM), and high (15.3% DM). In another experiment, we introduced gallic acid, a dietary phenolic, into the diets. Possums were offered a Choice PSM diet (cineole and gallic acid diets simultaneously) or a No-Choice PSM diet (containing either cineole or gallic acid). Detoxification products of cineole and gallic acid were examined in urine to determine that different detoxification pathways were utilized in the elimination of each compound. With increasing cineole levels, possums ate less, had smaller feeding bouts, and had a lower rate of intake, but did not extend their total nightly feeding time. Possums offered the Choice PSM diet, compared with the No-Choice diets, ate more, had larger feeding bouts, and tended to increase their rate of intake. Results from the urinary analysis indicated that gallic acid and cineole were not involved in competing detoxification pathways in brushtail possums. There was also a significant sex effect: females ate more overall, ate more per feeding bout, and ate at a higher rate than males. These results indicate that PSMs not only constrain overall intake, but that possums alter their feeding

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behavior in response to them. Altered feeding patterns may reduce the negative influence of PSMs on intake.

Key Words—*Trichosurus vulpecula*, plant secondary metabolites, cineole, gallic acid, feeding behavior, diet mixing, herbivore, generalist folivore.

INTRODUCTION

Plant secondary metabolites (PSMs) have been proposed to play a defensive role in protecting plants against herbivores (Fraenkel, 1959; Freeland and Janzen, 1974). To continue consuming a plant-based diet, herbivores must be able to deal with PSMs to avoid or minimize their deleterious effects. Strategies that herbivores use to cope with PSMs are a function of their physiological and behavioral capabilities (McArthur et al., 1991). Physiological strategies generally involve specific metabolic and detoxification pathways to detoxify and excrete PSMs (McLean and Foley, 1997). Herbivores may also possess specific postingestive feedback mechanisms affecting food selection and intake (Provenza, 1995). These feedback systems cause animals to increase energetic and nutrient consumption, and decrease toxic PSM intake (Provenza, 1995), thereby initiating behavioral responses.

On a broad scale, behavioral responses of herbivores to PSMs include avoiding plants containing them, eating a variety of plants to reduce the build-up of particular compounds, or selecting plants containing specific chemicals with which they are adapted to cope (Freeland and Janzen, 1974). The diet choice of herbivores is governed by a variety of factors, including nutritional requirements, an overall limit on the amount of food that can be processed within a given period, and the need to avoid ingesting large quantities of particular PSMs (Freeland and Janzen, 1974; Milton, 1979).

Freeland and Janzen (1974) hypothesized that, where possible, the feeding behavior of mammalian herbivores should "aim" to reduce the ingestion and impact of PSMs. Several studies have investigated the intake of herbivores in response to dietary PSMs (Pfister et al., 1997; Dearing and Cork, 1999), generally focusing on intake on a 24-hr basis. There has been limited work on more detailed behavioral responses of herbivores to PSMs within this time frame. Foley et al. (1999) described a preliminary model investigating intake in relation to the rate of elimination of a hypothetical PSM in brushtail possums. For a given total time period for feeding, and for a given PSM, simulations indicated that possums should be able to maximize intake using a range of feeding strategies. From these models, Foley et al. (1999) proposed that patterns of intake of plants containing PSMs within a 24-hr period are just as important as, and may even enhance or constrain, how much can be consumed by the herbivore overall. Relatively few investigations have attempted to quantify and directly measure the feeding behavior of herbivores, such

as the number and duration of feeding bouts in response to dietary PSMs (Morrison et al., 1992).

The general aim of this investigation was to determine patterns of feeding behavior in brushtail possums in relation to two PSMs, cineole and gallic acid.

The common brushtail possum (*Trichosurus vulpecula*), a generalist folivore, is perhaps the most adaptable of the Australian marsupials (Hume, 1999). It has an extensive distribution throughout a range of habitats, the widest of any Australian marsupial (Biggins, 1984; Hume, 1999). Brushtail possums do not have highly specific dietary preferences, and the dominant food species varies among habitat types (Kerle, 1984). Generally, their diet consists of *Eucalyptus* and often *Acacia* foliage (Owen and Thomson, 1965; Freeland and Winter, 1975; Fitzgerald, 1984). Brushtail possums are hindgut fermenters, further classified as caecum fermenters, with no selective retention of fine particles (Hume, 1999). They are nocturnal, hence the reference to "nightly" feeding behavior.

Cineole, a dietary terpene, is a major constituent of the essential oil in *Eucalyptus* species (up to 90%; Edwards et al., 1993; Stone and Bacon, 1994; Pass et al., 1999). Terpenes may act as toxins in a dose-dependent manner (Klaassen and Rozman, 1991). Brushtail possums naturally encounter cineole in their diet, largely from *Eucalyptus* species (Freeland and Winter, 1975).

Gallic acid is a low molecular weight phenolic. It occurs as gallic acid in plants, but also is a major product of the breakdown of gallotannins, which are simple esters of gallic acid commonly produced by plants (Hagerman and Butler, 1991; Hagerman et al., 1992). Its toxic effects have been demonstrated in rabbits, rats, sheep, and brushtail possums (Booth et al., 1959; Dollahite et al., 1962; Murdiati et al., 1992; Oswald, 1993). Gallic acid occurs in *Eucalyptus* foliage (Hillis, 1966a,b, 1967) and is naturally consumed by brushtail possums in the wild.

Increasing dietary cineole levels were used to examine detailed nightly feeding behavior of brushtail possums when they had no alternative food (i.e., no choice). We hypothesized that, with increasing cineole levels, possums would decrease their total intake, decrease their intake per feeding bout, and increase the time taken from their first to last feeding bout, including non-feeding activity. This alteration in feeding behavior was hypothesized to occur because detoxification pathways should reach a physiological constraint for dealing with the higher cineole levels. This, in turn, was expected to limit the amount and frequency with which food could be consumed, and to maintain cineole levels in blood plasma at or below the constraint level (Foley et al., 1999). Possums were also predicted to finish feeding later in the night with increasing cineole levels, in order to spread their feeding out over a longer period, again to maximize intake otherwise constrained by detoxification rates. Digestibility of the diet was also measured, in order to compare it with plant species commonly consumed by brushtail possums in the wild.

Feeding behavior was also compared when possums were offered either single PSMs (cineole or gallic acid) or a choice of PSMs (cineole and gallic acid). Presenting possums with a choice between different PSM diets allowed us to indirectly test the detoxification–limitation hypothesis. Freeland and Janzen (1974) hypothesized that generalist herbivores consume a varied diet to avoid overloading individual detoxification pathways, as mammalian herbivores are predicted to detoxify different classes of PSMs using separate metabolic pathways. When given the choice of diets containing either cineole or gallic acid, brushtail possums were hypothesized to eat both, rather than just one or the other diet. As a result, possums were predicted to increase total intake, and increase their intake per feeding bout compared with the No-Choice diets.

The choice of cineole and gallic acid was based on the assumption that detoxification pathways differed for each PSM in brushtail possums. We assessed this indirectly, by comparing the metabolic products excreted. Cineole metabolites excreted in brushtail possum urine are known (Boyle, 1999), but gallic acid metabolites have not been previously described for these possums.

METHODS AND MATERIALS

Six adult brushtail possums, three males and three females, body weight 3.41 ± 0.70 kg (Mean $\pm$ SD), were collected from Hobart and areas surrounding the University of Tasmania. Possums were housed individually in large-roofed cages (each 4.3 m long, 1.7 m wide, and 2.5 m in height) with wire mesh walls and sloping cement floors, at the School of Zoology, University of Tasmania, Hobart. Each cage contained logs to mimic above-ground runaways for environmental enrichment, a nest box, and fresh water.

Basal Diet and Its Digestibility. Possums were maintained on a basal diet prepared fresh each day. It consisted of (percent wet matter) 39.6% apple, 29.9% silver beet, 8.2% carrot, 4.5% ground lucerne (chaff-ground to pass a 1-mm sieve), 3.0% brown sugar, and 14.9% hot water. The diet was 16.8% dry matter, SE $\pm$ 0.2. Details of basal diet preparation are described in McArthur et al. (2000). Possums were provided with food at levels sufficient for maintenance (30–35 g DM/kg$^{-0.75}$ BM per day, where kg$^{0.75}$is metabolic body mass, BM) (McArthur et al., 2000).

Dry matter intake (DMI) was calculated daily for each possum (expressed as g/kg BM). Three, 100-g fresh matter (FM) subsamples of the diet were oven-dried at 55^{o}C for 48 hr (Van Soest, 1965) to determine %DM. This was used to convert FM offered into DM offered. Any remaining diet and spillage (orts) from each animal was collected the next morning, weighed, then dried as mentioned above. Individual DM intakes were calculated as DM offered minus DM remaining.

Apparent DM digestibility of the basal diet was calculated following Robbins (1983). All possums were fed basal diet during an 8-day collection period, and had

received the basal diet for 2 weeks prior. DM intake was calculated as subsamples of the basal diet as described, and DM output was calculated from pooled dried fecal samples collected daily for individual possums. Average digestibility of the basal diet was obtained as the mean of six possums.

Three samples of basal diet and three fecal samples for each of Possums B, C, E, and F were analyzed for nitrogen composition after grinding to pass through a 1-mm mesh using a laboratory mill. Samples for Possums A and D were not analyzed because of logistical constraints. Samples were dried at 70°C for 24 hr prior to digestion, then cooled to room temperature in a desiccator. A digest was performed on the samples following the methods outlined in Lowther (1980). After this, an ammonium analysis was performed based on the Berthelot reaction, using salicylate, hypochlorite, and nitroprusside (Reference number: TKN 10-107-06-2-D; Ann Wilkinson, personal communication). From these results, the percentage apparent digestibility of nitrogen was calculated (Robbins, 1983).

Subsamples of the basal diet and feces were analyzed for fiber content using the Ankom200/220 Fiber Analyzer, following procedures outlined in the Ankom200/220 Fiber Analyzer Operator's Manual (1997). Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL) procedures were performed. These samples were ashed at 500°C for 3 hr in crucibles. Between analyses, samples were stored in a desiccator. From these analyses, NDF, ADF, lignin, and ash content of the diet and fecal samples were determined. Digestibilities of each fraction were calculated (Robbins, 1983).

No-Choice PSM. Cineole (1,8-Cineole, purity 99%, Aldrich Chemical, Castle Hill, Australia) was added to the basal diet at three different levels: zero, medium (6.8% DM), and high (15.3 % DM). These levels were consistent with those used by Boyle (1999); representing 0, 2.0, and 4.5 g cineole/kg BM/day, respectively. Cineole was added to the diet daily and mixed thoroughly.

Possums received the basal diet containing no cineole for a preliminary 7-d period. The three treatments (zero, medium, and high cineole) were assigned to possums over three 7-d periods using a crossover design (Ratkowsky et al., 1993). This enabled the effect of carryover to be tested.

During the trial, individual possums were filmed nightly from 5:30 P.M.–8:30 A.M. Possums A and D were filmed from May 29, 2001 until June 27, 2001. Possums B, C, E, and F from June 27, 2001 until July 25, 2001. Timing was staggered due to limited equipment. Possums A and D were filmed simultaneously with a Panasonic® movie camera (NV-VS5A). With the addition of a second camera, which consisted of a basic lens constructed by University technical staff, Possums B and C were filmed simultaneously with one camera, and Possums E and F were filmed simultaneously with the other. A large wooden board (~2.4 m length × 1.1 m height) acted as a visual partition between the cages, preventing any interaction between possums. Each video camera was connected to a Panasonic® Video Cassette Recorder (NV-FJ600 Series). Video footage was recorded directly onto

5-hr cassette tapes using long-play (total 15 hr). Data from video footage were entered into The Observer® Video-Pro (Version 3.0, Noldus Information Technology Inc., Sterling, VA) computer program.

Behavioral observations recorded nightly were (1) number of feeding bouts; (2) size of feeding bouts; (3) rate of intake (feeding activity only); (4) time from the first to last feeding bout (incorporating nonfeeding activity); and (5) time taken to eat 10 g DM (incorporating nonfeeding activity [calculated as observation (4) $\times$ 10 per total DMI]). A single feeding bout was defined as the time from a possum's first bite to the end of its last chew, with no more than 1 min of nonfeeding directly following this last chew. Behavior (5) was measured as a way of standardizing feeding rate on the basis of a given intake.

Choice versus No-Choice PSM. Three treatment diets were used: cineole, gallic acid, and a choice diet of cineole and gallic acid. The cineole treatment diet used the high cineole level outlined above, which was at a level that constrains intake in brushtail possums (Boyle, 1999). The gallic acid treatment diet was at 4.6% of the total DM, a level predicted to constrain intake in brushtail possums (from Oswald, 1993). Gallic acid (3,4,5-trihydroxybenzoic acid; Aldrich Chemical, Castle Hill, Australia) was prepared and added daily. To avoid oxidation, 2.3% sodium ascorbate (nonacidic crystalline powder; Natures Works, Hobart) was first added to the gallic acid powder and mixed thoroughly. This was then added to the diet and mixed thoroughly. In the choice treatment, possums were offered two separate diets simultaneously, at levels described earlier. These diets were made equally available, placed side-by-side in separate feeding containers. Each day, the position of the feeding bowls was switched to avoid possible position preferences.

The trial was run as a crossover design, with 1 week per period for 3 weeks. Possums A, D, and F were tested from July 27, 2001 – August 16, 2001. Possums B, C, and E were tested from August 17, 2001 – September 6, 2001. This split was necessary because of limited filming equipment.

Within each period, possums were offered the basal diet for 2 d immediately before receiving a treatment, then offered the treatment diet for 5 d (Days 1–5). For days 1–4, possums were filmed. On Day 5, they were placed into individual stainless steel metabolism cages (60 $\times$ 60 $\times$ 45 cm) for 24 hr to collect urine. Urine was collected into containers held in liquid nitrogen and was frozen immediately. Samples were stored at -20°C until analysis.

Urinary Analysis. Urine from a single possum (Possum A) was analyzed to detect major urinary metabolites excreted after each of the diet treatments. Samples were analyzed for both free and total metabolites using methods derived from Boyle et al. (1999) with minor alterations, as described later. Difference between the two measurements indicates conjugation with glucuronic acid.

Free metabolites were measured from unhydrolyzed urine. These were prepared by placing 0.25 ml urine into a centrifuge tube along with 0.25 ml internal standard (0.125 mg 2,5-dimethylbenzoic acid in 0.25 ml of aqueous base)

and 0.50 ml distilled water. The sample was acidified to pH 1 with one drop of 5 M HCl. Urine was extracted with two washes of ethyl acetate (2 and 1 ml, respectively). Samples were vortexed for 1 min to ensure mixing before centrifuging for 5 min at 2000 rpm. Finally, the sample was derivatized by adding 20 μl *N*,*O*–bis(trimethylsilyl)-trifluoroacetamide (BSTFA; Alltech Associates, Inc., Deerfield, IL) to 20 μl of the sample extract, the vial was sealed and heated at 70°C for 20 min before analysis by gas chromatography (GC).

Hydrolyzed samples were prepared in the same manner as the free metabolites except that the urine was hydrolyzed prior to extraction. Urine (0.25 ml) was measured into the centrifuge tube along with the internal standard (as above), 0.20 ml acetate buffer (1.1 M; pH 5.2), 0.25 ml distilled water, and 50 μl extract of *Helix pomatia* (β-glucuronidase 141,000 units/ml; plus aryl sulphatase 3950 units/ml; Boehringer Mannheim, Germany). Samples were incubated overnight in a water bath at 37.5°C. Urine was then acidified to pH 1 using 5 M HCl, and was extracted and derivatized in the same manner as the unhydrolyzed samples.

Metabolites were identified with a Varian 3300 gas chromatograph fitted with a Varian 1077 split/splitless capillary injector, flame ionization detector (FID), and Star Chromatography Workstation (Version 5.31, Varian Pty Ltd, Walnut Creek, CA). Chromatography was on a 25 m HP1 capillary column. GC conditions used were injector 50°C; oven 100–160°C at 5°C/min, then 160–290°C at 30°C/min; and detector 300°C. Major metabolites derived from the urine samples were identified by their respective retention times.

The extent of glucuronidation was determined by comparing the ratios derived from respective metabolites and the internal standard peak areas between the hydrolyzed and unhydrolyzed urine samples from each diet.

A second ratio was calculated by dividing all metabolite peak areas by the corresponding peak area of 9-cineolic acid. This gave us the amount of each metabolite relative to 9-cineolic acid, making it possible to compare the excretion pattern between the variable diet treatments.

Statistical Analysis. The average of 7 days data for each possum was used in analyses. Dependent variables of DMI number of feeding bouts, DMI per feeding bout, rate of DMI, time from first to last feeding bout, and time taken to eat 10 g dry matter were tested against the independent variables of treatment, sex, period, and carryover, using the General Linear Model Procedure (PROC GLM) in SAS (Statistical Analysis System; SAS v 6, 1990). The Wilk-Shapiro statistic, normal probability plots and plots of the standardized residuals all indicated normality of the data. For significant results, pairwise comparisons were made using Tukey-Kramer adjustment. As the carryover effect was not significant for any variable, it was excluded from the final models.

In the second experiment, the average of 4-d data for each possum was used, incorporating the same variables as above. Log transformation of the DMI data

and square root transformation of the number of feeding bouts were required. The carryover effect was excluded because its effect was not significant.

Additional analysis was performed on the DMI data to further compare intakes of the treatment and basal diets. Intake on the basal diet was estimated from the three 2-d washout intervals between treatments, and was not included in the crossover design. Paired *t*-tests between intakes on the basal diet and each of the treatment diets were performed, using the Univariate Procedures (Proc Univariate) in SAS (SAS v. 6, 1990).

RESULTS

Basal Diet Characteristics and Digestibility. Results of the chemical composition of the basal diet and its digestibility in brushtail possums are shown in Table 1.

No-Choice PSM. There were no significant sex effects ($P \geq 0.170$) and weak or nonsignificant period effects ($P \geq 0.062$) for all variables in the final models. Therefore, the following results only consider the effect of main interest, i.e., treatment on intake and feeding behavior of possums.

The mean total DMI varied from 18.6 to 23.3 g/kg BW ($F_{1,2} = 5.33$, $P = 0.022$; Figure 1a). Possums ate less as dietary cineole increased. The mean number of feeding bouts was 8.9 on the zero cineole diet, 10.8 bouts on the medium cineole diet, and 17.1 bouts on the high cineole diet ($F_{2,12} = 2.56$, $P = 0.119$; Figure 1b). DMI per feeding bout varied from 4.60 to 10.75 g DM/kg BW ($F_{2,12} = 3.90$, $P = 0.049$; Figure 1c). Possums ate less per feeding bout as dietary cineole increased. The mean rate of DMI varied from 1.29 to 1.80 g DM/min between treatments ($F_{2,12} = 4.41$, $P = 0.037$; Figure 1d). With increasing cineole levels, possums ate at a slower rate. Possums on average spent between 7 and 9 hr of the night feeding, measured as the time taken from the first to last feeding bout and incorporating nonfeeding activity ($F_{2,12} = 0.66$, $P = 0.535$; Figure 1e). Possums took longer to eat the same quantity of food during a night (10 g DM, including nonfeeding

TABLE 1. COMPOSITION AND DIGESTIBILITY OF THE BASAL DIET

Constituent	Composition[a]	Digestibility (%)
Dry matter (DM)[b]	16.8 ± 0.2	75.6 ± 1.0
Nitrogen(N)	1.5 ± 0.1	41.5 ± 1.6
Neutral detergent fibre (NDF)	19.4 ± 0.7	50.3 ± 1.0
Acid detergent fibre (ADF)	12.9 ± 0.5	37.0 ± 0.7
Acid detergent lignin (ADL)	2.2 ± 0.3	14.5 ± 0.6
Ash	0.2 ± 0.0	0.5 ± 0.1

[a] Composition values, except dry matter, are percent DM, expressed as means ± SE and $N = 6$, except nitrogen digestibility, where $N = 4$.
[b] As a percentage of fresh matter (FM).

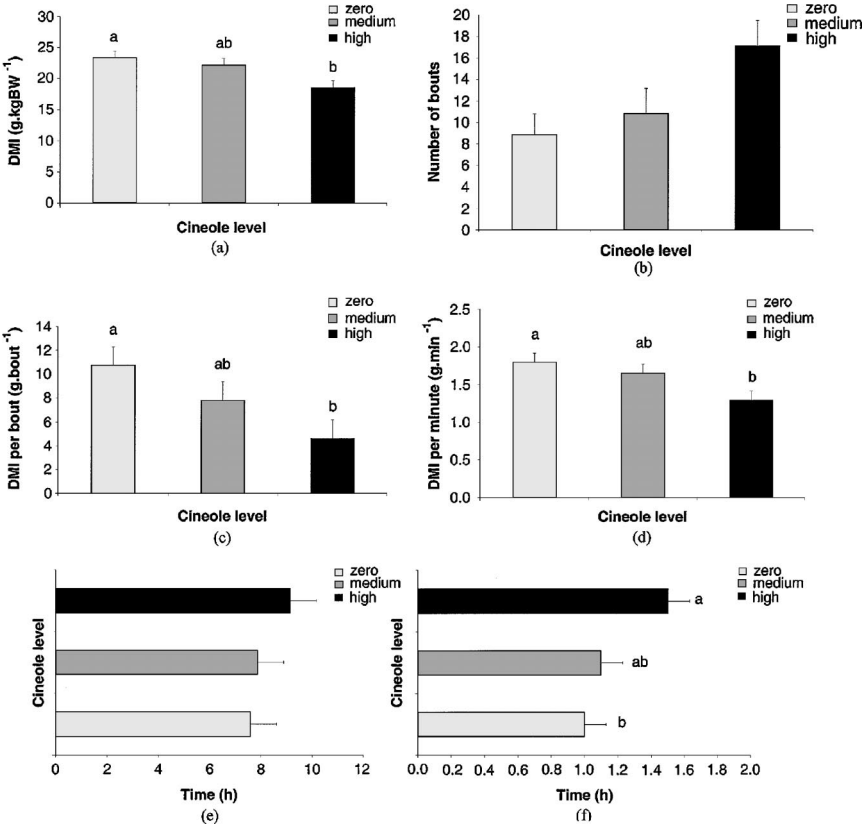

FIG. 1. Results showing (a) DMI, as grams per kg mass; (b) number of feeding bouts; (c) DMI per feeding bout; (d) rate of feeding per minute; (e) time taken from the first to last feeding bout, including nonfeeding activity; and (f) time taken to eat a specific quantity of food (10 g DM) by possums on treatment diets with varying cineole levels. All values are least squares means with SE bars. Letters that differ are significantly different ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons).

time) as dietary cineole increased ($F_{2,12} = 4.30$, $P = 0.039$; Figure 1f). Possums on the high cineole diet on average took 50% more time than on the zero cineole diet to consume the same quantity of food.

Choice Versus No-Choice PSM. There were no period effects for any variable in the final models ($P \geq 0.149$). The following results consider the effect of treatment and sex on intake and feeding behavior.

Possums ate more food on the Choice diet than on either of the single No-Choice diets ($F_{2,12} = 5.20$, $P = 0.024$; Figure 2a). On the Choice diet, $74 \pm 5.80\%$

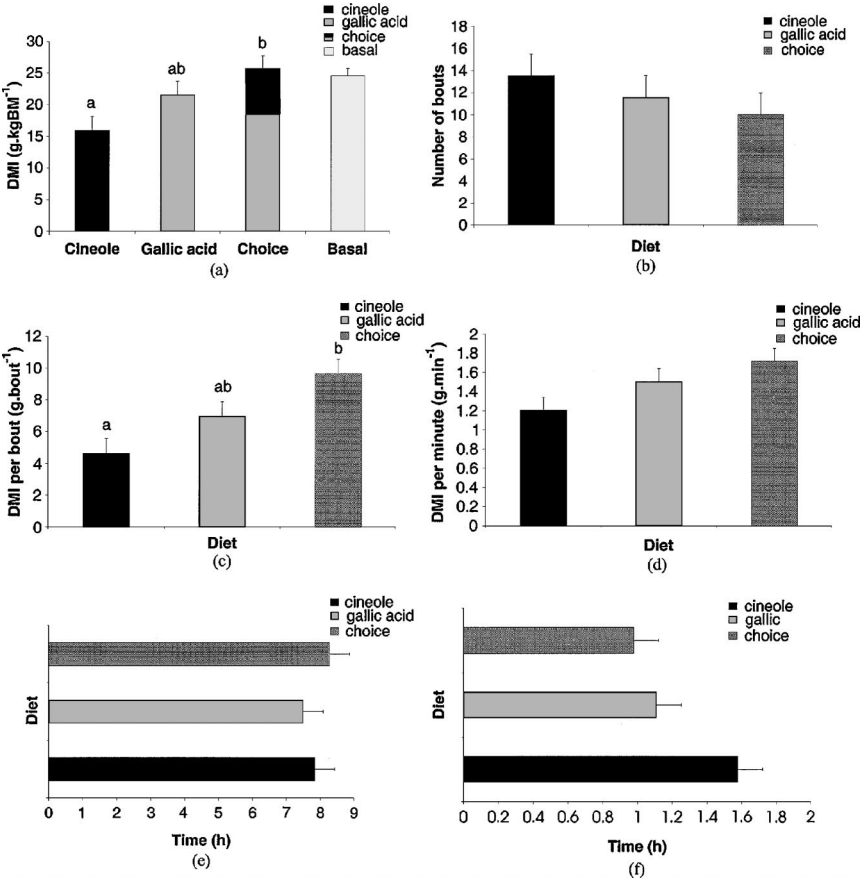

FIG. 2. Results showing (a) DMI as grams per kg body mass (showing intake on the basal diet as a comparison); (b) number of feeding bouts; (c) DMI per feeding bout; (d) rate of feeding per minute; (e) time taken from the first to last feeding bout, including nonfeeding activity; and (f) time taken to eat a specific quantity of food (10 g DM) by possums on treatment diets of Choice PSM and No-Choice PSM diets. Results of (a) were analyzed using log transformed data, (b) using square-root transformed data, and (c) to (f) all values are untransformed least-squares means, with SE bars. Letters that differ are significantly different ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons).

(mean $\pm$ SD) of the total food consumed was from the gallic acid diet, with the remaining $26 \pm 5.80\%$ (mean $\pm$ SD) from the cineole diet. Females ate 25% more than males (23.3 ± 1.8 g DM/kg BM compared with 18.7 ± 1.8 g DM/kg BM) ($F_{1,12} = 5.36$, $P = 0.039$).

When intake on treatment diets was compared with intake on the basal diet, possums consumed less of the cineole diet (mean difference = 8.60 g/kg BW ± 1.62, $t = 13.0$, $df = 5$, $P < 0.001$). The mean difference in intake between the gallic acid and basal diet was 3.01 ± 4.70 ($t = 1.570$, $df = 5$, $P = 0.177$). The mean difference in intake between the choice and basal diet was −1.00 ± 4.96 ($t = -0.498$, $df = 5$, $P = 0.639$).

On average, possums had between 10 and 14 feeding bouts per night ($F_{2,12} = 0.87$, $P = 0.444$; Figure 2b). There was a weak sex effect for number of feeding bouts per night ($F_{1,12} = 3.97$, $P = 0.070$). Possums ate more per feeding bout on the Choice diet than on either of the No-Choice diets ($F_{2,12} = 7.35$, $P = 0.008$, Figure 2c). Females ate more per feeding bout (8.38 ± 0.75 g DM/kg BM) than males (5.77 ± 0.75 g DM/kg BM) ($F_{1,12} = 5.99$, $P = 0.031$). Possums on the Choice diet tended to eat at a higher rate than on either of the No-Choice PSM diets ($F_{2,12} = 3.56$, $P = 0.061$, Figure 2d). Females ate at a higher rate (1.70 ± 0.11 g DM/min) than males (1.24 ± 0.11 g DM/min) ($F_{1,12} = 8.74$, $P = 0.012$). On average, there were 7.5–8.3 hr between the start of the first feeding bout to the end of the last feeding bout, including nonfeeding activity ($F_{2,12} = 0.44$, $P = 0.657$, Figure 2e). There was no sex effect ($F_{1,12} = 2.41$, $P = 0.147$). Possums took longer to eat the same quantity of food during a night on the No-Choice PSM diets than on the Choice PSM diet ($F_{2,12} = 4.81$, $P = 0.029$, Figure 2f). Possums on the cineole diet took on average 38% more time to consume 10 g DM than on the Choice diet. Possums on the gallic acid diet took on average 12% more time than on the Choice diet. There was no sex effect ($F_{1,12} = 3.01$, $P = 0.108$).

We found seven major metabolites of 1,8-cineole, but no unchanged 1,8-cineole. Metabolites varied in the degree of alteration they had undergone during detoxification. Three metabolites were identified by comparing their retention times to known standards and were 9-hydroxycineole (9OH), 9-cineolic acid (9COOH), and 7-cineolic acid (7COOH). The remaining four metabolites are likely to be hydroxycineolic acids (Boyle et al., 2000) although their structures were not characterized. They are noted by their retention times (Rt 15.00, 15.23, 15.28, 15.60 min). The most abundant metabolite of 1,8-cineole was 9 cineolic acid. Gallic acid was excreted unchanged. The excretory products associated with cineole and gallic acid are indicated in Figure 3a. There were no metabolites in the urine sample collected during the basal diet. Figure 3b shows the level of conjugation occurring in each experiment. We found partial conjugation of several 1,8-cineole metabolites on the No-Choice cineole diet. Cineole metabolites were apparently conjugated to a lesser extent in the Choice diet, where the cineole intake was significantly lower.

There was no major alteration in the excretion pattern of 1,8-cineole or gallic acid between the treatments (Figure 3c).

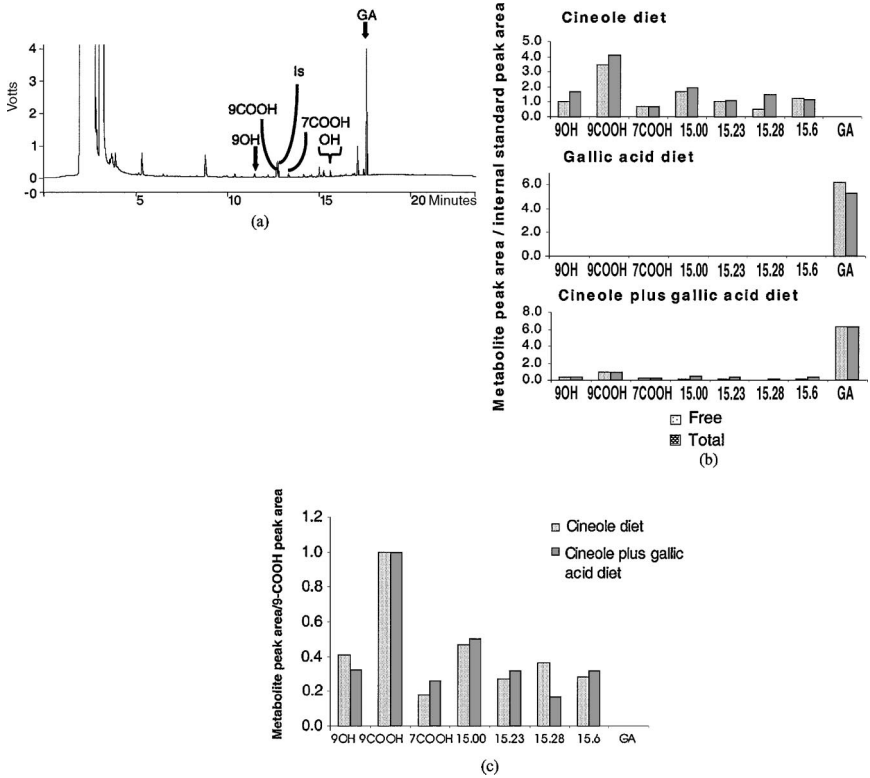

FIG. 3. GC analysis of urine extracts from a brushtail possum in the cineole–gallic acid choice experiment. (a) Chromatogram of hydrolyzed urine; (b) extent of glucuronidation, derived from the ratio of metabolite peak area to internal standard peak area; (c) relative amounts of metabolites excreted, derived from the ratio of metabolite peak area to 9-cineolic acid peak area. Metabolites are labeled by abbreviated names; 9OH – 9-hydroxycineole, 9COOH – 9-cineolic acid, 7-COOH – 7-cineolic acid, OH—15.00, 15.23, 15.28, and 15.60—unidentified hydroxy cineolic acids, GA – gallic acid, and Is – internal standard.

DISCUSSION

Basal Diet Characteristics. Nitrogen, neutral detergent fiber, and acid detergent fiber levels in the basal diet are similar to that in foliage of some tree species eaten by brushtail possums, although there were some differences in acid detergent lignin and ash content (Fitzgerald, 1978; Foley and Hume, 1987). Dry matter digestibility of the basal diet was considerably higher than digestibility of *E. melliodora* and *P. arboreum* foliage (Fitzgerald, 1978; Foley and Hume, 1987), species naturally consumed by brushtail possums. This result was expected,

as foliage contains tough fibrous plant cell walls and an array of other forms of mechanical and chemical defenses (Howe and Westley, 1988). Nitrogen digestibility was surprisingly low, but similar to some species (Foley and Hume, 1987).

Feeding Behavioral Responses to PSMs. The dietary terpene, cineole, altered nightly intake by brushtail possums. Possums consumed less food as dietary cineole increased, consistent with results from Boyle (1999) using similar cineole levels, and Lawler et al. (1999). These results are also consistent with those of Freeland and Winter (1975), who found that brushtail possums reduced their intake on diets containing PSMs compared with a PSM-free diet.

Our results also showed that possums altered their nightly feeding behavior in response to increasing cineole levels: they increased the number and decreased the size of feeding bouts, and decreased their rate of feeding. Possums may have been continually sampling and rejecting the diet throughout the night, a behavior described in previous studies with mammals (Freeland and Winter, 1975; Freeland and Saladin, 1989; Stapley et al., 2000). Sampling behavior is important in assessing the palatability of a diet, potentially improving the ability of herbivores to regulate their intake of PSMs (Stapley et al., 2000). However, a more comprehensive explanation for this alteration in feeding behavior may be associated with physiological feedback mechanisms (Provenza, 1995). When possums offered the high cineole diet started feeding each night, they may have initially consumed enough cineole to reach a physiological constraint, as modeled by Foley et al. (1999). Once reached, this constraining level would initiate feedback to decrease absolute intake and rate of intake. At each subsequent feeding bout, the constraining level would be reached relatively quickly again, reducing bout size, but increasing the number of bouts as possums attempted to maintain intake. Another possible explanation may be that the possums maintained a constant supply of cineole that matched the maximal capacity of the detoxification pathways. This would provide a constant supply of cineole to a rate-limited step, at a maximal rate, potentially maximizing the output over time. Current investigations are testing when possums reach this constraint, by analyzing blood samples taken during each meal (Rebecca Boyle, personal communication). This will allow a more thorough integration of the relationship between physiology and feeding behavior.

Possums generally used the same total amount of time during the night to consume their food, in contrast to our hypothesis. Our prediction was based on the assumption that they would increase the amount of time in-between feeding bouts, allowing longer periods for detoxification of ingested cineole (Freeland and Winter, 1975), by extending the total time from the first to last feeding bout. They did, however, increase the time taken to eat a specific quantity of food, incorporating both feeding and nonfeeding activity as dietary cineole increased. This suggests that they responded to cineole, but not sufficiently to maintain intake. We are unable to tell from our results whether the behavior changes observed with increasing cineole actually minimized negative effects on intake. Constraining diet

availability to particular times of night would allow us to determine this, but was beyond the scope of this study.

Choice versus No-Choice PSM. The level of gallic acid used in this study did not appear to reach a physiological threshold in possums, as intake was not significantly lower than on the basal or choice diets. The concentration used in the gallic acid diet was derived from previous work in which intake by brushtail possums was constrained (Oswald, 1993). It is possible that differences in the basal diets altered the capacity of animals to cope with the gallic acid. The previous basal diet had much lower nitrogen levels (0.51% N) and a higher fiber content (31.3% NDF) (Oswald, 1993) than our basal diet (1.5% N, 19.4% NDF). The capacity of animals to cope with PSMs can depend on their nutritional state (Bidlack et al., 1986), so they may have had a lower capacity to deal with gallic acid in Oswald's trial (1993). Although a significant feeding constraint was not reached, gallic acid may still have imposed a cost, as all feeding responses on the gallic acid diet were intermediate between the cineole and choice diets.

Feeding behavior of possums offered the Choice diet containing two separate PSMs differed from that on the No-Choice PSM diets: they ate more food, at a quicker rate, and had larger feeding bouts on the Choice diet. This supports the hypothesis that by mixing intake between diets with different PSMs, possums can increase their intake compared with when they do not have this choice. These results are consistent with those from previous investigations (Freeland and Winter, 1975; Dearing and Cork, 1999). This is also consistent with the concept that generalist herbivores consume a varied diet to avoid overloading individual detoxification pathways (Freeland and Janzen, 1974).

Interestingly, female possums ate significantly more food than males, at a quicker rate, and had larger feeding bouts. Females may have had higher energetic demands than males, but this does not explain how they were able to consume more cineole. We postulate that females had a higher detoxification capacity than males. However, no sex effects were detected, suggesting that increased energetic demands and/or detoxification capacity in female possums may vary over time. It is also possible that, because of the small sample size, the significant sex effect is not real.

Irrespective of treatment diets, possums spent a similar amount of the night feeding. However, they took less time to eat a specific quantity of food on the Choice diet and ate more overall than on the No-Choice PSM diets. This is consistent with Freeland and Janzen's hypothesis (1974), suggesting that they were able to detoxify a combination of PSMs more rapidly than a single PSM, hence were not constrained. Results from the urinary analysis offer support for the original assumption that gallic acid and cineole are not involved in competing detoxification pathways. Gallic acid was excreted unchanged and, therefore, does not compete with the metabolic pathways used for the detoxification of cineole.

From these results, we can infer something about the type of metabolic costs of these two PSMs. Detoxification of cineole in brushtail possums involves a series of oxidation reactions, followed by partial conjugation with glucuronic acid (Baudinette et al., 1980; Boyle et al., 2000). Glucuronidation is an important detoxification pathway used by many animals because of its high capacity and effectiveness in increasing excretion efficiency of metabolites (Hinks and Bolliger, 1957a,b; Boyle, 1999). Thus, cineole metabolism involves specific costs, in terms of energy and specific nutrients required for detoxification (Freeland and Janzen, 1974). Gallic acid metabolism has not been as extensively studied in brushtail possums as cineole. Murdiati et al. (1992) reported that in nonruminants, gallic acid is excreted in urine as free gallic acid, its 4-*O*-methyl ether, pyrogallol, and glucuronide conjugates of gallate. Booth et al. (1959) found similar major urinary metabolites of gallic acid in rats and rabbits. Metabolism of gallic acid in brushtail possums was investigated indirectly by Oswald (1993). From a basal diet to diets containing increasing gallic acid levels, possum urine decreased in pH, and increased in titratable acids and ammonium–nitrogen (Oswald, 1993). This implies that elimination of gallic acid is metabolically and energetically costly, even if it is excreted unchanged.

Detoxification of PSMs was investigated only in a single animal, as our major intention was to demonstrate that dissimilar metabolic pathways are used in the elimination of the chosen PSMs. Previous experience has shown little qualitative differences among possums during quantitative analyses of metabolic products of PSMs (e.g., Boyle et al., 1999). The semiquantitative interpretations of the chromatograms are an indication only of the metabolic fate of these compounds.

This study has shown that brushtail possums alter their feeding behavior, not just their absolute intake, in response to PSMs. This altered feeding behavior may simply be a response to physiological mechanisms, but we suggest it plays an important role in dealing with dietary PSMs. Either way, the results help in understanding and predicting feeding responses of possums in their natural environment, where the spatial distribution of plants and PSMs may vary. We suggest that plant species diversity and distribution across the landscape may strongly influence feeding patterns and foraging efficiency of relatively generalist folivores, such as brushtail possums, through the influence of PSMs on feeding.

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GLUCURONURIA IN THE KOALA

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Abstract—Glucuronuria is normal in marsupial folivores such as the koala (*Phascolarctos cinereus*), which excretes 2–3 g glucuronic acid daily. Although this has long been attributed to the metabolites of *Eucalyptus* terpenes, we have found that these are mostly excreted in the unconjugated form. We now report on the aglycones that account for most of the glucuronic acid in koala urine. Urine (24 hr) was collected from six male koalas (8.8 ± 0.4 kg, mean $\pm$ SE) that were maintained on *E. cephalocarpa* foliage. Urine samples were analyzed by liquid and gas chromatography (LC and GC) coupled with mass spectrometry (MS). Glucuronides were readily identified by LC-MS/MS, which generated characteristic product ions at m/z 113 and 175. From the corresponding parent glucuronide ions, the masses of the aglycones were calculated. Confirmation of identity was by GC-MS after hydrolysis with β-glucuronidase and comparison with standard compounds. Quantitation was by GC. The major non-terpene aglycones were 4-methylcatechol, resorcinol, salicyl alcohol, and two unidentified $C_7H_8O_2$ phenols. Smaller amounts of benzoic acid, benzyl alcohol, orcinol, *p*-cresol, phenol, and phloroglucinol were detected. We have previously reported that terpene metabolites account for about 10% urinary glucuronides in the same koalas fed *E. cephalocarpa*. The present study found that an additional 60% urinary glucuronic acid is conjugated with non-terpene, mainly phenolic,

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aglycones. It seems likely that these phenolic compounds are present in leaves as glycosides and are chiefly responsible for the glucuronuria in koalas.

Key Words—Glucuronic acid, *Phascolarctos cinereus*, koala, phenolics, *Eucalyptus*, terpenes, detoxification.

INTRODUCTION

Glucuronic acid is not excreted as the free form, but is found in urine conjugated with other molecules called aglycones. Glucuronide conjugates are formed with a variety of endogenous molecules such as steroid hormones, bile acids, and bilirubin, and from a virtually unlimited number of foreign compounds (xenobiotics), including dietary constituents and drugs (Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000; Parkinson, 2001). The role of glucuronidation is to convert lipophilic compounds into highly polar conjugates that can be more readily excreted in urine or bile. Additionally, as glucuronidation usually inactivates drugs and toxins, it is generally considered to be a detoxification reaction, although there are some exceptions where glucuronides are biologically active (Spahn-Langguth and Benet, 1992).

Glucuronides are formed by the reaction of UDP-glucuronic acid with a nucleophilic site on the aglycone molecule, especially the functional groups of phenols, alcohols, or carboxylic acids. The reaction is catalyzed by UDP-glucuronosyltransferase (UGT), of which over 50 forms have been described in vertebrates (Tukey and Strassburg, 2000). UGT activity is present in liver and kidney and also in the epithelia of the respiratory and intestinal tracts where it may have a role in detoxifying xenobiotic substances at the site of contact. As UGT is located in the lumen of the endoplasmic reticulum, substrates must be sufficiently lipophilic to penetrate this membrane barrier before they can be glucuronidated (Radominska-Pandya et al., 1999; Parkinson, 2001). The endoplasmic reticulum is also the site of cytochrome P450 and other phase 1 enzymes, so UGT is well-placed to conjugate the products of functionalization reactions.

The many forms of UGT have different but overlapping substrate specificities (Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000). In general, the UGT1A enzymes glucuronidate a wider range of xenobiotics than do the UGT2B forms. Simple phenols are conjugated most readily by human UGT1A enzymes, except for UGT1A4, especially in the liver and intestine (Tukey and Strassburg, 2000). These authors note that the UGT1A locus is highly conserved in mammals and expressed throughout the gastrointestinal tract, and they propose that the UGT1A enzymes have evolved to metabolize dietary constituents, especially phenols.

Thus, lipophilic dietary xenobiotics enter the cells of the digestive tract where glucuronidation can remove those with suitable nucleophilic sites and allow their excretion in urine or bile. Glucuronides excreted in the bile are liable to hydrolysis

by bacterial β-glucuronidases, but UGT is present at high levels in the colon where it can act to reconjugate the liberated aglycones.

The koala (*Phascolarctos cinereus*) excretes large quantities (2–3 g; 0.9–2.1 mmol/kg/day) of glucuronic acid per day (Hinks and Bolliger, 1957b; Eberhard et al., 1975; Cork et al., 1983). In contrast, humans normally excrete about 1000-fold less on a body weight basis (0.9–1.6 μmol/kg/day; estimated from the data of Lake et al. (1982). The glucuronuria in the koala and other eucalyptus leaf-eating marsupials has been attributed to the detoxification of eucalyptus oils, mixtures of monoterpenes that can comprise up to 10% of the dry leaf mass (Boland et al., 1991). The koala feeds exclusively on eucalyptus leaf, and consequently it is not possible to examine the effect of an artificial diet on glucuronic acid excretion. However, other marsupial folivores, such as the brushtail possum (*Trichosurus vulpecula*), which does not require an exclusively eucalyptus diet, permit manipulative experiments that show the association between the consumption of eucalyptus leaf, or terpenes, and urinary glucuronide excretion (Hinks and Bolliger, 1957a; Dash, 1988). As Dash (1988) reported for the brushtail possum, the ringtail possum (*Pseudocheirus peregrinus*) excreted almost no glucuronic acid when fed a non-eucalyptus artificial diet (McLean et al., 1993). However, after the ringtail was changed to *Eucalyptus radiata* foliage, the excretion of glucuronic acid rose over several days (as leaf consumption increased) to about 1.5 mmol/kg/day (McLean et al., 1993).

However, the excretion of terpene metabolites does not account for all of the glucuronide found in urine of eucalyptus-eating marsupials. In ringtail possum urine, virtually none of the urinary glucuronic acid was linked to terpene metabolites (McLean et al., 1993). The urinary metabolites of two dietary terpenes, *p*-cymene and 1,8-cineole, were found to be mostly or completely unconjugated in the koala, ringtail, brushtail, and greater glider (Boyle et al., 1999; 2000a, 2000b). Recently, we described the urinary metabolites of 1,8-cineole in the koala fed *E. cephalocarpa* leaf (Boyle et al., 2001). The koalas excreted about 2-g glucuronic acid daily (1.1 mmol/kg/day), but only about 10% of this was conjugated with cineole metabolites. Analysis by liquid-chromatography–mass-spectrometry (LC-MS) indicated that the glucuronic acid was conjugated with other small-molecular-weight compounds. The aim of this study was to identify the aglycones in urine that account for the excretion of glucuronic acid in the koala.

METHODS AND MATERIALS

Materials. Standard phenols, alcohols, and acids (Table 1) were obtained from Sigma or Aldrich. Extract of *Helix pomatia* (a mixture of β-glucuronidase 141,000 units/ml and aryl sulfatase 3,950 units/ml) was obtained from Boehringer Mannheim and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) from Alltech Associates. Other chemicals and solvents were of analytical reagent grade.

TABLE 1. AGLYCONES FOUND AS GLUCURONIDES IN KOALA URINE

Formula	MW	Aglycone	RT [a] (min)	Ions monitored[b] (m/z)	Amount excreted in 24 hr (mean ± SE) Total (μmol/kg)	% Conjugated
$C_7H_8O_2$	124	4-Methylcatechol	5.81	268, 253	357 ± 68	91 ± 1
		Phenol # 1	4.98	254	54 ± 7	86 ± 9
		Phenol # 2	6.07	254, 239	43 ± 5	40 ± 5
		Salicyl alcohol	6.59	268, 253	63 ± 15	87 ± 3
		Orcinol	6.48	268, 253	6 ± 1	100 ± 0
$C_7H_6O_2$	122	Benzoic acid	4.10	194	17 ± 5	100 ± 0
$C_6H_6O_2$	110	Resorcinol	5.83	254	157 ± 82	86 ± 8
C_7H_8O	108	Benzyl alcohol[c]	3.31	180	17 ± 3	100 ± 0
		p-Cresol	3.23	180	4 ± 3	100 ± 0
$C_6H_6O_3$	126	Phloroglucinol	Detected by LC-MS but not quantified			
C_6H_6O	94	Phenol				
				Total non-cineole aglycones	717 ± 128	89.3 ± 16.6
				Total conjugated non-cineole glucuronides	642 ± 119	

[a] GC-MS conditions under Methods and Materials. TMS derivatives were analyzed. The internal standard (3,5-dimethylphenol) had a retention time of 3.95 min and the ion at m/z 194 was monitored.
[b] The first ion was used for quantitation, and the others were used for peak confirmation.
[c] Analyzed by GC-FID using different GC conditions to resolve from the internal standard, which produced the same ions.

Animals and Urine Collection. Six male koalas (*Phascolarctos cinereus*) were used. Animals were maintained on a diet of *Eucalyptus cephalocarpa* and used to investigate the metabolic fate of 1,8-cineole, the major terpene in the leaf of this species. Details of animal care, experimental procedures, and ethical approval have been reported previously (Boyle et al., 2000b, 2001). The koalas (weight 8.8 ± 0.4 kg, mean ± SE) were offered fresh leaves daily, which were picked weekly from a plantation of 12 *E. cephalocarpa* trees. The animals had been feeding on this leaf diet for at least 3 days before urine was collected. Daily leaf consumption was measured (Boyle et al., 2001), enabling the 1,8-cineole intake to be calculated from its concentration in leaf. Urine was collected from koalas for 24 hr and stored at −18°C until analyzed.

Analyses. Glucuronides were identified in urine by MS. Urine samples were diluted (1:10), filtered (0.45 μm), and analyzed by LC-MS, using an LCQ instrument and chromatography conditions described earlier (Boyle et al., 2001). Negative electrospray ionisation (ESI) was used, because positive ESI did not readily detect phenolic glucuronides. ESI conditions were as follows: capillary temperature 300°C, needle voltage 4.5 kV, sheath gas 85 psi, auxillary gas 50 psi and capillary voltage −30 V. Data-dependent MS/MS scans were generated from the most intense ions, and other significant ions were subject to further analysis by MS/MS. Because glucuronides form strong dimer peaks, a series of three scans

was used to detect glucuronides and produce diagnostic product ions. The first was of the total ion current (TIC) from m/z 70–800, showing the major peaks. The second was another full TIC scan from m/z 70–340, excluding the cineole-derived glucuronides ($m/z > 345$) and glucuronide dimer ions ($m/z > 500$). The third scan was the data-dependent MS/MS analysis of the most abundant ion in the second scan. The software automatically selected the largest peak for MS/MS analysis, hence the need to limit the mass range scanned to avoid analysis of large extraneous peaks instead of the peaks of interest. The isolation window was 3 Da and collision energy 23%.

Aglycones were identified, after hydrolysis of glucuronides, using gas-chromatography–mass-spectrometry (GC-MS) and comparison with commercial standards of the phenols, alcohols, and acids likely to be derived from plants (Goodwin and Mercer, 1983). To an aliquot of urine (0.1–0.5 ml, as appropriate) were added 0.25-ml acetate buffer (1.1 M, pH 5.2), 50-μl extract of *Helix pomatia*, and sufficient distilled water to make 1.0 ml. The sample was incubated at 37°C overnight, then acidified to pH 1 with 20-μl 5 M HCl, and vortex mixed with 2.0-ml ethyl acetate for 1 min. The ethyl acetate phase was separated by centrifugation, and 20 μl was mixed with 20 μl BSTFA and heated at 60°C for 5 min to form trimethylsilyl (TMS) derivatives.

GC-MS was carried out with an Hewlett-Packard (HP) 5890 gas chromatograph and 5970B mass selective detector using HP 59970A Chemstation software. The GC was fitted with a 25-m bonded phase capillary column (0.5-μm cyanopropyl film, 0.32-mm i.d.; BP10, Scientific Glass Engineering, Melbourne, Australia). Chromatography conditions were as follows: injection volume 1 μl, split ratio 1:10, He carrier gas 25 psi, injector 215°C, detector 270°C, oven program from 100 to 240°C at 10°C/min. Masses were scanned in the range m/z 40–600 on a 1-sec cycle, and aglycones were identified from their full electron impact (EI) mass spectrum and GC retention time, in comparison with standards.

Aglycones were also quantitated by GC-MS as described earlier, except that internal standard was added to the urine sample (0.5-mg 3,5-dimethylphenol in 0.1-ml water) and the MS used selected ion monitoring (SIM) to ensure adequate selectivity and sensitivity. The following ions were monitored (at 2 cycles/sec): m/z 91, 165, 180 from 1.0 to 3.7 min; m/z 179, 194 from 3.7 to 4.4 min; m/z 108, 126, 139, 254 from 4.4 to 5.5 min; and m/z 239, 253, 254, 268 from 5.5 to 7.0 min. Each aglycone was quantitated using one ion, while others were used for confirmation of identity (Table 1). Calibration standards were prepared by adding suitable quantities of commercial aglycones to blank urine, collected from a brushtail possum that was fed an artificial, non-eucalyptus diet of fruits and vegetables (Boyle et al., 2000a).

Unconjugated metabolites were determined by the same method, except that the hydrolysis step was omitted. Total glucuronic acid was determined colorimetrically (Blumenkrantz and Asboe-Hansen, 1973; Boyle et al., 2000b).

Benzoic acid did not resolve well from the internal standard and, as these compounds also shared the same SIM ions, it was quantitated by flame ionization detection (FID) using different gas chromatography conditions. A Varian 3300 gas chromatograph was fitted with a 30-m Econocap capillary column (0.25-μm SE-54, 0.32-mm i.d.; Alltech Associates). GC conditions were as follows: injection volume 1 μl, split ratio 1:20, carrier He at 9 psi, injector 250°C, detector 290°C, oven held at 100°C for 2 min, then heated at 3°C/min to 135°C, then 30°C/min to 290°C, held for 2 min. Retention times of TMS derivatives were as follows: internal standard 7.65 min, benzoic acid 8.24 min.

RESULTS

Detection of Glucuronides by LC-MS. Figure 1 shows the negative ion mass chromatograms for a sample of koala urine analyzed by LC-MS. The total ion chromatogram (TIC) reflects the sum of all ions in the range m/z 70–800 (upper panel). MS/MS product ion scans can be generated automatically in the LCQ software, using data-dependent selection of the *n*th most intense ion from a normal

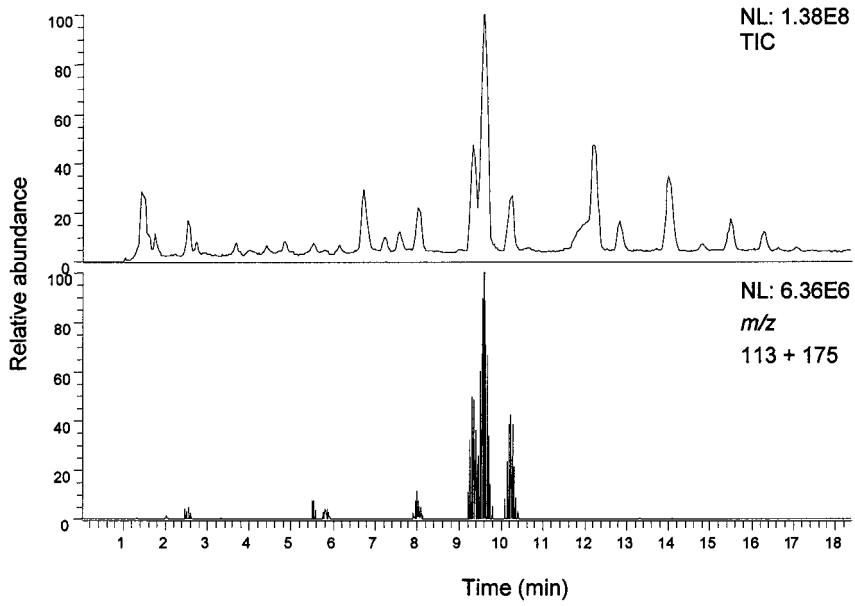

FIG. 1. Chromatograms from LC-MS analysis of koala urine showing non-cineole glucuronide peaks. Upper panel: total ion current (TIC), m/z 70–800. Lower panel: MS/MS product ions monitored at m/z 113 + 175. This trace is interrupted because only every third scan underwent MS/MS analysis. NL is the normalization level, a measure of the height of the largest peak.

scan as the precursor ion. However, most glucuronides formed dimers during the electrospray ionization process producing $[2M\text{-}H]^-$ ions as the most abundant species, which in turn after collisional activation yielded the $[M\text{-}H]^-$ ion corresponding to the original glucuronide. This futile cycle was avoided by including a second full scan, covering just the range m/z 70–340, thus excluding the dimers and ensuring the $[M\text{-}H]^-$ ion was the most abundant. A third scan cycle based on data-dependent selection of precursor ions from the second cycle was included to generate the MS/MS data. Mass chromatograms for the negative ions at m/z 113 and 175 were generated from this (lower panel), as these ions are indicative of glucuronides (Draper et al., 1989; Chen et al., 1998). As the MS/MS data were only produced every third scan, these chromatograms are discontinuous in nature. The full time between consecutive MS/MS scans was 2.4 sec. The molecular weights of the individual parent glucuronides were retrieved from the data, as the data-dependent precursor ion is recorded on each MS/MS spectrum.

Filtering out the higher masses also excluded the cineole glucuronide ions (hydroxy cineole glucuronide has a MW of 346) that appear in the TIC (Figure 1, upper panel) but not the MS/MS chromatogram, which is effectively a selective trace of non-cineole glucuronides (Figure 1, lower panel).

Further evidence of the presence of low-molecular-weight glucuronides is given in Figure 2, which shows selected individual mass chromatograms from LC-MS analysis of the same urine sample. The upper set of chromatograms shows the results for unhydrolyzed urine, and the lower set shows results of the same analyses on the same urine sample after hydrolysis with β-glucuronidase. The parent ions of the glucuronide peaks in Figure 1 were used to generate the corresponding $[M\text{-}H]^-$ ion chromatograms in Figure 2. Since H_2O is lost in the reaction between glucuronic acid and aglycone, the mass of each aglycone can be calculated by subtracting (glucuronic acid – H_2O) (i.e., 176) from the parent molecular weight. For example, the glucuronide $[M\text{-}H]^-$ ion at m/z 299 corresponds to an aglycone mass of 124, possibly methyl catechol, salicyl alcohol, orcinol, or a mixture of these compounds.

Most of the major peaks in the TIC chromatogram were glucuronides (Figure 2). Some were glucuronides of cineole metabolites whose identity was confirmed by positive ES and APCI data, as reported previously (Boyle et al., 2001). The major peaks were non-cineole glucuronides, and their ion chromatograms are displayed in the lower panels of Figure 2. There was also a significant hippuric acid peak, which was confirmed by MS/MS.

MS/MS analysis of each peak confirmed the characteristic glucuronide fragments at m/z 113 and 175 and usually also gave an ion corresponding to the deprotonated aglycone. For example, MS/MS analysis of the major peak shown in the ion chromatogram for m/z 299 (Figure 2) gave the ion m/z 123, corresponding to $C_7H_7O_2$ (Figure 3). Table 1 shows that there are several possible parent aglycones with the formula $C_7H_8O_2$. The glucuronide peaks in Figure 2 also disappeared after hydrolysis with β-glucuronidase (lower panels), although

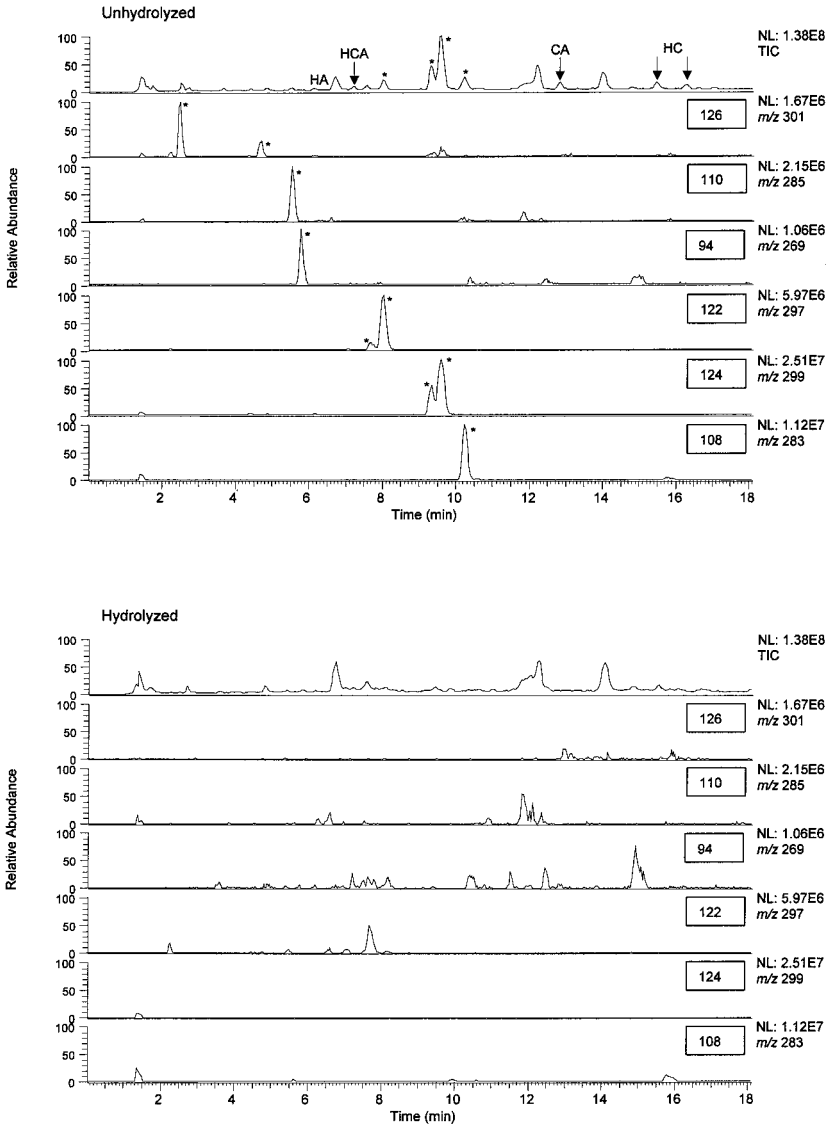

FIG. 2. Chromatograms from LC-MS analysis of koala urine showing TIC and individual mass chromatograms of non-cineole glucuronides. HCA = hydroxycineolic acid glucuronide, CA = cineolic acid glucuronide, HC = hydroxycineole glucuronide (Boyle et al., 2001), HA = hippuric acid, * = non-cineole glucuronide peaks. The upper panels show analyses of unhydrolyzed urine. Under the TIC trace are chromatograms of the $[M\text{-}H]^-$ ions of the non-cineole glucuronide peaks found in Figure 1. The masses of the corresponding aglycones (Table 1) are shown in the boxes. Lower panels: the analyses were repeated on the same sample after hydrolysis, using the same sensitivity settings, showing the absence of glucuronide peaks. NL: normalization level.

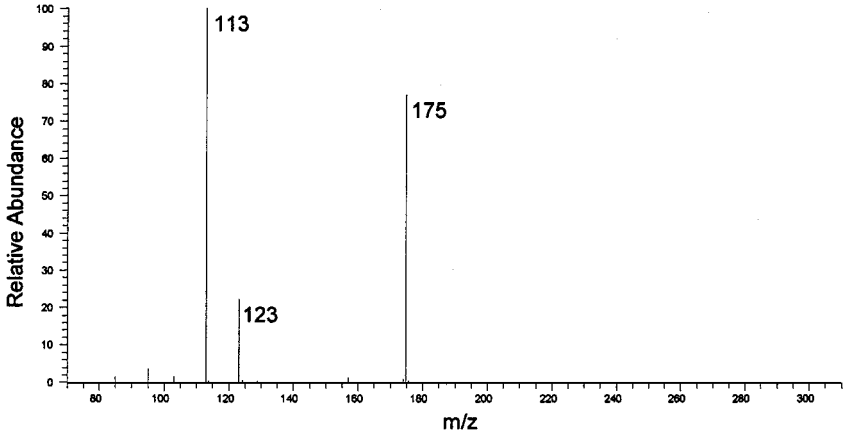

FIG. 3. MS/MS spectrum from $[M\text{-}H]^-$ at m/z 299 showing ions derived from glucuronic acid (m/z 113 and 175) and the deprotonated aglycone (m/z 123, i.e., $C_7H_7O_2$). The possible parent aglycones have the formula $C_7H_8O_2$ and are listed in Table 1.

some extraneous peaks with the same ions were produced. Note that similar sensitivity settings were used before and after hydrolysis, enabling direct comparison between corresponding upper and lower panels. However, the signal strength of each chromatogram depends on that of the formation of each ion and does not indicate the relative quantity of each metabolite.

Identification of Aglycones by GC-MS. The identities of aglycones could not be determined from the LC-MS data alone, so hydrolyzed urine was analyzed by GC-MS and comparison with standard phenols, alcohols, and acids likely to be found in plants. Metabolites were considered to match standards when the retention times and mass spectra were the same (data not shown). Nine metabolites were identified (Table 1), while another two were unknown isomers of methyl dihydroxy benzene (i.e., other than one of the standards). Searches were also made of the TIC chromatograms for possible aglycone molecules, other than the standard compounds selected, but none was found. Not all glucuronide peaks were identified; in particular, those that were present at low levels from the TIC chromatogram were not pursued.

After hydrolysis, the major aglycones in the urine of koalas fed *E. cephalocarpa* were 4-methylcatechol, two unidentified isomeric $C_7H_8O_2$ phenols, salicyl alcohol, and resorcinol (Table 1). No salicylic acid was detected, but there were small amounts of benzoic acid and benzyl alcohol, as well as orcinol and *p*-cresol. The glucuronides of phenol and phloroglucinol were also detected by LC-MS but not quantitated by GC. Phenol was not found by GC-MS and, in

comparison with a phloroglucinol standard, koala urine showed an insignificant peak in the GC-FID chromatogram.

Quantitation. Calibration curves were linear over the range of concentrations measured, with $r^2 \geq 0.99$. There were no interfering peaks present in the samples of blank urine used to prepare calibration standards, either before or after hydrolysis, nor were any of the measured aglycones present.

Comparison of urine before and after hydrolysis showed that, except for one of the unidentified phenols, conjugation was either complete or nearly so for all compounds in Table 1. Although urine was collected on two separate days, only the results from 1 day are presented, as one animal failed to urinate on the other day. However, the amounts of the metabolites excreted, free and conjugated, were not significantly different on the two collection days (paired t tests, $P > 0.05$, 4 df).

Quantitative data on cineole metabolites was taken from a previous study of urine from the same animals collected 2 days later (Boyle et al., 2001). The diet was the same, and the total glucuronic acid excreted (1148 $\pm$ 318 μmol/kg, mean $\pm$ SE) was similar to that found in the present study (1077 $\pm$ 149 μmol/kg, paired t test $P = 0.823$, 5 df). The aglycones identified were predominantly phenols and unrelated to the terpene metabolites. The total conjugated non-terpenes (642 μmol/kg) accounted for 58.9% $\pm$ 7.2% total urinary glucuronic acid in the koala.

DISCUSSION

The origin of most of the glucuronic acid in koala urine is attributable to dietary phenols. The phenols, together with small amounts of benzyl alcohol and benzoic acid, accounted for about 60% urinary glucuronides. Previously we have found that cineole-derived glucuronides accounted for 11% of urinary glucuronic acid in the same animals, maintained on the same diet (Boyle et al., 2001). Combining these findings, a total of about 70% of urinary glucuronic acid can be accounted for as phenolic and terpene derived conjugates in the koala maintained on a diet of *E. cephalocarpa* leaf. Although the remaining glucuronic acid is unaccounted for, it seems likely that it is present in conjugation with other, as yet unidentified, aglycones, since virtually no glucuronic acid is excreted when animals are fed a diet free from PSMs.

It seems likely that similar patterns occur in other eucalyptus-leaf-eating marsupials. The brushtail possum formed the same phenolic glucuronides (unpublished data) and, as in the koala, the major proportion of its terpene metabolites were excreted unconjugated. In the ringtail possum and greater glider, virtually none of the terpene metabolites are conjugated, so the urinary glucuronide is likely associated with the same (or similar) leaf-derived phenols.

Low-molecular-weight phenols (such as the aglycones reported here) occur in living cells in combined form with various sugars, mostly glucose (Harborne,

1964; Goodwin and Mercer, 1983). Therefore, the urinary glucuronides are probably derived from glycosides in the leaf. Glycosides which have a hydrophobic domain (e.g., the disaccharide amygdalin) are transported intact across the ileum, and hydrolyzed by a nonspecific hepatic β-glucosidase (LaMarco and Glew, 1986; Gopalan et al., 1992). The liberated aglycone can then be conjugated with glucuronic acid by UGT and UDP-glucuronic acid (Parkinson, 2001). The net result is the conversion of a mono- or disaccharide of glucose or another neutral sugar into the highly ionized glucuronide conjugate, which is excreted more efficiently by the kidney.

This is illustrated by the metabolic fate of salicyl alcohol glucoside (salicin, found in *Salix* and *Populus* spp.) after oral dosage in the brushtail possum (McLean et al., 2001). Some unchanged salicin (10–18% dose) was found in urine, showing that salicin can be absorbed and excreted unchanged. However, most had been hydrolyzed and the major metabolite (56–64%) was the glucuronide conjugate of the aglycone, salicyl alcohol. This is direct evidence of a hydrolysis-reconjugation mechanism for plant glucosides.

Glucuronidation is active in marsupials and appears to be the predominant conjugation reaction for phenols. The fate of [^{14}C] phenol was studied in nine marsupial species, and the major urinary metabolites were glucuronides in the koala (95%) and brushtail possum (90%) (Baudinette et al., 1980). However, a study of the fate of [^{14}C] benzoic acid in seven marsupials found that the glycine conjugate, hippuric acid, was the major metabolite, with benzoyl glucuronide being a minor metabolite in all species (6% dose in the brushtail possum) (Awaluddin and McLean, 1985). Hippuric acid was also the major metabolite of benzoic acid in the koala (Figure 2).

The LC-MS method described here has proven to be an efficient means of detecting glucuronide conjugates in urine. No workup was required, apart from dilution and filtration before liquid chromatography. Negative ESI resulted in satisfactory ionization of glucuronides from all compounds studied: phenols, benzoic acid, and benzyl alcohol as well as the varied terpene metabolites (alcohols, acids, and hydroxy acids) (Boyle et al., 2001). Glucuronide-selective chromatograms can be produced using data-dependent MS/MS scans from the most intense TIC peaks and monitoring for the characteristic ions at m/z 113 and 175, as in Figure 1. The m/z 175 ion corresponds to cleavage at the carbonyl carbon of the glucuronide residue. The mechanism for the formation of the 113 ion is postulated as the further loss of H_2O and CO_2 from the m/z 175 ion (Chen et al., 1998).

The aglycones in this study could not be identified from LC-MS data alone, because of the similarity of their masses and mass spectra. As is usually the case, there were no standards available for the glucuronide conjugates. Therefore, a list of likely compounds was drawn up from known leaf constituents and urinary phenols found in these animals, using the molecular weights that had been found by LC-MS. These standard compounds were analyzed by GC-MS, and their retention times and

mass spectra were compared with those of peaks from hydrolyzed urine extracts. Thus, the combination of LC-MS for detection of glucuronides and GC-MS for identification of aglycones was an efficient means of describing novel glucuronide metabolites. Quantitation was best achieved by analysis of the aglycones before and after hydrolysis to determine the amount excreted free and conjugated.

In the folivorous marsupials that have so far been studied (the koala, brushtail possum, ringtail possum, and greater glider), it appears that glucuronidation is required for xenobiotics that cannot be oxidized sufficiently for rapid excretion (Boyle et al., 1999, 2000a, 2000b, 2001). Thus, phenolic PSMs are mostly conjugated, whereas terpenes are mainly oxidized to metabolites, which are sufficiently polar to be excreted without conjugation. Marsupials use both phase I (oxidative) and phase II (conjugative) metabolic reactions, which target different types of dietary PSMs.

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CHEMICAL CUES IDENTIFY GENDER AND INDIVIDUALITY IN GIANT PANDAS (*Ailuropoda melanoleuca*)

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Abstract—The Giant panda communicates with conspecifics by depositing a mixture of volatile compounds (called scent marks) on trees and rocks. Using mass spectrometry, we identified 951 chemical components from scent glands, urine, vaginal secretions, and scent marks made by pandas. The scent marks of the two genders contained a similar array of chemicals but varied in concentration; specifically, males possessed a significantly greater amount of short chain fatty acids ($F(1, 29) = 18.4$, $P = 0.002$). Using stepwise discriminate analysis on the relative proportions of a subset of these chemicals, it was possible to classify gender (94% for males and females) and individuality (81% for males and 91% for females) from scent marks. The power to identify individual males was reduced due to the relatedness of two subjects. By cracking the identity code of Giant panda communication, we show insights into how these animals can match individuals with unique chemical profiles. Since radiocollaring is currently banned in China, the techniques described in this paper give field biologists a new means to identify and track pandas in the wild.

Key Words—Giant panda, scent mark, scent communication, olfaction, mass spectrometry, individual identity, urine analysis, short chain fatty acids.

INTRODUCTION

The Giant panda (*Ailuropoda melanoleuca*) is a rare solitary animal that inhabits and feeds on dense bamboo forests in isolated mountains of China. These animals have little social contact with each other, with the exception of a few days each spring during the female's estrous period. Pandas are believed to communicate with each other primarily by olfactory signals, and have been observed to leave

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behind a trail of chemical-rich scent marks deposited on environmentally prominent features, such as trees and rocks (Kleiman, 1983). The source of the scent is a specialized gland located near the urogenital region beneath the tail. Giant pandas use a variety of distinctive postures to rub chemicals from this gland onto substrates to form the marks (Schaller et al., 1985). Males mark sites throughout the year, using front handstands, cocking one rear leg, or by backing up against the substrate. Females, in contrast, form marks more commonly by squatting on the ground, or by rubbing their gland against a substrate. They scent mark much less frequently than males, with the exception of a 2 wk span leading up to their estrous period. Scent marks are believed to transmit messages of sexual receptivity and estrus, dominance and territory ownership, individuality, kinship and group membership, and thus may be the primary means of mediating social interactions in a solitary species like the Giant panda.

The abundance, novelty, and quantity of chemicals that relay pertinent information in scent communication appear to vary from species to species. The message of scent may be coded in the presence of a large quantity of a few chemicals, as in hamsters (Singer et al., 1976), a small amount of a novel chemical, as in elephants, (Rasmussen et al., 1997), or the interaction of several chemicals, such as in marmosets (Smith et al., 2001). In a few species, information coding for individuality has been attempted (Salamon and Davies, 1998; Lawson et al., 2001).

A recent surge of behavioral work has been conducted on olfactory communication in the Giant panda (Swaisgood et al., 1999, 2000). The pairing of this extensive behavioral work with a detailed chemical analysis of the olfactory signal (this study) will enhance our understanding of the information contained within these marks. Previous chemical research has focused on social species such as rodents and primates. Pandas are a solitary monoestrous species. Understanding the depth of information that is transmitted in panda scent compared to that of a social species may demonstrate the evolutionary flexibility of olfaction and illustrate the different roles scent plays under varying social situations.

METHODS AND MATERIALS

Scent samples were obtained from Giant pandas housed at the San Diego Zoo, National Zoo (Washington, DC), Mexico City Zoo (Mexico), and the Wolong Breeding Center (China). Scent mark samples were taken from environmental objects following observation of scent mark postures; anal gland and vaginal samples were taken while pandas were anesthetized for medical procedures, and urine was obtained either from drains in the bedroom or during medical procedures. All samples were taken during the spring breeding seasons 1997 to 2001, and all animals were of reproductive age as of 1997. A minimum of three scent mark samples

was collected from five adult females and five adult males during multiple years. These 10 animals represented 16% of the world's captive population at the time of sampling.

Analytical Determination. Samples were collected by rubbing over the site with a sterile Q-tip, which was stored in a glass vial at −20°C until analysis. Volatile compounds were extracted (35 min) from the Q-tip by using a solvent free solid phase matrix extractor (SPME) containing a 65-μ polydimethylsiloxane fiber (Supelco, Bellefonte, PA). Fiber contents were analyzed by capillary GC-MS, with an Hewlett-Packard 5890 Gas Chromatograph-5970 MSD, controlled by HP/UX Chem Station software. The column was a Supelco 60-m, 0.25-mm id, low-polarity SPB-octyl, operated using a gradient of 75°C held for 9 min followed by a 1.8°/min ramp up to 210°C. A splitless injection was used with an injection temperature of 250°C. Helium was used as the carrier gas with a 7-p.s.i column head pressure. Relative retention times and fragmentation spectra of peaks obtained by GC-MS were matched with those of known standards for identification. The remaining scientifically unnamed compounds were still used for analysis, because they possessed a reproducible retention time and mass fragmentation pattern and could be classified according to chemical function. These compounds were assigned a number based on their column retention time.

Several background objects were analyzed and their volatile pattern subtracted from the scent profile to minimize contamination from human direct and indirect sources. Identified contaminate sources included paint, cleaning supplies, disinfectants, tobacco, and foliage found in the enclosures.

Aerobic bacterial cultures and antibiotic sensitivity patterns were analyzed on anal gland swabs. Tests were performed by the Microbiology Laboratory at IDEX Veterinary Services, Inc., Sacramento, CA.

Statistics. A subset of volatile compounds that occurred in at least 10% of the samples was used in the analysis. Analysis was conducted on the relative proportion of the peak size to the overall total area of the chromatogram rather than on the absolute concentration of the peak. Fisher discrimination analysis was used to differentiate group membership, and cross validation percentage (also known as the leave-one-out method) is reported. The first and second canonical variables were plotted against each other. An analysis of variance (ANOVA) was conducted on specific chemicals based on functional groups. Statistical analysis was conducted using SPSS 10.0 and Statview 5.0.

RESULTS

We analyzed volatiles and semivolatiles obtained directly from Giant panda scent glands, from urine, vaginal secretions, and from naturally deposited scent

TABLE 1. CHEMICAL COMPOUNDS IN SCENT MARKS OF THE GIANT PANDA[a]

n-Butane	2-Butanol	Acetic acid	2-Methyl-1-propanol
0.184	1-Propanol	Propionic acid	0.228
Chloroform	1-Butanol	0.255	Isobutyric acid
n-Valeraldehyde	0.292	0.308	Butyric acid
Methyl butyrate	3-Methyl-1-butanol	2-Methyl-1-butanol	*n*-Heptane
0.355	Isopentanoic acid	2-Methyl butyric acid	Cyclopentanone
0.411	Hexyl aldehyde	Ethyl butyric acid	*n*-Pentanoic acid
4-Methyl-3-penten-2-one	Toluene	0.465	4-Hydroxy-4-methyl-penten-2-one
n-Octane	0.512	1-Hexanol	0.548
2-Heptanone	0.593	0.599	Cyclohexanone
0.615	Hexanoic acid	*o*-Xylene	3,4-Dimethyl heptane
Phenol	*p*-Xylene	Styrene	*m*-Xylene
0.717	1-Heptanol	0.723	0.742
Benzaldehyde	0.767	0.773	0.774
2,2-Dimethyloctane	0.768	3-Octanone	2-Octanone
0.806	0.816	Heptanoic acid	Hexyl acetate
Octyl aldehyde	0.834	0.852	*o*-Cresol
0.864	*m*-Cresol	*p*-Cresol	0.883
0.892	Phenyl acetaldehyde	β-Pinene	0.917
n-Decane	0.926	0.927	0.939
0.945	α-Terpinene	0.973	*p*-Cymene
Phenyl methyl ketone	1,8-Cineole	0.988	2-Nonanone
Limonene	*o*-Tolualdehyde	1.004	Octanoic acid
1.030	Nonyl aldehyde	1.074	1.085
γ-Valerolactam	1.147	*n*-Undecane	1.218
n-Decyl aldehyde	1.235	Methyl salicylate	1.246
1.256	α-Terpineol	*n*-Dodecane	Indole
1.390	1.449	1.501	

[a] Chemical compounds (111 total) isolated from Giant panda scent marks and used in the statistical analysis. Known compounds are identified by name. Unnamed compounds are recognized by their relative retention time number (listed in the table) and mass spectra fragmentation pattern (not shown).

marks, and recorded the presence of 951 separate compounds. From this list, 111 compounds (Table 1) met the minimum criteria of occurring in at least 10% of all samples and were used in subsequent statistical analysis. Both male and female gland samples contained many of the same compounds, with a 95% overlap in female compounds to all compounds and a 87% overlap in male compounds to all compounds. Concentration of five short-chain fatty acids (acetic, propionic, isobutyric, butyric, and isopentanoic) differed significantly between males and females ($F(1, 29) = 18.4$, $P < 0.001$), with these acids making up more than one half of the gland secretion in males and approximately one eighth in females. The

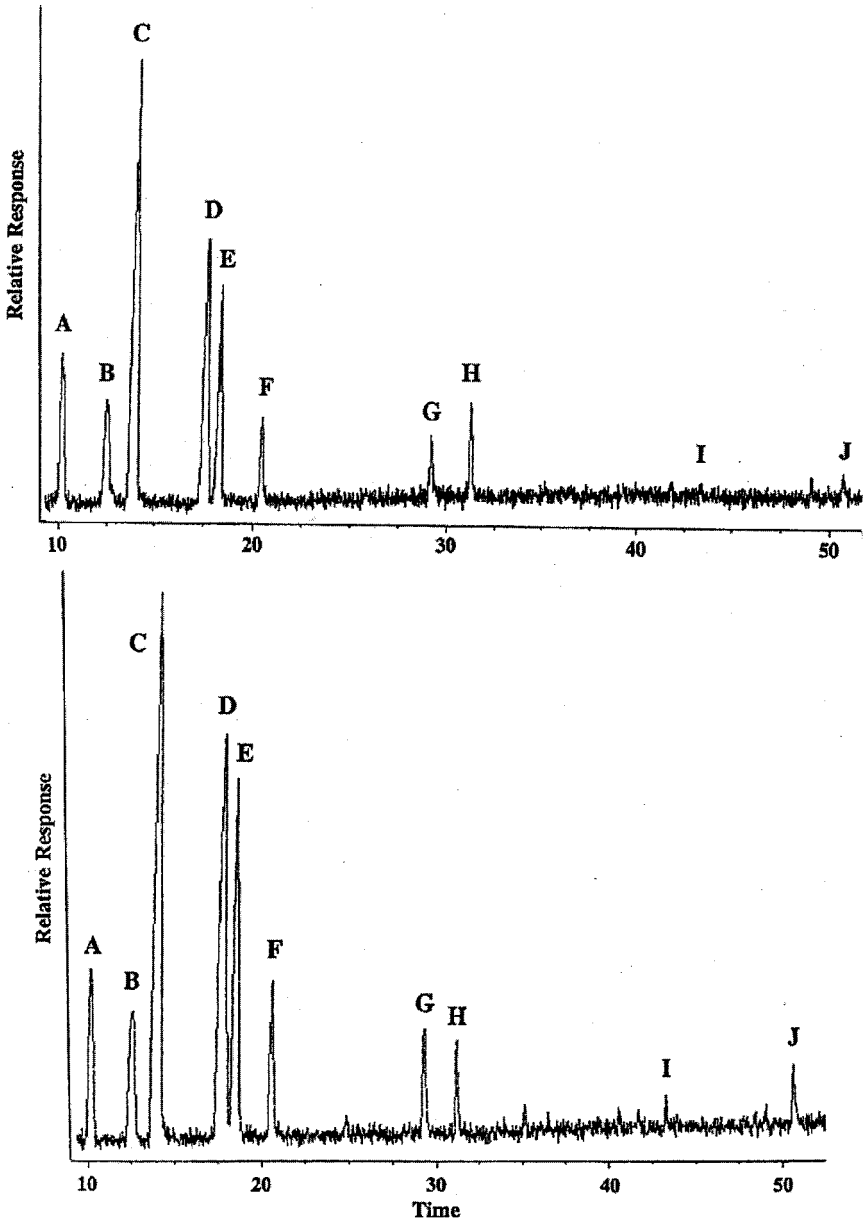


FIG. 1. Volatile chemical profile from a male Giant panda scent gland (upper) and scent mark (lower) made by the same animal, as visualized using GC-MS. The scent mark was left on pine tree bark and was collected 1 hr after deposit. Peak identities are as follows: (A) propionic acid; (B) isobutyric acid; (C) butyric acid; (D) isovaleric acid; (E) 2-methyl butyric acid; (F) valeric acid; (G) hexanoic acid; (H) phenol; (I) *o*-cresol; (J) δ-valerolactone.

dominance of these short-chain fatty acids in the overall scent gland profile is shown in the upper panel of Figure 1; a representative scent gland profile from a male panda. Using Fisher's stepwise discriminant analysis, it was possible to correctly classify the gender of samples obtained directly from scent glands with a cross validation rate of 100% (Wilk's $\lambda = 0.003$).

As shown in Figure 1 (lower panel) for a male panda, the chemical profile of male and female Giant panda scent marks contained the same volatiles as those obtained from their respective glands (100% of crossover for the males and 95% for female components). The concentration of the five major acids in male scent marks was less than the concentration in samples taken directly from the gland ($X_{\text{gland}} = 52.64$ and $X_{\text{scentmark}} = 12.47$). Additionally, three compounds were found in scent marks that were virtually absent in the gland but are present in high proportion in male urine (*n*-valeraldehyde, hexyl aldehyde, and 2-octane). A representative GC-MS profile of a female scent mark is shown in Figure 2. The overall pattern is more complex than those of males; female scent marks also contained short-chain fatty acids, but at a reduced level compared to males ($X_{\text{gland}} = 15.73$ and $X_{\text{scentmark}} = 4.869$). Additional compounds were also found in female scent marks not present in the gland. At least four of these compounds were also present in urine (*n*-valeraldehyde, hexyl aldehyde, 3-octane, 2-octane), and at least two compounds were present in high proportion in vaginal samples (1-butanol and 4-hydroxy-4-methyl-pentan-2-one). Stepwise discriminant analysis was again utilized to classify the gender source of scent marks. Both males and females could be correctly identified from scent marks with a cross-validation rate of 94% (Wilk's $\lambda = 0.001$).

The branched pattern of the short-chain carboxylic acids (C_2—C_6) found in panda scent glands and marks suggested that these compounds are not synthesized directly by the panda, but are instead the products of microbial fermentation. On the basis of the findings of Albone (1974) that short chain fatty acids in scent are produced by bacteria, we isolated and cultured bacterial strains obtained from the scent gland region of a male Giant panda. A mixture of three bacterial species (*Corynebacterium, α-Streptococci*, and *Escherichia coli*) were recovered, and metabolites from these species may directly participate in the formation of the scent profiles.

Following gender classification, stepwise discriminant analysis was again used to determine if scent marks contained sufficient information to correctly identify individual pandas. The canonical variable plot of group membership for females is shown in Figure 3a (upper panel). Individual females could be differentiated at a cross-validation value of 91% (Wilk's $\lambda = 0.002$). The corresponding group membership plot for males is shown in Figure 3b (lower panel), with a classification power of 81% (Wilk's $\lambda = 0.002$). Note that the squares of number 10 (Pan Pan) and squares of number 8 (Da Di) closely overlap.

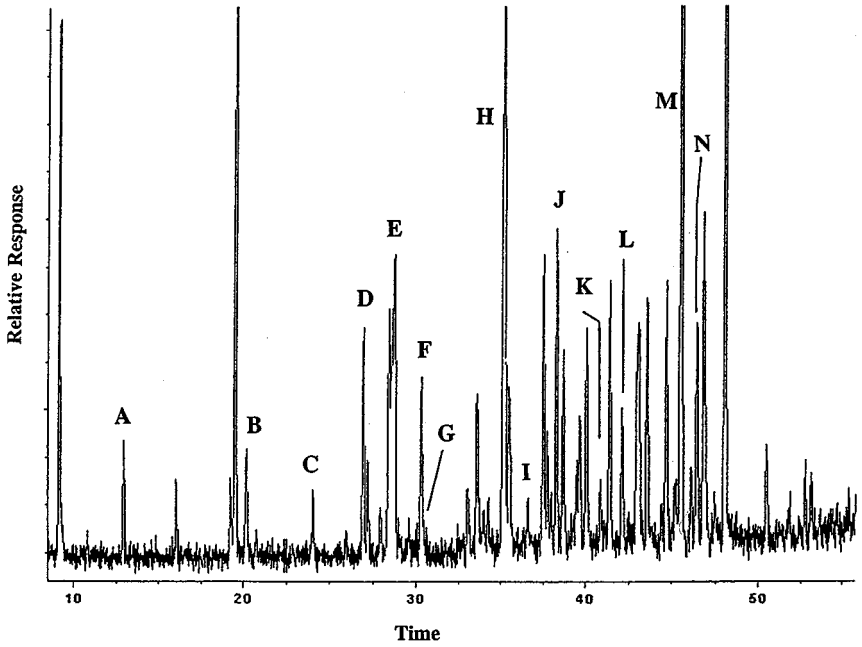
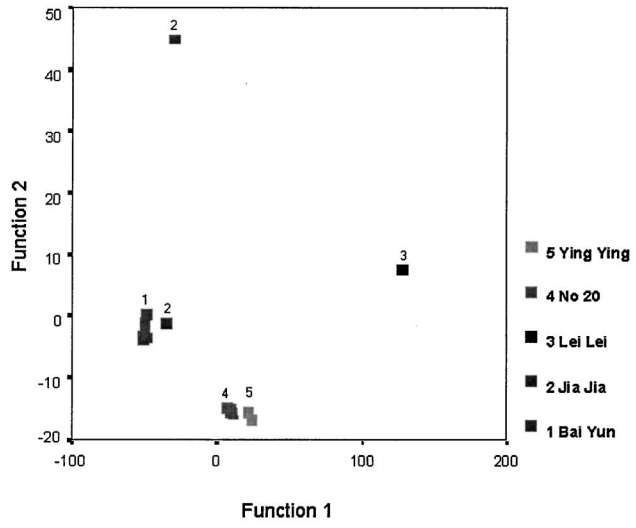


FIG. 2. Volatile chemical profile from a female Giant panda scent mark as visualized using GC-MS. Peak identities: (A) *n*-valeraldehyde; (B) toluene; (C) 1-hexanol; (D) 2-heptanone; (E) hexanoic acid; (F) phenol; (G) *m*-xylene; (H) benzaldehyde; (I) 2- and 3-octanone mixture; (J) hexyl acetate, octyl aldehyde; (K) *o*-cresol; (L) β-pinene; (M) acetophenone; and (N) limonene.

DISCUSSION

Analysis of Giant panda scent marks revealed that a major proportion of the volatiles present originate from and are transferred to the mark directly from the scent gland region. Most marks contained additional compounds not found in scent glands, including environmental contaminants and chemicals known to be present in urine (males and females) and in the vagina (females). Scent gland samples, and often those of scent marks, contained as a dominant component a series of short-chain fatty acids due to the metabolism of amino acids. Such fatty acids are also the dominant constituent of scent marks in other species, including carnivores like canids (Preti et al., 1976) and felids (Albone and Eglinton, 1974). These acids are believed to be formed by commensal bacterial residing in the scent gland (Gosden and Ware, 1976; Albone, 1984). It is possible that males have a greater amount of bacteria in the analgenital region than females, resulting in a greater manufacturing of acids (identification of bacteria from both sexes was

A



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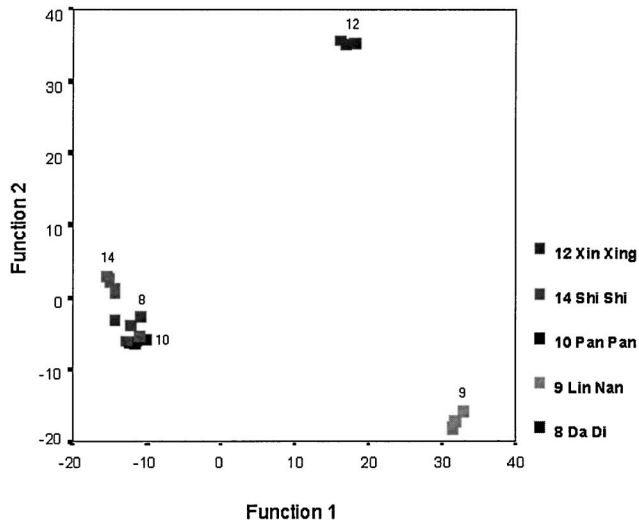

FIG. 3. Conical variable plot of group membership for female (upper panel) and male (lower panel) Giant pandas. The plots are generated by solving spacing equations that compact the data for each individual animal into a single point. Individuals are numbered, and their names are shown to the right of their respective plots.

conducted but not quantified). Additionally, males appear to have a "dirtier" gland than females (R. Swaisgood, personal communication), which could be linked to bacteria concentration and may be attributed to the physiology of the two sexes. Both sexes urinate while scent marking; when females urinate, it exits the urethra and dribbles posteriorly over the gland region and could be washing away resident bacteria on the gland region. In contrast, when males urinate and scent mark simultaneously, urine exiting the penis predominately avoids the gland region.

The extensive information present in scent marks, and our ability to distinguish gender and individuality from the marks, are consistent with behavioral observations on Giant pandas. Through habituation–discrimination paradigms, it has been shown that males and females can differentiate between conspecifics from urine samples (Swaisgood et al., 1999). Males and females also respond differentially to estrous and nonestrous scent and urine (White et al., in press), and exhibit signs of territorially, gender recognition, and age recognition in pen swapping experiments (Swaisgood et al., 2000, in press). Our study implies that the method of discrimination is through quantitative differences in the production of a distinct pattern of low molecular weight volatile compounds found primarily in the scent gland, with a subset of signals from urine and the vagina. Interactions among pandas are rare, but when it occurs it is aggressive. Learning to avoid others that are known to have been victorious in previous encounters can create confidence hierarchies and reduce injury (Barnard and Burk, 1979).

It was interesting to observe that the only two males with a direct family lineage in our sample set also shared a very similar scent profile. The extent to which panda odor (as well as the odor of other species) is under genetic control is poorly understood. Although it has long been popular to believe that there is a strong link between individual odor and MHC phenotype, based primarily on work with rodents (Yamazaki et al., 1982), these ideas have recently been challenged (Ehman and Scott, 2001).

Using the chemical profile of scent deposited on objects in the natural environment, it may be possible to identify and track pandas in the field without disturbing their behavior. This is particulariy important since radiocollaring of Giant pandas is currently banned in China. Using the techniques described in this study, we analyzed an unknown Giant panda scent mark that was collected from tree bark in 1994, 3 years before our study. We found that its depositor was female and that its composition differed markedly from any of the pandas in our database. In summary, the chemical analyses reported here provide insight into a mode of communication of the Giant panda and open the doors to numerous studies on panda behavior.

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ANTIAPHRODISIACS IN PIERID BUTTERFLIES: A THEME WITH VARIATION!

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Abstract—Male *Pieris napi* butterflies previously have been shown to synthesize and transfer an antiaphrodisiac, methyl salicylate (MeS), to females at mating. This substance curtails courtship and decreases the likelihood of female remating. Here, we show that similar systems occur in *Pieris rapae* and *Pieris brassicae*. In *P. rapae*, ^{13}C-labeling studies showed that males utilize the amino acids phenylalanine and tryptophan as precursors to MeS and indole, respectively. These volatiles are transferred to females at mating and function as antiaphrodisiacs, as demonstrated by field tests entailing painting MeS, indole, or a mixture on the abdomens of virgin females and assessing their attractiveness to wild males. With *P. brassicae*, ^{13}C-labeling studies showed that males use phenylalanine as a precursor to synthesize benzyl cyanide, which was demonstrated to function as an antiaphrodisiac by field tests similar to those for *P. rapae*. This communication system exhibits both similarities and differences among the three species; in *P. napi* and *P. rapae*, males are fragrant but transfer a volatile antiaphrodisiac to females that is completely different from the male odor, whereas in *P. brassicae* the antiaphrodisiac transferred by male to female is identical with male odor.

Key Words—Pieridae, pheromone, methyl salicylate, benzyl cyanide, phenyl acetonitrile, indole, phenylalanine, tryptophan.

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INTRODUCTION

Butterfly mating systems vary from monogamy to polygamy (Burns, 1968; Ehrlich and Ehrlich, 1978; Drummond, 1984; Wiklund and Forsberg, 1991). In butterflies, the mass of the ejaculate transferred from the male to the female at mating increases with female polygamy. There is evidence that the ejaculate functions as a nuptial gift, allowing females to increase their reproductive output (Boggs and Gilbert, 1979; Wiklund et al., 1993, 1998; Kaitala and Wiklund, 1994; Karlsson, 1998). Regardless of female remating frequency, male butterflies seem incapable of forcing matings on females, and female receptivity is a prerequisite to successful male courtship (Svärd and Wiklund, 1989). Hence, although last male sperm precedence is the rule in butterflies, as in most insects (Parker, 1970; Drummond, 1984; Bissondath and Wiklund, 1997; Wedell and Cook, 1998), males gain little from courting unreceptive females. Female receptivity is usually signaled by a combination of visual and olfactory signals, visual stimuli being more important at a distance and olfactory stimuli being more important close up (Silberglied, 1984). In the Pieridae, both virgin and mated females adopt a characteristic posture when courted, spreading the wings horizontally and lifting the abdomen vertically, a posture well designed for emitting chemical stimuli close to the location of the hovering courting male (Wiklund and Forsberg, 1985; Forsberg and Wiklund, 1989). Although there is little information on the actual chemicals involved in the signaling of female receptivity/unreceptivity, there is abundant evidence that males are able to distinguish between receptive virgin females and unreceptive mated females. Virgin females are courted intensively, but unreceptive mated females are quickly abandoned (Wiklund and Forsberg, 1985; Forsberg and Wiklund, 1989). Because a recently mated female gains from becoming unattractive, and mating males gain from inducing female monogamy, natural selection should select for male capability to induce female unreceptivity and unattractiveness. In line with this expectation, Gilbert (1976) suggested that males in the butterfly *Heliconius erato* (Nymphalidae) transfer an antiaphrodisiac (Happ, 1969) to females during mating to reinforce female monogamy. Recently, Andersson et al. (2000) demonstrated the existence of such a semiochemical in the green-veined white butterfly, *Pieris napi* (Pieridae), with males synthesizing methyl salicylate (MeS), which was transferred to females at mating and which rendered females unattractive to other males. Although male *P. napi* emit a strong fragrance of geranial and neral (citral), the volatile antiaphrodisiac MeS is biosynthetically different. In this paper, our objective was to test whether similar communication systems exist in *Pieris brassicae* and *Pieris rapae*, two close relatives of *P. napi*. Our results demonstrate that this is indeed the case, with males of both species producing antiaphrodisiacs that were transferred to females during mating, rendering them unattractive to other males. In *P. rapae*, MeS and indole were used as antiaphrodisiacs, whereas *P. napi* needed only one major compound. Furthermore, in *P. brassicae*, benzyl cyanide (BC) was used as an antiaphrodisiac, the same substance that is the characteristic male odor,

suggesting that male *P. brassicae* make females unattractive by cloaking their mates with their own male odor, thus making the females chemical mimics of males.

METHODS AND MATERIALS

Sampling Technique. To assess whether differences in female attractiveness to males was mediated by some volatile substance, we used the solid-phase microextraction [SPME, see analytical method] technique, and analyzed all SPME samples on a coupled gas chromatograph-mass spectrometer (GC-MS). To identify the odor emitted by virgin and mated females, and by males of *P. rapae* or *P. brassicae*, we used a glass cylinder (height: 18 cm, diameter: 8 cm, $V = 905\ \text{cm}^3$) with three small openings and one SPME fiber holder placed in each of the three openings. Sampling time was 1 hr for the first fiber, 2 hr for the second, and 3 hr for the third. To identify the odor from spermatophores, three dissected spermatophores were placed in a small sealed vial in which the SPME fiber was placed a few millimeters above the sample. The volatile compounds released from the spermatophores were trapped for 1 hr (Table 1).

Analytical Methods. Polydimethylsiloxane–divinylbenzene fibers (65 μm, Supelco™, Stockholm, Sweden) were used to sample the volatiles from live butterflies (Borg-Karlson and Mozuraitis, 1996). The SPME fibers were desorbed before each sampling by heating in the GC injector (250˚C, for 10 min) with a He gas flow. Analyses were conducted on a Varian 3400 gas chromatograph coupled to a Finnigan SSQ 7000 mass spectrometer (GC-MS). A DB-WAX column (30 m $\times$ 0.25 mm i.d., film thickness, 0.25 μm, J&W Scientific, Folsom, CA) was used, programmed for increases in temperature from 40°C (1 min) at 5°C/min to 110°C, and further through 10°C/min to 200°C (10 min), with an injector temperature of 225°C (splitless injection, 45 sec) and He carrier gas at 69 kPa. Identification of the compounds was made by comparison of retention times and mass spectra with authentic reference samples: MeS (98%, Lancaster), BC (99%, Aldrich), and indole (99.0%, Aldrich).

^{13}C-labeling Experiments. The GC-MS analyses showed that mated *P. rapae* females emitted MeS and indole, and mated *P. brassicae* females emitted BC when courted by males. To assess whether these substances could be transferred

TABLE 1. NUMBER OF ODOR COLLECTIONS INDIVIDUALS ANALYZED

	Virgin female		Mated female		Male		Spermatophore	
Species	R[a]	I[b]/R	R	I/R	R	I/R	R	I/R
P. rapae	8	5	10	5	5	5	9	3
P. brassicae	8	5	10	5	5	5	7	3

[a] Number of replicates.
[b] Number of individuals.

by the male to the female at mating, we dissected out spermatophores from newly mated females and analyzed the odors emitted by three spermatophores using the GC-MS and SPME techniques described above. To further assess which sex(es) synthesized MeS and indole (*P. rapae*) or BC (*P. brassicae*), we conducted a labeling experiment. Both male and female *P. rapae* larvae were fed leaves treated with labeled L-phenylalanine [ring 6^{13}C. 99% (GC), 0.3 μg, Cambridge Isotope Laboratories, Inc.] or L-tryptophan [1^{13}C. 99% (GC), Cambridge Isotope Laboratories Inc.]. *P. brassicae* larvae were fed leaves treated with only L-phenylalanine [ring 6^{13}C. 99% (GC), 0.3 μm Cambridge Isotope Laboratories, Inc.]. Larvae were fed in the final instar when weighing 100–150 mg, approximately 1–2 days prior to pupation. When the adult butterflies eclosed, the labeled males were mated with nonlabeled females and vice versa. All females were sampled as described in *Sampling Technique* and analyzed by GC-MS.

Laboratory and Field Experiments. We performed a field test to determine whether MeS and indole (*P. rapae*) or BC (*P. brassicae*) functioned as antiaphrodisiacs. We tested whether virgin females painted with hexane solutions of MeS and indole (*P. rapae*) or BC (*P. brassicae*) were less attractive than virgin females painted with only hexane. To ascertain that the amount of MeS and indole (*P. rapae*) or BC (*P. brassicae*) applied to virgin females was physiologically/behaviorally relevant, we painted five virgin females with 2 μl MeS and indole solution (*P. rapae*) or BC solution (*P. brassicae*) (1/100 dilutions in hexane, ca. 20 μg/female). The odor was sampled by SPME and analyzed by GC-MS in the same way as the five mated females in a glass cylinder (see above). The amount of MeS and indole (*P. rapae*) or BC (*P. brassicae*) obtained was compared to the amount of MeS and indole (*P. rapae*) or BC (*P. brassicae*) obtained from the SPME analyses of mated females. Comparison showed that the painted females emitted MeS and indole (*P. rapae*) or BC (*P. brassicae*) in amounts similar to those emitted by mated females.

In the *P. rapae* field test, 20 newly eclosed males were released in a small field in which a young crop of *Brassica napus* was growing. On the margins of the field there were plenty of flowers on which the butterflies could forage for nectar, and most of the males remained in the area in which they were released for 2–3 hr. During this time period, we released painted females one at a time close to a flying male. Eight females were painted with 2 μl MeS (1/100 in hexane, ca. 24 μg/female), seven females were painted with 2 μl indole (1/100 in hexane, ca. 20 μg/female), eight females were painted with 2 μl MeS and indole (ratio 1:1) (1/100 in hexane, ca. 20 μg/female), and eight females were painted with hexane as controls. The females were monitored individually, and the duration of each encounter with a male was measured. Each female was followed until mating or until she had been courted by males five times.

In the *P. brassicae* field test, 20 newly eclosed males were released in another field in which a young crop of *Brassica napus* was growing. The conditions were similar to those in the *P. rapae* test, and most of the released males remained in the area in which they were released for a couple of hours. During this time period,

we released the painted females one at a time in close vicinity of a flying male. Eight females were painted with 2μl BC (1/100 in hexane, ca. 20 μg/female), and six females were painted with hexane. The females were monitored individually, and time was measured for each encounter with a male. Each female was followed until mating or until she had been courted by males five times.

RESULTS

GC-MS analyses of the odors emitted by virgin and mated females showed that the main difference was that only mated females emitted MeS and indole (*P. rapae*) or BC (*P. brassicae*) (Figure 1). GC-MS analyses of spermatophores dissected from recently mated females showed that MeS and indole (*P. rapae*) or BC (*P. brassicae*) were emitted from spermatophores as well (Figure 1), suggesting that MeS and indole (*P. rapae*) or BC (*P. brassicae*) might be transferred by males to females.

This inference was shown to be correct in experiments in which male and female larvae were fed L-phenylalanine or L-tryptophan (*P. rapae*) and L-phenylalanine (*P. brassicae*). After labeled males had been mated with nonlabeled females, and vice versa, only nonlabeled females emitted MeS and indole (*P. rapae*) or BC (*P. brassicae*) containing the ^{13}C-labeled aromatic ring structure (Figure 2).

Hence, our results unequivocally demonstrate that MeS and indole (*P. rapae*) or BC (*P. brassicae*) emitted by mated females are transferred to the female by the male at mating. The results also show that L-phenylalanine is a precursor to BC in *P. brassicae*. In *P. rapae*, the results indicate that L-tryptophan is a precursor to indole, and that L-phenylalanine is a precursor to MeS (as previously demonstrated in the case of *P. napi* (Andersson *et al.*, 2000) (Figure 3).

MeS and indole (*P. rapae*) or BC (*P. brassicae*) were also tested as antiaphrodisiacs in field tests. The results showed that male *P. rapae* courted virgin females painted with either MeS or indole, or MeS + indole 1:1 blend a shorter time than virgin females painted with the solvent hexane [MeS $= 3.70 \pm 0.985$ sec (mean $\pm$ standard error), $N = 30$; indole $= 6.12 \pm 1.06$ sec, $N = 26$; MeS + indole $= 6.02 \pm 1.02$ sec, $N = 26$ vs. hexane $= 30.9 \pm 1.71$ sec, $N = 10$; $P < 0.001$, $F = 68.67$] (Figure 4). With *P. brassica*, males also courted the virgin females painted with BC a shorter time than virgin females painted with hexane [BC $= 1.31 \pm 5.73$ sec (mean $\pm$ standard error), $N = 16$; hexane $= 49.6 \pm 6.62$ sec, $N = 12$; $P < 0.001$, $F = 30.397$] (Figure 4). Moreover, none of the virgin females painted with MeS and indole (*P. rapae*) or BC (*P. brassicae*) were mated, whereas all virgin female controls painted with hexane were mated.

DISCUSSION

Although female *Pieris* spp. gain from remating through the nuptial gift transferred by males, experiments with *P. napi* have shown that females have a substantial refractory period after mating and only remate after an average of 4–6 days

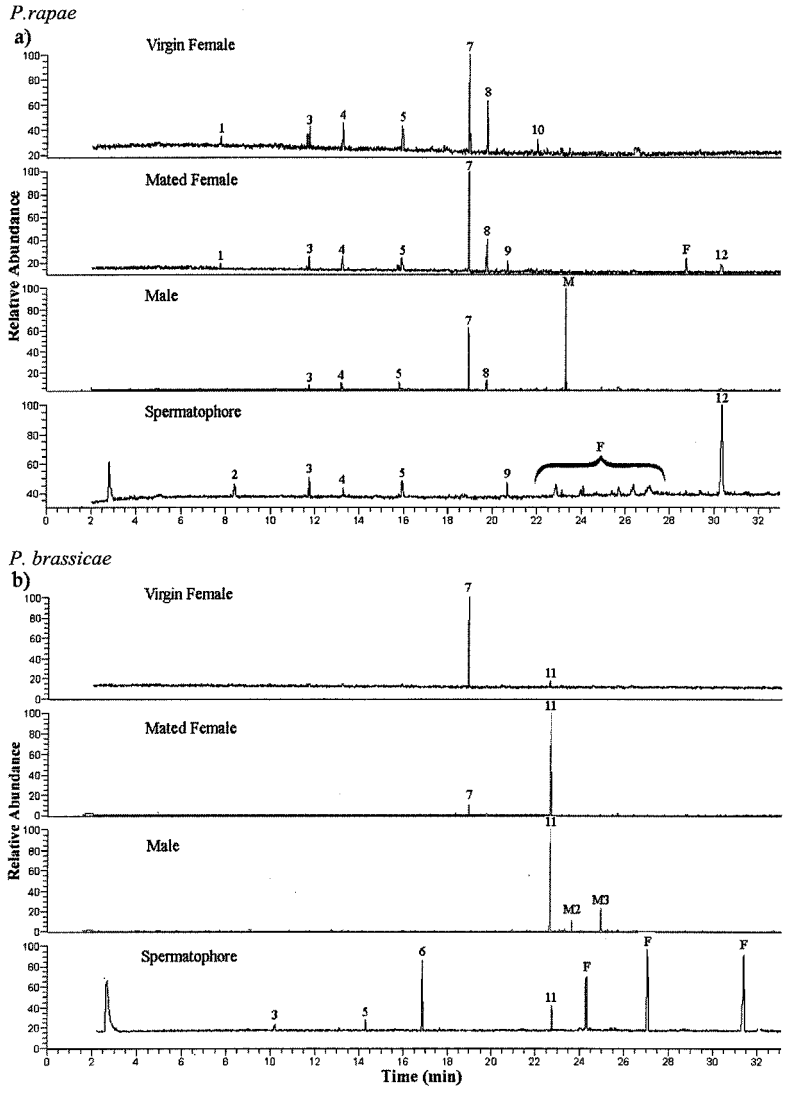

FIG. 1. (a) *Pieris rapae*: GC-MS analyses showed that MeS (9) and indole (12) were emitted by both mated females and spermatophores, but not by virgin females. Other components were heptanal (1), unidentified (2), sulcatone (3), nonanal (4), decanal (5), oxygenated monoterpenes (7, 8), and benzyl alcohol (10). "M" is only present in the male odor and "F" indicates background odors originating from the SPME fiber or the headspace area. (b) *Pieris brassicae*: GC-MS analyses showed that BC (11) was emitted by both virgin and mated females, males, and spermatophores, and virgin females emitted only trace amounts of this substance. Other compounds emitted were sulcatone (3), decanal (5), a carboxylic acid (6), an oxygenated monoterpene (7), and two unidentified male odors (M_2 and M_3). "F" indicates background odors originating from the SPME fiber or the headspace area.

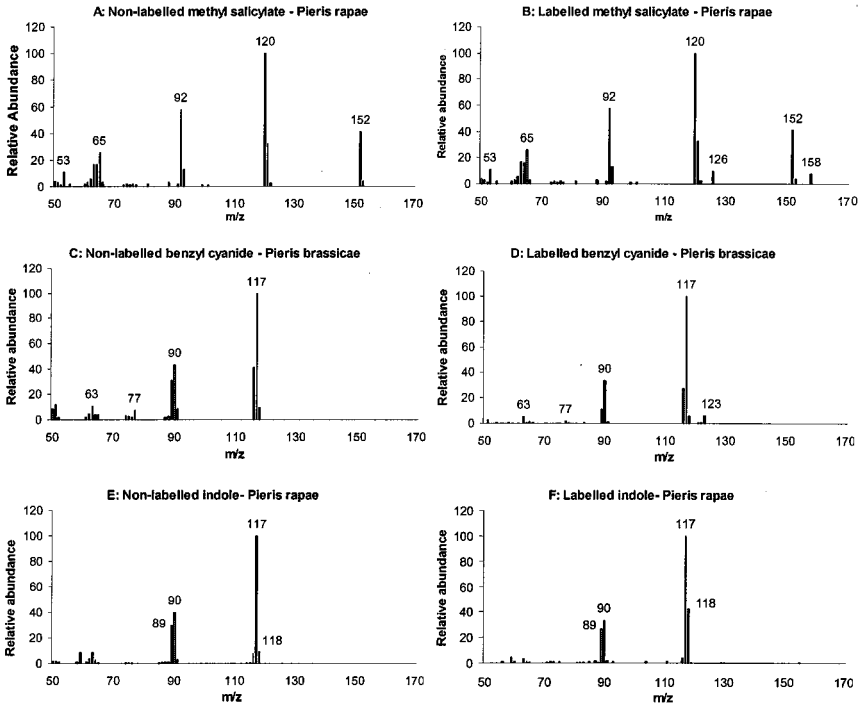

FIG. 2. Comparison of mass spectra of the antiaphrodisiacs MeS, indole (*P. rapae*), and BC (*P. brassicae*). The effluvia emitted by the labeled females after mating with a nonlabeled male (mass spectra A, C, E) and nonlabeled female after mating with a labeled male (mass spectra B, D, F) were collected on SPME fibers. Mass spectrum of MeS originating from ^{13}C-labeled phenylalanine fed male, B, contains two additional fragments of m/z 126 and 158, 6 amu larger than m/z 120 and 152 of synthetic MeS. Mass spectrum of indole, originating from ^{13}C tryptophan fed male, F, contains an enhanced fragment m/z 118, 1 amu larger than m/z 117 of synthetic indole. Mass spectrum of BC, D, originating from ^{13}C-labeled phenylalanine fed male contains an additional fragment m/z 123, 6 amu larger than m/z 117 in the mass spectrum of synthetic BC.

(Wiklund et al., 1993, 1998; Kaitala and Wiklund, 1994). There are at least two reasons why females wait several days until they remate. First, ejaculate mass transferred by male to female *Pieris* is extensive and can correspond to as much as 23% of the female's body mass so that a new ejaculate can only be received by a female when the first one has been absorbed (Forsberg and Wiklund, 1989). Second, there is a time conflict between egg-laying and mating—both activities require sunny weather, and time spent *in copula* interferes with egg-laying [and copula duration can be extensive in *Pieris*—copulation can last up to 21 hr (Kaitala

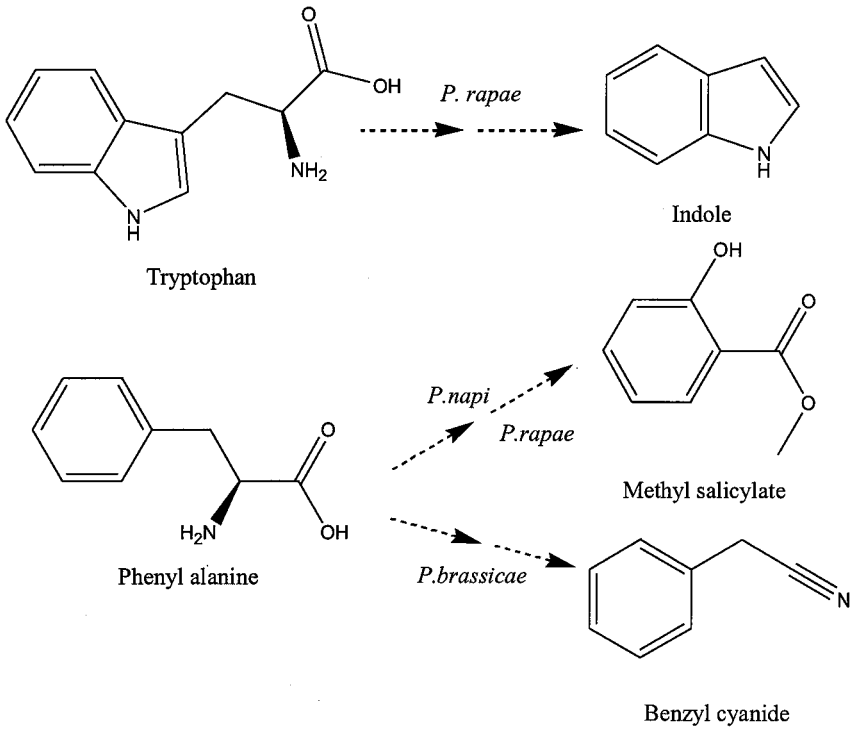

FIG. 3. Scheme of the biosynthetic origins of the antiaphrodisiacs. Our ^{13}C-labeling studies showed that indole emitted by *P. rapae* originated from tryptophan, and that MeS emitted by *P. rapae* and *P. napi* (Andersson et al., 2000) and BC emitted by *P. brassicae* originated from phenylalanine.

and Wiklund, 1995)]. The unattractiveness of recently mated females benefits both the male and the female (Forsberg and Wiklund, 1989). Males benefit by delaying female remating as long as possible to avoid sperm competition from other males, and females benefit by not being harassed by other males, thus increasing the time available for oviposition. This mutual interest between a female's first mate and the female turns into conflict as the female gradually becomes receptive to remating. Which sex has the upper hand in this conflict depends on whether females can control their attractiveness (i.e., the release of antiaphrodisiac) or not. If females do not have complete control and release whatever titer of antiaphrodisiac that remains when courted, this means that the female's first mate exercises some control over female remating frequency. However, if females do have complete control over antiaphrodisiac release, they should be able to realize a level of polyandry best suited to their own interests.

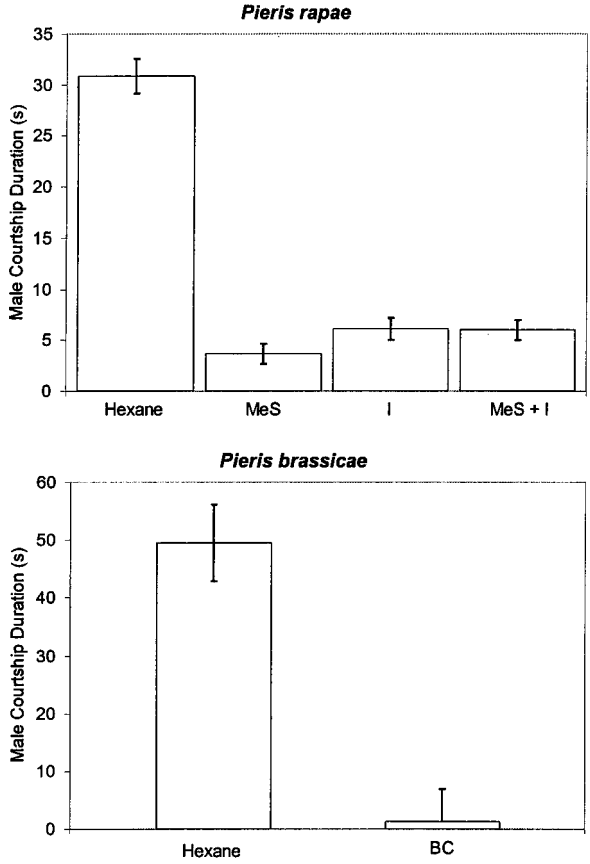

FIG. 4. Duration in seconds (s) of *Pieris* spp. male courtship of females painted with various treatments in field experiments. MeS = methyl salicylate, I = indole, BC = benzyl cyanide.

All of the three investigated *Pieris* species have a system for intersexual communication that is essentially similar: males synthesize a volatile substance (or substances) that is transferred to the female at mating and renders these females unattractive to males that subsequently court these females. From a functional viewpoint, this system has two particular characteristics of interest because males cannot force matings upon females. A volatile that is produced and transferred from male to female functions as an honest signal that emits the information that the recently mated female really has mated. Hence, courting males confronted with females that perform the typical pierid "spread wings—elevated abdomen," the so-called mate-refusal signal (Obara, 1964), can distinguish between mated and

virgin females that are displaying the unreceptive signal. Thus, males can make a "correct decision" and continue courting a virgin, albeit apparently unreceptive female, but quickly abandon an unreceptive female that has already mated.

Finally, it is interesting to speculate on the use of signaling substances and the information they convey. The antiaphrodisiacs emitted by *P. brassicae* and *P. napi* females are also present in some of their cruciferous host plants. Thus, we might expect interactions caused by the plant odors on the behavior of the butterflies. As pointed out in the introduction, male *P. napi* emit neral and geranial when disturbed, and eventually when courting females. These substances are not biosynthetically related to the volatiles that the males transfer as an antiaphrodisiac to the female. The same pattern prevails with *P. rapae*, where males also emit a characteristic, so far unidentified male odor. However, males transfer different volatiles to females as antiaphrodisiacs. In *P. brassicae*, males emit a strong odor of BC, which in effect constitutes male odor, and transfer this substance to females at mating to function as an antiaphrodisiac. Hence, in *P. brassicae* it appears that males make females unattractive by cloaking them in their own odor, thus turning mated females into chemical mimics of males.

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ANNOUNCEMENT

The International Allelopathy Foundation is organizing the IV International Congress on Allelopathy–Allelopathy in Sustainable Terrestrial and Aquatic Ecosystems–to be held August 23–25, 2004 at CCS Haryana Agricultural University, Hisar, India. There will be both oral and poster sessions. Fifteen symposia are being organized. The deadline for abstract submission is August 31, 2003.

The conference aims to bring together active allelopathy scientists working worldwide to review progress, identify constraints, and project future goals for basic and applied allelopathy research in Terrestrial an Aquatic Ecosystems. Previous conferences were attended by approximately 200 scientists representing 17 countries. Accommodations are included with the registration fee and will be in the Faculty House at the University. Hisar is a small ancient city about 165 km west of New Delhi.

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UNDERSTANDING GEOPHAGY IN ANIMALS: STANDARD PROCEDURES FOR SAMPLING SOILS

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Abstract—Geophagy or soil ingestion is a multidisciplinary phenomenon that has attracted the attention of many researchers in recent years; who have sought to understand why a large number of animals consume natural earths. To find out why animals ingest soils, it is of paramount importance to establish standard methods to analyze comestible soil. Researchers have used different methods to examine soils ingested by animals, often with incomplete or inconclusive results. To make meaningful comparisons among studies, it is necessary to perfect research designs and establish standard methods to evaluate and analyze geophagy in animals.

Key Words—Soil sampling, soil eating, chemical and mineral analysis, geophagy soil analysis.

INTRODUCTION

Soil ingestion or geophagy is a phenomenon that has attracted the attention of a number of researchers in recent years. Although geophagy is not exhibited everywhere, this phenomenon is widespread in many animals such as elephants (Weir, 1969; Ruggiero and Fay, 1994; Klaus et al., 1998), ungulates (Kreulen and Jager, 1984; Mahaney and Hancock, 1990; Mahaney et al., 1996a), carnivores (Schaller, 1967), primates (Hladik and Gueguen, 1974; Hladik, 1977; Oates, 1978; Mahaney, 1987; Davies and Baillie, 1988; Mahaney et al., 1995a; Krishnamani and Mahaney, 2000 and the references therein; Wakibara et al., 2001), birds (Diamond

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et al., 1999; Gilandi et al., 1999), humans (Laufer, 1930; Halstead, 1968; Danford, 1982; Vermeer and Ferrell, 1985; Aufreiter et al., 1997; Wiley and Katz, 1998) etc. Kreulen (1985) reviewed the benefits and banes of soil consumption, and Robbins (1983) cautioned about the adverse effects of geophagy. However, the benefits far outweigh the limitations and, hence, animals engage in geophagy for a number of reasons that are nonexclusive.

Geophagy possibly evolved because of an animal's innate ability to explore and taste the chemically hostile environment. Since geophagy always has some medicinal value (Krishnamani and Mahaney, 2000), it is akin to self-medication in animals (for a detailed review see Huffman, 1997, 2001; Wakibara et al., 2001; Engel, 2002). Although naturalists have known geophagy for a number of years, only now are wildlife biologists trying to understand this phenomenon. Earlier, in a seminal paper, Krishnamani and Mahaney (2000) outlined the possible reasons that primates might engage in geophagy. They emphasized the need for a coordinated effort between soil and behavioral scientists to understand the proximate and ultimate causes of geophagy. Without quantitative and comparable measures to explain geophagy in a variety of species, our understanding of the selection pressures on geophagic animals will necessarily be incomplete. Although previous authors attempted to explain the nature of geophagy in a variety of animals, each paper brought out seemingly different approaches and problems by estimating and analyzing the extent and kinds of soils ingested (Table 1). In short, different research designs and methods were employed in attempting to study the phenomenon.

The lack of standardized sampling protocols for analyzing and describing geophagic soils has certainly impeded our understanding of the reasons why animals consume soil (Table 1). Benefiting from the diversity of approaches in understanding geophagy, we outline here a new synthesis of the conceptual basis and methods required to evaluate and analyze this phenomenon in animals. Our paper has three specific goals: (1) to develop and standardize methods to understand geophagy; (2) to illustrate shortfalls in previous reports on geophagy, and ways and means to rectify them; and (3) to help coordinate future studies on geophagy between soil and behavioral scientists.

WHAT IS GEOPHAGIC SOIL?

To determine why an animal consumes soil, it is important to identify causal factors including the soil physical–mineral–chemical composition. The geophagic soil contains high quantities of clay and fine silt with little sand; it is often clay (>40% clay) or silty-clay to clay-loam (27–40% clay) that indicates a high degree of weathering and great age. The principal colloids in geophagic soil are similar to most soils and include clay minerals, viruses, bacteria, and fungi. Research on consumed natural soil shows that animals have been observed to ingest clay with minimum quantities of sand and silt (for a brief synopsis on geophagic soils see Krishnamani and Mahaney, 2000).

TABLE 1. ANIMAL SPECIES ENGAGING IN GEOPHAGY AND INSTRUMENTS USED TO STUDY GEOPHAGIC SOILS FOR EACH STUDY

Species	Instruments used to analyze minerals	Particle size analysis done?	Major/minor elements analyzed?	Conductivity-pH measured?	Uningested soil analyzed?	SEM and/or EDS techniques used?	Source
Mongoose lemur and Brown lemur, *Eulemur mongoz* and *Eulemur fulvus rufus*	AAS	No	Major	No	Yes	No	Ganzhorn, 1987
Moustached tamarin, *Saguinas mystax*	XRF	No	M and M	No	No	No	Heymann and Hartmann, 1991
Masked Titi monkey, }it Callicebus personatus melanochir	Colorimetry, XRF	Yes	M and M	Yes	Yes	No	Müller et al., 1997
Red howler monkey, *Alouatta seniculus*	AAS, FP	No	M and M	Yes	Yes	No	Izawa, 1993
Guereza monkey, *Colobus guereza*	FAAS, XRD	Yes	M and M	Yes	No	No	Oates, 1978
Japanese macaque, *Macaca fuscata*	XRD, INAA, MA	Yes	M and M	No	Yes	No	Mahaney et al., 1993

TABLE 1. CONTINUED

Species	Instruments used to analyze minerals	Particle size analysis done?	Major/minor elements analyzed?	Conductivity-pH measured?	Uningested soil analyzed?	SEM and/or EDS techniques used?	Source
Pigtailed macaque, *M. nemestrina*; Assamese macaque, *M. assamensis*; Long-tailed macaque, *M. fascicularis*; Rhesus macaque, *M. mulatta*	XRD	No	M and M	No	No	No	Eudey, 1978
Rhesus macaque, *M. mulatto*	None	No	None	No	No	No	Knezevich, 1998
	XRD, INAA	Yes	M and M	Yes	Yes	Yes	Mahaney et al., 1995b
Bonnet macaque, *M. radiata*	XRD, INAA	Yes	M and M	No	Yes	Yes	Voros et al., 2001
Hybrid macaques, *M. mulatta* × *M. fuscata* × *M. fascicularis* × *M. thibetana* × *M. nemestrina*	ICP-MS, XRD	Partly	M and M	Yes	Yes	No	Bolton et al., 1998
Red leaf monkey, *Presbytis rubicunda*	FP, AAS	No	Major	Yes	Yes	No	Davies and Baillie, 1988

Mountain gorilla, *Gorilla gorilla berengei*	INAA, MA	No	M and M	No	No	No	Mahaney et al., 1990
	XRD, INAA	No	M and M	No	No	No	Mahaney et al., 1995b
Chimpanzees, *Pan troglodytes troglodytes*	Colorimetry, FP, AAS	No	M and M	No	No	No	Hladik and Gueguen, 1974
Chimpanzees, *Pan troglodytes schweinfurthii*	XRD, INAA,	Yes	M and M	No	No	Yes	Mahaney et al., 1996b
	XRD, INAA, EM	Yes	M and M	No	Yes	Yes	Mahaney et al., 1997
	XRD, INAA, EM, TA, OA	Yes	M and M	Yes	Yes	Yes	Mahaney et al., 1999
	XRD, MB	Yes	No	Yes	Yes	No	Ketch et al., 2001
Orang-utan, *Pongo pygmaeus and Pongo abelli*	XRD, INAA, OA	Yes	M and M	Yes	Yes	Yes	Stambolic-Robb, 1997
Human, *Homo sapiens*	XRD, INAA, TA,	Yes	M and M	Yes	Yes	Yes	Mahaney et al., 2000
	XRD, INAA	Yes	M and M	Yes	Yes	Yes	Aufreiter et al., 1997
African Savannah elephant, *Loxodonta africana africana*	MA	Yes	Major	Yes	Yes	No	Ruggerio and Fay, 1994

TABLE 1. CONTINUED

Species	Instruments used to analyze minerals	Particle size analysis done?	Major/minor elements analyzed?	Conductivity-pH measured?	Uningested soil analyzed?	SEM and/or EDS techniques used?	Source
African Forest elephant, *Loxodonta africana cyclotis*	POL	Yes	M and M	Yes	Yes	No	Klaus et al., 1998
Birds	MA, XRD	Yes	M and M	Yes	No	No	Diamond et al., 1999
Primates, ungulates, carnivores etc.	MA	No	Major	Yes	Yes	No	Moe, 1993
Ungulates	MA	No	Major	No	No	No	Kreulen and Jager, 1984
	XRD, INAA	Yes	M and M	Yes	Yes	No	Mahaney et al., 1996a,b

Note: The instrument column is arranged according to different groups abbreviated as follows: FP = Flame Photometer; XRD = X-Ray Diffraction; INAA = Instrumental Neutron Activation Analysis; XRF = X-Ray Fluorescence; AAS = Atomic Absorption Spectrophotometer; FAAP = Flame Atomic Absorption Spectrophotometer; ICP-MS = Inductively Coupled Plasma-Mass Spectrometer; SEM = Scanning Electron Microscope; PO = Polarography; EDS = Energy Dispersive Spectrometry; EM = Electron Microprobe; TA = Toxin Analysis by Gas and Liquid Chromatograph; MB = Microbiological Analysis; MA = Mechanical Analysis or field texture. Mechanical analysis refers to simple textural analysis whereas particle size refers to a grain size curve where reliable estimates of sand, silt and clay are given to a tenth of a percent. INAA provides a chemical matrix including REEs (rare earth elements), which are important in determining the uniformity of the consumed material and in particular the clay minerals (where the bulk of the REEs reside). XRF and ICP-MS also provide a chemical matrix, the latter a complete chemical composition of the sample. The EM is particularly important in the analysis of thin sections where the chemical composition of mineral grains and matrix material is necessary. In some cases microtransects can be established to analyze variations in particular chemical elements including Na, Mg, Al, Si, P, S, K, Ca, Ti, Mn, Fe and Ba, with P, S, K, Fe and Ca being of particular importance to geophagy (see Mahaney et al., 1999). PO or polarography quantitatively or qualitatively measures the redox potential (oxidation or reduction capability) of a sample; almost 30 metals can be analyzed down to ppb levels. TA is achieved using combined gas and liquid chromatography (see Mahaney et al., 1999) to test for the concentrations of the alkaloids lupanine, sparteine, quinine, and atropine. MB, only rarely carried out in geophagy investigations, is particularly useful in assessing the role of fungi and bacteria in soil consumption (Mahaney et al., 1999; Ketch et al., 2001). OA refers to oxalic acid digestion of a sample that is used in gut simulation experiments (Mahaney et al., 1999).

Clay minerals are primarily crystalline hydrous aluminosilicates, the component silicon oxide tetrahedra and aluminum hydroxide octahedra sheets, which are associated either in a 2:1 ratio (Si—Al—Si) or a 1:1 ratio (Si—Al). In these associations, the unit layers, are held together tenaciously by oxygen–hydroxyl bonds (e.g., kaolinite–serpentine group), or are only weakly associated through van der Waals forces (e.g., smectite-vermiculite group). Intermediate and amorphous forms also exist. Since geophagy is mostly a tropical–subtropical phenomenon (for details see Krishnamani and Mahaney, 2000), soils are so well leached that 2:1 (Si—Al—Si) clay minerals are mostly eradicated or exist in small concentrations, making it difficult to form soil structures that rely on adherence of sticky or expandable clay (Krishnamani and Mahaney, 2000).

Geophagic soil may be segregated into biotic and abiotic components. The biotic component contains microorganisms, including viruses, bacteria, fungi, algae and protozoa, and chemical elements such as nitrogen and carbon derived from the nitrogen and carbon cycles. The abiotic components include inorganic minerals/chemicals originating from chemical weathering processes such as oxidation, hydrolysis, chelation, solution, and hydration. The organic component is composed of three distinct fractions: a macroscopic component represented by particulate plant, animal, and microbial debris in the early stages of disintegration and decay; a soluble component (e.g., carbohydrates, proteins) arising from degradation of materials such as plant, animal, and microbial debris; and a complex, dark-colored component, largely aromatic, of humic acids, fulvic acids, and humins, which form from the breakdown of lignin (Stotzky and Burns, 1982).

GEOPHAGY LANDSCAPE MODEL

In every geophagy study, there is one or more sites where natural earths (soils) are ingested (Figure 1). Surface soils or controls which animals have not been reported to ingest are also collected. These samples consist of a high percentage of coarse particles and/or light yellow-red colors, the hallmark of immature soils or soils that are only approaching a mature stage in their development. These controls often lack appreciable clay, or they may contain different species of clay minerals from ingested soils.

Ingested soils are usually situated on older, stable landscape sites (Mahaney, 1999) where they have had ample time to reach maturity and even old age, passing through many stages of weathering where primary minerals have been transformed into secondary (clay) minerals. At the same time, oxidation has affected the iron minerals in the soil to the extent that Fe^{+2} is transformed into Fe^{+3} giving the soils a bright reddish color that may or may not be accompanied by shades of brown, indicating the downward movement of organic compounds. In ancient soils on the landscape, red colors persist, whereas brown colors eventually fade as a result of hydrolysis and conversion of organic matter to carbon dioxide in the carbon cycle.

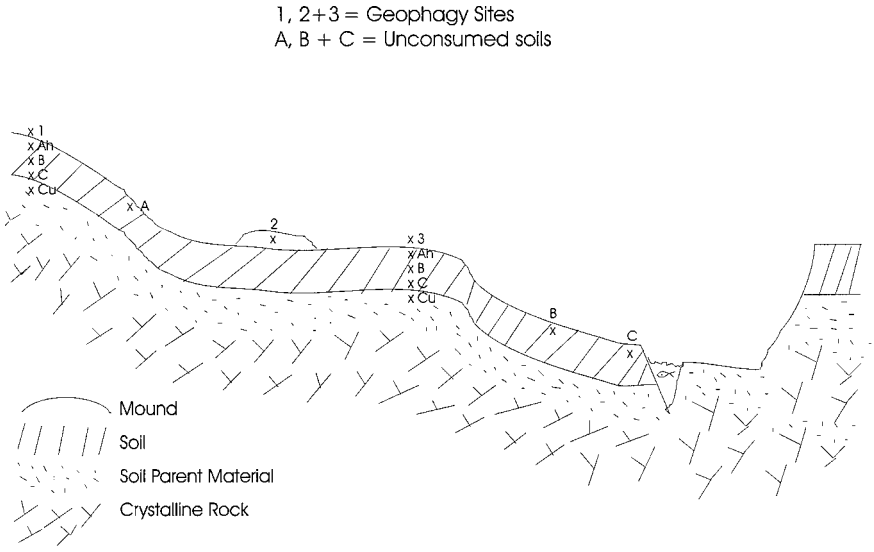


FIG. 1. Landscape model showing possible geophagy/control sites.

In collecting soils for study in a geophagy experiment, one is faced with the knowledge that a certain species of animal consumes a particular natural earth or earths. These sites must be carefully described and samples collected from the exact spot where they are ingested. In the model shown in Figure 1, soils are ingested only at sites 1 and 2 despite the occurrence of several adjacent sites where soils are available for ingestion along slopes or in cuts exposed by erosion.

The ingested soils are on higher, well drained, and presumably older segments of the landscape where they have been exposed to the subaerial atmosphere for long periods of time. One site (X1) exposes surface soil horizons and subsurface horizons, but only the B horizon is ingested. A second site is on a mound that might have been constructed by termites or ants and only the upper portion of the mound is ingested. The mound is certain to be a complex entity, so much so that it will have to be cut and sectioned in order to assess the degree of homogeneity of the ingested material. In collecting samples for laboratory analysis, some thought must be given to replicates, as single samples can produce false impressions and differences from the controls might be simply a matter of chance. It is absolutely essential to collect at least three, and possibly six, samples of the ingested material, to provide replicated data either from one site or several sites frequented by geophagic animals.

Geophagic animals do not consume control soils found at lower elevations and along slopes. In order to determine why these materials are not consumed, samples should be collected on the basis of color and particle size. The nonconsumption of

certain soils could have something to do with grain size, mineralogy, chemistry, and/or microbiology that can only be determined after detailed laboratory analysis. It is important to remember that the laboratory analysis is only as good as the field work and the field investigations must include relevant behavioral data indicating how often animals frequent a site to ingest a particular earth. The approximate mass of material ingested should also be noted.

Controls should be collected after analysis of different shades of color in the field using soil color chips (Oyama and Takehara, 1970) and by paying close attention to texture (particle sizes). Other relevant information includes soil structures—granular, blocky, and prismatic—as these often relate to texture. Soil consistency, along with plasticity and stickiness, is a function of clay content and provides an approximation of percent clay, providing important ancillary information that should not be neglected (Krishnamani and Mahaney, 2000).

If several controls are available for analysis, the researcher should select soils close to the geophagy sites as these, although not consumed, offer possibilities to ingesting animals. It is incumbent on the behaviorist to note which soils have been consumed and which ones are rejected or not consumed by geophagic animals. In such circumstances, it is important to fully analyze these avoided soils to determine the possible reasons for nonconsumption.

The site or sites need to be sketched out with respect to the full landscape. The ingested soils need to be fully described within the soil profile and individual horizons collected to compare the ingested material with refused earth. Once this is done, it is a matter of collecting multiple samples of the ingested material and a sufficient number of control samples to provide sufficient statistical data that differentiate comestible from noncomestible earth.

FIELD ANALYSIS

When collecting samples in the field, it is critical to do a complete soil description including the topsoil, subsoil, and parent material (unweathered substrate) with special attention to the particular horizon(s) that are consumed. The sites need to be sketched out with respect to the full landscape, the ingested soils fully described and differentiated from other soils in the same general area. Once this is done, it is a matter of collecting multiple samples of the ingested material and a sufficient number of control samples for statistical analysis that will help explain why animals opt for certain sites and consume earth.

The consumed horizon should be replicate sampled paying special attention to horizon thickness. If material in the ingested horizon is in a thin layer, it should be collected along the horizontal axis to insure that any variations in mineralogy and chemistry will be discovered. If the horizon is thick, multiple samples could be collected from top to bottom along a vertical transect. *Great care should be taken*

to ensure that the samples collected are the soils consumed by an animal. Within a thick soil horizon, it may be possible that a consuming animal might concentrate on just one part of it; for example, near the top where organic input might be slightly greater, or near the bottom where it might be less. These differences might only be detectable by close observation of the behavior of consuming animals in the field, i.e., from the senses they may detect slight variations in the composition of the soil that could be missed by the soil scientist.

The soil profile, when described in its entirety, may be expected to yield important information not only on the consumed material, but also on the surrounding material that is available, but not consumed (control samples). This unconsumed soil is as important as the ingested soil in geophagy studies because it must contain ingredients that are not beneficial to the consuming animal and hence it is important to determine precisely the constituents of the control samples.

Geophagy soils should be described according to the normal practice in pedology using color and particle size as the main criteria. Profile thickness and the thickness of individual horizons should be noted and the generic soil linked to the evolutionary history of the landscape in which it is formed. In addition, presence or absence of soil structures should be noted along with grade of consistency, plasticity, and stickiness, and such ancillary data as presence of pebbles, weathered state of pebbles, root systems, transitional or sharp contacts among horizons, and any other physical features considered relevant to understanding the geomorphic history of the soil. For a discussion of soil properties, see Soil Survey Division Staff (1993).

Controls should be collected after analysis of different shades of color in the field using soil color chips (Oyama and Takehara, 1970) and by paying close attention to texture (particle sizes). Other relevant information includes soil structures that are forms of granular, blocky, prismatic geometries, as these often relate to texture, although in tropical climates even mature/ancient soils often lack structure. Soil consistency is a function of clay content, and together with plasticity and stickiness, give an indication of clay content and provide important ancillary information that should not be neglected (Krishnamani and Mahaney, 2000)

The presence of datable material, when present, should be noted as it may provide valuable information as to the age of the profile. If, as is usually the case, the soil is open to the subaerial atmosphere, and still active in a weathering sense, it is important to determine its maximum age. It is possible to do this by obtaining organic material that can be radiocarbon dated in the soil or in the parent material from which it formed. If volcanic ash (tephra) is present, it may provide a means of absolute dating using potassium–argon concentrations or fission track methods (Mahaney, 1990). If various horizons of the soil contain fine and very fine sand, it may prove possible to use Optically Stimulated Luminescence (OSL) to obtain absolute age determinations (Mahaney et al., 2001). These dating methods provide ages that will assist in tracking the evolutionary history of the soil, a point

that is often important in geophagy investigations (Mahaney, 1999). Indeed, soils themselves may also provide relative age determinations simply by analysis of the primary and secondary mineral assemblages. The primary mineral suites may provide microscopic data that will aid in determining just how highly weathered the profile is and give some idea of the length of time it has been forming in its topographic setting.

In every geophagy study documented in the literature (Krishnamani and Mahaney, 2000), there is no evidence that animals, as diverse as mountain gorillas and birds, ingest surface horizons (topsoil). It may be that the microbial component in organic-rich soils contains bacteria and/or fungi that are toxic to the ingesting animal, or perhaps the A-horizon group is bereft of minerals that provide neither the raw chemical material or secondary weathering products that are the prime targets in geophagy. Whatever the reason, we have yet to document animals eating material in the zone of maximum biological activity i.e., the A-horizon (Figure 2).

Either by inference from published studies (Davies and Baillie, 1988), or from rare soil descriptions (Mahaney et al., 1990, 1995a,b, 1996a,b), the B-horizons, which occupy the zone of maximum chemical alteration in the soil (see Figure 2), are the prime targets of animals engaging in geophagy. As documented by Mahaney (1987), African Cape buffalo (*Syncerus caffer caffer*) always sought out

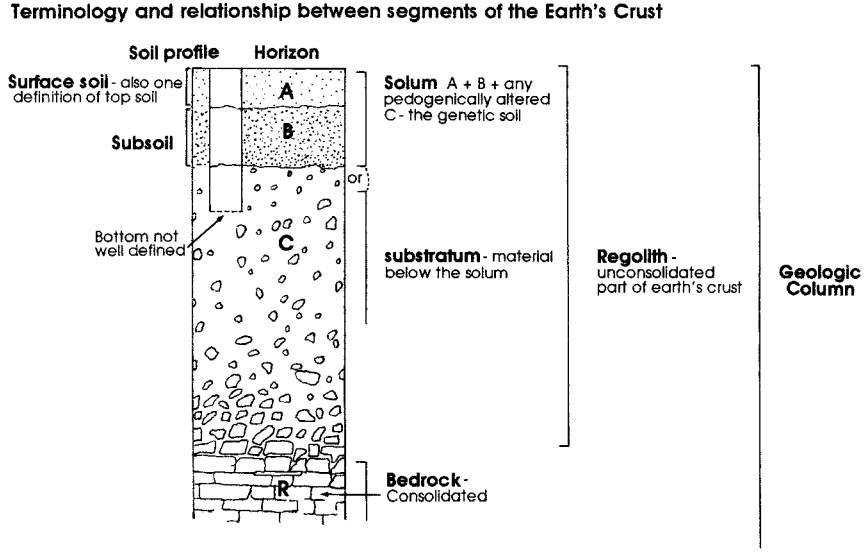


FIG. 2. Generalized soil profile showing individual horizons and relation to the regolith. Sampling of geophagy soils should include horizons not consumed by the animals since they are important and act as controls.

well-weathered, reddish B horizons on Mount Kenya over 12 years' observations of geophagic activity during the Mount Quaternary History Project (1976–1987). Buffalos always engaged in geophagy below the limit of the Last Glacial Maximum (LGM) where ancient soils (paleosols) were present (Mahaney, 1990). These soils provide clay-rich material containing kaolinite and metahalloysite, together with appreciable quantities of iron as secondary weathering products of goethite and hematite.

Evidence from Mt. Kenya (Mahaney, 1990) suggests a minimum age of ~100,000 years for soils to approach a weathering state sufficient to provide nutrients of interest to ingesting animals. Taking Eurasia and North America, for example, the analogous situation is that sites suitable for geophagy lie south of 35° north latitude in locations that escaped glaciation. Whether in mountains or in lowland continental positions, animals engaging in geophagy seek out older ancient soils (paleosols) that lie beyond the range of recent glaciation where weathering proceeded unimpeded, releasing active chemical ingredients and allowing the genesis of high concentrations of clay minerals.

Below the B-horizon in the soil, the thickness and state of the altered C-horizon group is important to describe and analyze, because it often contains material similar in kind to what is in the B, but in a less weathered state. Below the altered C-horizon is the parent material, usually termed the Cu horizon (u = unweathered). In some cases, the entire soil may form from this material over a certain length of time. In other cases, the soil may have several parent materials resulting from the additions of fluvial, aeolian (wind blown), glacial, and or mass wasted (gravitational) sediment. With respect to the latter scenario, analysis of the soil becomes more complex, and the investigator must consider the different chemical and mineral compositions of these diverse materials as they may influence a choice of geophagy soils.

Once the soil is described, the investigator will have collected multiple samples of the ingested material as well as several samples of material made available to, but not ingested by, the geophagic animals. If other geophagy sites exist in the same study area, the investigator should study them paying close attention to similarities and differences between sites as well as differences with the control soils.

LABORATORY ANALYSIS

In the laboratory, soil samples should be transferred from plastic bags to newspapers and left to air dry, a process that takes from a few hours, to several days depending on the clay content. Sand, for example, will dry quickly within a matter of hours, whereas clay-rich material may take several days to dry out fully. Once air dried, samples may be subjected to particle size analysis, a process that

involves fractionation of various grade sizes of material. First, pebbles, if present, need to be separated from the sand–silt–clay fraction by dry sieving using 2.0 mm brass sieves. The coarse fraction, consisting of pebbles (4–64 mm) and granules (2–4 mm), may be stored in paper bags for later analysis as dictated by additional laboratory analysis. The sand–silt–clay fraction may also be stored in paper bags and it is this fraction that will be continually used to perform analyses most closely related to geophagy (see Table 1, many studies have not done particle-size analysis, which means the ratios of sand/silt/clay are largely unknown).

The methods employed here are closely related to standard sedimentological methods previously described by Mahaney (1990, 2002) in the pursuit of lithostratigraphical and pedological problems. The normal procedure is to calculate the air-dried equivalent of 50 g oven-dried soil, a weight that is achieved by taking 50 g air-dried soil and heating to 110°C overnight, or for 15 hr. The weight loss is due to water loss, and the moisture factor is calculated from the ratio of weight of the air dried sample over the oven-dried sample. This quotient multiplied by 50 gives the weight of the air-dried sample in grams. Use of the air-dried equivalent weight avoids heating the sample to obtain 50 g oven-dried soil for particle size analysis (Day, 1965). Heating the sample is a procedure that alters the clay mineral components and requires the investigator to prepare two separate sample splits: one for particle size and another for clay mineralogical analysis.

Following this procedure, the <2 μm fraction can be drawn off from hydrometer jars after 24 hr sedimentation, stored in 1 l bottles, and later equilibrated by shaking for 1 hr. A 48-hr separation is also possible if one requires analysis of the <1 μm clay fraction (determination of fine vs. coarse clay might yield data important in understanding why certain soils are selected for ingestion). The agitated slurry may then be placed in centrifuge cups, fitted with special aluminum supports holding ceramic tiles of a size close to $45 \times 15 \times 2$ mm, and centrifuged at 2500 rpm for 15 min to obtain oriented mounts. After drying, the clay fraction may then be X-rayed using line diffraction equipment outfitted to produce Cu-K-alpha radiation. Normally, air-dried traces are required, followed by treatment with ethylene glycol, and heating in a furnace at 300°, 500°, and 550° (see Mahaney, 1981, 1990 for an explanation of the method).

Once the particle size analysis is complete, sand weights must be multiplied by 2 to give percentage values; these are then added together to give cumulative weight percents. The <63 μm fraction is analyzed using a hydrometer. Values are calculated from the sedimentation rate at intervals of 2, 5, 10, 40, 140, and 1440 min, and converted to phi diameters and cumulative weight percents according to procedures outlined by Day (1965) and Mahaney (1990). Once the phi diameters are known and correlated against percentages, they may be used to construct grain size curves (see Mahaney et al., 1995b and Figure 3). These curves are especially useful as they provide a visual image of the size–shape of the consumed

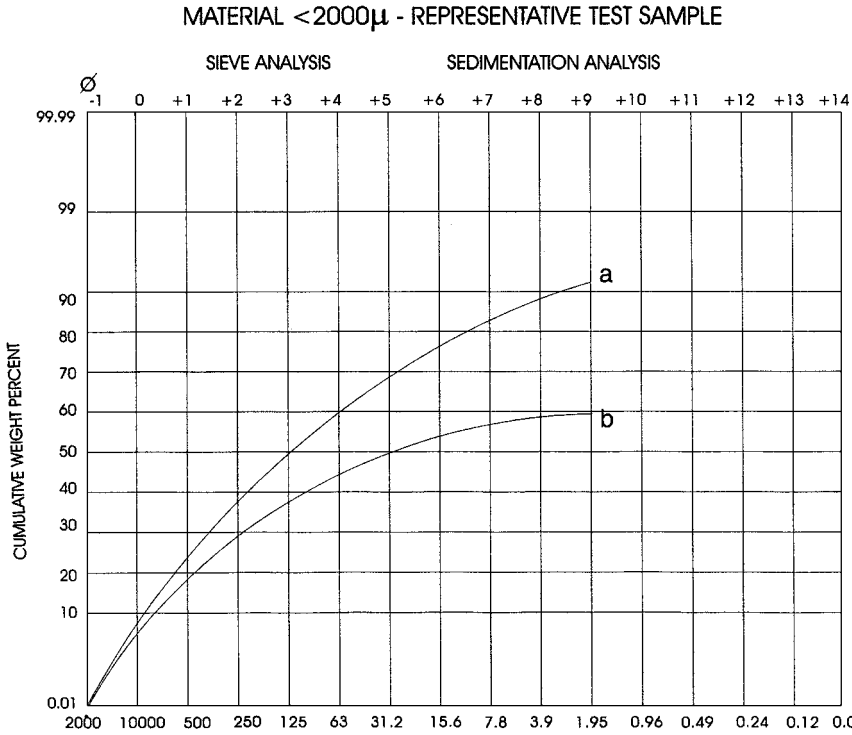


FIG. 3. Generalized grain-size curves showing hypothetical distributions of sand (2000-63:m), silt (63-2:m) and clay (<2:m) for geophagy soil (b) and control soil (a).

sediment/material. The left hand side of the curve gives the amount of sand present and the right hand side the amount of clay. The lower the percentage value on the right hand side, the greater the amount of clay present. Since the percentage of clay is one of the key factors in geophagy investigations, calculation of a precise grain size curve should be considered one of the cornerstones of soil ingestion studies.

The dry colors of the samples should be taken either under sunlight or warm fluorescent bulbs to approximate field conditions. Samples may then be loaded into plastic cups and weighed out to 20 g each for pH and electrical conductivity analyses. After adding 100 ml distilled H_2O and achieving a ratio of 1:5 (20 g soil: 100 ml H_2O), samples are placed in styrofoam plates, shaken for 1 hr, allowed to set for 30 min, after which pH and conductivity levels may be noted. Normally pH values of one tenth of a unit are sufficient and conductivity levels should be low in the range of 0–500 $\mu S/cm^{-2}$ (see Table 1, most studies have not reported pH and conductivity values of the ingested soils).

The organic constituents of the consumed and control material should be fully analyzed so that some estimate of the importance of carbon and nitrogen can be made. Nitrogen may be determined by Kjeldahl methods and carbon by ignition at 400°C overnight for 15 hr to give organic carbon. If automated equipment is available, carbon and nitrogen can be determined by use of a Leco apparatus, which gives total carbon and nitrogen. If no carbonate is present in the samples, the total carbon is the organic carbon. Normally, the value of organic carbon is considered to be 62% of total organic matter.

At some point, it is preferable to consider obtaining a chemical matrix on the consumed and control samples that will provide data on the distribution of macro- and microelements that are important in nutrition, dietary supplementation, zoopharmacognosy, and in assessment of parent material uniformity. One of the most reliable analytical methods is Instrumental Neutron Activation Analysis (INAA), which is used to bombard representative samples with a neutron stream, thus yielding a chemical matrix of some 35 chemical elements. Macroelements, such as calcium, sodium, magnesium, potassium, and iron, are of major importance in geophagy (Mertz, 1981; Robbins, 1983; Krishnamani and Mahaney, 2000). Concentrations of these elements can be obtained from the <2 mm fraction of the consumed and control material. Other microelements, such as arsenic, bromine, and copper, can be assessed for differences in concentration between the consumed and control groups. The concentration of aluminum, while not directly important in geophagy *per se*, yields important differences in clay mineral content, as usually the aluminum increases along with the clay content. If cadmium-lined vessels are used in the analysis, it is possible to obtain concentration levels of silicon to parallel the aluminum distributions, although it is a time consuming process. The rare earth elements (REEs), often considered to have little to do with nutrition, dietary supplementation, and/or zoopharmacognosy, are an important byproduct of INAA, and provide information on parent material uniformity and clay mineral variations (e.g., increases in REEs parallel increases in clay mineral content). As pointed out by Mertz (1981), many trace elements may prove to have an important role in human and animal nutrition, and their essentiality in primate physiology may eventually be shown by future research.

Close scrutiny of the collected material might reveal clumps of fine structures that form granules, blocks, or prisms. These soil structures contain a record of the evolutionary history of the soil. They also may contain clues as to why animals are ingesting the material. Collected as whole entities, they should be put aside in plastic bags for later analysis by Scanning Electron Microscope (SEM) and Energy Dispersive Spectrometry (EDS) to determine the chemistry of the enclosing material as well as the material in the nucleus of the structures.

Analysis of the sand fraction by SEM–EDS (Mahaney, 2002) is particularly useful for providing data on the weathered state of sands, and on coatings that

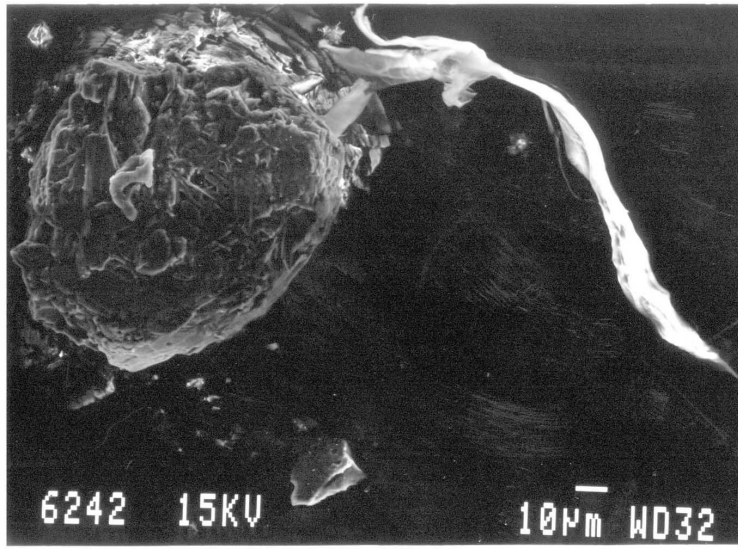
often envelope sand particles in consumed soils. While the sands themselves provide little if any benefit to animals ingesting earth, coatings on grain surfaces provide clues about the weathering history and lithology of the soil, and they may provide iron and other nutrients to consuming animals. Unlike the clay fraction that is mobile within the soil and prone to downward movement, sands >100:m are more or less fixed in the soil profile and provide a measure of the mineral and chemical uniformity of the parent material. The presence of iron, calcium, and silicon coatings precipitated on sands record the soil weathering history (Mahaney, 2002) and may prove important in assessing the chemical composition of soil water and the relative importance of iron and calcium to nutrition–zoopharmacognosy stimuli. Silicon, which may be important in bone connective tissue and skeleton breakdown, is also important in determining rates of leaching that affect clay mineral recrystallization. Because one of the important clay mineral combinations for alleviating diarrhea is kaolinite/metahalloysite or smectite, it is important to determine the ratio of silicon and aluminum (Si:Al).

The SEM provides imagery (Figure 4) that shows the weathered state of the sand fraction in comestible material, while EDS provides a chemical spectrum (example in Figure 5; and in Mahaney, 2002) useful in assessing the build up and loss of chemical elements. Analysis of the microbiological constituents in consumed soils suggest that animals may benefit from fungal components such as *Penicillium* spp. (Ketch, 1998; Mahaney et al., 1999; Ketch et al., 2001). Certainly, from what is known at present, antibiotic-rich earth might provide one of the main causes of geophagy, stimulating animals to ingest it and providing soil and behavioral scientists with a fertile area of interdisciplinary research.

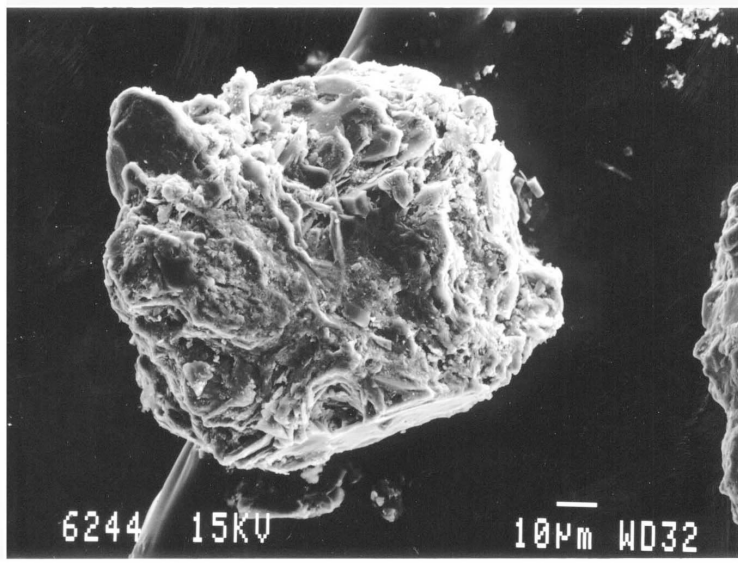
CONCLUSIONS

While advances in geophagy investigations have been made on the behavioral side, physical–chemical–mineralogical–pharmaceutical methods have generally not been employed by investigators to search for causal relationships that might explain the behavior. Ostensibly, this results from the training of behavioral investigators, which is generally heavily slanted towards biology. The main conclusion offered here is that geophagy is fully a multidisciplinary problem that requires a team effort, usually a research group that is coordinated to undertake detailed field and laboratory methods.

It would be beneficial, and the investigator would do well to empathize with the consuming animal, to closely observe the field site to determine the likely exposures where geophagy might occur. How many geophagy sites exist? How many different kinds of soil are exposed to animals, and what are the differences among this group? Is it possible for animals to “mine” soil (Mahaney, 1990), and how do they discover mining sites? Most geophagy sites are on flat and stable



A



B

FIG. 4. Examples of SEM imagery. (A) Rutile (TiO2) with attached root and slight coating of Fe (see Figure 5 for chemistry) from site Suci6 (Stambolic-Robb, 1997); (B) Orthoclase (K-feldspar) sand with Fe coating (site Suci6; Stambolic-Robb, 1997).

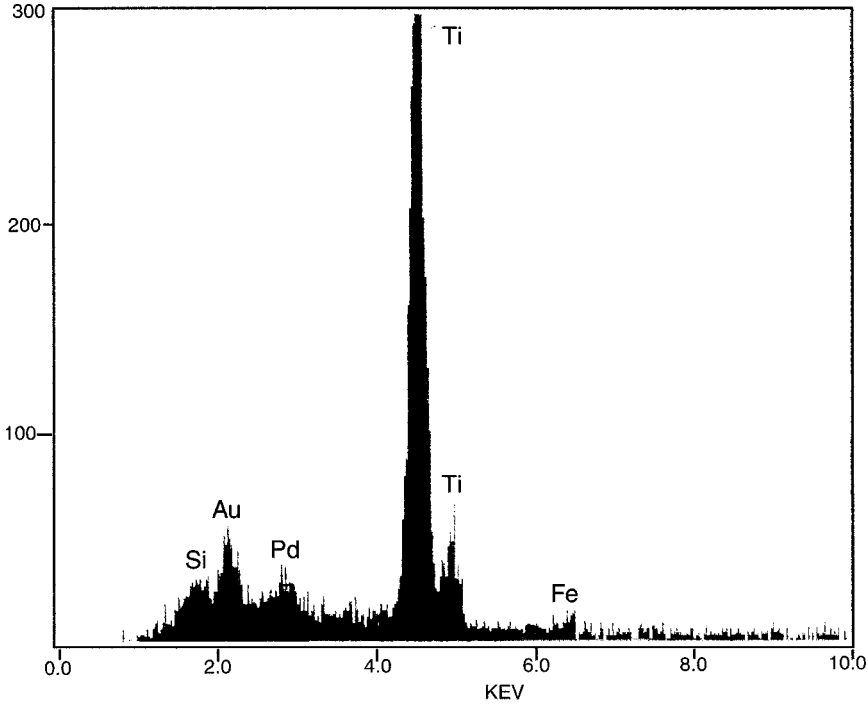


FIG. 5. Energy-dispersive spectrum for grain in Figure 4A showing thin coatings of Si and Fe on rutile. Gold (Au) and Palladium (Pd) are coatings applied to make the surface conductive.

surfaces, at the far end of the landscape evolutionary spectrum, and mostly of ancient age (e.g., older than the last glaciation or >100 ka). What are the differences between these sites and others on slopes or at different elevations? These are some of the questions that will aid finding answers to the perplexing question of why animals consume natural earths.

Organic-rich or even fully organic-depleted materials appear not to be eaten by animals engaging in geophagy. Yet, this should not discourage researchers from analyzing organic components in comestible soils, especially those with minute amounts of carbon that might provide an energy source for microbial populations, especially fungi such as *Penicillium.* If possible, a microbiologist should be part of a geophagy research team.

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CLAY MINERALOGICAL AND RELATED CHARACTERISTICS OF GEOPHAGIC MATERIALS

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Abstract—Geophagy is the deliberate consumption of soil and clay deposits by animals, including man. Various hypotheses have been advanced to explain geophagic behavior, the main ones being: detoxification of noxious or unpalatable compounds present in the diet, alleviation of gastrointestinal upsets such as diarrhea, supplementation of mineral nutrients, and as a means of dealing with excess acidity in the digestive tract. This review focuses on the mineralogical and chemical nature of the materials consumed, and considers whether this has been adequately characterized, particularly in the contexts of the above hypotheses. In many cases, the information provided is fragmentary and a number of inconsistencies and anomalies are identified. In particular, it is suggested that for these materials further information on their mineral constituents is required, particularly with respect to the kaolin minerals, interstratified kaolinite/smectite, the smectitic minerals and iron oxide and aluminous minerals. It is suggested that total chemical analyses of these materials are of little relevance unless supported by a physiologically based extraction test, this approach being essential in any study seeking to confirm the nutrient supplementation hypothesis.

Key Words—Geophagy, clay minerals, halloysite, kaolinite, soils, clays.

INTRODUCTION

Geophagy may be defined as deliberate and regular consumption by animals and man of earthy materials such as soils, clays, and related mineral substances. It is a practice that is found in all continents, but is most common in the tropics (Abrahams and Parsons, 1996). Geophagy was known in the ancient world, being first mentioned by Aristotle (Mahaney et al., 2000), and was more fully described when tropical countries began to be extensively explored and developed in the nineteenth century. However, one of the earliest and most perceptive accounts of

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geophagy in man was given by Alexander von Humboldt following his descent of the Orinoco in Venezuela during his expeditions of 1799–1804 (in Keay, 1993). Not only did von Humboldt describe the phenomenon of geophagy, as practiced by the Ottomac people during times of food scarcity, but he also identified, discussed, and speculated about many of the questions concerning geophagy that are still being debated today. Thus, he noted that the earth-eating habits of the Ottomacs appeared to cause no injurious effect to their health whereas peoples of other tribes who practiced, and became addicted to, geophagy became sick and wasted away. These observations anticipate the current debate as to whether geophagy confers definite medicinal benefits to its practitioners or whether it should be regarded as a particular form of "pica" due to psychological disturbance of the individual, akin, for example, to anorexia and related conditions. von Humboldt also noted that the "Ottomacs do not eat every kind of clay indifferently; they choose the alluvial beds or strata, which contain the most unctuous earth, and the smoothest to the touch" (Keay, 1993). He inquired as to the exact nature of this geophagic material and found that it was composed of alumina and silica with 3 or 4% lime, and could have no inherent nutritive value. On broadening the scope of his inquiry, he found that other geophagic peoples consume calcareous, ferruginous, or magnesian earths either by choice, or as a famine food to appease the pangs of hunger.

Recent research has highlighted the fact that geophagy is widespread throughout the animal kingdom. It has been reported for reptiles (Marlow and Tollestrup, 1982), birds (Diamond et al., 1999; Gilardi et al., 1999), mammals, particularly for generalist herbivores (Kreulen and Jager, 1984; Kreulen, 1985), and many nonhuman primates (Krishnamani and Mahaney, 2000). It may be regarded as a vestige of palaeonutrition so far as man is concerned (Ziegler, 1997) and may be of evolutionary significance. The question thus arises as to the driving reasons behind the geophagic behavior of animals. A wide variety of hypotheses have been proposed and, as it happens, many involve the exact nature, behavior, and properties of the clay minerals in the geophagic materials, so that clay mineralogy may be said to be an important component in the assessment of these various hypotheses. It is clearly important that the clay minerals in geophagic materials are characterized in a thorough and unequivocal way. The purpose of this review is to focus upon this particular aspect, with the aim of clarifying the role of these minerals in the phenomenon as a whole. In addition, some other chemical and physical aspects of geophagic materials are reviewed that also may be important in the selection of these materials for consumption.

CLAY MINERALOGY AND OTHER PROPERTIES OF GEOPHAGIC MATERIALS

Table 1 summarizes the reported clay mineralogy, as well as some important physical (color, clay content) and chemical [cation exchange capacity (CEC), pH,

TABLE 1. CHARACTERISTICS OF GEOPHAGIC MATERIALS CONSUMED BY VARIOUS ANIMALS

Reference and location	Animal	Source	Color	Clay (%)	CEC (cmol/kg)	pH	Clay mineralogy	High in total Nutrients (>2%)	High in available nutrients (>5%)
Diamond et al. (1999), New Guinea	Birds	Recent landslide	Not tested	13	5.5–17.1	6.5–7.0	Smectite + + + Illite ++ Kaolinite++ Chlorite +	Fe	Ca, Mg, K, Na, Mn, P
Gilardi et al. (1999), Peru	Birds	Clay exposed in river bank and cliff	Not tested	44–82	28.0	6.5–7.0	Smectite + + + Illite+ Kaolinite +	Fe	Ca, Mg K, Na P
Klaus et al. (1998), Central African Republic	Elephants and large mammals	Cave material Dolerite	Dark green Dark green	35–40 45–100	Not tested Not tested	5.09 5.19	Not tested Not tested	Not tested Not tested	All <5% All <5%
Abrahams (1999), Tanzania	Elephants and large mammals	Lick Soils L1 L2 L3	Red White-light grey Yellowish red	41 19 23	Not tested Not tested Not tested	4.3 11.0 8.2	Kaolinite + + + Smectite ++ Kaolinite++ Carbonate ++ Kaolinite ++ Illite ++ Smectite +	Fe Ca Fe	Ca, Mg, K, Na Na Ca, Mg, K, Na
Mahaney et al. (1993), Japan	Macaques	Soils from sediments and volcanics	Dark brown – very pale yellow brown	30–50	Not tested	Not tested	Halloysite ++ (sediments) Smectite ++ (volcanics)	Na, Fe	Not tested
Mahaney et al. (1995a), Puerto Rico	Macaques	Weathered volcanics	Strong yellow–brown	52–88	Not tested	5.2–7.0	Halloysite+ + + Illite + − ++ Smectite + − ++	Fe	Not tested
Bolton et al. (1998), Hong Kong	Macaques	Granitic soils	Red	Not tested	13.1–21.2	4.0–6.6	Kaolinite + + +	Mg, Fe	Fe

TABLE 1. CONTINUED

Reference and location	Animal	Source	Color	Clay (%)	CEC (cmol/kg)	pH	Clay mineralogy	High in total Nutrients (>2%)	High in available nutrients (>5%)
Heymann and Hartmann (1991), Peru	Tamarins	Soils and sands	Reddish brown to light brown	Not tested	Not tested	Not tested	Not tested	Fe	Not tested
Setz et al. (1999), Brazil	Golden-faced sakis	Termite nests	Reddish yellow	~50	38.7	4.3	Kaolinite + + + Gibbsite trace	All <2%	Not tested
Mahaney et al. (1997), Uganda	Chimpanzee	Soil from carbonatite tephra	Red to dark grey	50–65	Not tested	6.0–7.0	Halloysite+ + + Illite+	Fe	All <5%
Mahaney et al. (1999), Tanzania	Chimpanzee	Termite mounds granitic soils	Yellow brown – light brown	~30	2.6–6.7	6.1–8.6	Halloysite+ + +	Fe,K	All <5%
Aufreiter et al. (2001), Tanzania	Chimpanzee	Soils from granite/ gneiss	Brown to red brown	46	2.6–6.7	4.7–7.5	Halloysite+ + + Kaolinite + + + Illite++ Smectite Tr−+	Fe, K	All <5%
Mahaney et al. (1995), Rwanda	Gorillas	Weathered volcanic regolith	Yellow brown	10–20	Not tested	Not tested	Halloysite+ + + Illite+	Fe, Ca	All <5%
Aufreiter et al. (1997), China	Humans	Weathered rock	Pale yellow	>40	Not tested	Not tested	Smectite + + + Kaolinite+ − ++ Halloysite+	Fe,Ca	Mg
USA		Clayey subsoil	Light brown	35	Not tested	Not tested	Halloysite+ + + Illite/Smect +	Fe, K	All <5%
Zimbabwe		Termite Mounds	Red–yellow	10–20	Not tested	Not tested	Kaolinite++ Halloysite+	K	All <5%

Abrahams (1997), Uganda	Humans	Street market	Dark grey-red yellow	46–82	12–35	4.6–7.2	Kaolinite+ + + Kaol/Smectite +−trace	Fe	Ca, Mg Na, K
Mahaney et al. (2000), Indonesia	Humans	Soils from Leucite lava and sediment	Pale yellow brown	52–88	Not tested	6.0–8.1	Halloysite+ + + Smectite+ + + Kaolinite+ Illite +	Fe	Not Tested

Note: + + + = dominant (>50%), ++ = abundant (25–50%), + = subordinate (5–25%), Tr = trace (<5%).

total and available nutrient elements] properties of geophagic materials consumed by various animal groups.

Birds. There have been two recent studies of geophagy in birds (Diamond et al., 1999; Gilardi et al., 1999). Both concluded that the clay mineralogy of the consumed materials was dominated by smectite although other clay minerals are also present in substantial quantity. A curious feature of the X-Ray diffraction (XRD) traces of both materials is the relatively high spacing shown by smectite after solvation with glycerol. Smectites saturated with most cations yield a spacing of 17.8 Å after glycerol treatment but smectite in the geophagic materials expands to 18.9 Å. In addition, heating of the geophagic smectites does not appear to result in complete contraction to 10 Å as in normal smectites, suggesting the presence of nonexchangeable interlamellar material. This geophagic material from New Guinea only contains 13% clay, but, nevertheless, if the dominant clay mineral in this size fraction is a high-CEC smectite, then this would be consistent with the measured CEC of the bulk material. In considering the function of the geophagic behavior of birds, both studies concluded that this served to reduce the bioavailability of poisonous or bitter-tasting compounds, such as alkaloids and tannic acids, in a diet of fruits and seeds. Experiments showed that the geophagic materials were capable of binding high quantities of potentially toxic or unpalatable compounds. There was no evidence to support other explanations such as protection against diarrhea, buffering against acids, or mineral supplementation. The highly available state of the alkali and alkali earth cations suggests that these originate as exchangeable cations on the clay minerals.

Large Mammals. Geophagical behavior of mammalian herbivores has been reported from many sites (Klaus et al., 1998) but despite this research remains speculative and fragmentary to a high degree (Abrahams, 1999), at least in part due to inadequate characterization of the materials consumed. Such information is provided in recent papers of Klaus et al. (1998) and Abrahams (1999), summarized in Table 1. The study of Klaus and coworkers concerned the chemical and physical properties of geophagical material consumed by forest elephants and other mammalian species in the rain forest of the Dzanga National Park in the Central African Republic. This material was situated exclusively in areas of outcropping dolerite intrusions that yielded highly weathered material of a dark green color. It is extremely clay-rich and slightly acidic in nature. In considering the possible relationship between the physical and chemical properties of the geophagic soils and the reasons for their consumption, Klaus et al. (1998) conclude that mineral supplementation cannot be the only explanation as nongeophagic soils in the area often show higher concentrations of calcium, magnesium, and sodium. They attached more weight to the possibility that the clay-rich soil might act to reduce the bioavailability of toxins such as tannins and alkaloids. In general, it was concluded that consumption of clay- and nutrient-rich soils may relieve physiological stress and increase fitness of mammals living in poor-quality habitats such as rain forests.

The study of Abrahams (1999) concerned geophagical soils from three sites in the Mkomazi Game Reserve in Tanzania that were eaten by elephants and other wildlife. The three soils investigated were very different, the first (L1) being probably a highly weathered Ultisol, whereas the other two (L2 and L3) were alkaline in reaction, calcareous, and even sodic saline. The L1 soil is acidic, clay-rich, highly kaolinitic, and has a lower content of available nutrients than does L3. Both L2 and L3 are alkaline, or even highly alkaline, much less clay-rich, more smectitic, and contain high amounts of available sodium. Free halite can be detected in these soils by XRD. The general conclusion is that no single characteristic can explain the exploitation of these soils by animals, but that the kaolinitic soil could act to relieve gastrointestinal upsets, while the other soils may help to alleviate sodium deficiency and/or acidosis.

Soils consumed by Holstein hybrid cattle in the Northern Andes of Venezuela (not included in Table 1) were studied by Mahaney et al. (1996a). These soils are formed in a weathered regolith possibly of Tertiary age and are of a coarse sandy texture containing $<$20% clay. As this clay appears to contain elevated amounts of kaolinite and halloysite compared to nearby non-geophagic control soils, it was suggested that the eaten soils may alleviate diarrhea or perhaps play a role in adsorbing toxic or unpalatable compounds.

Macaque Monkeys. In the last decade, there have been many studies of geophagy among primates focusing particularly on the nature and function of the materials consumed. Two studies by Mahaney and coworkers of the geochemistry of geophagic materials consumed by macaque monkeys also contain data on clay mineralogy (Table 1). In their study of Japanese macaques (Mahaney et al., 1993) presented total elemental concentrations for the geophagic and control soils. The soils are Inceptisols developed on sedimentary or volcanic rocks and, judging from the textures given, contain between 30 and 50% clay. Halloysite was identified as the main clay mineral in both the geophagic and nongeophagic soils derived from sediments, whereas the $<$2-μm fraction in the geophagic soil derived from andesitic rock was dominated by either smectite or nonclay minerals, principally quartz and feldspar. Nonclay minerals were dominant or co-dominant in the $<$2-μm fractions of eight of the nine soils studied. In the authors' experience, this is a rather unusual finding and raises the question as to whether a true $<$2-μm fraction had been separated from the study soils. No clear geochemical stimuli that could explain the geophagic behavior could be discerned from the analytical results presented, and there was little evidence to support mineralogical stimuli either.

A more comprehensive study of geophagic materials consumed by the rhesus macaque monkeys on Cayo Santiago, Puerto Rico, was undertaken by Mahaney et al. (1995a). Soils were derived from weathered volcanic rock and compared with closely associated soils that were not eaten. The geophagic soils were more clay-rich than the non-eaten ones (52–88% compared with 19–46%), somewhat

more acidic with soil pH values often being <6, and had stronger, redder colors. Mineralogical analyses of the <2-μm fractions of both the eaten and the non-eaten soils showed that they often contained abundant halloysite and moderate-to-small amounts of illite, smectite, quartz, plagioclase feldspar, and orthoclase feldspar. Two samples of the eaten soils also contained abundant kaolinite. The authors considered that the eaten soils had a greater kaolin and smectite content than the non-eaten ones and suggested that this was consistent with the rhesus macaques using these materials as pharmaceutical agents for the prevention or treatment of gastrointestinal upsets. Analysis of the total chemical composition of the materials showed that they were geochemically homogeneous with respect to both major and trace elements. It was, therefore, concluded that the monkeys were unlikely to obtain any nutritional benefit from the eaten soils, although the question of element bioavailability was not addressed.

Further chemical and mineralogical studies were made of soils eaten by hybrid macaques in the Kowloon peninsula, Hong Kong (Bolton et al., 1998). The soils were classified as eaten, control, and refused, and were analyzed for soil pH, CEC, various forms of Al and Fe, organic C, acid extractable elements, particle size, and mineralogy. There was no significant difference in the mineralogy of any of the soils, all being dominated by kaolinite with small amounts of gibbsite. However, the weathered granite of Hong Kong contains abundant halloysite (Parham, 1969) and the distinction between halloysite and kaolinite is often difficult to make on the basis of XRD patterns alone, a topic discussed later in this review. The greatest differences were found between the eaten and refused soils, the latter representing material that was picked up by the monkeys and then discarded. The refused soils were more coarse textured and lighter colored than the eaten ones and, as might be anticipated, contained significantly less iron, in both total and extractable forms, and had a lower CEC. The refused soils also were lower in some extractable nutrients like Mg, but contained higher amounts of Ca as well as some trace elements such as Mo. Bolton et al. (1998) suggested that soil texture and color provided tactile and visual clues to enable monkeys to select preferentially suitable geophagic soils, and that the choice of finer-textured, redder soils may be related to their ability to aid digestive processes, to absorb various toxins and perhaps, to act as a supplementary source of Fe.

Moustached Tamarins. Heymann and Hartmann (1991) reported geophagy in moustached tamarins in Peruvian Amazonia, analyzing the material consumed for total chemical composition by X-ray fluorescence. One soil was from a termite mound and was fine-grained, whereas the two others were from bare patches on the forest floor and had a sandy texture. The forest floor samples were highly siliceous (>90% SiO_2), whereas the termite mound soil contained higher amounts of nutrient elements such as K, P, and Zn. The authors suggested that mineral supplementation was the most likely explanation for the geophagic behavior but, in the absence of results indicating the bioavailability of nutrient

elements under simulated physiological conditions, this suggestion is speculative.

Golden-Faced Saki Monkey. Geophagy in the golden-faced saki monkey was studied by Setz et al. (1999) in Central Amazonia. These monkeys consume soil from arboreal termite nests, and the geochemistry and mineralogy of this soil was compared with that of nearby topsoil and forest floor root mats that were not consumed. The clay mineralogy of all samples was dominated by kaolinite (Table 1) with small amounts of gibbsite, feldspars, and quartz. No data were given on the clay contents, but as the CEC of the termitaria samples was almost four times that of the topsoil samples, it is reasonable to suppose that the former were more clay-rich. All the materials were acidic (pH 4.1–4.3), but the termite nests were slightly richer in nutrient elements such as P, K, Ca, and Mg as extracted by ammonium acetate or ion exchange resins. However, the observed differences were not sufficiently great to support a mineral supplementation hypothesis.

Chimpanzees. Several papers have been published by Mahaney and coworkers on the chemistry and mineralogy of geophagic materials eaten by chimpanzees (Table 1). Soils consumed by chimpanzees from the Kibale Forest, Uganda, (Mahaney et al., 1997) are described as red to dark gray clays derived mainly from carbonatite tephra, with a clay mineralogy dominated by metahalloysite. It was suggested that this mineral could act as a stimulus because of possible pharmaceutical benefits that it confers. About 0.15–0.20% of the total iron could be extracted with oxalic acid at pH 2, the pH of the gastrointestinal tract of the chimpanzee, and it was thought that this could be of nutritional significance. It was concluded that bioavailable iron could act as a chemical stimulus for the geophagic behavior.

The geophagic soils from the Mahale Mountains of Tanzania are derived from termite mounds and granitic/gneissose soils (Mahaney et al., 1996b), and are of a clay loam texture with about 30% clay. The dominant clay mineral was again identified as metahalloysite. However, this identification was based upon the disappearance of the first-order basal reflection at $\sim$7.2 Å after heating at 500°C. However, this is not a generally accepted diagnostic criterion for the identification of halloysite as opposed to the more common kaolinite. Furthermore, most halloysite is of a tubular morphology leading to a rather intense hkl reflection at 4.5 Å even in oriented samples. In the XRD patterns of the clay fraction of a termite mound soil shown by Mahaney et al. (1996b), there is no indication of an intense non-basal reflection, suggesting a platy morphology more consistent with kaolinite rather than halloysite. Total chemical analysis of the termite mound material by neutron activation showed low levels of Na and Cl, indicating that it was not a good source of salt. It was suggested that concentrations of K and Fe in this material may be nutritionally useful, but in the absence of analyses under simulated physiological conditions this is speculation. Mahaney et al. (1996b) concluded

that geophagy in chimpanzees may be a strategy for the cure or alleviation of gastrointestinal upsets such as diarrhea, but acknowledged that more information was required to support this conclusion.

Further evidence on the nature of the geophagic soils eaten by chimpanzees in the Mahale Mountains was presented in a series of later papers (Mahaney et al., 1999; Aufreiter et al., 2001; Ketch et al., 2001). In the first of these, the physical, chemical, mineralogical, and microbiological properties of geophagic termite mound soils were compared with those of nearby topsoil that were not observed to be ingested by chimpanzees. The termite mound soils contained significantly more clay, 46% compared with 28% for the controls. However, there was no difference in the clay mineralogy of the two groups of soils, both being dominated by metahalloysite using the heating test described earlier. The geochemistry was described through total analyses of the bulk materials and electron probe microanalysis of selected mineral grains as observed under SEM. Soil pH, exchangeable cations, and organic matter were also determined. There were few geochemical differences although the termite mound soils were generally richer in most of the elements analyzed. This is hardly surprising since these soils usually derive from less weathered B horizons compared with the more intensively weathered control topsoils. Termite mound soils consistently gave higher pH values than controls, with 7 out of 10 samples being in excess of pH 7, but this was not reflected in any clear difference in amounts of exchangeable cations. As might be expected, the control topsoils contained much more organic matter. The adsorption capacity of the two groups of soils to adsorb potentially toxic alkaloids was also tested, but no differences were found. Microbial populations from the two soil sets did show some differences, but again this is hardly surprising bearing in mind the differences found for soil pH and organic matter content. Mahaney et al. (1999) concluded that geophagy in chimpanzees might serve a multifunctional purpose ranging through benefits related to mineral supplementation, alleviation of medical conditions (acidosis, diarrhea), and detoxification of dietary secondary metabolites.

These results were supported by another study of Tanzanian soils eaten by chimpanzees, compared with nearby control soils (Aufreiter et al., 2001). In addition to the analytical techniques used by Mahaney et al. (1999), this study also presented results of X-ray photoelectron spectroscopy, whereby the chemical composition of the surfaces of weathered minerals were analyzed, as well as amounts of elements extractable under acid (pH 2) conditions. The geophagic soils were more clay-rich and of a higher pH than their control counterparts, but there were no clear differences in mineralogy or geochemistry other than those that might be expected in a more clay-rich material. The soils contained very low levels of acid extractable nutrient elements, thus lending no support to explanations of geophagic behavior based upon mineral supplementation. In fact, as very low levels of Fe were extracted under acid conditions, it was suggested that this could be an adaptive advantage in so far as adsorption of Fe may stimulate a wide variety of pathogenic microflora.

Gorillas. Mahaney (1993) also studied the nature of the earthy material consumed by gorillas living in the Virunga Mountains in Rwanda (Table 1). These animals regularly mine and consume large quantities of weathered regolith, and samples of this were examined by SEM and other techniques. The material is weathered kivite, an olivine-rich, leucitic, volcanic rock, and SEM observations show an abundance of minerals derived from the parent rock, many of which are weathered and coated with iron oxides and clay minerals, said to be halloysite. Apparently, the gorrillas crush and grind the weathered material by hand, thus creating fresh mineral surfaces that can be seen under SEM. These observations were extended by a more detailed geochemical and mineralogical study (Mahaney et al., 1995b) of the geophagic material. The soil was shown to have a low K and Ca content, and identification of the main clay mineral was confirmed as halloysite. It had been previously suggested by Fossey (1983), that the weathered material could act as a supplementary dietary source of Ca, K, and Fe, but Mahaney et al. (1990) later showed that Ca and K were much higher in local non-eaten soils. Mahaney et al. (1995b), therefore, concluded that a chemical stimulus was probably not the main reason for the geophagic behavior of the gorillas, although they did not discount the possibility that the soils may be a source of Fe and other nutritionally important elements. A clay mineral stimulus, relating to the alleviation of gastrointestinal ailments, was thought to be a more likely cause and it was suggested that the eaten soils may help to adsorb plant toxins and to control dehydration in the dry season.

Humans. Possible explanations for geophagy in humans can, of course, be obtained merely by questioning those behaving in this way, although in many instances the replies are vague and ambiguous. The original observations of von Humboldt (Keay, 1993) with respect to the Ottomac peoples of the Orinoco suggested an obvious explanation based upon the alleviation of hunger pangs, as geophagy was only practiced during times of food shortage. However, other observations of human geophagy have brought forth additional explanations and have been coupled with chemical and mineralogical information of the geophagic material itself.

Johns (1986) found that four different geophagic clays consumed by Indian peoples from Bolivia, Peru, and Arizona were dominated by interstratified illite-smectite, sometimes with subsidiary amounts of other clay minerals like kaolinite and chlorite. He argued that in this instance geophagy functions as a detoxification technique to enable consumption of wild potato species. In fact, this is the specific reason for geophagy given by its practitioners. Eating clay with potatoes is thought to be effective in eliminating the bitterness of food, and in preventing stomach pains and vomiting. All the clays were effective in adsorbing the glycoalkaloid tomatine over a range of simulated physiological conditions. Tomatine adsorption capacities of the clays were usually intermediate between those of pure smectite and kaolinite, but for one clay it was significantly higher than smectite. A further example of the use of geophagic clays was given by Johns and Duquette (1991a) in their

study of the making of bread from acorns by Indians in California and by natives of Sardinia. The mineralogical composition of the clays from both localities was described as containing abundant kaolinite with subsidiary amounts of smectite. Traditionally, the clays are mixed with acorn meal to make it more palatable by reducing its bitter taste. Johns and Duquette (1991a) found that the clays used were able to reduce the tannic acid content of the acorn meal by up to 77%, thus providing a rationale for their consumption. Further experiments to determine the maximum adsorption capacity of the clays for tannic acid, taking account of the amounts of clay used in the traditional recipes for making acorn bread, showed that the addition of clay in the recommended amounts could only adsorb between 8 and 12% of the tannic acid in the acorns. It was concluded that the clay functions through enhancement of palatability rather than as a detoxification agent *sensu stricto*. It was also suggested that the heating involved in the baking of the bread might further enhance the tannic acid adsorption of the clays and could contribute to the catalytic activity of the clay in decomposing tannic acid.

Johns and Duquette (1991b) also examined the mineralogical composition of another series of geophagic clays from various countries in Africa and found that their clay mineral content was invariably dominated by kaolinite. The stated use of these clays, which were bought in local markets, was to alleviate diarrhea and sickness during pregnancy. Earlier, Vermeer and Ferrell (1985) had analyzed the mineralogy of a commercial geophagic clay from Nigeria. This clay derives from a deposit in Palaeocene shales in south-central Nigeria and consists of fine-grained, poorly crystallized, kaolinite. In this respect, it is strikingly similar to kaolinite-based western medicines that are marketed for the purpose of alleviating gastrointestinal upsets.

Geophagy is still a common practice among peoples living in tropical latitudes. It could have originated in Africa and was transferred to the New World with the slave trade (Hunter, 1973; Abrahams and Parsons, 1996), although the practice certainly existed on the American continent prior to this. One of the reasons commonly suggested for geophagy in tropical peoples is that it acts as a source of mineral nutrient supplementation. Abrahams and Parsons (1997) analyzed the chemical composition of three tropical geophagical soils to test this hypothesis. Soils, from Thailand, Uganda, and Zaire, were all reported as having low organic matter contents (0.2–1.5%) and clay contents (15–28%). No clay mineral data were given. The apparent dominance of the non-clay fractions in these soils is, however, rather anomalous, as the reported CECs of the soils ranged from 10.9 to 28.8 meq/100 g. This suggests that the reported particle size distribution is due to inadequate dispersion, a common problem in highly weathered tropical soils, and that the sand and silt fractions actually contain clay minerals in an aggregated form. The reported pH (in water) values for these soils vary widely, 4.4 for the Thailand soil, 6.8 for the Uganda soil, and 9.4 for the Zaire soil. The latter contains high values for Na and Mg extractable by 0.1 M HCl (3140 and 4100 mg/kg,

respectively) suggesting the presence of soluble salts. By analyzing the acid extractable mineral nutrient content of the soils, in conjunction with an assumed soil ingestion rate, Abrahams and Parsons (1997) concluded that the soils could supply a significant proportion of the recommended intake (United Kingdom, Department of Health) for some mineral nutrients, up to 17% in the case of Fe. This aspect was further investigated by Abrahams (1997) in a study of a sample set of 13 geophagic soils from Uganda, most of which were being sold as medicines in local markets. All these soils had a high clay content, usually >50%, and had a moderately acid pH (~5), except for four samples which had a pH of ~7. The mineralogy of the samples was invariably dominated (85%) by kaolin minerals, usually kaolinite itself, but despite this the CEC of these materials mostly ranged from 20 to 30 cmol/kg. These are anomalously high values for kaolinitic material. Abrahams (1997) suggested that this might be accounted for by the presence of interstratified smectitic material and noted that 7 of the 13 soil samples contained small amounts (<5%) of interstratified kaolinite—smectite. However, the basis for this identification, which could be difficult in a kaolinite-dominated clay, was not described. Abrahams (1997) concluded on the basis of geochemical analysis of one soil that the daily consumption of 5 g of this material was capable of contributing 19–25% of the daily recommended nutrient intake (RNI) of Fe. However, the bioavailability of Fe in all the other soils examined, as assessed by 0.1 M HCl extraction, was exceedingly low.

The prevalence of geophagy among schoolchildren and pregnant women in western Kenya was described by Geissler and coworkers. Over 70% of a sample of schoolchildren in Nyanza Province were found to consume soil at an average rate of ~30 g daily. The eaten soils came mainly from termite nests, the edges of paths and gullies, weathered stones, and the walls of huts (Geissler et al., 1997), but no mineralogical data were provided. Geophagy was identified as a possible risk factor in the transmission of various geohelminth parasitic infections (Geissler et al., 1998a) and also could be implicated in the development of iron depletion and anemia (Geissler et al., 1998b). The latter study found that an average of 28 g of soil was eaten daily by geophagous children, which, for Fe, could in principle supply 32% of the RNI for girls and 42% of the RNI for boys in this particular age group (10–18 years). However, Geissler et al. (1998b) were unable to come to any conclusion as to whether geophagy was a cause or a consequence of the incidence of anemia and iron depletion in this group of children. The consumption of soil may induce iron deficiency because it makes the mineral element less bioavailable, perhaps because other soil components may impair iron uptake or metabolism, but on the other hand it is possible that anemia or iron deficiency may induce a craving for soil.

Geissler et al. (1998c) also showed that geophagy is common among pregnant women on the coast of Kenya, with 56% of a sample population indulging in the practice. In this case, the source of the soil consumed was usually from the walls

of huts. The average daily intake of soil was sufficient to supply about 14% of the recommended dietary allowance of Fe for pregnant women (National Research Council, 1989). However, geophagous women tended to be more anemic and to suffer more from iron deficiency than non-geophagous individuals although, as for the previously described study undertaken on Kenyan children, no conclusion could be drawn from this tendency regarding causality.

The practice of geophagy among pregnant women in tropical Africa was discussed in detail by Wiley and Katz (1998). They hypothesized that it was related to a need for Ca, particularly for pregnant women in non-dairying peoples where there was a special requirement for Ca supplementation. In addition, it was suggested that clay consumption may reduce the discomfort and loss of nutrients experienced during the early stages of pregnancy. It was emphasized that geophagous materials often derive from termite nests, which are frequently high in Ca and represent sites of calcium carbonate accumulation in a landscape dominated by noncalcareous soils. Many of the geophagous soils described previously are notably low in Ca, and the hypothesis of Wiley and Katz is consistent with the work of Hunter and de Kleine (1984) in Central America. The latter involved analysis of the so-called "holy clay tablets," originating from Guatemala and widely consumed in Central America, particularly by pregnant Black Carib women in Belize. Comparing the total analysis of these tablets with the mineral nutrients that could be extracted by simulated human digestion (0.1 M HCl extraction), it was found that bioavailablity was 26% for Ca, 17% for Mg, 6.5% for K, 3% for Fe, 7% for Cu, and 14% for Zn. Consumption of two tablets per day could supply significant proportions of the recommended dietary allowance (National Research Council, 1989) for pregnant females, namely for Ca 18%, Mg 14%, Fe 19%, Cu 5–7%, and Zn 5%.

Other studies on human geophagic materials have been carried out by Mahaney and his coworkers in which the mineral constituents are specified (Table 1). Thus, Aufreiter et al. (1997) studied the chemistry and mineralogy of geophagic soils from Zimbabwe, North Carolina, U.S.A., and Hunan Province, China. Soils from Zimbabwe were from termite mounds and contained kaolinite as the main clay mineral. The amounts of Ca, Mg, K, and Na that could be extracted by weak acid (pH 2) treatment were low, as were the amounts of most other nutrient elements. The American samples were from a clay-rich subsoil. They were all dominated by halloysite and contained low amounts of Ca, which, like most nutrient elements, could barely be detected in the acid extraction treatment. Finally, the Chinese samples, which were from unspecified deposits, were generally smectitic in nature. Filtrate after acid extraction procedure could be obtained from only one sample in which the concentrations of nutrient elements were relatively low. Aufreiter et al. (1997) concluded that the results were consistent with the effects desired by the consumers of the soils, namely alleviation of diarrhea (Zimbabwe), general but unspecified health benefits (North Carolina), and as famine food (China).

The chemistry and mineralogy of soils consumed by humans in Indonesia was studied by Mahaney et al. (2000). Three of these geophagic soils were compared with two controls that were not eaten. The soils are derived either from volcanic material of an andesitic composition or from weathered Neogene sediments. Soils were clay-rich, usually containing over 70% clay, and low in organic matter. Soil pH values varied from 6.0 to 8.1 for the eaten soils and from 5.3 to 8.3 for the non-eaten ones. Kaolin minerals, both kaolinite and halloysite in its two hydration states, were major components of both the geophagic and control soils, although one of the latter was more strongly smectitic. There were differences in chemical composition between the volcanic and sediment-derived soils, but few differences when comparing eaten and control soils within each group. Similarly, there was no difference in the capacity of the eaten and control soils to adsorb alkaloids. On the basis of SEM examination of the sand fractions of the soils, Mahaney et al. (2000) speculated that some of the mineral constituents may have a role in mineral or trace element supplementation, and that the presence of sulphur-containing minerals, like marcasite, may be important in treating endoparasites.

DISCUSSION

It is widely agreed that there is no single explanation for geophagy in animals and that the practice may serve a variety of purposes (or even none at all). This is reflected in the diversity of physical, mineralogical, and chemical properties of the materials consumed and that are summarized in Table 1. Supporting evidence for the various proposed functions of geophagic behavior clearly requires that the soils and clays involved are thoroughly characterized and it is apparent that there are a number of anomalies, inconsistencies, and deficiencies that need further consideration.

Where it has been concluded that soils are consumed for the purpose of alleviating diarrhea or other gastrointestinal upsets, it is found that these soils are often rich in kaolin minerals, where the term “kaolin” is used in a nonspecific way. In a number of papers, however, the kaolin mineral is specifically identified as halloysite, in its hydrated, but more usually, in its dehydrated form (for example, Aufreiter et al., 2001). In some cases, both forms of halloysite are identified along with kaolinite and even small amounts of interstratified kaolinite–smectite (Abrahams, 1997).

The basis for identification of halloysite is not always specified, but seems to involve heating at 500°C (Mahaney et al., 1999). At this temperature halloysite it is claimed that is reduced to an amorphous form whereas kaolinite retains its crystalline structure. To the author’s knowledge this is not a recognized test for halloysite. The temperature of the endothermic dehydroxylation maximum on the differential thermal analysis curve for halloysite, which is indicative of its reduction

to an amorphous state, occurs at ~570 to 580°C, a somewhat higher temperature than where some poorly crystallized kaolinites dehydroxylate (Holdridge and Vaughan, 1957). Furthermore, where XRD patterns of kaolin-rich geophagic clays are shown (Mahaney et al., 1997, 1999), it is evident that the intense reflection at ~4.42 Å, which is characteristic of the tubular form of halloysite, the most common morphological form of this mineral, is not observed. This reflection arises from 11, 02 planes in a disordered tubular structure and is observed on the XRD pattern whatever the orientation of the sample. In the absence of further evidence, it can only be concluded that the identification of halloysite, rather than poorly crystallized kaolinite, as a major clay mineral of geophagic materials, is open to question. The use of supplementary XRD tests, such as intercalation with K-acetate (Wada, 1965), hydrazine (Range et al., 1969), or formamide (Churchman et al., 1984), to confirm the identification of halloysite in these materials would be useful, as would TEM observations of particle morphology.

With regard to the proposed function of kaolin-rich geophagic materials, the point has often been made that their XRD patterns resemble those obtained from western kaolin-based pharmaceutical preparations, such as Kaopectate, which help to cure or alleviate diarrhea and gastrointestinal upsets. However, Kaopectate is based upon kaolinite rather upon than halloysite (Vermeer and Ferrell, 1985), and there is no obvious reason to think that the latter mineral confers any special advantage over kaolinite. In any case, it seems unlikely that halloysitic clays as opposed to kaolinitic clays could act as a stimulus for the geophagic behavior of animals. This possibility cannot be altogether discounted because it may be that halloysite in an aggregate form may offer a tactile clue to an animal to enable its selection as appropriate geophagic material. Thus, palygorskite in aggregate form has the property of adhering strongly to the tongue, presumably because of capillary suction related to its fibrous structure and the fine micropores between the fiber bundles. A similar microporous structure may characterize halloysite aggregates consisting of bundles of tubes, although this surmise has not been tested.

Identification of interstratified kaolinite–smectite (Abrahams, 1997) must also be open to question, particularly where it is claimed to occur in minor (<10%) amounts and where no criteria for its identification are specified. It could be quite important to confirm the identification of this mineral because if present in significant quantities it would confer a moderately high CEC on the geophagic material in which it occurred. This could account for the anomaly of a clay dominated by kaolin minerals, but with a higher than expected CEC (Setz et al., 1999). Furthermore, a similar interstratified mineral (halloysite–smectite) has an enhanced capacity for fixing K ions (Delvaux et al., 1990). If this mineral is a major constituent of geophagic material, then this could be important in circumstances where changes in an animal's diet, as for example grazing lush grass during spring time, might lead to nutrient imbalances with an excess of K over Na (Mahaney et al., 1996a). In general, it would be difficult to distinguish between dehydrated halloysite and

interstratified kaolinite–smectite on the basis of XRD patterns alone without the use of supplementary tests.

There are also some unusual features in the mineralogy of geophagic soils that are considered beneficial to the consumer that ingests them by adsorbing toxins or unpalatable compounds present in the diet. These soils are likely to contain smectite because of the high CEC and absorbing capacity of the mineral. This is usually, but not always (Johns and Duquette, 1991b), found to be the case. Where smectite occurs in geophagic materials, it is usually diluted by other nonswelling minerals. Possibly, ingestion of a relatively pure smectitic clay, because of its swelling nature, may cause difficulty of movement in the digestive tract, as suggested by Aufreiter et al. (1997). In addition, the smectite in geophagic clays may be interstratified with illite (Johns, 1986) or it may be interlayered with material that would usually be attributed to nonexchangeable hydroxy aluminum. In the latter case, the smectite does not collapse completely to a 10 Å structure after heating, or it may show an anomalously high spacing after glycerol or ethylene glycol solvation, recalling the findings of Diamond et al., (1999) and Gilardi et al. (1999). It is possible that such an interlayered structure may act in the same way as synthetic pillared clays and preferentially absorb molecules of an appropriate size in the interlamellar space between the silicate sheets.

The hypothesis that geophagic soils act as a natural antacid helping to alleviate discomfort brought about by excess acidity in the digestive tract is dependent upon the chemical and mineralogical characteristics of these materials. An essential observation would seem to be to determine the buffering capacity of the ingested soil, but it is not often that this has been done. However, reasonable inferences about the effectiveness of geophagic soils in buffering acidity can be made from determinations of soil pH and from mineralogy. Where soil pH exceeds 7, then the likelihood is that the soil contains carbonate minerals, and where this pH is greatly exceeded, then the soil probably contains alkaline soluble salts. Both types of minerals would be effective as an antacid. A number of geophagic soils reviewed here have such elevated pH values (Abrahams and Parsons, 1997; Abrahams, 1999) but most do not. However, even the acidic soils could be effective in alleviating excess acidity because of the aluminous minerals that they contain. Thus, highly weathered soils of tropical environments, in which many geophagic soils are found, might be expected to contain the aluminum hydroxide mineral, gibbsite. Reactive aluminum hydroxide is used in many pharmaceutical antacid preparations, and it is possible that fine-grained gibbsite may behave similarly in the acid conditions (pH $\sim$2) of the digestive tract. Other hydroxylated aluminous minerals, such as kaolinite and hydroxy interlayered vermiculites, may also release hydroxyls under these conditions, but to a lesser extent. This aspect does not seem to have been considered in mineralogical studies of geophagic materials. Gibbsite is not often mentioned as a constituent of these soils, although it can be expected to occur in soils formed under tropical conditions characterized by intense weathering with

severe depletion of bases and silica. Such an environment is generally recognized as being ideal for the formation of gibbsite.

A further point concerns the observation that geophagic soils consumed by gorillas in the Virunga Mountains of Rwanda are sandy, weathered materials developed from base-rich volcanic rocks (Mahaney, 1993; Mahaney et al., 1995b). The gorillas crush and pulverize these materials by hand action before consuming them. This action could be effective in raising the pH values of these materials into the strongly alkaline range in that it simulates in a crude way the grinding involved in measuring the abrasion pH of minerals. When base-rich minerals are ground in a mortar under a little water, the measured pH of the slurry is highly alkaline following the release of bases from the freshly exposed mineral surfaces. For example, the abrasion pH of minerals like olivine, leucite, and labradorite, all of which occur in the parent kivite consumed by the gorillas, is 10 (Stevens and Carron, 1948). SEM observations of the crushed kivite consumed by the gorillas showed many fresh mineral grain surfaces (Mahaney, 1993), which could be effective in neutralizing excess stomach acidity.

An explanation of geophagy based upon the capacity of the ingested material to provide useful supplements of nutrient minerals follows naturally from the frequently observed animal behavior at mineral licks. This hypothesis requires substantiation by chemical analysis of the consumed material, but unfortunately many papers, even recent ones, provide only total analyses. These analyses may be of major nutrients, often with a variety of trace elements, many of which have no known physiological function. Such information is of dubious relevance to the question at hand, even when supplemented by analyses using sophisticated instrumental techniques, such as electron probe microanalysis of selected mineral grains or X-ray photoelectron spectroscopy. The approach would be analagous to an agronomist assessing the ability of a soil to grow crops on the basis of the total nutrient mineral content of the soil without considering what is available for uptake by the crop. In the same way, it is essential to assess the potential bioavailability of the nutrient minerals in geophagic soils under conditions approaching those of the consuming animal's digestive tract. In many papers, this is done by extracting with 0.1 M HCl or with acid ammonium oxalate at pH 2. These analyses provide relevant information and suggest that some geophagic materials are capable of supplying useful amounts of certain nutrient elements, particularly Ca and Fe. However, as pointed out by Smith et al. (2000), these extractants do not take into account changes in conditions of Eh/pH. Smith et al. (2000), therefore, developed a physiologically based extraction test (PBET) in their study of the bioavailability of essential and toxic elements from geophagic materials consumed by humans in Uganda. This method simulated gastric solution under different redox conditions and involved a cocktail of pepsin, and various organic acids adjusted to pH 2 by using 1% HCl. On the basis of the results from this extractant, Smith et al. (2000) concluded that the geophagic materials in question could provide a major portion

of the recommended daily intake of Fe, but were inadequate as supplements for other nutrient elements.

Mineralogical analysis of geophagic soils should enable some general conclusions to be drawn as to their potential to supply bioavailable nutrients. It would be expected, for example, that the Ca and Mg held on exchange sites on clays would be available, as might some of the interlayer K in weathered micas, particularly biotites. Soluble salts and carbonate minerals could be expected to decompose under the acid conditions of the digestive tract and to provide alkali and alkali earth cations. Similarly, weathered ferromagnesian minerals, such as olivine or pyroxene, as well as calcic plagioclase feldspars, might be acid-labile to a certain extent. With regard to the bioavailability of Fe from geophagic materials, this is likely to be related to the nature and reactivity of the constituent iron oxide minerals. For example, if the major iron oxide mineral is hematite (Fe_2O_3) and occurs in large crystals, then it is likely to be relatively stable during transit through the gut, even under acid reducing conditions. Ferrihydrite, a hydrous ferric oxide mineral, which is typically poorly crystalline and occurs in small particles, is much more reactive and more likely to be solubilized. Quantification of the proportion of more reactive to less reactive iron in a soil can be assessed from the ratio of oxalate soluble (Fe_o) to dithionite soluble Fe (Fe_d) iron (Schwertmann and Taylor, 1989). This has rarely been done for geophagic soils and in the majority of studies no information is given on the nature of the iron oxide minerals.

CONCLUSIONS

This review reflects the conclusions reached by many of the authors cited above, that there is no single explanation for geophagic behavior in animals, including man. There is good evidence to support all the major hypotheses outlined; namely, detoxification, or at least enhancement of the palatability, of foodstuffs containing undesirable components; alleviation of gastrointestinal upsets such as diarrhea; mineral supplementation, particularly Fe and Ca; and as an antacid to relieve excess acidity in the digestive tract. However, these hypotheses would have been even more strongly supported in many of the case studies reviewed here if the mineralogy and chemistry of the geophagic materials themselves had been more extensively characterized. In particular, more information is required with respect to the following: (a) the nature of the constituent kaolin minerals by using recognized supplementary tests and other investigational techniques such as TEM, perhaps with further characterization of the abilities of the kaolin minerals to coagulate, aggregate, and/or disperse under acid conditions; (b) the occurrence of interstratified kaolinite–smectite and whether this mineral could account for the anomalously high CECs of geophagic soils/clays; (c) the nature of the smectitic minerals, particularly the occurrence and extent of interstratification with other

layer silicates or interlayering with nonexchangeable hydroxy Al, especially as this relates to the ability of the clay to adsorb potential toxins; (d) characterization of the iron oxide minerals and determination of the ratio of reactive to nonreactive species in relation to supplementation of Fe in the diet; and (e) the occurrence and nature of aluminous minerals and whether they function effectively as natural antacids. With regard to geochemical analyses of geophagic materials, it seems that a reliable PBET, such as that used by Smith et al. (2000), is a *sine qua non* before any firm conclusions can be drawn regarding the potentialities for mineral supplementation. Such tests, in conjunction with refined mineralogical analyses, would provide comprehensive and convincing support for this hypothesis.

Turning to the possible stimuli that may account for geophagic behavior, in both humans and nonhuman animals, it seems unnecessary to endow them with an extraordinary capacity to be able to detect certain useful mineral types or major nutrients and/or trace elements that are deficient in their diet. A well-known exception to the latter case is salt-bearing mineral licks but most of the geophagic materials reviewed here are completely deficient in salt. However, many geophagic materials are notably high in clay, suggesting that its smoothness and unctuousness may provide an initial stimulus for ingestion, as was initially proposed by von Humboldt. Even where particle size distribution analysis shows that the geophagic material does not appear to be particularly clay-rich, care must be taken to ensure that such an analysis truly reflects the amounts of clay *versus* nonclay minerals present. Thus, many tropical soils are so strongly aggregated that they may be difficult to disperse by conventional techniques and they may, in fact, behave as if they have a sandy texture. More vigorous treatment can break down the clay aggregates. Once this is done, it is found that the soil is predominantly clay-rich. Possibly, such aggregates are broken down in the mouth of the animal following absorption of salivary liquids and mastication before being swallowed.

Soil/clay color has also been suggested as a simple stimulus to account for geophagic behavior. Most geophagic materials reviewed here have a reddish hue, although many do not. It seems unlikely, that color can be a primary stimulus, although it may have an important secondary effect. Thus, it could be envisaged that the initial stimulus for animal geophagy is the pleasant tactile sensation induced by the unctuous nature of the ingested clay. Such ingestion may then be associated with any of the benefits outlined above, so that geophagy becomes a learned behavior that is beneficial to the animal's welfare. The color of the geophagic clay/soil then becomes a secondary stimulus enabling the search for new sites and avoidance of the dark colors associated with organic rich top soils.

Explanations for geophagy in man take on the additional dimension that the practitioner can himself/herself give reasons for this behavior, although these often turn out to be vague or even bizarre. In some cases, however, the proffered explanations are perfectly credible, especially when they involve mineral supplementation or treatment for gastrointestinal complaints. For geophagic children, the primary

motivation seems to be the pleasant taste of the ingested material (Geissler et al., 1997; Geissler, 2000).

Finally, it is clear that research into geophagy requires a strong interdisciplinary approach and that input of the clay mineralogist, along with those of other specialists, has an important role to play in this respect.

NOTE ADDED IN PROOF: Another possible reason for geophagy has recently been drawn to the author's attention. Robertson (1996), in discussing the work of Stumpf, a forensic pathologist, on treating cholera more than one hundred years ago, mentions that Stumpf had concluded that useful properties of clays in the field of medicine are bacteriostasis, sterilization, membrane coating, and adsorption of toxins. Furthermore, Stumpf had recommended the use of large doses of clay as being effective in sweeping through the alimentary canal so combining these properties in a single operation. Roberston (1996) coins the term *abverrant* to describe the clays that are useful in this respect. He also mentions that historically important geophagic clays preserved in the British Musem have a very mixed clay mineralogy. This suggests that in many cases geophagic clays are consumed because of their abverrant properties and that there is no necessarily strict relationship between geophagic clays and their mineralogy.

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LOW-MOLECULAR-WEIGHT ORGANIC ACIDS AND HORMONE-LIKE ACTIVITY OF DISSOLVED ORGANIC MATTER IN TWO FOREST SOILS IN N ITALY

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Abstract—Concentrations of aliphatic acids, phenolic acid, and inorganic nutrients, as well as hormone-like activity, were determined in soil dissolved organic matter (DOM) from two forest sites in N Italy showing differing degrees of silver fir regeneration. In the site where silver fir recruitment was abundant, humification processes prevailed, and the soil DOM had a high content in aliphatic and phenolic acids. This enhanced the hormone-like activity in the soil, which could in turn promote growth of silver fir seedlings. In the site with poor fir recruitment, the soil DOM underwent rapid mineralization and was richer in inorganic nutrients, but had lower concentrations of aliphatic and phenolic acids, and lower hormone-like activity.

Key Words—Recruitment, silver fir, soil moisture, phenolics, humus, phytosociology.

INTRODUCTION

Dissolved organic matter (DOM), consists of humic substances and other organic compounds dissolved in the soil solution, such as amino acids, carbohydrates, aliphatic acids, aromatic acids, and hydrocarbons (Thurman, 1985; Kalbitz et al., 2000a,b). DOM plays an important role in the cycling of carbon and nutrients, especially N, P, and S, because the nutrients in the soil solution are more available,

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both to soil microbes and plants, than those occurring in the soil organic matter (Herbert and Bertsch, 1995). Low-molecular-weight (LMW) organic aliphatic and phenolic acids, although representing only a small fraction of DOM, exert a disproportionate influence on soil processes since they are among the most reactive forms of C in the soil (Fox, 1995).

LMW organic aliphatic acids affect soil chemical processes through complexation with metals in solution and through ligand exchange reactions at the soil surface (Martell et al., 1988). They also enhance plant nutrient availability in forest soils (Jones and Darrah, 1994). In natural soils, organic acids control the dissociation of humic structures into smaller molecules (Nardi et al., 1996). The resulting small-size humic fractions stimulate various biochemical processes in plants, such as nitrate uptake, ATPase activity, and hormone-like activity (Nardi et al., 1996, 2000, 2002). Besides aliphatic acids, LMW phenolic acids, released by plants and soil microbes, also represent an important group of compounds having major ecological functions. Phenolics are synthesized in plants with a great specificity (Haslam, 1998), but relationships between vegetation and forest soil organic matter characteristics are not fully understood (Gallet and Lebreton, 1995). Little information is available as to what chemical properties of soils are affected by phenolic compounds and how they influence soil processes. Vance et al. (1986) reported concentrations of monomeric phenolics in soils to be correlated with soil podzolization. Moreover, phenolics have significant effects on nutrient cycling, by forming complexes with nutrients. Therefore, phenolics can influence rates of nutrient turnover in soils (Inderjit and Mallik, 1997).

Since LMW organic acids and phenolic acids may have a strong impact on ecosystem processes, knowledge of those occurring in the soil solution of forest soils is of major ecological interest. In particular, we determined concentrations of LMW organic aliphatic and phenolic acids in the soil of two forest sites showing differing degrees of silver fir (*Abies alba* Mill.) recruitment. We also determined the auxin (IAA) and gibberellin (GA)-like activities of soil DOM since IAA and GA play important roles in controlling seed germination as well as in the adaptation of plants to environmental stress (Vaughan and Malcolm, 1985). Upon germination, IAA esters accumulate in the developing seed and move from the endosperm to the shoot where they are deconjugated to yield free IAA (Normanly, 1997). Phenolics may act as modulators of plant development by regulating IAA catabolism (Volpert et al., 1995). GA induces degradation of storage products in the early germination stage not only in starch-storing seeds, but also in lipid-storing seeds such as pine and fir seeds (Hammer and Murhy, 1993).

METHODS AND MATERIALS

Study Area and Site Choice. The study area (Pian de1 Cansiglio, Province of Belluno) is situated in the Ventian Praealps, N Italy, at ca. 1050 m elevation. The area consists of a gently sloping (10° on average), E-facing carbonate plateau. The

climate is temperate-montane oceanic, with a mean annual temperature of 7.1°C. The mean temperature of the coldest month is −1.2°C and that of the warmest 15.7°C. The mean annual precipitation is 1869 mm with two maxima, in late Spring and mid-Autumn (Fliri, 1975).

Two sites were chosen for study, after a preliminary field survey using 1 : 50,000 vegetation maps. The two sites were similar with respect to geomorphology, topography, and forest management. The basal area ranged between 26 and 30 m^2/ha and the mean height of the dominant trees from 25 to 27 m. The two sites differed remarkably from each other in regard to regeneration of silver fir: Pian Canaie (PC) showed a high degree of silver fir recruitment; Col Campon (CC) showed a poor recruitment, with almost all seedlings dying within the first year.

Vegetation Analysis and Soil Sampling. The vegetation was recorded in two homogeneous plots (200 × 100 m) at each of the two sites. The percentage cover of all vascular and moss species occurring in each plot was estimated visually, and the data were used for typification based on the Braun-Blanquet (1964) syntaxonomy. Species nomenclature follows Pignatti (1982) for vascular plants and Frahm and Frey (1987) for mosses.

Five pits were dug at each site with a soil auger. The soil profile was described morphologically in each pit, and triplicate samples were taken from each horizon in the five pits. One of the soil subsamples was placed in a sealed container that was weighed within 6 hr and reweighed after drying for 48 hr at 105°C. Soil moisture content was determined gravimetrically based on the loss in weight after oven drying. The second soil subsample was air-dried and sieved to <2 mm for chemical analyses (SISS, 2000). The third soil subsample was freshly extracted for DOM analysis.

Soil Microbial Biomass. Soil microbial biomass was estimated by the fumigation–incubation method (Vance et al., 1987). Moist soil was exposed to $CHCl_3$ for 24 hr, the fumigant was removed, and the soil extracted with 0.5 M K_2SO_4. A nonfumigated control was extracted under the same conditions at the time fumigation commenced. Organic carbon in the extracts was determined by dichromate digestion (Walkley and Black, 1934).

DOM Extraction and Characterization. DOM extraction was carried out on fresh soil samples by using double-deionized water with a solid/volume ratio of 1:2 for the A and B horizons and 1:4 for the L and F horizons (Corre et al., 1999). Suspensions were shaken for 2 hr at room temperature under N_2 atmosphere and then centrifuged at 7000 g for 15 min at 10°C. Extracts were filtered through Whatman microfiber glass filters (Whatman, Maidstone, England) and then 0.45 μm Millipore filters (Millipore, Milford, MA, USA). Dissolved organic carbon (DOC) content was determined by dichromate oxidation (Walkley and Black, 1934).

NO_3^-, SO_4^{2-}, and PO_4^{3-} concentrations in DOM were determined by HPLC on an AS 4S-SC anion exchange column (Dionex, Sunnyvale, CA), with a Dionex suppressor, and a 431 Waters conductivity detector (Waters-Millipore, Milford, MA).

A solution of sodium bicarbonate and sodium carbonate (1.7 mM $NaHCO_3$/1.8 mM Na_2CO_3) was used as the eluent (2 ml/min flow rate). As reference standards, sodium nitrate, potassium sulphate, and potassium phosphate, were obtained from Fluka (Fluka, Buchs, Switzerland).

To determine LMW organic acids, an aliquot of DOM was preliminarily purified by passing onto an anion exchange solid-phase extraction column (SAX, Alltech, Deerfield, IL). Anionic fractions were eluted with 500 mM H_2S0_4. Free organic acids were separated by HPLC using a 400 Perkin-Elmer high-pressure pump (Perkin-Elmer, Norwalk, Connecticut), a 234 Gilson autoinjector (Gilson, Middlestone, WI), and a LC 90 Perkin-Elmer variable wavelength UV detector set at 210 nm, with 10-μm flow cell. Ion-exchange chromatography was carried out using an HPX-87-H Aminex column (Biorad, Richmond, CA; 300 mm $\times$ 7.8 mm ID) and isocratic elution with 6 mM H_2SO_4 as mobile phase (0.4 ml/min) at room temperature (Pecina et al., 1984). Reference standards were supplied by Sigma Chemical Co. (Sigma, St. Louis, MO).

To determine phenolic acids, a standard volume of DOM was acidified (pH = 3.0) with acetic acid and then extracted 3 times with ethyl acetate. After evaporation to dryness, the residues were redissolved in water. These water solutions were used for phenolic HPLC determination and quantification. Gallic, protocatechuic, gentisic, phthalic, syringic, *p*-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, and ferulic acids were separated by HPLC and detected with a Perkin-Elmer UV detector set at 275 nm, with 10-μl flow cell. RP chromatography was carried out by using a Luna Phenomenex (Phenomenex, Torrance, CA) column (250 mm $\times$ 4.6 mm ID) containing C_{18} bonded-phase on silica and isocratic elution with water–acetic acid–*n*–butanol (342:1:11, v/v/v) and a flow rate of 1.2 ml/min (Hartley and Buchan, 1979). Reference standards were supplied by Sigma Chemical Co. (Sigma, St. Louis, Mo). Semiquantitative results were obtained because only identified phenolic acids were integrated. Chrom-Card (CE Instruments, Thermo-Quest, Milan, Italy) for Windows (Version 1.19) was utilized to integrate known peaks.

Gas Chromatographc/Mass Spectrometric (GC/MS) Analyses. Two milliliter of each DOM sample were placed into a 4-ml vial and freeze-dried using a Modulyo 4K system (Edwards). Two hundred microliter of 2,2-dimethoxypropane and 20 μl of concentrated HCI were added. This reagent permits a high-yield derivatization of all the acidic species, by producing the corresponding methyl ester (Rachelle, 1963). The modified acids, when analyzed by GC/MS, showed improved detectability and gas chromatographic performance compared to the underivatized acids. After 30 min, the liquid phase was dried under nitrogen and methanol (20 μl) was added. A portion of the final liquid phase was injected into the GC/MS for analysis.

All sample were analyzed on a HP 5890 II gas chromatograph coupled with quadruple HP 5971 A mass spectrometer. Chromatographic separation of analytes was accomplished on a capillary column HP 50+ with the following

dimensions: length 30 m; film thickness 0.5 μm, and internal diameter 0.25 mm.

The temperature program was from 100°C × 1 min to 250°C—5°C/min—250°C × 10 min with injector and transfer-line temperatures, 250°C and 28O°C, respectively. The mass spectrometer operated in SCAN mode detecting the ions generated by electron ionization (70 eV) at the ion source temperature of 180°C, with a mass range 50–320 Da. Identification of the acid species was performed by using certified linear standards submitted to the same derivatization treatment and by comparing the mass spectra with those reported in the NBS mass spectra library.

Hormone-Like Activities. IAA- and GA-like activities were assessed by checking the reduction in growth of watercress (*Lepdium sativum* L.) roots and the increase in length of lettuce (*Lactuca sativa* L.) epicotyls (Audus, 1972). Watercress and lettuce seeds were surface-sterilized by immersion in 8% H_20_2 for 15 min. After rinsing 5 times with sterile distilled water, 10 seeds of watercress were placed onto atersile filter paper in a sterile Petri dish containing 1.2 ml of 1 mM $CaSO_4$ as control, or 1.2 ml of 20, 10, 1, and 0.1 mg/l IAA (Sigma, St. Louis, MO) for the calibration curve, or 1.2 ml of 10, 5, 2.5, 1, 0.5, 0.2, and 0.1 mg C/l DOM. For lettuce, the experimental design was the same except that the sterile filter paper was wetted with 1.4 ml instead of 1.2 ml and the calibration curve was a progression of 100, 10, 1, and 0.1 mg/l GA (Sigma, St. Louis, MO). Seeds were germinated in the dark at 25°C in a germination room. After 48 hr for watercress and 72 hr for lettuce, the seedlings were removed and the root or epicotyl lengths measured. The values obtained were the means of five samples and three replications, and the standard errors was always $\leq$5% of the mean.

Phenolic Acid Bioassay. Silver fir seeds, after a stratification period to break dormancy (Gradi, 1980), were grown in sterile conditions in Petri dishes containing filter paper (Whatman No. 3) wetted with 0.1, 1, 10, 50, and 100 μM of the phenolic acids present in the soil solution, or with 20, 10, 1, and 0.1 mg/l indoleacetic acid (IAA) (Sigma, St. Louis, MO) for the IAA calibration curve, or with 100, 10, 1, and 0.1 mg/l gibberellic acid (GA) (Sigma, St. Louis, MO) for the GA calibration curve, or with 1 mM $CaSO_4$ as control. Seeds were germinated in the dark at 25°C in a germination room. After 12 d, seedlings were removed and the roots or epicotyl lengths were measured. Values obtained were the means of five samples and three replications, with the standard errors always $\leq$5% of the mean.

Statistical Analyses. All data were treated by univariate statistical methods. Significance of differences was assessed by the Student–Newman–Keuls test (Sokal and Rohlf, 1969).

RESULTS

Vegetation and Soil Classification. Silver fir was abundant in the tree layer of both sites, but the co-dominant species differed with beech being more frequent at PC and spruce at CC (Table 1). The shrub layer was poorly developed in both

TABLE 1. PERCENTAGE COVER OF VASCULAR AND MOSS SPECIES IN THE TWO SITES (PC, SITE WITH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

	PC	CC
Tree layer		
Abies alba	30	30
Fagus sylvatica	40	10
Picea excelsa	15	30
Shrub layer		
Abies alba	5	
Acer pseudoplatanus		1
Fraxinus excelsior		1
Lonicera alpigena	R	10
Picea excelsa	10	
Field layer		
Abies alba	10	
Actaea spicata	R	2
Adenostyles glabra		15
Cardamine trifolia	10	1
Circaea lutetiana	2	5
Cystopteris montana		5
Daphne mezereum	1	1
Dryopterix filix-mas		15
Fagus sylvatica	15	R
Festuca altissima	5	1
Fragaria vesca	5	2
Impatiens parvifora		30
Luthyrus vernus	10	
Luzula nivea	10	R
Milium effusum		5
Mycelis muralis	R	5
Oxalis acetosella	10	40
Picea excelsa	R	
Polygonatum verticillatum		5
Polystichum aculeatum		5
Prenanthes purpurea	5	5
Rubus saxatilis	5	R
Senecio fuchsii		5
Sorbus aucuparia	5	
Stellaria nemorum		5
Veronica montana	5	
Moss layer		
Eurhynchium striatum	10	<1
Hylocomium splendens	10	<1
Plagiomnium affine		5
Plagiomnium undulatum		5
Polytrichum formasum	10	10
Rhytidiadelphus triquetrus	10	10

Note: The figures are the means of the cover values of each species in two sampling plots. Only the species showing a mean cover >1% in at least one of the sites were included in the table. R = rare species (cover <1%).

TABLE 2. SOIL PROPERTIES IN THE A AND B HORIZONS AT THE TWO SITES (PC, SITE WITH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

Site	Soil classification (FAO)	Horizon	Depth (cm)	pH (H_2O)	SOM (%)	C:N	Sand (%)	Silt (%)	Clay (%)	CEC (meq/100 g)
PC	Calcaric Cambisol	A	0–4	5.2ab	23.3a	14.5a	43a	18c	39b	71a
		Bw	>40	5.5a	2.2c	8.8b	31c	24b	45a	22c
CC	Eutric Cambisol	Ah	0–8	4.9b	7.2b	9.1b	37b	28a	35c	30b
		Bw	>40	5.2ab	2.0c	7.4c	33c	30a	37bc	18d

Note: Values are the means of five samples, with standard errors always ≤5% of the mean. The means within each column followed by the same letter do not differ at $P = 0.05$ based on Student–Newman–Keuls tests.

sites. The floristic relief reflected the high degree of silver fir renovation at PC, as shown by the high cover of fir seedlings in the field layer. However, the field layer of both sites was characterized by shade tolerant species indicating optimal soil-moisture content, e.g., *Actaea spicata*, *Cardamine trifolia*, *Festuca altissima* and, especially, *Circaea lutetiana* (Table 1). The high percentage of cover of *Oxalis acetosella* and *Impatiens parviflora* in the field layer of CC indicates somewhat more acidic soil conditions at that site. The moss layer was well developed, with highest cover at PC. Overall, the vegetation of both sites can be included into the same syntaxonomical type, i.e., the *Dentario–Fagetum abietetosum albae* (Del Favero, 1998).

Soil pH was similar at the two sites, but soil organic matter and C : N ratio were highest in the A horizon of PC (Table 2). The A horizon in PC was somewhat coarser and had a much greater cation exchange capacity than at CC (Table 2). Overall, the soil at PC could be classified as a Calcaric Cambisol (FAO-UNESCO, 1990) with a mormoder humus form (Green et al., 1993), and that at CC as an Eutric Cambisol with a vermimull humus form. Soil moisture content was higher at PC compared to CC, while the microbial biomass had a higher carbon content in the CC soil (Table 3).

Dissolved Organic Matter and Hormone-Like Activity. The soil DOM pH was moderately acidic in the upper horizons and slightly alkaline in the Bw horizon both at PC and CC (Table 4). The soil DOC content (Table 4) was highest in the F horizon of both soils, and showed a significant positive correlation with soil moisture content (Pearson's correlation coefficient = 0.781; $P < 0.05$). Concentrations of inorganic nutrients were, overall, higher in the CC soil, with phosphate being clearly enriched in the upper soil horizons and sulphate showing an opposite trend in the soil profile at the two sites (Table 4). Nitrate peaked in the F horizon of both soils, in relation to microbial biomass (Pearson's correlation coefficient = 0.89; $P < 0.01$).

Total concentrations of LMW aliphatic acids were always higher at PC, except in the Bw horizon where concentrations of aliphatic acids were very low at both

TABLE 3. SOIL MOISTURE CONTENT (MC) AND SOIL MICROBIAL BIOMASS CARBON CONCENTRATION (MBC) IN THE TWO SITES (PC, SITE WITH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

Site	Depth (cm)	MC (%)	MBC (μg C/g soil)
PC			
L	1	61.33b	103f
F	4	70.55a	1230b
A	4	59.33bc	312d
Bw	>40	29.75e	36h
CC			
L	1	54.71c	215e
F	2	68.89a	2109a
Ah	8	37.94d	784c
Bw	>40	27.06f	52g

Note: Values are the means of five samples, with standard errors always $\leq$5% of the mean. Means within each column followed by the same letter do not differ at $P = 0.05$ based on Student–Newman–Keuls tests.

sites (Table 5). The L and F horizons were rich in oxalic acid and tartaric acid. The L horizon at PC also exhibited high concentrations of citric and succinic acid. Concentrations of fumaric acid were low in both soils (Table 5). GC/MS analyses revealed the presence of $C_5(COOH)_2$, $C_7(COOH)_2$, $C_{14}(COOH)$, $C_{15}(COOH)$,

TABLE 4. PH, CONCENTRATIONS OF DOC AND INORGANIC NUTRIENTS IN THE SOIL DOM OF THE TWO SITES (PC, SITE WITH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

				DOM[a]		
Site	pH	DOC (%)	DOC (mg C/1)	mg NO_3^-/l	mg PO_4^{3-}/l	mg SO_4^{2-}/l
PC						
L	6.2b	0.105c	102c	0.29d	16.94bc	2.54d
F	6.5b	0.183b	131b	0.67b	15.36c	2.41d
A	6.3b	0.034d	69d	0.11f	0.77e	7.54a
Bw	8.2a	0.009e	33e	0.04g	1.95d	2.53d
CC						
L	6.0b	0.174b	197b	0.27d	23.58a	6.63b
F	5.3c	0.280a	218a	4.24a	18.61b	6.48b
AH	6.3b	0.007f	23f	0.45c	0.01g	2.85
Bw	7.8a	0.002g	6g	0.13e	0.03f	1.28e

[a] The retention time was 7.43 for nitrate, 8.92 for phosphate, and 10.45 for sulphate. Values are the means of five samples, with standard errors always $\leq$55% of the mean. The means within each column followed by the same letter do not differ at $P = 0.05$ based on Student–Newman–Keuls tests.

TABLE 5. CONCENTRATIONS OF LMW ALIPHATIC ACIDS IN THE SOIL DOM OF THE TWO SITES (PC, SITE WITH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

	Low-molecular-weight organic acids (μM)						
Site	Oxalic (10.84)	Citric (11.97)	Tartaric (12.72)	Succinic (18.27)	Fumaric (23.59)	1-Malic (14.44)	Total (μM)
PC							
L	218b	448a	266a	371a	1.76a	n.d.	1305a
F	615a	n.d.	100c	n.d.	0.98b	n.d.	716b
A	12.12e	n.d.	16.30d	n.d.	0.12d	n.d.	41e
Bw	2.59h	n.d.	3.40e	n.d.	n.d.	n.d.	6g
CC							
L	153c	n.d.	172b	n.d.	0.92b	n.d.	326c
F	87d	n.d.	96c	81b	0.72c	n.d.	265d
Ah	6.67f	10.40b	n.d.	n.d.	0.03e	n.d.	17f
Bw	2.90g	n.d.	1.82f	n.d.	0.02f	1.53a	6g

Note: The retention times are reported in brackets. Values are the means of five samples, with standard errors always $\leq$5% of the mean. The means within each column followed by the same letter do not differ at $P = 0.05$ based on Student–Newman–Keuls tests; n.d. = not detected.

and C_{17}(COOH) acids in the DOM from the L and F horizons, and C_{14}(COOH) and $C_{15}(COOH)_2$ acids in the A and Bw horizons of both soils (Table 6). The DOM from the F horizon at CC differed from that at PC owing to the presence of $C_5(COOH)_2$ (pimelic acid) and $C_6(COOH)_2$ (suberic acid) (Table 6).

The phenolic content ranged from 6773 nM to 1334 nM in PC and from 1124 nM to 76 nM in CC, with higher concentrations of all phenolic acids in the PC soil (Table 7). Most of the phenolic acids exhibited highest concentrations in the top soil horizons, with *p*-coumaric acid and ferulic acid occurring only in the L horizon at both sites. In contrast, protocatechuic acid was most abundant in the A horizon in the PC soil. Soil DOM had a very modest hormone-like activity at both sites (Table 8), except the F horizons. CC had only a GA-like activity, while PC showed both GA- and IAA-like activities. Gallic and protocatechuic acids had an IAA-like activity, while *p*-hydroxybenzoic acid, vanillic acid, and syringic acid had a GA-like activity on silver fir seedlings (Table 9).

DISCUSSION

Soil DOM is controlled by several factors such as degree of mineralization, organic matter content, and pH (Ross and Barlett, 1996). The soils investigated had a DOC content of 0.280–0.002%, i.e., within the ranges reported in the literature (Burford and Bremner, 1975; Nambu and Yonebayashi, 1999). DOC content decreased with depth in both soils, the decline being fivefold greater at CC, the site

TABLE 6. LMW ALIPHATIC ACIDS DETECTED IN THE SOIL DOM OF THE TWO SITES BY GC/MS ANALYSIS (PC, SITE WITH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

Horizon	Site: PC	Site: CC
L	$C_5(COOH)_2$ (15.15)	$C_5(COOH)_2$ (15.12)
	$C_7(COOH)_2$ (20.04)	$C_7(COOH)_2$ (20.00)
	$C_{15}COOH$ (25.67)	$C_{15}COOH$ (25.67)
F	$C_7(COOH)_2$ (20.01)	$C_5(COOH)_2$ (15.07)
	$C_{14}COOH$ (22.68)	$C_6(COOH)_2$ (17.56)
	$C_{15}COOH$ (25.67)	$C_7(COOH)_2$ (19.96)
	$C_{17}COOH$ (27.73)	$C_{14}COOH$ (22.62)
		$C_{15}COOH$ (25.61)
		$C_{17}COOH$ (29.47)
A	$C_{14}COOH$ (23.61)	$C_{14}COOH$ (23.56)
	$C_{15}COOH$ (25.75)	$C_{15}COOH$ (25.65)
Bw	$C_{15}COOH$ (25.75)	n.d. n.d.

Note: The retention times of each organic acid isomer is reported in brackets; n.d. = not detected.

without fir regeneration, than at PC, the site with fir regeneration. This may be due to a different evolution of the soil organic matter. Soil organic matter undergoes rapid mineralization in the Eutric Cambisol (CC), giving vermimull as the humus form. In contrast, soil organic matter undergoes humification–immobilization in the Calcaric Cambisol (PC), as shown by the C : N ratio and the presence of mormoder humus. Moreover, the lower pH and higher DOC content in the F horizon at CC are in accordance with results of Ross and Barlett (1996), reporting a negative correlation between DOC and pH in a Spodosol. As regards the evolution of the organic matter, particularly mineralization, the high nitrate content in the F horizon at CC may be linked to the high microbial biomass, as also reported by Lipson et al. (1999) for an alpine ecosystem. Soil microbes act as allelochemical mediators (Pellissier and Souto, 1999) and exert important effects on key processes that affect ecosystem functioning and, ultimately, the structure of plant community (Wardle et al., 1998).

Among the various LMW organic acids, oxalic acid has been detected in different forest ecosystems (Pohlman and McCall, 1988). Furthermore, high concentrations of oxalic and citric acids have been detected in other Cambisols under beech forests (Tani and Higashi, 1999). Large amounts of citric acid in the top soil horizons of PC were probably released by fungi and plant roots in the litter layer (Huang and Violante, 1986). Our two soils also differed from each other with regard to the occurrence of pimelic and suberic acids (Table 6). Pimelic acid is an intermediate in biotin biosynthesis, both in microorganisms and plants (Baldet et al., 1993). It is noteworthy that pimelic acid has been found to exert a toxic

TABLE 7. CONCENTRATIONS OF PHENOLIC ACIDS IN THE SOIL DOM OF THE TWO SITES (PC, SITE WITH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

Horizon	gal (3.55) nM	proto (6.12) nM	gen (8.30) nM	pht (9.00) nM	syr (9.80) nM	*p*-hydro (10.40) nM	van (10.72) nM	caf (12.48) nM	*p*-coum (22.81) nM	fer (23.65) nM	Total nM
Site: PC											
L	240a	502b	532a	1588a	53b	1535a	418a	499b	1020a	386a	6773a
F	127b	451b	302b	612b	38c	897b	216b	373c	626b	n.d.	3642b
A	217a	1464a	85c	190c	48b	36g	18e	23g	n.d.	n.d.	2081c
Bw	n.d.	254c	9f	43f	32d	96e	65d	835a	n.d.	n.d.	1334d
Site: CC											
L	17e	158d	n.d.	n.d.	23e	269d	122c	139d	275c	121b	1124e
F	52d	66e	33d	121d	29d	605c	131c	113e	n.d.	n.d.	1150e
Ah	93c	52f	95c	86e	140a	79f	4f	121e	n.d.	n.d.	670f
Bw	1f	7g	14e	n.d.	n.d.	15h	4f	35f	n.d.	n.d.	76g

Note: gal, gallic; proto, protocatechuic; gen, gentisic; pht, phthalic; syr, syringic; *p*-hydro, *p*-hydroxybenzoic; van, vanillic; caf, caffeic; *p*-coum, *p*-coumaric; fer, ferulic acids. The retention times are reported in brackets. Values are the means of 5 samples, with standard errors always $\leq$5% of the mean. The means within each column followed by the same letter do not differ at $P = 0.05$ based on Student–Newman–Keuls tests; n.d. = not detected.

TABLE 8. HORMONE (IAA AND GA)-LIKE ACTIVITY IN THE SOIL DOM OF THE TWO SITES (PC, SITE WTH FIR RECRUITMENT; CC, SITE WITHOUT FIR RECRUITMENT)

Site	Horizon	IAA-like activity	GA-like activity
PC	L	2941d	n.d.
	F	13.51g	222d
	A	1000f	n.d.
	Bw	1250e	n.d.
CC	L	19608b	1000b
	F	21739a	256c
	Ah	9091c	1538a
	Bw	10000c	213d

Note: The data are concentrations (as mg C/1) of DOM which have a hormone-activity corresponding to 1 mg/l of the original hormones. The means within each column followed by the same letter do not differ at $P = 0.05$ based on Student–Newman–Keuls tests.

effect on crop species, while biotin does not affect plant metabolic processes even if it actively enters living cells (Watanabe et al., 1982). The presence of pimelic acid in the F horizon may have a detrimental effect on silver fir seedlings at CC. However, our data can neither support nor contrast the hypothesis that pimelic acid exerts allelopathic effects in the CC soil.

TABLE 9. EFFECT OF IAA, GA, AND SOME PHENOLIC ACIDS ON ROOT AND EPICOTYL LENGTH OF SILVER FIR SEEDLINGS

Treatment	Root (mm)	Treatment	Epicotyl (mm)
Control	30a	Control	21f
IAA (20 mg/l)	5d	GA (10 mg/l)	27a
gal (1 μM)	18c		
proto (1 μM)	20b	gen (1 μM)	24c
—		*p*-hydro (10 μM)	25b
—		van (1 μM)	24c
		fer (1 μM)	23d
—		syr (0.1 μM)	22e

Note: gal, gallic acid; pyro, pyrogallic acid; proto, protocatechuic acid; gen, gentisic acid, p-hydro, p-hydroxybenzoic acid; van, vanillic acid, fer, ferulic acid; syr, syringic acid. Values followed by the same letter in a given column are not statistically different at $P = 0.01$, using the Student–Newman–Keuls test (Sokal and Rohlf, 1969).

The regeneration of natural forests can be affected by phenolics released into the soil either by trees or by understory plants (Mallik and Pellissier, 2000; Nilsson et al., 2000; Souto et al., 2000). In our soils, concentrations of phenolic acids were lower when compared with those found in silver fir forest soils in a nearby region (Pizzeghello et al., in press). In particular, the low level of phenolic acids at CC may be related to leaching of phenolics during senescence of spruce needles (Gallet and Lebreton, 1995), as well as to microbial or chemical degradation of phenolics and/or sorption onto soil particles (Inderjit and Mallik, 1999).

The role of phenolic acids in different ecosystems has been the object of intensive studies, with special focus on their phytotoxic action (Inderjit, 1996). At concentrations of 0.1–1 mM, many phenolic compounds are toxic to plants, especially to seedlings. At concentrations of 0.1 μM–0.1 mM, phytotoxic effects are less apparent and the growth of some plant species may even be enhanced (Rice, 1984). Hrubcová et al. (2000) found that phenolic compounds can stimulate plant growth by regulating the activity of IAA oxidase. Our data showed that gallic and protocatechuic acids could account for the IAA-like activity of soil DOM, while *p*-hydroxybenzoic acid could account for GA-like activity. The high content of *p*-hydroxybenzoic acid at both sites may indicate recalcitrance (Baziramakenga et al., 1995). Conversely, the lower content in procatechuic and caffeic acids is probably due to their higher degradability (Gallet and Pellissier, 1997). Overall, the phenolic acids detected at both sites are widespread in the plant kingdom, none of them being specific to silver fir (Inderjit and Mallik, 2002). The hormone-like activity of soil DOM, although much lower than that of pure humic substances (Pizzeghello et al., 2001), was appreciable, probably because the organic carbon in soil DOM is more promptly available to plants compared to that in humus.

In conclusion, it is difficult to demonstrate what was the main cause accounting for the failure of silver fir regeneration at our CC site. A complex interplay of various mechanisms probably played a role with this respect (Inderjit and Mallik, 2002). However, we stress that, among the various factors, the hormone-like activity of DOM is likely to play an important role in enhancing regeneration of silver fir.

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TRADE-OFF BETWEEN SYNTHESIS OF SALICYLATES AND GROWTH OF MICROPROPAGATED *Salix pentandra*

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Abstract—We studied the relationship between biosynthesis of salicylates, the main chemical defenses in willow and growth of *Salix pentandra* by cultivating plants in the presence of 2-aminoindan-2-phosphonic acid (AIP), a powerful inhibitor of phenylalanine ammonia-lyase (PAL: EC 4.3.1.5.). AIP inhibited efficiently, though not totally, the endogenous synthesis of salicylates. This inhibition markedly increased plant growth. Exogenous application of the precursors of salicylates, benzoic acid (BA), salicylic acid (SA), and helicin, increased the levels of several individual salicylates, but decreased the growth of plants cultivated in the presence of AIP. These results suggest a trade-off between plant growth and the synthesis of salicylates in *S. pentandra*. Phenylalanine, which accumulated in response to inhibitor treatment, but was decreased by precursor treatments, may be a common and limiting precursor for both plant growth and salicylate synthesis. The biosynthesis of salicin is suggested to proceed mainly via benzoyl-glucose, an intermediate in the synthesis of salicylic acid. Salicin is the most obvious precursor of more substituted salicylates, salicortin, acetylsalicortin, and tremulacin. In addition, we found that the salicylate pools of mature plant parts of *S. pentandra* were not subject to turnover, implying that the maintenance of salicylates does not demand high resources of plants, although their initial construction is costly.

Key Words—Salicylates, biosynthesis, PAL, phenolic glycosides, defense, herbivory, trade-off, willow, *Salix*.

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INTRODUCTION

Many secondary compounds have important defensive roles in plants and are effective in reducing the damage to plant foliage inflicted by a variety of plant-eating animals. However, the cost to the plant of producing and maintaning these defensive compounds may be substantial (Gershenzon, 1994). Resources used for defense are not available for plant growth and reproduction; this may lower their competitive ability, especially in resource-rich environments (Coley et al., 1985; Herms and Mattson, 1992), Under herbivore pressure, investments in defense may increase plant fitness compared to less well defended individuals (Coley et al., 1985). for example, the induced synthesis of nicotine in *Nicotiana* spp. has been shown to reduce both growth and the competitive ability of plants in the absence of herbivores (Baldwin and Hamilton, 2000), but to benefit plants under herbivore pressure (Baldwin, 1998). Natural selection favors the production of defensive compounds if the cost of defense is lower than the benefit (Coley et al., 1985).

Because of a trade-off between defense and growth, it has been claimed that inherently fast-growing plants in resource-rich environments are more weakly defended than inherently slow-growing plants inhabiting resource-poor environments (Coley et al., 1985). This genetic trade-off was observed in two Salicaceous species, *Populus tremuloides* (Hwang and Lindroth, 1997) and *Salix sericea* (Nichols-Orians et al., 1993)-inherently slow-growing clones contained higher levels of salicylates than inherently fast-growing clones and *vice versa*. In addition, fertilization increased growth, but reduced the level of secondary compounds in willows and birches (e.g., Hakulinen et al., 1995; Ruohomäki et al., 1996; Hakulinen, 1998; Haukioja et al., 1998). In spite of many correlative results, there is still little direct evidence for an actual trade-off (Herms and Mattson, 1992).

The Northern Hemisphere is inhabited by a high diversity of willow species, providing food for a great variety of herbivores. Salicaceae are characterized by specific phenolic glycosides, salicylates, that are the major defensive compounds found in *Salix* and *Populus* species. These compounds deter the feeding of a variety of generalist herbivores (e.g., Tahvanainen et al., 1985a,b; Lindroth et al., 1988; Lindroth and Peterson, 1988; Ruuhola et al., 2001a). Many northern willows, such as *Salix pentandra*, are fast-growing, early successional species that grow in marshy fields and on the banks of ditches and rivers that are often rich in nutrients.

The metabolic cost to synthesize a defensive compound is determined by the structure, stability, and concentration of the compound in plant tissues (Skogsmyr and Fagerström, 1992; Gershenzon, 1994) and by the length of the biosynthetic pathway (Gershenzon, 1994). Salicylates, as carbon-based compounds, are thought to be inexpensive compounds to produce, especially when nitrogen limits plant growth and the overflow carbon can be used for production of carbon-based chemicals (Bryant et al., 1983). In contrast to the carbon–nutrient balance (CNB) hypothesis (Bryant et al., 1983) the protein competition model (PCM) states that nitrogen

needed for the synthesis of phenylalanine (Phe), a precursor of many phenolics, is the limiting factor for both plant growth and the synthesis of phenolics (Jones and Hartley, 1999).

In the leaves of certain willow species, such as *S. pentandra* and *S. myrsinifolia*, the concentration of salicylates can be high (Julkunen-Tiitto, 1989; Rank et al., 1998; Ruuhola and Julkunen-Tiitto, 2000; Ruuhola et al., 2001a,b), and the biosynthesis of salicylates may be demanding in terms of plant resources. Moreover, phenolic glycosides, such as salicylates, are thought to be dynamic compounds that undergo marked metabolic turnover (Reichardt et al., 1991; Basey and Jenkins, 1993); this would further increase the metabolic cost of defense (Gershenzon, 1994).

Salicin, the most common salicylate found in northern willows (Julkunen-Tiitto, 1989), is synthesized from Phe via the shikimate pathway (Zenk, 1967). Two pathways have been suggested: (1) *t*-cinnamic acid → *o*-coumaric acid → *o*-coumaryl-CoA → salicyloyl-CoA → salicylaldehyde → helicin → salicin; and (2) BA → benzylalcohol → benzaldehyde → salicylaldehyde → helicin → salicin (Zenk, 1967). In addition, the biosynthesis of salicylic acid (SA), an important mediator of systemic acquired resistance (SAR) and hypersensitive response (HR), has been shown to occur either via benzoic acid (BA) or *o*-coumaric acid (El-Basyouni et al., 1964; Ellis and Amrhein, 1971; Chadha and Brown, 1974). Surprisingly, the biosynthesis of the salicylates most efficient in defense against generalist herbivores, i.e., salicortin, acetylsalicortin, and tremulacin (Tahvanainen et al., 1985a,b; Lindroth et al., 1988; Lindroth and Peterson, 1988; Clausen et al., 1989), have not been investigated.

The purpose of this study was to examine the biosynthesis of salicylates and their relationship to the growth of willows. The biosynthesis of salicylates was studied by applying potential precursors, BA, SA, helicin, and salicin, to micropropagated *S. pentandra* L. plants cultivated in the presence of 2- aminoindan-2-phosphonic acid (AIP; Zón and Amrhein, 1992), an inhibitor of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5). PAL catalyzes the first reaction in phenylpropanoid metabolism, an elimination of ammonia from Phe forming *t*-cinnamic acid (Strack, 1997).

METHODS AND MATERIALS

Experimental Procedure. S. pentandra (clone RM 1) was micropropagated using axillary shoot multiplication. The plants were cultivated for 3 weeks in solidified woody-plant medium (Lloyd and McCown, 1980) with 3% sucrose content; no plant growth regulators were added. After precultivation, plants were transferred aseptically into culture vessels (five plants per vessel) on cotton pads moistened with liquid woody-plant medium.

The stem segment above the first mature leaf was marked in each plant so that newly produced shoot parts could be separated from mature shoot parts (see Ruuhola and Julkunen-Tiitto, 2000). Four putative precursors of salicylates, salicin, helicin, BA, and SA, were added at a concentration of 2.5 mM into the liquid medium containing 30 μM AIP. After transfer into the liquid woody-plant medium for six different treatments ($-$ALP control, $+$AIP control, $+$AIP $+$ salicin, $+$AIP $+$ BA, $+$ALP $+$ helicin, and $+$AIP $+$ SA), plants were cultivated for an additional 17 days . Control plants ($+$AlP) had no precursors added to them. The effect of AIP treatment was controlled with plants cultivated in the absence of the inhibitor ($-$AIP).

Plants were split into shoots and roots. Shoots were split into old shoots (below the mark) and new shoots (above the mark). Mature leaves from old shoots were separated from the stems and petioles. New shoot parts were split into shoot tips, containing two to three unfurled leaves, and intermediate segments. Roots and intermediate segments were dried at room temperature and extracted following the method of Ruuhola and Julkunen-Tiitto (2000). Other parts were extracted in 100% MeOH immediately after cutting and weighting (see Ruuhola and Julkunen-Tiitto, 2000). Plants from one culture vessel were pooled and analyzed together. Each treatment had six replicates. One plant per vessel was used for determination of dry weights ($+105^{\circ}$C, overnight).

HPLC and MS-HPLC Analyses. Samples were analyzed by high performance liquid chromatography, HPLC (Hewlett-Packard, Palo Alto, CA) and HPLC/API-ES mass spectrometer (HP 1100 Series LC/MSD, Hewlett-Packard, Palo Alto, CA). The device, elution gradient, and conditions for HPLC were described in Ruuhola et al. (2001b), and those for MS-HPLC as in Julkunen-Tiitto and Sorsa (2001). The fragmentation voltages used for mass spectra were 60, 120, and 200 V.

Hydrolysis of BA- and SA-Glucosides by Almond β-Glucosidase. Root samples of the BA- and SA-treated plants were hydrolyzed with almond β-glucosidase. The reaction mixture consisted of 5 U of enzyme (7 U/mg protein, Sigma) per 0.1 μmol glucoside and 0.1 M potassium buffer (pH 5.0). The incubation time was 15 min at $+37^{\circ}$C, after which the samples were analyzed by HPLC.

Calculations and Statistics. Salicylate concentrations in whole shoots were calculated as follows. The amounts of individual salicylates in stems, leaves, intermediate segments, and shoot tips were summed as were the dry weights of these plant parts. The total amounts of individual compounds found in whole shoots were divided by the dry weights of whole shoots. Salicylate concentrations in new shoots were calculated by summing the amounts found in the shoot tips and intermediate segments, and dividing by the combined mass of those shoot parts.

The biomass of whole shoots, roots, and plants, the growth of new shoots, and the shoot/root ratio were analyzed by one-way ANOVA followed by Dunnet's test for multiple comparisons of $+$AIP controls with other treatments (SPSS). The concentrations of salicylates and Phe found in the stems, leaves, shoot tips, and

whole shoots were tested similarly. The concentrations of secondary compounds found in roots were tested by the Kruskall–Wallis test (SPSS) followed by non-parametric comparisons of of a control (+AIP) to the other groups (Zar, 1999). Correlations between growth and concentrations of salicylates and cholorogenic acid were tested by Pearson's correlation test (SPSS).

RESULTS

Phenolic Compounds in Micropropagated S. pentandra. The main identified phenolic compounds in micropropagated *S. pentandra* plants were salicylates. The plants also contained substantial amounts of chlorogenic acid, but the amounts of cinnamic acid derivatives and flavonoids remained near or below detectable limits. The concentrations of condensed tannins were negligible in shoots, and low in roots.

In addition to salicin, salicortin, and acetylated salicylates, *S. pentandra* clone RM1 also contained tremulacin, which has not been found in *S. pentundra* in earlier studies (Julkunen-Tiitto, 1989; Julkunen-Tiitto et al., 1995; Rank et al., 1998; Ruuhola et al., 2001a). We also found two novel derivatives of 2′-*O*-acetylsalicortin that had spectra identical to 2′-*O*-acetylsalicortin, but with longer retention times. These compounds had putative molecular ion peaks at m/z 531 $[M+Na]^+$ and at m/z 627 $[M+Na]^+$. The first compound, tentatively 2′,6′- *O*-diacetylsalicortin, contains two acetyl groups attached to the glucose moiety. Fragmentation of the compound released hydroxycyclohexenone saligenin ester (262 Da), producing a positive fragment ion at m/z 269; this represents the molecular weight of the diacetylated glucose moiety. The mass spectrum also contained a fragment ion at m/z 179, representing the molecular weight of the hydroxycyclohexenone carboxylic acid.

The second novel compound is believed to contain two 1-hydroxy-6-oxo- 2-cyclohexen-1-carbonyl moieties, one moiety attached to the aglycone part of glucoside and the other to the sugar moiety. The compound was named acetylhydroxycyclohexenone salicortin. Fragmentation of this compound released cyclohexenone saligenin ester (262 Da), producing a fragment ion at m/z 365; this represents the molecular weight of the acetylated glucose moiety esterified with a 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety. The mass spectrum also contained a fragment ion at m/z 489, representing the molecular weight of acetylsalicortin, and a fragment ion of the hydroxycyclohexenone carboxylic acid at m/z 179.

No free SA or BA was detected in roots or other parts of the SA- or BA-treated plants grown in the presence of AIP. However, two compounds with spectra resembling those of free SA and BA, but with longer retention times, were detected in the roots at marked levels.

The mass spectrum of the BA derivative contained a molecular ion peak at m/z 307 $[M+Na]^+$, representing the molecular mass of benzoyl-glucose. Fragmentation of the compound released free BA (122 Da), producing a fragment ion of the glucose moiety at m/z 185. The SA derivative contained a molecular ion peak at m/z 323 $[M+Na]^+$, representing the molecular mass of SA-glucoside, and a fragment ion at m/z 139, representing the molecular mass of free SA. Hydrolysis of these putative glucosides with almond β-glucosidase released free BA and SA, confirming their identification.

Willow Growth and Salicylate Concentrations Were Inversely Related. The dry weights of new shoots from the +AIP control treatment were significantly higher than the dry weights of new shoots from the −AIP control treatment (Figure 1a), indicating enhanced plant growth when the synthesis of phenolics was inhibited. The growth of inhibitor-treated plants, however, was significantly reduced by BA, SA, and helicin treatments (Figure 1a). In the case of SA, growth was totally inhibited; salicin, on the other hand, had no effect on plant growth.

The dry weight of shoots, root, and whole plants also differed among treatments (Table 1). AlP treatment tended to increase the dry weights of shoots, while application of SA and BA to inhibitor-treated plants reduced the dry weight (Figure 1b). Interestingly, AIP treatment increased the dry weight of roots, whereas BA, SA, and helicin added to +AIP plants decreased it (Figure 1c). Because of enhanced root growth, +AIP controls had lower shoot/root ratios than did −AIP plants (Figure Id). Salicin-treated plants also had an abnormal shoot/root ratio, but BA, SA, and helicin treatments increased the shoot/root ratio to a level close to that found in the −AIP controls. +AIP control plants had higher whole plant biomass than did −AIP controls. The biomass of SA- and BA-treated plants was lower than that of +AIP control plants (Figure 1e).

New shoots developed in the presence of PAL inhibitor contained low levels of salicylates, and the growth of these shoots was enhanced. We found a clear negative correlation between the total concentration of salicylates and the biomass of new shoots (Figure 2a). The correlation between the concentration of chlorogenic acid and the biomass of new shoots, on the other hand, was nonsignificant (Figure 2b).

Biosynthesis of Salicylates. In shoot tips of +AIP control plants, the concentration of salicylates was low (Figure 3a–g). The shoot tips, in contrast contained high level of free Phe (Figure 3h), a substrate of PAL. This accumulation, however, was reduced by BA and helicin treatments (Figure 3h). The −AIP controls did not contain detectable amounts of Phe in any organs.

The concentrations of all salicylates in shoot tips differed significantly between treatments (Table 2). The concentrations of salicin and 2′-*O*-acetylsalicin were increased by BA treatment, and those of diglucoside of salicin by the helicin treatment (Figure 3a–c), while the level of acetylhydroxycyclohexenone salicortin was decreased by the salicin treatment (Figure 3f). However, the total

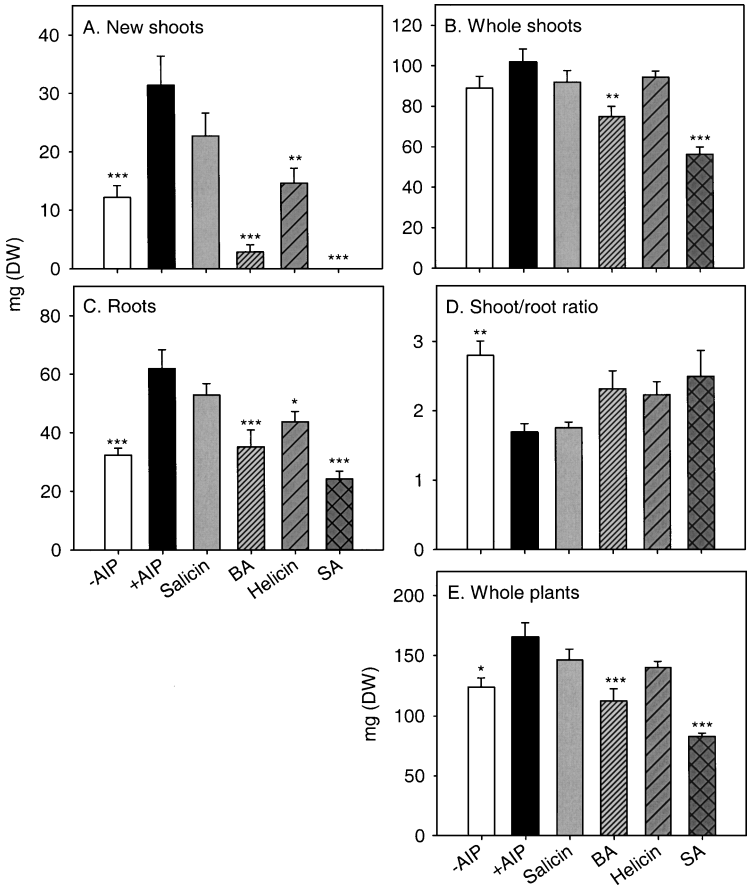

FIG. 1. Effect of different treatments on the dry weight of new shoots (A), whole shoots (B), roots (C), shoot/root ratio (D), and dry weights of whole plants (E). Data tested by ANOVA followed by Dunnet's test for comparing a control (+AIP) with other treatments (SPSS). Statistically significant cases are marked with asterisks: $^{*} \leq 0.05$, $^{**} \leq 0.01$, $^{***} \leq 0.001$. Error bars represent SE.

concentration of salicylates did not change significantly (Figure 3g). The shoot tips of SA-treated plants were excluded, since unlike the plants in other treatments, they did not produce new shoot tips during the AIP treatment. Salicortin and tremulacin remained below detection limits in the shoot tips.

In mature leaves, significant differences between treatments in the concentrations of salicin, diglucoside of salicin, salicortin, 2′-*O*-acetylsalicortin, total salicylates, and Phe were found by analysis of variance (Table 2). Application of

TABLE 1. ANOVA TABLE OF THE EFFECTS OF DIFFERENT TREATMENTS ON PLANT DRY WEIGHT

Source of variation	*df*	MS	*F*	*P*
New shoots				
Bg	5	700.521	13.460	<0.001
Wg	30	52.045		
Whole shoots				
Bg	5	1644.251	10.889	<0.001
Wg	30	151.003		
Roots				
Bg	5	1166.517	10.294	<0.001
Wg	30	113.322		
Whole plants				
Bg	5	0.354	15.590	<0.001
Wg	30	0.268E-02		
Shoot/root ratio				
Bg	5	1.093	3.576	0.012
Wg	30	0.306		

Note: Bg = between groups; Wg = within groups. Whole plant data were ln-transformed prior to the analyses.

helicin to AIP-treated plants increased the concentrations of salicin (Figure 4a), diglucoside of salicin (Figure 4b), 2′-*O*-acetylsalicortin (Figure 4e), and total salicylates (Figure 4i). The BA treatment increased the concentrations of diglucoside of salicin (Figure 4b) and salicortin (Figure 4d).

AIP treatment caused accumulation of Phe in mature leaves, (Figure 4j). Precursor treatments did not affect the accumulation of Phe (Figure 4j). Inhibitor treatment had no effect on the concentrations of any salicylates in the mature leaves of +AIP controls compared to concentrations observed in the −AIP controls

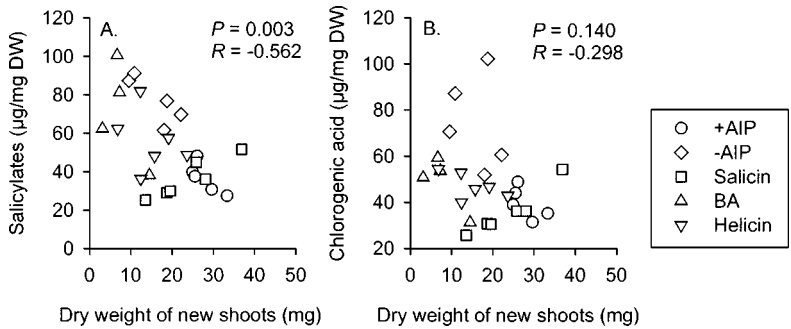

FIG. 2. Correlation between dry weight of new shoots and concentration of salicylates (A) and chlorogenic acid (B). Data tested by Pearson's correlation test (SPSS).

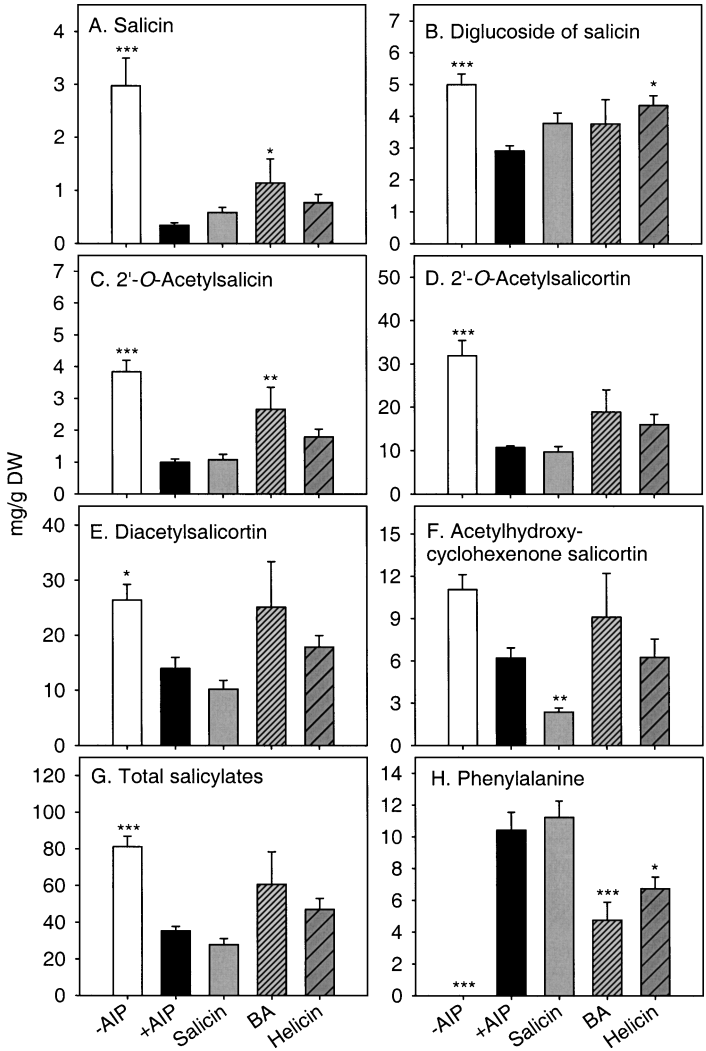

FIG. 3. Effects of differents treatments on the concentration of salicylates and Phe in shoot tips of *S. pentandra*. Precursors were applied to plants grown in the presence of 30 μM AIP. Data tested by ANOVA, followed by Dunnet's test for comparing a control (+AIP) with other treatments (SPSS). Cases marked with asterisks were significant: $^{*} \leq 0.05$, $^{**} \leq 0.01$, $^{***} \leq 0.001$. Error bars represent SE.

TABLE 2. ANOVA TABLE OF THE EFFECT OF DIFFERENT TREATMENTS ON THE CONCENTRATION OF SALICYLATES AND PHE IN SHOOT TIPS, MATURE LEAVES AND STEMS

	Shoot tips				Mature leaves				Stems			
Source of variation	*df*	MS	*F*	*P*	*df*	MS	*F*	*P*	*df*	MS	*F*	*P*
Salicin												
Bg	4	3.733	13.324	<0.001	5	3.151	2.793	0.035	5	1.173	11.09	<0.001
Wg	23	0.280			30	1.128			30	0.106		
Diglucoside												
Bg	4	3.520	4.175	0.006	5	19.42	7.940	<0.001	5	1.037	4.537	0.003
Wg	23	0.747			30	2.446			30	0.229		
Acetylsalicin												
Bg	4	8.384	15.74	<0.001	5	0.563	1.959	0.114	5	0.570	4.570	0.003
Wg	23	0.533			30	0.288			30	0.125		
Salicortin												
Bg	—	—			5	4.799	8.766	<0.001	5	9.975	11.33	<0.001
Wg	—	—			30	0.547			30	0.881		
Acetylsalicortin												
Bg	4	1.292	9.936	<0.001	5	241.0	2.914	0.029	5	11.32	0.744	0.597
Wg	23	0.130			30	82.71			30	15.21		
Diacetylsalicortin												
Bg	4	270.8	4.503	0.008	5	32.69	1.348	0.272	5	0.294	0.838	0.534
Wg	23	60.15			30	24.25			30	0.351		
Acetyl-HCH-salicortin												
Bg	4	1.956	10.94	<0.001	5	27.20	1.742	0.156	5	10.22	5.150	0.002
Wg	23	0.179			30	15.61			30	1.984		
Tremulacin												
Bg	—	—			5	4.358	2.248	0.075	5	4.082	29.68	<0.001
Wg	—	—			30	1.938			30	0.138		

Total salicylates											
Bg	4	1.021	8.701	<0.001	5	1279	2.810	0.034	5	0.046	1.263	0.305
Wg	23	0.117			30	455.3			30	0.037		
Phe												
Bg	4	122.6	27.94	<0.001	5	36.20	11.540	<0.000	5	76.69	76.69	<0.001
Wg	23	4.389			30	3.220			30	1.000		

Note: Bg = between groups; Wg = within groups; — = not detected.

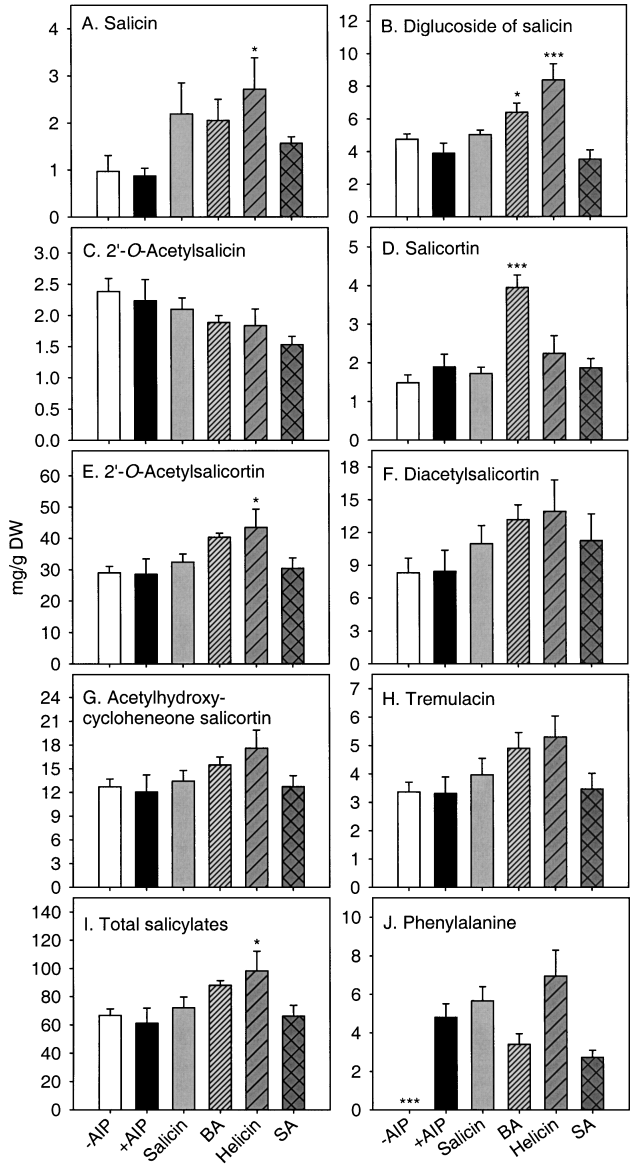

FIG. 4. Effect of different treatments on the concentration of salicylate and Phe in mature leaves of *S. pentandra*. Precursors were applied to plants grown in the presence of 30 μM AIP. Data tested by ANOVA, followed by Dunnet's test for comparing a control (+AIP) with other treatments (SPSS). Cases marked with asterisks were significant: $^{*} \leq 0.05$, $^{**} \leq 0.01$, $^{***} \leq 0.001$. Error bars represent SE.

(Figure 4a–i), suggesting that turnover did not occur in the salicylate pools of mature leaves.

In stems, the concentrations of all other compounds except those of 2′-*O*-acetylsalicortin, diacetylsalicortin, and total salicylates differed among treatments (Table 2). All four precursor treatments increased the concentration of salicin (Figure 5a). The concentration of the diglucoside of salicin was increased by both salicin and helicin treatments (Figure 5b), those of 2′-*O*-acetylsalicin and salicortin by the BA and SA treatments (Figures 5c and d), and that of tremulacin by the BA, helicin, and SA treatments (Figure 5h). Despite the increase in concentrations of individual salicylates, the total level of salicylates remained unchanged in all treatments (Figure 5i). Phe accumulated markedly in the stems of +AIP controls, while BA, SA, and helicin treatments reduced this accumulation (Figure 5j). Salicylate content of mature stems of +AIP plants did not differ from −AIP plants (Figure 5a–i).

Root of both −AIP and +AIP control plants did not contain any salicylates, but the roots of precursor-treated plants contained marked amounts of these compounds (Figure 6). Salicin was detected in the roots of all four precursor-treated plants (Figure 6a), and the diglucoside of salicin was present in the roots of salicin-, helicin-, and BA-treated plants (Figure 6b). Salicortin and 2′-*O*-acetysalicortin were detected only in the roots of BA-treated plants (Figure 6c and d). Phe accumulated in the roots of AIP-treated plants, but precursor treatments attenuated this accumulation (Figure 6f). Exogenously applied salicin (Figure 7a) and helicin (Figure 7b) were converted into isosalicin, which was detected at marked levels in roots, but not in other plant parts. Helicin was not found in any plant part, suggesting the rapid metabolism of this compound. BA and SA were converted in to their respective glucosides in the roots, where their levels were considerable. These glycosides, however, were present in low concentrations in the shoot tissues (Figure 7c and d).

DISCUSSION

Inhibition of Phenolic Synthesis Led to Accumulation of Phenylalanine. The endogenous synthesis of salicylates was inhibited efficiently, though not completely by AlP treatment; this was seen in the low level of salicylates in new shoot parts developed in the presence of inhibitor. In our earlier study, biosynthesis of salicylates in *Salix myrsinifolia* was also inhibited efficiently by AIP treatment (Ruuhola and Julkunen-Tiitto, 2000). In both studies, AIP treatment caused a marked accumulation of Phe, confirming the efficient inhibition of PAL. This accumulation also suggests the absence of a feedback control in the synthesis of Phe in willows. A similar result was obtained with buckwheat hypocotyls, where another PAL-inhibitor, L-AOPP, caused a marked accumulation of Phe (Holländer et al., 1979).

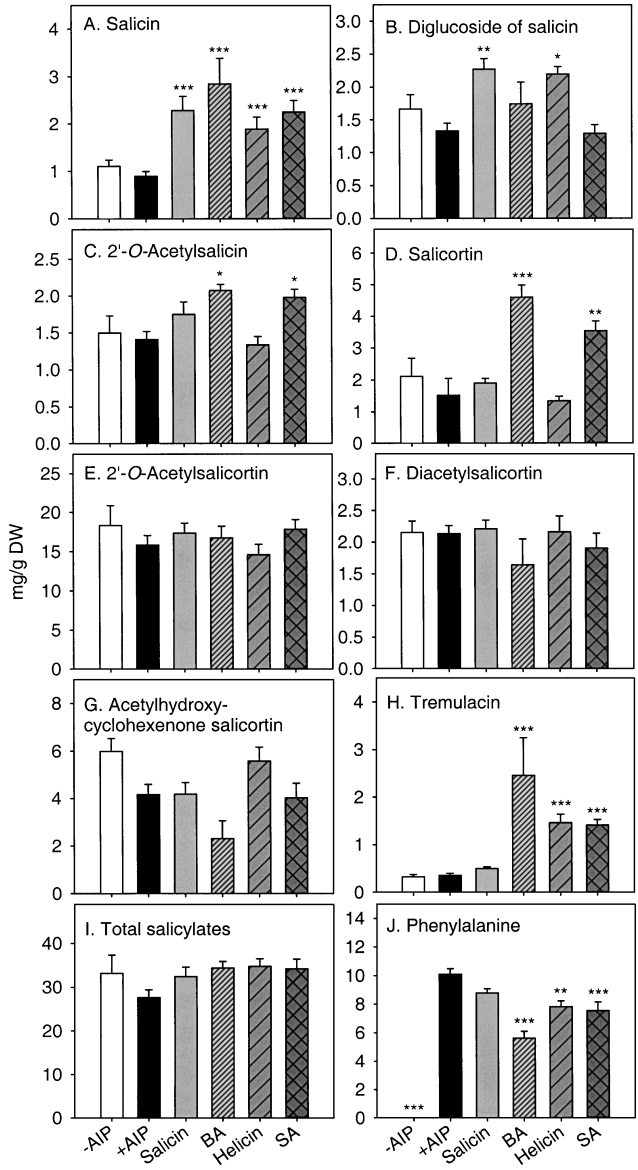

FIG. 5. Effect of different treatments on the concentration of salicylate and Phe in stems of *S. penkmiru*. Precursors were applied to plants grown in presence of 30 μM AIP. Data tested by ANOVA, followed by Dunnet's test for comparing a control (+AIP) with other treatments (SPSS). Cases marked with asterisks were significant: $^{*} \leq 0.05$, $^{**} \leq 0.01$, $^{***} \leq 0.001$. Error bars represent SE.

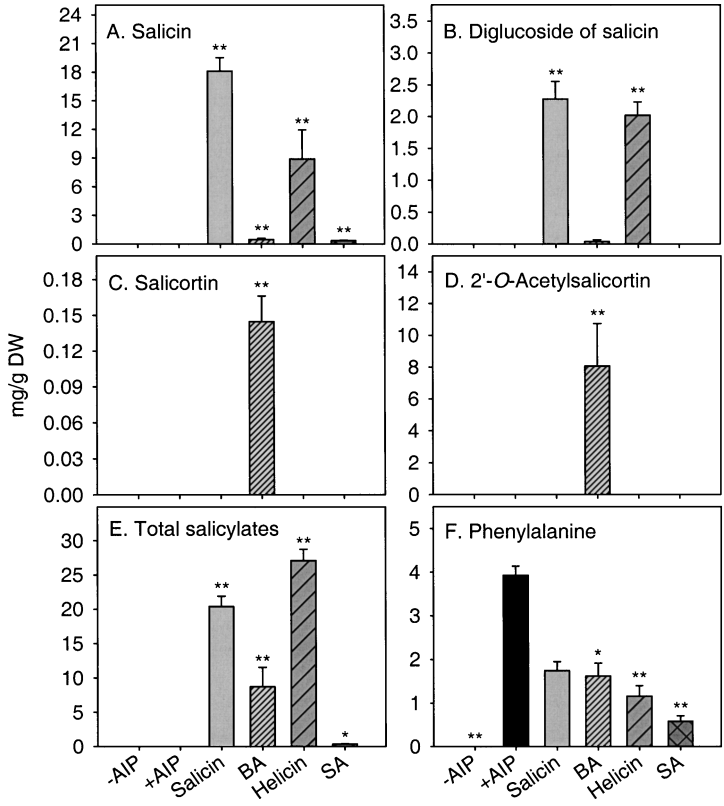

FIG. 6. Effect of different treatments on concentration of salicylate and Phe in roots of *S. pentundra*. Precursors were applied to plants grown in presence of 30 μM AIP. Data tested by the Kruskal–Wallis test (SPSS) followed by nonparametric comparisons of a control (+AIP) with other treatments (Zar, 1999). Cases marked with were significant: $^{*} \leq 0.05$, $^{**} \leq 0.01$, $^{***} \leq 0.001$. Error bars represent SE.

Application of BA, SA, and helicin decreased the accumulation of Phe, suggesting that these small phenolics may act as down-regulators of the shikimate pathway. Metabolic pathways are often controlled by feedback inhibition, whereby accumulating intermediates or end products down-regulate their own synthesis. For example, exogenously applied *t*-cinnamic acid down-regulates PAL-activity by reducing the transcription of PAL-encoding genes in bean (Mavandad et al., 1990).

Trade-Off between Salicylate Synthesis and Plant Growth. Inhibition of the endogenous synthesis of salicylates, the main secondary compounds of micropropagated *S. pentandra*, was associated with a marked increase in plant biomass,

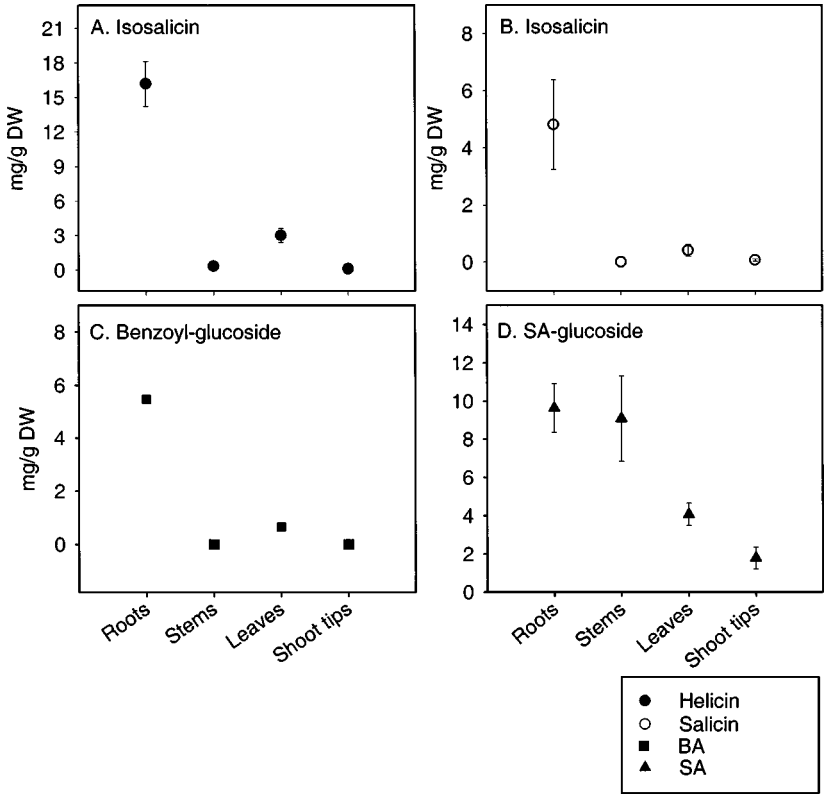

FIG. 7. Concentration of isosalicin (A,B), benzoyl-glucose (C), and SA-glucoside (D) in different plant parts of precursor treated plants. Error bars represent SE.

suggesting a trade-off between salicylate synthesis and plant growth. Interestingly, AIP treatment enhanced the allocation of resources to roots rather than to shoots, leading to a reduced shoot/root ratio in the +AIP controls.

Application of exogenous precursors of salicylates to AIP-treated plants increased the concentration of salicylates and, concomitantly, decreased the biomass of plants. SA treatment totally inhibited the growth of willows. SA may either induce or promote plant cell growth, depending on the context (Vanacker et al., 2001). Induced defense against pathogens by benzathiadiazole, which mimics the function of SA, reduced growth and fruit set of *Tritium aestivum*, suggesting a trade-off between defense and growth and reproduction (Heil et al., 2000). Thus, growth of SA-treated plants might be reduced because of induced defense reactions. However, SA was readily metabolized in roots, i.e., no free SA was detected in any plant parts. However, the glucosylation of SA and also of BA may be costly

for photomixotrophic plants, since the glucose consumed is not available for the synthesis of amino acids and growth of plants.

The enhanced growth of +AIP controls could be explained by the accumulation of Phe, not detected in the smaller −AIP control plants. In addition, both the accumulation of Phe and the growth of plants were repressed by precursor treatments, further suggesting that Phe may be a growth-limiting factor. However, compared to the excessive accumulation of Phe in +AIP control plants, growth was promoted only slightly, indicating that Phe is not the only growth-limiting factor.

Synthesis of proteins and phenolics, is claimed to compete for a common and limiting resource, Phe (Haukioja et al., 1998; Jones and Hartley, 1999). However, protein synthesis and plant growth are believed to be prioritized over the synthesis of phenolics in the allocation of resources (Bryant et al., 1983; Herms and Mattson, 1992; Jones and Hartley, 1999). Thus, inhibition of phenolic synthesis should not lead to enhanced growth of plants. On the other hand, excessive accumulation of Phe predicts that a markedly high amount of Phe will be used for the synthesis of phenolics. This is consistent with the results of Hall and Yeoman (1991), who showed that proteins were relatively minor sinks for Phe; the majority of labeled Phe ends up in the cell wall in covalently bound phenolics.

Salicylate-rich willows, such as *S. pentandra* and *S. myrsinifolia*, grow rapidly and produce high concentrations of salicylates in their growing shoot tips in spite of the apparent trade-off. Also, *S. myrsinifolia* increases synthesis of salicylates in expanding leaves as a response to herbivory (Ruuhola et al., 2001b). These results suggest that synthesis of proteins and phenolics is not necessarily inversely related (see Herms and Mattson, 1992; Jones and Hartley, 1999). On the contrary, these willow species seem to synthesize both phenolics and proteins simultaneously, similar to mountain birch (Riipi et al., 2002).

Phenolic glycosides such as salicylates are considered to be mobile defenses that have low construction costs, but high maintenance costs due to their rapid metabolic turnover (Coley et al., 1985). However, our previous findings (Ruuhola and Julkunen-Tiitto, 2000) and the present results both show that the salicylate pools in mature leaves and stems are not subject to marked turnover, suggesting low maintenance costs. It is the synthesis of salicylates that seems to be costly. This is seen as a trade-off between salicylate synthesis and plant growth. Thus, salicylates should be considered as immobile defenses, with high construction and low maintenance costs (see Coley et al., 1985).

Exogenously Supplied Precursors Did Not Restore Salicylate Content in New Shoot Tips. Exogenously supplied precursors failed to restore salicylate concentrations in new shoot tips of AIP-treated plants to the level found in −AIP controls. Coquoz et al. (1998) found that BA and *t*-cinnamic acid supplied to AIP-treated potato plants did not increase SA concentrations to the level observed in control plants. They suggested that parts of these precursors were used in the biosynthesis

of lignin. However, SA, salicin, and helicin are not precursors of lignin; thus, lignification does not explain the failure to restore the salicylate concentration by exogenous precursors in our study. However, impaired lignification in AIP-treated plants may cause problems in translocation of the precursors. Amrhein et al. (1983) showed that xylem vessels of mung bean seedlings grown in the presence of another PAL inhibitor, L-AOPP, collapsed because of impaired lignification.

Biosynthesis of Salicin May Proceed via Benzoyl- and SA-Glucoside. BA, SA, and helicin all acted as precursors of salicin by markedly increasing salicin concentrations, especially in *S. pentandra* roots and stems. BA is a more efficient precursor than *t*-cinnamic or *o*-coumaric acids (data not shown). BA also seems to be a more important precursor of SA than *o*-coumaric acid (Klämbt, 1962; Yalpani et al., 1993; Léon et al., 1993, 1995; Coquoz et al., 1998). BA is synthesized from *t*-cinnamic acid. Several pathways have been proposed for the biosynthesis including: an oxidative, CoA-dependent pathway that proceeds via 3-hydroxy-3-phenylpropanoic acid (Jarvis et al., 2000), and a non-oxidative pathway via benzaldehyde (Schnitzler et al., 1992). As a third alternative it has been suggested that phenylpropanoid glucose esters may act as activated intermediates in side chain shortening (Funk and Brodelius, 1990).

In the roots of *S. pentandra*, both BA and SA were converted to their respective glucosides, benzoyl-glucose and SA-glucoside, as found in several earlier studies (Klämbt, 1962; Yalpani et al., 1990, 1993; Malamy et al., 1992; Enyedi and Raskin, 1993; Thulke and Conrath, 1998; Chong et al., 2001). In addition, Yalpani et al. (1990) found SA-UDP-glucosyl transferase activity in oat roots, which was responsible for detoxifying the high level of exogenously supplied SA.

In our experiment, benzoyl-glucose was rapidly metabolized, only low amounts of the compound were detected in shoot tissues. Concomitantly the level of salicylates increased in shoots, suggesting that benzoyl-glucose was converted into salicylates. Unlike control roots, the roots of BA-treated plants contained salicylates, confirming that BA is indeed a precursor of salicylates. In BA-fed roots, a compound with a spectrum similar to SA-glucoside was detected, but the retention time of this compound was slightly longer. The glucose group of the compound may be attached to the carboxylic group rather than to the hydroxyl group of 2-C as in SA- glucoside. This change in the position of glucose could increase the retention time, as in the case of isosalicin, a corresponding isomer of salicin. Thus, the unknown compound would likely be salicyloyl-glucoside ester (Figure 8), a precursor of SA and SA-glucoside (Chong et al., 2001).

We suggest that biosynthesis of salicin proceeds via the same pathway (Figure 8) as that of closely related SA in tobacco in tobacco (Chong et al., 2001), i.e., via benzoyl-glucose, which is synthesized either from cinnamoyl-CoA or from cinnamoyl-glucose. Benzoyl-glucose can then be hydroxylated to salicyloyl-glucose ester, which would be the most immediate precursor of SA (Figure 8). Salicyloyl-glucose ester may be further glucosylated to salicyloyl-diglucose ester

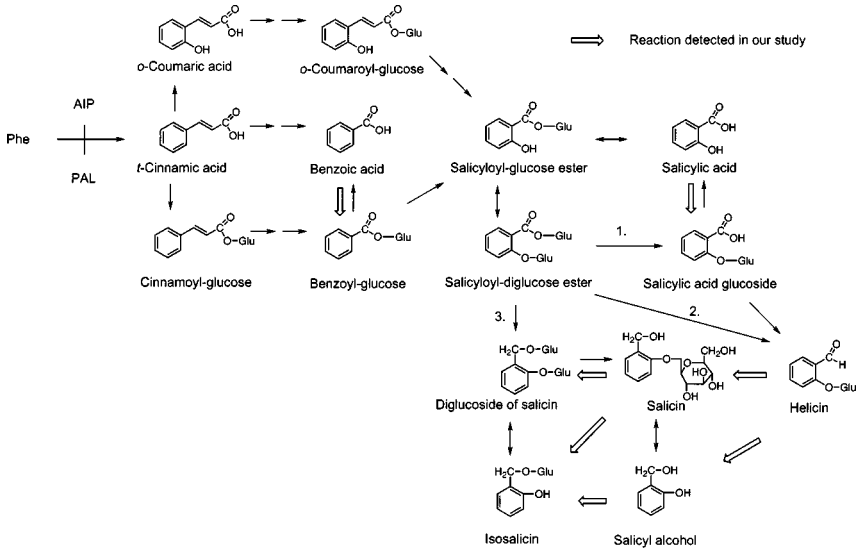

FIG. 8. Hypothetical pathways leading to salicin and closely related SA. Salicin and SA may have common intermediates, such as benzoyl-glucose and salicyloyl-glucose ester. The biosynthesis of salicin is thought to has proceed mainly via salicyloyl-diglucoside ester, which has three putative fates: it can be converted to SA-glucoside (1) helicin (2), or reduced to diglucoside of salicin (3). The biosynthesis of SA is based on that proposed by Chong et al (2001).

(see Pierpoint, 1994), which has three putative fates: it can be converted to SA-glucoside, to helicin, or reduced directly to the diglucoside of salicin, which would be immediate precursor of salicin (Figure 8).

Salicin may also be synthesized from SA-glucoside via helicin, but free SA is probably not a major intermediate in the endogenous biosynthesis of salicylates. SA-glucoside has been suggested to act as a precursor of salicin (Ibrahim and Towers, 1959; Pridham and Saltmarsh, 1963). However, Zenk (1967) claimed that neither SA nor SA-glucoside was the precursor of salicin in *S. purpurea*.

Both salicin and helicin were converted to isosalicin, in the root tissue. Apparently as a result of hydrolysis of compounds to salicyl alcohol (saligenin), a precursor of isosalicin (Pridham and Saltmarsh, 1963; Zenk, 1976; Tabata et al., 1976). Trace amounts of isosalicin found in the shoot tissue suggest that isosalicin was rapidly metabolized probably to salicin, by a putative isosalicin:salicin-transglucosylase (Dombrowski, 1993) or via the diaglucoside of salicin (Figure 8).

Salicin treatment failed to increase the levels of higher substituted salicylates, e.g., salicortins, acetylsalicortins, and tremulacin, while their levels were increased by BA, SA, and helicin, the precursors of salicin. Salicin is the only reasonable

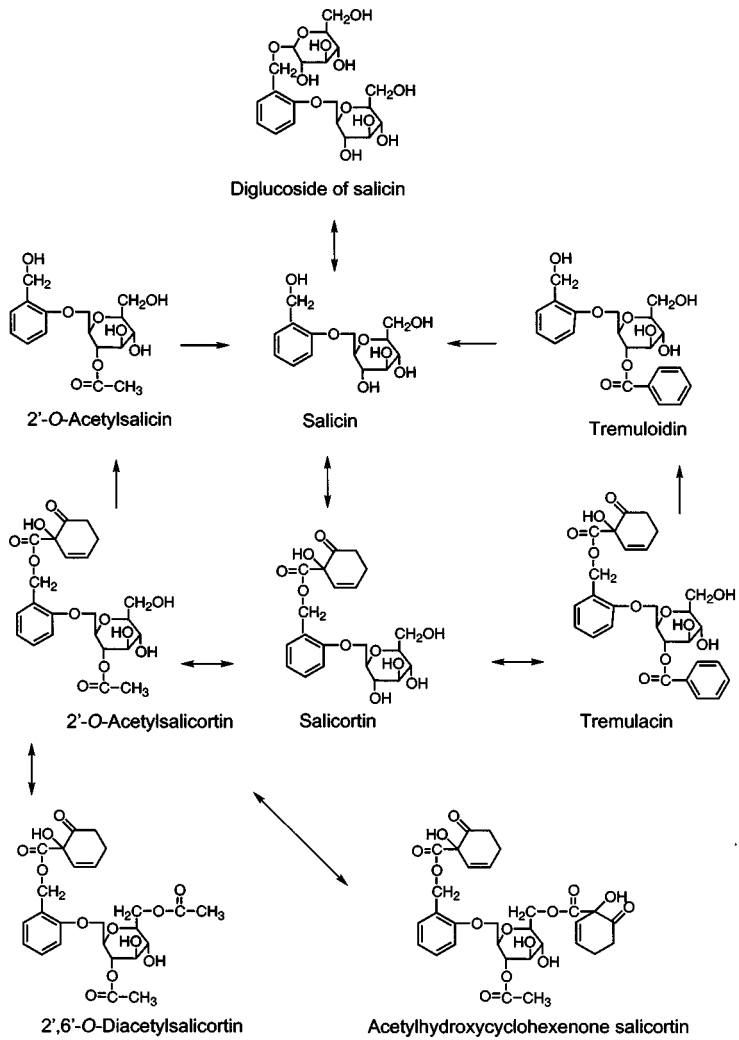

FIG. 9. Hypothetical biosynthesis of higher substituted salicylates. Salicin is a degradation product of salicortin and its derivatives and, thus, plays a key role in the putative metabolic grid of salicylates where the substances can be converted to each other.

structure where the hydroxycyclohexenone carboxylic acid might be esterified (Vainiotalo, personal communication). AIP treatment seems to deactivate esterification of hydroxycylohexenone carboxylic acid. Small molecular weight phenolics such as BA, SA, and helicin either activate this step or act as precursors of the hydroxycyclohexenone carbonyl moiety. In cell suspension cultures, the putative

precursors of salicylates increased the level of salicin, but not the levels of higher substituted salicylates (Dombrowski, 1993; Julkunen-Tiitto et al., unpublished results), indicating that biosynthesis of higher substituted salicylates needs some activator.

We suggest that the synthesis of higher substituted salicylates proceeds via salicin, which is esterified with hydroxycyclohexenone carboxylic acid, producing salicortin, which is probably the immediate precursor of 2′-*O*-acetylsalicortin and tremulacin (Figure 9). The chemical structure of the benzoyl group of tremulacin suggests that this moiety may be BA-derived. Indeed, BA was the most efficient precursor of tremulacin. The levels of diacetylsalicortin and acetylhydroxycyclohexenone salicortin responded more weakly to the AIP and precursor treatments than the level of 2′-*O*-acetylsalicortin, leading to the conclusion that these compounds are located downstream from 2′-*O*-acetylsalicortin in the biosynthetic pathway (Figure 9).

Salicortin and other higher substituted salicylates are easily degraded to salicin (e.g., Pearl and Darling, 1971; Lindroth and Pajutee, 1987; Julkunen-Tiitto and Tahvanainen, 1989; Julkunen-Tiitto and Sorsa, 2001; Ruuhola et al., unpublished). However, salicin is also a potential precursor of these compounds, suggesting the existence of a metabolic grid salicylates and may account for the stability of salicylates in intact willows.

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VOLATILES FROM WHITEFLY-INFESTED PLANTS ELICIT A HOST-LOCATING RESPONSE IN THE PARASITOID, *Encarsia formosa*

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Abstract—The blend of volatile compounds emitted by bean plants (*Phaseolus vulgaris*) infested with greenhouse whitefly (*Trialeurodes vaporariorum*) has been studied comparatively with undamaged plants and whiteflies themselves. Collection of the volatiles and analysis by gas chromatography revealed more than 20 compounds produced by plants infested with whitefly. Of these, 4 compounds, (*Z*)-3-hexen-1-ol, 4,8-dimethyl-1,3,7-nonatriene, 3-octanone, and one unidentified compound were emitted at higher levels than from the undamaged control plants. Synthetic (*Z*)-3-hexen-1-ol, 4,8-dimethyl-1,3,7-nonatriene, or 3-octanone all elicited a significant increase in oriented flight and landing on the source by the parasitoid, *Encarsia formosa*, in wind tunnel bioassays. Two-component mixtures of the compounds and the three-component mixture all elicited a similar or, in most cases, a better response by the parasitoid, the most effective being a mixture of (*Z*)-3-hexen-1-ol and 3-octanone. These results demonstrate that *E. formosa* uses volatiles from the plant-host complex as olfactory cues for host location.

Key Words—*Trialeurodes vaporariorum, Encarsia formosa, Phaseolus vulgaris*, host location, plant volatiles, wind tunnel, air entrainment.

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INTRODUCTION

The greenhouse whitefly, *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae), is an economically important pest of many kinds of ornamental plants and vegetables including potato, tomato, cucumber, and aubergine. Not only does it have a direct effect on plant health and consequently the final yield, it can also be a vector for a number of plant diseases, e.g., potato yellow vein disease (Salazar et al., 2000). In 1926, Speyer reported that a parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) emerging from "black pupae" of the whitefly would be a good candidate for a biological control agent for the pest (Speyer, 1927). Since the 1940s, however, synthetic organic pesticides have been the major pest management agents for many insects, including the greenhouse whitefly. Unfortunately, the long-term and unsuitable use of synthetic insecticides has caused severe side-effects, such as the development of resistance in pest insects and accumulation of residues in crops and the agricultural environment. These undesirable side-effects have led to the search for techniques that are more environmentally safe for pest management, including the use of biological control agents. As a result, the use of *E. formosa* for whitefly management in glasshouses is now universally accepted (van Lenteren and Woets, 1988; Hoddle et al., 1998). Another parasitoid, *Amitus fuscipennis* MacGown and Nebeker (Hymenoptera: Platygasteridae), is also used in crops such as bean and snap bean (both *Phaseolus vulgaris* L.) in South America.

Recent studies have shown that many parasitoids locate their hosts by using volatile semiochemicals from their hosts or from food plants infested by their hosts (Noldus, 1989; Vet and Dicke, 1992). The tritrophic system of plant, spider mite, and predatory mite has been particularly well investigated (Dicke et al., 1999b). These and other studies have led to the suggestion that volatile compounds that regulate host location could be used in pest management strategies to enhance natural enemies' fitness, by attracting them to and/or maintaining them in crop fields, or by priming their responses to their hosts (Du et al., 1997).

Although the literature contains several reports dealing with host location by *E. formosa* (Guerrieri, 1997; Sütterlin and van Lenteren, 2000), and the searching and oviposition behavior of *A. fuscipennis* has been studied recently (Manzano et al., 2002), none of the chemical cues involved in host location by either species have been identified. The reason for this, at least in part, may be the difficulty in working with small insects that have a propensity to escape and contaminate controls. We report here the identification of some volatile compounds from the *P. vulgaris*/*T. vaporariorum* complex and their role in the attraction of the parasitoid *E. formosa*.

METHODS AND MATERIALS

Plants and Insects. Bean plants (*P. vulgaris*) cv. Canadian Wonder, greenhouse grown in small pots, were used when 8 d old for infestation and entrainment. The

greenhouse whiteflies (*T. vaporariorum*) that were used in entrainment experiments were reared on bean plants at 22±1°C with a 16:8 hr L:D photoperiod. The culture was renewed every 14 d, which means that the ages of the whiteflies were in the range 1–14 d. Empty pupal cases were collected from bean leaves using fine forceps.

For the bioassays, all of which were carried out at Portici, Italy, whiteflies were reared on *P. vulgaris* cv. Borlotto nano at 25±1°C, 65±5% relative humidity, and an 18:6 hr L:D photoperiod. Parasitoids (*E. formosa*) were reared in a separate environmental chamber on whiteflies feeding on bean plants, also at 25±1°C with an 18:6 hr L:D photoperiod. Parasitoids were reared as synchronized cohorts by exposing heavily infested plants for 24 hr to 1 day-old females of *E. formosa*; after a week, the resultant black pupal cases were clipped from the plant and isolated in glass test tubes (50 × 5 mm) plugged with cotton wool. Experimental females were used within 24 hr of their emergence, between the 3rd and 9th hr after the onset of photophase and were starved before use.

Volatile Collection from Whitefly-Infested and Uninfested Bean Leaves. The collection apparatus for volatile compounds, which is described in detail elsewhere (Agelopoulos et al., 1999), used a positive/negative pressure system. Briefly, the system comprised two glass half-cells (6 cm ID × 3 cm deep and 6 cm ID × 4.5 cm deep), which when put together formed a chamber around part of the plant, the petiole, or stem passing through a small hole. The remaining space around the petiole or stem was packed with glass wool, held in place using PTFE sealing tape. Air was pumped through molecular sieve and activated charcoal filters at 600 ml min^{-1} into the chamber to provide a positive pressure of clean air. In another outlet was placed a glass tube (8 cm × 0.3 cm ID; actually an inlet liner for the gas chromatograph, see below) containing Tenax TA (50 mg) sandwiched between glass wool plugs. This tube had previously been baked in a stream of clean nitrogen for 2 hr at 200°C. The end of this tube inside the chamber was wrapped with PTFE tape, and 7 small holes were made in the tape with a pin so that the insects could not get into the absorbent tube. Air was drawn from the chamber through the tube under negative pressure at a flow rate of 300 ml min^{-1} (the remaining 300 ml min^{-1} was vented to the atmosphere via the non-gastight closure around the petiole). In this way, volatile compounds were collected on the Tenax TA for 18 hr. Entrainments were carried out under artificial lighting at 22°C with a 16:8 hr L:D photoperiod.

Volatile entrainment was conducted with 8-day old plants of *P. vulgaris* when they had two fully developed leaves. At approximately 3.5 hr into the light period, the plant and apparatus was set up as described above with one leaf contained within the chamber. Whiteflies (approximately 1000 or 20 cm^{-2} of leaf) were introduced into the chamber 1 hr later. A control plant was set up in exactly the same way but without the insects. Entrainment of the volatiles was begun 5 hr after introducing the whiteflies on the 1st d and at the same time on the 2nd and 3rd d,

in each case for 18 hr. Volatiles were also collected on other days up to the 8th d of the experiment (Day 4+).

Volatile Collection from Adult Whitefly and Empty Pupal Cases. The collection apparatus consisted of a pair of ground glass joints (Quickfit B19), one closed with a straight glass tube (3 cm × 0.5 cm OD) and the other with a screw threaded glass tube which can take a PTFE ferrule and a plastic screw cap. When they were put together, they formed a small cylindrical chamber (approximately 8 cm × 1.2 cm ID). Approximately 1000 whiteflies or empty pupal cases were placed in the chamber, and clean air from molecular sieve and charcoal filters was pumped in at 300 ml min^{-1} through the glass tube. At the other end of the chamber, a tube packed with Tenax TA as described above was inserted through the PTFE seal and air drawn through it at 300 ml min^{-1} for 18 hr.

Gas Chromatography (GC) and Gas Chromatography—Mass Spectrometry (GC/MS). Analysis, of the volatile compounds entrained, by GC was performed on an AI 94 gas chromatograph fitted with an Optic 400 programmed temperature vaporization (PTV) inlet (Atas, UK) and a flame ionization detector (FID). The column used was a polyethylene glycol Zebron ZB-Wax (30 m × 0.32 mm ID × 0.50 μm, film thickness; Phenomenex, UK), and the carrier gas was nitrogen. The oven temperature was set at 30°C for 1 min and then programmed to increase at 10°C to 200°C and maintained for 20 min.

Volatiles trapped on the Tenax TA were released directly onto the GC column by thermal desorption, the whole tube being inserted into the unit as the inlet liner and the temperature of the PTV unit being programmed to rise from 30°C to 220°C in 40 sec.

Selected samples were analyzed by GC/MS using either a VG 70-250 or a VG Autospec (VG Analytical, UK) combined with an HP5890 (Hewlett-Packard, UK) GC, which was fitted with the same PTV unit as described above. The column used was an HP-1 (100% dimethyl polysiloxane, 50 m × 0.25 mm ID × 0.25 μm film thickness, Hewlett-Packard, UK), and the oven temperature was programmed to stay at 30°C for 5 min and then rise at 5°C min^{-1} to 250°C.

Compounds were tentatively identified by comparison of mass spectra with spectra in the NIST database and with spectra from authentic compounds. Identification was confirmed by peak enhancement on coinjection on GC with authentic compounds.

Wind Tunnel Bioassays. Experiments were carried out in a wind tunnel similar to that described by Miller and Roelofs (1978) and modified according to Guerrieri et al. (1993). The wind speed, monitored with a hotwire anemometer (Alnor, model CGA-26), was adjusted to 20 ± 1 cm sec^{-1}. Exhaust air was vented outside the wind tunnel room. Light intensity in the flight chamber was maintained at approximately 50 μmol m^{-2} sec^{-1} at the take-off point, and a black and white striped pattern was placed beneath the flight chamber floor to aid visual orientation.

Bioassays were carried out at 20±1°C and 65 ± 5% relative humidity. Individual parasitoids were used once, released from an open-ended glass test tube, which was held on a plastic base so that the take-off point was exactly in the middle of a cross section of the flight chamber. For each target, 50 parasitoids were tested by releasing them individually in the odor plume 25 cm downwind from the target. Parasitoids were observed for a maximum time of 10 min, and flight behavior data were recorded and analyzed with the aid of event-recording software (The Observer, Noldus Information Technology, Wageningen, The Netherlands). A flight response was considered as oriented towards the experimental target when the female flew directly upwind and landed on the target or within 5 cm of it. Experiments were conducted on a minimum of 3 d, and targets were presented in a random order to reduce any effect of temporal variability of behavioral responses.

Individual compounds (commercial or synthetic samples) were tested by placing a solution, in redistilled hexane (10 μl, 1 mg ml^{-1}), onto a small piece (3 × 1 cm) of filter paper (Whatman No. 1). This was inserted into an open-ended glass tube (6 × 1 cm) with the end facing the parasitoid surrounded by a green circular target (8 cm diam). This was to reduce any negative effect due to the absence of physical stimuli normally provided by the plant. A fresh aliquot of solution and a new piece of filter paper was used for each female parasitoid. A similar target, which was dosed with 10 μl redistilled hexane, served as a control.

Mixtures of compounds were tested by applying a solution of each component in hexane (10 μl, 1mg ml^{-1}), in turn, onto the same piece of filter paper before releasing the parasitoid. The components were applied in a random order on each day of the experiment.

The daily mean percentage responses to each compound or mixture were calculated. The proportions responding (i.e., showing oriented flights and landings on the target) were subject to logit transformation to normalize the data, which was then analyzed by using a one-way analysis of variance to test the effect of the target on the response. A Tukey–Kramer multiple comparison test (Sokal and Rohlf, 1981; Rohlf and Sokal, 1995) was used to compare the means. The proportions of oriented flights resulting in landings on the target for each compound or mixture were analyzed by using the simultaneous test procedure of the unplanned test of homogeneity of replicates for goodness of fit (Sokal and Rohlf, 1981; Rohlf and Sokal, 1995).

RESULTS

Volatile Compounds from Whitefly-Infested Bean Leaf. By GC and GC/MS, more than 20 compounds were identified in both infested and uninfested bean leaves (Figure 1 and Table 1). Most were common plant volatiles. Despite large variations among plants in the amounts of volatiles collected (Table 1), comparisons of GC peak areas for volatiles from the two leaves show a marked and

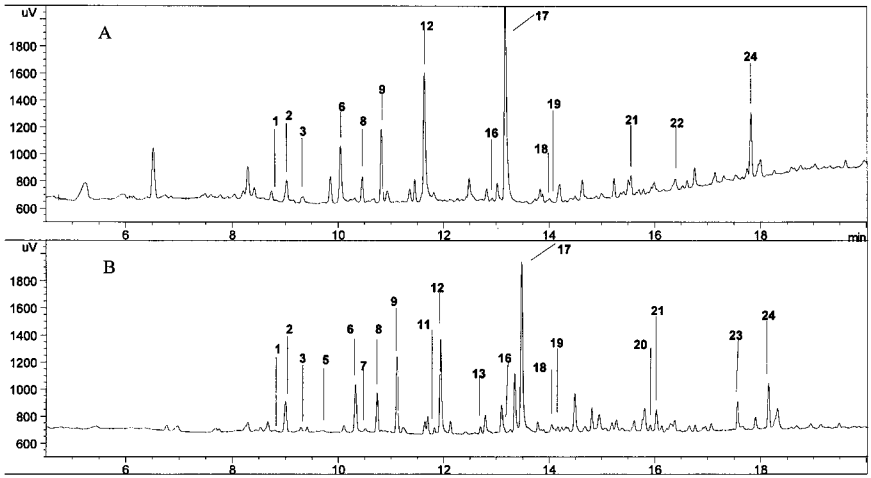

FIG. 1. Typical gas chromatograms (on an HP-wax column) of volatile compounds emitted from undamaged (A) and whitefly-infested (B) leaves of *Phaseolus vulgaris*. For an explanation of peak numbers see Table 1.

consistent increase in the amounts of 3-octanone, (*Z*)-3-hexen-1-ol, 4,8-dimethyl-1,3,7-nonatriene, and an unknown compound (Kovats retention index, KI = 1472 on HP-wax column; 1214 on HP-1) in the infested leaf. Some of the other compounds (Table 1), especially methyl salicylate, appeared to show a similarly large increase, but as the figures given represent a mean of seven experiments they do not always represent a consistent result. The compounds chosen for further experiments gave largely consistent results.

The method of absorption onto Tenax, followed by thermal desorption of the volatiles directly onto the GC column, does not lend itself to the use of an internal standard. However, previous studies have shown the reproducibility of these methods of collection and analysis (Agelopoulos and Pickett, 1998; Agelopoulos *et al.*, 1999). Chromatograms of an *n*-alkane series (20 ng each, C_8 to C_{24}) were used to assess the approximate amounts of volatiles detected, with middle of the range alkanes giving 180 peak area units ng^{-1}.

The unknown compound mentioned above gave the following major ions, *m/z* (intensity) by MS: 27 (23%), 41 (46%), 43 (26%), 55 (100%), 56 (32%), 57 (46%), 70 (95%), 83 (27%), 85 (28%), 97 (28%), and 112 (30%). These data are consistent with the compound being an octyl acrylate, which is supported by *m/z* 73 (9%) that would arise from $CH_2{=}CHC(OH){=}OH^+$. It will be fully characterized in further studies.

Figure 2 shows the changes in the emission of the four compounds from infested and uninfested leaves over the period of the experiment. High levels of

TABLE 1. COMPOSITION OF VOLATILES COLLECTED FROM WHITEFLY-INFESTED AND UNDAMAGED BEAN LEAVES, WHITEFLIES ALONE, AND EMPTY PUPAL CASES

Peak	Compound	GC peak area units[a]				
		Infested leaf	Undamaged leaf	Whitefly bodies	Empty pupal cases	Blank
1	limonene	103.0 (0–973)	132.7 (0–954)	90.7 (0–453)	25.3 (0–126)	N.D.[b]
2	3-methyl-1-butanol	510.2 (0–1498)	388.6 (0–1061)	115.2 (0–280)	85.7 (46–208)	128.3 (0–257)
3	(*E*)-2-hexenal	353.1 (0–5040)	120.8 (0–787)	73.4 (0–137)	89.0 (0–208)	N.D.
4	(*E*)-ocimene	178.7 (0–1364)	123.8 (0–1459)	N.D.	20.3 (0–57)	43.4 (0–87)
5	3-octanone	189.4 (0–1633)	17.4 (0–226)	N.D.	22.7 (0–113)	N.D.
6	octanal	2223.0 (221–5839)	2032.0 (784–5794)	668.0 (487–988)	757.0 (598–1009)	1114.4 (973–1256)
7	4,8-dimethyl-1,3,7-nonatriene	667.9 (0–6648)	143.5 (0–1154)	81.6 (0–130)	93.2 (75–117)	47.8 (0–96)
8	(*Z*)-3-hexenyl acetate	734.5 (0–5569)	718.1 (0–9818)	31.4 (0–84)	N.D.	N.D.
9	6-methyl-5-hepten-2-one	1993.7 (296–4467)	1649.7 (0–3748)	492.7 (299–762)	1167.8 (376–1525)	1143.8 (573–1715)
10	(*Z*)-3-hexenyl *iso*-butyrate	51.6 (0–796)	26.9 (0–430)	N.D.	N.D.	N.D.
11	(*Z*)-3-hexen-1-ol	299.7 (0–3278)	14.1 (0–270)	N.D.	N.D.	N.D.
12	nonanal	4910.9 (260–5839)	3885.6 (1356–8877)	7017.1 (1802–20486)	3065 (1665–6116)	2928.5 (2855–3001)
13	unknown (KI = 1472)	896.5 (0–9639)	15.0 (0–118)	N.D.	N.D.	N.D.
14	(*Z*)-3-hexenyl *n*-butyrate	119.9 (0–930)	18.6 (0–522)	117.9 (0–422)	48.1 (0–181)	38.2 (0–76)
15	(*Z*)-3-hexenyl 2-methylbutyrate	83.2 (0–1718)	17.6 (0–230)	17.1 (0–86)	N.D.	N.D.
16	(*Z*)-3-hexenyl 3-methylbutyrate	106.1 (0–611.2)	55.0 (0–274)	139.3 (103–193)	22.9 (0–114)	N.D.
17	decanal	7968.3 (821–17310)	7524.4 (1830–14857)	2214.1 (1538–2685)	3414.2 (1739–5412)	6063.1 (5140–6985)
18	linalool	270.2 (0–1547)	160.7 (0–953)	22.1 (0–110)	N.D.	N.D.
19	benzaldehyde	944.3 (0–8948)	500.5 (0–1985)	918.7 (693–1622)	605.4 (443–826)	949.0 (539–1359)
20	acetophenone	459.6 (0–1389)	378.4 (0–1965)	788.6 (0–1491)	216.8 (0–543)	441.5 (0–883)
21	$\alpha-$humulene	390.7 (0–1061)	303.1 (0–1417)	195.3 (0–475)	65.1 (0–209)	171.3 (72–271)
22	$(E, E) - \alpha$-farnesene	245.8 (0–2741)	231.8 (0–1025)	784.8 (216–2669)	N.D.	N.D.
23	methyl salicylate	1002.5 (0–17566)	198.5 (0–1076)	170.0 (0–618)	N.D.	N.D.
24	geranylacetone	1783.4 (252–5588)	1557.5 (422–4719)	1088.1 (464–1959)	609.0 (276–1021)	1027.1 (955–1099)

Note: The range of values is in parantheses.
[a] Data represent the mean of seven replicates except for the blank (two replicates).
[b] N.D., not detected.

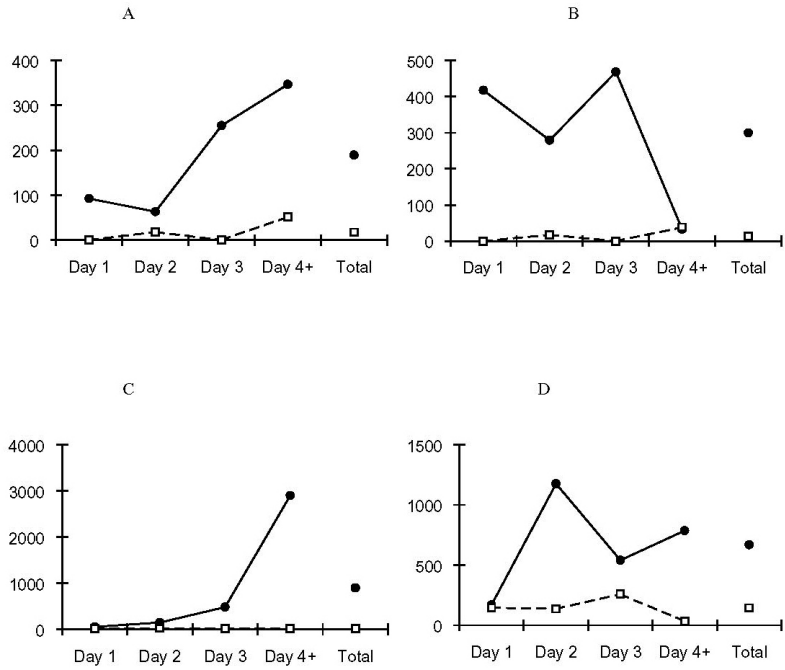

FIG. 2. Emission of 3-octanone (A), (*Z*)-3-hexen-1-ol (B), an unknown compound (C), and 4,8-dimethyl-1,3,7-nonatriene (D) from whitefly-infested (-●-, solid lines) or undamaged (- -, dotted lines) bean leaves. Data represent the mean of seven replicates.

the green-leaf volatile, (*Z*)-3-hexen-1-ol, were produced during the first 3 d after the whiteflies were introduced, but after that levels fell to those of the uninfested plants. 3-Octanone, 4,8-dimethyl-1,3,7-nonatriene, and the unknown compound were present in low amounts on the 1st d but all showed increases thereafter.

Behavioral Responses of Parasitoids to Induced Compounds in the Wind Tunnel. Synthetic samples of the three identified compounds that increased in infested plants were tested in the wind tunnel, individually and in mixtures, for their ability to induce behavioral responses in *E. formosa*. The results of these bioassays are shown in Figure 3. Single compounds and the mixtures all elicited a higher response than redistilled hexane. However, there were significant differences in oriented flights only between hexane and (*Z*)-3-hexen-1-ol and between hexane and any of the mixtures containing this compound. Landings on the target were significantly different from hexane for any compound or mixture. The mixture of (*Z*)-3-hexen-1-ol and 3-octanone elicited the largest number of oriented flights (Figure 3A) and landings on the source (Figure 3B), but the proportion of those

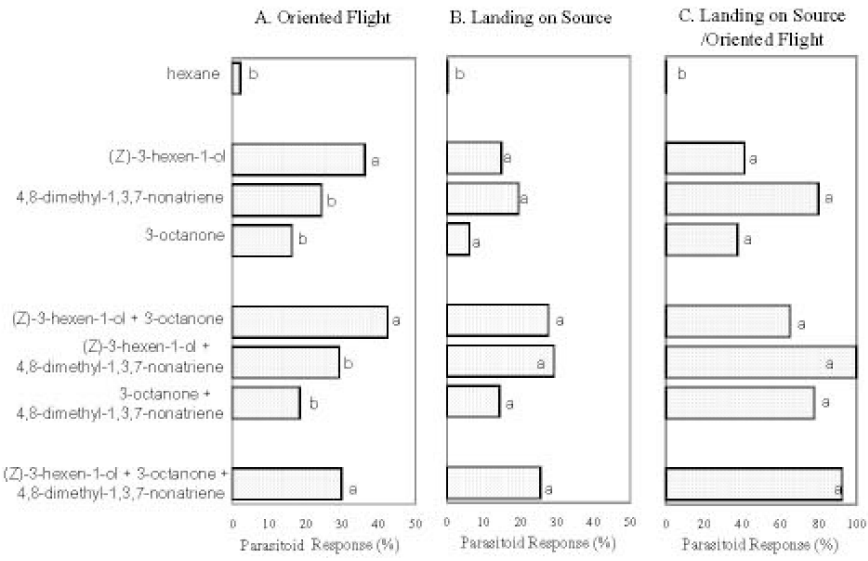

FIG. 3. Response of the parasitoid *Encarsia formosa* in a wind tunnel to (*Z*)-3-hexen-1-ol, 4,8-dimethyl-1,3,7-nonatriene, and 3-octanone, singly and as mixtures. In each case 50 insects were tested. (Statistical analysis; a—significantly different than hexane control, b—not significantly different than hexane control).

showing oriented flight that landed on the source was highest (100%) with the mixture of (*Z*)-3-hexen-1-ol and 4,8-dimethyl-1,3,7-nonatriene (Figure 3C). The three-way mixture also showed a good response in terms of the proportion of oriented flights resulting in landings (92.3%).

DISCUSSION

This study demonstrates that plants of *P. vulgaris* infested by the greenhouse whitefly, *T. vaporariorum*, emit at least three compounds that influence the flight behavior of the parasitoid *E. formosa*. The levels of these compounds, (*Z*)-3-hexen-1-ol, 4,8-dimethyl-1,3,7-nonatriene, and 3-octanone, together with an unidentified compound, were all consistently higher in the infested than in the uninfested leaves, and this increase was not accounted for by the presence of the whiteflies themselves or their pupal cases. The compounds may, therefore, be considered as resulting from whitefly infestation.

(*Z*)-3-Hexen-1-ol is a product of fatty acid metabolism via the octadecanoid or lipoxygenase pathway, and 4,8-dimethyl-1,3,7-nonatriene of the isoprenoid pathway (Paré and Tumlinson,1996). Together with 3-octanone these compounds have been shown to be induced in Lima bean, *P. lunatus*, by treatment with jasmonic

acid (Dicke, *et al*., 1999a), which is believed to be involved in the signal transduction of induced defenses in many plants. It is possible that the induction of the jasmonic acid pathway is a key factor in the emission of volatiles by *P. vulgaris* following whitefly infestation and the three compounds characterized herein, together with the unidentified one, could be considered to be herbivore-induced. However, the possibility of induction by mechanical damage has not been ruled out.

It is apparent from the wind tunnel results that when (*Z*)-3-hexen-1-ol was not present the number of oriented flights was less (Figure 3A), while 4,8-dimethyl-1,3,7-nonatriene caused more landings on the target (Figure 3B), especially when mixed with (*Z*)-3-hexen-1-ol in which case all the parasitoids that showed oriented flight also landed on the target (Figure 3C). It is interesting to note that a lower proportion of oriented flights resulted in landings on the target for (*Z*)-3-hexen-1-ol than for any other compound or mixture. These results suggest that (*Z*)-3-hexen-1-ol is a key component for the long distance attraction of *Encarsia formosa* while 4,8-dimethyl-1,3,7-nonatriene becomes attractive at a closer range. Such a difference could arise from the difference in volatilities of the two compounds, the former being the more volatile. From a behavioral point of view, the distance from the source of a semiochemical, at which it can be detected by the insect, regulates the transition from flying to landing during the parasitoid's foraging behavior.

The mixture of 3-octanone and (*Z*)-3-hexen-1-ol elicited the best response in terms of oriented flight and landing on source (Figure 3A and B). However, 3-octanone alone elicited the smallest response of any treatment. It is possible that 3-octanone acts as a synergist for the other compounds.

The results of wind tunnel tests were similar to those reported previously on flight behavior of *E. formosa* (Guerrieri, 1997). The percentage of oriented flights towards a host damaged plant (HDP, 27%, as reported by Guerrieri, 1997) was not significantly different from the percentage towards the most attractive mixture of compounds tested in this work (30% to the (*Z*)-3-hexen-1-ol/3-octanone mixture). The response of *E. formosa* to the compounds identified in the plant-host complex cannot be attributable to the physical attraction of the green target, as shown by the absence of any oriented response towards control targets (2%). This confirms that flight searching of *E. formosa* is not random but is regulated by compounds from the plant-host complex.

The temporal emission pattern of (*Z*)-3-hexen-1-ol was very different from the other three induced compounds (Figure 2). High levels of (*Z*)-3-hexen-1-ol were released from the damaged leaf in the early stages of infestation and then declined after 3 d. This could explain the higher response, in terms of landings on the source, reported here for the most attractive mixture (26%) than for the HDP source (0%) reported by Guerrieri (1997) that had been infested for at least 10 d prior to the bioassay.

It is possible that the plant recruits the parasitoid with emission of the attractive (*Z*)-3-hexen-1-ol when the degree of damage is low, but that as the colonies of *T. vaporariorum* enlarge and the plant deteriorates due to increasing damage other compounds mediate host location by *E. formosa*. Further experiments are necessary to demonstrate these hypotheses and to discover the most attractive blends of volatiles for *E. formosa* to improve performance in biological control programmes.

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FIELD EVALUATION OF HERBIVORE-INDUCED PLANT VOLATILES AS ATTRACTANTS FOR BENEFICIAL INSECTS: METHYL SALICYLATE AND THE GREEN LACEWING, *Chrysopa nigricornis*

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Abstract—Synthetic methyl salicylate (MeSA), a herbivore-induced plant volatile (HIPV), was demonstrated to be an attractant for the green lacewing, *Chrysopa nigricornis*, in two field experiments conducted in a Washington hop yard. Significantly greater numbers of *C. nigricornis* were trapped on MeSA-baited sticky cards (mean: 2.8 ± 0.4/card/week) than on unbaited cards (0.45 ± 0.15) during June–September. Cards baited with two other HIPVs, hexenyl acetate and dimethyl nonatriene, did not attract more *C. nigricornis* than did unbaited traps (0.30 ± 0.10, 0.44 ± 0.15, respectively). MeSA-baited Unitraps captured 1.9 ± 0.5 *C. nigricornis*/trap/week during July–August compared to 0.20 ± 0.20/trap/week in methyl eugenol-baited traps and 0.03 ± 0.03/trap/week in unbaited traps. The potential use of MeSA in enhancing *C. nigricornis* populations in Washington hop yards as an aid to conservation biological control of aphids and mites is discussed.

Key Words—Herbivore-induced plant volatiles, methyl salicylate, *Chrysopa nigricornis*, attractant, predators, biological control, hops.

INTRODUCTION

Plants respond to herbivore damage by producing volatiles that attract natural enemies of the herbivores responsible for the damage (Dicke et al., 1990; Turlings et al., 1990; Vet and Dicke, 1992; Stowe et al., 1995; Takabayashi and Dicke,

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1996). The qualitative and quantitative characteristics of herbivore-induced plant volatiles (HIPVs) can vary according to the herbivore involved, the plant species, and even the genotype (Turlings et al., 1993; Takabayashi et al., 1994). The phenolic compound, methyl salicylate (MeSA), has been identified in HIPV blends from at least 10 plant species attacked by herbivores, including lima bean damaged by spider mites, *Tetranychus urticae* Koch (Dicke et al., 1990; Ozawa et al., 2000a), tomato [*T. urticae*] (Dicke et al., 1998), cucumber [*T. urticae*] (Agrawal et al., 2002), cabbage [caterpillars, *Pieris* spp] (Geervliet et al., 1997), pear [Psyllidae] (Scutareanu et al., 1997), hops [hop aphid, *Phorodon humuli* Schrank] (Campbell et al., 1993), bird cherry [bird cherry oat aphid, *Rhopalosiphum padi* (L.)] (Glinwood and Pettersson, 2000; Ninkovic et al., 2003), potato [Colorado potato beetle, *Leptinotarsa decemlineata* (Say)] (Bolter et al., 1997), *Nicotiana attenuata* [caterpillars, leaf bugs, flea beetles] (Kessler and Baldwin, 2001), and *Lotus japonicus* [*T. urticae*] (Ozawa et al., 2000a). Tobacco plants innoculated with tobacco mosaic virus also produced MeSA (Shulaev et al., 1997). However, MeSA is not universally produced by herbivore-damaged plants, as evidenced by its absence in volatiles from caterpillar (*Spodoptera exigua* (Hubner)) damaged corn (Turlings et al., 1990) and lima bean (Ozawa et al., 2000b), spider-mite-infested apple trees (Llusia and Penuelas, 2001) and cotton and maize plants damaged by western tarnished plant bugs, *Lygus hesperus* Knight (Rodriguez-Saona et al., 2002). Other common HIPVs reported, particularly from spider-mite-damaged plants, include the terpene 3,7-dimethyl-1,3,6-octatriene, the methylene terpene 4,8-dimethyl-1,3,7-nonatriene, and the ester *Z*-3-hexenyl acetate (Dicke et al., 1990; Pare and Tumlinson, 1996).

Laboratory studies have demonstrated MeSA to be attractive to the predatory mite, *Phytoseiulus persimilis* Athias-Henriot (Phytoseiidae) (Dicke and Sabelis, 1988; Dicke et al., 1990; Ozawa et al., 2000a) and the predatory bug, *Anthocoris nemoralis* (Fabricius) (Anthocoridae) (Drukker et al., 2000). In contrast, olfactometer studies showed MeSA to be repellent to the aphid plant pests, *Aphis fabae* Scop. and *Sitobion avenae* (Hardie et al., 1994; Petterson et al., 1994). Similarly, field experiments showed MeSA reduced trap catches of the aphid, *Phorodon humuli* Schrank, during spring colonization of hop yards (Losel et al., 1996). Ninkovic et al. (2003) demonstrated that MeSA significantly delayed establishment and reduced infestation of the bird cherry oat aphid (*Rhopalosiphum padi*) in oats treated with a pellet formulation of the semiochemical.

Most of the research to date on HIPVs and response by natural enemies has been conducted under laboratory conditions (Hunter, 2002). If these compounds are to realize their potential in applied entomology (Sabelis et al., 1999), then we need to conduct more studies on HIPVs and natural enemy responses in the field environment. This study provides the first field evidence for attraction of a generalist predator in the Pacific Northwest of the United States, the green lacewing, *Chrysopa nigricornis* Burmeister (Neuroptera: Chrysopidae), to synthetic methyl salicylate.

METHODS AND MATERIALS

One experiment was conducted in an unsprayed 1-ha hop yard at Washington State University – Prosser from April 11 to September 5, 2002 to determine whether selected HIPVs are attractive to field populations of beneficial arthropods. Yellow sticky cards (23 × 18 cm, Trece Incorporated, Salinas, CA) baited with 2-ml glass vials of candidate HIPV solutions were tied to wooden poles ~2 m above the ground (poles in hop yards are used to carry wires for supporting hop plants). HIPVs tested were methyl salicylate [MeSA] (99%, Sigma-Aldrich, St. Louis, MO), (*Z*) – 3 - hexenyl acetate (98%, Sigma-Aldrich, St. Louis, MO), 3,7- dimethyl – 1,3,6 – octatriene (Sigma-Aldrich, St. Louis, MO, 8 mg in 2-ml hexane) and 4,8 – dimethyl – 1,3,7 – nonatriene (synthesized by R. J. Bartelt, USDA, Peoria, IL, 10 mg in 2-ml hexane). Dimethyl octatriene was used until June 13. Thereafter, it was replaced by dimethyl nonatriene. These compounds were replaced weekly; MeSA and hexenyl acetate were replaced fortnightly. Bait vials were taped to the lower edge of the cards that were placed in a 3 × 4 grid with 10 m between the randomized treatments. Three replicates were run for each treatment. Sticky cards were replaced weekly and examined under a stereomicroscope for green lacewings (Chrysopidae). Lacewings were identified using the keys of Agnew et al. (1981) and Penny et al. (2000).

A second experiment was conducted in the same hop yard as the first from July 2 to September 10 by using Universal Moth traps or Unitraps (Agrisense, Fresno, CA). Six traps were placed in separate rows, six rows apart with a minimum distance of 10 m between traps. Traps were alternately baited with MeSA or left unbaited (three replicates of each). Glass vials containing 2 ml of 99% MeSA were taped to the interior wall of traps. All traps were filled to a depth of ~5 cm with water to drown trapped insects. Traps were checked weekly for insects, which were removed, identified, and counted. MeSA baits were changed monthly, and water was renewed weekly. From August 6, an additional three traps were placed in the yard (within the experimental area in "vacant" rows with a minimum distance of 10 m from other traps). These were baited with 2-ml glass vials of methyl eugenol (98%, Sigma-Aldrich, St. Louis, MO), a compound previously described as an attractant for two species of *Chrysopa* (Suda and Cunningham, 1970; Umeya and Hirao, 1975). Data were $\log(x + 1)$ transformed to equalize variance and analyzed using analysis of variance and Fisher's least significant difference procedure.

RESULTS

Sticky Cards. Significantly greater numbers of the green lacewing, *C. nigricornis*, were trapped on MeSA-baited sticky cards than on the other HIPV-baited or unbaited cards ($F = 20.2$, df $= 3, 42$, $P = 0.00$; Figure 1). A mean of 2.8 (±0.4) *C. nigricornis*/card/week was captured during 13 June – 5 September on the MeSA- baited cards compared to 0.45 (±0.15), 0.30 (±0.10), and 0.44 (±0.15)

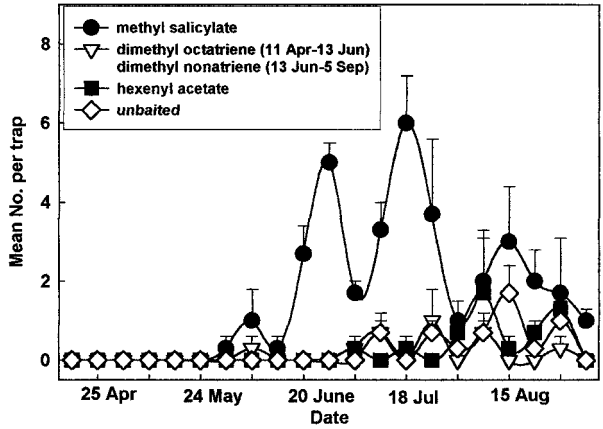

FIG. 1. Weekly mean (±SE) number of adult *Chrysopa nigricornis* trapped on yellow sticky cards baited with methyl salicylate, dimethyl octatriene, dimethyl nonatriene, hexenyl acetate, or left unbaited, during April 11 – September 5, 2002 in a Washington hop yard.

on the unbaited, dimethyl nonatriene-, and hexenyl acetate-baited cards, respectively. Attraction of *C. nigricornis* to MeSA-baited cards appeared to be greatest from mid-June to mid-July, decreasing in August. *Chrysoperla plurabunda* (Stephens) was seen occasionally in the hop yard, but none were trapped on sticky cards.

Unitraps. During the 10-week period that the second experiment was conducted, only one *C. nigricornis* was recorded from the unbaited Unitraps (mean = 0.03/trap/week). In contrast, a total of 67 *C. nigricornis* was recorded from the MeSA-baited traps (mean = 1.9/trap/week). Both sexes of *C. nigricornis* were trapped (female- to-male ratio = 2:1, $N = 33$) with numbers declining during late August (Figure 2). Three *C. nigricornis* were recorded from a methyl eugenol trap on August 20. No *C. plurabunda* were trapped.

DISCUSSION

Hunter (2002) in a review of plant volatile–natural enemy interactions makes the point that although laboratory evidence for plants manipulating the foraging of natural enemies by volatile emission is now overwhelming, few studies have been published showing natural enemy responses to plant volatiles under field conditions. In addition, the few outdoor studies that have been published are often correlative (higher densities of predators around pest-infested plants than uninfested plants (e.g., Drukker et al., 1995; Shimoda et al., 1997), or involve inducing HIPVs from plants (without other prey-associated cues) and measuring responses by natural enemies (e.g., Thaler, 1999; Ockroy et al., 2001). The current study,

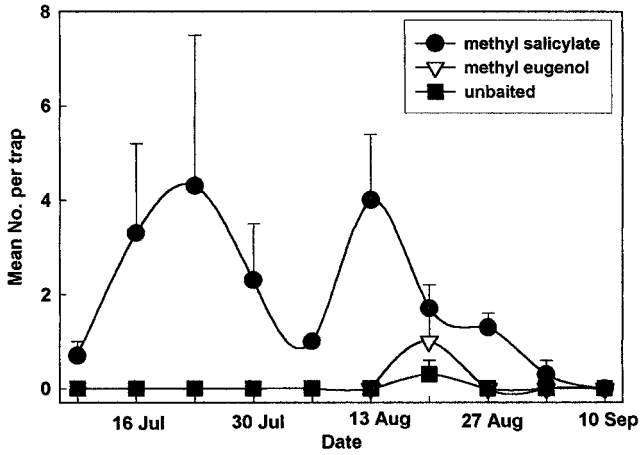

FIG. 2. Weekly mean (±SE) number of adult *Chrysopa nigricornis* captured in Unitraps baited with methyl salicylate, methyl eugenol or left unbaited, during July 2 – September 10 2002 in a Washington hop yard. Note, methyl eugenol used only during August 6 – September 10.

demonstrating field attraction of a beneficial insect species to a synthetic formulation of an HIPV, joins a much smaller list of similar studies using synthetic formulations (e.g., Flint et al., 1979; Sabelis et al., 1999; Zhu et al., 1999). Demonstrating bioactivity of commercially available, synthetic HIPVs in field experiments is an important step towards practical application of these semiochemicals in pest management. This study is also the first demonstration of attraction of a green lacewing species to MeSA.

Chrysopa nigricornis is a large green lacewing, distributed throughout the United States (Agnew et al., 1981) that is predatory as an adult as well as a larva. It is common in the Pacific Northwest and is a useful component of Insect Pest Management (IPM) programs in tree and vine crops and hops. In hops, early season recruitment of predators like *C. nigricornis* has been identified as a key factor in better utilizing conservation biological control of aphids and mites within an IPM program (James et al., 2001, 2003). The use of commercially available HIPVs, like MeSA, may have potential for enhancing spring recruitment of beneficial arthropod populations in crops. However, developing semiochemical formulations that combine practicality, efficacy, and longevity remains a challenge (Dicke et al., 1990).

The results of this study indicate that MeSA is an attractant for *C. nigricornis.* In contrast, there was no evidence that *C. nigricornis* was attracted to hexenyl acetate or dimethyl nonatriene. Dimethyl octatriene also appeared not to be attractive although results for this HIPV are less conclusive because of the limited

period of use in the experiment. This study was designed to answer the simple question of whether any of four common components of identified HIPV blends possess field attraction to any beneficial insect(s) in a Washington hop yard. Now that bioactivity of synthetic MeSA to at least one predatory insect species in the field has been established, it will be prudent to investigate effective concentration levels/release rates and the effect of using HIPV blends. Although the population of *C. nigricornis* in the hop yard was small (weekly canopy beating samples (9 vines/week) yielded only two *C. nigricornis* adults during June – August), significant numbers of adults were trapped on MeSA-baited sticky cards and in the Unitraps. Interestingly, the smaller green lacewing species, *C. plurabunda*, was also present in the yard (similar low numbers in canopy samples) but was not attracted to MeSA-baited traps. The decline in trapped *C. nigricornis* observed during August may have been the result of a diminishing population rather than a decline in response to MeSA. Canopy sampling indicated greatly increasing populations of *C. plurabunda* during September (>100 individuals), but this species was still not detected in MeSA-baited traps (James, unpublished observation). *C. nigricornis* was absent in September canopy samples. A possible explanation for MeSA attraction of *C. nigricornis* and not *C. plurabunda* may lie in the fact that the former is a carnivore as an adult while the latter is only carnivorous as a larva. HIPVs presumably have more immediate benefits to predators that are carnivorous in the prey-locating adult life stage than to predators that are carnivores only as larvae.

C. nigricornis has not been studied previously for its response to HIPVs, although Caltagirone (1969) showed that a mixture of terpenyl acetate, dark brown cane sugar, and water was attractive. *C. plurabunda* is known to respond to 2-phenylethanol and (E)-β-farnesene (Zhu et al., 1999), isomers of tryptophan and its breakdown products (Van Emden and Hagen, 1976) and β-caryophyllene (Flint et al., 1979). Monoterpene alcohols isolated from a Japanese vine (*Actinidia polygama* Miq) were shown to be attractive to males of *Chrysopa septempunctata* Wesmael and *Chrysopa japana* Okomoto (Sakan et al., 1970). Methyl eugenol was reported by Suda and Cunningham (1970) and Umeya and Hirao (1975) to attract *Chrysopa basalis* Walker and an unidentified *Chrysopa* sp., respectively. In the current study, no clear evidence of attraction of *C. nigricornis* to methyl eugenol was obtained, although this compound was only used for a limited period.

Campbell et al. (1993) showed that aphids (*Phorodon humuli*) feeding on hop leaves induced release of MeSA, (E)-2-hexenal and β-caryophyllene. The latter two components were attractive to aphids in laboratory tests, while MeSA appeared to be repellent. Subsequently, Losel et al. (1996) showed MeSA reduced trap catches of *P. humuli* in a hop yard. A similar result was achieved by Ninkovic et al. (2003), who delayed field establishment and reduced population levels of the bird cherry oat aphid, *Ropalosiphum padi*, by distributing wax pellets containing MeSA. Aphid (*P. humuli*) numbers in the hop yard used in this study were low (0–2 per leaf)

for most of the season apart from three weeks in June when numbers reached 20 per leaf. Elevated numbers of *C. nigricornis* in MeSA-baited traps were first seen at this time (presumably as a consequence of greater numbers of aphids) but continued through July and August when aphids were virtually absent in the yard. The spider mite, *Tetranychus urticae*, was the other major herbivore in the yard during the experiments and although mites are known to induce production of MeSA in other plants (e.g., Dicke et al., 1990; Ozawa et al., 2000a), it is unknown whether they do in hops. The use of synthetic MeSA in hop yards may conceivably have two benefits to pest management; enhanced recruitment of beneficial insects like *C. nigricornis* and repellence of the aphid pest, *P. humuli*. Field evidence for attraction of other beneficial insect species to MeSA and hexenyl acetate is being collected and will be reported in due course (James, in preparation, 2003). The efficacy, potential, and practicality of using controlled release dispensers containing MeSA as an enhancement for conservation biological control in Washington hops is currently being examined.

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EFFECTS OF SUBLETHAL DELTAMETHRIN ON THE CHEMICAL COMMUNICATION SYSTEM AND PBAN ACTIVITY OF ASIAN CORN BORER, *Ostrinia furnacalis* (GÜENEE)

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Abstract—Topically applied sublethal doses of deltamethrin may interrupt chemical communication between the two sexes of the Asian corn borer, *Ostrinia furnacalis* (Güenee), by affecting the probability of calling behavior, sex pheromone titer and PBAN-like activity. At the 1st, 2nd, and 4th day of post-treatment with deltamethrin (1 ng/moth), female *O. furnacalis* showed 32, 36, and 46% calling probabilities of control female moths, while the sex pheromone titer of females was only 15, 20, and 25% the amount of control females, respectively. The chemical communication system was significantly affected even at the 4th day post-treatment. By injections of 1, 1/2, 1/4, and 1/8 female equivalent of brain and subesophageol ganglion homogenate of 1 ng deltamethrin-treated females into decapitated females, female pheromone production was reduced to 64, 31, 30.7, and 8.1% of that of control female moths, respectively. This result appears to indicate that sublethal deltamethrin affected the activity of the PBAN-like factor of *O. furnacalis*.

Key Words—*Ostrinia furnacalis*, deltamethrin, sublethal, sex pheromone, calling behavior, pheromone biosynthesis activating neuropeptide (PBAN).

INTRODUCTION

The behavior of insects, as of all animals, is governed by complex interactions among neurons within their nervous systems. Insecticides have been selected and sometimes designed for their remarkable abilities to kill insects. Most of them

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attack specific sites within the insect nervous system (Matsumura, 1985). Sublethal effects occur when insufficient amounts of an insecticide to cause death reach the sites of insecticide action.

In recent years, there have been only a few detailed studies concerning the potential behavioral effects of sublethal doses of insecticides. For example, sublethal doses have shown the ability to change calling behavior in *Pectinophora gossypiella* (Haynes and Baker, 1985), the wing fanning response of male *P. gossypiella* to pheromones (Floyd and Gatehouse, 1981), and the pheromone-mediated flight behavior of *Grapholita molesta* (Linn and Roelofs, 1984). The potential for control of behavior with a sublethal quantity of a neurotoxic insecticide is just beginning to be realized. These early studies have already revealed that further research on the recovery of calling behavior and pheromone production affected by sublethal neurotoxicants, and on the mechanisms by which sublethal effects on chemical communication systems are brought about are especially necessary today.

The production of sex pheromone in a number of moth species follows a diel rhythm, which may be regulated by neural (Tang et al., 1987), neuroendocrine (Raina and Klun, 1984), or hormonal factors (Cusson and McNeil, 1989). The production of sex pheromone in the Asian corn borer, *Ostrina furnacalis*, appears to be under the control of a neuroendocrine regulation factor, pheromone biosynthesis activating neuropeptide (PBAN; Qiu and Cao, 1989). However, it is not clear if and how the sublethal neurotoxicants may affect this process. In this paper, we describe effects of sublethal doses of insecticide on calling behavior, sex pheromone production, and PBAN-like activity in *O. furnacalis*.

METHODS AND MATERIALS

Insect. Larval *O. furnacalis* were reared on artificial diet modified from that described by Qiu Qiu and Cao (1989). Pupae were separated by sex. Male and female moths were held in $25 \times 15 \times 35$ cm screened cages housed within separate environmental chambers (14:10 light–dark photoregime, $26 \pm 1^{\circ}$C). Moths had access to 10% sugar water.

For experiments using decapitated females, freshly emerged virgins were decapitated with scissors at the onset of scotophase. The decapitated females were kept in a box at 26°C with a light–dark cycle of 14:10 hr and normally used for experiments after 24 hr.

Chemicals and Sublethal Dose. Technical grade deltamethrin (ROUSSEL-UCLAF, France) was diluted in distilled acetone. Solutions of deltamethrin were prepared at 0.1, 1, and 10 ng/μl within 1 week of use. Adult female moths were dosed respectively with 0.1 ng ($N = 50$), 1 ng ($N = 50$), and 10 ng ($N = 50$) of deltamethrin in acetone (1 μl) on the ventral portion of the abdomen to determine

sublethal doses. The control females were dosed with 1 μl of acetone ($N = 50$). The mortality for each group was recorded at 24 hr and 100 hr after treatment. The dose that caused little mortality (<5%) by 24 hr after treatment and less than 30% mortality by 100 hr was chosen as a sublethal dose for further research.

Calling Behavior Observation. Calling behavior of deltamethrin-treated or control virgin female *O. furnacalis* was observed in an environmental chamber (26°C, 14:10 light–dark photoregime) with dim incandescent red backlighting in an otherwise dark room. Calling behavior was defined as gland protrusion. At 30-min intervals throughout the 10 hr scotophase, female moths were noted as calling or not calling.

GC Analytical Methods. The samples used for analyses by gas chromatography (GC) were taken from the sex pheromone gland of female *O. furnacalis* at the peak period of calling (about 8 hr after the initiation of scotophase). The female Asian corn borer moth possesses a sex pheromone gland located at the intersegmental membrane between the 8th and 9th abdominal segments. Abdominal tips with pheromone gland of 2-day old calling females were removed and placed in redistilled hexane. After extraction for 30 min, tips were removed, and the extract was maintained at 0°C for analyses. Female moths (30) at the peak period of calling in their second scotophase were used for studies of sex pheromone biosynthesis.

Gas chromatographic analyses of the extracts were performed on an Hewlett Packard 5890 capillary gas chromatograph equipped with a 25 m $\times$ 0.2 mm i.d. HP-17 column and a flame ionization detector. The carrier gas was nitrogen, and the GC injector was set at 200°C and the detector at 250°C. GC oven temperature was maintained at 100°C for 2 min, and then raised at 10°C/min to 200°C for a final hold time of 15 min.

Brain-Subesophageal Ganglion (Br-SOG) Extracts. At least 50 Br-SOG complexes of *O. furnacalis* were dissected from 2- or 3-day-old normal female moths and 1 ng deltamethrin-treated female moths at the 5th hr of scotophase. Br-SOG complex was homogenized in a cold sodium phosphate buffer (188.0 mM NaCl, 20.1 mM KCl, 9.0 mM $CaCl_2$ and 1.0 mM $MgSO_4$, pH 6.6) containing 35 mM sucrose in a 2-ml glass homogenizer. The homogenate was centrifuged at 10,000g for 10 min, and the supernatant was kept on ice for injection at a dose of 1 Br-SOG equivalent/10 μl.

Different doses of supernatant were injected into the 5–6th abdominal segment of decapitated moths to induce sex pheromone production.

RESULTS

Sublethal Dosage. Mortality caused by different dosages of deltamethrin is summarized in Table 1. Doses of 0.1 and 1 ng of deltamethrin caused little mortality (<5%) by 24 hr after treatment, and mortality observed 100 hr after treatment was less than 30%. We chose 1 ng of deltamethrin treatment as a sublethal dosage in

TABLE 1. MORTALITIES OBSERVED BY 24 HR AND 100 HR AFTER DELTAMETHRIN TREATMENT ($N = 50$)

Time after treatment	Mortality			
		Deltamethrin (ng)		
	Control	0.1	1	10
24	0	0	2%	4%
100	2%	16%	24%	60%

our experiments to determine the sublethal effects of insecticide on the chemical communication system and PBAN-like activity of *O. furnacalis*.

Sublethal Effects of Deltamethrin on Calling Behavior. One-day-old adults were treated with deltamethrin at 1 hr before the beginning of scotophase. Moths were cold anesthetized on ice during the dosing procedure. The calling behavior of the virgin females treated with sublethal deltamethrin (1 ng) was observed in an environmental chamber (26±1°C, 14:10 light–dark photoregime) with dim red backlighting in a dark room. The observations were conducted at half-hour intervals, and the tested females were designated as either calling or not calling (Figure 1).

The calling behavior of females was significantly affected by sublethal doses of deltamethrin (Figure 1). At the peak of calling (about 8 hr after the initiation of scotophase), there was a significant difference of calling probabilities between control females and sublethal deltamethrin-treated female moths. Only 32% of deltamethrin-treated female moths performed calling behavior, while 100% calling was observed for control female moths. We also studied the recovery of calling behavior of females treated with sublethal deltamethrin. The experimental results showed that the percentages of calling at the 2nd and 4th day after treatment with deltamethrin (1 ng) were only 36 and 46%, respectively, comparing to 100% calling for control females (Figure 1).

Interestingly, the peak of calling came at almost the same time for both control females and sublethal deltamethrin-treated female moths (Figure 1). The calling peak occurred at 8.1 ± 1.35, 7.8 ± 1.35, and 7.8 ± 1.36 hr at the 1st, 2nd, and 4th day after treatment, respectively. Meanwhile, from the 1st to the 4th day, control females also had a similar calling peak time at 7.8 ± 1.38 hr. No significant difference of calling peak time was found between the 1 ng deltamethrin-treatment females and control moths (ANOVA followed by Duncan's multiple range test). Treatment with sublethal deltamethrin apparently does not change calling periodicity, but rather the calling probability.

The recovery of calling behavior of females was incomplete 4 days after treatment with deltamethrin. However, there was a trend toward recovery. This

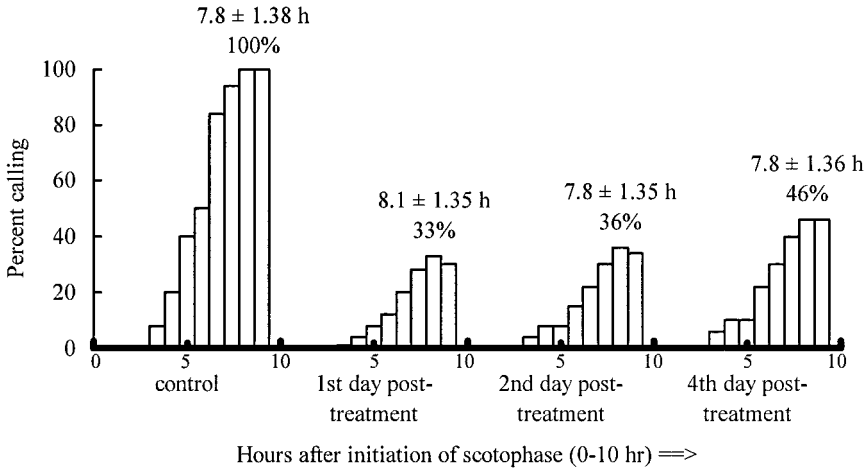

FIG. 1. Sublethal effects of deltamethrin (1 ng per female) on calling behavior of female *O. furnacalis*. Calling peak occurred at 8.1 ± 1.35, 7.8 ± 1.35, and 7.8 ± 1.36 hr at the 1st, 2nd, and 4th day after treatment, respectively. From the 1st–4th day, the calling percentages of control females were 100%, with a similar calling peak time occurring at 7.8 ± 1.38 hr. No significant difference of calling peak time was found between the 1 ng-deltamethrin-treated females and control moths (ANOVA followed by Duncan's multiple range test). Control females and the 1st-day posttreatment females were dosed 1 h before the beginning of scotophase.

result indicated that sublethal deltamethrin interfered with female calling over a relatively long period. In the field, a reduced probability of calling would be expected to cause a decrease of mating rate. As a result, the offspring population density of insects should be reduced.

Sublethal Effects on Sex Pheromone Biosynthesis in O. furnacalis. The above results show that sublethal deltamethrin interferes with female calling behavior. Next, we asked whether a sublethal deltamethrin dose would also affect sex pheromone biosynthesis in *O. furnacalis* females. The sex pheromone of female *O. furnacalis* contains both *E*- and *Z*- isomers of 12-tetradecenyl acetate. We determined the sex pheromone titer and the *E*/*Z* ratio of pheromone components by GC analyses.

Mean (±SE) sex pheromone titers are given in Table 2. A significant difference between deltamethrin treatment and control female moths ($P < 0.05$, by *t*-test assuming equal variances) was observed. The sex pheromone titer at the 1st day posttreatment was only 15% of that of control females. At the 2nd and 4th day posttreatment, sex pheromone titers were 20% and 25% of those of control females. It is clear that the chemical communication system was affected by sublethal insecticide even at the 4th day of posttreatment.

TABLE 2. RECOVERY OF CHEMICAL COMMUNICATION SYSTEM OF *O. furnacalis* DOSED AT 1 NG PER FEMALE OF DELTAMETHRIN ($N = 30$)

Days after treatment	Chemical communication system	Control[a]	1 ng deltamethrin treatment[a]	Percent of control	t-test[b]
1	Sex pheromone titer	9.10 ± 3.42	1.39 ± 0.60	~15%	$P < 0.05$
	$E/(E + Z)$	0.43 a	0.45 a		
	C.V.%	4.1	9.7		
2	Sex pheromone titer	7.99 ± 3.92	1.55 ± 1.18	~20%	$P < 0.05$
	$E/(E + Z)$	0.44 a	0.41 a		
	C.V.%	5.5	6.8		
4	Sex pheromone titer	4.79 ± 2.74	1.19 ± 0.84	~25%	$P < 0.05$
	$E/(E + Z)$	0.43 a	0.41 a		
	C.V.%	9.6	9.1		

[a] The treatment in each experiment were compared after ANOVA by Duncan's multiple range test. Values followed by the same letter were not significantly different.
[b] t-Test assuming equal variances.

Interestingly, the E/Z ratio of pheromone components was not significantly changed (ANOVA followed by Duncan's multiple range test). The variation coefficients (C.V.%) of E/Z ratio of pheromone components at the 1st, 2nd, and 4th day of posttreatment were 9.7, 6.8, and 9.1%, respectively (Table 2). The results indicated that the sexual chemical communication system could be controlled within a narrow range.

Sublethal Effects on PBAN-Like Activities of Female O. furnacalis. PBAN-like activities might well be influenced by deltamethrin, since pheromone production is controlled by PBAN, and our above-described research results indicate that deltamethrin affected pheromone production. In order to verify this, the following experiments were carried out.

The homogenates of Br-SOG complex of normal female moths and 1 ng deltamethrin-treated female moths were serially diluted to 1, 0.5, 0.25, and 0.125 female brain equivalent (FE) per 10 μl of saline, respectively. These different doses of homogenate were injected into decapitated females to induce sex pheromone production. The sex pheromone glands were taken from these moths after 3 hr and extracted with redistilled hexane for 30 min. The extract was used for GC analyses. The dose-response bars of control moths and 1 ng deltamethrin-treated ones are given in Figure 2.

Pheromone productions of 8.2 ± 3.3, 7.8 ± 2.8, 7.4 ± 2.7, and 4.1 ± 1.6 ng per female, respectively, were determined when 1, 1/2, 1/4, and 1/8 FE of Br-SOG homogenate of normal females (2- or 3-day-old) were injected into decapitated females. In comparison, pheromone productions were induced to 5.8 ± 2.5, 2.8 ± 1.3, 2.8 ± 1.2, and 0.7 ± 0.5 ng per female when 1, 1/2, 1/4, and 1/8 FE of Br-SOG homogenate of 1 ng deltamethrin-treated females were injected in

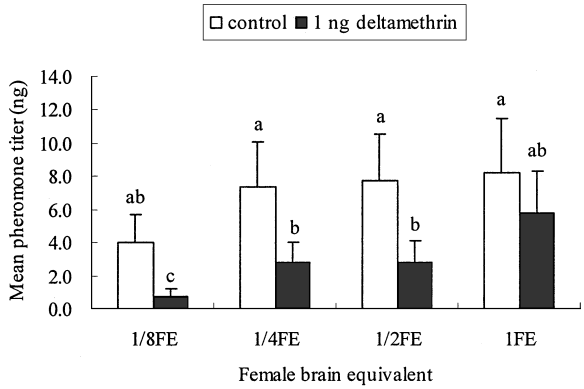

FIG. 2. Comparison of pheromone production in decapitated females injected with Br-SOG homogenate of normal females and sublethal deltamethrin-treated females. Bars represent the mean ± SE. Different letters at the top of bars within each group indicate means which are significantly different at $P < 0.05$ (ANOVA followed by Duncan's multiple range test).

decapitated females (Figure 2). The observed decrease of pheromone emission in dose-response bars of Br-SOG could be due to the difference of PBAN-like activity of female *O. furnacalis*.

DISCUSSION

The release of pheromone in *O. furnacalis* and most other species of moths depends on an active behavioral process of everting the pheromone gland. We found that sublethal doses of deltamethrin substantially decreased the probability that females would call, without changing the periodicity of this activity (Figure 1). Similar results were found in *Glossina austeni* Newst and *Pectinopora gossypiella* (Haynes and Baker, 1985). The effects of deltamethrin on the behavior of calling in female *O. furnacalis* would be expected to reduce the chance of mate location since the response from males depends on the volatile pheromone released by the females.

As far as we know, an effect of sublethal deltamethrin on female sex pheromone biosynthesis has not been documented previously. Sex pheromone biosynthesis in insects involves a complex coordination of physiological activities. Pheromone emission by *O. furnacalis* is controlled by interrelated physiological and behavioral factors. It was suggested that calling behavior and transport of pheromone to the gland surface are synchronized in time (Hunt and Haynes, 1990). These activities are under hormonal and neuronal controls. Our data suggest that sublethal deltamethrin could interfere with this process.

The ratio of *Z*- and *E*-isomers of sex pheromone are important for reproductive isolation between species (Cardé and Baker, 1984). The pheromone components, (*E*)- and (*Z*)-12-tetradecenyl acetates of *O. furnacalis*, are biosynthesized from palmitic acid by Δ14 desaturation, followed by chain shortening (β-oxidation), reduction, and acetylation (Zhao et al., 1995). Our data reveal that sublethal deltamethrin does not interfere with this regulation model.

As in other moth species, PBAN controls sex pheromone biosynthesis in *O. furnacalis*. However, the process is rather complicated. Our study indicates that sublethal deltamethrin affects not only pheromone production but also the activity of PBAN-like factor. Certainly, the decrease of PBAN-like factor activity will reduce pheromone production. Further studies in this area are under way.

This research indicates that the application of sublethal doses of insecticides may be useful for the control of pest insect populations. In some cases, chemicals may have been rejected for economic or environmental reasons when they were assessed solely on the basis of mortality. It now appears that some chemicals might be effective in Integrated Pest Management at a fraction of the cost and with minimal environmental impact. Sublethal effects of deltamethrin on female calling and pheromone production of female *O. furnacalis* could be expected to interfere with female reproductive capacity by decreasing the mating rate. As a result, the pest insect population density could be reduced. We hope that our research in this area may help develop a new technique for pest control.

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OVIPOSITION STIMULANTS FOR THE TROPICAL SWALLOWTAIL BUTTERFLY, *Papilio polytes*, FEEDING ON A RUTACEOUS PLANT, *Toddalia asiatica*

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Abstract—In nature, *Papilio polytes* utilizes a limited range of rutaceous plants as hosts. We isolated and identified oviposition stimulants for the butterfly from the foliage of its primary host plant *Toddalia asiatica*. Females readily deposited eggs in response to a methanolic extract of the plant. Partition of the extract with organic solvents revealed that chemicals responsible for eliciting egg-laying resided in a water-soluble fraction. Further bioassay-guided fractionation of the active fraction by column chromatography, preparative TLC, and HPLC led to the isolation of two oviposition stimulants. One was isolated from an amphoteric fraction and identified as *trans*-4-hydroxy-*N*-methyl-L-proline [(−)-(2*S*,4*R*)-4-hydroxy-1-methyl pyrrolidine-2-carboxylic acid; HMP]. The other, isolated from an acidic fraction, was identified as 2-*C*-methyl-D-erythronic acid [(−)-(2*R*,3*R*)-2-methyl-2,3,4-trihydroxybutanoic acid; MEA]. HMP alone evoked significant oviposition-stimulatory activity, although this was much lower than that of the original water-soluble fraction. MEA, on the other hand, alone did not elicit positive responses from females. However, HMP, when assayed in combination with MEA, markedly enhanced the female response, and the mixture was as active in stimulating oviposition as were the original water-soluble fraction and the plant foliage. We conclude that HMP is a substance crucial for host recognition by females, while MEA is a synergistic stimulant involved in host recognition and/or preference.

Key Words—Oviposition stimulants, host selection, *Papilio polytes*, Papilionidae, *Toddalia asiatica*, Rutaceae, *trans*-4-hydroxy-*N*-methyl-L-proline, 2-*C*-methyl-D-erythronic acid.

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INTRODUCTION

During the last two decades, studies of chemical interactions between herbivores and plants have established that plant secondary metabolites in general play significant roles in host utilization by phytophagous insects (Miller and Strickler, 1984; Feeny, 1992; Städler, 1992). A number of studies conducted on phytochemicals responsible for acceptance or rejection of potential hosts by ovipositing females of lepidopterans have also revealed that stimuli evoked by particular secondary metabolites determine host choice by females (Renwick and Chew, 1994; Honda, 1995; Honda and Nishida, 1999). In butterflies, assessment of potential suitable hosts is achieved mainly by drumming on the surface of foliage with foretarsi, during which plant chemicals are perceived by chemotactile sensory receptors. Extensive work on butterfly oviposition has been performed with the Papilionidae, Pieridae, and Danaidae families, and the cumulative results clearly demonstrate that the dominant factors, serving at close range as the crucial cues that permit females to discriminate and to recognize their hosts, are the chemical attributes of plants that can be characterized as mostly nonvolatile secondary metabolites (Honda and Nishida, 1999), though olfactory cues from host plants also stimulate alighting on plants, and hence egg-laying by females (Feeny et al., 1989; Baur et al., 1993; Baur and Feeny, 1995).

An array of oviposition stimulants has been reported for *Papilio* butterflies feeding on Rutaceae, Apiaceae, or Lauraceae (e.g., Ohsugi et al., 1985; Honda, 1986, 1990; Nishida et al., 1987; Feeny et al., 1988; Ohsugi et al., 1991; Nishida, 1995; Carter et al., 1998, 1999; Ono et al., 2000a,b). Major compounds identified include amino acid derivatives, sugar-related acids, alkaloids, flavonoids, and hydroxycinnamic acid derivatives. Among the stimulants for *Papilio* butterflies, some are common stimulants for other species, and others are structurally related within a given class of compounds, thereby strongly suggesting that phytochemicals mediate host specialization on one hand, and expansion of host range on the other. Relationships between phylogeny and the host range of Papilionidae have long been an unsettled issue that has gained enthusiastic attention of many scientists and amateur researchers (e.g., Miller, 1987a,b; Aubert et al., 1999). Even so, insufficient knowledge of a chemical basis for differential acceptance of potential hosts by both larvae and ovipositing females has been accumulated. Comprehensive investigations on the plant chemicals that govern behaviors (feeding and egg-laying) are thus needed to clarify whether or not papilionids have coevolved with their host plants, and how they have accomplished drastic host shifts, probably from Aristolochiaceae, through Magnoliaceae, Lauraceae, Annonaceae, and Rutaceae, eventually to Apiaceae and Asteraceae.

Papilio polytes Linnaeus is a Rutaceae-feeding papilionid butterfly inhabiting Southeast Asia and the Southwestern Islands of Japan. The butterfly specializes on *Toddalia asiatica* Lam., but occasionally infests another rutaceous plant, *Citrus*

depressa Hayata in Japan. Larvae perform no worse on *C. depressa* than on *T. asiatica* (Murakami et al., 2003). According to a chemotaxonomic examination of the Rutaceae (Dreyer, 1966), *T. asiatica*, belonging to Toddalioidae, is deemed one of the primitive plants of the family. *T. asiatica* is also utilized as a host by many other *Papilio* species distributed in Southeast Asia. The associations between *Papilio* butterflies and the plant, and its phylogenetic disposition, prompted us to study what kind of phytochemicals in *T. asiatica* stimulate egg-laying by females of *Papilio* butterflies. The outcome will be of great importance for deducing the ancestral system of host recognition exploited by the Papilionidae, and for elucidating the physiochemical causes for their host shifts. The present paper addresses oviposition stimulants for *P. polytes* identified from *T. asiatica*.

METHODS AND MATERIALS

Insects. Adults of *P. polytes* used for the behavioral bioassay were gravid females derived from laboratory stock cultures that originated from a natural population collected in the Yaeyama Islands and fed on *T. asiatica*. Larvae were reared at 25°C under a 16L: 8D regime on potted plants of *T. asiatica* and some *Citrus* spp., on which immatures are known to complete their larval and pupal development. Females were fed once daily with 15% aqueous sucrose solution.

Extraction and Fractionation of Plant Materials. Young foliage of *T. asiatica* (800 g), collected in Okinawa Prefecture, was extracted with methanol (6 l) at room temperature for 1 month. The methanol extract was filtered and concentrated *in vacuo* below 50°C, and an aliquot of the whole concentrate, after being dispersed in water, was extracted successively with chloroform and isobutanol to give three fractions (two organic and one aqueous fractions). The aqueous layer was fractionated by column chromatography on porous polymer gel (MCI gel CHP20P, 75–150 μm, Mitsubishi Kasei Co. Ltd.) and eluted stepwise with H_2O (fraction 1), 25% aq. MeOH (fraction 2), 50% aq. MeOH (fraction 3), and MeOH (fraction 4). Fraction 1 was further separated into acidic (A), neutral/amphoteric (N), and basic (B) fractions employing cation exchange resin (Accell Plus CM, Waters) and anion exchange resin (Accell Plus QMA, Waters). The general procedure for ion exchange chromatography was as follows. An aqueous solution of the sample deposited on CM resin (H^+ form) was eluted successively with water and 3% aq. NH_3 solution to give an N plus A fraction and fraction B, respectively. The former fraction (N plus A) was subjected to anion exchange chromatography on QMA resin (OH^- form), eluted successively with water (fraction N) and 50 mM aq. KH_2PO_4 solution (fraction A). Each fraction, once lyophilized, was stored in 50% aq. MeOH below 0°C until use.

Isolation of Active Compounds. Compound **1** was isolated from fraction N. Fraction N was first subjected to rough separation with a TLC stick (30 × 30 ×

130 mm, GL Science). The stick was developed with MeOH and a fraction (R_f: 0.35–0.53) was collected. This fraction was further fractionated by preparative TLC (Merck PLC plate Silica gel 60, MeOH, R_f: 0.29), and final purification of compound **1** was achieved by HPLC on a reversed phase column (Mightysil RP-18 GP Aqua, 4.6 mm i.d.$\times$250 mm, Kanto Chemical Co., Inc.), eluted with water at a flow rate of 0.5 ml/min (R_t: $\sim$6 min). HPLC was carried out with a JASCO GULLIVER PU-986 liquid chromatograph, monitoring the column eluates with a differential refractometer (Shodex RI SE-51).

Compound **2** was isolated from fraction A by a similar method. Fraction A was separated by preparative TLC (Merck PLC plate Silica gel 60; MeOH, R_f: 0.56–0.81), and subsequently, the corresponding fraction was purified by HPLC (Mightysil RP-18 GP Aqua, 10 mm i.d. $\times$ 250 mm, Kanto Chemical Co., Inc.), eluted with water at a flow rate of 1.0 ml/min to yield compound **2** (R_t: 14.3 min).

Bioassay for Oviposition Response. Behavioral bioassays were conducted according to a previously reported method (Honda, 1986) employing artificial green plastic leaves as ovipositional substrates. A leaf surrogate treated with a test sample was presented to a free-flying female kept in a transparent plastic chamber externally illuminated with an incandescent lamp (3500 lux). The amount of test materials applied to the leaf surrogate was estimated at $\sim$50 μg/cm^2 when the leaf was treated with a 0.5% solution of a test sample. Before testing, females were screened daily to assess their responsiveness, and only those that showed positive responses to the foliage of *T. asiatica* and negative responses to water alone (control) were chosen. Unresponsiveness of the females to the control was confirmed every three trials of sample presentation to remove any misleading outcome brought about by carry-over effects. Appraisal of female responsiveness to each sample was based on the criteria given in a previous paper (Honda et al., 1997a): in each trial (sample presentation), the response of an individual was scored as 100% for actual egg-laying or an equivalent behavior, 50% for half-curling the abdomen with continuous drumming (this behavior takes place much less frequently), and 0% for drumming only with no positive response. Trials were replicated more than five times for each individual, and the responses of an individual to a given sample were averaged. For all trials, merely alighting on ovipositional substrates without drumming was not included. Oviposition response to each sample was finally represented as the mean percentage of responses recorded from 15 females. Samples were tested at 0.5% w/v unless otherwise noted.

Instrumentation for Structural Elucidation. ^{1}H and ^{13}C NMR spectra were measured in D_2O on a JEOL JNM-A400 FT-NMR spectrometer with tetramethylsilane as the external standard. Chemical shifts were represented by δ units. Multiplicities of proton signals or carbon resonances determined by DEPT were abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). FAB-mass (FAB-MS) and high resolution FAB-mass (HR-FAB-MS) spectra

were obtained with a JEOL SX 102A mass spectrometer using glycerine as a matrix. Optical rotation was measured with a JASCO DIP-370 digital polarimeter at 23°C in H_2O.

Synthesis of Trans-4-Hydroxy-N-*Methyl*-L-*Proline.* Authentic *trans*-4-hydroxy-*N*-methyl-L-proline (HMP) was prepared by the method of Sondengam et al. (1973). A solution of *trans*-4-hydroxy-L-proline (200 mg) and 35% aq. formaldehyde (2 g) dissolved in MeOH (4 ml) was refluxed for 2 hr. The reaction mixture had 300 mg of $NaBH_4$ added and was then held at room temperature with stirring for 1 hr. After evaporating the solvent to dryness *in vacuo*, the residue was redissolved in MeOH and filtered. Removal of MeOH from the mother liquor by evaporation gave crude HMP, which was purified by preparative TLC (Merck PLC plate Silica gel 60, MeOH: 28% aq. $NH_3 = 85$: 15, R_f; ~0.75) to afford pure HMP as colorless crystals.

RESULTS

Our previous work (Nakayama et al., 2002) revealed that the water-soluble fraction derived from a methanolic extract of *T. asiatica* strongly evoked a positive response (90.0%) from ovipositing *P. polytes* females when assayed at a concentration of 0.5%, suggesting the presence of oviposition stimulant(s) exclusively in aqueous media. In this study, the aqueous fraction was separated by column chromatography into four fractions (fractions 1–4). The relative proportions of the fractions to the fresh weight of the foliage were approximately 3.2%, 0.31%, 0.35%, and 0.22%, respectively. Fraction 1 elicited a potent positive response, whereas the other three exhibited no appreciable stimulatory activity as shown in Figure 1. In the next experiment, three subfractions [acidic (A), neutral/amphoteric (N), and basic (B)] prepared from fraction 1 by ion exchange chromatography were tested singly or in combination. Fractions A and B were inactive by themselves,

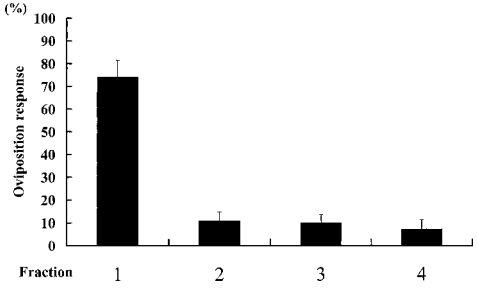


FIG. 1. Oviposition responses (mean ± SE) of *P. polytes* to fractions prepared from the aq. fraction by column chromatography on porous polymer gel. Each sample was tested at 0.5%. $N = 15$.

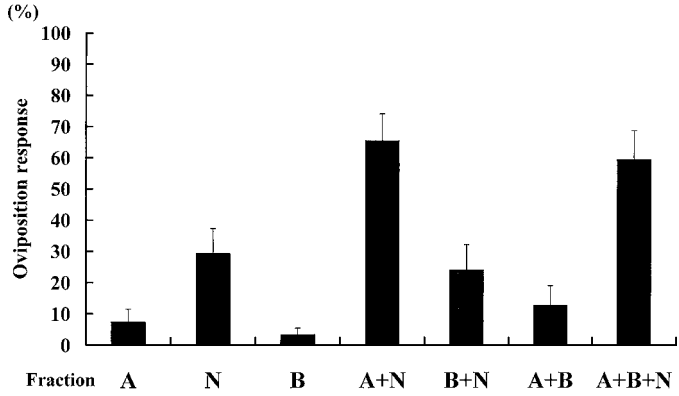


FIG. 2. Oviposition responses (mean ± SE) of *P. polytes* to fractions and their mixtures prepared by ion exchange chromatography. A: acidic fraction, N: neutral/amphoteric fraction, B: basic fraction. Binary and ternary mixtures consist of an equivalent quantity of individual fractions. Each sample was tested at a sum total of 0.5%. $N = 15$.

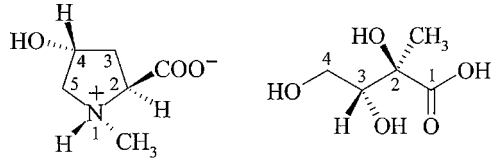
while only fraction N was found weakly stimulative (Figure 2), thereby suggesting the involvement of more than one compound in releasing oviposition. In fact, when the three fractions were recombined, the sample resumed an activity comparable to that of the original fraction (fraction 1). Moreover, Figure 2 clearly indicates that a particular combination of two fractions, N and A, suffices to induce egg-laying by females and that fraction B had no synergistic effect, although a mixture of fractions A and B appeared only slightly stimulative. Since fraction A alone exerts no activity, it was concluded that the main oviposition stimulant(s) are present in fraction N and that fraction A contains at least one distinct stimulant that acts as the synergist. The chemical constituents in the two fractions were examined in detail. Investigation of fraction N by ^{13}C NMR spectroscopy revealed the occurrence of a characteristic compound (**1**) as a dominant component as well as relatively smaller amounts of ubiquitous sugars such as sucrose and glucose. Fractionation of the fraction by TLC and HPLC followed by preliminary bioassays showed that compound **1** was the active principle. In contrast, fraction A was found to comprise compound **2** (a major component), quinic acid, and other unidentified minor substances. Through oviposition bioassays, quinic acid and the minor miscellaneous compounds were shown to conduce little to the enhancement of the activity of compound **1**. Structural elucidation of compounds **1** and **2** was attempted on the basis of their spectroscopic and physicochemical properties.

Compound 1. FAB-MS: $(M-H)^-$ at m/z 144. ^{1}H NMR, δ(ppm): 1.83 (H-3b, ddd, $J = 4.8, 10.9, 14.0$ Hz), 2.05 (H-3a, broad dd, $J = 7.4, 14.0$ Hz), 2.59 (N-CH_3, s), 2.73 (H-5a, broad d, $J = 12.7$ Hz), 3.51 (H-5b, dd, $J = 4.7, 12.7$ Hz),

3.72 (H-2, dd, $J = 7.4$, 10.9 Hz), and 4.21 (H-4, m)("a" denotes a *trans* proton to the carboxyl group and "b" *cis*). ^{13}C NMR δ(ppm): 38.2 (t), 43.0 (q), 62.6 (t), 69.4 (d), 70.0 (d), and 173.2 (s). $[\alpha]_D^{23} = -74.0^\circ$ (H_2O, c1.0). The molecular formula of this compound, having a mol wt of 145, was determined to *definitely* be $C_6H_{11}O_3N$ by exact mass measurement (HR-FAB-MS; Calc. for $C_6H_{10}O_3N$, 144.0661. Obs., 144.0662). ^{13}C NMR signals suggested the existence of one methyl carbon (38.2 ppm) attached to a nitrogen atom and one carboxyl carbon (173.2 ppm). The other resonances seemed to be assignable to a proline skeleton. Since all these spectral data and the optical rotation coincided with those previously reported for *trans*-4-hydroxy-*N*-methyl-L-proline ($[\alpha]_D^{25} = -82.7^\circ$; Sciuto et al., 1983; Jones, 1987), compound **1** was identified as [(−)-(2*S*,4*R*)-4-hydroxy-1-methylpyrrolidine-2-carboxylic acid; HMP]. The identity was further corroborated by direct comparison with an authentic sample prepared from *trans*-4-hydroxy-L-proline.

Compound 2. FAB-MS: $(M-H)^-$ at m/z 149. ^{1}H NMR, δ(ppm): 0.95 (CH_3,s), 3.18 (H-4, d, $J = 5.6$ Hz), 3.39 (H-3, t, $J = 5.6$ Hz). ^{13}C NMR δ (ppm): 22.6 (q), 62.5 (t), 76.1 (d), 77.1 (s), 181.0 (s). This compound, an acidic substance of molecular weight 150, possessing one methyl carbon, three hydroxy-substituted carbons, and one carboxyl carbon, was deemed to be one of the four possible stereoisomers of 2-methyl-2,3,4-trihydroxybutanoic acid, which should have two asymmetric carbons. To determine the configuration at C-2 and C-3, this compound was converted to the corresponding lactone (**2′**) (2,3-dihydroxy-2-methyl-γ-butyrolactone) with BF_3 in aqueous media, because the lactone has already been reported from some plants. Compound **2′** gave the following NMR data. ^{1}H NMR δ (ppm): 1.04 (CH_3, s), 3.83 (H-3, d, $J = 3.7$ Hz), 3.89 (H-4a, d, $J = 11.0$ Hz), 4.17 (H-4b, dd, $J = 3.7$, 11.0 Hz)("a" denotes a *cis* proton to the hydroxy group and "b" *trans*). ^{13}C NMR, δ (ppm): 20.3 (q), 73.0 (d), 73.2 (t), 74.2 (s), 180.7 (s). These parameters were in good agreement with those of a known compound, (−)-2-*C*-methyl-D-*erythrono*-1,4-lactone (Teresa et al., 1980; Ford, 1981; Ahmed et al., 1995; Ono et al., 2000b). Compound **2′** was, therefore, considered to have either (*R*, *R*) or (*S*, *S*) configuration. Although attempts to measure an accurate value of optical rotation of compound **2′** were unsuccessful because of the paucity of the sample available, the compound proved to be a (−)-isomer. This indicated that compound **2′** had an (*R*, *R*) configuration, which, in turn, substantiates that compound **2** was also an (*R*, *R*)–isomer. Consequently, compound **2** was concluded to be (−)-(2*R*,3*R*)-2-methyl-2,3,4-trihydroxybutanoic acid [2-*C*-methyl-D-erythronic acid; MEA]. The chemical structures of compounds **1** and **2** are shown in Figure 3.

The averages of HMP and MEA concentrations in young leaves of *T. asiatica* were estimated at ∼0.4% and 0.1%, respectively (based on data obtained from three sample collections at different localities). The oviposition-stimulatory activity of the two compounds was examined in the next experiment (Figure 4), in which synthetic HMP was used. HMP alone displayed moderate activity (34.8%



1 2

FIG. 3. Chemical structures of oviposition stimulants for *P. polytes* identified from *T. asiatica*. **1**: *trans*-4-hydroxy-*N*-methyl-L-proline (HMP), **2**: 2-*C*-methyl-D-erythronic acid (MEA).

response) in releasing egg-laying, and female response increased to 70.8% even at a lower concentration (0.25%) after the addition of an equivalent amount of fraction A, despite the inertness of the fraction (Figure 2). This undoubtedly indicates that such marked enhancement of female responsiveness was due to the synergistic action evoked by fraction A. On the other hand, MEA, isolated from fraction A was, as predicted, almost inactive in itself (below 10% response at 0.5%, $N = 5$). However, HMP and MEA, when tested in combination at their respective concentrations found in living plants, strongly stimulated oviposition by females, and the stimulatory activity (95.3% response) of the mixture exceeded by far that of the parent aqueous fraction (1 in Figure 1) and reached the level comparable to that (94.5% response) evoked by the foliage of *T. asiatica* (Nakayama et al., 2002). These results suggest that *P. polytes* females make use of only two

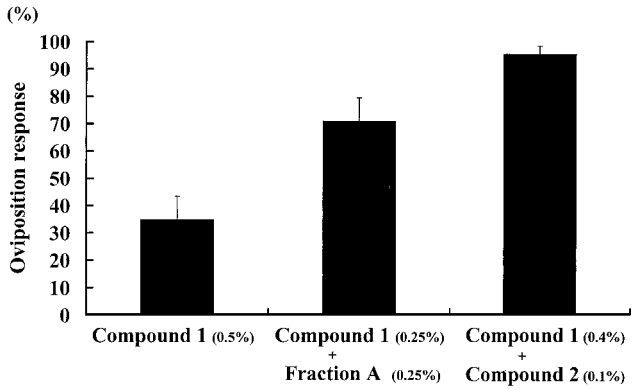


FIG. 4. Oviposition responses (mean ± SE) of *P. polytes* to compounds identified from *T. asiatica*. Compound **1**: HMP (synthetic), compound **2**: MEA (isolated). Concentrations of respective components in each sample are given in parentheses. $N = 15$.

simple compounds as the crucial chemical cues in recognizing *T. asiatica* as a host.

DISCUSSION

In the present work, two compounds from *T. asiatica* were identified as semiochemicals that impart crucial cues to ovipositing *P. polytes* females. The results indicate that HMP present in *T. asiatica,* exhibiting moderate oviposition-stimulatory activity by itself, is the stimulant responsible for host recognition by ovipositing *P. polytes* females. MEA, which, though inactive alone, concurrently promoted female responses together with HMP, may also be regarded as a significant synergist involved in host recognition and/or host preference. These two compounds are new oviposition stimulants for the Papilionidae. The fact that a combination of only two components, viz. HMP and MEA, was as active in eliciting oviposition as was the living foliage of the host plant strongly suggests that apart from possible implication of olfactory stimuli (volatile chemicals) for host seeking, the chemotactile stimuli invoked by just the two compounds virtually regulate egg-laying by *P. polytes* on *T. asiatica*. In many *Papilio* butterflies, extreme synergism of multiple components has been reported to feature in their stimulatory system of oviposition (e.g., Nishida et al., 1987; Feeny et al., 1988; Honda, 1990; Ohsugi et al., 1991). Although there have been a few examples in which a single component moderately induced oviposition (Honda et al., 1997b; Ono et al., 2000a), which is also the case with *P. polytes*, it seems to be a general notion that more than four or five compounds are required to elicit sufficient oviposition responses from females, particularly, of Rutaceae-feeding species. The two-component system on which *P. polytes* females assess their host, therefore, appears to be the simplest ever documented for oviposition by *Papilio* butterflies colonizing rutaceous plants.

HMP has been reported from several plant sources: the bark of *Croton gubouga* (Euphorbiaceae; Goodson and Clewer, 1919), the heartwood of *Afromosia elata* (Fabaceae; Morgan, 1964), *Copaifara* (Fabaceae; Figliuolo et al., 1987), and the red alga *Chondria coerulescens* (Sciuto et al., 1983). Certain nitrogenous compounds such as nonprotein amino and imino acids, which are synthesized and stored in plant tissue, occasionally in substantial amounts, are known to be toxic to insects, vertebrates, and microorganisms (Bell, 1977, 1981; Rosenthal and Bell, 1979; Rosenthal, 1982; Bernays, 1983; Brenner and Romeo, 1986; Romeo and Simmonds, 1989; Romeo, 1998). Figliuolo et al. (1987) have demonstrated that HMP, a nonprotein imino acid derivative, effectively inhibits larval development of the seed-eating bruchid beetle *Callosobruchus maculates* and deters larval feeding of the generalist moth *Spodoptera littoralis*. As a matter of course, larvae of *P. polytes* did not seem to be at all deterred from feeding or growing by the substance. However, that *P. polytes* females utilize HMP, which might possibly be

a potential autotoxin, as a crucial cue in host selection is intriguing and informative in understanding the mechanism underlying the coevolutionary interactions between butterflies and plants. As for MEA, its occurrence has been reported only from a few leguminous plants (Schramm et al., 1979), and the physiological effect of the compound on herbivores appears to be unelucidated.

HMP is similar in chemical structure to L-stachydrine (L-proline *N*, *N*-dimethylbetaine), already shown to be one of the oviposition stimulants for both *P. protenor* (Honda, 1990) and *P. xuthus* (Ohsugi et al., 1991), identified from *Citrus unshiu*. MEA is a compound closely related to 2-*C*-methyl-D-*erythrono*-1,4-lactone (γ-lactone of MEA), which has recently been reported as an oviposition stimulant for *P. bianor* feeding on another rutaceous plant *Orixa japonica* (Ono et al., 2000b). Moreover, MEA as well as D-quinic acid, which is also an important oviposition stimulant for *P. protenor* present in *C. unshiu* (Honda, 1990), are polyhydroxycarboxylic acids with short (5–7) carbon chains. The foliage of *T. asiatica* contained a considerable amount of quinic acid, and in the field *P. polytes* sometimes infests *C. depressa*, which preliminary chemical analysis has found to also have the acid (Honda, unpublished). In our experiments, however, quinic acid alone neither stimulated egg-laying nor exerted any synergistic effect on the action of HMP. These findings may provide useful clues to the elucidation of sensory mechanisms by which differential acceptance of potential hosts and shifts in host affiliation take place.

Haribal and Feeny (1998) reported that an Annonaceae-feeding swallowtail butterfly, *Eurytides marcellus* (tribe Graphiini), was induced to oviposit by a single compound, 3-caffeoyl-*muco*-quinic acid (3-C*m*QA), present in its primary host, *Asimina triloba*. To the best of our knowledge, this may be the simplest host-recognition system among the papilionids of which oviposition stimulants have hitherto been studied. From the fact that *E. marcellus* depends exclusively on 3-C*m*QA in contact assessment of its host and that some other cinnamic acid derivatives are utilized as stimulants by ovipositing females of several papilionids, the authors inferred that responses to hydroxycinnamic acid derivatives (hydroxycinnamates) may have been a primitive feature of Papilionidae. Indeed, *P. polyxenes* (an Apiaceae feeder; Feeny et al., 1988), *P. troilus* (a Lauraceae feeder; Carter et al., 1999), and a few Rutaceae feeders such as *P. protenor, P. macilentus*, and *P. bianor* (Honda, 1990; Honda and Nishida, 1999; Ono et al., 2000a) are known to be stimulated to oviposit by the action of certain hydroxycinnamates (different chemicals depending on the species involved) in synergy with other co-occurring stimulants (most of them uncharacterized). Nevertheless, it seems too early to argue that hydroxycinnamates might have constituted the major oviposition stimulants for ancestral papilionid butterflies, because knowledge available on other important synergists responsible for host selection by these species is limited. In Papilionidae, the genus *Papilio* is the largest taxon comprising about 40% of the family, and the majority (80%) of extant *Papilio* species

utilize rutaceous plants as larval food plants (Tyler et al., 1994). Although the ancestral host plants of the genus *Papilio* are presumed to be members of the Lauraceae (Aubert et al., 1999), for now it is difficult to make an in-depth discussion of key substances on which ancestors of papilionids relied in recognizing and choosing suitable hosts; published literature demonstrates that at least two *Citrus* feeders, *P. protenor* and *P. xuthus*, distributed in temperate Asia make best use of an amino acid derivative (stachydrine), a cyclitol (*chiro*-inositol), or a sugar acid (quinic acid) in their host recognition (Honda, 1990; Ohsugi et al., 1991). Recently, Ono et al. (2000b) reported that γ-lactone of MEA (a sugar-related compound) identified from *Orixa japonica* (Rutaceae) served as one of the oviposition stimulants for *P. bianor*. Our present findings conform well to these results in that females of *P. polytes*, inhabiting tropical Asian regions and feeding on *T. asiatica* (Rutaceae), rely exclusively on an amino acid derivative with a hydroxy group (HMP) and a kind of sugar acid (MEA) in egg-laying. In addition, oviposition of the primitive papilionids, *Atrophaneura alcinous* (Nishida and Fukami, 1989) and *Battus philenor* (Sachdev-Gupta et al., 1993), both feeding on *Aristolochia* plants (Aristolochiaceae), also exemplifies the importance of cyclitols (sequoyitol and pinitol). All these phytochemicals are closer to primary metabolites in their structures, rather than being categorized as secondary metabolites, and hence, in view of the evolutionary process of biosynthesis in plants, are more primitive than the hydroxycinnamates mentioned above. Furthermore, it should be noted that most such hydroxycinnamates are composed of aliphatic hydroxyacid and hydroxycinnamic acid moieties: mono- to polyhydroxylated acids (quinic, *muco*-quinic, threonic, and malic acids) esterified with caffeic or related aromatic acids. Therefore, the "hydroxyacid" or "polyol" structure *per se* may possibly play a crucial role in the assessment of potential hosts by ovipositing females. All this information, taken together, might allow for another interpretation that female responses to short-chain polyhydroxylated compounds, including the response to hydroxyacids, are more likely to be a plesiomorphic trait of the family Papilionidae.

T. asiatica, widely distributed in tropical Southeast Asia, is surmised to be a relatively primitive member of the Rutaceae (Dreyer, 1966) and is known to be utilized as a host plant by many other *Papilio* butterflies as well. The present finding that oviposition of *P. polytes* on *T. asiatica* is readily induced by the synergistic action of only two simple compounds closely related to amino acids or sugars seems to provide a clue to the roots of plant chemicals that have mediated host recognition and discrimination by the ancestors of the Papilionidae and to promote an understanding of the sensory systems associated with host range evolution.

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IDENTIFICATION OF THE FEMALE-PRODUCED SEX PHEROMONE OF THE SCARAB BEETLE, *Hoplia equina*

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Abstract—*Hoplia equina* LeConte (Coleoptera: Scarabaeidae: Melolonthinae) is a beetle pest of cranberry beds in Massachusetts. Larvae feed on the roots of the cranberry plant, reducing yield as well as vine density. The female sex pheromone was identified as 2-tetradecanone. There were eight compounds found in the airborne volatiles collected from females that elicited antennal responses from males. Of the eight compounds tested (nonanal, decanal, dodecanal, 2-dodecanone, 2-tridecanone, 2-tetradecanone, 2-pentadecanone, and 2-hexadecanone), 2-tetradecanone was the only one that attracted male beetles in the field. Combining any of the other seven antennally active compounds with 2-tetradecanone did not increase male capture.

Key Words—Ketone, 2-tetradecanone, cranberries, electroantennogram.

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INTRODUCTION

Five species of scarab beetles have been recorded as pests in cranberry beds in Massachusetts. They include the cranberry white grub, *Phyllophaga anxia* (LeConte), the cranberry root grub, *Lichnanthe vulpina* (Hentz), the oriental beetle, *Anomala orientalis* (Waterhouse), the Japanese beetle *Popillia japonica* Newman, and *Hoplia equina* LeConte, a small (6- to 8-mm long), native, melolonthine species for which there is no common name. Larvae of the cranberry white grub and the cranberry root grub have a long history as cranberry root feeding pests in Massachusetts (Franklin, 1950). Japanese beetle grubs are regarded as minor pests of cranberry beds, almost always being found in weedy areas of the beds (Dunn and Averill, 1996), although an authenticated damaging population has been noted (P.S.R. and D.C.W., personal observations). *Hoplia* grubs had never been observed as cranberry root pests until an employee of Ocean Spray Cranberries, Inc., discovered them causing feeding damage in a cranberry bed in 1990. The adults reared from those larvae were originally identified as *Hoplia modesta* Haldeman, but were correctly identified as *H. equina* in 2001 by P.S.R. Every beetle examined by P.S.R. (unpublished observations) from a number of bogs from collections made over several years was found to be *H. equina*.

The last revision of the genus *Hoplia* indicates 12 species in America north of Mexico (Hardy, 1977). Hardy (1977) reported the range of *H. equina* as from Maryland and the eastern portion of Pennsylvania through Delaware and New Jersey to Connecticut, Massachusetts, and New Hampshire. The oriental beetle and *H. equina* (*modesta*) were first reported as new and important cranberry pests by Dunn and Averill (1996). Surveys of Massachusetts cranberry beds in 1994 and 1995 indicated that in those sampled beds where grub species were present, *H. equina* was found in 23% (5/22) in 1994 and in 30% (11/33) in 1995. Aside from the summer flood and total bog renovation, there are no recommended methods of control for any cranberry feeding scarab beetle larvae. Dunn and Averill (1997) recorded behavior indicating the presence of a female-produced sex pheromone in the mating system of *H. equina* and also demonstrated that this species is diurnal and has a 2-year life cycle.

METHODS AND MATERIALS

Pheromone Collections. Third-instar of *H. equina* were collected by digging from an infested cranberry bog in Rochester, Massachusetts, in late April 1999. Recovered larvae were housed individually in ~30-ml plastic cups in a 3:1 mix of greenhouse sand and screened peat moss raised to about 12% moisture in a controlled environment room at 25°C during the 16-hr photophase and 20°C during the 8-hr scotophase. After pupation and adult emergence, individuals were separated

by sex and about 100 females were placed into an all-glass collection vessel. During photophase, pump-drawn air was filtered through charcoal, bubbled through distilled water, passed over and among the females, and finally through a glass tube filled with adsorbent Super Q polymer material (Alltech, Deerfield, IL). Captured volatiles were eluted from the Super Q using about 2 ml of dichloromethane and then condensed under a nitrogen stream to a volume of about 20 μl.

Electrophysiological Recordings. A Hewlett Packard 5890 Series II plus gas chromatograph equipped with a polar DB-WAXetr capillary column (60 m $\times$ 0.25 mm ID, 0.25-mm film thickness, J&W Scientific, Folsom, CA) in the splitless mode was used for coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Zhang et al., 1997). The oven temperature was programmed at 80°C for 2 min, then raised 10°C/min to 250°C, and held for 15 min. Injector and flame ionization detector (FID) temperatures were 260°C. Nitrogen was the carrier gas with a flow rate of 2.6 ml/min and inlet pressure was set at 276 kPa. An antenna plucked from a beetle was placed tip and scape between two gold wire electrodes immersed in saline-filled wells in a small acrylic holding station so that the three lamellae of the antennal club were spread. The gold wire output recording electrodes were connected by a short coaxial cable to a high-impedance 1:300 amplifier with automatic baseline drift compensation. The temperature of the antennae on the acrylic station was maintained at about 5°C by flushing 0°C water from a benchtop refrigerated circulator (RTE-100, NESLAB Instruments, Inc., Portsmouth, NH) through the insulated layer of the modified condenser containing the acrylic holding station mounted on top of the GC. An HP dual channel ChemServer/ChemStation A/D interface, 35900E, was used for the EAD recordings.

GC–mass spectrometry (GC-MS) was carried out with a Hewlett Packard 6890 gas chromatograph coupled to an HP 5973 Mass Selective Detector using the same 60-m DB-WAXetr or 60-m DB-5 capillary column and conditions as above, but with helium as the carrier gas.

Chemicals. All synthetic aldehydes, ketones, and the butylated hydroxytoluene (BHT) were purchased from Lancaster Synthesis, Inc. (Windham, NH), or Aldrich (Milwaukee, WI). Purities were $>$98% based on GC analysis. The 2-tetradecanone was synthesized from the corresponding alcohol by PCC (pyridinium chlorochromate) oxidation (Corey and Suggs, 1975).

Preparation and Field Evaluation of Synthetic Lures. EAD active components were tested on rubber septa in the field in 1999, 2001, and 2002. Lures were formulated by dissolving the neat compounds in hexane, dispensing appropriate amounts into 5-mm rubber stopper septa (Thomas Scientific, Swedesboro, NJ), and allowing the hexane to evaporate in a fume hood. Lures were deployed in the field in lab-constructed cross-vane traps. The bottom of the trap was hung about 50 cm from the ground. Traps were placed at about 20-m intervals. Trap placement was randomized at initial deployment.

TABLE 1. RELATIVE PERCENTAGES OF THE EAD-ACTIVE COMPOUNDS FOUND IN THE AIRBORNE VOLATILES COLLECTED FROM FEMALES OF *Hoplia equina*, AS DETERMINED BY GAS CHROMATOGRAPHY

Compound	Percentage
Nonanal	27.7
Decanal	21.4
Dodecanal	10.0
2-Dodecanone	2.1
2-Tridecanone	1.7
2-Tetradecanone	24.0
2-Pentadecanone	4.0
2-Hexadecanone	9.1

In 1999, the nine treatments included each of the five ketones tested alone at 300-μg/septa, a blend of the five ketones formulated in the ratio determined from the captured volatiles (Table 1) tested at 100 μg/septa and 300 μg/septa, 2-tetradecanone and 2-hexadecanone combined in the exact amounts found in the 300-μg mix, and a solvent control. One replicate of the nine treatments was deployed at each of four Massachusetts cranberry bogs during June and July. Traps were checked and emptied two to three times each week and rerandomized each time they were checked.

In 2001, the seven treatments included 2-tetradecanone at 300 μg/septum, the 300 μg/septum mix of the five ketones as above, the ketone mix + nonanal, the ketone mix + decanal, the ketone mix + dodecanal, the ketone mixture + all three aldehydes, and a solvent control. The aldehydes were added to the 300 μg of five ketone mixtures according to the percentages in Table 1. BHT was added (0.5%) to these lure mixes to slow trimerization of aldehydes. Six replicates of the treatments were tested during June and July at a cranberry bog that was infested with *H. equina*. Trap catch was recorded nine times and traps were rerandomized twice.

In 2002, three treatments were deployed including 2-tetradecanone alone at both 300 μg/septa and 176 μg/septa, and the mix of the five ketones together at 300 μg/septa. Four replicates of the three treatments were tested during June and July at an infested Massachusetts bog. Traps were checked three times and rerandomized each time.

Statistics. Numbers of beetles caught in the field in the various treatments were analyzed using ANOVA and Fisher's LSD test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Pheromone Identification. Using the GC-EAD system, seven EAD active compounds ($t_R = 8.60$, 9.94, 12.49, 13.58, 14.72, 15.89, 16.83 min) were

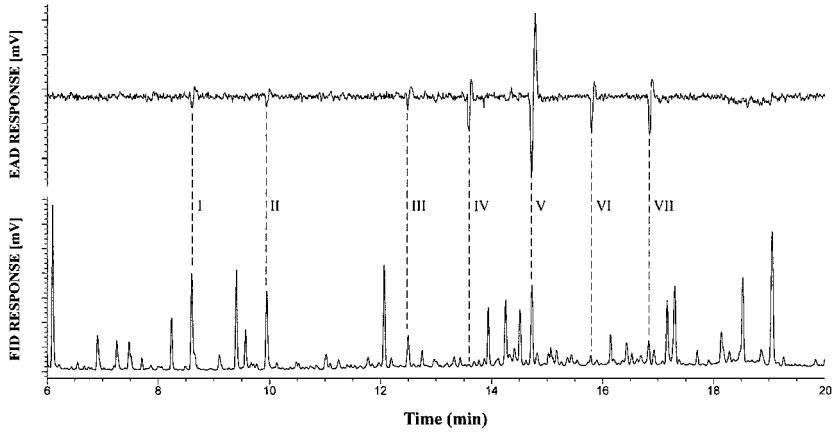

FIG. 1. Simultaneous responses of flame ionization detection (FID) and electroantennographic detection (EAD) of an adult male *Hoplia equina* antenna stimulated with female volatiles on a 60-m DB-WAXetr capillary column. I – nonanal. II – decanal, III – dodecanal and 2 – dodecanone (see text), IV – 2-tridecanone; V – 2-tetradecanone; VI – 2-pentadecanone; VII – 2-hexadecanone.

revealed using male antennae stimulated with female volatiles (Figure 1). These same volatiles did not elicit any significant response from female antennae. The EAD active compounds with peak numbers as in Figure 1 and relative ratios as in Table 1 were identified as nonanal (I), decanal (II), dodecanal (III), 2-tridecanone (IV), 2-tetradecanone (V), 2-pentadecanone (VI), and 2-hexadecanone (VII) by comparison of mass spectra and GC retention times on both polar and nonpolar capillary columns with synthetic standards. When a selected ion monitoring method (m/z 184, 198, 212, 226, and 240) was used to check the series of 2-ketones in female airborne extract, a tiny amount of 2-dodecanone was found to elute just before the third EAD active peak at $t_R = 12.35$) under the GC conditions used.

Field Evaluation of Synthetic Lures. No female beetles were found in the traps during the 3 years of field testing of the various compounds. In 1999, 2-tetradecanone at 300 μg was the only single compound to catch more males than the control (Figure 2). Septa loaded with the 300-μg blend of the five ketones attracted more beetles than did the 100-μg blend. There were no differences among captures in traps baited with 300 μg of the 2-tetradecanone alone, the 300-μg blend of all five ketones, and the blend of 2-tetradecanone and 2-hexadecanone. Traps baited with the 100-μg ketone blend did not capture significantly more male beetles than the solvent baited control traps (Figure 2).

In 2001, traps baited with 300 μg of 2-tetradecanone alone caught more beetles than the 300-μg five ketone blend (containing 176 μg of 2-tetradecanone).

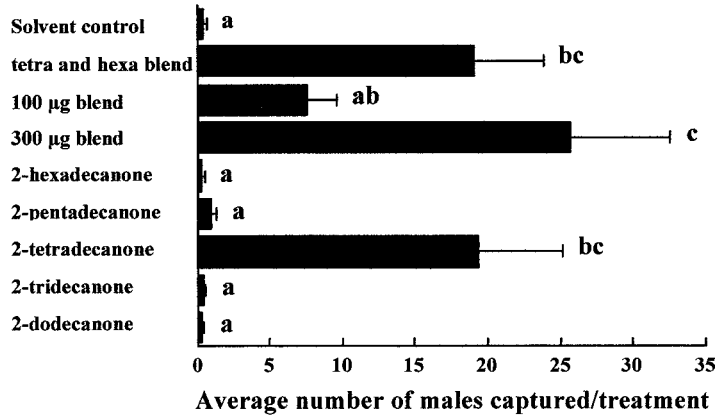

FIG. 2. Mean number of male *Hoplia equina* beetles caught/treatment in 1999. Bars with the same letters are not significantly different, $P < 0.05$, Fisher's LSD test. Total male beetles trapped = 1261.

Addition of any of the aldehydes to the 300-μg blend of ketones, alone or in combination, resulted in a trend towards reduced trap catches (Figure 3). The data from this test raised the question of whether the increased capture in the 300-μg dose of 2-tetradecanone resulted a dose response to the smaller amount of 2-tetradecanone in the 300-μg blend of ketones. However, in 2002, there were no significant differences among the three treatments of 300 μg of 2-tetradecanone alone, 176 μg

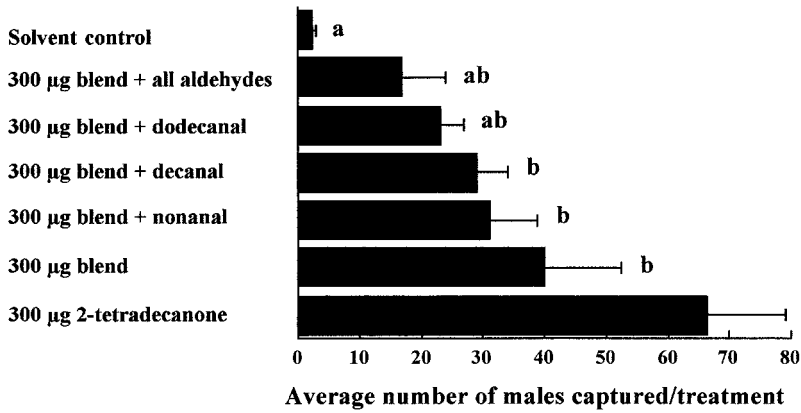

FIG. 3. Mean number of male *Hoplia equina* beetles caught/treatment in 2001. Bars with the same letters are not significantly different, $p < 0.05$, Fisher's LSD test. Total beetles trapped = 2505.

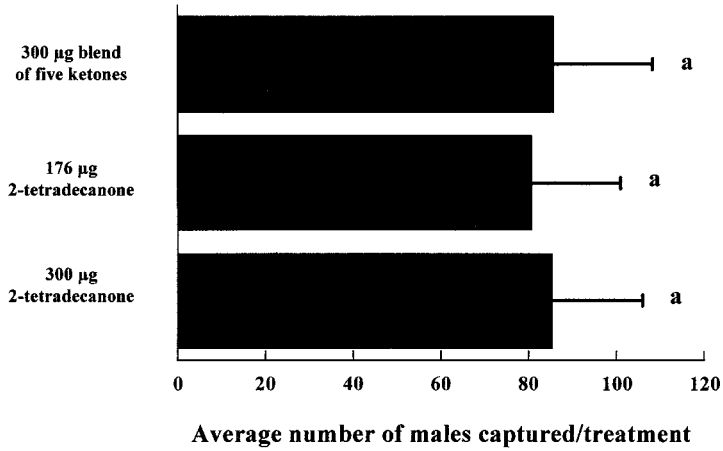

FIG. 4. Mean number of male *Hoplia equina* beetles caught/treatment in 2002. Bars with the same letters are not significantly different, $p < .05$, Fisher's LSD test. Total beetles trapped = 3020.

of 2-tetradecanone alone, and the 300-μg five ketone blend (containing 176 μg of 2-tetradecanone). Furthermore, addition of other EAD active ketone compounds to 2-tetradecanone did not result in increased male catches relative to 2-tetradecanone alone (Figure 4).

In three seasons of field testing, addition of any of the other EAD active components, either alone or in combinations, to 2-tetradecanone, did not result in increased male capture rates relative to 2-tetradecanone alone. We conclude that 2-tetradecanone is the sex pheromone of *H. equina*.

This is the second report of a ketone employed as the sex pheromone of a scarab beetle. Two similar ketones, 7-(Z)- and 7-(E)-2-tetradecanone, constitute the sex pheromone of *Anomala orientalis*, the oriental beetle (Leal, 1993; Zhang et al., 1994). Other known melolonthine sex pheromones include phenol (Henzell and Lowe, 1970), L-isoleucine and linalool (Leal et al., 1992), anisole (Leal et al., 1996a; Ward et al., 2002), (R,Z)-7,15-hexadecadien-4-olide (Leal et al., 1996b), (Z,E)-α-farnesene (Yarden et al., 1996), and a combination of L-isoleucine and L-valine (Zhang et al., 1997) (for a discussion of the sex pheromones of other scarab beetles, see Leal, 1998).

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A SEX ATTRACTANT FOR THE SCARAB BEETLE *Anomala solida* ER.

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Abstract—(*R*, *Z*)-5-(-)-(Oct-1-enyl)oxacyclopentan-2-one (*R*-buibuilactone) attracted male *Anomala solida* Er. (Coleoptera: Scarabaeidae, Rutelinae), a vineyard and orchard pest in Southeastern Europe. The presence of the corresponding (*S*) enantiomer or of 2-(*E*)-nonen-1-ol (a frequently found pheromone component in other *Anomala* spp.) in the bait did not influence catches. Traps baited with (*R*, *Z*)-5-(-)-(oct-1-enyl)oxacyclopentan-2-one were successfully used for monitoring the flight of *A. solida*, and may have practical applications for detection, monitoring, and mass trapping of the pest.

Key Words—*Anomala solida*, Coleoptera, Scarabaeidae, Rutelinae, (*R*, *Z*)-5-(-)-(oct-1-enyl)oxacyclopentan-2-one, sex attractant, field trapping.

INTRODUCTION

Several pheromone structures have been described among scarab beetles (Coleoptera: Scarabaeidae) in the subfamily Rutelinae, mostly from pest species occurring in Japan and North America (Leal, 1998). In Hungary, 2-(*E*)-nonen-1-ol

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(E2-9OH) has been found to attract male *Anomala vitis* Fabr. and *A. dubia* Scop. (Coleoptera: Scarabaeidae, Rutelinae), defoliating pests of grapes and some orchard trees in Europe (Tóth et al., 1994). In the course of screening tests, two specimens of another *Anomala* species, namely *A. solida* Er., were also caught, one in a trap baited with (*R*, *Z*)-5-(-)-(oct-1-enyl)oxacyclopentan-2-one (*R*-buibuilactone) and another in a trap baited with a 1:1 mixture of the same compound and (*R*,*Z*)-5-(-)-(dec-1-enyl)oxacyclopentan-2-one (*R*-japonilure) (Tóth et al., unpublished). *A. solida* is a rare species in Hungary (Endrödi, 1956), but is reported to cause occasional damage to vineyards and other plant cultures in the southeastern part of Europe (especially on the Balkan peninsula) (Tadic, 1950; Popov, 1958; Hurpin, 1962; Bobirnac and Somogy, 1971; Manolache et al., 1974). Consequently, an attractant-baited trap would be useful for plant protection purposes. The above chance captures prompted us to investigate in more detail the activity of possible attractant candidates in geographical areas with more abundant *A. solida* populations. Because *R*-buibuilactone was the common component in the two baits showing chance captures in our previous study (Tóth et al., unpublished), tests concentrated on the verification of the activity of this compound towards *A. solida.* The influence of the (*S*) enantiomer was also tested, because among scarabs enantiomeric configuration of pheromone components can be of crucial importance (Tumlinson et al., 1977; Leal, 1996). The influence of the presence of E2-9OH was also tested because *A. solida* co-occurs in several areas with *A. vitis* and *A. dubia* (Hurpin, 1962), and we wanted to check whether there was any indication of interaction in the chemical communication of these taxa.

METHODS AND MATERIALS

Chemicals. (*R*,*Z*)-5-(-)-(oct-1-enyl)oxacyclopentan-2-one(*R*-buibuilactone) and (*S*,*Z*)-5-(+)-(oct-1-enyl)oxacyclopentan-2-one (*S*-buibuilactone) were prepared as previously reported (Leal, 1999). The optical purity of these compounds was higher than 99.6% by GC analyses with a γ-cyclodextrin-based chiral column (Leal, 1991). 2-(*E*)-Nonen-1-ol (E2-9OH) was purchased from Bedoukian Inc. (Danbury, USA) and was $>$95% pure by GC.

Bait Dispensers. Two dispenser types were used. Rubber: baits for the tests were prepared by using pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ 9691/6; extracted 3 times in boiling ethanol for 10 min, then 3 times in methylene chloride overnight, prior to usage). PE vial: 0.7 ml polyethylene vials with lid (No. 730, Kartell Co., Italy). The lid of the vial was kept closed when setting up the trap in the field. All types of baits were attached to 8 $\times$ 1 cm plastic handles for easy manipulation when assembling the traps. For loading the baits,

the required amounts of compounds were administered to the surface (rubber) or into (PE vial) the dispensers in hexane solutions. After evaporation of the solvent, the lids of the PE vial dispensers were closed, and dispensers were wrapped singly in pieces of aluminum foil and stored at -65°C until use.

Traps. The traps and baits used were standard *Anomala* funnel traps (CSALOMON® VARb2) produced by the Plant Protection Institute, Hungarian Academy of Science, Budapest, Hungary. The trap consisted of a plastic funnel (top opening outer diameter: 20 cm, funnel hole diameter 3 cm, height of funnel 19 cm), under which a transparent plastic catch container was attached by a rubber band. Beetles falling through the funnel were caught in the ca. 1-l plastic container. The bait dispenser was attached on the inner wall in the middle of the funnel opening.

Field Trapping. Tests were set up in cherry and apple orchards of the Institute of Fruit Growing, Kyustendil, Bulgaria and in mixed fields of strawberries, raspberries, currants, and cherry orchards of the Station of Small Fruits, Kostinbrod, Bulgaria.

One replicate of each treatment was incorporated into a block so that individual treatments were 5–10 m apart, and blocks were sited 50–100 m apart. Traps were moved one position forward within a block at each occasion when the traps were inspected; at the same time, captured beetles were recorded and removed.

Capture data were transformed to $(x + 0.5)^{1/2}$ and analyzed by ANOVA. Treatment means were separated by Games-Howell tests. In case one of the treatments was catching nil, the difference of the catch of other treatments from zero catch was analyzed by Dunnet's Post hoc Procedure (one-tailed). All statistical procedures were conducted using the software packages StatView® v4.01 and SuperANOVA® v1.11 (Abacus Concepts, Inc., Berkeley, USA).

Single Field Experiments

Test 1. Kyustendil: June 14–July 26, 1999; traps were inspected each 3–4 days over 35 days, providing 22 data points (11 inspections of 2 block replicates).

Test 2. Kostinbrod: June 14–July 26, 1999; traps were inspected twice weekly over 28 days, providing 24 data points (8 inspections of 3 block replicates).

Dosage Test. Kyustendil: June 14–July 26, 1999; traps were inspected each 3–4 days over 35 days, providing 22 data points (11 inspections of 2 block replicates).

Monitoring of Flight. Kyustendil: May 30–July 27, 2000; and June 1–July 30, 2001; traps were inspected at 2–3 days intervals; in each test 2 traps baited with 1 mg of *R*-buibuilactone were operated at a distance of ca. 20 m from each other.

TABLE 1. CATCHES OF *A. solida* IN TRAPS BAITED WITH SCARAB PHEROMONE COMPOUNDS IN BULGARIA IN 1999

Bait (mg)			No. of beetles caught			
			Kyustendil		Kostinbrod	
R-buibuilactone	*S*-buibuilactone	E2-9OH	Mean catch/trap/ inspection ± SE	Total	Mean catch/trap/ inspection ± S.E.	Total
1.0	—	—	10.2 ± 1.7 a	224	0.5 ± 0.2 a	13
1.0	0.1	—	10.1 ± 1.8 a	222	0.2 ± 0.1 a	5
1.0	—	0.1	14.2 ± 2.5 a	313	0.4 ± 0.2 a	10
—	—	—	0.0 ± 0.0	0	0.0 ± 0.0	0
—	—	10.0[a]	0.0 ± 0.0	0	0.0 ± 0.0	0

Note: Means with same letter within one column are not significantly different at $P < 0.05$ by ANOVA followed by Games-Howell test. All means were significantly different from zero catch (unbaited and E2-9OH-baited) at $P < 0.05$ by Dunnett's Post hoc Procedure (one-tailed).

[a] *A. vitis/dubia* commercial bait (CSALOMON®; Plant Protection Institute, Budapest, Hungary).

RESULTS AND DISCUSSION

In the preliminary tests, *R*-buibuilactone was tested alone and in mixtures with the *S*-enantiomer or E2-9OH vs. unbaited control traps. Another treatment was included with E2-9OH alone to check for the presence of the related *A. vitis* or *A. dubia*. At both sites, all baits containing *R*-buibuilactone caught significantly more *A. solida* beetles than the unbaited controls (Table 1). Catches in traps with the binary mixtures of *R*-buibuilactone and 10% of *S*−buibuilactone or E2-9OH were similar to those with *R*-buibuilactone alone. Unbaited traps or those baited with the *A. vitis/dubia* attractant (E2-9OH) on its own caught no beetles. The results clearly show that *R*-buibuilactone attracts male *A. solida* confirming the indication in the earlier trials in Hungary.

In a second test, doses of 0.1 to 5.0 mg of *R*-buibuilactone were tested vs. unbaited controls. Increasing doses attracted increasing numbers of *A. solida* (Table 2). with the highest catches recorded with 5.0 mg. All doses attracted significantly more beetles than unbaited traps, which captured nil. Of the sample of ca. 100 beetles checked, all were male.

R-buibuilactone seems to be a common pheromone component in *Anomala*. It has been identified in the sex pheromones of *A. cuprea* Hope (Leal, 1991; Leal et al., 1993), *A. albopilosa sakishimana* Nomura (Leal et al., 1994b), and *A. octiescostata* Burm. (Leal et al., 1994a). In *A. daimiana* Harald (Leal et al., 1993) and *A. albopilosa albopilosa* Hope (Leal et al., 1996), both *R*-buibuilactone and

TABLE 2. CATCHES OF *A. solida* IN TRAPS BAITED WITH DIFFERENT DOSES OF *R*−BUIBUILACTONE IN BULGARIA

Dose of *R*-buibuilactone (mg)	Mean catch/trap/inspection ±SE	Total caught
0.1	7.7 ± 1.6 a	209
0.5	12.2 ± 2.3 ab	330
1.0	14.5 ± 2.2 bc	392
5.0	23.3 ± 3.2 c	629
Unbaited	0.0 ± 0.0	0

Note: For significance levels see Table 1. Means with different letters are significantly different at $P < 0.05$.

E2-9OH are present in the pheromone. In the case of *A. solida*, the presence of E2-9OH in the bait did not seem to influence the activity of *R*-buibuilactone at the ratio tested (Table 1).

It has long been known that responses of male Japanese beetles *Popillia japonica* Newman to their pheromone *R*-japonilure were inhibited by the *S*-enantiomer (Tumlinson et al., 1977). This was elucidated to be part of an interesting mechanism of reciprocal agonist–antagonist activities of enantiomeric pheromone (Leal, 1996). The Osaka beetle, *A. osakana* Sawada produces and responds only to *S*-japonilure, the activity of which is completely inhibited by the *R*-enantiomer (Leal, 1996). Thus, we tested whether *S*-buibuilactone might affect catches of *A. solida*. However, captures in traps baited with *R*-buibuilactone alone or in combination with *S*-buibuilactone were not significantly different, suggesting that *S*-buibuilactone is neither beneficial nor a behavioral antagonist.

The seasonal flight of *A. solida* was reliably monitored by traps baited with *R*-buibuilactone in both 2000 and 2001 (Figure 1). In both years, the flight started at the beginning of June and beetle abundance peaked between June 10 and July 5. According to Grigorov (1972) *A. solida*'s flight period begins in Bulgaria at the end of June and the flight lasts until, the end of July. In the present study, the flight in both years started ca.1 month earlier. Whether this difference with literature data is due to warmer temperatures in the given years or to other factors remains to be determined.

We conclude that traps baited with *R*-buibuilactone can be used for trapping *A. solida* in regions of Europe where it is a pest . Furthermore, such traps might be applied for beetle control through mass trapping, building on promising results obtained recently with the closely related *A. vitis/dubia* in Hungary (Voigt and Tóth, 2000, 2002).

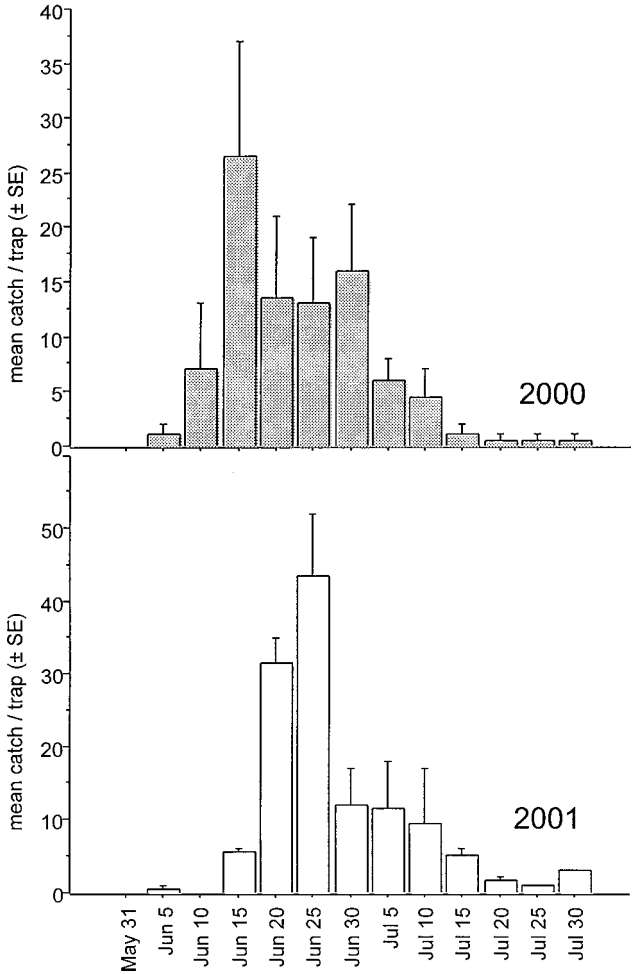

FIG. 1. Monitoring of the flight of *A. solida* by two traps baited with 1 mg of *R*-buibuilactone on rubber dispenser in 2000 and 2001 in Bulgaria.

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PHLOEOPHAGOUS AND PREDACEOUS INSECTS RESPONDING TO SYNTHETIC PHEROMONES OF BARK BEETLES INHABITING WHITE SPRUCE STANDS IN THE GREAT LAKES REGION

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Abstract—Tree killing and saprophytic bark beetles exert important ecological and economic roles in North American spruce forests. Chemical signaling among bark beetles, and responses by associate insects such as predators and competitors, have significant effects on the population dynamics and ecology of this community. Synthetic pheromones of primary (tree killing) and secondary (saprophytic) bark beetle species and blank controls were tested using multiple funnel and lower stem flight traps in white spruce forests in Wisconsin, Michigan, and Minnesota. Six phloeophagous and four predaceous species were collected with significant attraction by the bark beetles *Dryocoetes affaber, Dryocoetes autographus*, and *Polygraphus rufipennis*, and the predatory checkered beetles (Coleoptera: Cleridae) *Thanasimus dubius* and *Enoclerus nigrifrons*. In general, trap catches to synthetic lures resembled the species composition obtained by felling trees and collecting emerging beetles in a companion study, although several species showed differing trends. Some cross attraction occurred among bark beetles and between bark beetles and predatory beetles. For example, *P. rufipennis* was abundant in traps baited with *Dryocoetes* spp. pheromones. *Thanasimus dubius* and *E. nigrifrons* were collected in significant numbers in traps baited with the pheromone of the spruce beetle (*Dendroctonus rufipennis*), frontalin plus α-pinene. This is a new observation for *E. nigrifrons*. Attraction of *T. dubius* to the pheromones of at least three bark beetle species in the Great Lakes region, as well as to several southern and western species, reflects its role as a habitat specialist and feeding generalist. Several other important predators and competitors commonly obtained in pine forests in this region were not obtained in these spruce stands, either in response to synthetic pheromones of spruce colonizing beetles, or in host material colonized by these beetles. Potential differences in

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predator prey dynamics between spruce and pine ecosystems in the Great Lakes region are discussed.

Key Words—*Dendroctonus, Thanasimus, Dryocoetes, Polygraphus, Enoclerus,* aggregation pheromones, kairomones, Scolytidae, white spruce, cross attraction, froatalin, α-pinene, breviconim, bark beetles.

INTRODUCTION

Spruce ecosystems extend across the northern portion of North America and are major components of boreal forests. The structure of spruce forests ranges from relatively homogenous stands in the northwest and Rocky Mountains to more fragmented stands in the midwest and east (Burns and Honkala, 1990). Bark beetles can exert important pressures on spruce ecosystems. The roles of these phloeophagous herbivores appear to vary with region. In western North America, the spruce beetle, *Dendroctonus rufipennis* (Kirby), undergoes intermittent outbreaks and is responsible for high mortality over large areas of spruce forests (Werner and Holsten, 1983, 1984; Bentz and Munson, 2000). Outbreaks last for several years and beetles may colonize and kill most of the trees within 125,000 ha during a single year (Schmid and Freyes, 1977; Werner and Holsten, 1984; Werner, 2002). Spruce beetles also undergo outbreaks in New England, but these are smaller and more closely linked to severe host stress than in the west (McCambridge and Knight, 1972). Less aggressive beetles such as *Dryocoetes affaber* (Mannerheim), *Dryocoetes autographus* Ratzeburg, *Polygraphus rufipennis* (Kirby), and *Ips perturbatus* (Eichoff) are often associated with the spruce beetle. These species arrive at standing trees after spruce beetles kill them or are often the first to colonize slash and wind thrown trees (Gara et al., 1995). *Polygraphus rufipennis* has occasionally caused mortality in patches of declining black spruce in Canada (Bowers et al., 1991). Tree killing (primary) and saprophytic (secondary) bark beetles affect important ecological processes such as nutrient cycling, food for wildlife, and succession. They also cause serious economic losses arising from mortality to living trees and damage to stored forest products, respectively.

Relatively little is known about the bark beetles associated with spruce forests in the Great Lakes region. Some tree mortality occurs from the spruce beetle, but it is not common and is usually associated with prior outbreaks of spruce budworm or weather damage (Drooz, 1985). *Dendroctonus rufipennis* has recently been reported in Wisconsin arriving at felled white spruce in very small numbers (Haberkern et al., 2002). *Dryocoetes affaber, P. rufipennis, Ips pini* (Say), and *D. autographus* were the most abundant insects arriving at these trees.

Chemical signaling among bark beetles plays an important role in their population dynamics and ecology (Wood, 1982a). Aggregation and antiaggregation pheromones are essential for mate finding, tree colonization, and resource

partitioning. The degree of specificity varies among species. Sometimes there is cross attraction, but in other cases there are refined mechanisms for preventing interspecific exploitation (Lanier et al., 1980; Stock et al., 1990; Schroeder, 1996; Greenwood and Borden, 2000). Interspecific exploitation commonly takes two forms: prey finding by predators and host tree finding by phloeophagous and xylophagous competitors (Wood, 1982a; Mizell et al., 1984; Poland and Borden, 1997).

Responses of bark beetles and predators to pheromones in spruce forests are not well characterized in the Great Lakes region. The objective of this project was to identify the phloeophagous and predaceous insects responding to synthetic pheromone lures of three bark beetle species, and their degree of cross-attraction in white spruce forests in Wisconsin, Minnesota, and Michigan, USA.

METHODS AND MATERIALS

Site Description. Ten sites, 0.13–0.20 ha each, were established in white spruce stands throughout the Great Lakes region. Four sites were located in the Upper Peninsula of Michigan, four were located in northern Wisconsin, and two were located in northern Minnesota. Two sites were located in each stand. Stands were separated by a minimum of 8 km, contained at least 80% *Picea glauca* Moech Voss, ranged from 0.8 to 56.6 ha in size, and averaged 44 years in age. The surrounding landscape was predominantly forested with an even mixture of conifer and deciduous trees. Detailed descriptions are in Haberkern (2001) and Haberkern et al. (2002).

Sample Design. Synthetic pheromone lures were selected to emulate those produced by the two most abundant insects obtained in a companion host-colonization study, *D. affaber* and *P. rufipennis*, and by *D. rufipennis* because of its economic importance and recent collection in Wisconsin (Haberkern et al., 2002). Because of a lack of studies on these species in the Great Lakes Region lures were selected based on results from the only studies conducted, in western populations. All data on pheromone lure compositions, purities, and release rates were provided by Phero Tech, Inc., Delta, British Columbia, Canada.

Three experiments were conducted to assay responses of phloeophagous and predaceous insects to synthetic bark beetle pheromones. In experiment 1, seven 12-unit funnel traps (Lindgren, 1983) were deployed within each site during the first week of May, 1998. These flight traps were suspended on ropes between white spruce trees, approximately 2 m above the ground, and were used to collect insects that colonize the main stem. Traps were positioned 17–20 m apart. Four traps per site were baited with a membrane release lure containing racemic frontalin (>99% purity) and α-pinene (>99% purity, 94% (−): 6% (+)), to simulate the aggregation pheromone of *D. rufipennis* (Wood, 1982a). The emission rates were

2.6 mg and 1.5 mg/d, respectively, at 23°C. Lures were attached to each trap approximately 1.5 m above the ground. Three unbaited traps per site served as controls. Treatments were assigned randomly. Funnel traps were sampled weekly and sometimes biweekly from May 11, 1998 to July 6, 1998. Treatments were rerandomized at the end of each sampling period. A 6 × 7 cm piece of corrugated cardboard was placed in each collection cup (10 × 12 cm) to provide a substrate for bark beetles to evade predatory beetles. The corrugated cardboard was replaced after each collection.

In experiment 2, the funnel traps from experiment 1 included an additional pheromone treatment. Two traps contained a lure with racemic frontalin and α-pinene (94% (−): 6% (+)), three traps contained a lure with 3-methyl-3-buten-1-ol (the aggregation pheromone of *P. rufipennis*) (Bowers et al., 1991), and two unbaited traps served as controls. Membrane release lures with 3-methyl-3-buten-1-ol had an emission rate of 100–200 mg/d at 20°C (Phero Tech Inc., Delta, British Columbia). Traps were sampled from the first week of July until August 26, 1998 as described above.

In experiment three, a lower stem flight trap (Klepzig et al., 1991; Rieske and Raffa, 1993) was used to sample lower stem and root colonizing insects during the same period as experiment two. This trap was constructed from a 4.78-l plastic milk jug by removing all of three sides except for two support columns at the front corners, and retaining one large flat side that served as a striking surface. The jug was inverted, and the striking surface was attached to the stem of the tree approximately 25 cm above the ground. Collection cups, 3.5 × 5 cm, were snapped onto the bottom of the jug and a 2.5 × 2.5 cm piece of corrugated cardboard was placed in the bottom of each cup. Five jug traps per site were placed 17–20 m apart from each other. A membrane release lure containing a mixture of racemic *endo*-brevicomin plus racemic *exo*-brevicomin, to simulate the pheromone of *D. affaber* (Poland and Borden, 1998a,b), was attached to three traps, and two remained unbaited to serve as controls. These lures had a ratio of 92 *endo*: 7 *exo*-brevicomin, and emission rates of 1.7 mg/d at 20°C. Treatments were assigned randomly and the jug traps were sampled at the same times as the funnel traps in experiment two. All jug traps were at least 10 m from funnel traps.

Arriving beetles were identified to species using keys (Wood, 1982b). Insect identifications were confirmed by Steven Krauth of the University of Wisconsin—Madison Insect Research Collection, in which voucher specimens were deposited.

Statistical Analysis. A nonparametric Chi-squared analysis was used to compare the abundance of insects in traps with synthetic pheromone lures versus controls. Each experiment was analyzed separately. Experiment 1 contained two treatments: control ($N = 258$) and frontalin plus α-pinene ($N = 343$). Experiment 2 contained three treatments: control ($N = 95$), frontalin plus α-pinene ($N = 95$), and 3-methyl-3-buten-1-ol ($N = 143$). Experiment 3 contained two treatments: control ($N = 99$) and racemic 92% *endo*- 7% *exo*-brevicomin ($N = 149$). The

null hypothesis was that $p1 = p2$, where p was the proportion of zeros for each treatment. Statistical analysis was limited to those species for which the total sample size was at least 20 insects.

RESULTS

A total of 2430 insects from six phloeophagous and four predaceous species were obtained (Table 1). All were beetles. Bark beetles (Coleoptera: Scolytidae) comprised 66%, predatory checkered beetles (Coleoptera: Cleridae) comprised 33%, and weevils (Coleoptera: Curculionidae) comprised less than 1% of the total. Over 95% of all insects were collected in baited traps vs. unbaited controls.

In experiment 1, the predaceous beetles *Thanasimus dubius* (Fabricius) and *Enoclerus nigrifrons* (Say) were significantly more abundant in traps baited with racemic frontalin plus α-pinene than in controls (Table 2). Ninety-two percent of each species was collected in baited traps (Figure 1). The temporal pattern of these clerids appeared to differ. Half of all *T. dubius* were collected between May 11 and May 30, whereas the number of *E. nigrifrons* remained relatively uniform throughout the experiment. Insufficient *D. rufipennis* were obtained for statistical analysis (Table 1). *Polygraphus rufipennis* showed no preference for controls vs. traps baited with racemic frontalin plus α-pinene, the synthetic pheromone simulating that of *D. rufipennis*. Neither *D. affaber* nor *D. autographus* arrived in appreciable numbers to these baited or control traps.

TABLE 1. TOTAL NUMBER OF INSECTS COLLECTED IN MULTIPLE FUNNEL AND LOWER STEM FLIGHT TRAPS BAITED WITH SYNTHETIC BARK BEETLE PHEROMONE LURES IN THE GREAT LAKES REGION IN THE SUMMER OF 1998

Insects	Numbers
Coleoptera: Scolytidae	
Polygraphus rufipennis	1447
Dryocoetes affaber	77
Dryocoetes autographus	69
Dendroctonus rufipennis	4
Crypturgus borealis (Swaine)	12
Coleoptera: Curculionidae	
Pissodes nemorensis (Germar)	4
Coleoptera: Cleridae	
Thanasimus dubius	475
Enoclerus nigrifrons	316
Thanasimus undulatus	24
Zenadosus sanguineus (Say)	2

TABLE 2. CHI-SQUARED ANALYSIS OF PHLOEOPHAGOUS AND PREDACEOUS INSECTS RESPONDING TO BAITED VERSUS UNBAITED FLIGHT TRAPS IN WHITE SPRUCE STANDS IN GREAT LAKES REGION

Insects	Treatments	*df*	χ^2	*P*
Experiment 1	Control, frontalin plus α-pinene[a]			
Thanasimus dubius		1	64.55	<0.001
Enoclerus nigrifrons		1	51.33	<0.001
Polygraphus rufipennis		1	1.22	0.269
Experiment 2	Control, frontalin plus α-pinene, 3-methyl-3-buten-1-ol[b]			
Thanasimus dubius		2	34.12	<0.001
Enoclerus nigrifrons		2	36.28	<0.001
Polygraphus rufipennis		2	132.65	<0.001
Experiment 3	Control, *endo*-brevicomin[c]			
Dryocoetes affaber		1	23.59	<0.001
Dryocoetes autographus		1	23.59	<0.001
Polygraphus rufipennis		1	7.67	0.006

Note: See text for exact lure composition.
[a] Synthetic pheromone lure to simulate *Dendroctonus rufipennis.*
[b] Synthetic pheromone lure to simulate *Polygraphus rufipennis.*
[c] Synthetic pheromone lure to simulate *Dryocoetes autographus* and *Dryocoetes affaber.*

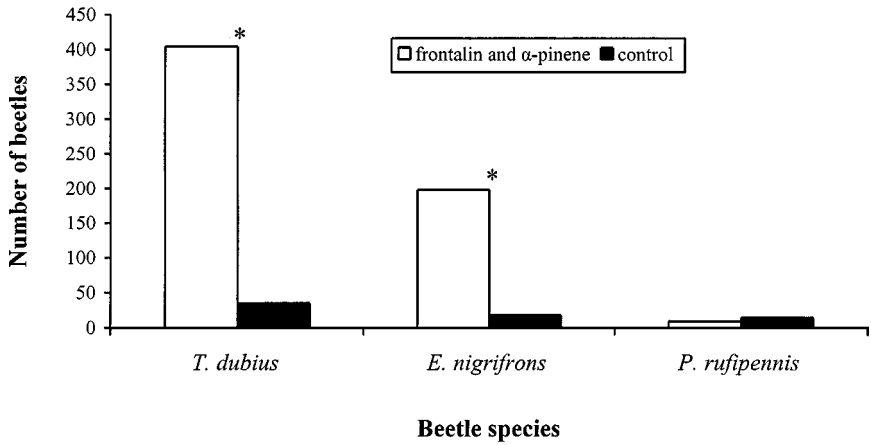

FIG. 1. Phloeophagous and predaceous insects responding to baited vs. unbaited multiple funnel flight traps (lures suspended 1.5 m above ground) in the Great Lakes region, May–June of 1998 (racemic frontalin and 94% (−): 6% (+) α-pinene: $N = 343$, Control: $N = 258$). *Significantly different at $P < 0.05$.

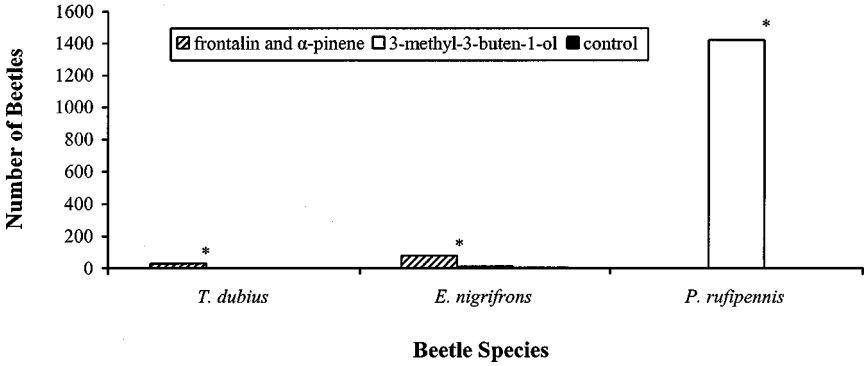

FIG. 2. Phloeophagous and predaceous insects responding to baited vs. unbaited multiple funnel flight traps (Lures suspended 1.5 m above ground) in the Great Lakes region, July–August of 1998 (racemic frontalin and 94% (−): 6% (+) α-pinene: $N = 95$, 3-methyl-3-buten-1-ol: $N = 95$, Control: $N = 143$). *Significantly different at $P < 0.05$.

In experiment 2, *T. dubius*, *E. nigrifrons*, and *P. rufipennis* were significantly more abundant in baited vs. unbaited traps (Table 2). Ninety-four percent of *T. dubius* and 87% of *E. nigrifrons* were collected in traps baited with racemic frontalin plus α-pinene (Figure 2). *Thanasimus dubius* and *E. nigrifrons* were collected consistently throughout experiment 2, although overall numbers were lower than during experiment 1. Neither predator showed significant attraction to 3-methyl-3-buten-1-ol. Ninety-nine percent of all *P. rufipennis* were collected in traps baited with 3-methyl-3-buten-1-ol (Figure 2). *Polygraphus rufipennis* showed no attraction to racemic frontalin plus α-pinene relative to controls. They exhibited two collection peaks, one in late July and one in mid-August. Neither *D. affaber* nor *D. autographus* were caught in appreciable numbers in these baited or control traps.

In experiment 3, *D. affaber*, *D. autographus*, and *P. rufipennis* were significantly more abundant in traps baited with racemic 92% *endo*- 7% *exo*-brevicomin than in controls (Table 2). No insects were collected in unbaited control traps (Figure 3). All three species were collected consistently throughout this experiment. No predators were caught in experiment 3.

DISCUSSION

The species composition and relative proportions of beetles obtained by trapping generally corresponded with emergence data from felled white spruce trees in these same stands. In particular, the two most abundant phloeophagous herbivores were *P. rufipennis* and *D. affaber* using both methods (Figures 2 and 3) (Haberkern

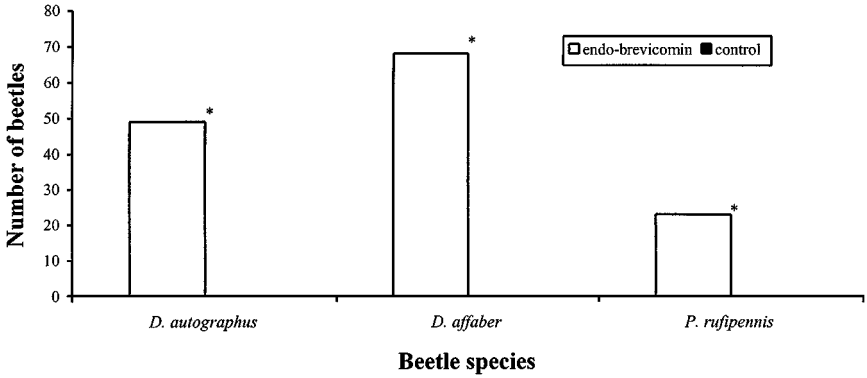

FIG. 3. Phloeophagous and predaceous insects responding to baited versus unbaited lower stem flight traps (lures suspended 0.4 m above ground) in the Great Lakes region, July–August of 1998 (racemic 92% *endo*- 7% *exo*-brevicomin: $N = 99$, Control: $N = 149$). *Significantly different at $P < 0.05$.

et al., 2002). Numbers of *D. rufipennis* were low in both studies. However, there were also some differences between trap catch and emergence data. *Ips pini*, a beetle typically associated with *Pinus*, was the third most common species emerging from felled spruce trees (Haberkern et al., 2002), but it was not caught in baited or control traps in the current study. Likewise, small numbers of *Trypodendron lineatum* (Olivier), *Orthotomicus caelatus* (Eichhoff), and *Ips grandicollis* (Eichhoff) emerged from felled spruce trees, but were not caught in traps in the same stands. *Dryocoetes autographus* was obtained in higher numbers using baited lower stem traps than by tree felling. The utility of lower stem traps for sampling *D. autographus* likely reflects its preference for the basal section of trees, a correspondence between colonization behavior and trap type that also occurs in pine colonizing beetles (Klepzig et al., 1991; Rieske and Raffa, 1993).

Some cross attraction occurred at the intergenus and interspecies levels. *Polygraphus rufipennis* was attracted to its aggregation pheromone as expected, and also to the pheromone of *Dryocoetes spp*. Both *D. affaber* and *D. autographus* were attracted to the synthetic pheromone, racemic 92% *endo*- 7% *exo*-brevicomin. This agrees with previous observations that brevicomins are attractive to *Dryocoetes* species in western populations (Camacho et al., 1993, 1994; Poland and Borden, 1998a,b). However, these results also suggest that regional differences may occur. Specifically, Jeans-Williams (1999) concluded that racemic *endo*-brevicomin was not attractive to *D. autographus* based on subtractive elimination, yet in the Great Lakes region strong attraction occurred to 92% *endo*– 7% *exo*- brevicomin relative to controls (Figure 2, Table 2). No phloeophagous beetles were attracted to the synthetic spruce beetle pheromone, frontalin plus α-pinene,

relative to controls. This is consistent with the observation by Greenwood and Borden (2000) that cobaiting for *D. rufipennis* and *Dryocoetes confusus* Swaine does not cause interference between these species' pheromones. However, some *D. rufipennis* populations experience exploitation of their pheromones by secondary beetles, such as attraction of *Ips perturbatus* (Eichhoff) to frontalin plus α-pinene in Alaska (Werner, 1993).

Predatory beetles are often attracted to bark beetle pheromones and infested hosts (Wood, 1982a; Mizell et al., 1984; Schroeder, 1996). To the best of our knowledge, this is the first report of attraction of *E. nigrifrons* to frontalin plus α-pinene. The predator *T. dubius* is known to be attracted to ipsdienol and ipsenol in Great Lakes region pine forests (Raffa and Klepzig, 1989; Herms et al., 1991; Aukema et al., 2000a,b) where it is a major predator of *I. pini* and *I. grandicollis*, respectively, and to frontalin plus α-pinene in southern pine (Mizell et al., 1984) and western spruce forests (Gara et al., 1995; Bentz and Munson, 2000) where it is a major predator of the southern pine beetle *Dendroctonus frontalis* Zimmermann and *D. rufipennis*, respectively. Attraction to the pheromones of multiple bark beetle species is consistent with the characterization of *T. dubius* as a habitat (i.e., bark beetle-colonized tree) specialist, but feeding generalist (Erbilgin and Raffa, 2001a,b).

In contrast to *T. dubius*, which responds to pheromones of both pine- and spruce-colonizing beetles, several other important pine-colonizing species were not obtained in spruce stands, either by trapping or tree felling (Haberkern et al., 2002). In particular, the predators *Platysoma cylindrica* (Paykull) and *Platysoma parallelum* (Say), and the competitors *Monochamus carolinenis* Oliver and *Monochamus titillator* (Fabricius), which are consistently among the most abundant species attracted to ipsdienol and ipsenol (Raffa, 1991; Aukema et al., 2000a; Erbilgin and Raffa, 2001b) and to hosts colonized by *Ips* spp. in the Great Lakes region (Aukema et al., 2000b; Wallin and Raffa, 2001), were not found in these spruce stands. Interestingly, some of these associates are attracted to the southern pine beetle, a source of frontalin plus α-pinene (Billings and Cameron, 1984; Stephen et al., 1989; Dodds and Stephen, 2000) and a compound that did not attract these insects in our study (Table 1). This suggests several possibilities. First, our stands may be beyond the northern range of these predators and competitors. That seems unlikely, as *P. cylindrica* has been reported in Ontario to at least 46°05′ (Mazur, 1997; Bousquet and LaPlante, 1999), and *P. cylindrica* and *P. parallelum* were listed by Ayres et al. (1999) as the second and third most abundant predators attacking *I. pini* in a Minnesota pine forest north (47°12′) of our northernmost stand (46°46′). Moreover, the above *Platysoma* and *Monochamus* species were widely recovered in surveys of red pine forests in Wisconsin, extending to 45°17′ (Erbilgin and Raffa, 2002). Another possibility is that these associates are locally present but are not attracted to the pheromones tested. Given the attraction of these species to southern pine beetle, any lack of attraction to frontalin plus α-pinene in

the Great Lakes region would suggest a regional preference for *Ips*. A third possibility is that the above associates are closely associated with *Pinus* habitats and chemistry, and specialize on prey within that habitat. We are currently exploring these possibilities.

Responses of bark beetles and associates are known to vary with geometric- and stereo-isomers, component mixtures, and host allelochemicals (Wood, 1982a, Seybold et al., 2000). Geographic variation in optimal responses is common (Lanier et al., 1980). Thus, subsequent studies specifically addressing such sources of behavioral complexity and specificity are needed to fully characterize the responses of phloeophagous beetles and predators inhabiting spruce forests in the Great lakes region.

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MATE CHOICE AND TOXICITY IN TWO SPECIES OF LEAF BEETLES WITH DIFFERENT TYPES OF CHEMICAL DEFENSE

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Abstract—Evidence for the use of defensive compounds for sexual purposes is scarce, even though sexual selection might have some importance for the evolution of defensive traits. This study investigates the effect of defense-related traits and body size on mating success in two sister species of leaf beetle differing in their type of chemical defense. *Oreina gloriosa* produces autogenous cardenolides, whereas *O. cacaliae* sequesters pyrrolizidine alkaloids from its food plant. Larger *O. gloriosa* males with more toxin or higher toxin concentration had a mating advantage, likely due to direct or indirect female choice. In the laboratory, particular pairings recurred repeatedly in this species, indicating mate fidelity. *O. gloriosa* females were also subject to sexual selection, possibly by male choice, because larger females and those with higher toxin concentration mated more readily and more often. In *O. cacaliae*, in contrast, sexual selection for toxicity and body size was not detected, or at best was much weaker. Because toxicity is heritable in *O. gloriosa* but environment-dependent in *O. cacaliae*, individuals of the former species could be choosing well-defended partners with "good genes." Our study suggests that sexual selection may contribute to the maintenance of heritable defensive traits.

Key Words—Sexual selection, chemical defense, cardiac glycosides, pyrrolizidine alkaloids, sequestration vs. *de novo* synthesis, assortative mating, *Oreina*, Chrysomelidae.

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INTRODUCTION

A wide variety of insect species exploit chemical defenses to protect themselves against natural enemies (Euw et al., 1967; Brower, 1984; Bowers, 1992). Defensive traits such as toxic compounds most likely evolve in response to predation pressure, and may be constrained by various physiological parameters. An additional mechanism selecting for toxicity may arise if individuals benefit from mating with a well-defended partner. Some authors have proposed that defensive secretions may have a direct pheromonal effect, provided that glands are not sealed and volatile compounds can diffuse (Attygalle et al., 1991; Eggenberger and Rowell-Rahier, 1993a). In other species, defensive compounds have been shown to be precursors of sexual pheromones (Trigo and Brown, 1990; Dussourd et al., 1991; Amano et al., 1999). Toxicity may be a reliable trait for assessing overall mate quality, as it is costly (Zahavi, 1975; Pasteels et al., 1990; Bowers, 1992; Andersson and Iwasa, 1996). However, evidence for the use of defensive compounds for sexual purposes in insects in most cases concerns plant-derived compounds (Nishida and Fukami, 1990; Trigo and Brown, 1990; Dussourd et al., 1991; Amano et al., 1999). Sexual selection for defensive compounds may occur when individuals of the choosing sex benefit from this choice. This is the case with the moth *Utetheisa ornatrix* (Dussourd et al., 1991), where females choose males advertising a toxin-rich spermatophore, a nuptial gift that females use to defend their offspring. In turnip sawflies, more distasteful females (i.e., containing greater amounts of diterpenes) have greater mating success (Amano et al., 1999), which may indicate male choice for toxic females. Clearly, more evidence is needed to evaluate the role of sexual selection in the evolution of chemical defenses.

Two fundamentally different means of chemical defense occur within the alpine leaf beetle genus *Oreina* Chevrolat (Coleoptera, Chrysomelidae). Some species, like *O. gloriosa*, defend themselves by synthesizing cardenolides (autogenous defense: Van Oycke et al., 1987; Eggenberger and Rowell-Rahier, 1993a,b). Other species, like *O. cacaliae*, are defended by pyrrolizidine alkaloids (PAs) acquired from their food-plant, which they store in defensive glands (toxin sequestration: Rowell-Rahier et al., 1991; Pasteels et al., 1992). *Oreina cacaliae* is, thus, dependent on its food-plant for defense against predators. In neither species are defensive compounds provided to offspring by their mother (Eggenberger and Rowell-Rahier, 1992; Dobler and Rowell-Rahier, 1994). However, in *O. gloriosa*, the concentration of 8 out of 16 cardenolides in the defensive secretion is heritable ($h^2 = $ ca.0.5, Eggenberger and Rowell-Rahier, 1992), and these components account for 31% of the total intrapopulation variation. Defensive capability is, therefore, at least partly genetically determined. Thus, in this species, there is the potential for selection for "good genes" to operate (Andersson, 1994), and toxicity may be the basis for mate choice if detectable by the partner. Indeed, an individual that chooses a well-defended partner would be favored by natural selection

because this enhances the survival of its offspring through higher resistance to predators. In contrast, this would be less likely in sequestering species such as *O. cacaliae*, where toxicity is primarily related to host plant content of PAs (Isman, 1977; Isman et al., 1977; Brower et al., 1984; Bowers, 1992). Toxicity will also be influenced by foraging or physiological conversion capacity, so may serve as an indicator of "good genes," but nothing is known about the heritability of these traits.

Here, we investigate sexual selection in these two leaf beetle species in the laboratory and the field, using modern standardized methods for quantifying sexual selection (Lande and Arnold, 1983; Arnold and Wade, 1984a,b; Brodie et al., 1995). In addition to the defensive traits of toxin volume and concentration (which are physiological traits rarely assessed in the sexual selection context; Kingsolver et al., 2001), body size was also analyzed because it has been shown to affect mate choice in many species (Andersson, 1994; Bonduriansky, 2001). Although supposedly rare and poorly documented (Andersson, 1994), male choice for females may occur when males are in some way limited in their number of matings, or if the quality of their mates strongly influences their fitness. This may be the case in toxic insects, so we specifically investigate this possibility. Lastly, we provide a general description of the mating behavior of both species.

MATERIALS AND METHODS

Collection of Beetles. Laboratory Experiment. This experiment was designed to record the mating pattern for each individual *O. gloriosa* and *O. cacaliae*. For both species, we collected 60 adults of each sex. *O. cacaliae* was sampled near La Fouly in the Val Ferret (Valais, Swiss Alps, 45.56 N, 7.05 E, alt. 1500 m) in early May 2000, when individuals emerge from the ground, dig out from the snow, and start feeding on *Petasites paradoxus* (Asteraceae). Individuals of this species were sexed using their sexually dimorphic tarsi (Lohse and Luche, 1994). *O. gloriosa* was sampled in Saas Grund (Valais, Swiss Alps, 46.08 N, 7.57 E, alt. 1800 m) at the beginning of June 2000, when they start feeding on *Peucedanum ostruthium* (Apiaceae). As sexual dimorphism of tarsi does not occur in this species, beetles were sexed using body weight. Previous studies have documented the weight distribution of both sexes, females being heavier than males (mean weight and standard deviation of females and males, respectively, 0.094 ± 0.013 g and 0.067 ± 0.006 g). We avoided using individuals weighing between 0.077 and 0.091 g. Beetles lighter than 0.077 g were taken as males, and those heavier than 0.091g as females. In another experiment in which sex could be checked because females larviposited, this method produced only a 2% error rate. To minimize disturbance during transportation, beetles along with leaves of their food-plant were placed in plaster-bottomed boxes with high humidity.

Field Sampling. An instant sampling was performed in order to document the mating pattern of both species in the field. Adult beetles were collected at the same sites and dates as for the laboratory experiment. Two categories of beetles were collected for each species: mating couples ($N = 31$ in *O. gloriosa*, $N = 25$ in *O. cacaliae*), and beetles that were not mating at the time (31 beetles of each sex in *O. gloriosa*, 25 in *O. cacaliae*). The sampling dates correspond to their peak mating time in the field. We collected beetles that were active (feeding and mating) on the leaves of their food plants, which should represent a random sample of the individuals in the population active at the time, as inspection on the ground under the food plants revealed the presence of few beetles and no mating. The sex ratio in the field is typically male-biased, with up to 84% of individuals being males (Kalberer et al., unpublished data). Each mating pair and single beetle was carefully placed into a separate vial to minimize disturbance while transporting them to the laboratory. All vials were plaster-bottomed to ensure high humidity, and provided with a fresh piece of the food-plant. Field-sampled beetles were milked for their defensive secretion within 24 hr after arrival in the laboratory (see below). All beetles were released at their respective field sites at the end of the experiment.

Experimental Procedure. The laboratory experiment lasted from 1 to 30 June, 2000, for *O. gloriosa*, and from 3 to 30 May, 2000, for *O. cacaliae*. These dates correspond to the mating period in the field, as indicated by field observations from the previous 4 years (Knoll, Kalberer and Nessi, unpublished data). For each species, we placed 30 males and 30 females in each of two 30- $\times$50-cm trays. This approximately reflects the natural density on plant patches in the field. Beetles were individually marked with correction fluid (Tipp-Ex) and bee labels on the elytrae. Defensive secretions and weights of all individuals were taken within 2 days at the end of the experiment. The holding trays had wet filter paper at the bottom to ensure humidity, and were placed in the laboratory at room temperature (range: 19–25°C), away from direct sunlight. Fresh food-plants were provided every day for food and shelter.

We recorded every mating by inspection every 4 hr (point sampling) over the entire period of the experiment. As preliminary observations indicated that matings last on average 6 hr in both species, we were confident that nearly all copulations were observed. After six nights of observation, we confirmed that nocturnal copulations are rare (only one mating was observed); hence, no survey was done between 11 p.m. and 6 a.m. Each mating pair was carefully removed from the tray and kept in a vial until the partners separated. Afterwards, beetles were released into the trays in order to keep insect density approximately constant in both trays. At the end of the experiment, each beetle that survived (62% of *O. gloriosa* and 48% of *O. cacaliae*) was weighed and milked for its defensive secretions as described below. As every copulation was recorded, chronological order of mating and the total number of copulations could be determined for all

individuals, allowing us to correlate these mating attributes to the measured traits. To estimate the duration of the different phases of mating, 12 mating pairs of *O. gloriosa* and 13 of *O. cacaliae* were observed every hour for the duration of the mating.

Measurement of Defensive Secretions. Defensive secretions were collected by holding the beetle under the microscope and gently tapping its pronotum and elytrae with fine forceps until drops of secretion appeared from the gland openings. The stimulus was applied as long as defensive liquid could be emitted. Drops were collected with a calibrated glass capillary, and the volume of the secretion (as represented by the height of the liquid in the capillary tube) was measured with a graduated lens on the microscope. Each secretion was stored individually in 150 μl of methanol in the freezer. Beetles were weighed to the nearest 10^{-4}g.

Sample Preparation and Chromatographic Analysis of Cardenolides. A preliminary trial revealed that the spectrophotometric method described by Dobler and Rowell-Rahier (1994) is not sensitive enough to analyze individual secretions. Thus, the samples were prepared and the concentration of total cardenolides in the secretions of *O. gloriosa* determined using reverse-phase high pressure liquid chromatography (HPLC) as described by Eggenberger and Rowell-Rahier (1993b), using a Varian Star chromatography workstation system with automated injection. We used ouabain (Sigma) as an internal standard, and concentrations are expressed as μg equiv. ouabain/μl. The minimum detected value was 0.95 $\mu g/\mu$l of secretion, and the standard deviation equalled 0.7% of the mean.

Sample Preparation and Chromatographic Analysis of PAs. Samples were prepared, and the concentration of total PAs in the secretions of *O. cacaliae* determined, using capillary gas chromatography (GC) as described by Rowell-Rahier et al. (1991), using a HP1-MS 30-m $\times$ 0.025-mm $\times$ 0.25-μm column, and senecionin as external standard. Concentrations are expressed as μg equiv. senecionin/μl. The minimum detected value was 0.01 μg/μl, and the standard deviation equalled 6.4% of the mean. Gas chromatography coupled with mass spectrometry (GC/MS) was used to confirm that the peaks corresponded to PAs.

Statistical Analysis. Sexual selection was quantified by calculating uni- and multivariate linear and quadratic selection coefficients (or gradients) using regression, following Lande and Arnold (1983) and Arnold and Wade (1984a,b). For each sample, we produced standardized z-scores for all measured traits (body weight, toxin concentration, and secretion volume) by subtracting the sample mean from each value and dividing the difference by the standard deviation: $z_i = (x_i - \bar{x})/SD_x$. For the field data, we estimated the effect of all traits on pairing success (yes or no) and on assortative mating given pairing. Relative pairing success (i.e., relative fitness $w_i = W_i/\bar{w}$) of males and females was calculated as absolute pairing success (1 or 0) divided by an estimate of the operational sex ratio [(i.e., mean fitness: Brodie and Janzen (1996)]. Because of the complex structure of habitat, the operational sex ratio could not be reliably estimated in the field

at the time of sampling. We, therefore, obtained an estimate of the proportions of paired and unpaired males and females from the laboratory experiment. This estimate was calculated as the average proportions determined at 9 point samples in time corresponding to the first 9 days of the experiment. We used the univariate models of relative fitness on standardized body size $w = c + \beta_1 z$ to estimate the linear (β_1) and $w = c + \beta_1' z + (\gamma_1/2)z^2$ to estimate the quadratic (γ_1) coefficients. The resulting coefficients β_1 and γ_1 are the linear and nonlinear (quadratic) selection differentials, reflecting the combined effects of direct and indirect selection on body size (Endler, 1986; Brodie et al., 1995). We used the corresponding trivariate models $w = c + \beta_{2,\mathrm{bw}} z_{\mathrm{bw}} + \beta_{2,\mathrm{tc}} z_{\mathrm{tc}}$ for the linear ($\beta_{2,j}$) and $w = c + \beta'_{2,\mathrm{bw}} z_{\mathrm{bw}} + (\gamma_{2,\mathrm{bw}}/2) z^2_{\mathrm{bw}} + \beta'_{2,\mathrm{tv}} z_t + (\gamma_{2,\mathrm{tv}}/2) z^2_{\mathrm{tc}} + \gamma_{2,\mathrm{tc}} z^2_{\mathrm{tc}}$ for the quadratic ($\gamma_{2,j}$) coefficients, where the subscripts bw, tv, and tc refer to body weight, secretion volume, and toxin concentration, respectively. These coefficients are the multivariate linear and nonlinear selection gradients (Brodie et al., 1995). The difference of the regression coefficients from a slope of zero (the null hypothesis of no selection) was tested for each species, whereby for the binary field data, least-squares regression was used to derive the estimate, but logistic regression was used to test for significance (Brodie et al., 1995; Blanckenhorn et al., 1999). Assortative mating (homogamy) with regard to all traits was assessed by analogously regressing the relative trait value in one sex on the z-standardized trait value in the other. Since fecundity is typically positively correlated with body size in insects (Honek, 1993), female size estimates male reproductive success. Analogous models were used in the laboratory experiment, using two measures of mating success reflecting different selection episodes, separately for each sex. Relative fitness $w_i = W_i/\bar{w}$ was computed as (1) the total number of matings of an individual divided by the mean number of matings (which were the same for both sexes because the sex ratio in the laboratory cages was unity); and (2) the rank order of the first mating of an individual, reflecting time to first mating, divided by the mean rank of all matings that occurred during the entire experiment (reflecting multiple matings of many individuals). Assortative mating was not assessed in the laboratory, as most beetles that mate also multiply. Although there were two replicate population cages for each species, all selection coefficients were calculated for both data sets combined because the estimates did not differ significantly. All measured traits were square-root–transformed to fit model assumptions.

Mate Fidelity and Random Mating Simulation. From the results, we suspected that matings were not random in *O. gloriosa*, because certain combinations of partners were observed several times. In order to see if this pattern might have arisen under random mating, we performed a simulation using the S-PLUS 2000 software. The program displayed two lists of 182 randomly chosen numbers (because there were an average of 182 matings per tray) ranging from 1 to 30 (because each tray contained 30 individuals of each sex). Each list represented beetles of one sex, and mating events were represented by the two numbers on the same line.

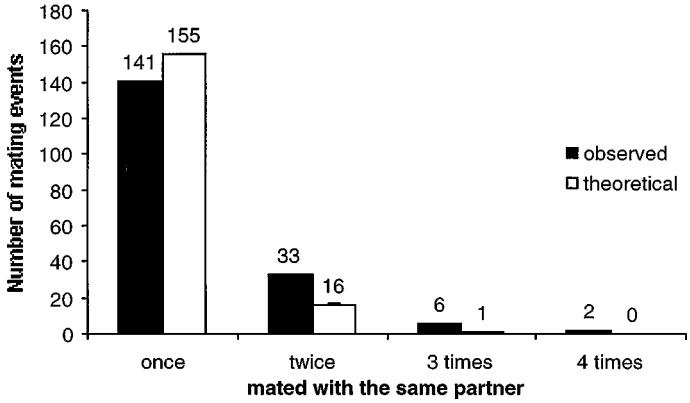

FIG. 1. Observed and calculated numbers of occurrences of repeated mating with a particular partner one to four times. For *O. gloriosa*, calculated numbers were obtained by a simulation assuming random mating. Note that standard errors of theoretical means are too small to be visualized on the figure.

The program then scored the number of "pairings" (i.e., combinations) occurring once, twice, or more often. We repeated this simulation 300 times, and recorded the mean numbers of expected matings in each category, and their standard errors (Figure 1). A goodness-of-fit Chi-square test was performed to see if the differences between observed and calculated values were significant. For this test, we used only two categories ("once the same partner" and "more than once") because the expected values were too small in the other categories.

RESULTS

Field Study. Our field sample consisted of 31 and 25 mating pairs, and 31 and 25 single beetles of each sex for *O. gloriosa* and *O. cacaliae*, respectively. Because of technical problems, there are some missing values for the concentration measurements (Table 1). Toxin volume and concentration were negatively correlated in *O. gloriosa* ($R = -0.30$, $P < 0.05$), whereas all other traits were uncorrelated. In *O. cacaliae*, weight and toxin concentration were positively correlated ($R = 0.33$, $P < 0.01$), and volume and concentration negatively ($R = -0.58$, $P < 0.001$).

In *O. gloriosa*, large males and those with large toxin volumes had a mating advantage, whereas toxin concentration did not influence pairing success (Table 1). These effects were independent, as uni- and multivariate selection differentials were congruent. Furthermore, males and females paired assortatively with regard to body weight and toxin concentration, but not toxin volume (Table 1). Therefore,

TABLE 1. MEAN (±SE) TRAIT VALUES OF PAIRED AND UNPAIRED INDIVIDUALS, UNIVARIATE (β_1) AND MULTIVARIATE (β_2 LINEAR SEXUAL SELECTION COEFFICIENTS FOR MALE AND FEMALE PAIRING SUCCESS AND ASSORTATIVE MATING IN THE FIELD FOR TWO SPECIES OF LEAF BEETLE

Trait	Mean ± SD paired (N)	Mean ± SD unpaired (N)	Pairing success		Assortative mating, Univariate β_1
			Univariate β_1	Multivariate β_2	
O. gloriosa females					
Body weight (g)	0.101 ± 0.01 (31)	0.100 ± 0.011 (31)	+0.055 ± 0.241	+0.139 ± 0.470	+0.063 ± 0.016*
Toxin volume (μl)	0.112 ± 0.047 (31)	0.123 ± 0.089 (31)	−0.102 ± 0.195	−0.664 ± 0.303*	−0.107 ± 0.076
Toxin concentration (μg/μl)	18.68 ± 10.88 (14)	23.18 ± 8.95 (12)	−0.733 ± 0.547	−0.878 ± 0.548	+0.392 ± 0.163*
O. gloriosa males					
Body weight (g)	0.070 ± 0.006 (31)	0.064 ± 0.005 (31)	+1.035 ± 0.232*	+1.072 ± 0.318*	+0.048 ± 0.012*
Toxin volume (μl)	0.138 ± 0.058 (31)	0.094 ± 0.065 (31)	+0.623 ± 0.220*	+0.719 ± 0.276*	−0.107 ± 0.076
Toxin concentration (μg/μl)	22.33 ± 7.64 (12)	26.82 ± 8.36 (15)	−0.461 ± 0.319	−0.253 ± 0.230	+0.199 ± 0.083*
O. cacaliae females					
Body weight (g)	0.092 ± 0.008 (24)	0.090 ± 0.011 (24)	+0.128 ± 0.245	−0.579 ± 0.366	+0.008 ± 0.019
Toxin volume (μl)	0.048 ± 0.039 (24)	0.073 ± 0.038 (24)	−0.606 ± 0.279*	−0.637 ± 0.572	+0.111 ± 0.172
Toxin concentration (μg/μl)	0.075 ± 0.089 (19)	0.147 ± 0.161 (8)	−0.657 ± 0.434	−0.337 ± 0.681	−0.214 ± 0.286
O. cacaliae males					
Body weight (g)	0.066 ± 0.009 (24)	0.061 ± 0.007 (25)	+0.544 ± 0.304	+0.800 ± 0.694	+0.013 ± 0.031
Toxin volume (μl)	0.078 ± 0.045 (24)	0.060 ± 0.033 (25)	+0.499 ± 0.313	+0.768 ± 0.857	+0.078 ± 0.121
Toxin concentration (μg/μl)	0.118 ± 0.142 (19)	0.161 ± 0.131 (17)	−0.367 ± 0.359	−0.524 ± 0.345	−0.214 ± 0.286

* $P < 0.05$.

all three traits positively affected male mating success in *O. gloriosa*. Aside from the assortative mating, which is reciprocal (however, male and female estimates of course vary quantitatively), and a negative multivariate linear selection gradient for toxin volume, female mating success appeared random with regard to all traits (Table 1).

In *O. cacaliae* males, sexual selection differentials for pairing success were qualitatively similar to those in *O. gloriosa*, but lower and nonsignificant, perhaps because of the lower sample size (i.e., there tended to be a positive effect of body weight and toxin volume on mating success; Table 1). There was no assortative mating. Toxin volume negatively affected the mating success of *O. cacaliae* females, but this was not significant in the multivariate analysis (Table 1). So again, sexual selection on females with regard to the three traits was largely absent, whereas sexual selection on male body weight and toxin volume may be present. Non-linear selection differentials were largely non-significant and are not presented in Table 1.

Laboratory Study. General Mating Behavior. Mean pairing duration was similar in both species (6.1 ± 2.8 hr and 6.7 ± 2.4 hr in *O. gloriosa* and *O. cacaliae*, respectively). We could distinguish two phases: true copulation, during which the male copulatory organ was fully inserted into the female's reproductive tract, and "guarding," during which the male clings onto the female's back but no intromission occurs. The duration of these two pairing phases was different in the two species: in *O. gloriosa* true copulation usually lasted less than 1 hr (less than 10 min on average), whereas in *O. cacaliae* all mating time consisted of true copulation. Although the switch between copulation and guarding was observed only once out of the 12 matings observed, we think that the guarding phase occurred after true copulation. Neither of the two species showed any apparent courtship. In both species, males often tried to mate with females that were running away. Most of the time these unwilling females were successful in escaping. Direct contest for access to mates was not evident in males or females. Although in some cases potential male competitors approached a mating pair and even climbed onto the back of the male already in place, this never caused the current pairing to end. All this suggests a mating system of male scramble competition for access to females, combined with some sort of (direct or indirect) female choice (Andersson, 1994; Rowe et al., 1994; Wiley and Poston, 1996).

Sexual Selection. We observed a total of 384 matings in *O. gloriosa* and 77 in *O. cacaliae* during the laboratory experiment. Again, the toxin concentration sample size was reduced because of technical problems. In *O. gloriosa*, all males mated and only 3% of the females did not, whereas in *O. cacaliae*, 28% of males and 59% of females remained unmated. The estimated average proportion of beetles mating at any point in time was 9% for *O. gloriosa* and 6% for *O. cacaliae*. This suggests that *O. cacaliae* might be less active overall (at least in the laboratory) than *O. gloriosa*.

There were no significant phenotypic correlations among the three traits in the laboratory experiment. In contrast to the field data, body weight and toxin volume had no effect on the number or order of mating in *O. gloriosa* males (Table 2). Only toxin concentration positively affected mating order, i.e., males with high concentration mated first (Table 2). Furthermore, females with higher toxin concentrations mated more often, and heavier females mated sooner (Table 2).

In *O. cacaliae*, there were no significant effects of any of the three traits on the number and order of mating in the laboratory (Table 2). Nonlinear selection coefficients were nonsignificant and are not presented.

Mate Fidelity and Random Mating Simulation. Remating with the same partner was never observed in *O. cacaliae*. In *O. gloriosa*, repeated pairings of the same two partners made up 21% (82 of 384) of all pairings observed (Figure. 1). There were 12 mates that remated three times, and 4 mates that remated four times. (Note that in the figure these numbers were divided by 2 to represent the situation in only one of the two trays.) Our simulation showed that we would expect fewer rematings under random mating ($\chi^2 = 32.01$, $df = 1$, $P < 0.001$; Figure. 1).

DISCUSSION

Our study suggests that defensive toxins and body size play a role in sexual selection in *O. gloriosa*, a species that autogenously produces toxins (Eggenberger and Rowell-Rahier, 1992). Sexual selection on physiological traits in general, and on toxic defensive compounds in particular, is rarely documented in the literature (e.g., Kingsolver et al., 2001). Although field and lab results are not entirely congruent, sexual selection apparently acts on male *O. gloriosa*: larger males with either greater secretion volume (field) or higher toxin concentration (laboratory) have a mating advantage. This is possibly due to direct (active) or indirect (passive) female choice (Rowe et al., 1994; cf. Wiley and Poston, 1996).

Moreover, there are indications that *O. gloriosa* females are subject to sexual selection as well, perhaps by male choice, as larger females mated more readily and those with higher toxin concentration more often. Males might compete for the largest receptive females, which typically have higher fecundity in many insects (Honek, 1993). Additionally, since toxicity is heritable in this species (Eggenberger and Rowell-Rahier, 1992), individuals could increase the chances of survival of their offspring by choosing well-defended partners with "good genes." Although poorly documented, male choice for females has been shown to operate in numerous species (Waring-Wilde, 1996; Cunningham and Birkhead, 1998; Amano et al., 1999; Gwynne and Bailey, 1999; Bonduriansky, 2001). Although male choice is in theory more likely to operate when the OSR is balanced or even female-biased (as is rare in nature but was the case in our lab experiment), the OSR does not in any straightforward way predict observed differences in choosiness (Bonduriansky,

TABLE 2. MEAN (±SE) TRAIT VALUES, UNIVARIATE (β_1) AND MULTIVARIATE (β_2) LINEAR SEXUAL SELECTION COEFFICIENTS FOR THE NUMBER OF MALE AND FEMALE MATING PARTNERS AND THE SPEED OF THE FIRST MATING (MATING ORDER) IN THE LABORATORY FOR TWO SPECIES OF LEAF BEETLE

		Number of matings		Mating order	
Trait	Mean ±SD, paired (N)	Univariate β_1	Multivariate β_2	Univariate β_1	Multivariate β_2
O. gloriosa females					
Body weight (g)	0.099 ± 0.009 (28)	$+0.169 \pm 0.093$	$+0.176 \pm 0.091$	$+0.278 \pm 0.132^*$	$+0.263 \pm 0.127^*$
Toxin volume (μl)	0.049 ± 0.029 (28)	-0.010 ± 0.099	-0.098 ± 0.094	$+0.094 \pm 0.152$	-0.013 ± 0.152
Toxin concentration (μg/μl)	22.93 ± 14.64 (28)	$+0.179 \pm 0.092$	$+0.189 \pm 0.092^*$	$+0.206 \pm 0.147$	$+0.183 \pm 0.149$
*O. gloriosa*males					
Body weight (g)	0.072 ± 0.006 (31)	-0.040 ± 0.089	-0.066 ± 0.095	$+0.105 \pm 0.119$	-0.035 ± 0.116
Toxin volume (μl)	0.040 ± 0.023 (30)	$+0.076 \pm 0.091$	$+0.085 \pm 0.095$	$+0.158 \pm 0.124$	$+0.118 \pm 0.120$
Toxin concentration (μg/μl)	16.63 ± 12.74 (31)	$+0.051 \pm 0.089$	$+0.053 \pm 0.093$	$+0.301 \pm 0.119^*$	$+0.281 \pm 0.126^*$
O. cacaliae females					
Body weight (g)	0.092 ± 0.010 (16)	$+0.142 \pm 0.412$	-0.344 ± 1.174	-0.276 ± 0.479	$+0.116 \pm 0.506$
Toxin volume (μl)	0.074 ± 0.053 (16)	$+0.132 \pm 0.413$	-0.143 ± 0.966	$+0.234 \pm 0.416$	-0.040 ± 0.422
Toxin concentration (μg/μl)	0.109 ± 0.111 (7)	$+0.882 \pm 0.599$	$+0.818 \pm 0.928$	$+0.593 \pm 0.259$	$+0.646 \pm 0.408$
O. cacaliae males					
Body weight (g)	0.075 ± 0.007 (25)	-0.045 ± 0.192	$+0.216 \pm 0.252$	-0.288 ± 0.156	-0.233 ± 0.210
Toxin volume (μl)	0.125 ± 0.072 (25)	-0.178 ± 0.189	-0.253 ± 0.370	$+0.290 \pm 0.267$	$+0.260 \pm 0.291$
Toxin concentration (μg/μl)	0.156 ± 0.110 (15)	$+0.154 \pm 0.226$	$+0.079 \pm 0.249$	$+0.104 \pm 0.186$	$+0.181 \pm 0.199$

$^*P < 0.05$.

2001). However, reports of male mate preferences are primarily from species and situations with balanced or female-biased sex ratios, especially when males are limited in the number of copulations they can perform, when males do not have to invest a lot in searching for females (as occurs in gregarious insects like *Oreina*) and there is ample choice, and when there is large variation in female quality (which is the case in *O. gloriosa*). Furthermore, in *Oreina* spp., toxins are costly to produce/sequester (for both sexes), and mating might be costly (e.g., in terms of predation) even for males. All these conditions together may limit the number of female partners a male can typically obtain in a season, potentially selecting for male (in addition to female) choice based on toxicity. Additionally, in the laboratory experiment, particular pairings recurred repeatedly in *O. gloriosa*, clearly indicating nonrandom mate fidelity. Contrary to the laboratory experiment, sexual selection on females was largely absent in the field sample, where more pronounced male–male scramble competition may have primarily favored large, fit males and rendered mate choice for toxicity secondary. However, the evidence for male choice in the lab experiment remains weak, and there are alternative interpretations possible, including a lab artifact.

In *O. cacaliae*, in contrast, which sequester their toxins from their food plants (Rowell-Rahier et al., 1991; Pasteels et al., 1992), such selection is absent or at least much weaker. The smaller samples for this species limit the interpretation of the data, as the field estimates of sexual selection in *O. cacaliae* were roughly similar to those of *O. gloriosa,* albeit a bit lower (Table 1). Results for *O. cacaliae* should be regarded as preliminary. However, sexual selection for "good genes" would not necessarily be expected in *O. cacaliae*, in which toxin sequestration is highly dependent on the PA content of their host plants (cf. Isman, 1977; Isman et al., 1977; Brower et al., 1984; Bowers, 1992). First, because toxin sequestration is often thought to be less costly than *de novo* synthesis (even though this is difficult to show: Pasteels et al., 1990; Bowers, 1992), mate choice for toxicity may be less likely to evolve in *O. cacaliae* than *O. gloriosa*. Second, even though physiological sequestering efficiency or food-plant choice might also be heritable, environmental variation should be large in comparison. Indeed, in *O. cacaliae*, toxicity was not consistent over seven samplings in a 6-week experiment (Labeyrie, unpublished data). Nevertheless, most other examples of sexual selection in relation to chemical defense concern sequestering species (Nishida and Fukami, 1990; Trigo and Brown, 1990; Dussourd et al., 1991; Amano et al., 1999). In these studies, this could be associated with spermatophores offered by males to females, which do not occur in our leaf beetles.

Mean pairing duration was long (ca. 6 hr) and approximately similar in both species. However, in *O. gloriosa*, actual intromission (as opposed to guarding) duration was much shorter (< 1 hr) than in *O. cacaliae*, where it lasts for the whole time. We also found that *O. cacaliae* mated much less frequently than *O. gloriosa*, although we have no data on differential activity rates of males or

females for either species. It is possible that these differences in copulation frequency and duration between the two species are related, suggesting more sexual conflict in *O. cacaliae* (cf. Rowe et al., 1994). Copulation duration is typically correlated with insemination and fertilization rates and/or spermatophore size (He and Tsubaki, 1992; Birkhead and Møller, 1998; Micholitsch et al., 2000), although genital contact does not always imply that sperm are being transferred (Rubenstein, 1989). Longer copulations are also often indicative of greater sperm competition and should lead to faster sperm depletion. Everything else being equal, this should allow *O. gloriosa* males to mate more often than males of *O. cacaliae*.

The field and laboratory observations revealed that females often successfully avoid matings by running away. As there appears to be no courtship, and females show no other rejection behavior (such as shaking; e.g., Rowe et al., 1994), this would be their primary expression of mate choice. However, the use of aerial or contact pheromones for sexual attraction is not inconceivable in leaf beetles (Edwards and Seabrook, 1997; Shu et al., 1999; Ruther et al., 2000). For toxicity to serve in mate choice, some such cue is necessary that individuals may use for assessing toxicity of potential mates. As far as we could observe, the beetles only emit their defensive liquid when disturbed by a predator, and not when they mate. This makes it unlikely that toxin concentration is directly assessed by mates before or during mating. However, as defense glands are under neural regulation (Schooneveld et al., 1992), it is possible that small amounts of secretions are released from the glands in a sexual context as a (contact) pheromone (Trigo and Brown, 1990; Attygalle et al., 1991; Dussourd et al., 1991; Eggenberger and Rowell-Rahier, 1993; Amano et al., 1999). We have no direct evidence for this process in *Oreina* leaf beetles.

In this study, the mean secretion volume differed between the field sample and the lab experiment. This is most likely due to different physical conditions, including air humidity, water availability, and temperature. Indeed, the lab air humidity (ca. 60%) was considerably lower than in the field. Beetles may have been limited by the quantity of liquid available for producing large volumes of secretion and consequently may have reduced the volume produced to a threshold (40–43% of the volume in the field) necessary for effective defense. This could explain why no effect of secretion volume was detected in the laboratory.

In conclusion, reciprocal male and female choice for well-defended, in addition to large, partners can to some degree explain the mating patterns observed in *O. gloriosa*, but probably not in *O. cacaliae*. Even though the exact mechanism and signals involved in sexual selection are currently unclear, the study suggests that sexual selection may contribute to the maintenance, although not necessarily the origin, of defensive traits in the leaf beetle *O. gloriosa* and probably other species as well.

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α-ACARIDIAL A FEMALE SEX PHEROMONE FROM AN ALARM PHEROMONE EMITTING MITE *Rhizoglyphus robini*[1]

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Abstract—The female sex pheromone of *Rhizoglyphus robini* Claparède (Astigmata: Acaridae) was identified as α-acaridial [2(*E*)-(4-methyl-3-pentenyl)-butenedial], which stimulated males sexually and enhanced the frequency of male mounting behavior. Although a hexane extract of females manifested alarm pheromone activity against tested males due to the presence of the alarm pheromone neryl formate, silica gel column fractions containing α-acaridial evoked increased mounting behavior by males at a dose of 0.1 female equivalent. Synthetic α-acaridial at a dose of 10 ng showed a peak of activity as a sex pheromone, with a convex dose–response relationship. Its content was determined to be 388 ± 244 ng per female and 163 ± 97 ng per male by GC. This is the first time that two pheromones (the alarm pheromone neryl formate, and the sex pheromone α-acaridial) have been demonstrated to be components of the same opisthonotal gland secretion in astigmatid mites. A mechanism for the appropriate expression of the two pheromones by the mites under different conditions is proposed.

Key Words—α-acaridial, sex pheromone, neryl formate, alarm pheromone, *Rhizoglyphus robini*, opisthonotal gland, mite.

INTRODUCTION

Since the first identification of neryl formate as the alarm pheromone of the cheese mite *Tyrophagus putrescentiae* (Kuwahara et al., 1975), alarm pheromones in a total of 16 species, as well as aggregation pheromones in 2 species and female

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sex pheromones in 7 species, have been identified among the 52 species of Astigmata examined (Kuwahara, 1999a, Ryono et al., 2001). However, until recently, no mite species had been found to possess two pheromones or to demonstrate two pheromone functions, with the exception of *Lardoglyphus konoi*, which possesses the aggregation pheromone 1*R*,3*R*,5*R*,7*R*-1,3,5,7-tetramethyldecyl formate (Kuwahara et al., 1994) and the alarm pheromone citral (Kuwahara et al., 1980). All pheromone components in Astigmata seem to be from the secretion of the opisthonotal gland, because no other secretory gland is known in this suborder. Two species have been found to display dual pheromone functions elicited by a single compound, depending upon either the emitted dose range of the active principle(s) or living conditions. One is *Schwiebea elongata*, which releases neral [(*Z*)-3,7-dimethyl-2,6-octadienal] that functions as an alarm pheromone at a high dose, and as an attractant (possibly an aggregation pheromone) at relatively low doses (Nishimura et al., 2002). The second is *Caloglyphus polyphyllae*, which deploys the sex pheromone β-acaridial [2(*E*)-(4-methyl-3-pentenylidene)-butanedial] that also plays a role as an aggregation pheromone at the same dose range in response to an unfamiliar environment (Shimizu et al., 2001).

Many species of Astigmata consist of males and females, and mating is often observable between sexes in stock cultures, suggestive of the presence of sex pheromones even in a species possessing alarm pheromones. The bulb mite *Rhizoglyphus robini* is one such species reproducing by mating and possessing the alarm pheromone neryl formate (Kuwahara et al., 1988; Akiyama et al., 1997). The hexane extract of the mite, however, only demonstrated alarm pheromone activity, and there were no behavioral indications of the presence of a sex pheromone. When neryl formate was removed by silica gel column chromatography and the other fractions combined, an isolated group of males exposed to the mixture subsequently displayed mating behavior, such as tapping each other's abdomen with a foreleg and trying to mount one another. However, no peculiar behavior was observed among females upon the introduction of a male or exposure to the alarm pheromone–removed extract. On the basis of these observations, the presence of a female sex pheromone was suggested. The alarm pheromone neryl formate in the extract seemed to block the sex pheromone.

Here, we report the isolation and structural elucidation of the female sex pheromone from the bulb mite, which possesses the alarm pheromone neryl formate. It was demonstrated that the two pheromones were products of the same gland. A mechanism for the expression of the two, depending upon the quantity of secretion discharged and their dose–response relationships, is also proposed.

METHODS AND MATERIALS

Mites. Rhizoglyphus robini Claparède (Astigmata: Acaridae) is a strain derived from a colony collected originally from rotten lily bulbs by Dr. M. Kuwahara

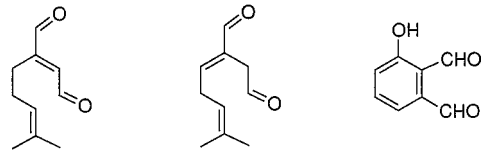
at the Tea and Vegetable Research Institute, Ministry of Agriculture and Fishery, Anou, Mie Prefecture, Japan. The culture has been kept in our laboratory for more than 15 years by feeding moistened dry onion powder under conditions of high humidity at 25°C, as previously described (Kuwahara et al., 1985). Part of the culture was maintained aseptically on agar medium (Kuwahara, 1999b).

Bioassay. Sex pheromone activity was evaluated as detailed by Mori et al. (1996), with minor modifications, using small Petri dishes (7 mm id × 5 mm in height) and a cover glass plate (18 × 18 mm). Groups of 10 males with a small lump of moistened dry yeast as food was placed into each assay chamber, whose bottom surface was covered with moistened filter paper. After 1 hr of conditioning, a living female or a piece of filter paper (1 × 2 mm) impregnated with a candidate compound at each dose was introduced. Frequencies of subsequent mating behavior among males were counted under a binocular microscope for 3 min at room temperature and ambient humidity. Observations were replicated 7–10 times using new groups of males, and filter paper treated with hexane was used as a control. Statistical significance was evaluated with the Mann–Whitney *U* test or Kruskal–Wallis test followed by Dunn's multiple-comparison test.

GC/MS and GC Analyses. Gas liquid chromatography—mass spectrometry (GC/MS) analysis was carried out using an Hewlett Packard HP-5890 Series II Plus gas chromatograph/mass spectrometer operated at 70 eV in a splitless mode, with an HP-5 MS capillary column (0.32 mm × 30 m, 0.33 μm in film thickness). The carrier gas was helium delivered at 1.23 ml/min, and the oven temperature was programmed to increase from 60°C (2 min hold) to 290°C at a rate of 10°C/min. Gas liquid chromatography (GC) was performed using a Hewlett Packard HP-5890 series II Plus by total ion count detection (TIC) and with a flame ionization detector (FID) on an HP-5 and an HP-1 capillary column (0.2 mm × 25 m, 0.33 μm in film thickness), respectively, under the same conditions as those used for GC/MS analysis.

Direct Sampling of the Secretion from the Opisthonotal Gland. In addition to the conventional method of extracting mite compounds (dipping mites in hexane for 3 min), a method to collect the secretion directly from the gland of female mites was employed. A filter paper tip was brought into contact with the gland's orifice, visible in the present species under a binocular microscope, and the secretion that oozed out was instantly collected by absorption. The paper was dipped into hexane (4 μl), and the extract was subsequently subjected to GC and GC/MS analyses.

Extraction and Isolation of Sex Pheromone. Three hundred females collected with a needle from the stock culture were dipped into 300 μl of hexane for 3 min. The extract was then applied to a silica gel column (Wakogel C-200, 300 mg), which was eluted successively with 3-ml aliquots of hexane, 1, 3, 5, 10, 20, and 50% ether in hexane and 100% ether. All fractions were bioassayed to locate the active fraction, the composition of which was then determined by GC and GC/MS.



α-Acaridial β-Acaridial γ-Acaridial

FIG. 1. Structures of acaridials.

Quantitative Determination of α-Acaridial and Neryl Formate. One mite (male or female) was dipped in hexane (3-μl, containing 10 ng of octadecane as the internal standard) for 3 min, and the extract was subjected to GC analysis using FID on an HP-1 capillary column under the same conditions as those described above. Contents (ng per mite) of α-acaridial [2(E)-(4-methyl-3-pentenyl)-butenedial] and neryl formate were calculated by response factors relative to octadecane [peak area ratios (x or x') of α-acaridial or neryl formate (NF) to octadecane] using the following equations: y (ng of α-acaridial) $= 31.36x + 0.113$, $r = 0.999$, for α-acaridial; y' (ng of neryl formate) $= 5.60x' + 9.08$, $r = 0.999$, for NF. Mann–Whitney U test was employed for statistical analysis.

Chemicals. The following compounds (Figure 1) were prepared as reported: α-acaridial (Suzuki et al., 1992), neryl formate (Kuwahara et al., 1980), β-acaridial [2(E)-(4-methyl-3-pentenylidene)-butanedial] (Leal et al., 1989b), and γ-acaridial (3-hydroxybenzene-1,2-dicarbaldehyde) (Sakata and Kuwahara, 2001).

RESULTS

GC and GC/MS Analysis. GC profiles (recorded by TIC) of the hexane extract of males on an HP-5 capillary column were composed of the following 10 compounds, with 9 peaks listed in decreasing order of recorder responses; tridecane (t_R 10.28 min), a mixture of neryl formate (t_R 10.04 min), and α-acaridial (t_R 10.06 min, identified by Leal et al., 1989a), γ-acaridial (t_R 10.21 min, identified by Sakata and Kuwahara, 2001), robinal (3-oxo-4-isopropylidene-1-cyclohexene-1-carbaldehyde, t_R12.05 min, identified by Leal et al., 1990a), β-acaridial (t_R 11.03 min, identified by Leal et al., 1989b), geranial (t_R 9.66 min), rosefuran (t_R 7.30 min), neral (t_R 9.29 min), and hexyl rhizoglyphinate (hexyl 2-formyl-3-hydroxybenzoate, t_R 17.28 min, identified by Leal et al., 1990b, not detected in the male extract) (Figure 2). The female extract consisted of the same mixtures as the male, but relative amounts of some components were different. Compounds showing the most female-biased occurrence (more than two-fold) were α-acaridial and hexyl rhizoglyphinate, suggesting they might be the female sex pheromone. In contrast, geranial was distributed mainly in males. As a mixture of neryl formate and α-acaridial gave unresolved peaks on GC and GC/MS analysis, quantitative

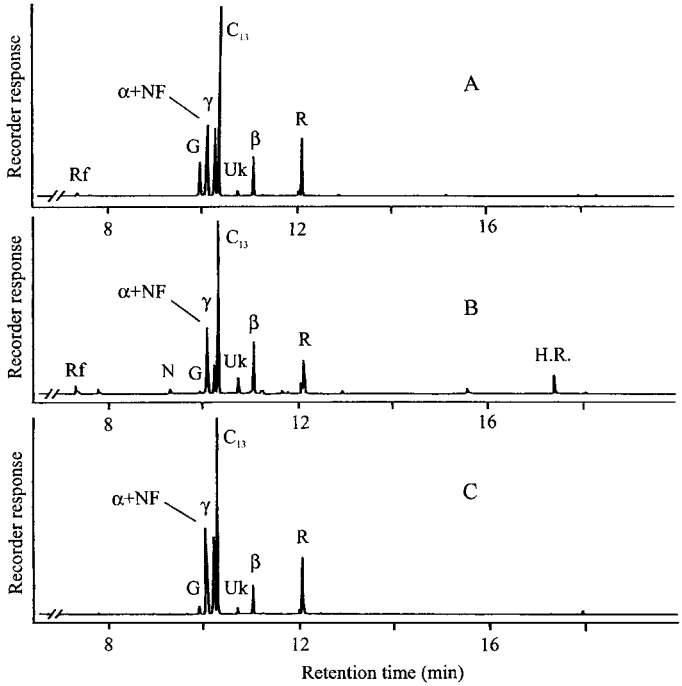


FIG. 2. Typical gas liquid chromatograms of *Rhizoglyphus robini* extracts, from 5 males (A), 5 females (B), and the secretion of the female opisthonotal gland (C). (See text for conditions. Rf, rosefuran; N, Neral; G, geranial; α + NF, a mixture of α-acaridial and neryl formate; γ, γ-acaridial; C13; tridecane; Uk, unknown; β, β-acaridial; R, robinal; H.R., hexyl rhizoglyphinate).

determination was performed using an HP-1 capillary column as described below. GC/MS analysis of the opisthonotal gland secretion from a female mite showed almost the same results as that of the female extract. No unusual differences were observed, and it was confirmed that all compounds detected in the hexane extract were those discharged from the opisthonotal gland.

Demonstration of Female Sex Pheromone Activity. The hexane extract of females was separated into eight fractions by silica gel column chromatography. Two fractions (5 and 10% ether in hexane eluates) at doses of 0.1 female equivalent elicited sex pheromone activity (significant at $P < 0.01$, $N = 87$) from isolated males, triggering mounting behavior among males, while no activity was observed against isolated females. Bioassay results of all column fractions are summarized in Figure 3. Alarm pheromone activity was detected in the 3% ether in hexane fraction, and the presence of neryl formate together with hexyl rhizoglyphinate was confirmed by GC and GC/MS analysis (data not shown).

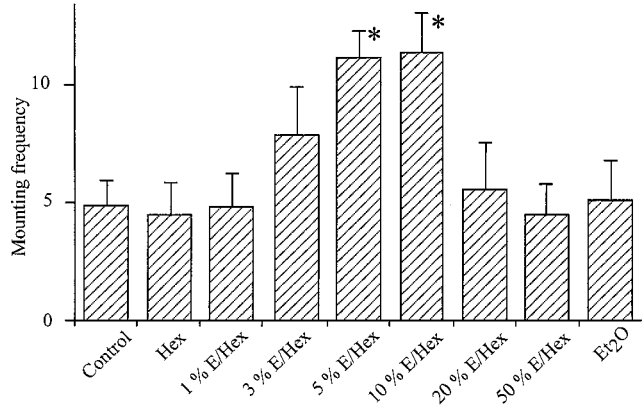


FIG. 3. Sex pheromone activities of SiO_2 column eluates, assayed at 0.1 female equivalent. (*Significant differences ($P < 0.01$) determined by the Kruskal–Wallis test followed by Dunn's multiple-comparison test relative to the control).

Three compounds α-, β-, and γ-acaridial were common to the two active fractions, among which α-acaridial was the major component. These observations together with the female-biased distribution suggested that α-acaridial might be the sex pheromone. α-Acaridial was active at a dose of 10 ng with a convex dose–response relationship. No significant activities were detected at doses lower or higher than 10 ng (Figure 4). The other two compounds in the active fraction (β- and γ-acaridial) showed no activity in the same dose range.

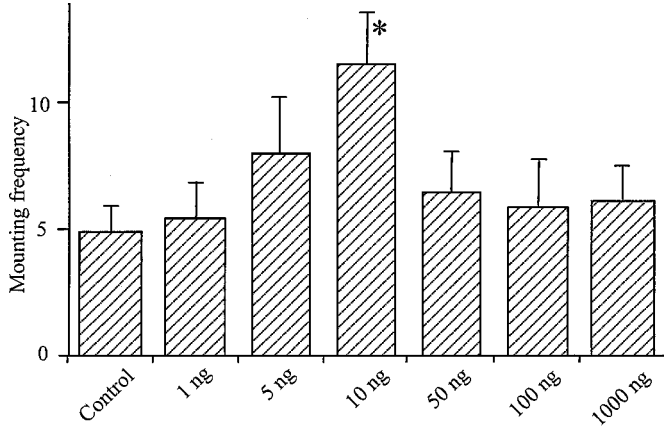


FIG. 4. Dose–response relationships of synthetic α-acaridial. (*Significant differences ($P < 0.01$) determined by the Kruskal–Wallis test followed by Dunn's multiple-comparison test).

Quantitative Determination of Neryl Formate and α-Acaridial. Females contained 388 ± 24 ng of α-acaridial and 10.1 ± 6.3 ng of neryl formate on average, while males contained 163 ± 97 ng and 11.4 ± 5.3 ng on average. The relative female/male ratio was 2.38 for α-acaridial, while neryl formate was present at almost equivalent levels in both sexes with a ratio of 0.89. Therefore, 1/39 (less than 3%) of the α-acaridial content in the female body is sufficient to elicit the sex pheromone activity. On the other hand, almost all of the neryl formate present in the body may be required to elicit alarm pheromone activity (active at a dose of 10 ng; Akiyama et al., 1997).

DISCUSSION

The female sex pheromone was identified as α-acaridial, which showed peak activity at 10 ng on a convex dose–response relationship (Figure 3), similar to other mite sex pheromones, and was also detected in males (Mori and Kuwahara, 2000). The compound was a component of the opisthonotal gland secretion, together with the alarm pheromone neryl formate (also active at 10 ng, Akiyama et al., 1997) as shown in Figure 1. The quantity of α-acaridial was almost 39-fold higher in females and 16-fold higher in males than the threshold level required to activate males. This relatively high level in males may explain why they so often try to mate with each other. On the other hand, the alarm pheromone neryl formate was distributed equally between both sexes, and almost the same dose was needed to manifest its activity. This explains why all of the gland's contents (10 ng of neryl formate) need to be discharged to elicit the alarm pheromone activity (active at 10 ng, Akiyama et al., 1997).

The amounts of α-acaridial and neryl formate in the female opisthonotal gland are roughly 400 ng and 10 ng, respectively. The following mixtures [α-acaridial (ng) and neryl formate (ng)], therefore, were prepared to simulate the gland contents and used in bioassays with males; 10–0.25, 10–1, 10–10, 20–0.5, 30–0.8, 50–1.3, 100–2.5, and 400–10, as indicated in Figure 5. Significant sex pheromone activity was only detected with 10–0.25 ($P < 0.01$), while males exhibited escape behavior when exposed to both 10–10 and 400–10 (results not evaluated statistically). Thus, the sex pheromone activity of the mixture was demonstrated only in a narrow range of the α-acaridial dose as shown in Figure 5, consistent with the bioassay results using pure α-acaridial.

Such a restricted emission or leakage of the gland contents (ca. 1/40th of total quantity) might occur when mites remain under good conditions in stock culture. The quantity of the alarm pheromone neryl formate discharged from the mold mite *T. putrescentiae* disturbed by 1 hr of shaking by hand was 102-fold times greater than that of controls, as determined using the Porapak Q air trapping method (Kuwahara et al., 1979). Similar experiments on citral trapping from disturbed

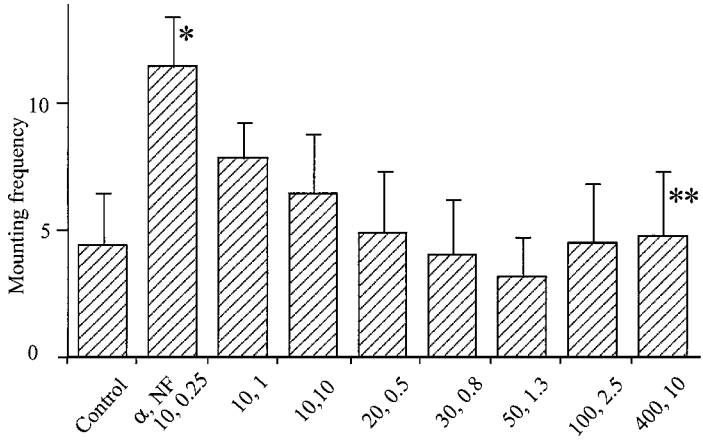


FIG. 5. Dose–response relationships of mixtures of synthetic α-acaridial and neryl formate, tested as mixtures of ng, each. (α α-acaridial; NF, neryl formate. (*Significant differences ($P < 0.01$) determined by the Kruskal–Wallis test followed by Dunn's multiple-comparison test. (**Alarm pheromone activity was observed).

mites using *Aleuroglyphus ovatus* and *Carpoglyphus lactis* showed levels 16- and 48-fold greater than those of undisturbed controls, respectively (Kuwahara et al., 1980). Therefore, in the bulb mite, it is possible that one-fortieth of the total content of the gland leaks out continuously, and α-acaridial at this level may function as the sex pheromone, while a complete discharge of the gland's contents at one time following a disturbance may result in neryl formate functioning as the alarm pheromone.

The dose–response relationships of various sex pheromones have been well studied in many acarid mites; 2-hydroxy-6-methylbenzaldehyde (HMB) in *Acarus immobilis* (Sato et al., 1993), undecane in *C. rodriguezi* (Mori et al., 1995), (2R,3R)-epoxyneral in *Caloglyphus* sp. "MJ" (Mori et al., 1996), rosefuran in *Caloglyphus* sp. "HP" (Mori et al., 1998), HMB in *Cosmoglyphus hughesi* (Ryono et al., 2001), β-acaridial in *C. polyphyllae* (Leal et al., 1989c), and HMB in *Al. ovatus* (Kuwahara et al., 1992). In the former five species, the relationships are convex, while the effect of the pheromone in *C. polyphyllae* seems to be sigmoid, and that in the last species is not clear. Although there may be exceptions, the sex pheromones in Astigmata generally indicate a convex dose–response relationship, while the alarm pheromone seems to function in a sigmoid dose–response. As *R. robini*, many astigmatid mites with alarm pheromones might also deploy sex pheromones.

α-Acaridial has been shown to be widely distributed in 20 species (as one of the major compounds in the genera *Rhizoglyphus* and *Schwiebea*) of Astigmata

among the 52 species examined; however, its pheromonal role, if any, has not been determined (Kuwahara, 1999a). It is the first pheromone whose function has been identified among Astigmata, despite the fact that it occurs as a major component in the genera *Rhizoglyphus* and *Schwiebea*.

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ANTENNAL RESPONSES OF THE TWO HOST RACES OF THE LARCH BUD MOTH, *Zeiraphera diniana*, TO LARCH AND CEMBRAN PINE VOLATILES

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Abstract—The larch bud moth (LBM) *Zeiraphera diniana* Guenée causes defoliation on larch in the Alps at 8- to 10-year intervals, after which populations crash. There are two LBM host races, one on larch and the other on cembran pine. These host races are morphologically indistinguishable as adults but they differ genetically in larval color types. Furthermore, females of each host race produce distinct pheromone blends and show oviposition preferences for their respective hosts. It is not clear to what extent host choice contributes to assortative mating in the LBM. Here, we compare the olfactory sensitivities of the two host races to the odors of fresh foliage of the host plants using the electroantennogram (EAG) technique, and the responses of the two host races to volatiles collected from the two host plants as analyzed by gas-chromatography-linked antennographic detection (GC-EAD). Both sexes of the larch and cembran host races show the same EAG responses to vapors of fresh larch and cembran pine foliage. Fifteen plant volatiles identified as chemostimuli by GC-EAD from larch and cembran pine odors elicited the same antennogram responses from the two host races. However, the GC-EAD analyses indicate that the number and quantity of chemostimuli emanating from each host plant is different. It is, therefore, most probably the array of olfactory receptors responding to the bouquet of volatiles unique to each host plant that underlies the host preferences of the two races. What remains open is the extent to which the similarity of the olfactory systems may contribute to cross-attraction. The fact that LBM individuals with intermediate characteristics between the two host races exist, suggests

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that olfactory perception does not hinder gene flow and contributes to sustained genetic diversity within the species *Z. diniana.*

Key Words—Larch bud moth, *Zeiraphera diniana,* host races, *Pinus cembra, Larix decidua,* plant volatiles, antennogram.

INTRODUCTION

The larch bud moth (LBM) *Zeiraphera diniana* Guenée (Lepidoptera: Tortricidae) is renowned for its regular outbreaks in larch forests in the Alps at 8–10-year intervals, causing conspicuous defoliation (Baltensweiler et al., 1977). Population density climbs in four to five generations to some 20,000-fold at peak density. There are two LBM host races, one feeding on larch (*Larix decidua*) and the other on cembran pine (*Pinus cembra*). Populations on pine also appear to be cyclic (Baltensweiler, unpublished), and one outbreak is reported for Northeastern Asia on *Pinus pumila* (Khomentovsky et al., 1997). Morphologically, these host races are distinguishable only at the fifth instar; larvae on larch are black and those on pine are light yellow-orange. This color polymorphism in the larvae varies during population cycles (Baltensweiler et al., 1977; Baltensweiler, 1993).

The major factors governing reproductive isolation in most sympatric phytophagous insect host races are host fidelity and/or other assortative mating traits such as those governed by pheromones. Host fidelity acts as an effective premating barrier between a wide variety of sympatrically speciating insect species across different insect orders. There are only a few reports on host fidelity in the LBM. One incident of host fidelity is known for the larch host race when moths emigrating from the Engadine Alps were grounded by a cold front over the Lake of Constance and reoriented subsequently to larch trees within the deciduous forest (Baltensweiler and von Salis, 1975). Studies on host alighting preference have revealed a strong preference by the LBM host races to alight on their own host plants, both in laboratory (Bovey and Maksymov, 1959; Drès, 2000) and field experiments (Emelianov et al., 2003). However, "infidelity" has been estimated; the overall probability of the larch race adults to alight on cembran pine is 13%, and the probability that pine race adults will alight on larch is 11% (Emelianov et al., 2003).

Assortative mating mediated by sex pheromones has been hypothesized to play a role in host race maintenance in the LBM (Guerin et al., 1984). The major sex pheromone components of the two races were identified as *E*11-14: Ac and *E*9-12: Ac, with the larch host race producing largely *E*11-14: Ac and the pine race *E*9-12: Ac (Baltensweiler and Priesner, 1988). Females produce the two compounds in their sex pheromone glands in ratios corresponding to the response spectra observed for males (Guerin et al., 1984), and F1 hybrid males show the same electroantennogram (EAG) response amplitudes to the two pheromone

components (Priesner, 1979; Priesner and Baltensweiler, 1987). Using a quartet mate choice design in the laboratory (one male and one female of each of the two races per cage), Drès (2000) estimated the overall degree of hybridization between larch and pine host races to be 28%. Long-range host-associated premating isolation governed by pheromones has recently been tested in the field, suggesting an incomplete premating isolation between the two host races (Emelianov et al., 2001).

What allows the cross-attraction between the host races? Gene flow is estimated to be between 2% and 4% per generation (Emelianov et al., 1995; Drès, 2000). Incomplete specificity of the LBM pheromone system and/or the host plant volatiles could be implicated. Although short- and long-range pheromone attraction has been studied (Drès, 2000; Emelianov et al., 2001), no study has been made on the role of host plant volatiles in the sensory ecology of the LBM. To study this, we compared the antennal responses of the two host races to larch and cembran pine volatiles by using the electroantennogram technique (EAG) (Schneider, 1957) and by gas-chromatography-linked EAG analysis (Arn et al., 1975) of host plant volatiles. In addition, we compared the antennal responses of the two host races to volatiles emanating from larch foliage damaged by larval feeding. Larch needles, even only nibbled at, quickly desiccate in the dry subalpine climate and turn red-brown. Commonly occurring plant volatiles were also tested to assess the broader antennal discrimination capabilities of the two LBM host race antennae. The aim of the study was to determine if the antennal olfactory receptor sensitivity of the LBM host races is selectively tuned to the detection of odors that are associated with their respective host plants.

METHODS AND MATERIALS

Insects. Cembran pine and larch branches infested with LBM late instar larvae were collected at the end of June 1999 and on July 1, 2000, in the Engadine Valley. Larvae were reared on the respective host plants at 20°C, 80% relative humidity (RH), in the laboratory to permit pupation and adult emergence. Moths were sexed after emergence and separated to prevent mating.

EAG Recordings. Recordings were made from excised antennae of the moths. The tip of the antenna was cut to facilitate electrical contact. Chloridized silver wires in drawn-out glass capillaries filled with 0.1% KCl + 1% polyvinylpyrrolidone were used as reference and recording electrodes. The antenna was held in a humidified airstream (90%–100% RH, 23°C±2°C) delivered at 1 m/sec via a water-jacketed glass tube (6-mm i.d.) whose outlet was about 1 cm from the preparation. The EAG signal was fed into an AC/DC amplifier (×100) via a high impedance preamplifier (×10), recorded on the hard disk of a PC via a 16-bit analogue-digital IDAC card (Syntech, The Netherlands), and monitored simultaneously with an oscilloscope (Tektronix 5103, USA).

TABLE 1. SYNTHETIC PLANT VOLATILES TESTED AS CHEMOSTIMULI FOR LBM ANTENNAL RECEPTORS

No.[a]	Compound	Source	Purity (% GC)
1	(−)-Bornyl acetate	Firmenich	Unknown
2	(−)-Camphene	Fluka	85
3	(+)-Camphor	Fluka	97
4	(+)-3-Carene	Fluka	98
5	(+)-Carvon[a,b,c]	Fluka	99
6	(−)-β-Caryophellene[b]	Fluka	99
7	Citral (cis+trans)	Fluka	96
8	Citronellal	Fluka	>98
9	Eugenol[b,c]	Fluka	99
10	Geraniol[b,c]	Fluka	99.3
11	(*E*)-2-Hexenal[b,c]	Aldrich	99
12	(*E*)-2-Hexenol[b,c]	Fluka	95
13	Isoprene	Fluka	99.5
14	(*R*)-(+)-Limonene[b,c]	Fluka	99
15	*p*-(−)-Menthyl acetate[b,c]	Fluka	99
16	β-Myrcene[b,c]	Sigma	90
17	(*E*)-β-Ocimine	Firmenich	Unknown
18	(1*R*)-(+)-α-pinene[b]	Fluka	99
19	(+)-β-Pinene	Fluka	99
20	γ-Terpinene[b,c]	Fluka	99
21	(+)-α-Terpineol[b,c]	Fluka	99
22	α-Terpinolene[b]	Fluka	90
23	Sabinene[b]	Unknown	Unknown
24	(*E*)-β-farnesene[b,c]	Bedoukian	Unknown

Note:Except where indicated, compounds with chiral center(s) were racemic mixtures.
[a] Compounds 1–22 were included in the 22-component mixture tested by GC-EAD and EAG (see text).
[b] Compounds tested by EAG.
[c] Compounds included in the 11-component mixture.

Antennae were stimulated as described in Guerenstein and Guerin (2001) by passing 1 ml of charcoal-filtered air through a 5 ml polypropylene syringe containing the stimulus. The latter consisted of either a synthetic plant volatile at 1 μg source dose (Table 1), the LBM pheromone components *E*11-14: Ac and *E*9-12: Ac (Institute for Pesticide Research, Wageningen, NL) at 100 ng source doses, mixtures of 11 and 22 synthetic plant volatiles (Table 1) with each compound at a source dose of 0.1, 1, and 100 μg, fresh or LBM-defoliated larch, and cembran pine needles (2 g each). An aliquot of a stimulus chemical dissolved in dichloromethane (DCM, Merck, analytical grade) was deposited on a filter paper strip that was placed in the syringe after evaporation of the solvent; DCM alone

was used as a control. The EAG amplitudes presented are the absolute amplitudes in millivolts generated by the stimuli minus the control value (if any).

Gas-Chromatography-Coupled Electroantennogram Detection (GC-EAD). The methodology is described in Steullet and Guerin (1994). LBM antennae were employed as detectors to locate biologically active volatiles in the odors of fresh and LBM-defoliated larch, and cembran pine. These odors were separated on a high-resolution gas chromatography capillary column (30 m DBWAX, 0.25-mm i.d., 0.25-μm film thickness, J&W Scientific, CA, USA) in a gas chromatograph (Carlo Erba Instruments 5160, Mega series, Milan, Italy) with a split–splitless injector at 200°C (for direct headspace vapor injection), an on-column injector (for analysis of extracts), and a flame ionization detector (FID, at 260°C). The carrier gas was H_2 (30 cm/sec at 40°C). On-column injections were made at 40°C, and the column temperature was programmed at 5°C/min to 230°C (held for 10 min). The column effluent was split (50:50) between the FID and the antennographic detector (EAD) and simultaneously monitored by the FID and a LBM antenna, and both signals were recorded simultaneously on a PC using a GC-EAD software program (Syntech, The Netherlands). Kovats retention indices (KIs) for the biologically active plant volatiles detected were calculated with reference to *n*-alkanes (C_{10}–C_{30}) injected under the same GC conditions as the analyte.

Direct Analysis of Plant Volatiles. Some 20 g of fresh foliage of *P. cembra* (Pinaceae), fresh and LBM-defoliated *L. decidua* (Pinaceae), or fennel *Foeniculum vulgare* (Apiaceae) were placed in airtight glass bottles (200 ml) with a rubber septum in the lid for 1 hr at room temperature to allow headspace vapor sampling with a 5 ml gas sampling syringe. A 2 ml sample was injected splitless (method described in McMahon et al., 2001) onto the column for GC-EAD analysis.

Porous Polymer Extracts of Plant Volatiles. Some 800 g of fresh foliage of cembran pine and fresh and LBM-defoliated larch foliage (800 g each) were put in airtight dessicators (2 l) fitted with inlet and outlet tubes. Charcoal-filtered air entered through one tube, and a glass cartridge containing 5 g preconditioned (Byrne et al., 1975) Porapak Q® (60–80 mesh, Millipore Corporation, USA) was attached to the other. A water pump sucked air (50 ml/min) over the plant material to the adsorbent. The porous polymer was extracted with 500 μl DCM (Merck, analytical grade) and 2 μl of the extract were injected on-column.

Gas-Chromatography-Coupled Mass Spectrometry (GC-MS). Plant odor extracts analyzed by GC-EAD were subsequently analyzed by GC-MS in as HP 5890 series II chromatograph linked to a HP 5971A mass selective detector (MSD; Hewlett Packard, USA), with the column and conditions as in the GC-EAD analysis (above). Blank controls were analyzed as for the respective headspace extracts. Two microliters of extract were injected on-column; the column was connected via a 1-m deactivated fused-silica capillary (0.25-mm i.d.) to the MSD ion source (temperature 160°C, ionization energy 70 eV) with helium as carrier gas at constant

flow (linear velocity ~30 m/sec at 40°C). Headspace vapor analysis by GC-MS with splitless injection was performed as described in McMahon et al. (2001) under the same conditions as for GC-EAD, but in a Varian 3400-Saturn 3 (CA, U.S.A.), with the MSD, column, and carrier gas flow as above.

Biologically active components of headspace vapors and volatile extracts located by GC-EAD analysis were relocated by GC-MS using KIs, and by comparison of chromatogram profiles. Identification of an electrophysiologically active peak in an extract was first based on the match of its mass spectrum with that of a known product stored in a computer-based library using the HP-Chemstation software. The KI of an unknown chemostimulus from larch and pine was then compared with that of the library-proposed synthetic analogue injected under the same conditions. Biological activity with synthetic analogues of four chemostimuli was established by GC-EAD with LBM antennae (Table 2). Specific enantiomers of compounds identified in extracts with chiral center(s) were not determined, but enantiomers of synthetic chiral products used are indicated in Table 1.

Statistical Analysis. Because of the variation in EAG responses between LBM antennae, responses of a given antenna to different chemostimuli in either EAG or GC-EAD experiments were normalized by summing the responses in millivolts to all the chemostimuli. The percent contribution of each compound to this sum was then square root transformed for statistical analysis. Where responses of the sexes did not differ significantly ($P > 0.05$, ANOVA), male and female antennal responses were pooled within a host race. EAG and GC-EAD response amplitudes of the host races were then compared by ANOVA. All the chemostimuli identified in the GC-EAD analyses of larch and cembran pine volatiles were included in paired comparisons, except the cubebol/epicubebol peak in the cembran pine bouquet that elicited varied responses from the two host race antennae, and an unidentified peak (unidentified 1, M^+ 150) in the larch foliage bouquet that selectively elicited responses from the cembran pine race only. The EAG responses to fresh and LBM-defoliated larch foliage, and to fresh cembran pine foliage, were compared by *t* test (unpaired). The vapors emanating from LBM-defoliated larch were analyzed by GC-EAD with both larch and cembran pine host race antennae, but the porous polymer collected volatiles from LBM-defoliated larch were analyzed only with cembran pine race antennae (the only ones available).

RESULTS

EAG Responses to Larch and Cembran Pine Foliage. Both sexes of the larch and cembran pine LBM host races responded similarly to larch and cembran pine foliage vapors collected in the stimulus cartridge: cembran pine foliage elicited EAG responses of 2.25 ± 0.86 (mean EAG response $\pm$ standard deviation) and 1.71 ± 0.97 mV from the cembran pine and larch host races, respectively, whereas

TABLE 2. ANTENNAL CHEMOSTIMULI IDENTIFIED FOR THE LARCH BUD MOTH AND FREQUENCY OF THEIR DETECTION BY LBM HOST RACE ANTENNAE IN THE ODOR OF HOST PLANTS (LARCH AND CEMBRAN PINE), AND IN THAT OF A NONHOST PLANT (FENNEL)

	Direct headspace vapor injection[a]								Porous polymer trapped volatile extract[b]					
	P. cembra		*L. decidua*				*F. vulgare*[d]		*P. cembra*		*L. decidua*			
	Fresh		Fresh		Defoliated		Fresh		Fresh		Fresh		Defoliated	
Compound	LR ($N = 7$)	PR ($N = 12$)	LR ($N = 7$)	PR ($N = 8$)	LR ($N = 3$)	PR ($N = 6$)	LR ($N = 3$)	PR ($N = 2$)	LR ($N = 4$)	PR ($N = 4$)	LR ($N = 4$)	PR ($N = 4$)	PR ($N = 3$)	Identification criteria[c]
Camphene	7	8	1		1									MRE
β-Myrcene	7	12	1	1	1		3	2	4	4	4	4	3	MRE
Limonene	7	11	1	1	1				4	4	2	4	3	MRE
β-Phellandrene											3	3	3	MR
β-Ocimene							3	2						MR
γ-Terpinene											2	4	3	MRE
p-Cymene	7	12	7	8	3	6	3	2			3	4	3	MR
Unidentified 1 (M^+ 150)											0	4	3	
Unidentified 2									4	4				
Unidentified 3									4	2				
Isopinocamphone											4	4	3	M
Thymol methyl ether									4	4				M
Trans-pinocarveol											4	4	3	M
β-Farnesene											4	4	3	MR
Methyl chavicol							3	2						MR
α-Terpinyl acetate									4	4				MR
Isogermacrene-D											4	4	2	M
α-Farnesene											4	4	3	M
Cubebol/epi-cubebol									3	4				M

Note: Pooled data from male and female antennae; LR = larch race and PR = pine race. Absence of a compound down a column denotes no response from antennae to the dose present in the headspace or extract in GC-EAD analysis (see text).

[a] Foliage headspace injected directly.

[b] Foliage headspace components trapped over a porous polymer.

[c] Criteria for identification of an EAD active peak: M: matching mass spectra with products in the HP-Chemstation database, R: matching retention time of the synthetic analogue, and E: matching electrophysiological activity with that of the synthetic analogue.

[d] Analysis only by females of two host races.

the larch foliage elicited clearly smaller responses of 0.91 ± 0.58 and 1.06 ± 0.35 mV, respectively, ($N = 12$; 6 males and females for each host race). These responses were not different either between sexes of a given host race or between the host races ($P > 0.05$). The higher responses recorded from the antennae of both host races to cembran pine foliage can be explained by the higher amount of volatiles emanating from this host plant (Figures 1 and 2). Using the direct headspace sampling method described here, we estimated camphene at 3 times more, β-myrcene at 10 times more, limonene at 75 times more, and *p*-cymene at 2 times more in the headspace of cembran pine over larch. LBM-defoliated larch elicited EAG responses of 0.69 ± 0.31 mV, no different from the 1.05 ± 0.16 mV responses generated in pine host race antennae to fresh larch foliage ($N = 6$, $P > 0.05$).

GC-EAD Analysis of Larch, Cembran Pine, and Fennel Headspace Vapors. In fresh larch foliage headspace vapor, only the aromatic *p*-cymene elicited a conspicuous and consistent EAG response from male and female antennae of both host races; the response was recorded from all 7 larch and 8 cembran pine host race antennae employed (Figure 1 and Table 2). The EAD responses of 3 larch and 6 cembran pine host race antennae to LBM-defoliated larch vapors were similar to those to fresh larch, where *p*-cymene was again the most active chemostimulus in LBM-defoliated vapors. As with fresh larch, responses were recorded occasionally to camphene, β-myrcene, and limonene in LBM-defoliated larch vapor whenever the injected sample contained the compounds in sufficient quantity.

In cembran pine headspace vapor, in addition to *p*-cymene, the monoterpenes camphene, β-myrcene, and limonene elicited antennal responses from both sexes of the two host races (Figure 2, Table 2). Despite the similarity of the responses of the two host races to chemostimuli from both host plants, cembran pine released a greater number of chemostimuli at a sufficiently high dose to stimulate antennae of both the host races. The vapors released from the two host plants therefore induce a specific array of olfactory responses. GC-EAD analysis of odors of a nonhost plant, fennel, demonstrated the presence of another array of chemostimuli for the LBM composed of monoterpenes β-myrcene and β-ocimene, and the aromatics *p*−cymene and methyl chavicol; these four compounds elicited responses in all the female antennae of the two host races that were used (Table 2).

GC-EAD Analysis of Volatiles Trapped from Larch and Cembran pine. Ten constituents of the porous polymer trapped volatiles from larch elicited responses from antennae of both sexes of the two host races and were identified as the monoterpenes β-myrcene, limonene, β-phellandrene, γ-terpinene, the sesquiterpene β-farnesene, and the aromatic hydrocarbon *p*-cymene (Figure 3 and Table 2). In addition, isopinocamphone, trans-pinocarveol, isogermacrene-Δ, and α-farnesene were tentatively identified by mass spectral matches with computerized database spectra. An unidentified peak (M^+ 150, KI 1316, unidentified 1; Table 2) elicited responses from antennae of both sexes of the cembran pine host

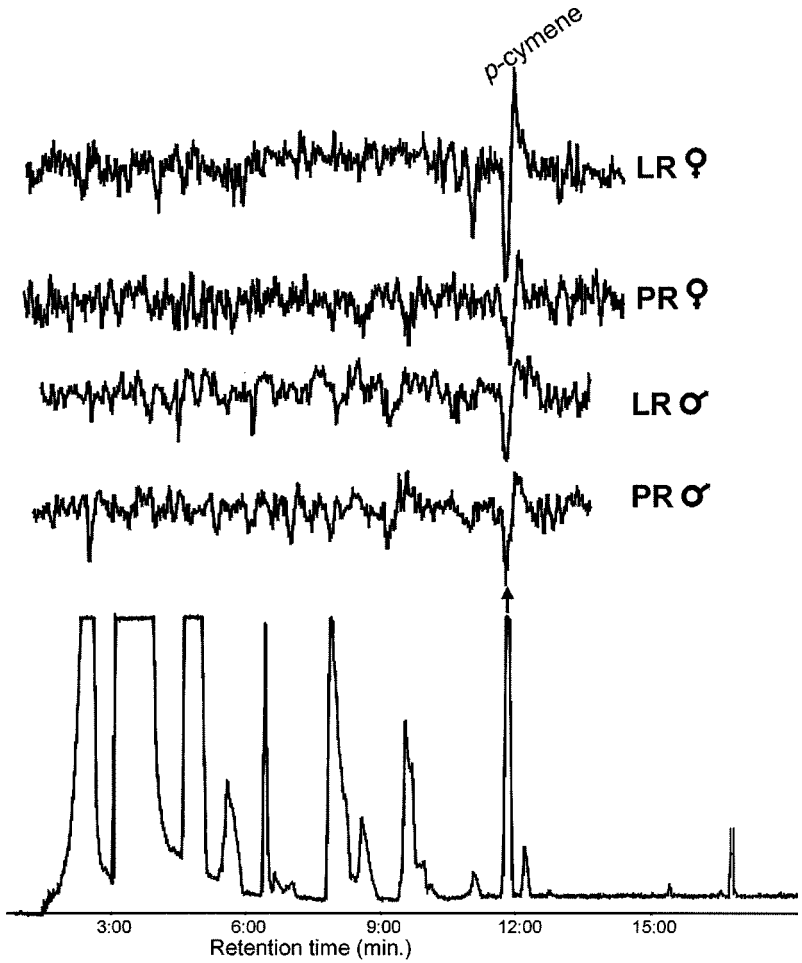

FIG. 1. Analysis of directly injected larch foliage headspace volatiles by GC-EAD with LBM larch (LR) and cembran pine host race (PR) antennae. The *lower trace* is the flame ionization detector (FID) response and the *upper four traces* are EAD responses generated during elution of the biologically active constituents of the headspace odor from the gas chromatographic column.

race only. This product was not included in the paired comparisons of response amplitudes (Figure 5). The porous polymer extract of LBM-defoliated larch volatiles induced similar EAD response profiles as induced by fresh larch foliage in the three cembran pine race female antennae employed. Moreover, the FID detector response of the GC revealed that the amounts of 10 chemostimuli in the porous

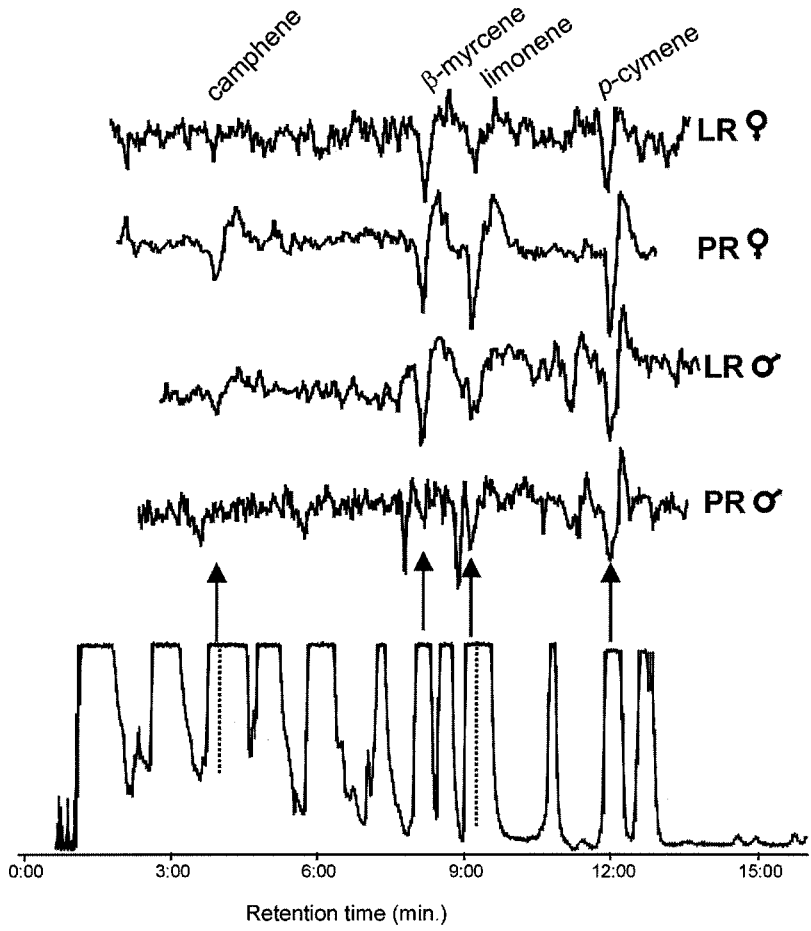

FIG. 2. Analysis of directly injected cembran pine foliage headspace volatiles by GC-EAD with LBM larch (LR) and cembran pine (PR) host race antennae. For further details see text and legend to Figure 1.

polymer extract of fresh larch volatiles were approximately the same in the vapors of LBM-defoliated larch, but the latter did contain additional components.

Constituents of the volatiles trapped from cembran pine elicited EAD responses and were identified as β-myrcene and limonene, and the aliphatic ester α-terpinyl acetate. Thymol methyl ether and a sesquiterpene, which was either cubebol or epicubebol (Figure 4 and Table 2), were also tentatively identified by matches with database spectra. The cubebol/epicubebol peak (KI 1932) induced the strongest antennal response from both host races. However, the response was

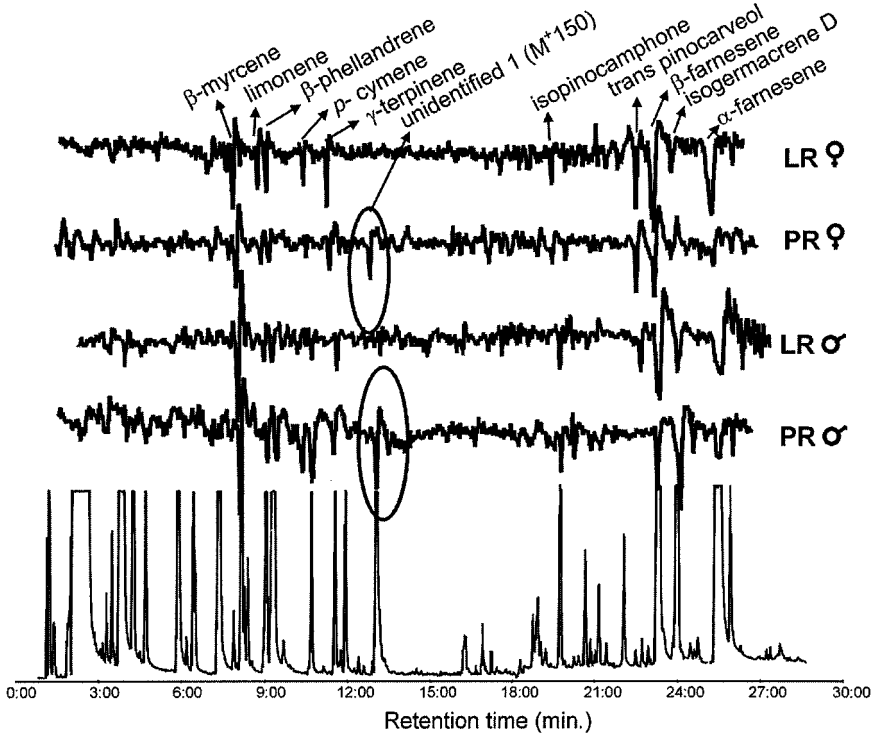

FIG. 3. Analysis of larch foliage odor as collected on a porous polymer by GC-EAD with LBM larch (LR) and cembran pine host race (PR) antennae. For further details see text and legend to Figure 1.

not consistent (observed in 4 of 4 analyses with the cembran pine host race but in only 3 of 4 analyses with larch host race antennae), and so the cubebol/epicubebol peak was not included in the paired comparisons (below). Exact chemical identity of the two chemostimuli with KIs of 1361 (unidentified 2) and 1512 (unidentified 3) could not be established (Table 2, Figure 4). Comparisons of the EAD response amplitudes of male and female antennae of each host race to the 10 larch- and 6 cembran-pine-identified chemostimuli indicated no significant differences either between sexes of the same host race or between the two host races ($P > 0.05$; Figure 5). It is clear, however, that the number and quantity of volatile chemostimuli collected from each host plant is unique, and correspondingly the olfactory response profiles of the LBM host races.

GC-EAD Analysis and EAG Screening of Synthetic Plant Volatiles and Pheromone Components. In the 22-component mixture of generally occurring plant volatiles analyzed by the GC-EAD, 13 compounds elicited electrophysiological

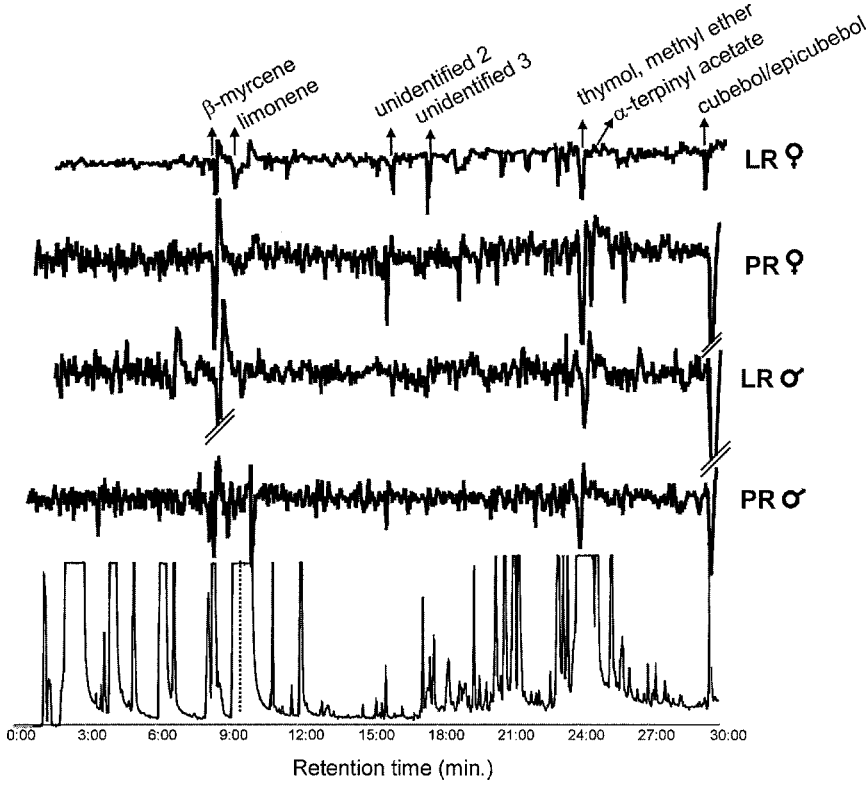

FIG. 4. Analysis of cembran pine foliage odor as collected on a porous polymer by GC-EAD with LBM larch (LR) and cembran pine host race (PR) antennae. For further details see text and legend to Figure 1.

responses from male and female antennae of both host races. In addition to three of the chemostimuli identified above from both larch and cembran pine, i.e., β-myrcene, limonene, and γ- terpinene, 10 other compounds elicited antennal responses, i.e., citronellal, (+)-camphor, (+)-α-terpineol, (+)-carvone, geraniol, (*E*)-2-hexenal, (*E*)-2-hexenol, *p*-(−)-menthyl acetate, (−)-bornyl acetate, and eugenol. In follow-up EAG tests, 15 compounds identified as chemostimuli for the LBM (above) were tested at a 1 μg source dose in the stimulus cartridge. Each of these compounds elicited similar EAG responses from antennae of either host race (Figure 6; $P > 0.05$ between sexes and between the host races). Two mixtures of synthetic plant volatiles containing 11 and 22 components (Table 1) elicited linear dose-dependent EAG responses, covering 3 orders of magnitude (0.1, 1, and 10 μg) from both male and female antennae of the two host races; the 22-component mixture elicited higher responses at all doses tested.

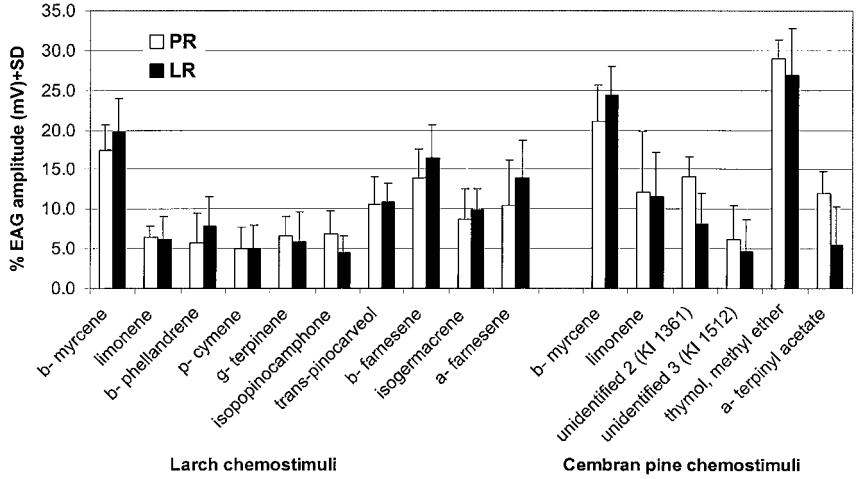

FIG. 5. Electroantennograhic detector responses (mean EAD responses + SD) of LBM larch (LR, $N = 4$) and cembran pine host race (PR, $N = 4$) antennae to chemostimuli eluting from the chromatographic column in the GC-EAD analysis of larch and cembran pine headspace volatiles as collected on a porous polymer (from Figures 3 and 4). Values are percent contribution of each stimulus to the pooled amplitudes generated by all the chemostimuli from a given antenna, and the standard deviation. Male and female responses were pooled for each host race as the effect of sex was not significant ($P > 0.05$).

In order to establish that we were dealing with LBM host races similar to those previously described (Priesner, 1979), the responses of the male antennae were recorded to the LBM host race pheromone components. Male antennae of the two host races showed higher responses to the respective host race principal pheromone components, i.e., antennae of the larch host race responded best to *E*11-14: Ac, whereas those of the cembran pine host race responded best to *E*9-12: Ac ($N = 6$ in each case).

DISCUSSION

The two LBM host races were equally sensitive to the particular suites of volatile chemostimuli released from larch and cembran pine. Mono- and sesquiterpenes accounted for over 80% of the chemostimuli identified for the LBM from larch and cembran pine volatiles. Because both host races feed exclusively on conifers (Baltensweiler et al., 1977), the predominance of terpenes as chemostimuli is not surprising. Antennae of both host races also responded to green leaf volatiles such as (*E*)-2-hexenol and (*E*)-2-hexenal and to the aromatics *p*-cymene,

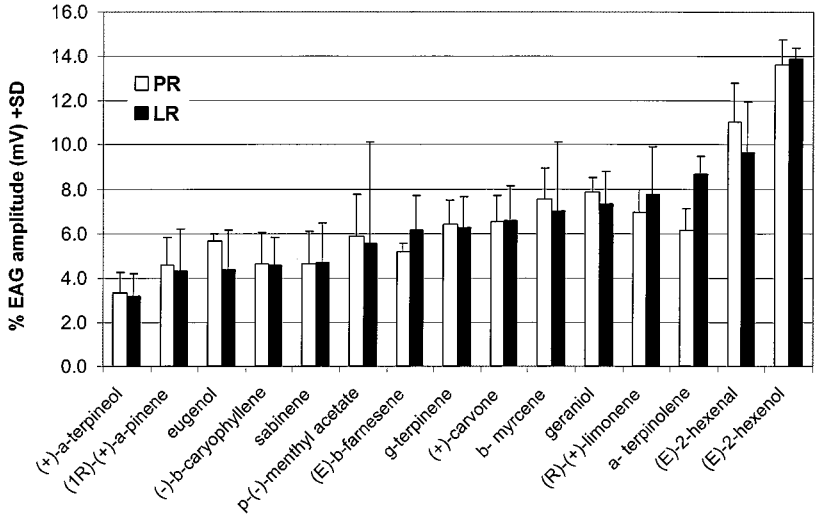

FIG. 6. EAG responses of LBM larch (LR) and cembran pine host race (PR) antennae to a range of plant volatiles. Values are percent contribution of each stimulus to the pooled amplitudes generated by all the compounds tested on a given antenna, and the standard deviation ($N = 7$ and 8 for cembran pine and larch host races, respectively). Male and female responses were pooled for each host race, as the effect of sex was not significant ($P > 0.05$).

eugenol, and methyl chavicol. The responses induced by the green leaf volatiles were the highest for both host races.

However, there was one unidentified compound in the larch porous polymer volatile extract that elicited EAD responses only from male and female cembran pine host race antennae. Otherwise there was no difference in EAG responses of the sexes of either host race to any of the chemostimuli. Furthermore, females of both host races responded equally to a suite of chemostimuli from fennel, a nonhost plant.

Among the 10 compounds chemically identified as stimuli in the porous polymer extracts of larch, 8 of these could be located in cembran pine by single ion monitoring (SIM) (isopinocamphone and α-farnesene were not detected), whereas of the 5 identified cembran pine chemostimuli, 4 were detected by SIM in larch (thymol methyl ether was not detected). High yields of chemostimuli sometimes interfered with the resolution of products: two distinct FID and corresponding EAD responses were recorded from both host races to limonene and β-phellandrene from the porous polymer extract of larch, but these chemostimuli were not resolved in the cembran pine extract because of overloaded nonresolved peaks. The presence of both products in cembran pine was, however, confirmed by SIM in GC-MS.

Antennae of both the LBM host races showed dose-dependent responses to mixtures of plant volatiles, underlining the graded responses of the antennal receptors to the chemostimuli. Clearly, behavioral tests are required to investigate how host plant odors affect LBM host race behaviors.

The EAD response profiles of the LBM larch race antennae to vapors of freshly cut larch foliage and LBM-defoliated larch were the same. However, the EAD profiles induced by direct injection of LBM-defoliated larch vapors or by the porous polymer trapped extract of these volatiles were similar to those induced by fresh larch, but not to those induced by cembran pine. Furthermore, the quantities of the chemostimuli were approximately the same in the LBM-defoliated porous polymer extract as in the fresh larch extract. This would suggest that the LBM-defoliated larch is probably perceived as "larch" by the LBM. Despite this, the LBM-defoliated larch appears not to provide the LBM with an adequate substrate, especially contact chemostimuli, to induce oviposition (Baltensweiler and Rubli, 1999). This may explain the mass migration previously noted from LBM-defoliated larch stands in the Engadine (Baltensweiler and von Salis, 1975).

Cross-attraction between the LBM host races in the field has been documented (Emelianov et al., 2001): larch race females calling from pine attracted significantly more alien males (37% of the total number of males attracted) than did larch race females calling from larch (2.4% alien males). Pine females calling from either pine or larch invariably attracted only 3.6% alien males. This indicates two important points. First, females of both host races could be mated on either host plant. The pheromone of either host race is perceived by at least some males, independent of host odors, i.e. the specificity of chemostimuli from larch or cembran pine does not interfere with pheromone perception. Second, and more important, assortative mating for the larch host race is enhanced when females call from their own host plant, but this almost breaks down when these females call from cembran pine. However, the attraction of pine race males to larch race females is probably more related to the mixed pheromone signals of some larch host race females, which may contain small amounts of *E*9-12: Ac (Guerin et al., 1984; Baltensweiler and Priesner, 1988), than to the context of host plant volatiles in which the pheromone is perceived. Nevertheless, in mixed forests with equal numbers of larch and cembran pines, the two host races showed 80%–90% alighting preferences for their own hosts, and in laboratory choice experiments, larch and pine races preferred their own host of 63%–69% (Emelianov et al., 2003). Even though the olfactory systems of two host races share a common array of receptors responding to a variety of rather nonspecific host plant odors, it is the response of an array of olfactory receptors to the bouquet unique to each host plant that may allow each race to discriminate among different host plants by the across-fiber pattern of activated peripheral receptors (Visser, 1986).

The best-studied insect host races show remarkable similarity in their transition patterns between host plants. The apple maggot fly, *Rhagoletis pomonella*,

has host races on hawthorn and apples, and volatiles identified from apples that attract the apple maggot fly (Fein et al., 1982) were also identified from hawthorn (Carle et al., 1987). The close similarity in volatile profile between apple and hawthorn led Carle et al. (1987) to suggest that the chemical similarity in volatile composition facilitated the shift of *R. pomonella* from hawthorn to apple. Despite the differences in the suites of chemostimuli we collected from larch and cembran pine, a close similarity in the volatile profile has been reported by Rappaport et al. (1996) for alpine larch and cembran pine. We did not, however, investigate the details of the volatile profile of larch and cembran pine, but compared the olfactory responses across both sexes and host races of the LBM to chemostimuli from either host plant.

There is evidence in phytophagous insects for the evolution of monophagy from polyphagy (Ehrlich and Raven, 1964; Bernays and Graham, 1988), with the possible benefit of reduced predation (Jeffries and Lawton, 1984). Among sympatrically evolving host races, the more nutritive resource is usually preferred. This is equivalent to the exploitation of larch with its high nutritive value by the larch host race [nitrogen in larch reaches 25 mg/g needles but only 15 mg/g in evergreen Norway spruce (Baltensweiler, 1992)]. By contrast, LBM larvae exploiting cembran pine must cope with higher amounts of toxic oleoresins (Norin, 1972). It has been suggested that the more generalist cembran pine race surviving on evergreens represents the original form because of its more adaptive characteristics like slower rate of post-diapause development, significantly smaller adult size, and greater survival under nutritional stress (Baltensweiler, 1993; Khomentovsky et al., 1997). Seasonal and annual variation in relative host abundance and suitability, a common occurrence in nature (Boughton, 2000), tends to oppose the increase in host choice. Given a choice between two constant plant resources, an insect should evolve increased fidelity for higher quality or the more abundant host (Fry, 1996). However, predictable seasonal variation in host abundance makes it difficult to eliminate completely the vestiges of choice of an alternative plant (Berlocher and Feder, 2002). In the LBM system, the disadvantage for the larch host race is that larch, despite being more nutritive, is susceptible to defoliation at high moth densities. This results in a less suitable resource for the larch race at regular 8-year intervals. At this point in the cycle, however, the proportion of light morphs capable of exploiting cembran pine (Baltensweiler, 1993) is highest on larch (Baltensweiler, unpublished). Since our data suggest that the larch host race does perceive chemostimuli from cembran pine, we have to assume the host plants may still function as rendezvous sites for both host races, but whether olfactory perception serves to permit utilization of the alternate resource is an open question. Nevertheless, olfactory perception does not act as a definite selective barrier for either host race to restrict genetic diversity within the species *Z. diniana*.

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TASTE RECEPTORS FOR PYRROLIZIDINE ALKALOIDS IN A MONOPHAGOUS CATERPILLAR

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Abstract—Caterpillars of *Utetheisa ornatrix* are monophagous on species of *Crotalaria* from which they obtain pyrrolizidine alkaloids (PAs) for defense and which the males convert to a pheromone. We show that a taste receptor neuron in each of the lateral and medial galeal styloconic sensilla responds to PAs of three different types. Monocrotaline, commonly present in *Crotalaria* species, is the most strongly stimulating with thresholds of response below 10^{-11} and 10^{-9} M in the two sensilla. These are among the lowest known taste thresholds in any insect and are similar to the thresholds for PAs in a polyphagous arctiid caterpillar that also sequesters PAs and uses them as the source of male pheromone. The receptors also respond to heliotrine, a type of PA that is probably never encountered by the insects. Monocrotaline and senecionine *N*-oxide are shown in behavioral assays to be phagostimulants. The data show that there is no tight link between taste receptor sensitivity to specific PAs and hostplant selection in these caterpillars. Perhaps the adults are primarily responsible for hostplant selection.

Key Words—Caterpillar, *Crotalaria*, host specificity, monocrotaline, phagostimulation, pyrrolizidine alkaloid, senecionine *N*-oxide, taste receptor, threshold of response, *Utetheisa*.

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INTRODUCTION

The caterpillars of the arctiid moth, *Utetheisa ornatrix* (L.), are monophagous on species of *Crotalaria*. From the host they acquire pyrrolizidine alkaloids (PAs) that are sequestered for defense (Doussourd et al., 1988; Eisner and Eisner, 1991; Hare and Eisner, 1993; Gonzalez et al., 1999; Eisner et al., 2000) and from which the male moth synthesizes its pheromone (Conner et al., 1981). The species is widely distributed in the tropics of Central and South America and extends northwards into the southern United States. It has been recorded as feeding on at least 10 species of *Crotalaria* (Robinson et al., 2002, list eight in addition to the two known North American hosts), and PAs have been identified from six of these species. Three of them contain PAs of the monocrotaline type, with an 11-membered ring (Figure 1), one species contains the senecionine type, with a 12-membered ring, and two species contain PAs of both structural types (Hartmann and Witte, 1995). The two principal hostplant species in the southern United States are *Crotalaria spectabilis* and *Crotalaria mucronata*, which contain monocrotaline and usaramine, respectively, as their principal PAs (Johnson et al., 1985; Williams and Molyneux, 1987). Thus, *U. ornatrix* appears to be adapted to use both monocrotaline and the

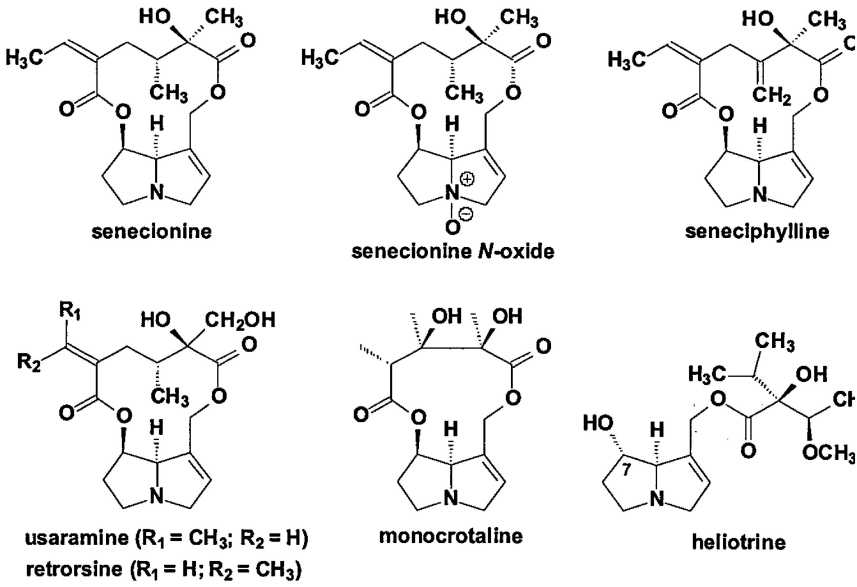

FIG. 1. Structural formulae of some pyrrolizidine alkaloids illustrating the different types of PAs used in the experiments. Monocrotaline is a monocrotaline type, heliotrine a lycopsamine type, and all the others are senecionine type PAs.

senecionine type PAs, and both have been shown to be behavioral phagostimulants for its larvae (Bogner and Eisner, 1991; Kelley et al., 2002) with the implication that the larva possesses taste receptor cells for their detection. Such cells are present in both the lateral and medial galeal styloconic sensilla of larvae of the polyphagous arctiid, *Estigmene acrea*, a species that also uses PAs for defense and pheromone production (Bernays et al., 2002a,b). In *E. acrea* and another polyphagous arctiid, *Grammia geneura*, the neurons respond to a number of different PAs and are very sensitive; that in the lateral sensillum of *E. acrea* is sensitive to concentrations down to at least 10^{-9} M.

In this study, we investigated whether PA-sensitive cells are also present in the galeal sensilla of the larva of *U. ornatrix*. Given that the *Crotalaria* hosts of this species differ qualitatively in their PA content, we examined the responses to a number of structurally different PAs to determine the extent to which the neural responses might be adapted to specific hostplant chemicals. Finally, since the receptors were shown to be sensitive to a number of PAs that are not major constituents of the *Crotalaria* eaten by the population from which our caterpillars originated, we compared the behavioral responses to one of them, senecionine *N*-oxide, with the responses to monocrotaline, a known phagostimulant for *U. ornatrix*.

METHODS

Insects. Adult female moths were collected at the Archbold Research Station in Florida where *Crotalaria mucronata* and *C. spectabilis* are present, although the population from which the insects used in this study originated is generally associated with *C. mucronata*. Eggs laid by the females were allowed to hatch and the larvae were placed on diet (Tobacco Budworm Dry Mix: BioServ® F9915B) with the addition of crushed seed pods of *C. mucronata*. The caterpillars were sent to the University of Arizona, Tucson, where each insect was reared individually in a 200-ml plastic cup containing a small cube of the same diet that was replaced daily. The cups were kept in an environment chamber with a 14:10 1ight–dark cycle and temperature constant at 25°C. Insects used for experiments were in an active feeding stage in the final larval stadium, although their precise age was not always known. Individuals were removed from their diet cups and tested without any imposed period of food deprivation.

Chemicals. Monocrotaline free base was the main PA tested as a representative monocrotaline-type PA, and because it is one of the principal alkaloids in some species of *Crotalaria* eaten by *U. ornatrix*, although it is not present in *C. mucronata* in which usaramine is the principal PA. Usaramine was not tested because it was not available at the time of the experiments. However, its diasteromere retrorsine (Figure 1) was among the senecionine-type PAs that were tested, the others being senecionine and seneciphylline. Heliotrine, a PA monoester of the

lycopsamine type (Figure 1), was included as an example of a structure that is not present in any hosts of *U. ornatrix*. Within-insect comparisons of the responses to different PAs were made at 10^{-7} M on the lateral sensillum and 10^{-5} M on the medial, concentrations at which monocrotaline produced moderate rates of firing in the sensory neurons. Heliotrine, monocrotaline, senecionine, and seneciphylline were all tested as both free bases and *N*-oxides.

All the chemicals were dissolved in 5×10^{-2} M KCI to improve the signal to noise ratio. The PA free bases were first dissolved in ethanol, and KCI solution was added to give a stock solution with a PA at 5×10^{-3} M concentration that contained 5% ethanol. Experiments with *E. acrea* and *G. geneura* had demonstrated that even at this concentration the ethanol did not affect the sensory response (Bernays et al., 2002a,b). Because nearly all the experiments with *U. ornatrix* were performed with the stock solution diluted at least 100-fold, we have assumed that the ethanol had no effect on the responses of this insect.

The pyrrolizidine alkaloids were obtained and prepared as follows. Seneciphylline (final purity >98% containing <1% senecionine) and monocrotaline (>98%) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Heliotrine (>99%) was a gift from Dr Michael Boppré. Senecionine (>90% containing <10% seneciphylline) was isolated from *Senecio vernalis* according to Hartmann and Zimmer (1986). The identity and purity of all PA free bases were confirmed by gas chromatography/mass spectrometry (Witte et al., 1993). If necessary, the alkaloids were purified by silica gel column chromatography (Weber et al., 1999). The PA *N*-oxides were prepared by oxidation of the respective purified free base with 3-chloroperoxybenzoic acid (Craig and Purushothaman, 1970).

Electrophysiology. Electrophysiological recordings were made from the styloconic sensilla on the galea of the caterpillar with the tip-recording method (Hodgson et al., 1955) using live insects immobilized by immersion in a vial of 0.1 M KCI with a rubber gasket around the neck so that the head was exposed (Gothilf and Hanson, 1994). The indifferent electrode was sealed through the glass of the vial so that it made contact with the KCI in which the insect was immersed. Immediately prior to each stimulation, the stimulating solution was drawn from the tip of the recording electrode with absorbent paper to reduce concentration increases due to evaporation. After each stimulation, the insect's mouthparts were rinsed with distilled water and then wiped with absorbent tissue. A Johnson baseline-restoring preamplifier was used to provide high input resistance to reduce the stimulus artifact (Frazier and Hanson, 1986), and the signal was amplified and filtered with a band width set at 130–1200 Hz. The first 1 sec of the response was recorded directly onto a computer in the spike analysis program, SAPID (Smith et al., 1990). Only records from one side of each insect, and only a single record of the response by an insect to each chemical or combination of chemicals, were used for analysis. At least 3 min were allowed to elapse between successive stimulations of a sensillum to ensure complete disadaptation of the receptor cells. Subsequent

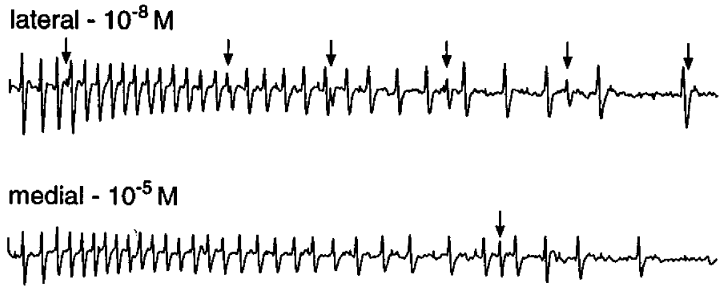

FIG. 2. Examples of the first 250 ms of responses to monocrotaline in the lateral and medial sensilla. Each trace is dominated by action potentials from a single cell (the PA-sensitive cell) with occasional firing from one of the salt-sensitive cells (arrows).

analysis was made in the spike train analysis STA program version 3.0 (courtesy E. Städler), which permits examination of the records at different degrees of temporal resolution.

Action potentials (spikes) were categorized from their regular, and different patterns of firing and by differences in spike height (see Figure 2 and Bernays et al., 2002a). The spikes of PA-sensitive cells were easily identified because they were large relative to any other cells firing at the same time, and their firing rates increased with increasing PA concentration in the stimulating solution. They were easily counted because of their clarity and so are accurate representations of the number of action potentials actually produced. Counts of small spikes from cells with lower firing rates were sometimes less accurate because their signal became confused with that of the PA-sensitive cells, nevertheless their presence could usually be detected (see how spikes show different degrees of overlap in Figure 2A, top trace).

Behavior. Because the ages of the insects were not known with certainty, it was important to use a within-insect design in examining their behavioral responses. This was achieved by comparing the feeding behavior of each individual first on a 2% agar block without additives (control), and then on a similar block containing one of the stimulating chemicals (test). It was then possible to compare the time feeding on test with the time feeding on control by each individual. Because, in the interval between control and test feeding, the insects were becoming more deprived of food and so might eat more for that reason alone, one set of insects was given a second control block following the initial control feeding. If there was no difference in the amounts of agar control and agar only test blocks eaten, it was assumed that any increase in feeding when the test block contained a PA was an indication of phagostimulation and not just of increasing food deprivation. Monocrotaline, known to be a phagostimulant, was used as a measure of a positive response. Senecionine *N*-oxide was tested because it produced a similar response

to monocrotaline in the PA-sensitive cell of the lateral sensillum, but also caused a much greater response from the deterrent cells (see results). It was possible that the input from these cells was sufficient to offset the phagostimulatory effect of the PA-sensitive cell. The PAs were thoroughly mixed with the agar while it was still setting and well below the temperature at which they would decompose. The amount added was equivalent to a concentration of 10^{-7} M in the aqueous fraction.

Feeding behavior was recorded continuously using the Observer Program (Noldus, 1991) from the time an insect first encountered an agar block. The duration of feeding in the following 10-min period was used as the measure of feeding. After this, the agar block was removed and replaced by a second (test) block of agar only, agar with monocrotaline, or agar with senecionine *N*-oxide. Feeding was then recorded for a second 10-min period, and the durations of feeding in the control and test periods were compared using Wilcoxon's sign rank test. The experiments were carried out blind to avoid observer bias.

Statistical analyses were carried out using the IMP 3.2.1 Software program (SAS Institute, 1996) except for the Wilcoxon's sign rank test.

RESULTS

Sensory Responses. The responses to monocrotaline in both the lateral and medial galeal sensilla were dominated by action potentials from a single cell (Figure 2). The threshold of response in the lateral sensillum was between 10^{-12} and 10^{-11} M (paired *t*-test compared with KCI alone: 10^{-12} M, $P = 0.233$, $N = 5$; 10^{-11} M, $P = 0.006$, $N = 9$), and in the medial sensillum was between 10^{-10} and 10^{-9} M (paired *t*-test compared with KCI alone: 10^{-10} M, $P = 0.060$, $N = 9$; 10^{-9} M, $P = 0.009$, $N = 10$) (Figure 3). The firing rate increased with increasing concentration up to at least 10^{-5} M in the lateral sensillum and 10^{-3} M in the medial.

Two other cells also usually responded to the PA in KCI at a low firing rate (Figure 2). The same two cells responded to 5×10^{-2} M KCI alone. Because it was not always possible to distinguish between these cells, especially when the PA-sensitive cell was firing strongly, data for these two cells are pooled in Figure 3. The pooled values did not differ from the response to KCI alone up to 10^{-7} M in the lateral sensillum (ANOVA: $F_{6,62} = 2.246$, $P = 0.052$). At higher concentration, the activity of these cells was often obscured by that of the PA-sensitive cell, but the few data available suggest that their firing rates were higher with 10^{-6} and 10^{-5} M monocrotaline, following the trend suggested by the point at 10^{-7} M in Figure 3. The responses of the salt-sensitive cells in the medial sensillum did not differ from the response to KCI alone up to 10^{-3} M monocrotaline ANOVA: $F_{10,92} = 0.674$, $P = 0.746$).

Comparisons of the responses to different PAs were made using each compound at a concentration of 10^{-7} M on the lateral sensillum and 10^{-5} M on the

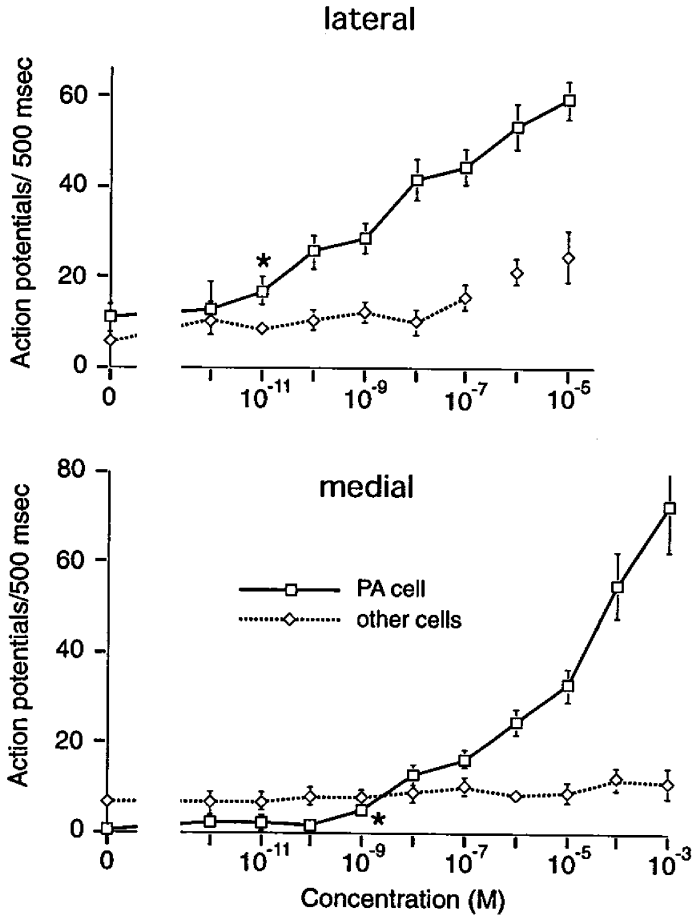

FIG. 3. Concentration/response curves for neurons in the lateral and medial sensilla when stimulated with monocrotaline. Asterisks show the lowest concentration at which the response differs significantly from that to KCI alone. Each point is based on data from 8–11 insects, except for the lowest and highest concentrations (5–7 insects). Two isolated points for salt-sensitive cells (other cells) in the lateral sensillum are means based on only three and four insects because of the difficulty of recognizing the action potentials clearly. Vertical lines are standard errors.

medial. The use of these different concentrations was necessitated by the differences in sensitivity of the neurons in the two sensilla. All the PAs tested produced a response from the PA-sensitive cell in both sensilla (Figure 4). The neuron in the lateral sensillum was equally sensitive to monocrotaline and its *N*-oxide,

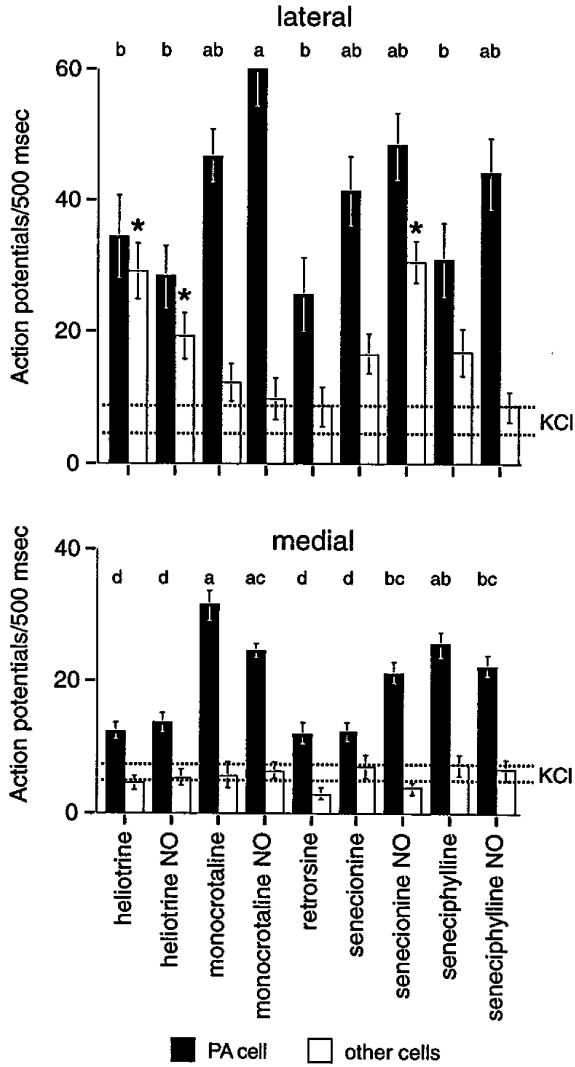

FIG. 4. Comparisons of responses to a range of PAs tested at 10^{-7} M on the lateral sensillum, and 10^{-5} M on the medial sensillum. Vertical lines are standard errors. Responses of the PA-sensitive cell with the same letter above are not significantly different (Tukey–Kramer test). Horizontal dotted lines show the upper and lower limits of standard errors of the response to KCl alone. Asterisks show responses of the salt-sensitive cells that differ from the response to KCl alone (Dunnett's test). Data for lateral sensilla are based on responses of seven insects except that retrorsine was tested on only four insects. Data for the medial sensillum are based on responses of 11 insects.

TABLE 1. MEAN RANKS OF RESPONSES TO STIMULATING PAs ON THE MEDIAL AND LATERAL SENSILLA

Pyrrolizidine alkaloids	Lateral	Medial
Heliotrine	6.6 ± 0.84	7.5 ± 0.33
Heliotrine *N*-oxide	7.9 ± 0.35	7.3 ± 0.31
Monocrotaline	3.3 ± 0.61	1.3 ± 0.14
Monocrotaline *N*-oxide	1.1 ± 0.14	3.0 ± 0.38
Retrorsine	7.4 ± 0.75	7.8 ± 0.54
Senecionine	4.8 ± 0.31	7.1 ± 0.37
Senecionine *N*-oxide	2.8 ± 0.34	4.4 ± 0.34
Seneciphylline	6.7 ± 0.36	2.5 ± 0.33
Seneciphylline *N*-oxide	3.6 ± 0.32	4.1 ± 0.29

Note: Columns are means ± standard errors. Low ranks indicate high responses. Same insects as in Fig. 4.

senecionine and its *N*-oxide, and seneciphylline *N*-oxide. Because there were considerable differences in sensitivity among individual insects, the responses to the PAs were ranked within each insect. Monocrotaline *N*-oxide produced the highest firing rate in the lateral sensilla of six of the seven insects examined, and its average ranking was significantly lower than that of any other compound (Table 1: low rank indicating high firing rate). Retrorsine, in contrast, at the same concentration produced a low firing rate and had a high rank value. With some of the PAs, the firing rate of the salt-sensitive cells also increased (Figure 4, asterisks). With heliotrine and its *N*-oxide and with senecionine *N*-oxide, firing rates were significantly higher than to KCI alone (ANOVA: $F_{9,62} = 6.385$, $P < 0.001$). In a few cases, the firing rates of the salt-sensitive cells were similar to that of the PA-sensitive cell, and it was not possible to count their action potentials.

The neuron in the medial sensillum responded strongly to monocrotaline and its *N*-oxide, and to seneciphylline. Monocrotaline produced the highest firing rate in 8 of the 11 insects with a mean rank of 1.3 (Figure 4, Table 1). Retrorsine, again, was relatively less effective and ranked highest (lowest firing rate) of the nine compounds. Heliotrine and its *N*-oxide, and senecionine produced similar firing rates to retrorsine. In the medial sensillum, the firing rates of the salt-sensitive cells were always low and did not differ with different PAs (ANOVA: $F_{9,109} = 0.939$, $P = 0.495$).

Behavior. When the agar control was followed by plain agar as the test, most insects rejected both blocks (Figure 5), and there was no tendency for the insects to eat more of the test block ($N = 11$, $P > 0.5$). With both monocrotaline and senecionine *N* oxide, the insects usually rejected the plain agar control but fed on the PA-treated block (monocrotaline: $N = 12$, $P < 0.01$; senecionine *N*-oxide: $N = 11$, $P < 0.005$). The average intervals between the end of the control feeding period and first contact with the test block were similar, although there

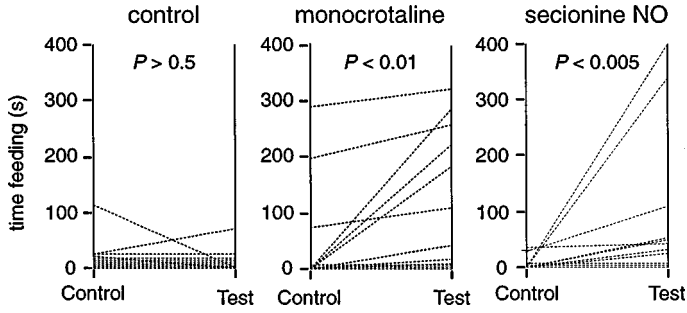

FIG. 5. Results of behavior experiment. Each panel shows the amount of time feeding by the same insects when first offered a block of control diet (left axes) followed by a test block (right axes) each at 10^{-7} M. The test block consisted of a second control block, a block containing monocrotaline, or a block containing senecionine NO (as labeled above each graph). Each line shows the results for one insect. Parallel lines near the *x*-axis should all be on that axis, that is, both control and test values were zero, but have been displaced upwards for clarity. There were seven such insects in control experiments, and three on both monocrotaline and senecionine NO. Control and test values were compared using Wilcoxon's sign-rank test. *P* values are shown in the figure.

was considerable variation (plain agar: 28 ± 5 min [mean ± SE]; monocrotaline: 29 ± 7 min; senecionine NO: 30 ± 6 min).

DISCUSSION

Caterpillars of *U. ornatrix* have sensory neurons in both the lateral and medial styloconic sensilla of the galea that respond to PAs. As in *Estigmene acrea* (Bernays et al., 2002a,b), the neuron in the lateral sensillum has a lower threshold of response than that in the medial sensillum, and the thresholds of both neurons are similar to those in the polyphagous species, slightly below 10^{-11} and 10^{-9} M, respectively. It might have been expected that the threshold for the monophagous *U. ornatrix* would be lower than that for the polyphagous *E. acrea*, and the similarity may reflect the essential importance of the PAs as defensive compounds and pheromone precursors in both species. The dynamic ranges of the neurons over which firing rate is proportional to concentration are even broader than in *E. acrea*, extending over at least six orders of magnitude of concentration (Figure 3). Our experiments do not allow us to determine whether the neurons are dedicated to the perception of PAs, as is the case in the medial sensilla of *E. acrea* and *G. geneura* (Bernays et al., 2002b).

Iyengar et al. (2001) show that female *U. ornatrix* select males on the basis of the amounts of hydroxydanaidal they produce. This is correlated with the PA

content of the insects (Conner et al., 1990; Doussourd et al., 1991) and the amount available for transfer to the females, so that male larvae that consume and sequester greater quantities of PA will enjoy a greater probability of successful mating when they become adult. There is also good experimental evidence that the effectiveness of PAs in defense is proportional to the concentration of PA present in the prey (Silva and Trigo, 2002), although the data relating specifically to *U. ornatrix* are generally unclear on this point. The implication is that larvae should maximize their intake of PAs, and it is recorded that they feed "avidly" on the seeds that have the highest concentrations of PA in the plant (Conner et al., 1990). Larvae that are deficient in PAs also exhibit a greater tendency to cannibalize conspecific eggs and pupae containing PAs (Bogner and Eisner, 1991,1992). The extended dynamic ranges of the PA receptors provide the insect with the capacity to distinguish differences in concentration over a wide range, though whether or not they do so behaviorally has not been investigated. The very low concentrations that the insects can detect may allow them to respond to low concentrations in the plant. Leaves of *C. spectabilis* have much smaller amounts of PAs than flowers and seeds, with averages as low as 0.24% dry weight (Johnson et al., 1985). Much of the feeding of *U. ornatrix* occurs on the leaves of the plants, so they must have the capacity to detect very low concentrations of PAs. Soldaat et al. (1996) did not detect PAs on the leaf surface of *Senecio jacobaea* and concluded that if they were present, their concentration did not exceed 10 nmol/m^2, or about 6×10^9 molecules mm^2. This must be close to the lower margin of sensitivity of the PA-sensitive cell in the lateral galeal sensillum of *U. ornatrix*.

We had expected that the insects' sensory responses to PAs might reflect the occurrence of these compounds in their hosts. Usaramine, the principal PA in *C. mucronata*, was not tested, but retrorsine, a diasteromere of usaramine, was less stimulating than most other PAs (Figure 4, Table 1). On the other hand, monocrotaline, a major constituent of *Crotalaria spectabilis*, which supports other populations of *U. ornatrix* in the United States and is present in several other host species (Hartmann and Witte, 1995), was maximally stimulating for both sensilla either as the free base or the *N*-oxide. This may suggest some degree of adaptation of the insect's PA receptors for compounds that are abundant in the hostplants. However, both lateral and medial sensilla also responded to all the other PAs tested, including some, such as senecionine and seneciphylline *N*-oxides, that are most abundant in species of Asteraceae which are not hosts for *U. ornatrix*. This may not be surprising since these other PAs have 12-membered rings like usaramine. Heliotrine is not known to be present in any host of *U. ornatrix* that is unable to convert it to hydroxydanaidal, the male pheromone (Schulz et al., 1993). Schulz et al. (1993) suggest that this inability may reflect a lack of evolutionary exposure to plants containing this or other PAs with the 7S configuration (see Figure 1). Despite this, heliotrine produces a moderate sensory response especially in the lateral sensilla. Thus, *U. ornatrix* caterpillars are stimulated by a variety of PAs,

some from nonhostplants, which argues strongly against a role for PAs as the only chemicals involved in hostplant selection by the larvae of *U. ornatrix*. However, on the lateral sensillum, several of the stimulating PAs other than monocrotaline also produced high firing rates from the salt-sensitive cells. Based on our studies of another arctiid, *Grammia geneura*, we have suggested that these cells are deterrent cells (Bernays and Chapman, 2001). If this is also true in *U. ornatrix*, it is possible that the input from these cells offsets the input from the PA-sensitive cells, but our behavioral experiment demonstrates that seneciphylline *N*-oxide is a phagostimulant even at a low concentration, and any negative impact of the deterrent cells must be slight. We do not mean to imply that the larvae are necessarily unable to differentiate hostplants from non-hostplants, but our data suggest that PAs alone are not responsible for such distinctions.

Host selection may result primarily from adult behavior. It is known that the eggs are laid on the leaves of *Crotalaria* (Hare and Eisner, 1993), and adults of both sexes are sensitive to hydroxydanaidal (Grant et al., 1989; Bogner et al., 1992). PAs in plants are known to break down to volatile compounds that may include hydroxydanaidal (Bogner and Boppré, 1989), and Bogner at al. (1992) suggest that these products may attract the insects to their hostplants. Whether these products provide host-specific cues is not known.

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BOOK REVIEW

Chemoecology of Insect Eggs and Egg Deposition. By Monika Hilker and Torsten Meiners (editors). Blackwell Publishing, Oxford. ISBN 1-4051-00087 $124.95 US, £60 UK

Who cares about insect eggs? Given the dearth of books devoted to the subject since H. E. Hinton's three-volume work over 20 years ago, one might conclude that the answer is "very few entomologists." However, the various chapters in this book make it eminently clear that while they may not attract quite as much attention as larvae and adults, eggs have been and are the subject of an incredible amount of fascinating research. This multiauthored work edited by Hilker and Meiners provides an excellent, up-to-date review of the work associated with the different facets of "egg" chemical ecology. The volume has been divided into two, not mutually exclusive, sections: the chemical ecology of the eggs themselves and the chemical ecology of egg laying.

In section one, the first two chapters provide the background necessary to actually understand egg production. Trougakos and Margaritis start with an excellent introduction to egg morphology, as well as to the developmental physiology associated with processes including vitellogenesis, the assembly of egg shells, and programmed cell death. Gillott then provides an overview of the male and female reproductive systems, concentrating particularly on the structure and function of the accessory glands. This is understandable for, as he concisely documents, accessory gland secretions not only play many important roles in the production and protection of eggs, but also in behaviors affecting the reproductive success of both sexes. The next two chapters expand on the defensive roles mentioned by Gillott. Chapter 3, by Blum and Hilker, provides an introduction to the vast array of chemical compounds associated with the defense of eggs against natural enemies and, in the case of parasitic insects, the immune reactions of their hosts. In addition, they discuss the origin of compounds: produced *de novo* by adult or egg stages, or acquired from host plants. The parental origin of egg defenses is considered in greater detail in Chapter 4, by Eisner et al. The authors discuss adult behaviors associated with the acquisition of chemicals, introduce the concept of chemically mediated mate choice, and how male compounds transferred during mating may subsequently serve for the defense of the female and her progeny. A number of the same examples are found in Chapters 2–4, but this is not a real

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issue as they are presented from somewhat different perspectives. In Chapter 5, Ayasse and Paxton provide an excellent overview for social insects, where eggs are concentrated in one place but themselves contain no chemical defenses. There is, however, an array of mechanisms present to defend the eggs, from the deposition of repellents at strategic points within the nest, to the production of defense secretions acting as glues, irritants, or toxins. In addition to natural enemies, the survival of eggs in social insects may be affected by nest usurpation and conflicts among nestmates. The potential roles of chemical cues modulating these interactions are also discussed. Chapter 6 introduces the reader to the chemical ecology of positive and negative insect–microorganism interactions that directly or indirectly may affect the egg stage. Topics include the role of microorganisms on female fecundity, in the selection of oviposition sites, the acquisition of essential endosymbionts through the ingestion of the egg chorion (or unhatched conspecifics) by neonate larvae, and the chemical defenses used to reduce pathogenic infections.

Chapter 7, the first in the section dealing with the chemoecology of egg deposition, presents an up-to-date review of oviposition site selection in herbivorous insects. Stadler considers the subject from several different perspectives: (i) where the different chemicals stimulating or deterring oviposition are found, the types of compounds implicated, as well as the intra- and interspecific variability in potential oviposition sites; (ii) the sequential behaviors involved in the location and selection of suitable plants, including the concept of "search images," the role of previous experience and learning; and (iii) the encoding of chemical cues by the adult. The insect is generally perceived as the active participant when eggs are laid, but in Chapter 8, there is convincing evidence that plants are not just passive oviposition sites. Hilker et al. initially discuss direct plant defenses, such as the formation of neoplasms and hypersensitive responses, which serve to reduce egg survival. Then, they present a section devoted to gall formation, underlining the fact that while insects obviously benefit, so also might the plant if the production of a gall localizes herbivore damage. The authors then address the induction of plant defenses in response to insect oviposition, a relatively new research area in which they have played a pioneering role. Anderson, in Chapter 9, discusses how chemical cues of insect origin may influence the selection of a suitable oviposition site. While oviposition-stimulating pheromones are discussed, the majority of this chapter is dedicated to the more intensely studied oviposition-deterring pheromones (ODPs). Topics include the species and developmental stages known to produce ODPs, the site of production and chemical composition of these pheromones, the sensory detection of these cues, and the factors affecting their persistence and efficacy. In addition to a discussion on the intra- and interspecific behaviors that may be influenced by ODPs, their potential use in management programs is also addressed. In Chapter 10, there is a similar treatment for insects of

medical and veterinary importance, with particular emphasis on the Diptera. McCall considers the role of oviposition attraction pheromones in species that oviposit directly on their host (e.g., blowflies), as well as in those where larval development occurs in aquatic (e.g., mosquitoes, blackflies) and terrestrial (tsetse flies, bugs) habitats. The ways in which habitat-related allelochemicals may interact with pheromones and other stimuli influencing (positively or negatively) female oviposition behavior are also presented. Finally, there is a brief discussion of deceitful pollination by plants, based on chemicals that mimic cues used by carnivorous and blood-feeding insects. In Chapter 11, Steidle and van Loon consider the chemoecology of egg laying in parasitoids and predators. They address the similarities (and differences) in habitat location by these two groups of natural enemies, as well as the role of chemical cues in the recognition and acceptance of hosts by parasitoids. These authors also treat current theory dealing with the use of infochemicals to locate oviposition sites, which include aspects of signal reliability, and response plasticity to chemical cues, as well as the importance of innate versus learned responses to infochemicals in specialist and generalist natural enemies.

The last two chapters of this book consider oviposition from an evolutionary perspective, and fit well with previous chapters addressing the more proximal aspects of the same subjects. In Chapter 12, Hoffmeister and Roitberg use the framework of communication theory to address four questions: Why produce an ODP? Where should the insect deposit an ODP? When should an oviposition site be marked? and What information should be transmitted? The authors then examine how natural selection may shape both the use and composition of these cues. In the final chapter, Janz considers the evolutionary ecology of oviposition strategies, with major emphasis on phytophagous insects. Topics include the use of phylogenetic patterns to examine large-scale patterns of host utilization, the potential of mother–offspring conflicts in the selection of oviposition sites, the pros and cons of laying eggs singly or in batches, factors determining oviposition preference, as well as the complexities of host specificity and correlations between oviposition preference and subsequent larval performance.

The overall presentation of this volume is excellent, with clear figures and tables. Furthermore, I detected very few typographic or spelling mistakes. On the basis of the science content alone I would not hesitate in recommending this book as an invaluable reference for those working in chemical ecology and to all entomologists. Collectively the contributors to this edited volume have provided convincing evidence that the chemical ecology of insect eggs has not been neglected. However, they have also provided a second compelling reason to recommend this book. Several authors specifically pointed out avenues of future work, and in all chapters phrases such as “we have only scratched the surface,” “actual proof has not been obtained,” “certainly warrant further investigation,” and “to our knowledge there

are no studies available" abound. Clearly, this field is a veritable goldmine for those seeking interesting and challenging research topics. Therefore, it is hard to imagine we will be waiting another 20 years for the next volume dedicated to eggs.

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ANNOUNCEMENT

The International Allelopathy Foundation is organizing the IV International Congress on Allelopathy—Allelopathy in Sustainable Terrestrial and Aquatic Ecosystems—to be held August 23–25,2004, at CCS Haryana Agricultural University, Hisar, India. There will be both oral and poster sessions. Fifteen symposia are being organized. Deadline for abstract submission is August 31, 2003.

The Conference aims to bring together active allelopathy scientists working worldwide to review progress, identify constraints, and project future goals for basic and applied allelopathy research in Terrestrial and Aquatic Ecosystems. Previous conferences were attended by approximately 200 scientists representing 17 countries. Accommodations are included with the registration fee and will be in the Faculty House at the University. Hisar is a small, ancient city about 165 km west of New Delhi.

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TETRODOTOXIN LEVELS IN EGGS OF THE ROUGH-SKIN NEWT, *Taricha granulosa*, ARE CORRELATED WITH FEMALE TOXICITY

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Abstract—We quantified the amount of the neurotoxin tetrodotoxin (TTX) present in females and newly deposited eggs of the rough-skin newt, *Taricha granulosa*, to examine the relationship between the toxicity of an individual female and the toxicity of her eggs. We found high levels of TTX in individual eggs as well as substantial variation among clutches. Variation in the amount of TTX per egg within individual clutches was extremely low. Female skin toxicity was positively correlated with the mean egg toxicity of her clutch. Neither egg volume nor female size was significantly correlated with egg TTX levels. Tetrodotoxin stereoisomer–analog profiles were identical for females and their eggs. The presence of high levels of TTX in individual eggs coupled with the relationship between levels of TTX in female skin and levels of TTX in her eggs suggests that the TTX present in eggs of *T. granulosa* is maternally derived. The lack of correlation between egg size and TTX levels in individual eggs, as well as the low levels of within clutch variation, may indicate that deposition of TTX in eggs of *T. granulosa* is not linked to the deposition of other egg resources (e.g., lipids or other yolk components).

Key Words—Tetrodotoxin, chemical defense, egg toxicity, *Taricha granulosa*, Salamandridae, Caudata.

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INTRODUCTION

The rough-skin newt, *Taricha granulosa*, possesses high levels of the neurotoxin tetrodotoxin (TTX) in its skin (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974; Hanifin et al., 1999). Tetrodotoxin serves as a defensive compound in adult newts (Brodie, 1968; Brodie et al., 1974; Daly et al., 1987). Although this toxin is concentrated in the granular glands of the skin (Wakely et al., 1966; Brodie et al., 1974; Tsuruda et al., 2002), it is also present in other tissues, including ovaries, ova, and eggs (Twitty, 1937; Wakely et al., 1966). Tetrodotoxin has also been reported in the eggs or ovaries of other TTX-bearing amphibians, including the frog *Atelopus chiriquiensis* (Pavelka et al., 1977) and the salamander *Notophthalmus viridescens* (Brodie et al., 1974), as well as puffer fish (Goto et al., 1965; Mahmuda et al., 2003). Additionally, Daly et al. (1987) state that TTX is likely present in the eggs of other frog species that possess TTX, but whose eggs and ovarian tissues have not been examined (e.g., other *Atelopus* spp.).

The role of TTX in eggs and reproductive tissues of any species is unclear. In puffer fish, TTX may function as a pheromone (Matsumura, 1995), possibly explaining the high concentrations of TTX found in their ovaries. Tetrodotoxin may serve as an antibacterial or antimycotic agent in amphibian eggs (Brodie et al., 1974; Daly et al., 1987; but see Kim et al., 1975), but the general view is that its primary function is as a chemical defense against egg predation (Twitty, 1966; Daly et al., 1987). Early estimates of egg toxicity in *Taricha* (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974) also indicate that levels of TTX present in newt eggs are probably sufficient for TTX to play a defensive role in those eggs. The defensive role of toxins present in eggs of other taxa has been demonstrated to various degrees. *Bufo* eggs are lethal or unpalatable to known or potential predators of those eggs (Licht, 1967, 1968, 1969; Brodie et al., 1978; Crossland and Alford, 1998). Eggs of the marine gastropod *Dolabella auricularia* possess compounds that increase their unpalatability to possible predators (Pennings et al., 1999), and the importance of pyrrolizidine alkaloids as a defensive mechanism in the eggs of *Utetheisa ornatrix* has been elegantly documented (Dussord et al., 1988; Eisner and Meinwald, 1995; Eisner et al., 2000).

In many of the species that produce toxic eggs, the toxins present are either maternally derived (Licht, 1967, 1968; Brodie et al., 1978) or, in cases where the toxin is of exogenous origin, toxin deposition is under active maternal control (Dussord et al., 1988; Eisner and Meinwald, 1995; Eisner et al., 2000). Brodie et al. (1974) proposed that the TTX present in eggs of *T. granulosa* originates in the female and is secondarily deposited in eggs. Thus, the toxin present in *Taricha* eggs may be a result of maternal endowment. This maternal endowment of TTX does not require that newts are producing their own TTX; if the ultimate origin of TTX is exogenous, females could still control the deposition of TTX in eggs. Extensive work in a range of taxa (including *Taricha torosa*) has demonstrated that variation in maternally endowed egg resources can have a significant effect on

offspring quality (Kaplan, 1985; Sinervo, 1990, 1999; Gil et al., 1999; Komoroski and Congdon, 2001). The primary focus of this work has been the distribution of energetic resources in eggs and the effect that variation in resource distribution has on offspring quality. However, Komoroski and Congdon (2001) make the point that other maternally allocated egg components (e.g., protective chemicals such as TTX) can affect offspring quality independently of either egg size or offspring size. This type of differential investment of nonenergetic egg resources independent of egg size was demonstrated in zebra finches (Gil et al., 1999) and is well documented in *Utethesia ornatrix* (Dussord et al., 1988; Eisner and Meinwald, 1995; Eisner et al., 2000), but has not been shown in amphibians. If TTX is an important defensive compound in eggs of *T. granulosa*, and the proximate source of egg TTX is maternal (e.g., produced by the mother or, if the ultimate origin of TTX is exogenous, sequestered by the mother), variation in maternal allocation of TTX in eggs could have a significant effect on egg survivorship, and thus on offspring fitness.

Understanding the origin of TTX, as well as its role and the contribution of differential maternal allocation, in the eggs of *T. granulosa* requires detailed information on levels of TTX in individual eggs as well as the females that produced those eggs. Previous work has demonstrated the presence of TTX in eggs (or ova) of *T. granulosa* and *T. torosa* (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974), but these studies could not examine individual female toxicity or directly measure individual egg toxicity. Nor did these studies measure variability in TTX levels within individual clutches or variation between clutches within populations. We measured the TTX toxicity of individual *T. granulosa* eggs with a known "family structure," (i.e., from clutches for which the female toxicity was known). This sampling strategy allowed us to quantify the variance in TTX toxicity within and among clutches of eggs from the same population and to evaluate some of the possible factors important in that variance.

METHODS AND MATERIALS

Sampling. We measured the TTX levels in 68 *Taricha granulosa* eggs and 11 females from a previously studied population (Hanifin et al., 1999, 2002) located in the central Willamette Valley (Benton County, Oregon). Gravid female newts were collected in the spring of 2000 ($N = 10$) and the spring of 2001 ($N = 1$). Females collected in 2000 spontaneously deposited eggs, but were also injected with 10 μl LHRH (de-Gly^{10}, [d-His(Bzl)6]-Luteinizing Hormone Releasing Hormone Ethylamide; Sigma #12761) to prolong egg deposition in captivity; the female from the 2001 collection deposited eggs spontaneously. A subset of the eggs deposited by each female were collected within 15 hr of deposition and frozen at -20°C. Eggs from this sample were used for toxin analysis (10 eggs each from 3 clutches, 5 eggs each from 7 clutches in 2000, and 3 eggs from a single clutch in 2001). An additional four gravid females were collected from Benton County in 2001. These females were euthanized and preserved in formalin. Each of these

animals was dissected and all ova counted and used to estimate a typical Benton County clutch size for *T. granulosa*.

Female Morphological and Egg Measurements. Egg diameters were measured to the nearest 0.1 mm, using an ocular micrometer. Because previous reports (Twitty, 1966) and preliminary observations (Hanifin, unpublished data) indicated that TTX is concentrated in the yolk and cytoplasm of the oocyte rather than the gel coat surrounding the egg, we measured the diameter of the oocyte rather then that of the total egg (oocyte + gel coat). Females (except for the animal collected in 2001) were weighed and both total length (TL) and snout-vent length (SVL) were measured. Two circular skin samples (0.2 cm^2) were removed from the dorsal midbody (one on either side of the vertebral column) with a human skin biopsy punch (Acupunch™, Acuderm Inc.). This methodology allows for a high degree of accuracy and repeatability when measuring skin toxicity levels (Hanifin et al., 2002).

Toxin Extraction. Extracts from individual eggs were prepared by grinding a single egg in 100 μl of extraction buffer (0.1 M acetic acid). This homogenate was mixed thoroughly and heated for 5 min in a boiling water bath. After heating, the homogenate was cooled to 32°C and spun at 13,000 rpm for 20 min in a microcentrifuge. The supernatant was removed and spun again at 13,000 rpm for 20 min in 0.5-ml Millipore centrifuge tubes (Ultrafree-MC, 10,000 NMWL filter units). Aliquots of 20 μl were used for analysis. Extracts from each skin sample were prepared in the same manner after grinding and extracting in 800 μl of 0.1 M acetic acid using a 1-ml glass tissue grinder (Kontes). This extraction procedure has been shown to be highly repeatable (Hanifin et al., 1999) and is not a significant source of variance in our analysis.

TTX Assay. The levels of TTX present in our samples were quantified by fluorometric HPLC, modified from Yasumoto and Michishita (1985) and Yotsu et al. (1989). Separation of TTX and TTX analogs was performed on a Develosil RP-Aqueous (0.46 × 25 cm or 0.46 × 15 cm, Phenomenex, USA) reverse-phase C30 column with a 25 mM ammonium acetate and 35 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 1% acetonitrile run at a flow rate of 0.5 ml/min. The eluate was mixed with an aqueous 5 N NaOH solution from another pump (0.9 ml/min) and passed through an ASI 330 postcolumn reactor (1-ml reaction chamber) heated to 150°C to derive analogs to fluoresce. After cooling in a 10-cm water jacket, the fluorescent derivatives were detected by a Rainin Dynamax FL-2 fluoromonitor. The excitation wavelength of the detector was set at 365 nm and the emission wavelength was set at 510 nm. The peak areas were measured and integrated with a Hewlett-Packard 3390a integrator. Peak area concentration curves were calculated using standards prepared from commercial TTX (Sigma).

Statistical Analyses. All analyses were performed using either Statview 4.02, Super Anova (Abacus Concepts), or SAS version 8.1 (SAS Institute). We calculated Pearson product–moment correlations between female skin toxicity and mean egg toxicity of her clutch as well as among the female size estimates (mass,

SVL, TL) and female toxicity. These analyses did not include the female collected in 2001 because morphological data were not available for that female. In addition, we used linear regression to examine the relationship between female skin toxicity and the mean egg toxicity of her clutch. We used a one-way analysis of variance (ANOVA) with clutch as the factor to test for differences in toxicity among clutches as well as differences in egg size among clutches. We used analysis of covariance (ANCOVA) with egg volume as a covariate of TTX to test for the effect of egg volume on individual egg toxicity within and among individual clutches.

RESULTS

The TTX levels of the 11 female newts in our study sample ranged from 0.046 to 0.487 mg TTX/cm^2 skin (Table 1), with a mean of 0.200 ± 0.042 (SE) mg TTX/cm^2 skin. Female toxicity was not correlated with any of the female morphological measurements (mass, SVL, or TL) (Table 2). The stereoisomer profile of our animals was also identical to that previously reported (Hanifin et al., 1999, 2002) (Figure 1). Only TTX (peak **A**) and its apparent equilibrium products (tetrodonic acid (peak **D**), 4-*epi*TTX (peak **B**), and *anhydro*TTX (peak **C**)) were present in the skin of newts from this locality.

Tetrodotoxin also was present, in remarkably high levels, in each of the eggs assayed. The mean egg TTX per clutch ranged from 804 to 2767 ng (Table 1). The stereoisomer profiles of eggs matched the stereoisomer profiles of adults (Figure 1). Tetrodotoxin was present in only trace amounts in isolated gel coats of eggs.

TABLE 1. MASS, STANDARD LENGTH (SVL), TOTAL LENGTH (TL), MEAN EGG TETRODOTOXIN PER CLUTCH, AND FEMALE SKIN TETRODOTOXIN LEVELS FOR *Taricha granulosa*

Female mass (g)	SVL (cm)	TL (cm)	#Eggs	Mean TTX (ng)/egg $\pm$ SE	TTX (mg/cm^{2}skin)
6.21	6.5	14.0	$N = 10$	1314.8 ± 38.3	0.184
9.0	6.6	14.7	$N = 10$	1642.9 ± 45.0	0.182
9.0	6.6	15.9	$N = 10$	820.0 ± 32.2	0.221
11.44	7.4	16.1	$N = 5$	858.5 ± 29.0	0.092
9.33	6.4	13.6	$N = 5$	2630.6 ± 65.9	0.190
7.13	6.6	15.1	$N = 5$	2767.4 ± 25.1	0.272
10.23	6.7	15.7	$N = 5$	804.0 ± 30.1	0.058
10.48	7.1	15.8	$N = 5$	672.8 ± 44.1	0.082
10.5	6.8	15.2	$N = 5$	1823.7 ± 69.2	0.384
8.18	6.6	13.6	$N = 5$	967.5 ± 54.5	0.046
NA	NA	NA	$N = 3$	2506 ± 80.1[a]	0.487[a]

[a] Morphological data were not taken for this female.

TABLE 2. PEARSON PRODUCT–MOMENT CORRELATIONS (ρ) FOR FEMALE MORPHOLOGICAL MEASURES AND FEMALE TOXICITY, MEAN EGG TTX PER CLUTCH, AND EGG VOLUME

	Female	ρ	P	N
Female toxicity	Mass	−0.17	0.65	10
	SVL	−0.28	0.43	10
	TL	−0.006	0.99	10
Mean egg TTX/clutch	Mass	−0.36	0.301	10
	SVL	−0.50	0.140	10
	TL	−0.45	0.190	10
Egg volume	Mass	−0.51	0.136	10
	SVL	−0.26	0.464	10
	TL	−0.10	0.779	10

Mean levels of egg TTX per clutch varied significantly between clutches ($F_{9,55} = 237.7$, $P < 0.001$) (Table 1, Figure 2), and this variation was correlated with female TTX levels ($R = 0.69$, $N = 11$, $P = 0.019$); (Figure 2). No other factors that we measured were important correlates of egg toxicity. None of the female size measures were correlated with mean egg TTX levels per clutch (Table 2). Although egg volume varied significantly among clutches ($F_{9,55} = 3.628$, $P = 0.001$), this variation in egg volume was not correlated with variation of TTX levels among clutches ($F_{1,45} = 0.18$, $P = 0.673$) or within clutches ($F_{9,45} = 0.134$, $P = 0.999$). None of the female size measures were correlated with egg volume (Table 2). Within clutch variation in TTX levels was extremely

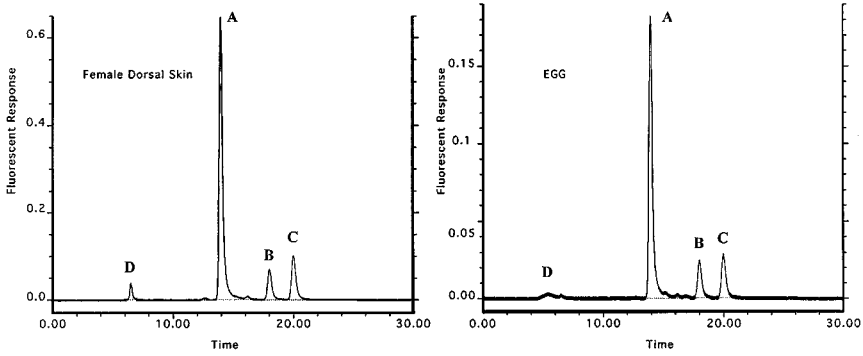

FIG. 1. HPLC-FLD chromatograms of a single typical female *Taricha granulosa* from Benton County, OR (left) and one of her eggs (right) showing the similarity in TTX isomer profiles between female and egg. Note that all analogs present in the female are present in the egg (A = TTX, B = 4-epiTTX, C = anhydroTTX, D = tetrodonic acid).

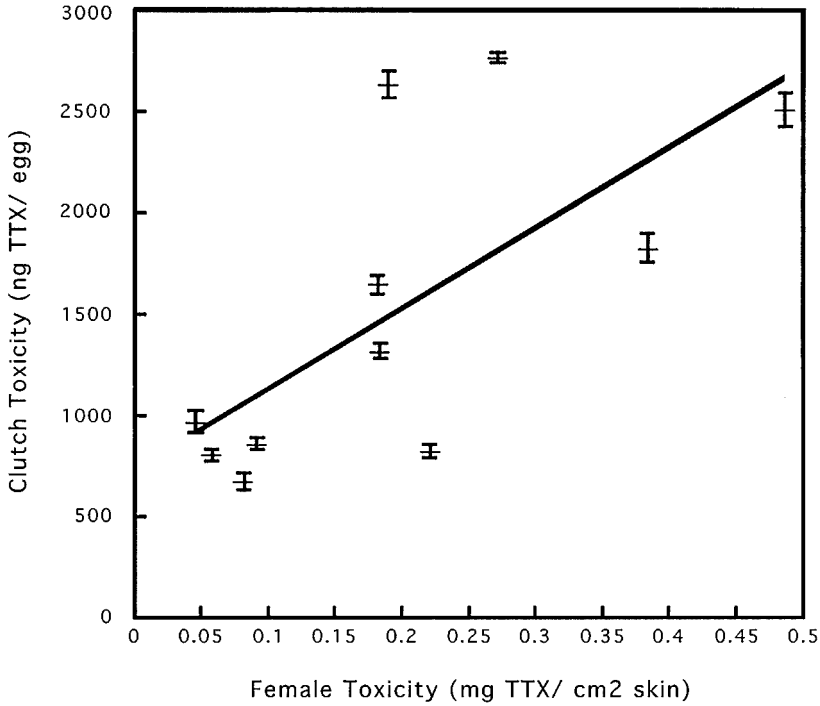

FIG. 2. Graph of egg toxicity versus female toxicity showing a correlation between female toxicity (on the *x* axis) and mean egg toxicity per clutch (on the *y* axis). Individual clutches are represented as the mean egg toxicity (light line) with standard error bars. The regression line: Mean Clutch TTX (MCT) = Female TTX*3968 + 735 is significant ($F_{1,9} = 8.128$, $R^2 = 0.48$, $P = 0.019$).

low (Table 1, Figure 2). Mean clutch size (four clutches) was 542 (SD = 110) eggs.

DISCUSSION

Female *Taricha granulosa* with higher levels of TTX in their dorsal skin produced eggs with higher levels of TTX. This correlation between female toxicity and egg toxicity suggests that the TTX present in *T. granulosa* eggs is maternally derived and is allocated by the female. A maternal origin of TTX is further supported by the early developmental stages of the eggs we sampled. In our samples, eggs were frozen within a short time of deposition (no later than 15 hr), and it is unlikely that the TTX present in eggs could be synthesized by developing embryos during that period. Additionally, levels of TTX in *T. torosa* eggs and larvae are

known to decrease as fertilized eggs develop (Twitty, 1937), indicating that new synthesis of TTX does not occur in the eggs while the yolk is being absorbed. Although the TTX present in eggs appears to be a result of maternal allocation, we have no evidence for differential maternal investment of toxin. The correlation between female toxicity and clutch toxicity may indicate that even if females are provisioning their eggs with TTX, they may not be actively controlling the process.

The fact that larger eggs do not posses more TTX than smaller eggs is, however, suggestive that the deposition of TTX in the eggs of *T. granulosa* may be independent of other egg provisioning pathways and, possibly, under maternal control. Other maternally invested resources in *Taricha* are positively correlated with egg size (Kaplan, 1985), while TTX is not, indicating that TTX deposition may not be linked with the deposition of other resources. Likewise, passive diffusion of TTX into eggs should result in larger eggs possessing higher levels of TTX, yet this is not the case in eggs of *T. granulosa*. There is a precedent for active deposition of resources independent of egg size in zebra finches (Gil et al., 1999), but it has not been demonstrated in salamanders. If deposition of TTX in the eggs of *T. granulosa* is independent of other egg provisioning, it could allow selection to act on the process of TTX deposition more easily and also could allow for differential investment of TTX independent of energetic resources.

The amount of TTX present in a single egg, or clutch, of this species is greater than that reported for any other amphibian species in which TTX is present. The average toxicity of a single egg from the most toxic clutch of our study population is 2.767 μg or roughly 14 Mouse Units (M.U.) (Hashimoto, 1979) of TTX per egg. Given that the range of clutch sizes we measured in four females was 400–655 eggs, the total amount of TTX present in a single *T. granulosa* clutch might be as high as 1.8 mg (or approximately 8000 M.U.) of TTX. This level is substantially larger than that reported for *Atelopus chiriquiensis*, which possesses approximately 870 M.U. of TTX per clutch and about 2.5 M.U. of TTX per egg (Pavelka et al., 1977). Estimates from *T. torosa* are more comparable to our results (Mosher et al., 1964; Wakely et al., 1966). Mosher et al. (1964) report that a pooled sample of 100 kg of *T. torosa* eggs (13,000 clutches with a total of $\approx$250,000 individual eggs) contained 330 mg of TTX or approximately 1.320 μg of TTX per egg, nearly identical to the 1.315 μg of TTX per egg present in our median clutch. Estimates of TTX levels in eggs of other species that posses TTX are less comparable to our results. Brodie et al. (1974) reported that the toxicity of extracts taken from *T. rivularis* ova are roughly similar to those of *T. granulosa* and *T. torosa*, but that extracts of *Notophtalmus viridescens* ova possessed roughly 1/100th the toxicity of *T. granulosa* extracts. This is not surprising given that the skin from adult *N. virdescens* is known to have substantially less toxin that *T. granulosa* (Brodie et al., 1974; Yotsu-Yamashita and Mebs, 2001). The skin TTX levels of females in our study sample were comparable to those previously reported for newts from the Benton County locality. Our mean level of TTX (0.200 ± 0.042 (SE) mg TTX/cm^2

skin) is similar to that previously reported (0.169 ± 0.02 (SE) mg TTX/cm^2 skin from Hanifin et al., 2002).

Levels of TTX in individual eggs are virtually identical within a given clutch. It is impossible to compare within clutch variation in toxicity in *T. granulosa* to that of other toxin bearing amphibians because other studies have not quantified variation within clutches. However, in insects, one species has been tested for within clutch variation in egg toxin levels. In the moth *Utheisa ornatrix*, low levels of variation in within clutch toxicity are an important defensive factor for the eggs (Eisner et al., 2000). Because *T. granulosa* disperses its eggs and *U. ornatrix* deposits a single clutch in one place, the low levels of within clutch variation that we see in *T. granulosa* are not as indicative of a defensive role for TTX in the eggs of *T. granulosa.* Although the similarity of TTX levels within a clutch of *T. granulosa* eggs may not be evidence for a defensive role for TTX in the eggs, it provides additional evidence that toxin deposition in *T. granulosa* eggs may be under some type of maternal control.

The positive correlation between female skin toxicity and egg toxicity, as well as the apparent independence of TTX deposition from yolk deposition, raises the intriguing possibility that adult skin toxicity might experience indirect selection. In adult newts, the efficacy of skin TTX as a defensive compound against vertebrate predators is well understood (Brodie, 1968). If the TTX present in the eggs of *T. granulosa* is also defensive (functioning to deter other vertebrate or invertebrate egg predators) then selection on egg TTX levels would generate correlated selection on maternal toxin levels. Likewise, egg TTX levels could evolve indirectly through the reciprocal pathway. This sort of indirect selection would occur regardless of whether maternal deposition of TTX into eggs is active or passive, and requires only the phenotypic correlation between maternal and egg traits to occur (Lande and Arnold, 1983). Further ecological studies of predation on *T. granulosa* eggs and adult newts are needed to understand the ecology of TTX in the eggs of *T. granulosa* as well as understand the driving forces behind the evolution of egg toxicity in this species.

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VENOM VARIATION AND CHEMORECEPTION OF THE VIPERID *Agkistrodon contortrix*: EVIDENCE FOR ADAPTATION?

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Abstract—Previous studies of chemoreceptive behavior in vipers suggest that snakes focus on the scent of envenomated tissue to track their prey following envenomation. Other studies have indicated a correlation between qualitative differences in venom biochemistry and geographic variation in diet. The North American copperhead (*Agkistrodon contortrix*) varies geographically in diet and venom biochemistry; snakes were collected from three populations (Kansas, Texas, and Louisiana) that are known to have different prey preferences. Behavioral experiments were conducted to assess whether copperheads preferred envenomated prey more than nonenvenomated prey, as do other species of vipers studied thus far. Additional experiments tested the ability of copperheads to distinguish between envenomated prey from different geographic populations, and between geographic populations of copperheads and two other species of viper. Results indicated that copperheads prefer envenomated prey to nonenvenomated prey. In envenomated-prey discrimination experiments, copperheads distinguished between envenomated prey from different geographic populations, and some snakes distinguished envenomated prey of *A. contortrix* from those of *A. piscivorus* and *Sistrurus catenatus*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the variation

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of venom biochemistry in this species and two other taxa (*A. piscivorus* and *S. catenatus*), and confirmed intraspecific and interspecific variation of venom proteins. Relative potency of the venom from different populations as indicated by time to immobilization experiments was in the order: Louisiana > Texas > Kansas. The relative potency of the venom from each population matched the order of preference in the chemoreception experiments. These results suggest that chemoreception is sensitive to subtle differences in venom biochemistry and may reflect adaptation to improve efficiency of finding envenomated prey.

Key Words—*Agkistrodon contortrix*, *Sistrurus*, chemoreception, venom, snake, SDS-PAGE, behavior, adaptation.

INTRODUCTION

Vipers are sit-and-wait, venomous predators that typically strike, envenomate, and release their prey (but see Kardong, 1986 for exceptions), and then use tongue-flicking (chemoreception) to trail and locate prey (Chiszar et al., 1992). Tongue-flicking has been used in numerous studies of squamate behavior as an index of chemosensory investigation (Font and Desfilis, 2002, and citations within). Studies of chemoreception in venomous snakes have demonstrated that individuals tongue-flick mammalian prey envenomated by a conspecific significantly more often than prey that is not envenomated—this phenomenon of envenomated prey preference is common in viperids (e.g., Baumann, 1927, 1928, 1929: *Vipera aspis* and *V. berus*; Chiszar et al., 1992: *Agkistrodon piscivorus* and *Gloydius blomhoffi*; Duvall et al., 1980; Robinson and Kardong, 1991; Chiszar et al., 1992; Lavin-Murcio and Kardong, 1995; Chiszar et al., 1999: *Crotalus atrox*, *C. durissus*, *C. enyo*, *C. horridus*, and *C. viridis*). These studies suggest that after striking prey, vipers use chemical cues from envenomated tissue to a larger extent than chemical cues produced by the prey (e.g., prey scent, urine, or alarm pheromones) to locate their prey (Chiszar et al., 1992). Only conspecifics were used in these experiments; thus, it was not determined whether snakes could differentiate among venoms produced by different species.

Viper venom consists mainly of a complex mixture of peptides and proteins, which immobilize, kill, and digest prey (Thomas and Pough, 1979; Russell, 1980). Crotaline venom proteins have numerous deleterious effects on prey including, but not limited to, local tissue and systemic vascular damage, hemolysis, and fibrinolysis (Johnson and Ownby, 1993; Gomez and Dart, 1995). Venom composition and mode of action can vary at all taxonomic levels from populations to families (Jones, 1976; Broad et al., 1979; Mebs, 1999). Presumably, tissue envenomated by different species should produce different chemical cues owing to variation in the chemical composition of the venoms. Because species with venoms of relatively high toxicity can kill prey faster (León et al., 1999; Mebs, 1999), the distance needed to travel to envenomated prey is decreased. We hypothesize that as venom toxicity increases, the ability of snakes to detect envenomated prey from a distance (i.e., salience), or ability to follow envenomated prey trails increases. Thus, the

ability to distinguish between envenomated prey from snakes with disparate venom composition could reflect adaptations of local populations to improve chemoreceptive ability. Chiszar et al. (1999) demonstrated the minimal perceptible venom dose required for effective rattlesnake chemoreception (~6.0 mg), but the effects of qualitative venom variation on the chemoreceptive ability of vipers has never been tested.

Agkistrodon contortrix is a medium-sized crotaline viper, which ranges from extreme northeastern Mexico to Massachusetts. Currently, five subspecies are recognized (Gloyd and Conant, 1990). Rodents are the main prey item throughout its range, although local populations may concentrate on other types of prey (e.g., Louisiana: frogs, Dundee and Rossman, 1989; Texas: cicadas and lepidopteran larvae, Lagesse and Ford, 1996; Kansas: mammals, Fitch, 1960). Populations of *A. contortrix* vary both in venom biochemistry and diet (Fitch, 1960; Jones, 1976; Dundee and Rossman, 1989; Lagesse and Ford, 1996). Correlations between venom and diet have been suggested by studies of genes controlling venom in *Crotalus* and *Trimeresus* (Forsdyke, 1995, and citations within), and were demonstrated in the Asian viperid *Calloselasma rhodostoma* (Daltry et al., 1996). This general information provides the basis for testing several hypotheses related to variation in venom biochemistry and chemoreception in populations of the copperhead, *A. contortrix*.

To address whether variation in snake venom can affect chemoreceptive searching, we tested the behavioral responses of copperheads (*Agkistrodon contortrix*) to: (1) conspecific envenomated vs. nonenvenomated mouse prey; (2) conspecific envenomated prey from snakes in different populations (Kansas, Texas, Louisiana) with documented variation in venom biochemistry and diet; and (3) conspecific envenomated prey vs. envenomated prey from *A. piscivorus* and *Sistrurus catenatus*. We also tested how quickly a bite from snakes in the three copperhead populations could immobilize mammalian prey. We analyzed the venom biochemistry of three species of crotaline snakes (*A. contortrix, A. piscivorus,* and *S. catenatus*) to determine intraspecific and interspecific variation, and to identify proteins that might be responsible for differences in chemoreceptive behavior.

METHODS AND MATERIALS

Animal Collection and Maintenance. Adult copperheads (*Agkistrodon contortrix*) from three populations (Kansas, Texas, Louisiana) with documented differences in diet preferences (mammal, lepidopteran larvae, and amphibians, respectively) were collected from May 15 to June 30, 2000 (Greenbaum, 2003). Nine snakes were collected from the vicinity of Lawrence, Kansas; 13 from the vicinity of Whitehouse, Texas; and eight from the vicinity of Monroe, Louisiana. Individual snakes were housed in 10-gal glass cages (26 × 31 × 51 cm) with opaque sides to prevent distractions to the animals. The bottom of each cage was lined with newspaper, and a plastic water bowl allowed *ad libitum* drinking. All animals were housed

in a limited-access room with a relatively constant temperature ($27.0 \pm 1.0^{\circ}$C), humidity ($50.0 \pm 5\%$), and fluorescent lighting set for a daily 12 hr L:D cycle. Cages and water bowls were cleaned regularly with disinfectant. Snakes were allowed to acclimate to captivity for 6 wk prior to behavioral experiments, which commenced in mid-August 2000. Two adult *A. piscivorus* from Bastrop, Louisiana, and one adult *Sistrurus catenatus* from Kansas were housed in the same room and used as venom donors for envenomated-prey experiments.

Behavioral Experiments. The following experimental protocols were modified from those of Chiszar et al. (1999) and Main and Bull (1996). We began a trial by removing the cage lid of the test snake and placing a small, 60-W lamp over the cage; this allowed us to observe the snake and made it difficult for the snake to see us. Each snake's cage served as a test arena for each experiment. After each snake was acclimated for 1–2 min, it was allowed to strike a live mouse, which was removed immediately (Chiszar et al., 1999). Then a prey-presentation device was placed into the arena opposite the snake. The prey-presentation device consisted of two mouse carcasses (both lab-raised, 15–20 g), each in separate nylon bags that were suspended (10 cm apart and 2 cm from floor of cage) from a wooden beam across the top of the aquarium. Location of each prey item in either the right or left nylon bag was determined randomly. Nylon bags were washed with detergent and allowed to air dry between trials to remove prey scent and venom residue.

Prior to placement in nylon bags, envenomated mice were observed for 10 min to ensure that venom had been injected, and all physical movements had ceased. The number of tongue flicks directed toward each prey item (within 2 cm of the bottom of the nylon bag) was recorded for 10 min. At the end of each trial, we removed both prey from the bags and offered them to snakes; the purpose was to evaluate whether the initial strike was a defensive or predatory response to the prey item. If the snake ate one of the items within 5 min, we classified its behavior as "predatory" (Chiszar et al., 1999). If a snake attempted to consume a prey item through a bag during the final 2 min of a trial, we also interpreted the behavior as "predatory," the trial was terminated, and the bitten prey item was recorded as "consumed" prey. If a snake crawled out of the cage, or remained stationary for the first 5 min, the trial was terminated and not counted.

One experiment tested whether snakes could distinguish between envenomated vs. nonenvenomated prey. We hypothesized that *Agkistrodon contortrix* would possess envenomated prey preference, as do other crotaline species (Chiszar et al., 1999). A nonenvenomated mouse (killed by CO_2 overdose) was placed in one bag, and a mouse killed by envenomation from a snake in the same population as the test subject was placed in the remaining bag. The control experiment recorded the number of tongue flicks directed at two nonenvenomated mice. We also tested whether the snakes could distinguish between envenomated prey from conspecifics in their own population vs. conspecifics in one of two other populations, or from individuals of *A. piscivorus* and *Sistrurus catenatus*. We

hypothesized that copperheads would direct more tongue flicks at prey envenomated by local conspecifics than at prey envenomated by conspecifics from other populations. We also hypothesized that specimens of *A. contortrix* would direct more tongue flicks at prey envenomated by local conspecifics than at prey envenomated by *A. piscivorus* or *S. catenatus*.

All studies followed a randomized, complete-blocks design to minimize differences in behavior caused by time of year or other nonexperimental factors (Sokal and Rohlf, 1995). Because some individual snakes rarely cooperated for experiments, there are small differences in sample sizes among experiments within each population; some individuals were tested more than once for a given experiment. A minimum of 10 wk elapsed between duplicate trials for individual snakes, thus minimizing the chance of one trial affecting the outcome of another. To test this assumption, we subjected these data to runs tests to search for autocorrelations among snakes (Sokal and Rohlf, 1995). To reduce variability between individual snakes and facilitate comparisons on the same scale, we converted behavioral data (number of tongue flicks directed toward each prey item) to percentages of total number of tongue flicks for each trial (Duvall et al., 1980; Chiszar et al., 1999). We performed an arcsine transformation on these percentages to ensure normality and homogeneity of variance (Duvall et al., 1980). We analyzed these data with a one-way ANOVA to test whether individual snakes differed in preference; if this test and runs tests did not indicate significant differences we combined trials from each population. We analyzed these data with the Student's 1-sample *t* test. Because of small sample sizes, it was not possible to make adjustments to the significance level ($\alpha = 0.05$) to account for multiple *t* tests; thus, it is possible that a small number of Type I errors were made. Data on the type of prey consumed at the end of each trial were analyzed with the sign test.

Prey Immobilization. During random trials of behavioral experiments for each population of *Agkistrodon contortrix*, we timed how long it took an envenomated mouse (15–20 g) to become immobilized from a bite; immobilization occurred when a mouse could no longer walk. If immobilization did not occur within 5 min, the trial was discounted. To minimize variation from amount of venom injected or location of envenomation, five trials were conducted for each snake and averaged before the analysis. Data from these experiments were analyzed with a one-way analysis of variance with unequal sample sizes (ANOVA).

Venom Biochemistry Analysis. To examine the venom biochemistry of the snakes, venom samples were collected from one *Agkistrodon piscivorus* and one *Sistrurus catenatus*, as well as three individuals from each copperhead population. Venom was collected by allowing snakes to bite plastic collection cups with parafilm covers three times. Samples were collected from snakes in all populations at the same time; all individuals had eaten exactly 7 d prior to venom collection. Samples were lyophilized and stored at 4.0°C; crude venom was dissolved in 10 mM Tris-HCl (pH 8.6) (Johnson and Ownby, 1993). Total protein concentration

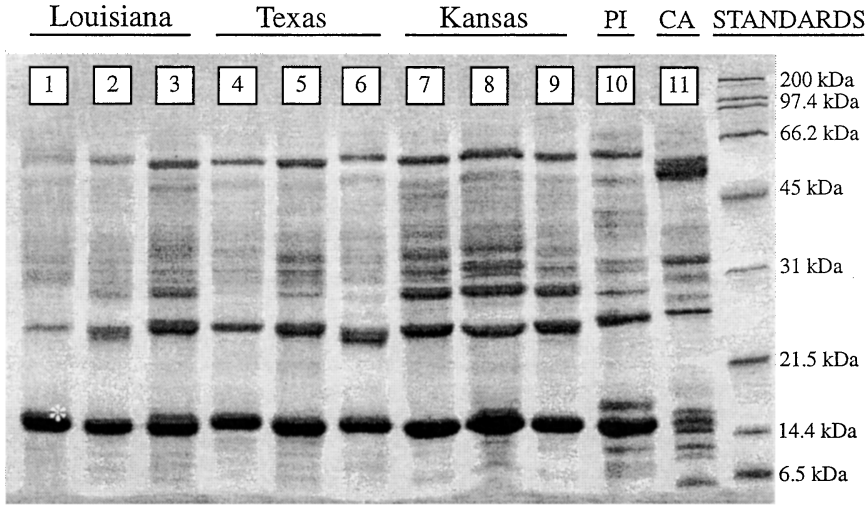

FIG. 1. Results of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude snake venom using a Criterion 15% Tris-HCl gel. Lanes 1–3, *Agkistrodon contortrix* from Louisiana; Lanes 4–6, *A. contortrix* from Texas; Lanes 7–9, *A. contortrix* from Kansas; Lane 10, *A. piscivorus* (PI); Lane 11, *Sistrurus catenatus* (CA). Note the variation in presence and relative concentrations of venom proteins among the populations of *A. contortrix*, and between the species. Asterisk denotes Phospholipase A2 Homolog Myotoxin precursor (ca. 16.0 kDa); present in all *A. contortrix*. The last lane consists of molecular weight standards.

in the venom solutions was determined spectrophotometrically (595 nm detection) with the Bio-Rad® protein assay dye reagent. Sample aliquots containing 20 μg of protein were diluted with denaturing sample buffer (5% mercaptoethanol, 2% sodium dodecyl sulfate, 0.06 M Tris-HCl [pH 6.8], 10% glycerol), heated at 95°C for 5 min, and examined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a Criterion 15% Tris-HCl gel (Bio-Rad). The electrophoresis was performed at 200 V for 60 min, and gels were stained with Coomassie Brilliant Blue G-250 in 20% methanol to facilitate viewing of bands. The gel image was acquired with a Molecular Imager FX (Bio-Rad). Protein molecular weights were estimated by visual alignment to molecular weight standards on the SDS-PAGE gel and their relative concentrations were quantified by the densitometry method (Quantity One Software, Bio-Rad).

For the most intense bands on the gel (approximately 16, 25, 29, and 60 kDa; Figure 1), we utilized the peptide mass-fingerprinting approach to identify the main protein components (Henzel et al., 1993). This method involves a mass spectrometric analysis of peptide fragments from in-gel digested proteins followed by a search with the generated mass data in protein databases. The protein

bands (16, 25, 29, and 60 kDa) were excised by hand and digested with trypsin (Promega) as previously described by Rosenfeld et al. (1992). Extracted peptide mixtures were analyzed with Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (Voyager-DE STR, PerSeptive Biosystems), with CHCA (10 mg/ml solution of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.3% trifluoroacetic acid [50% MeCN/0.3% TFA]) as a matrix. Internal mass calibration was performed using trypsin autolysis peaks (MH+ 842.5021 and 2211.0968). The peptide mass fingerprinting data were analyzed with the MS-Fit program (Protein Prospector, http://prospector.ucsf.edu) in the protein databases SWISS-PROT (http://www.expasy.ch/sprot) and NCBInr (http://www.ncbi.nlm.nih.gov). The following parameters were used: molecular weight range 10–100 kDa; peptide mass tolerance 100 ppm; maximum missed cleavages 3; acrylamide modified Cys.

RESULTS

Chemoreception Experiments. In all experiments, the one-way ANOVA test and runs tests did not indicate significant differences; thus, individial snakes did not differ in preference and trials from each respective population were combined. Each population of *Agkistrodon contortrix* directed more tongue flicks at envenomated prey than at nonenvenomated prey (Kansas: $t = 2.26$; df $= 12$; $P < 0.05$; Texas: $t = 2.24$; df $= 11$; $P < 0.05$; Louisiana: $t = 3.65$; df $= 10$; $P < 0.01$; Figure 2); this result supported our hypothesis. In the control experiment, there were no significant differences in number of tongue flicks directed at the two nonenvenomated prey items for each population (Kansas: $t = 0.04$; df $= 9$; $P > 0.05$; Texas: $t = 0.96$; df $= 10$; $P > 0.05$; Louisiana: $t = 1.26$; df $= 5$; $P > 0.05$). Despite these results, snakes did not consume significantly more envenomated prey than nonenvenomated prey (all sign tests: $P > 0.05$); the first prey encountered most often was the one consumed.

Snakes from Kansas preferred Texas-envenomated prey to their own ($t = 4.91$; df $= 11$; $P < 0.01$), and Louisiana-envenomated prey to their own ($t = 2.74$; df $= 9$; $P < 0.05$; Figure 3). However, snakes from Kansas did not differ in number of tongue flicks between prey envenomated by Kansas snakes and those of *Agkistrodon piscivorus* ($t = 0.33$; df $= 8$; $P > 0.05$) or *Sistrurus catenatus* ($t = 0.55$; df $= 10$; $P > 0.05$). Snakes from Texas did not prefer either prey item in the Texas vs. Louisiana trials ($t = 0.12$; df $= 28$; $P > 0.05$), but Texas snakes preferred their own envenomated prey over those from Kansas ($t = 3.80$; df $= 10$; $P < 0.01$), *A. piscivorus* ($t = 2.62$; df $= 8$; $P < 0.05$), and *S. catenatus* ($t = 2.95$; df $= 10$; $P < 0.05$). Snakes from Louisiana directed more tongue flicks at envenomated prey from their own population than envenomated prey from Texas ($t = 3.72$; df $= 15$; $P < 0.01$), Kansas ($t = 2.68$; df $= 10$; $P < 0.05$), *A. piscivorus* ($t = 2.92$; df $= 10$; $P < 0.05$), or *S. catenatus* ($t = 2.59$; df $= 10$;

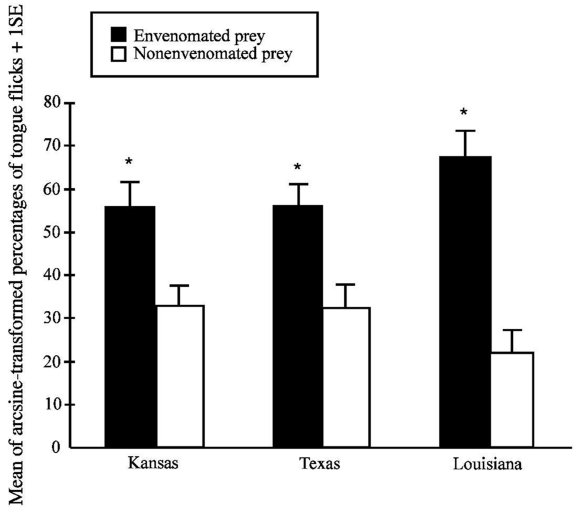

FIG. 2. Mean (+SE) of arcsine-transformed percentages of tongue flicks of three populations of *Agkistrodon contortrix* directed at envenomated or nonenvenomated prey items. Asterisks indicate significant differences ($P < 0.05$).

$P < 0.05$). As with the experiments to test ability to discern envenomated vs. nonenvenomated prey, snakes often consumed the first prey item encountered (all sign tests: $P > 0.05$).

The amount of venom collected from cooperative snakes did not differ among populations ($F = 2.21$; df $= 2, 23$; $P > 0.05$; Table 1). Because there was no significant difference in length ($F = 2.34$; df $= 2, 27$; $P > 0.05$), weight ($F = 1.94$; df $= 2, 27$; $P > 0.05$), or venom yields among the populations of snakes in this study, it is assumed that the average amount of venom injected during behavioral experiments did not differ among populations. During these trials, mice succumbed differently to venom from each population of *Agkistrodon contortrix*. The average number of seconds required for a 15–20 g mouse to become immobilized after a bite from the snakes differed among populations (Table 1). A one-way ANOVA confirmed that these means were significantly different from each other ($F = 5.34$; df $= 2, 27$; $P < 0.05$; Table 1). Therefore, it is assumed that the venom of snakes from Louisiana is most potent and that of snakes from Kansas is least potent.

Venom Biochemistry. Analysis of the SDS-PAGE image of the venom samples (Figure 1) revealed generally similar protein compositions for the three populations of *Agkistrodon contortrix*. Some variation in relative protein concentration was noted between venom samples of Kansas snakes (16 kDa > 29 kDa > 25 kDa > 60 kDa) and those of Louisiana and Texas (16 kDa > 25 kDa > 60 kDa > 29 kDa).

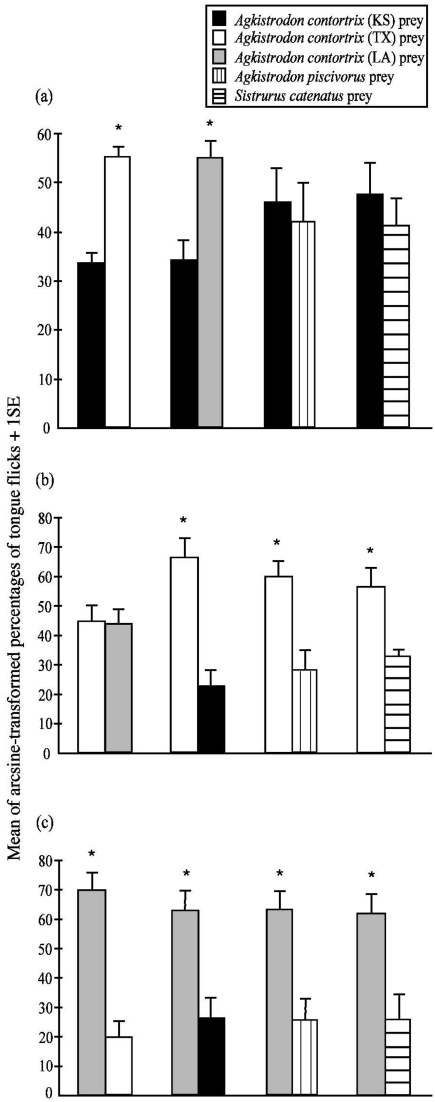

FIG. 3. Mean (+SE) of arcsine-transformed percentages of tongue flicks of three populations of *Agkistrodon contortrix* directed at envenomated prey items. a: Kansas, *A. contortrix*; b: Texas, *A. contortrix*; c: Louisiana, *A. contortrix*. Asterisks indicate significant differences ($P < 0.05$).

TABLE 1. COMPARISON OF AVERAGE LENGTH, WEIGHT, AND VENOM YIELD OF SNAKE TEST SUBJECTS, AND TIME TO IMMOBILIZATION OF MICE ENVENOMATED BY COPPERHEADS (*Agkistrodon contortrix*) FROM KANSAS ($N = 9$), TEXAS ($N = 13$), AND LOUISIANA ($N = 8$)[a]

	Kansas	Texas	Louisiana
Length (cm)	49.88 ± 11.01	45.37 ± 5.97	53.54 ± 9.08
Weight (g)	126.67 ± 92.87	77.69 ± 39.98	87.37 ± 27.19
Venom yield (g)	0.043 ± 0.019	0.025 ± 0.015	0.034 ± 0.02
Mouse immobilization time (sec)	62.83 ± 20.28	39.5 ± 31.07	26.25 ± 11.74

[a] Data are Means $\pm$ 1 SD.

The 16 kDa protein with the highest concentration in all populations of *Agkistrodon contortrix* has been identified as Phospholipase A_2 Homolog Myotoxin precursor (SWISS-PROT accession number P49121, MW 15775 Da). Nine peptide matches covered 51% of the protein sequence. The highest concentration protein (14 kDa) from the *A. piscivorus* sample has been identified as Phospholipase A_2 precursor (SWISS-PROT accession number P51972, MW 13989 Da). Eight peptide matches covered 44% of the protein sequence. Phospholipase A_2 is a major component of most snake venoms, and numerous deleterious effects on prey have been noted (Forsdyke, 1995; Meier and Stocker, 1995).

Although mass spectra of other in-gel digested protein bands identified 19–35 molecular peaks, each with a mass range of 700–3000 Da, none of these proteins were matched to database proteins as of May 30, 2002. This result is not surprising because no *Agkistrodon* species have completely sequenced genomes at this time. Although our analysis was not limited to the genus *Agkistrodon*, no homologous sequences from other species were identified in our database search. Two venom proteins of *A. contortrix*, including a hemorrhagic metalloprotease at 29 kDa (Johnson and Ownby, 1993) and a hyaluronidase at 61 kDa (Kudo and Tu, 2001) have been isolated and characterized, but their sequences have not been determined and deposited in these databases. These proteins could be possible matches with our high band intensities at 29 kDa and 60 kDa, respectively.

DISCUSSION

The results indicate that *Agkistrodon contortrix* can distinguish between envenomated and nonenvenomated prey, a finding consistent with those of similar studies that examined other viperids (Baumann, 1927, 1928, 1929; Duvall et al., 1980; Robinson and Kardong, 1991; Chiszar et al., 1992, 1999; Lavin-Murcio and Kardong, 1995). Behavioral observations confirm that *A. contortrix* exhibits

typical viperid poststriking behavior, which includes an increased rate of tongue flicking (Radcliffe et al., 1986), strike-induced chemosensory searching (Chiszar et al., 1992; Stiles et al., 2002), poststrike head orientation toward the departing path of struck prey (Keith et al., 1985; Lee et al., 1988), and a tendency to strike and hold relatively smaller prey (Kardong, 1986).

Relative to rattlesnakes, which are dietary specialists (Klauber, 1956), the copperhead (*Agkistrodon contortrix*) is a dietary generalist; however, several populations seem to prefer certain types of prey (e.g., Louisiana: frogs, Dundee and Rossman, 1989; Texas: cicadas and lepidopteran larvae, Lagesse and Ford, 1996; Kansas: mammals, Fitch, 1960). We do not know whether this geographic and populational variation is an artifact of prey availability or some other factor. There is no likely significance to the observation that all test subjects consumed the first prey item they encountered at the termination of each trial—if a snake discovered a suitable prey item on the floor of the cage, the prey item was consumed. Additional experiments are needed to test the significance of prey consumption (but see Duvall et al., 1978). For the purposes of the current study, consumption indicated that the behavior was predatory.

The significance of the ability of *Agkistrodon contortrix* to distinguish between envenomated prey from different populations may not be understood until future workers can demonstrate a correlation between specific venom components and chemoreceptive behavior. It is possible that the venom components affect the tissue of envenomated prey and that the resulting tissue damage is sensed by snakes during chemoreceptive searching. Moreover, it is likely that there is an adaptive value to higher-potency venom in relation to effectiveness of prey immobilization and digestion (Thomas and Pough, 1979; Pough and Groves, 1983) and ability of snakes to find envenomated prey. If prey are envenomated with a relatively potent venom, they travel a shorter distance before immobilization; thus, the snakes find prey more efficiently after envenomation. This may be especially important in mesic habitats such as Louisiana, because water exacerbates chemoreceptive trailing of prey, at least in rattlesnakes (Brock, 1980). Because the relative potency of the venom from each population matches the order of preference in the chemoreception experiments, we hypothesize that the specific proteins responsible for the increased onset of death are also responsible (perhaps indirectly) for the increased preference of the envenomated prey to snakes during chemoreception. The relatively weak potency of the venom in snakes from Kansas may explain why this population could not distinguish between their envenomated prey and those of the other species. However, caution is needed in interpreting these results because of the dearth of venom samples from *A. piscivorus* and *Sistrurus catenatus*.

One question arises. Why do not all populations of copperheads have the most potent (Louisiana) venom if it is potentially adaptive? We propose four possible explanations: (1) Disparate environmental effects resulting from climate or habitat may affect venom toxicity (Russell, 1980); (2) The more potent venom in snakes

from Louisiana arose from random genetic drift in an isolated population (Thorpe et al., 1995); (3) Intraspecific variation in venom is a result of antipredator defense against predator resistance to venom (Kilmon, 1976; Ovadia and Kochva, 1977; Werner and Vick, 1977; Perez et al., 1979; Rodriguez-Acosta et al., 1995; Oliveira and Santori, 1999); and (4) Potent venom is energetically costly and may be necessary only in populations that need to overpower prey resistance to the venom (Perez et al., 1978, 1979; De Wit, 1982; Daltry et al., 1996; Heatwole et al., 1999; Martinez et al., 1999). Prey may vary intraspecifically in immunological resistance or physiological susceptibility to venom (Poran et al., 1987; Mebs, 1999); therefore caution is needed in interpreting results based on laboratory mice that likely do not have a natural resistance to venom.

Although our venom biochemistry analysis did not identify venom protein components that explain the observed differences in venom potency and behavior among the populations of copperheads in this study, it is hoped that our preliminary data will serve as a basis for future research into this interesting link. If individual venom components can be isolated and injected into prey items, it may be possible to identify the specific components that are responsible for the behavior elucidated herein.

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ISOLATION, CHARACTERIZATION, AND QUANTITATIVE ANALYSIS OF MICROVIRIDIN J, A NEW *Microcystis* METABOLITE TOXIC TO *Daphnia*

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Abstract—This paper describes the purification and characterization of microviridin J, a newly discovered metabolite of *Microcystis* that causes a lethal molting disruption in *Daphnia* spp., upon ingestion of living cyanobacterial cells. Microviridin J consists of an acetylated chain of 13 amino acids arranged in three rings and two side chains. Unlike other known isoforms of microviridin, microviridin J contains arginine that imparts a unique solution conformation

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characterized by proximal hydrophobic interactions between Arg and other regions of the molecule. This eventually results in the formation and stabilization of an additional ring system. Microviridin J potently inhibits porcine trypsin, bovine chymotrypsin, and daphnid trypsin-like proteases. The activity against trypsin is most likely due to Arg and its distinctive conformational interactions. Overall, the data presented for microviridin J emphasize once again the ability of cyanobacteria to produce numerous and potent environmental toxins.

Key Words—*Microcystis*, cyanobacteria, *Daphnia*, microviridin, NMR, microcystin, environmental toxins.

INTRODUCTION

Many freshwater cyanobacteria share the ability to produce bioactive compounds that may affect other organisms. In particular, the bloom-forming taxa, members of the orders Nostocales and Chroococcales, including *Microcystis*, *Anabaena*, *Aphanizomenon*, and *Planktothrix* species, are known as rich sources of potent enzyme inhibitors, cellular disrupters, and compounds with a diverse range of other biological activities (Carmichael, 1992; Codd, 1995). Some of these metabolites also present serious threats to animal and human health. Prominent examples are the microcystins, which have been linked to the poisoning of farm animals and, in at least one incident, to human fatalities (Jochimsen et al., 1998; Pouria et al., 1998).

In nature, bioactive compounds may threaten all aquatic organisms that coexist with or feed on cyanobacteria. Of particular interest are the effects on the microcrustaceans *Daphnia* spp., which are potential grazers of *Microcystis*, *Planktothrix*, and most other planktonic cyanobacteria. Daphnids constitute a key group of organisms in freshwater systems (Riemann and Christoffersen, 1993), and disturbance of these animals may have effects throughout the aquatic community. Various studies have highlighted the ability of cyanobacteria to produce "*Daphnia* disturbing" metabolites, including feeding deterrents (Haney et al., 1994), protease inhibitors (Agrawal et al., 2001), and potent toxins (Jungmann, 1992; Jakobi et al., 1996; Reinikainen, 1997). However, most of these compounds have, never been fully identified or lack a comprehensive analysis of their effects on *Daphnia*. Among the few exceptions are the microcystins, the *Daphnia* toxicity of which has been extensively studied by a number of authors (e.g. DeMott and Dhawale, 1995; Christoffersen, 1996; Rohrlack et al., 1999, 2001).

Recently, Kaebernick et al. (2001) have noted a lethal molting disruption in *Daphnia* spp. when fed with cells of the *Microcystis* strain UWOCC MRC. Such symptoms have not been previously reported for cyanobacteria and point to the occurrence of a new toxin in *Microcystis* UWOCC MRC. On the basis of mass spectroscopic analyses of UWOCC MRC cells, the authors suspected a peptide with a molecular mass of m/z 1684.8 Da $[M + H]^+$ to be that novel toxic compound. This was later confirmed by Rohrlack et al. (unpublished data), who

showed that the purified 1684.8 Da peptide caused exactly the same lethal molting problems as described by Kaebernick et al. (2001) for *Microcystis* UWOCC MRC. Further, a relationship between the rate with which the 1684.8 Da peptide is taken in and the median survival time of *Daphnia* has been found. Rohrlack et al. have also found another producer of the 1684.8 Da peptide, *Microcystis* strain UWOCC CBS, which disrupts molting as well.

This study describes the purification and chemical characterization of the newly discovered peptide that poisons *Daphnia* by causing a lethal molting disruption. Analytical methods, including amino acid analysis and NMR, were used to clarify the structure and solution conformation of the toxin. Since pilot experiments indicated that the new toxin might be a protease inhibitor, this study also includes *in vitro* tests employing mammalian and daphnid proteases. In addition, a simple quantification method is presented and validated. *Microcystis* UWOCC CBS, which produces the new toxin in large amounts, was used as the basis for the experiments presented.

METHODS AND MATERIALS

Mass-Culturing of Cyanobacteria and Toxin Extraction. Microcystis aeruginosa strain UWOCC CBS originated from the University of Wisconsin Culture Collection (Oshkosh, USA) and was kindly provided by D. L. Parker (Scripps Institute for Oceanography, San Diego, USA). This strain was grown in batch cultures by using Z8 medium (Kotai, 1972) in 3 to 10 l culture vessels. The cultures were maintained under continuous light (mean light intensity of 30 μmol photons/m^2/sec) at $20 \pm 1^\circ$C and continuous aeration. Measurement of culture optical density at 800 nm was used to monitor growth. Once the stationary growth phase had been reached, cyanobacterial cells were harvested by centrifugation at $2000 \times g$ for 10 min and lyophilized (final amount about 6 g dry weight). The cell material was extracted twice with 50% MeOH (solvent concentrations are based on volumetric units throughout this paper). Each extraction step involved 60 min of intensive shaking and a subsequent centrifugation at $2000 \times g$ for 10 min. Afterwards, the liquid phases of both extraction steps were pooled.

Toxin Purification. Purification of the toxic compound was based on reversed-phase HPLC (Waters 600 photodiode array detector at 210 nm, 717 auto sampler, 600E controller, Symmetry C18 5 μm 3.9 $\times$ 150 mm column, flow rate of 1 ml/min, column temperature 40°C), which was performed using a linear gradient (20% acetonitrile in 10 mM ammonium acetate, pH 5.0, to 28% acetonitrile) and a gradient time of 26 min. To detect the toxin peak, major HPLC fractions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (see Erhard et al., 1999, for a description). The toxin was identified by its molecular mass of m/z 1684.8 Da $[M + H]^+$ (Kaebernick et al., 2001). MALDI-TOF-MS was also used to confirm the purity of the toxin-

containing fraction. Once the toxin peak was found, a routine sample procedure was initiated. Finally, all fractions containing the compound were pooled, mixed with the same volume of deionized water and desalted using C18 cartridges (International Sorbent Technology, Hengoed, United Kingdom). The pure toxin was eluted with 100% MeOH and dried using a constant flow of sterile air and a subsequent freeze-drying step. The yield was determined by means of a microbalance (Mettler AE163, Copenhagen, Denmark).

Amino Acid Analysis. The toxin was chemically digested using hydrochloric acid. Briefly, 20 μg substance was dissolved in 500 μl 6 N HCl, which was then transferred into a 1 ml glass ampoule. After evacuation and sealing, the sample was subjected to 110˚C for 16 hr. A second sample was prepared with 500 μl 6 N HCl containing 1% phenol and kept at 100˚C for 10 hr. Afterwards, the hydrolyzed toxin was dried under a constant flow of nitrogen. The amino acid composition of the hydrolyzed samples was determined by HPLC as described previously (Koppitz et al., 2000).

NMR Analysis. Nine milligrams of toxin was dissolved in 0.7 ml dimethylsulfoxide (DMSO)-d_6 and the NMR spectra recorded at 30°C on a Varian Unity-Inova 600 MHz NMR spectrometer. TMS served as the internal reference for chemical shift. DQF-COSY (Piantini et al., 1982), TOCSY (Braunschweiler and Ernst, 1983) with a spin lock time of 120 ms, NOESY (States et al., 1982) with mixing time of 70 and 150 ms, and ^{1}H, ^{13}C HSQC (John et al., 1992; Kay et al., 1992) spectra were recorded with a recycle time of 2–3 sec. All NMR data were processed with VNMR software (version 6.1C) and analyzed with XEASY (Bartels et al., 1995).

Modeling of Toxin Solution Conformation. Molecular mechanics calculations were done using the Hyperchem 5.01 program and MM^{+} potentials (Allinger, 1997) using a Polak-Ribiere gradient and a cutoff at a RMS gradient of 0.2 KJ/Å mol.

Extraction of Proteases from Daphnia. Tryspin-like proteases were extracted from *Daphnia magna* as described by Hasler (1935). Briefly, a *D. magna* clone (isolated from Lake Langedamm, Denmark) was maintained in a mass culture with 4 mg C/l *Scenedesmus acutus* (kindly supplied by W. Lampert, Max Planck Institute for Limnology, Plön, Germany) as sole food. The food suspension, which was prepared with synthetic zooplankton medium (Klüttgen et al., 1994), was exchanged every 3rd day. The temperature was kept constant at 22°C. To extract daphnid trypsin-like proteases, 30 adult animals were taken from the mass culture and washed with zooplankton medium to remove attached *Scenedesmus* cells. Afterwards, the animals were dehydrated in 10 ml acetone, washed three times with 5 ml *n*-hexane, homogenized with an ultrasonic needle, and dried in a constant flow of air. The homogenate was dissolved in 1 ml of 50% glycerol. After 2 hr at 7°C, the extract was centrifuged (10, 000 $\times$ *g*, 10 min). The supernatant contained the required proteases. The total protein content was determined by applying a Roti-Nanoquant system (Roth, Germany) according to the methods of Bradford (1976) and Zor and Selinger (1996) and was found to be 0.1 g protein/l.

Protease Inhibition Assays. The effects on porcine trypsin (Roth, Germany), bovine α-chymotrypsin (Sigma, Germany), and porcine elastase (Sigma, Germany) as well as on daphnid trypsin-like enzymes were analyzed. Trypsin activity was measured with a 1 mM aqueous solution of *N*-α-benzoyl-DL-arginine-*p*-nitroanilide as substrate (Erlanger et al., 1961) and 10, 30, and 50 mg/l solutions of porcine trypsin dissolved in 50% glycerol or the undiluted *Daphnia*-trypsin extract. The activity of chymotrypsin was determined with *N*-succinyl-phenylalanine-*p*-nitroanilide as the specific substrate, dissolved in 1 ml EtOH and mixed with chymotrypsin buffer (100 mM Tris-HCl, pH 7.2; 100 mM NaCL; 1 mM $CaCl_2$; and 1 mM HCl) to obtain a final concentration of 1 mM (Nagel et al., 1965). Chymotrypsin was dissolved in chymotrypsin buffer at a concentration of 1 g/l. *N*-succinyl-alanine-alanine-alanine-*p*-nitroanilide served as substrate in the elastase experiments (Bieth et al., 1974). The substrate was dissolved in 1 ml dimethylformamide and brought to a final concentration of 1 mM by adding elastase buffer (100 mM Tris-HCl, pH 7.2; 100 mM NaCl; and 1 mM $CaCl_2$). The same buffer was used to prepare the porcine elastase solution to a concentration of 50 mg/l. All enzyme activity measurements were performed by mixing 1 ml 100 mM Tris-HCl (pH 7.2), 100 μl enzyme solution, and a defined amount of toxin dissolved in 100-μl 50% MeOH. Control measurements were performed using 100 μl 50% MeOH without toxin. The reaction components were mixed carefully, incubated at 20°C for 30 min and finally transferred into standard cuvettes (1 cm length). To initiate the enzymatic reactions, 300 μl of the respective substrate solution was added directly to the cuvettes. The production of *p*-nitroanilide was monitored photometrically at 410 nm for 10 min, and the enzyme activity was determined by linear regression as *p*-nitroanilide production rate. Solutions of pure *p*-nitroanilide were used to calibrate the system. On the basis of measurements with different toxin concentrations, the concentration, which caused a 50% inhibition of the enzyme activity (IC_{50}), was determined.

Quantification of the Toxin in Cyanobacterial Material. Microcystis UWOCC CBS was grown in Z8 medium in semicontinuous 300 ml cultures under 11 μmol photons/m^2/sec continuous light at 20 $\pm$ 1°C. Cultures were diluted daily to an optical density of 0.13 at 800 nm. To obtain defined cyanobacterial culture material, all samples were taken after 3 weeks of constant growth. The toxin was extracted from UWOCC CBS cells collected on glass fiber filters. A culture volume corresponding to 9×10^5 *Microcystis* cells (cell counts performed microscopically) was filtered through a 22 mm GF/C filter. This filter was then transferred into a 1.5 ml microcentrifuge vial, which served as the sample vessel throughout the entire extraction procedure, and was lyophilized. To extract the toxin and to test the effect of different MeOH concentrations on extraction efficiency, 0.7 ml of either deionized water, 20, 40, 50, 60, 80, or 100% MeOH was added to the lyophilized UWOCC CBS samples and shaken for 30 min. Afterwards, the liquid phases were individually transferred into new microcentrifuge vials, and the

extraction procedure was repeated once. Finally, both liquid phases of a sample were combined and centrifuged at 12,300 × g for 10 min. Three replicates were performed for each solvent concentration extraction. The sample temperature was kept at approximately 5°C throughout the entire extraction procedure in order to avoid methylation of the toxin (see Discussion). For the same reason, all extracts were stored at −18°C until analyzed. The toxin concentration of the extracts was determined by reversed-phase HPLC (Waters 600 PDA, 717 auto sampler, 600E controller, Symmetry C-18 5 μm 3.9 × 150 mm column, flow rate of 1 ml/min, column temperature 40°C). Separation was achieved with a linear gradient starting with 20% acetonitrile in 10 mM ammonium acetate solution (pH 5.0) and ending after 26 min with 28% acetonitrile. The UV absorption spectrum of the toxin was recorded between 200 and 300 nm. Solutions of the purified toxin with different concentrations served as references and were used to calibrate the system. Quantification was done at 220 nm, being the UV maximum of the toxin (see Results section).

Statistics. All IC_{50} value calculations and regression analyses were performed using the regression package of STATGRAPHICS 2.1. The mean values of cellular toxin amounts were compared with the Student's t test with the data revealing a normal distribution. All statistical tests or calculations were performed at the 95% level of significance.

RESULTS

Chemical Characterization. The purification procedure yielded 15.77 mg of the new *Microcystis* toxin. By visual inspection it was a white, amorphous substance. HPLC analysis of the purified compound did not show any UV absorbing contaminants. The amino acid and NMR analyses suggested the occurrence of one residue each of Arg, Asp, Ile, Lys, Pro, Thr, Tyr, and two Glu, Ser, and Trp moieties per toxin molecule. Only minor contaminations (<1%) with amino acids other than those occurring in the toxin molecule were found in the hydrolyzed samples. Using DQF-COSY, TOCSY, and NOESY spectra an almost complete ^{1}H resonance assignment of the toxin molecule was achieved (Table 1, see a complete list at www.fbl.ku.dk/kchristoffersen/microviridin/microviridin_J_NMR.htm). Ten of the total 12 backbone amide protons were in slow exchange with water and their complete spin systems were identified. The missing backbone amide protons of Asp 10 and Trp 11 were either in fast exchange with water or experienced severe broadening due to conformational averaging. The sequential mapping of all 14 residue spin systems was derived from typical sequential NOEs between N_pH, $C_\alpha H$, $C_\beta H$ of the i-th residue and N_pH of the $(i + 1)$-th residue, or from sequential NOEs between side chain protons when the N_pH was missing. Most of the 217 assigned NOEs were intraresidue NOEs or those between neighboring residues.

TABLE 1. ^{1}H AND ^{13}C (IN PARENTHESES) CHEMICAL SHIFTS OF THE TOXIN DISSOLVED IN DMSO-D_6 (ALL DATA ARE GIVEN IN PPM AND IN RELATION TO TMS)

Residue	NH	$C_\alpha H$	$C_\beta H$	Others
Ac 1				$CH_3$1.85 (22.3)
Ile 2	7.96	4.25 (56.6)	1.72 (36.3)	$C_\gamma H_2$ 1.08, 1.42 (24.2); $C_\gamma H_3$ 0.85 (15.3); $C_\delta H_3$ 0.80 (10.9)
Ser 3	8.15	4.43 (61.6)	3.60 (61.2)	OH 5.07
Thr 4	7.61	4.59 (54.4)	5.34 (70.6)	$C_\gamma H_3$ 1.09 (16.3)
Arg 5	8.47	4.16 (52.3)	1.49, 1.69 (28.5)	$C_\gamma H_2$ 1.36 (24.9); $C_\delta H_2$ 2.99 (40.1); $N_\varepsilon H$ 8.13; $N_\eta H$ 7.56
Lys 6	6.79	4.15 (52.3)	1.55 (32.1)	$C_\gamma H_2$ 1.11, 1.16 (21.2) $C_\delta H_2$ 1.26, 1.36 (28.7); $C_\varepsilon H_2$ 2.95, 3.11 (37.7); $N_\zeta H_3$ 7.05
Tyr 7	8.35	4.35 (51.7)	2.64, 2.75 (37.3)	$C_\delta H_2$ 6.93 (129.9); $C_\varepsilon H_2$ 6.66 (115.0); OH 9.39
Pro 8		3.51 (60.2)	1.51, 1.62 (30.6)	$C_\gamma H_2$ 1.50, 1.61 (30.6) $C_\delta H_2$ 3.21, 3.40 (46.1)
Ser 9	6.74	4.50 (51.7)	4.12 (61.6)	
Asp 10		4.38 (54.7)	2.59, 2.68 (34.6)	
Trp 11		4.51 (53.2)	3.14, 3.23 (25.7)	$C_{\delta1}H$ 7.21 (123.5) $N_{\varepsilon1}H$ 10.86; $C_{\varepsilon3}H$ 7.46 (117.9); $C_{\zeta3}H$ 6.96 (118.3); $C_{\eta2}H$ 7.04 (120.9); $C_{\zeta2}H$ 7.26 (111.3)
Glu 12	6.48	4.08 (52.4)	1.56, 1.84 (24.4)	$C_\gamma H_2$ 1.30, 2.05 (29.4)
Glu 13	7.34	4.08 (52.4)	1.55 (28.1)	$C_\gamma H_2$ 1.95, 2.05 (32.0)
Trp 14	7.42	4.13 (55.0)	2.98,3.19 (27.6)	$C_{\delta1}H$ 7.06 (123.1); $N_{\varepsilon1}H$ 10.56; $C_{\varepsilon3}H$ 7.51 (118.3); $C_{\zeta3}H$ 6.89 (117.7); $C_{\eta2}H$ 6.98 (120.2); $C_{\zeta2}H$ 7.25 (110.8)

A few long-range NOEs were found between Ser 3 and Tyr 7 as well as between Lys 6 and Asp 10. On the basis of all structural data, the amino acid sequence was suggested to be as shown in Figure 1. To model the solution conformation of the toxin, a molecular mechanics simulation without experimental restraints was performed. Since NOESY cross peaks among Arg 5 $N_\varepsilon H$, $N_\eta Hs$, and Tyr 7 OH resonances indicated that the side chain of Arg 5 pointed towards Tyr 7, an artificial bond was incorporated into the initial calculation to bring Arg 5 and Tyr 7 side chains into closer proximity. At a later stage, the artificial bond was broken

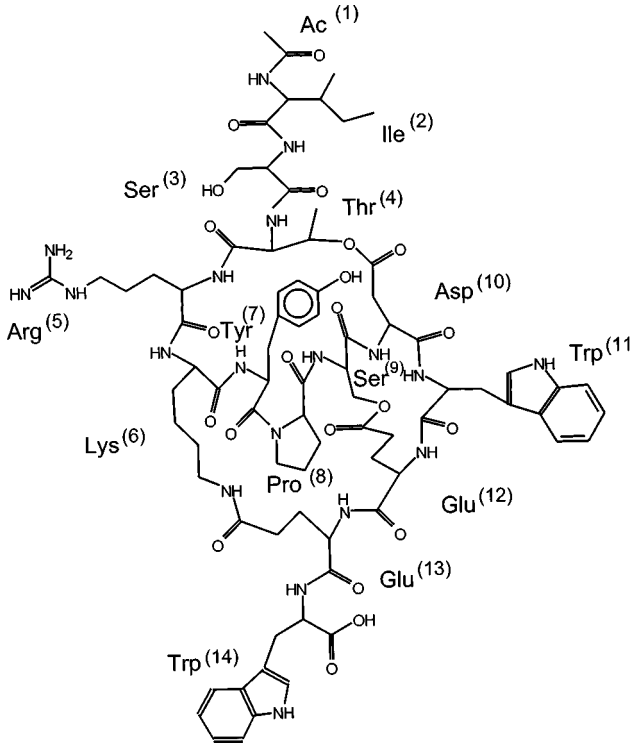

FIG. 1. Proposed amino acid sequence and 2D structure of the toxin.

and the final structure was energy-minimized (Figure 2). The calculated structure confirmed the presence of long-range NOE contacts between Ser 3 and Tyr 7, as well as between Lys 6 and Asp 10. The structure also indicated close hydrophobic contacts between the Ser 3, Arg 5, and Tyr 7 side chains. This was in good agreement with the occurrence of labile protons Ser 3 OH, Arg 5 $N_{\varepsilon}H$, $N_{\eta}Hs$, and Tyr 7 OH, which indicated unique rigid conformations of the Ser 3, Arg 5, and Tyr 7 side chains. Overall, consistent prediction of important structural features using both experimental data and theoretical calculations strongly suggested that the structure shown in Figure 2 closely resembles the actual solution conformation of the novel compound.

Protease Inhibition Assays. The toxin turned out to be a potent inhibitor of porcine trypsin, daphnid trypsin-like enzymes, and bovine chymotrypsin. The activity of porcine elastase, on the other hand, was not affected at a toxin concentration of 10 mg/l. The IC_{50} values for the 10, 30, and 50 mg/l porcine trypsin stock solutions were 0.034, 0.096, and 0.150 mg/l, respectively, and showed a

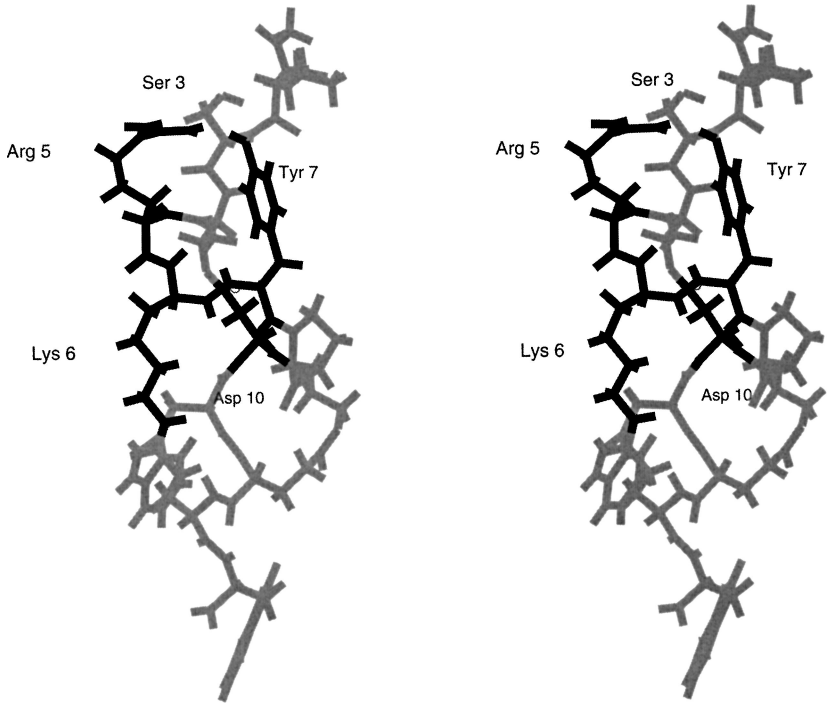

FIG. 2. Calculated solution conformation of the toxin (stereographic plotting).

significant linear correlation to the actual enzyme concentrations used in the experiments ($r = 0.99$). The regression analysis suggested that, irrespective of the trypsin concentration, a molar toxin:trypsin ratio of 0.65 (confidence interval of 0.58–0.72) was sufficient to inhibit 50% of the enzyme activity. *Daphnia* trypsin-like proteases were inhibited with a IC_{50} of 0.0039 mg/l (confidence interval of 0.0021–0.0075). Fifty percent of bovine chymotrypsin activity was inhibited at a toxin concentration of 2.80 mg/l, equating to a molar toxin:chymotrypsin ratio of 0.62 (confidence interval of 0.49–0.80).

Quantification of the Toxin. The HPLC analysis of the purified compound yielded a single peak with a retention time of 21 min. The UV absorption spectrum showed a distinct maximum at 220.6 nm and weak maxima at 280.6 and 288.7 nm. At 220 nm, a significant linear correlation between the injected amount of toxin and the peak area was found ($r > 0.99$). This was confirmed using toxin quantities between 2.5 and 100 ng. Injection of amounts greater than 2.5 ng afforded reliable identification and quantification of the toxin. All HPLC chromatograms of *Microcystis* UWOCC CBS extracts showed reliable toxin peaks with a retention time of 21 min together with a number of minor peaks. The UV spectrum of the toxin peak

from the cell extracts was identical to that of the purified compound. Quantitative analyses indicated that MeOH concentrations between 40 and 80% were equally suitable for toxin extraction. Significantly lower amounts were extracted with either water or 20% MeOH. The lowest extraction efficiency was found with 100% MeOH. Considering all data from the extraction with 40–80% MeOH, *Microcystis* UWOCC CBS was found to contain 85.8 ± 7.5 fg toxin/cell (mean value $\pm$ SD, $N = 12$).

DISCUSSION

In this paper we describe a *Microcystis* metabolite that causes, once ingested as a component of living cyanobacterial cells, a lethal molting disruption in *Daphnia* (Kaebernick et al., 2001). Detailed chemical analyses have characterized this toxin as a cyclic peptide with a structure that closely resembles that of the microviridins (see Murakami et al., 1997; Fujii et al., 2000, for a description of all known microviridins). This identification is supported by the typical amino acid sequence and by ^{1}H NMR chemical shifts of large regions of the molecule, which compare well with those of other microviridins (Ishitsuka et al., 1990; Okino et al., 1995; Shin et al., 1996; Murakami et al., 1997; Fujii et al., 2000). However, the toxin also possesses unique structural features and should, therefore, be recognized as a previously undescribed microviridin. Following the classification system used for other microviridins, we propose the toxin name microviridin J.

Microviridins constitute a diverse group of cyclic depsipeptides (Ishitsuka et al., 1990; Okino et al., 1995; Shin et al., 1996; Murakami et al., 1997; Fujii et al., 2000). Their usual structure consists of an acetylated chain of 13 or 14 L-amino acids arranged in one to three rings and two side chains. The rings are closed by amino and ester linkages, which can also partially undergo methylation. The general residue sequence of microviridins is Ac-Y-(Y*)-Y-Thr-Y-Lys-Tyr-Pro-Ser-Asp-X-Glu-Y-X with X being always aromatic amino acid residues and Y being variable molecule regions (Y* is only present in microviridins A, B, C, D, G, H, but not in E, F, J). Thus, more than 60% of the microviridin molecular structure either is conserved or shows only minor variations. The variability of the remaining residues is extensive and points to the probable existence of many more than the 10 microviridins characterized to date.

Microviridin J differs from all other described microviridins due to the occurrence and unique conformation of Arg at the position between Thr and Lys. The NMR analysis strongly suggested that the side chain of Arg was in close hydrophobic contact to other regions of the molecule, which results in the formation and stabilization of a so far unknown ring system. This novel conformation of Arg may also confer new bioactivities in comparison to other microviridin isoforms. To date, microviridins have been described as tyrosinase, elastase, and/or

chymotrypsin inhibitors (Murakami et al., 1997; Fujii et al., 1999). The potent inhibition of trypsin by microviridin J is new for this group of compounds and, we propose, because of Arg since this residue constitutes the major novel feature in the microviridin J molecule and an important binding side for trypsin (Laskowski and Qasim, 2000).

A comparison of the structure and biological activity of all known microviridins highlights the significance of the amino acid at the position between Thr and Lys. Microviridins with Leu at that position are very potent inhibitors of elastase (microviridins B, C, G, H, I), while a Met, Tyr or Phe at the same place results in weak (microviridins D, E, F) or no inhibitory effects on elastase (microviridin A) (Murakami et al., 1997; Fujii et al., 1999). The present data regarding microviridin J shows that an Arg at the position between Thr and Lys may be essential for strong trypsin inhibition and, at the same time, negates the inhibitory effect on elastase. In addition to the amino acid between Thr and Lys, a hydrophilic residue at the position just before Thr, and an ester linkage between Thr and Asp appear crucial for an inhibitory effect on elastase and maybe also trypsin (Murakami et al., 1997).

As already mentioned, microviridin J taken in with *Microcystis* cells interferes with *Daphnia*'s molting process and causes rapid death of the animals (Kaebernick et al., 2001). This lethal molting disruption might be due to the inhibitory effect of microviridin J on *Daphnia*-trypsin established in the present study. In fact, an inhibition of proteases in the midgut by microviridin J released from digested *Microcystis* cells may well result in an incomplete protein digestion and consequently in a lack of amino acids, which are essential for a production of chitin or other components of the daphnid integument. A direct effect of microviridin J on trypsin-like proteases in *Daphnia*'s molting fluid also seems possible (Gimenez *et al*., 2001). In both cases, serious molting problems are likely to occur.

However, microviridin J is not the only protease inhibitor produced by cyanobacteria. Many other metabolites with activities against trypsin, chymotrypsin, elastase, and other proteolytic enzymes have been isolated from a diverse range of cyanobacterial species (Weckesser et al., 1996; Radau, 2000). Protease inhibitors occur in many laboratory strains and field populations of *Microcystis*, *Planktothrix*, and other genera of cyanobacteria (Fastner et al., 2001). This distribution and data on the toxicity of microviridin J to *Daphnia* (Kaebernick et al. 2001) emphasizes the potential significance of cyanobacterial protease inhibitors in nature. In addition, few data relate to the effects of compounds, such as microviridin J, on organisms other than daphnids. Protease inhibitors present a potential threat to all herbivorous animals that feed on cyanobacteria. In these animals, the release of protease inhibitors from cyanobacteria into the digestive tract may result in incomplete protein digestion leading to amino acid imbalances, molting problems, reduced growth, lower reproductive activity, and early death (Jongsma and Bolter, 1997).

An important objective of the present paper was to propose an efficient method for the quantification of microviridin J in cyanobacterial cells. Diluted MeOH, which has been used in many studies on cyanobacterial toxins, extracts high amounts of microviridin J and appears to be a reliable solvent for the peptide. Care must be taken, however, to avoid derivatization of the toxin molecule. According to our experience, the incubation of purified microviridin J with MeOH results in the gradual production of a new peptide with a molecular mass of m/z 1716 $[M + H]^+$. MALDI-TOF-MS analyses suggested that MeOH causes methylation of one of the ester linkages and high temperatures ($> 30^\circ C$) accelerate this reaction. However, this methylation could be prevented by keeping the sample temperature around 5˚C throughout the entire extraction procedure and by using 50% MeOH as solvent. Use of 50% MeOH furthermore ensures high extraction efficiency of microviridin J as well as other bioactive cyanobacterial metabolites (Fastner et al., 1998).

The proposed HPLC method afforded reliable and sensitive analyses of microviridin J. It has also been used to quantify other toxic compounds such as the microcystins (Rohrlack et al., 2001). Using the described HPLC procedure, *Microcystis* UWOCC CBS was found to contain 86 fg microviridin J per cell, which is a rather high but not unusual cellular amount for a bioactive cyanobacterial compound (Chorus and Bartram, 1999). Further studies may determine whether microviridin J is a common metabolite of *Microcystis*. Since microviridins have been isolated from diverse cyanobacterial genera (Murakami et al., 1997), microviridin J may not be restricted to species of *Microcystis*.

In conclusion, our data highlight the ability of freshwater cyanobacteria to produce potent environmental toxins. Considering the data on microviridin J, it is evident that microviridins and other protease inhibitors are of potential ecological significance. These compounds should receive considerable attention in future studies of cyanobacterial ecology.

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VULGARONE B, THE ANTIFUNGAL CONSTITUENT IN THE STEAM-DISTILLED FRACTION OF *Artemisia douglasiana*

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Abstract—Antifungal activity of the steam distilled essential oil fraction of *Artemisia douglasiana* was detected by bioautography on silica gel TLC plates against three *Colletotrichum* spp. The active principle was isolated by bioassay-directed fractionation using column chromatography followed by crystallization and was characterized as vulgarone B by ^{1}H and ^{13}C NMR and GC-MS. Antifungal activity of vulgarone B was further evaluated using 96-well microtiter assay against *Colletotrichum acutatum, C. fragariae, C. gloeosporioides*, and *Botrytis cinerea*. In addition, the antifungal activity of vulgarone B and verbenone, and their corresponding alcohols was tested by bioautography and microtiter assay. Structure–activity studies revealed that the α, β-unsaturated carbonyl functionality is a prerequisite for the antifungal activity of these mono and sesquiterpene ketones. This is the first report of antifungal activity of vulgarone B. The yield of essential oil from *A. douglasiana* is about 0.6–0.8% by weight of the dry material, including plant stems.

Key Words—Antifungal activity, vulgarone B, bioautography, *Artemisia douglasiana*, microtiter assay, α, β-unsaturated ketones, *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, *Botrytis cinerea.*

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INTRODUCTION

Filamentous fungi of the genus *Colletotrichum* and its telemorph *Glomerella* are major plant pathogens worldwide (Farr et al., 1989). *Colletotrichum* species often cause typical symptoms of anthracnose, a disease characterized by sunken necrotic lesions usually bounded by a red margin (Freeman et al., 1998). Anthracnose diseases of strawberry (*Fragaria x ananassa* Duch.) are serious problems for fruit and plant production in many areas of the world (Mass and Palm, 1997; Mass, 1998). The pathogens, *Colletotrichum acutatum* J. H. Simmonds, *C. gloeosporioides* (Penz.) Penz., and *C. fragariae* A. N. Brooks, can occur singly or in combination, and can infect flowers, fruits, leaves, petioles, stolons, and crowns of the strawberries (Howard et al.,1992; Smith, 1998a,b,c).

Botrytis fruit rot is among the most destructive diseases of strawberry fruits. Fruits are infected just before harvest, especially when the crop experiences persistent wetness. The causal fungus, *Botrytis cinerea* Pers. Fr. also affects blossom blight and may infect strawberry leaves and petioles (Sutton, 1998). Fungicidal sprays have been widely used for the control of *Botrytis* fruit rot, but in some areas *B. cinerea* has become resistant to many fungicides. Resistance to the most commonly used classes of fungicides, the benzimidazoles and dicarboximides, has seriously compromised the effectiveness of these fungicides. Few fungicides are now available for effective control of *Botrytis* diseases. New approaches for disease control have become necessary as the effectiveness and availability of commercial fungicides decrease.

Among different procedures used to minimize crop losses, chemical control of causative fungi remains the most common and effective method. A number of synthetic fungicides are used for this purpose (Vyas, 1988). Owing to emergence of resistant strains of certain pathogenic fungi, some currently used fungicides are less effective. There is a need for more selective, safe, and environment friendly fungicides. Natural products offer a pool of structurally diverse antifungal agents. Following natural product leads, more potent compounds and a better understanding of the mode of action of the fungicides can be achieved.

In our continuing search for antifungal compounds of natural origin against the fungi that are responsible for anthracnose disease of strawberries, the steam-distilled oil fraction of the aerial parts of *Artemisia douglasiana* Besser, a shrub from Oregon was investigated. *Artemisia* belongs to the family Asteraceae. Many members of the Asteraceae have been shown to possess diverse biological activities (Wat et al., 1980, 1981; Hudson et al., 1991, 1993). *Artemisia* species are often considered weeds in many parts of the world. Many weeds are ecologically important and contain bioactive compounds such as allelopathic and antifungal constituents in order to survive in the ecosystem. In this paper we report the isolation and identification of the antifungal compound from the steam distillate of *A. douglasiana* and the structural requirements for the antifungal activity.

METHODS AND MATERIALS

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. GC-MS analysis was carried out on HP5790 MSD spectrometer (Hewlett Packard, USA) equipped with 5890 GC using a DB-1 column 20-m $\times$ 0.2-mm, 0.18-μm film thickness (J & W Scientific Inc., Folsom, CA). The oven was temperature programmed from 60°C (5 min) to 280°C (20 min) at 5°C/min, total run: 69 min. The carrier gas was helium.

Materials. Steam distilled oil of aerial parts of *A. douglasiana*, a bluish colored oil, was provided by Aromagen, Albany, Oregon, USA. The steam distillation was performed on dry aerial parts of the plant grown in Albany, Oregon, USA, from 1998 harvest. The oil sample was stored in the freezer at -25°C until use. Verbenone and verbenol standards and $LiAlH_4$ (LAH) were purchased from Aldrich Chemical. Fungicide standards thiophanate methyl, captan, and thiabendazole were purchased from Chem Service, West Chester, PA, USA. Thin layer chromatography (TLC) was performed on silica gel GF plates, 0.25-mm thickness (Analtech Inc., Newark, DE, USA) using 20% ethyl acetate in hexane. Anisaldehyde spray reagent, iodine vapor, and UV light were used for the detection of compounds on TLC plates. Column chromatography was performed on Kieselgel 60 (particle size 0.063–0.2 mm) silica gel (Merck, Germany) with hexane and methylene chloride in increasing amounts. Solvents were reagent grade and were used without further purification.

Pathogen Production and Inoculum Preparation. Pathogen production and inoculum preparation for *B. cinerea*, *C. gloeosporioides*, *C. fragariae*, and *C. acutatum* were carried out according to the published procedures (Wedge and Kuhajek, 1998; Wedge et al., 2000). Conidial concentrations were determined photometrically (Wedge and Kuhajek, 1998) from a standard curve and suspensions were then adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/ml.

Bioautography. Bioautography using *Colletotrichum* spp. was used to identify the antifungal activity according to the previously published method (Wedge et al., 2000). Crude steam distillate of *A. douglasiana* was separated on silica TLC plates using 20% ethyl acetate and hexane, and then air-dried. Each plate was subsequently sprayed with a spore suspension (10^6 spores/ml) of the fungal species of interest and then incubated in a moist chamber for 3 days at 24°C with a 12 hr photoperiod under 60 ± 5 μmol/m^2/sec light. The regions where the antifungal constituents were present on the TLC plate were detected as clear zones, in a dark background on the TLC plate due to the inhibition of fungal growth.

Microbioassay. The steam distillate and the purified active compound isolated was further evaluated for quantitative antifungal activity using a 96-well format according to previously published methods (Wedge and Kuhajek, 1998; Wedge et al., 2000). Thiabendazole and captan with two different modes of action

were used as standards in this experiment. Thiophanate methyl was used as the standard in evaluating the activity of the crude steam distillate. Steam distillate was tested at 20, 50, and 100 μg/ml, whereas purified compound and standards were tested at 0.3, 3.0, and 30 μM concentrations against *B. cinerea*, *C. fragariae*, *C. gloeosporioides*, and *C. acutatum* in 96-well microtiter plates (Nunc Micro Well, untreated; Roskilde, Denmark). The steam distillate was tested at 20, 100, and 500 μg/ml. Microtiter plates were covered with a plastic lid and incubated in a growth chamber at 24 $\pm$ 1°C and 12 hr photoperiod under 60 $\pm$ 5 μmol/m^2/sec. Fungal growth was monitored photometrically by measuring absorbance at 620 nm at 24, 48, and 72 hr. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 hr. Analysis of variance of the Least Square Means for percent inhibition of each fungus at each dose of test compound relative to the untreated controls was used to evaluate fungal growth inhibition. The experiment used a repeated measures design and was repeated once. Data presented were pooled from two independent experiments.

Isolation of the Antifungal Constituent. The steam distilled oil (5 g) was column chromatographed on silica gel column (30-cm $\times$ 35-mm id) using hexane and increasing amounts of CH_2Cl_2(0–80% CH_2Cl_2 in hexane, with hexane 2l, 20% with 31, 40% with 41, and 80% with 21). Fractions of 250 ml were collected, concentrated at 40°C, and the fractions with similar TLC profiles were combined to afford 22 fractions. Each fraction was tested on TLC bioautography to select the antifungal active fractions. Fractions that showed antifungal activity were pooled according to TLC profile and further purified by silica gel column chromatography (20-cm $\times$ 25-mm id) using CH_2Cl_2 in hexane. The antifungal compound, vulgarone B (**1**), was eluted in fractions 6–14 with hexane–CH_2Cl_2 (1:1). This was crystallized with 1% CH_2Cl_2 in hexane at 4°C to afford 580 mg of white crystalline compound. ^{1}H NMR: δ ($CDCl_3$) 5.71 (1H, br s, 2-H), 2.73 (1H, d, J = 6.4 Hz, 11-H), 2.52 (1H, d, J = 6.4 Hz, 4-H), 2.11 (1H, s, 5-H), 1.98 (3H, br s, 15-H_3), 1.68 (2H, m, 9-H_2) 1.62 (2H, m, 8-H_2), 1.39 (2H, m, 7-H_2), 0.92 (6H, s, 13-H_3, 14-H_3), 0.83 (3H, s, 12-H_3). ^{13}C NMR: δ ($CDCl_3$), 204.9 (C-1), 172.6 (C-3), 122.5 (C-2), 66.8 (C-5), 57.9 (C-11), 55.1 (C-10), 49.9 (C-4), 41.5 (C-9), 38.5 (C-7), 33.8 (C-6), 27.7 (C-12), 27.2 (C-13), 24.6 (C-14), 23.3 (C-15), 21.3 (C-8). $M^+ = 218$. The identity was confirmed by NMR and $[\alpha]_D$ with those reported in the literature (Uchio et al., 1977).

Reduction of Vulgarone B. To an ice cold suspension of LAH (30 mg) in anhydrous ether (15 ml) vulgarone B (**1**) (20 mg) in 10 ml of anhydrous ether was added and the mixture was stirred for 45 min at room temperature. The reaction was quenched by pouring the mixture into 0.5% HCl in H_2O (100 ml). The mixture was extracted with ether (3 $\times$ 150 ml), the ether layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield a white crystalline product (19 mg) that was further purified by TLC using 10% ethyl acetate–hexane. The allylic alcohol vulgarol B (**2**) with its β-orientation was obtained as white crystals and

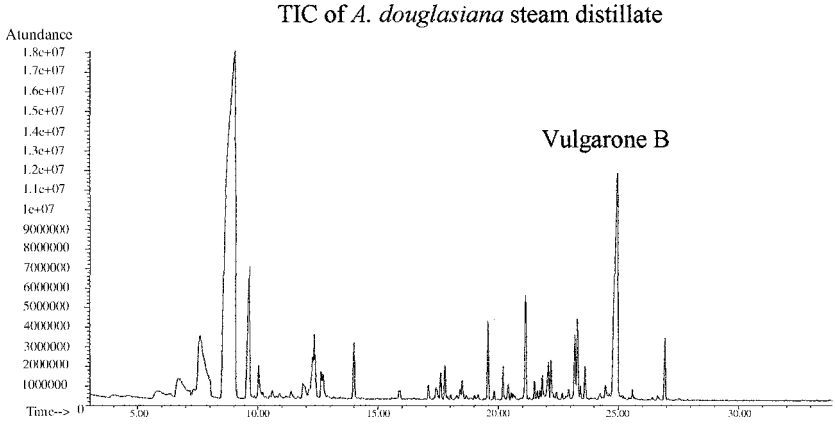

FIG. 1. A sample gas chromatogram of *A. douglasiana* steam distillate.

was identified by comparison of its NMR data and $[\alpha]_D$ with those reported in literature (Uchio et al., 1977). ^{1}H NMR: δ ($CDCl_3$) 5.32 (1H, br s, 2-H), 4.38 (1H, br s, 1-H), 2.35 (1H, m, 11-H), 2.03 (1H, d, $J = 6.3$ Hz, 4-H), 1.92 (1H, s, 5-H), 1.71 (3H, br s, 15-H_3), 1.68–1.53 (4H, m, 8-H_2, 9-H_2), 1.33 (2H, m, 7-H_2), 1.00 (3H, s, 14-H_3), 0.86 (3H, s, 12-H_3), 0.80 (3H, s, 13-H_3). ^{13}C NMR: δ ($CDCl_3$), 150.0 (C-3), 120.2 (C-2), 75.4 (C-1), 61.7 (C-5), 47.4 (C-11), 47.1 (C-4), 41.3 (C-9), 40.4 (C-10), 38.5 (C-7), 32.6 (C-6), 28.1 (C-12), 27.1 (C-13), 24.8 (C-14), 22.4 (C-15), 21.5 (C-8). $M^+ = 220$.

GC-MS Analysis of Vulgarone B. The concentration of vulgarone B in the crude oil sample of *A. douglasiana* was determined by GC-MS analysis using selective ion mode. A calibration curve was obtained using standard solutions of pure vulgarone B and plotted using linear regression ($R^2 = 0.99$). The concentration of vulgarone B in the crude oil was *ca.* 11% by weight (Figure 1).

RESULTS AND DISCUSSION

Preliminary screening of the steam distilled oil of *A. douglasiana* by bioautography on silica gel TLC plates revealed the presence of antifungal agents inhibitory to *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* (Figure 2). Microbioassay of the crude steam distillate also indicated inhibitory activity against *C. acutatum*, and *B. cinerea* where thiophanate-methyl was used as the fungicide standard (Table 1). Bioassay guided fractionation of the steam distilled oil by column chromatography facilitated the isolation of the antifungal compound that was identified by ^{1}H and ^{13}C NMR and GC-MS as vulgarone B (**1**), a sesquiterpene ketone, previously been isolated from *Chrysanthemum vulgare* (Uchio et al., 1977). The concentration of

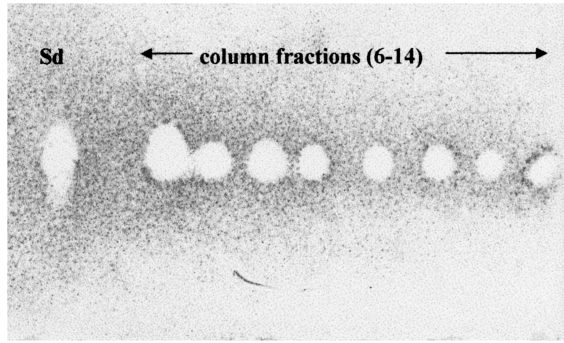

FIG. 2. Bioautography of *Artemisia douglasiana* steam distillate (Sd) and silica gel column fractions. The plate was sprayed with spores of *Colletotrichum fragariae.* Clear zones demonstrate area of fungal growth inhibition and indicate the presence of antifungal constituents.

vulgarone B in the steam-distilled oil of *A. douglasiana* was determined by GC-MS to be *ca.* 11%.

In order to establish some structure–activity relationships, verbenone (**3**), vulgarone B (**1**), and the corresponding allylic alcohols, verbenol (**4**) and vulgarol B (**2**), obtained by $LiAlH_4$ reduction of (**1**) (Figure 3), were assessed by bioautography. Only compounds possessing an α, β-unsaturated carbonyl group had antifungal activity (Figure 4). Hence, it is evident that this functional group is essential for the antifungal activity of vulgarone B and verbenone. Therefore, we postulate that one of the possible modes of action of these antifungal compounds may be due to their roles as Michael-type acceptors for biological nucleophiles (Ahn and sok, 1996). Evaluation of the quantitative antifungal activity of vulgarone

TABLE 1. PERCENT INHIBITION OF FUNGAL SPECIES BY STEAM-DISTILLED OIL OF *Artemisia douglasiana*

Organism	Sample	Mean ± SD (48 hr fungal growth)[a]		
		20 μg/ml	100 μg/ml	500 μg/ml
B. cinerea	Thiophanate-methyl[b]	24.0 ± 10.3	5.1 ± 3.6	−36.7 ± 36.5
	A. douglasiana steam distillate	35.2 ± 5.7	−26.1 ± 35.2	−98.5 ± 10.3
C. acutatum	Thiophanate-methyl	18.2 ± 50.2	−20.3 ± 37.6	−86.3 ± 7.9
	A. douglasiana steam distillate	23.2 ± 23.2	−44.5 ± 11.6	−95.5 ± 6.3

[a] Negative values represent the strength of fungal growth inhibition and positive values represent growth stimulation relative to the untreated controls. Data are pooled from two different experiments repeated in time.

[b] Commercial fungicide standard.

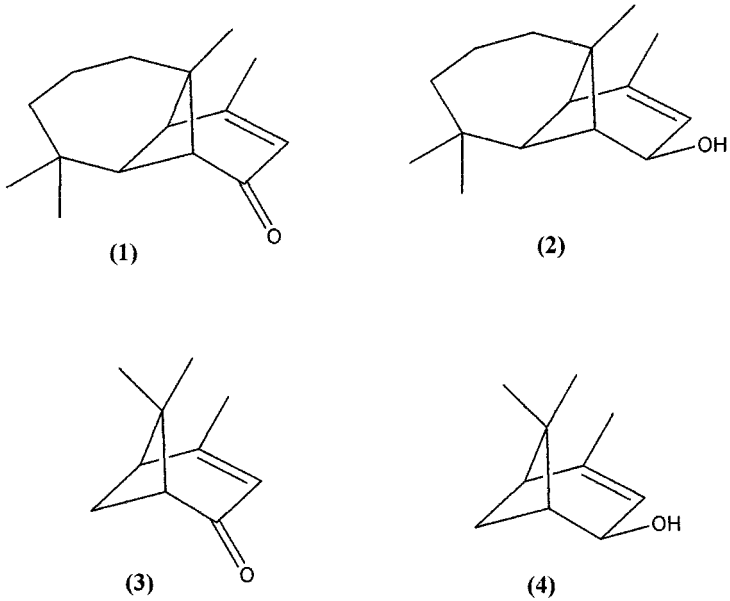

FIG. 3. Structures of the compounds: (1) Vulgarone B, (2) Vulgarol B, (3) Verbenone, (4) Verbenol.

B and verbenone was carried out using 96-well microbioassay system in comparison with commercial fungicide standards thiabendazole and captan according to published methods (Wedge and Kuhajek, 1998; Wedge et al., 2000). Growth inhibition results indicated that vulgarone B and verbenone had some selectivity toward *B. cinerea,* (Figure 5) but had marginal activity at concentrations $\leq$30 μM (data not

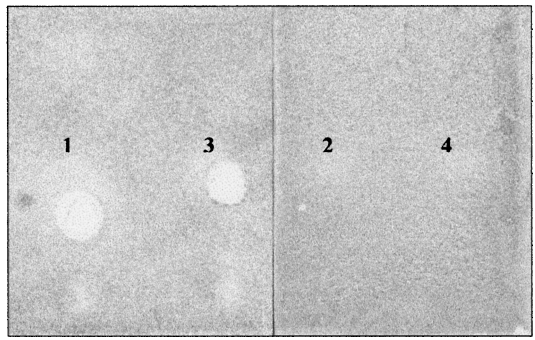

FIG. 4. Bioautography of vulgarone B (**1**), verbenone (**3**), and their corresponding alcohols **2** and **4**. Only **1** and **3** showed antifungal activity.The plate was sprayed with spores of *Colletotrichum fragariae.*

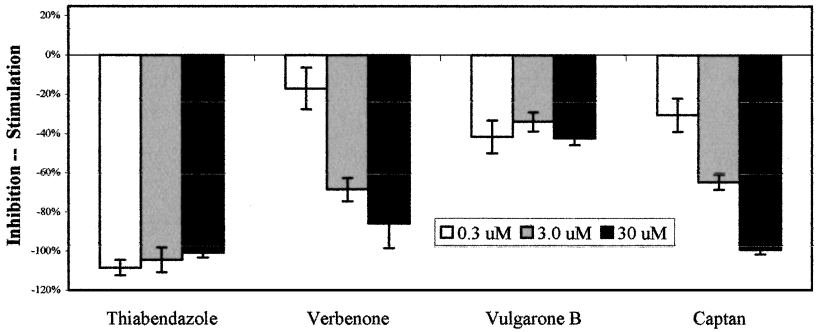

FIG. 5. Growth inhibition of *Botrytis cinerea* at 48 hr using a 96-well microbioassay format in response to verbenone (**3**) and vulgarone B (**1**) and the commercial fungicide standards thiabendazole and captan. Means for percent growth inhibition were pooled from two experiments replicated in time.

shown) against *C. gloeosporioides*, *C. fragariae*, *C. acutatum*, and *F. oxysporum*. At 30 μM concentrations, vulgarone B showed *ca*. 40% inhibition of *B. cinerea* growth and 20% inhibition of *C. fragariae* growth. Verbenone showed much greater antifungal activity against *B. cinerea* (*ca*. 80% growth inhibition) and lower activity against *C. fragariae* (20% growth inhibition). At 30 μM concentrations, the commercial fungicides thiabendazole and captan showed *ca*. 90% inhibition of *B. cinerea* growth. These results indicate that vulgarone B and verbenone are moderately active against *B. cinerea* and marginally active against the *Colletotrichum* species that we tested. While the antifungal mode of action of vulgarone B and verbenone is yet to be determined, this is the first report of antifungal activity of vulgarone B and verbenone against *Colletotrichum* and *Botrytis* species. Future greenhouse studies will determine the potential commercial applications for these compounds to control fungal diseases.

In summary, there are numerous reports in the literature about the antifungal activity of essential oils but reports on isolation and identification of the individual components that are responsible for the biological activity are limited (Pattnaik et al., 1997; Meepagala et al., 2002; Vila et al., 2002). This research has demonstrated the antifungal activity of steam-distilled oil of *A. douglasiana* against *B. cinerea*, and marginal activity against *C. acutatum*, *C. fragariae*, and *C. gloeosporioides*. The antifungal component of *A. douglasiana* was isolated and identified as vulgarone B (**1**). This is the first report demonstrating the antifungal activity of vulgarone B to agriculturally important plant pathogenic fungi. Moreover, we have also shown the importance of the α, β-unsaturated carbonyl group for the antifungal activity of this class of compounds. Further investigation of the mode of action and use of this compound as a postharvest fumigant shall be performed.

Acknowledgments—We thank Mr. Dewayne Harries and Ms. Amber L. Hale for their valuable technical assistance and Ms. Linda Robertson for assistance in bioautography and microtiter assay. Mr. Don Stanford and Mr. Frank Wiggers in the NCNPR at the University of Mississippi are acknowledged for their assistance with GC-MS and NMR facilities.

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NITROGEN OXIDES ELICIT ANTIPREDATOR RESPONSES IN JUVENILE CHANNEL CATFISH, BUT NOT IN CONVICT CICHLIDS OR RAINBOW TROUT: CONSERVATION OF THE OSTARIOPHYSAN ALARM PHEROMONE

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Abstract—Recent studies with cyprinid and characin (superorder Ostariophysi) fishes suggest that purine-*N*-oxides function as chemical alarm cues (alarm pheromones) and that the nitrogen oxide functional group acts as the chief molecular trigger. To further test the hypothesis that the nitrogen-oxide functional group is evolutionarily conserved as an active component of the Ostariophysan alarm pheromone system, we exposed juvenile channel catfish (*Ictalurus punctatus*, Siluriformes) to conspecific skin extract, hypoxanthine-3-*N*-oxide (the putative alarm pheromone) and a suite of structurally and functionally similar compounds. Conspecific skin extract and hypoxanthine-3-*N*-oxide elicited significant increases in species typical antipredator behaviors. A structurally dissimilar compound possessing a nitrogen oxide functional group (pyridine-*N*-oxide) elicited a significant, but less intense alarm response. Compounds lacking a nitrogen oxide functional group were not significantly different from control stimuli. In addition, two non-Ostariophysan species known to possess chemical alarm cues (convict cichlids, *Acrchocentrus nigrofasciatus*, Cichlidae, Acanthopterygii and rainbow trout, *Oncorhynchus mykiss*, Salmonidae, Protacanthopterygii) did not show any increase in antipredator behavior in response to

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hypoxanthine-3-*N*-oxide. These data demonstrate the conservation of chemical alarm cues within at least three orders of the superorder Ostariophysi.

Key Words—Ostariophysan fishes, chemical alarm cues, channel catfish, antipredator behavior, hypoxanthine-3-*N*-oxide, nitrogen oxides.

INTRODUCTION

The superorder Ostariophysi is a diverse group of fishes, incorporating over 6500 described species (Moyle and Cech, 1996) and accounting for approximately 65% of all freshwater species (Nelson, 1994). The superorder consists of five extant orders; Gonorynchiformes, Cyprinidformes, Characiformes, Siluriformes, and Gymnotiformes. A common character of most Ostariophysan fishes are the specialized epidermal club cells (Pfeiffer, 1977), which produce and store a chemical alarm cue (alarm pheromone; Smith, 1992, 1997). This chemical is released into the water column, following mechanical damage to the skin. Conspecifics and some sympatric heterospecifics, respond with dramatic, short-term increases in species typical antipredator behavior (Smith, 1992; Chivers and Smith, 1998). Such antipredator responses have been shown to lead to significant increases in the probability of surviving an encounter with a potential predator, and hence fitness potentials (Mathis and Smith, 1993; Chivers et al., 1996; Mirza and Chivers, 2001a).

The putative Ostariophysan alarm pheromone has been identified as hypoxanthine-3-*N*-oxide (H3NO; Argentini, 1976; Pfeiffer et al., 1985; Brown et al., 2000). H3NO is characterized by a purine skeleton and a nitrogen-oxide functional group at the third position (Figure 1). However, recent work suggests that the alarm pheromone system of the superorder Ostariophysi may actually consist of a suite of aromatic compounds, which are related through a common nitrogen oxide functional group (Brown et al., 2000, 2001a). Two cyprinid species (fathead minnows, *Pimephales promelas* and finescale dace, *Phoxinus neogaeus*) and two characin species (glowlight tetras, *Hemigrammus erythrozonus* and neon tetras, *Paracheirodon innes*) significantly increase their antipredator responses when exposed to conspecific skin extracts (natural alarm pheromones), H3NO, a

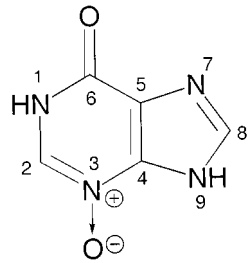


FIG. 1. Hypoxathine-3-*N*-oxide, with standard purine numbering system.

bicyclic heteroaromatic compound, or pyridine-*N*-oxide, a monocyclic heteroaromatic compound, both containing a nitrogen-oxide. No significant response was found following exposure to a suite of structurally similar compounds lacking a nitrogen oxide functional group (Brown et al., 2000, 2001a). In addition, replacing the carbonyl oxygen at the sixth position (Figure 1) with progressively larger alkoxy groups, methoxy, ethoxy, *iso*-propoxy, and *tert*-butoxy, respectively, appears to have no effect on the ability of the purine-3-*N*-oxide family to elicit a significant alarm response (G. E. Brown, J. C. Adrian Jr., and J. M. Tower, unpublished data). Finally, under mildly acidic (pH 6.0) conditions, natural skin extract and H3NO fail to elicit a behavioral response (Brown et al., 2002); an apparent consequence of a covalent bond change leading to the loss of the N—O functional group (Kawashima and Kumashiro, 1969; Wölke and Brown, 1969). These data, taken together, implicate the N—O functional group as the chief molecular trigger, eliciting an increase in antipredator response.

Cyprinds likely diverged from the Euteleostian lineage during the Eocene period (Nelson, 1994), while characins diverged more recently, during the Miocene period (Fink and Fink, 1981; Orti and Meyer, 1997). Siluriformes are thought to have diverged much later than either the cyprinids or characins (Nelson, 1994; Figure 2). Siluriformids, like most Ostariophysian fishes, possess epidermal club cells and an alarm pheromone (i.e., channel catfish, *Ictaluris punctatus*, Ictaluridae, Pfeiffer, 1977; pintado, *Pseudoplatystoma coruscans*, Pimelodidae, Giaquinto and Volpato, 2001; hardhead catfish, *Arius felis*, Ariidae, Smith, 2000). It remains unknown, however, if a Siluriforme species (i.e., channel catfish) will respond to compounds containing a N—O functional group, as do cyprinids and characins. To test our model, we conducted the current series of experiments. We examined the response of juvenile channel catfish to conspecific skin extract, H3NO, and a suite of structurally similar compounds lacking a nitrogen oxide functional group. We also examined the responses of two non-Ostariophysan (Euteleosti) species known to possess analogous damage released alarm signals (convict cichlids, *Archocentrus nigrofasciatus*, Cichlidae, Acanthopterygii; Wisenden and Sargent, 1997; Alemadi and Wisenden, 2002) and rainbow trout (*Oncorhynchus mykiss*, Salmonidae, Protacanthopterygii, Brown and Smith, 1997, 1998; Mirza and Chivers, 2001b) to conspecific skin extracts and H3NO (Figure 2).

METHODS

Response of Channel Catfish. Juvenile channel catfish were obtained from a commercial supplier and held in a 195 l glass aquaria, filled with continuously filtered dechlorinated tap water (pH 7.8, 20°C, 12:12 Light:Dark cycle) and a gravel substrate. Green swordtails (*Xiphophorus helleri*) were also obtained from a commercial supplier and held in a 110 l aquaria under similar conditions, except that the temperature was maintained at approximately 25°C. Catfish and swordtails

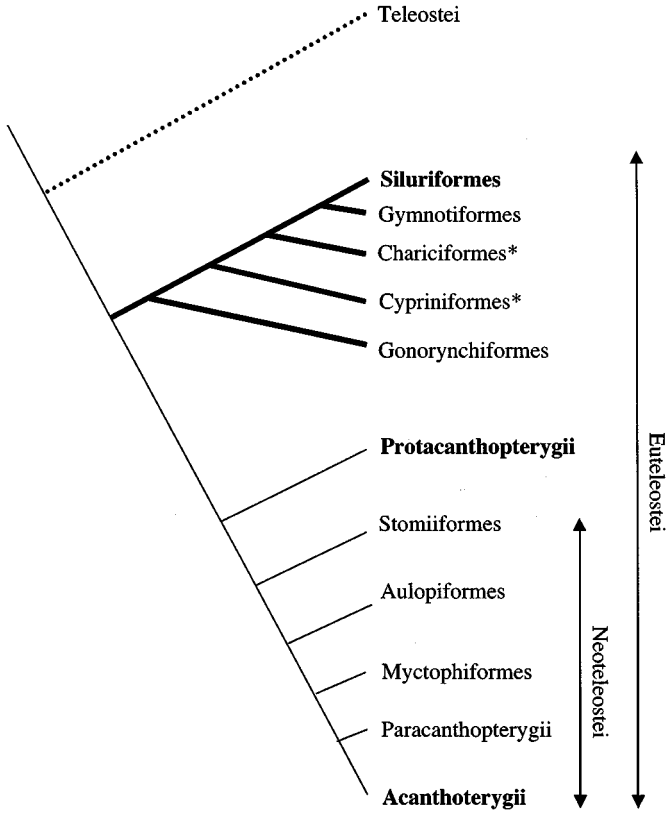


FIG. 2. General phylogeny of Euteleost fishes, showing relationship within the superorder Ostariophysi (heavy lines). Asterisks denote orders previously tested for responses to purine-*N*-oxides. Names in bold type denote groups tested in the current study. Phylogeny based on Moyle and Cech (1996).

were fed *ad libitum*, twice daily with brine shrimp (*Artemia* spp.) and commercial flake food. Swordtails were used as a control for the odor of any injured prey, as they are allopatric and phylogenetically distant from channel catfish.

Experimental Stimuli. We generated a total of nine experimental stimuli, including two natural skin extracts (conspecific skin extract (CSE) and swordtail skin extract (SWT)) and seven synthetic stimuli (hypoxanthine-3-N-oxide (H3NO), pyridine-N-oxide (PNO), pyridine (PN+), 4(3*H*)-pyrimidone (PYR), guanine (GUAN), hypoxanthine (HXAN), and xanthine (XAN)), and an additional distilled water control (DW).

We collected natural skin extracts from five catfish donors (mean $\pm$ S.E. standard length $= 5.70 \pm 0.45$ cm) and six swordtail donors (3.47 ± 0.18 cm). Donor

fish were killed with a blow to the head, in accordance with Concordia University Animal Care Committee protocol # AC-2002-BROW. Skin fillets were removed from either side of the fish and immediately placed into 50 ml of chilled, glass distilled water. Skin fillets were then homogenized and filtered through glass wool, to remove any remaining tissue, and diluted to final volumes with distilled water. A total of 28.36 cm^2 (in 320 ml) of catfish skin and 24.54 cm^2 (in 275 ml) of swordtail skin was collected. Skin extracts were frozen in 25-ml aliquots at approximately -20°C until required. In addition, we froze distilled water in 25-ml aliquots.

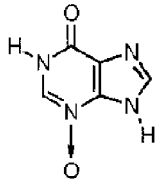
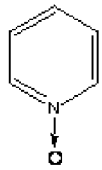
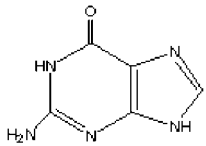
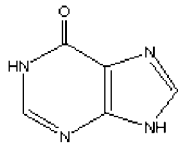
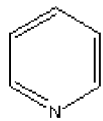
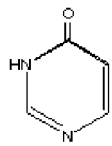
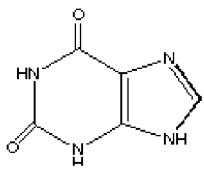
We tested a total of seven synthetic compounds (Table 1). H3NO was synthesized as described in Brown et al. (2000). The remaining six compounds were obtained from a commercial supplier and used as received. We prepared the synthetic stimuli by dissolving 0.002 g of the compound of interest in 200 ml of distilled water and stirring for 15 min. The resulting solutions were frozen in 25 ml aliquots until needed. Concentrations of natural and synthetic stimuli were consistent with those used for cyprinid (Brown et al., 2000, 2001b) and characin (Brown et al., 2001a) species.

Experimental Protocol. Trials were conducted in a series of 37 l glass aquaria, filled with 35 l of dechlorinated tap water (pH 7.8, 20°C), surrounded on three sides with black plastic and containing a gravel substrate and a single airstone. Connected to the airstone was an additional 2 m length of tubing, allowing us to inject the test stimuli without disturbing the fish.

Twenty-four hr prior to trials, we placed individual catfish into the test tanks to acclimate. During this period, catfish were fed, *ad libitum*, with flake food. Trials consisted of a 5 min prestimulus and 5 min poststimulus observation period. Prior to the prestimulus observation, we withdrew and discarded 60 ml of tank water from the stimulus injection tube (to remove any stagnant water) and then withdrew and retained an additional 60 ml. Following the prestimulus observation period, we injected 5 ml of an experimental stimulus (or distilled water control) and slowly (approximately 30 sec) flushed it into the test tank using the retained 60 ml of water. After the stimulus was completely introduced, we began the poststimulus observation period. All trials ($N = 15$ for CSE and DW, $N = 10$ for all other stimuli) were conducted between 11:00 and 14:00 daily and the order of stimuli tested was randomized. We videotaped all trials for subsequent analysis of behavioral responses. Test tanks were drained and rinsed between each trial. Individual catfish were tested only once.

During pre- and poststimulus observation periods, we recorded the time spent by individuals in each of four behavior patterns: slow movement, fast movement, motionless, and resting. Slow movement was typical of normal foraging behavior, with an individual moving slowly (<2 body lengths per second) at or near the substrate, in a head down posture (Valentincic and Caprio, 1994). Fast movement consisted of rapid swimming (dashing; Valentincic and Caprio, 1994; Giaquinto and Volpato, 2001), off the substrate, often with an individual covering the entire

TABLE 1. CHEMICAL STRUCTURES OF THE SYNTHETIC STIMULI USED IN EXPERIMENT 1

	Hypoxanthine-3-*N*-oxide
	Pyridine-*N*-oxide
	Guanine
	Hypoxanthine
	Pyridine
	4(3H)-Pyrimidone
	Xanthine

length of the tank in a single burst. Motionless was defined as an individual remaining on the substrate without moving, in a head down posture and the dorsal spine erect ($>45^\circ$ to dorsal line). Resting was defined as an individual resting at or near the substrate, with its head up. A decrease in slow movement and time spent resting and an increase in fast movement and time spent motionless are consistent with an antipredator response in catfishes (Valentincic and Caprio, 1994; Smith, 2000; Giaquinto and Volpato, 2001).

Statistical Analysis. For each behavioral measure, we calculated the difference between the pre- and poststimulus observation periods and used these values as dependent measures in a MANOVA, with stimulus type as the independent variable. Subsequent one-way ANOVAs were used to test for differences between chemical treatments for each behavior pattern.

Response of Convict Cichlids. Juvenile convict cichlids (mean $\pm$ S.E. S.L. $=$ 3.89 ± 0.22 cm) from our laboratory stock were held and fed as described above for green swordtails. We collected 40.62 cm^2 of cichlid skin (in 460 ml of distilled water) as described above. Test tanks were identical to those described above.

Two cichlids (matched for length) were placed into each of the test tanks, 24 hr prior to testing. The experimental protocol was as described above, with the exception that pre- and poststimulus observation periods lasted 10 (rather than 5) min. We videotaped all trials for subsequent analysis. During both pre- and poststimulus observation periods, we recorded area use and distance between the two fish every 15 sec. Area use was recorded as the position of each cichlid ($1 =$ bottom quarter of the test tank, $4 =$ top quarter). Distance between individuals was measured from the mid point of each fish. In addition, we recorded frequency of aggressive interactions, defined as biting, chasing for a minimum of two body lengths and directed approaches (Wisenden and Sargent, 1997). For all behavioral measures, we recorded the difference between pre- and poststimulus observation periods and used these difference scores as dependent variables in a MANOVA, with stimulus type as the independent variable. Decreased area use, distance between individuals and frequency of aggressive interactions is indicative of an antipredator response in juvenile convict cichlids (Wisenden and Sargent, 1997; Alemadi and Wisenden, 2002).

Response of Rainbow Trout. Juvenile rainbow trout (mean $\pm$ SE SL at testing $= 6.52 \pm 0.49$ cm) were obtained from a commercial hatchery and held in a 390 l recirculating tank, maintained at 14°C. Trout were fed *ad libitum* daily with commercial trout pellets. We collected 24.92 cm^2 (in 280 ml) of trout skin, as described above, to generate our conspecific skin extract. H3NO was also prepared as described above.

Experimental stream channels consisted of opaque Plexiglas oval ($2.5 \times$ 0.66 m), divided with a watertight barrier down the center line. This created two experimental channels per tank. Each channel was provided with individual water inflow and outflows, minimizing mixing between the two channels. We generated a weak laminar current (approximately 5 cm/sec) by continuously pumping water

into the upstream end of the channel and allowing it to drain over a standpipe at the downstream end of each channel. Test fish were constrained to a 1.6×0.33 m section by mesh screens at the up and down stream ends of the channels. Temperature within the stream channels was maintained at 14°C throughout the experiment. Each channel contained a cover object, consisting of a 12.5 cm^2 ceramic tile supported by three glass cylinders (5.25-cm high), positioned at the mid point of the stream channel.

We placed two trout into each stream channel 24 hr prior to testing. Trials consisted of a 10-min prestimulus and a 10-min poststimulus observation periods. All observations were videotaped using low light cameras at approximately 10 lux. Following the prestimulus observation period, we injected 20 ml of trout skin extract or H3NO (experimental cues) or distilled water (control) directly into the inflow hoses. From the videotapes, we arbitrarily selected one of the two trout as a focal animal and recorded time spent moving, time under shelter, and time in the front half of the test channel (i.e., nearest the point of stimulus injection). We calculated the difference between pre- and poststimulus observation periods and used these difference scores as dependent measures in a MANOVA. Decreased movement and time in the front area of the test channel and increased time under shelter are indicative of an antipredator response (Brown and Smith, 1997; A. Leduc and G. E. Brown, unpublished data).

RESULTS

Response of Channel Catfish. We found a significant overall effect of stimulus type on the change in behavioral response patterns ($F_{(9,100)} = 18.38$, $P < 0.001$). We also found significant effects of stimulus type on the change in individual behavioral measures (slow movement: $F_{(9,100)} = 2.29$, $P < 0.03$, Figure 3A; fast movement: $F_{(9,100)} = 2.42$, $P < 0.02$, Figure 3B; time motionless: $F_{(9,100)} = 11.64$, $P < 0.001$, Figure 4A; time resting: $F_{(9,100)} = 2.98$, $P < 0.004$, Figure 4B). Individual catfish exposed to CSE and H3NO significantly decreased time slow moving and resting and increased fast movement and time motionless (Figures 3 and 4). Conversely, individuals exposed to DW, SWT, PN+, PYR, GUAN, HXAN, and XAN exhibited an increase in slow movement time and only minor changes in time resting and fast movement (Figures 3 and 4). The response of individuals exposed to PNO was characterized by a significant increase in fast movements and time spent motionless and intermediate decreases in slow movements and time resting (Figures 3 and 4).

Response of Convict Cichlids. We found a significant overall effect of stimulus type on the behavioral responses of juvenile convict cichlids ($F_{(6,50)} = 6.59$, $P < 0.001$; Figure 5). Cichlids exposed to conspecific skin extract significantly decreased area use ($F_{(2,27)} = 3.43$, $P < 0.05$; Figure 5A), distance between individuals ($F_{(2,27)} = 12.58$, $P < 0.001$; Figure 5B), and frequency of aggressive

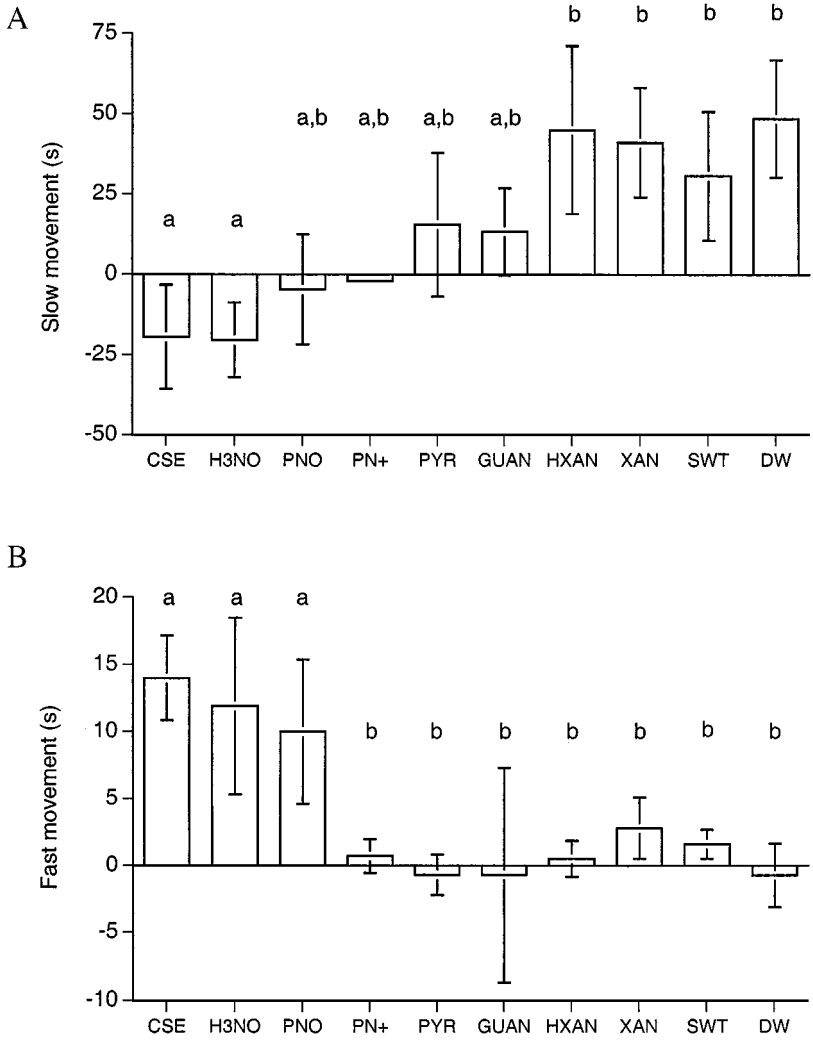


FIG. 3. Mean (±SE) change in slow movement (A) and fast movement (B) time (sec) by juvenile channel catfish. Different letters denote significant differences based on Fisher's Probability of Least Squared Differences ($P < 0.05$). CSE = conspecific skin extract, H3NO = hypoxanthine-3-*N*-oxide, PNO = pyridine-*N*-oxide, PN+ = pyridine, PYR = 4(3*H*)-pyrimidone, GUAN = guanine, HXAN = hypoxanthine, XAN = xanthine, SWT = swordtail skin extract, DW = distilled water.

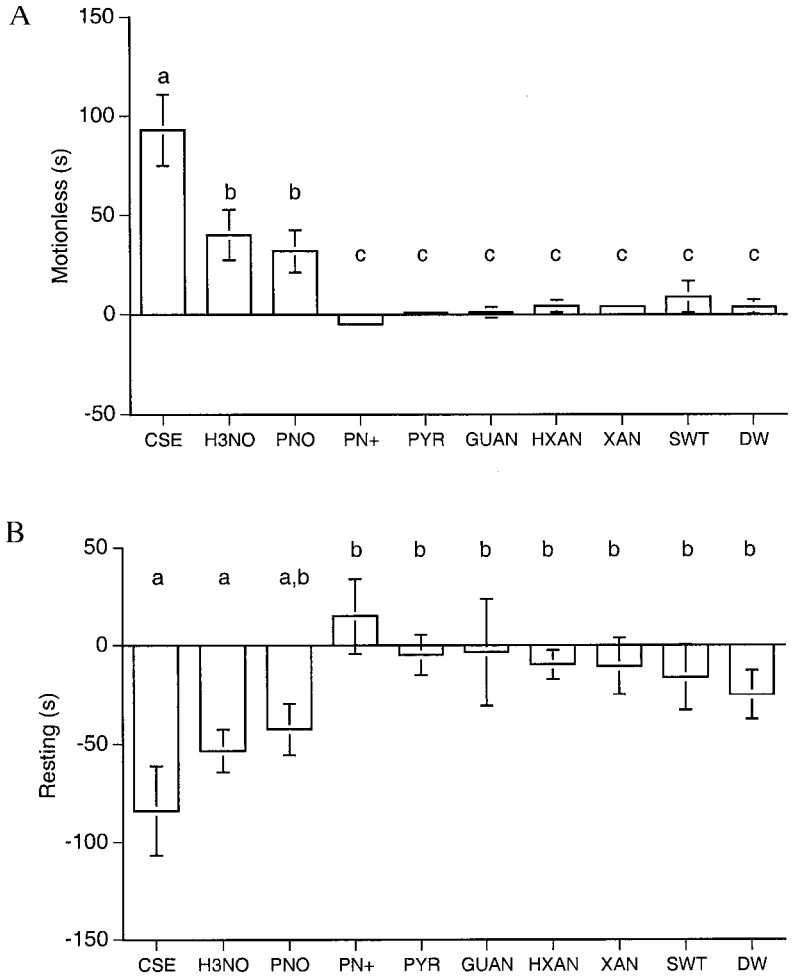


FIG. 4. Mean (±SE) change in time (sec) spent motionless (A) and resting (B) by juvenile channel catfish. Different letters denote significant differences based on Fisher's Probability of Least-Squared Differences ($P < 0.05$). CSE = conspecific skin extract, H3NO = hypoxanthine-3-*N*-oxide, PNO = pyridine-*N*-oxide, PN+ = pyridine, PYR = 4(3*H*)-pyrimidone, GUAN = guanine, HXAN = hypoxanthine, XAN = xanthine, SWT = sword-tail skin extract, DW = distilled water.

interactions ($F_{(2,27)} = 6.21$, $P < 0.006$; Figure 5C) compared to either distilled water or H3NO.

Response of Rainbow Trout. We found a similar overall effect of stimulus type on behavioral responses by juvenile rainbow trout ($F_{(6,50)} = 4.75$, $P <$

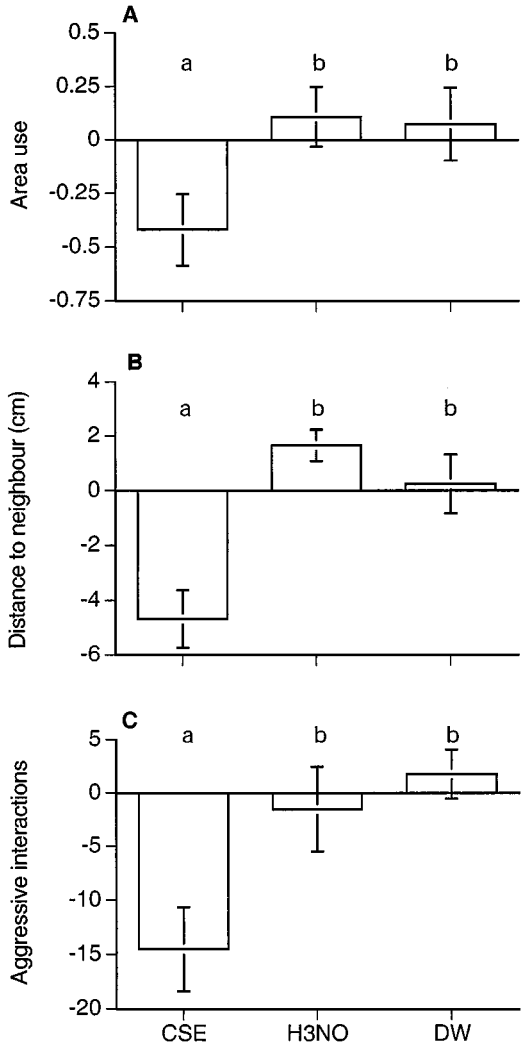


FIG. 5. Mean (±SE) change in area use (A), distance to neighbor (cm, B) and number of aggressive interactions (C) for juvenile convict cichlids exposed to conspecific skin extract (CSE), hypoxanthine-3-*N*-oxide (H3NO) or distilled water (DW). Different letters denote significant differences based on Fisher's Probability of Least-Squared Differences ($P < 0.05$).

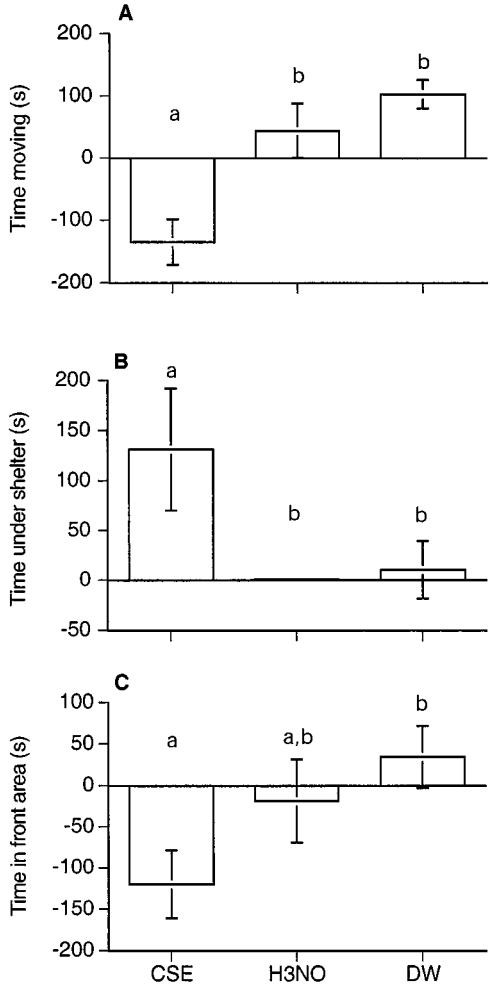


FIG. 6. Mean (±SE) change in time spent moving (sec, A), time under shelter (sec, B) and time in front area (sec, C) for juvenile rainbow trout exposed to conspecific skin extract (CSE), hypoxanthine-3-*N*-oxide (H3NO) or distilled water (DW). Different letters denote significant differences based on Fisher's Probability of Least-Squared Differences ($P < 0.05$).

0.001; Figure 6). Trout exposed to conspecific skin extract decreased time spent moving ($F_{(2,27)} = 12.29$, $P < 0.001$; Figure 6A), and increased time under shelter ($F_{(2,27)} = 3.47$, $P < 0.05$; Figure 6B) relative to either distilled water or H3NO. We found a marginally significant difference in time spent in the front area of

the test tank (i.e., near point of stimulus injection; $F_{(2,27)} = 3.27$, $P = 0.054$; Figure 6C).

DISCUSSION

These data clearly demonstrate that H3NO elicits an increase in antipredator behavior (decreased slow movement and time resting, and increased fast movement (dashing) and time spent motionless) of a similar intensity to conspecific skin extract in juvenile channel catfish. In addition, a structurally dissimilar compound with a N—O functional group (pyridine-*N*-oxide) elicited a significant, but less intense increase in antipredator behavior. Swordtail skin extract and the remaining synthetic compounds lacking a N—O functional group were not different from our distilled water control. These data are in full agreement with previous work with cyprinids (Brown et al., 2000) and characin (Brown et al., 2001a) prey species. When exposed to H3NO, two non-Ostariophysan species known to possess damage-released chemical alarm signals, convict cichlids (Wisenden and Sargent, 1997) and rainbow trout (Brown and Smith, 1997), did not exhibit any significant change in species typical antipredator behavior. Taken together, these results support our current model of the conservation of the N—O molecular trigger within the Ostariophysan alarm signaling system.

Schutz (1956) demonstrated significant heterospecific responses within the superorder Ostariophysi, but demonstrated decreasing response intensities with increasing phylogenetic distance. This suggests, that while the N—O molecular trigger may be highly conserved within the superorder (Brown et al., 2000, 2001a), there exists some species specificity within the Ostariophysan alarm pheromone system. Brown et al. (2000) suggested that the alarm pheromone system may be comprised of a suite of purine compounds sharing a common N—O functional group and that species-specific ratios of these compounds might account for the observed decline in response intensity. Alternatively, purine-*N*-oxides might be bound to a carrier molecule, such as a protein or a peptide (Kasumyan and Ponomarev, 1987). Species specific carrier molecules, associated with a common active compound, presumably a purine-*N*-oxide, would likewise account for the observed response trends.

At least two additional examples of highly conserved alarm cues have been demonstrated. Mirza and Chivers (2001b) exposed hatchery-reared rainbow trout, brook charr (*Salvelinus fontinalis*), and brown trout (*Salmo trutta*) to skin extracts from all three species and that of swordtails. They report significant increases in antipredator responses to each of the salmonid alarm cues, and no significant change in behavior towards swordtail skin extracts. Similarly, Mirza et al. (2001) found a significant response between two allopatric poeciliid species They found that red swordtails exhibited a significant increase in antipredator behavior when exposed to conspecific skin extract and that of allopatric guppies (*Poecilia reticulata).*

Interestingly, Brown and Godin (1999) failed to find a significant response of guppies towards swordtail skin extract, suggesting that within the Poecillids, the alarm cues are not as strongly conserved.

A variety of additional examples of cross-species responses have been demonstrated (Chivers and Smith, 1998; Smith, 1999; Mirza and Chivers, 2001a). While these may represent conservation of alarm cues within taxonomic groups, they may also simply be due to learned responses between prey guild members. For example, fathead minnows and brook sticklebacks (*Culaea inconstans*) respond to each other's alarm cues with significant increases in antipredator behavior, but only following previous interactions (Chivers and Smith, 1994; Brown et al., 1995). Likewise, minnows respond to the alarm cues of Iowa darters (*Etheostoma exile*) only if they have been reared sympatrically (Chivers et al., 1995). Strong selection pressures should exist for cross-species responses, either through evolutionary conservation or learned associations, as they would likely dramatically increase an individual's probability of surviving encounters with potential predators (Smith, 1999; Chivers and Mirza, 2001; Brown et al., 2001c).

The evolutionary history of the Ostariophysan alarm cue is still largely unknown (Magurran et al., 1996; Smith, 1997, 1999), however, our current data (and that of Brown et al., 2000, 2001a) suggest that the N—O functional group has been conserved as the chief molecular trigger. Though speculative (Smith, 1997) the ostariophysan alarm signaling system likely originated as an antipathogen/antiparisite or wound protection compound and was secondarily selected as an alarm cue (Smith, 1997, 1999). The conservation of N—O functional groups as the molecular trigger was likely selected because they are metabolically inexpensive, would allow for reliable cross-species responses among taxonomically related species independent of prior experience and are stable under natural environmental conditions (Brown et al., 2001a).

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES OF THE BROWNBANDED COCKROACH, *Supella longipalpa*, TO STEREOISOMERS OF ITS SEX PHEROMONE, SUPELLAPYRONE

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Abstract—Females of the brownbanded cockroach, *Supella longipalpa*, release a sex pheromone (supellapyrone) during a calling behavior and attract males from a distance. Supellapyrone has four possible configurations resulting from two asymmetric carbons at positions 2 and 4 (i.e., 2*R*,4*R*; 2*R*,4*S*; 2*S*,4*R*; and 2*S*,4*S*), but only the *RR* isomer is produced by females. Using pure synthetic stereoisomers in field tests, we showed that males are attracted to *RR* but also to high concentrations of the isomer *SR*. To study the activity of the stereoisomers in more detail we developed behavioral and electroantennogram (EAG) dose–response curves for each. Behaviorally, *RR* was the most active isomer with just 0.3 pg delivered on a filter paper being sufficient to elicit 50% male response in the olfactometer. Males were also attracted to *SR* and *SS* in the olfactometer, but at much higher dosages (100×) than the natural compound; *RS* did not elicit behavioral responses at any of the doses tested. In EAG assays, the antenna of male *S. longipalpa* showed high and similar sensitivity to *RR* and *SR*, but a much lower (10%) sensitivity to *SS* and practically no response to *RS*. The

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lack of agreement between behavioral and electrophysiological data suggested either that *RR* and *SR* stimulate different antennal sensory neuron types, or that some aspect of the interaction between the pheromone and the sensillum environment or the receptor neuron itself is different. To test the first hypothesis we examined the response of the antenna before and after adaptation with each of the four stereoisomers. Positive cross-adaptation between *RR* and *SR* suggests that these two compounds stimulate the same receptor cells. Therefore, the lack of agreement between behavioral and EAG dose–response curves could be explained by isomer-specific molecular interactions between the pheromone and the receptor neuron. Although *RR* and *SR* produced the same EAG amplitude, stimulation with *SR* resulted in a slower recovery rate (i.e., wider peaks) than stimulation with *RR*. To gain further understanding of the response specificity of the antennae to the different stereoisomers we compared EAG responses (amplitude and recovery time) in response to individual stereoisomers and binary mixtures of isomers. These tests showed additive responses of the EAG amplitude to mixtures of compounds, but nonadditive responses of EAG recovery time. Therefore, peak height and width are independent parameters of the EAG, probably representing different intrasensillar events, and likely resulting in the expression of different behavioral responses.

Key Words—*Supella longipalpa*, cockroach, Dictyoptera, sex pheromone, supellapyrone, chirality, electroantennogram, dose–response, EAG.

INTRODUCTION

Chemical communication with sex pheromones requires specificity of signals and receptors. Signal specificity is normally achieved by combinations of two or more pheromone components in specific blend ratios, whereas specificity of response normally requires the presence of several receptor neuron types, each responding to a different pheromone compound. A further refinement of the olfactory system consists of the ability to discriminate between absolute configurations of odor molecules. In communication systems that utilize stereoisomeric signal chemicals, the receptor system must also be stereochemically discriminating. Furthermore, in pheromone-mediated mate-finding in which reproductive isolation among species may be determined primarily by the chirality of a single pheromone component, it is not uncommon for responders to perceive antagonistic stereoisomers (Leal, 1998; Mori, 1998). Stereospecificity of odorants is probably more widespread in nature than earlier recognized, because only in recent years have new techniques routinely allowed resolution of trace amounts of enantiomers.

Females of the brownbanded cockroach, *Supella longipalpa* (F.) (Dictyoptera, Blattellidae), emit a volatile sex pheromone 5-(2′,4′-dimethylheptyl)-3-methyl-2*H*-pyran-2-one, or supellapyrone (Charlton et al., 1993), that attracts walking or flying males from a distance of several meters. This compound has four possible configurations at positions 2 and 4 of the alkyl side chain: (2*R*,4*R*), (2*R*,4*S*), (2*S*,4*R*), and (2*S*,4*S*). Electroantennographic detection coupled with chiral capillary gas chromatography (GC-EAD) has shown that the natural product is the

(2*R*,4*R*)-isomer (*RR*) and that male antennae also respond to the *SR*, but not to the *RS* or *SS* stereoisomers (Leal et al., 1995).

A mixture of supellapyrone stereoisomers effectively attracts *S. longipalpa* (Liang et al., 1998), but the attractant efficiency of the individual isomers has not been investigated. Synthesis of a mixture of stereoisomers is more economical than synthesis of the *RR* enantiomer, but whether the attractancy of *RR* in such a mixture is impaired by the presence of other stereoisomers is unknown. The four stereoisomers have been synthesized recently (Fujita and Mori, 2001), which prompted us to study the response of *S. longipalpa* males to each isomer and to their mixtures in the laboratory and in the field.

METHODS AND MATERIALS

Insects and Pheromones. The colony of *Supella longipalpa* was reared in plastic cages under a 12:12 (light:dark) photoregime at $27 \pm 1^{\circ}$C, and was provided with water and food (Purina Rat Chow # 5012, Purina Mills, St. Louis, MO) *ad libitum*. Newly-emerged adults were collected from the colony every 3–4 days, the sexes were placed in different containers under a reversed photoperiod, and males were used in tests ~30 days later. Stereoisomers of supellapyrone (*RR*, *SR*, *RS* and *SS* >99.9% chemically and diastoreomerically pure) were synthesized following the procedures of Fujita and Mori (2001). The enantiomeric excesses of *RR*, *SR*, *RS*, and *SS* were 97.6, 99.8, 98.4, and 95.6%, respectively, as determined on a Chiraldex GTA chiral capillary column (20-m × 0.25-mm × 0.12-μm; Chrompack, Raritan, NJ) operated at 142°C in a Hewlett-Packard 6890 gas chromatograph. The pheromone glands in tergites 4 and 5 (Schal et al., 1992) were dissected from 5 to 6-day-old females, extracted in CH_2Cl_2 for 24 hr, and the pooled extract was concentrated under N_2 to 1 and 0.1 female equivalents (FE) per 10 μl. All solutions were stored at -20°C in glass vials with Teflon-lined caps.

Field Test. Field trapping experiments were conducted in the Department of Genetics at North Carolina State University in December, 2001. Hexane-rinsed rubber septa (Thomas Scientific, Swedesboro, NJ) were loaded with 1 μg of *RR*, *SR*, *SS*, or *RS* in 50 μl CH_2Cl_2. Septa loaded with solvent only served as controls. In a second experiment, septa were loaded with 15 ng of *RR*, *SR*, or *SS*. Septa were placed individually in the bottom of 0.5-l glass jars, the inner wall of which was coated with petroleum jelly to prevent cockroaches from escaping. The jars were wrapped in a paper towel held in place by a rubber band to facilitate cockroach climbing. Placement of the traps in the room each day was based on a randomized complete block design so that each treatment was tested at least once in each trapping location during the test (experiment 1: $N = 5$; experiment 2: $N = 6$). Trapped cockroaches were counted and released in the room daily.

Olfactometer Tests. We used 16 olfactometers, each consisting of a clear Plexiglas tube (60 cm long × 3 cm ID). The olfactometers were connected symmetrically

to a vacuum pump, which was set to provide a wind velocity of 20 cm/s in each tube. The downwind end of the tube was covered with a metal screen (1-mmz mesh), and males were restrained 7 cm from the downwind end of the tube, which was covered with a metal screen (1-mm^2 mesh), by means of a metal screen gate (0.5-mm^2 mesh). Males were acclimated in the olfactometers 30–60 min before the stimulus was introduced. The pheromone (0.01–1000 pg) was applied to a 0.5-cm^2 filter paper disk ~30 min before the first individual was tested. Each disk was attached to a cork with an insect pin and kept inside a glass vial until used. New samples were prepared each day and used for a maximum of 6 hr. The stimulus was introduced through a 7-mm diameter orifice located at the top of, and 1.5-cm from the upwind end of the tube, and the filter paper was positioned 0.5 cm from the floor of the tube. Two fluorescent lights wrapped in red photographic filters were placed 50 cm above the olfactometers to facilitate observations, which took place during the second half of the scotophase, the period of maximum sexual activity in this species (Liang and Schal, 1990). A test sequence consisted of opening the gate, introducing the sample, and recording behavioral events with a microcomputer-based time–event recorder until the males left the cage or for up to 2 min if no response was observed. For each set of males placed in the olfactometers, one was tested with solvent, another with 0.1 FE, and the rest were tested with the four stereoisomers of supellapyrone. We tested the lowest doses first and gradually increased the doses throughout the day. The tubes were rinsed with tap water at the end of the day. Each naïve male was tested with a single treatment, then discarded. The percentage of males ($N = 29$–30) responding was analyzed using probit analysis and logistic regression of log-transformed data (SAS Institute, 2000).

Electrophysiology. Males were taken out of the rearing chamber during the scotophase shortly before being tested, anesthetized with a short pulse of CO_2 and one of their antennae was detached with fine forceps. The proximal end of the antenna was placed in the narrow end of a Pasteur pipette filled with cockroach saline BG-SSA (Kurtti and Brooks, 1976). Several terminal segments of the distal end of the antenna were excised, and this end was placed in a second capillary. Ag–AgCl wires, 0.5-mm diameter, connected the saline-filled capillaries to a Grass P-16 amplifier (Astro-Med, Inc., West Warwick, Rhode Island) with coaxial cables and BNC connectors. With this setup we experienced little environmental noise so no further shielding was necessary. The antenna was slightly curved between the electrodes, forming a horizontal arch, which was positioned within a 1-cm-diameter glass tube, and clean humidified air was passed continuously over the antenna at 1.5 l/min. The test samples were delivered through a perforated rubber septum that was fitted at the end of a lateral branch of the glass tube 8-cm upwind from the antenna. The signal was acquired through an A/D board installed in an HP5890 GC and recorded and analyzed with ChemStation software (Agilent Technologies, Palo Alto, CA).

Fresh samples were prepared daily. Each sample was loaded onto a folded rectangular filter paper (1.5 cm^2), which was placed into the wide section of a Pasteur pipette. Two militers of room air were taken into a calibrated glass syringe and delivered as a rapid puff to the antenna through the pipette containing the test compound. The calibration curve started with the solvent blank (CH_2Cl_2), followed by 1 FE and then the samples in ascending doses. Solvent and 1 FE were puffed every 2–3 test samples and at the end of the run. Each sample was puffed three times and the average constituted the experimental unit. A different syringe was used for each treatment (stereoisomer and dose) and the syringes, along with all glass components of the apparatus were rinsed daily with acetone. Each treatment (isomer $\times$ dose) was tested on six different antennae. To control for variation among antennae, the average amplitude of each set of three responses was divided by the average amplitude in response to the previous 1 FE sample. Comparison among treatments was performed on square-root-transformed data using a general linear model (SAS Institute, 2000).

Cross-Adaptation EAG. Adaptation of antennae using several stimuli was tested. After recording the response of an antenna to the test stimuli (four isomers, solvent, and the general odorant geraniol (four isomers, solvent, and the general food odorant geroniol [Aldrich, Milwaukee, WI]) a filter paper containing the adapting odor also was placed in the 1.5 l/min airstream flowing continuously over the antenna. The response of the antenna to puffs of test stimuli was then recorded in the presence of the adapting odor. We used concentrations of adapting odors (1 ng of *RR* and *SR*, and 10 ng of *SS* and *RS*) that caused some adaptation but did not impair antennal response, and concentrations of test stimuli (10 ng of *RR* and *SR*, and 100 ng of *SS* and *RS*) that produced discernible EAG responses under these conditions. Pipettes and syringes were reused for several days and were kept at -20°C between tests. The section of the pipette exposed to the adapting stimulus was rinsed with acetone after each set of three puffs. For delivery of the puffs in this and the following test, we used a straight 10-cm-long glass tube with a single orifice 3 mm in diameter and 6-cm upwind from the antenna preparation.

An adaptation index (AI) was calculated such that AI = postadaptation ratio/preadaptation ratio, where the postadaptation ratio = EAG amplitude of test stimulus/geraniol after introduction of the adapting stimulus, and the preadaptation ratio = EAG amplitude of test stimulus/geraniol before the introduction of the stimulus. The lower the AI the higher the effect of the adapting stimulus, with an AI = 1 meaning no adaptation. The data were square-root-transformed and analyzed with ANOVA followed by a planned means comparison test (Tukey's test) (N = 7–8).

Individual Compounds and Mixtures. To determine the effect of the interaction between *RR* and *SR* on the response of antennae (peak width at half height and peak amplitude) we performed EAGs using 10 μl CH_2Cl_2 (negative control), 1 FE (positive control), 5 ng of *RR*, 5 ng of *SR*, a mixture of 5 ng of *RR* plus 5 ng of *SR*, 10 ng of *RR*, and 10 ng of *SR*. Six independent sample loadings were tested

on 18 antennae ($N = 17$–18). Peak height (mV) and peak width at half height were measured and normalized by the response to 1 FE. The data were square-root-transformed and differences among treatments were analyzed with ANOVA followed by a planned means comparison test (Tukey's test).

RESULTS

Field Tests. In Experiment 1 (1 μg per septum), traps baited with *RR*, *SR*, *SS*, *RS*, and solvent control trapped 10, 11, 0, 1, and 1 males, respectively. One ootheca-carrying female was collected in a trap baited with *RS*. In Experiment 2 (15 ng per septum), traps baited with *RR*, *SR*, *SS*, and control trapped 8, 0, 0, and 0 males, respectively. Thus *RR* was most attractive and *SR* also attracted males at high doses, whereas *SS* and *RS* were no different from the solvent control.

Behavioral Dose–Response Studies. Insects were generally quiescent at the start of each behavioral assay, and males that were not quiescent were not tested. The percentage of males responding increased with the dose of the test stimulus. *RR* elicited the highest percentage of responses at all concentrations and reached a plateau at ~0.003 ng (Figure 1). Males were 100-fold less responsive to *SR* and *SS*, and did not respond to any amount of *RS* or to the solvent control. *RS* was, therefore, excluded from statistical analyses. The response curves for *RR* and *SS* were not different from each other ($\chi^2 = 0.84$, df $= 1$, $P = 0.36$), but differed from *SR* ($\chi^2 = 4.8$, df $= 1$, $P = 0.028$), indicating similarity in curve shape for *RR* and *SS*. The 95% fiducial limits for the 50% response level of *RR* (0.0002–0.0015 ng) did not overlap with the fiducial limits of *SR* (0.005–0.087 ng) or *SS* (0.032–0.088 ng), but there was overlap between these last two treatments (Figure 1).

EAG Dose–Response Curves. EAG responses increased with stimulus strength (Figure 2). *RR* elicited the highest EAG amplitudes, followed by *SR*. Unlike the behavioral results, EAG responses to *SR* and *SS* differed. *SR* elicited higher EAG amplitudes than *SS* throughout the range of doses tested. Overall, EAG amplitudes with *SS* and *RS* as a group were much lower than EAG amplitudes with *RR* and *SR* as a group ($F = 12.53$, df $= 1, 130$, $P < 0.001$), but *RR* and *SR* had similar peak amplitudes ($F = 1.51$, df $= 1, 66$, $P = 0.22$). Lack of significant interaction between concentration and isomer in this pair ($F = 0.07$, df $= 1, 66$, $P = 0.79$) indicated that the slopes of *RR* and *SR* were similar. The EAG amplitude of *RS* was indistinguishable from the solvent control at all doses except the highest.

Although the EAG responses to *RR* and *SR* had similar amplitudes at equivalent concentrations, the EAG deflection from *SR* had a much slower recovery time than the EAG response to *RR* (Figure 3, inset; $F = 121.43$, df $= 1, 60$, $P < 0.001$), and consequently had a significantly lower ratio of peak amplitude to peak width than *RR* (Figure 3; $F = 47.41$, df $= 1, 60$, $P < 0.001$). EAG amplitude increased

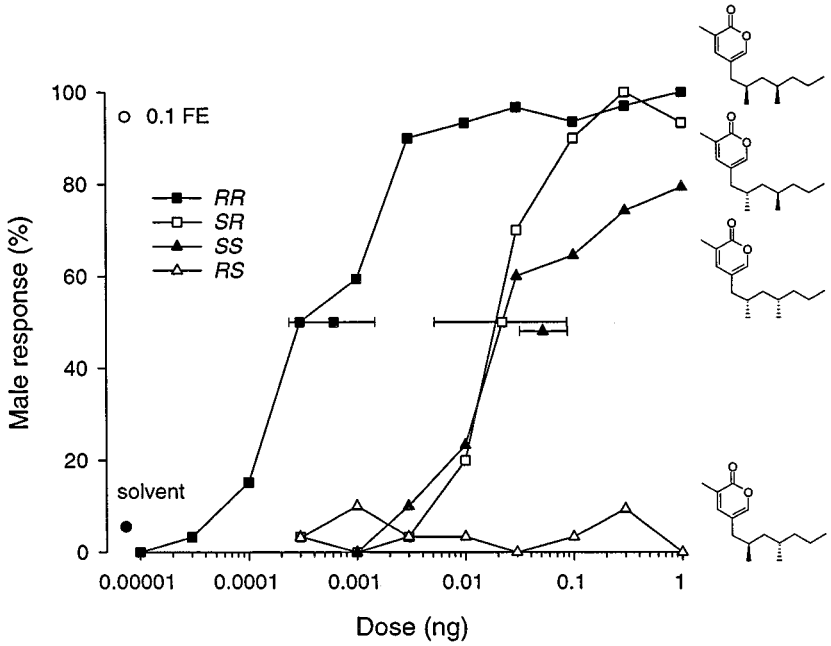

FIG. 1. Dose–behavioral response curves of *S. longipalpa* males to the four stereoisomers of supellapyrone. The percentage of males that started walking toward a source of supellapyrone, 0.1 female equivalents (FE), or a solvent control in an olfactometer was recorded ($N = 29$–30). Horizontal error bars indicate 95% fiducial limits for the 50% response levels to *RR*, *SR*, and *SS* determined from probit analysis (*SS* and *SR* fiducial limits have been separated for convenience). The symbol in the middle of the fiducial limits line marks the 50% response of the predicted response curves.

more with dose than did recovery time, and while this trend was steeper for *RR* than *SR* the slopes for these two isomers were marginally nonsignificant (no isomer $\times$ concentration interaction: $F = 2.24$, df $= 5, 60$, $P = 0.06$)

Cross-Adaptation EAG. The introduction of adapting odorants or the solvent control into the EAG air stream resulted in cross-adaptation among some of the compounds (Figure 4). Antennae exposed to a continuous stimulus of geraniol became less responsive to this compound but their responsiveness to the other compounds did not diminish (AI > 1, no difference among treatments; $F = 0.96$, df $= 5, 24$, $P = 0.46$). (*RS*)-supellapyrone and the solvent, on the other hand, did not cause any adaptation to any compound (AI ≈ 1, no difference among the treatments; $F = 1.22, \text{df} = 5, 37, P = 0.33$ and $F = 2.03, \text{df} = 5, 42, P = 0.094$, respectively). The *RR*, *SR*, and *SS* isomers adapted the antenna to themselves, to each other, and to 1 FE of natural supellapyrone (AI < 1), but not to solvent or

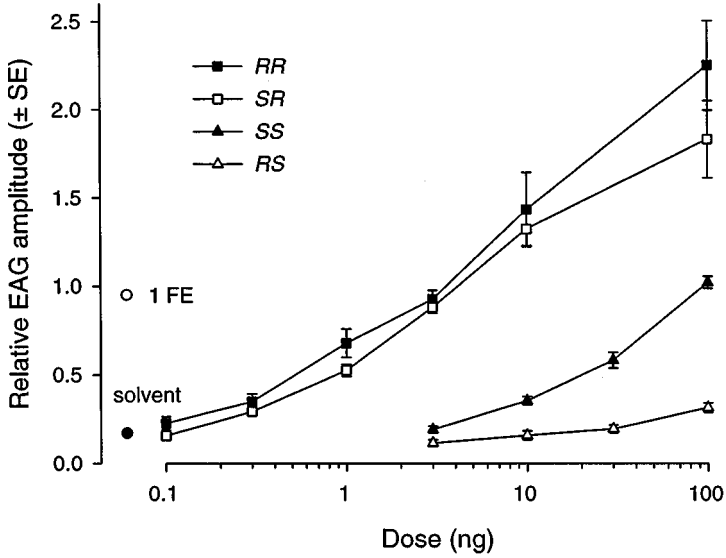

FIG. 2. Electroantennogram responses (mean ± SE) of *S. longipalpa* males to the four supellapyrone stereoisomers, 1 FE of extract, and a solvent control. To standardize the response of the antennae the amplitude generated by each test sample was divided by the amplitude generated by an extract of tergites 4 and 5 of a virgin female (1 FE).

to the *RS* isomer (AI $\approx$ 1) ($F = 6.81$, df $= 5, 41$, $P < 0.001$; $F = 11.33$, df $= 5, 42$, $P < 0.001$; and $F = 4.93$, df $= 5, 36$, $P = 0.001$, respectively).

Individual Compounds and Their Mixture. The *RR* stereoisomer generated significantly greater EAG deflections at 10 ng than at 5 ng, whereas *SR* produced deflections of similar amplitude at both concentrations (Figure 5; $F = 17.63$, df $= 4, 84$, $P < 0.001$). The EAG amplitude due to 5 ng *RR* was higher than with 5 ng *SR*. A blend of 5 ng of each of these two compounds produced an intermediate EAG amplitude between 5 and 10 ng *RR*, but not significantly different from either one. The EAGs with *SR* were significantly wider than with *RR* or the blend ($F = 19.57$, df $= 4, 84$, $P < 0.001$). The *RR*–*SR* blend produced EAGs of intermediate width between *RR* and *SR*, but not significantly different from *RR*.

DISCUSSION

This study supports our previous findings that although the *RR* stereoisomer of supellapyrone is the natural pheromone of *S. longipalpa*, male antennae respond to both the *RR* and *SR* isomers (Leal et al., 1995). Under field conditions, males are attracted to low doses of *RR*, and to higher doses of *SR*. Olfactometer tests

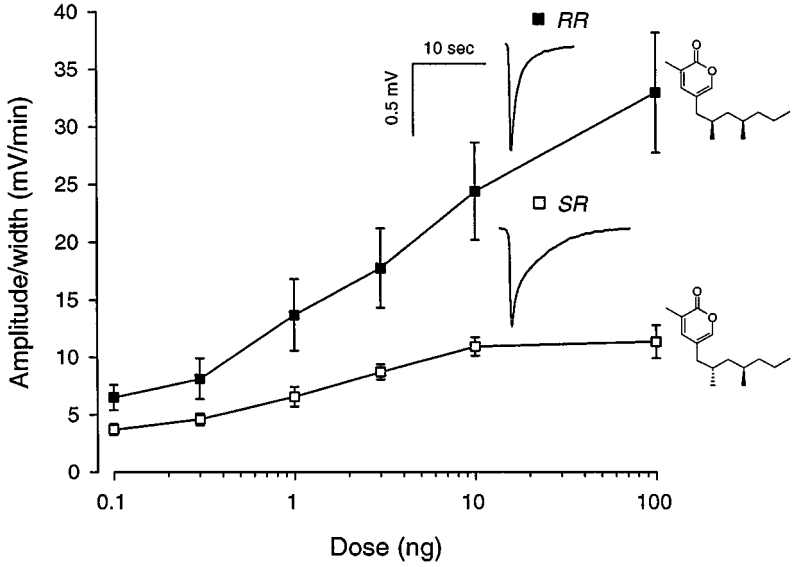

FIG. 3. Analysis of the shape of the EAG responses of male *S. longipalpa* antennae to *RR*- and *SR*-supellapyrone. EAG amplitude increases more than EAG recovery (width) with dose for both compounds, but this trend is steeper with *RR*. Also shown are two EAG responses to 10 ng of *SR* and *RR* delivered to the same antenna a few minutes apart.

revealed that males respond behaviorally to *SR* and *SS* also but at much higher doses than with the natural isomer. No response (behavioral or EAG) was elicited by *RS*. *RR* also induced a behavioral response in the highest percentage of males and the largest EAG amplitudes. Changing the methyl group farthest from the ring (position 4) from the (4′R)- to the (4′S)-configuration (i.e., *RR* to *RS*) resulted in complete suppression of the behavioral response. The antennae of males were, in fact, anosmic to *RS*, as determined by the lack of EAG responses to this isomer, except at exceptionally high concentrations. In contrast, changing the methyl group in position 2 from the (2′R)- to the (2′S)-configuration (i.e., *RR* to *SR*) reduced but did not eliminate the behavioral response. Interestingly, maintaining the syn-configuration by changing both methyl branches from *R* to *S* (i.e., *RR* to *SS*) did not result in the same dramatic reduction in behavior as changing from *RR* to *RS*. In fact, *SS* and *SR* induced behavioral responses in a similar percentage of the males, although *SS* produced significantly smaller EAGs. A 100-fold higher dose of *SR* was needed to stimulate behavioral responses of the same magnitude as *RR*. However, *RR* and *SR* produced similar EAG amplitudes. This last result showed a clear disparity between the antennal and the behavioral responses to the *SR*-isomer.

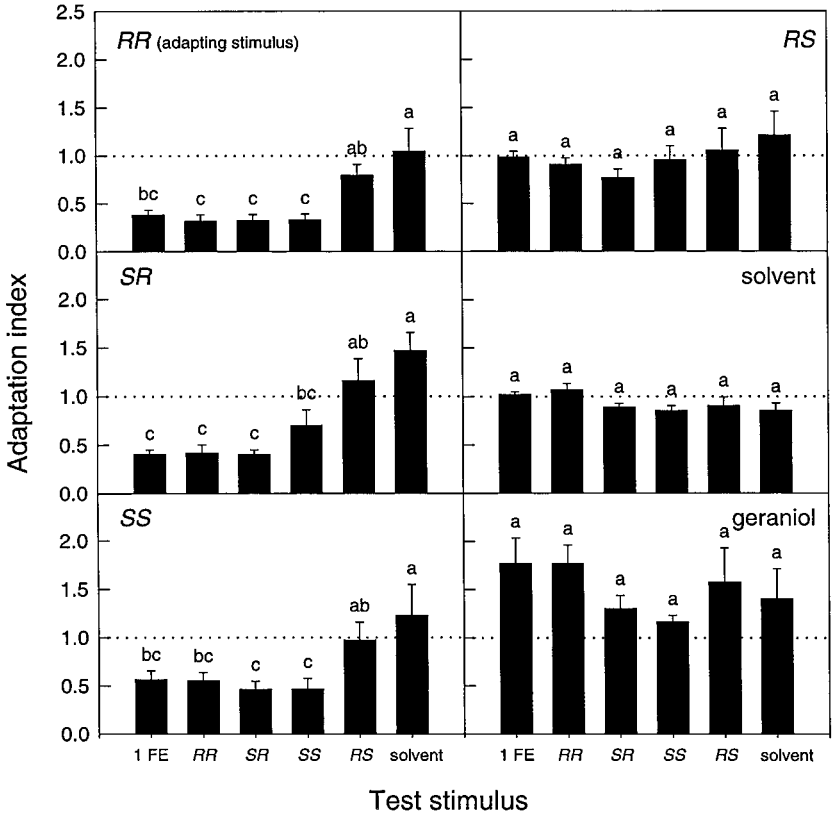

FIG. 4. Effect of adaptation of *S. longipalpa* male antennae on the responses to the stereoisomers of supellapyrone and to geraniol. Adaptation with *RR*, *SR*, and *SS* (left) caused a reduced EAG response (AI < 1) to 1 FE, *RR*, *SR*, and *SS*, but not to solvent or to *RS*. *RS*, solvent, and geraniol (right) failed to adapt the antenna to any of the test stimuli (AI ≥ 1).

Such a disparity between behavioral and EAG responses can be explained by an isomer acting as an antagonist of the natural pheromone. In the sympatric scarab beetles *Anomala osakana* Sawada (Osaka beetle) and *Popilia japonica* Newman (Japanese beetle), for instance, (*S*)-japonilure and (*R*)-japonilure, respectively, serve as the natural pheromones that release sexual responses in males (Tumlinson et al., 1977; Leal, 1996). However, because the (*R*)-enantiomer inhibits male response in the Osaka beetle, its antennae must recognize both isomers to accommodate such agonist–antagonist enantiomeric discrimination (Leal, 1998). In *S. longipalpa*, on the other hand, the nonnatural isomers do not appear to either suppress or promote male sexual responses. This is readily apparent in behavioral

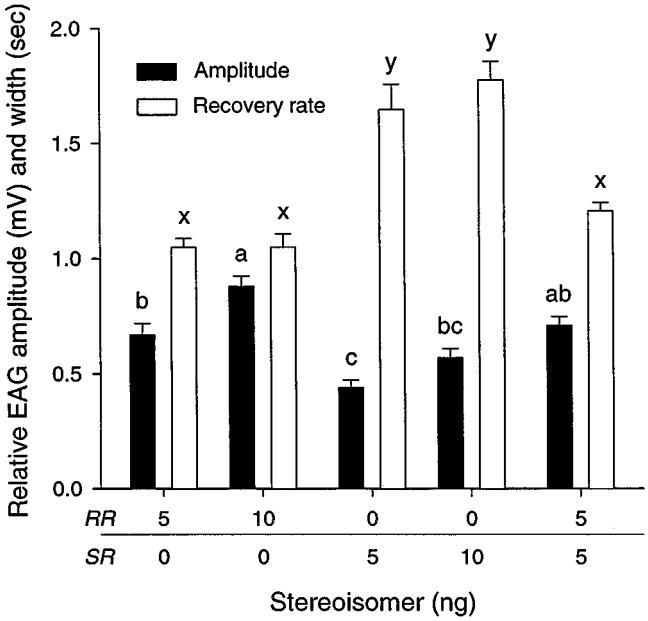

FIG. 5. EAG peak amplitude and width (mean + SE) in response to *RR*- and *SR*-stereoisomers and their blend. *SR*-supellapyrone produced the wider peaks and *RR* the highest amplitudes. The EAG amplitude of the blend was similar to that of *RR*. However, its width was reduced compared with the width of *SR* alone. Peak amplitude and width are standardized relative to the extract of a virgin female (1 FE). Different letters indicate significant differences ($P < 0.05$, Tukey's) for separate tests for amplitude and recovery time.

assays with mixtures of stereoisomers, which do not inhibit the response to *RR*-supellapyrone (Charlton et al., 1993) and in our preliminary experiments showing that the *SR*-isomer, the least active of the four stereoisomers, also does not diminish behavioral responses to *RR*-supellapyrone (personal observation). Further behavioral and trapping assays, especially with mixtures of the *RR*- and *SR*-isomers, are warranted before we may conclude unequivocally that attraction of males to the natural *RR*-supellapyrone is not modulated by its stereoisomers.

The *SR*- and *RR*-isomers give almost equivalent EAG responses, but 100-fold more *SR* is required for similar behavioral responses, so these two isomers could stimulate different olfactory receptor neurons, and this, in turn, could explain the disparity between EAG and behavior. EAG cross-adaptation has been used to test a similar hypothesis in other insect species (Dickens and Payne, 1977; Nagai et al., 1977; Lucas et al., 1994). In the American cockroach, *Periplaneta americana* (L.), cross-adaptation EAG results demonstrated that periplanone-A and periplanone-B

stimulate different receptor cell types (Nishino and Manabe, 1983; Tsuchiya and Takahashi, 1991), a finding corroborated by single cell recordings (Sass, 1983). Our data showed cross-adaptation between *RR* and *SR*, strongly suggesting that these two compounds stimulate the same odorant receptors. Hence, the hypothesis that *RR* and *SR* stimulate different receptor neuron types and that this results in different behavioral responses but similar EAGs, was not supported.

However, EAG cross-adaptation data must be interpreted with caution. Lack of cross-adaptation between two compounds is a clear indication that each interacts with different sensory neuron types tuned to specific compounds, but cross-adaptation may occur even if there are receptor neurons tuned to each of the compounds. For example, each receptor neuron type may respond predominantly to one of the odorants, but may exhibit some response to the other, sufficient to result in EAG cross-adaptation at relatively high doses. In the *S. longipalpa* system, however, the most likely explanation of cross-adaptation is that males possess only one type of pheromone receptor neuron because females produce only the *RR* isomer. Possibly, as discussed earlier, closely related sympatric species use one of the other isomers as a sex pheromone, and *S. longipalpa* males have receptors to detect it, and thus avoid mating with heterospecific females. This seems to be the case in *Periplaneta* cockroaches (Gemeno and Schal, 2003), but not in *S. longipalpa* because the behavioral tests do not indicate antagonism with the nonnatural isomers, but rather differential sensitivity to the four isomers of supellapyrone. Also, the addition of *SR* to *RR* did not attenuate the response to *RR*. However, the only definitive way to determine if each isomer stimulates the same or different receptor cells is to record from individual antennal receptor neurons.

If, as assumed, *SR*- and *RR*-supellapyrone do interact with the same receptor neurons, how do they produce similar EAGs, yet differ so dramatically in the behaviors elicited? The insect brain cannot discriminate between identical sensory inputs projecting from the same sensory neurons. Therefore, in order to elicit different behavioral responses, the two isomers must be discriminated within the sensillum. There are several ways in which this could be achieved (Kaissling, 2001). Once the hydrophobic pheromone molecule enters the sensillum, it must be transported to the neuronal receptors through the aqueous sensillum lymph. This transport is mediated by pheromone binding proteins (PBPs), which may exhibit high specificity for certain ligands. For example, in the gypsy moth, *Lymantria dispar* (L.), two PBPs differ in their enantiomeric binding preference: PBP1 has a higher affinity for (−)-disparlure while PBP2 has a higher affinity for the (+)-enantiomer (Plettner et al., 2000). The odor molecules must then interact with olfactory receptors at the neuronal plasma membrane, and also with pheromone-degrading enzymes, which clear the pheromone to favor further neuronal stimulation by new pheromone molecules. Any of these protein–ligand interactions can contribute to or diminish the specificity of response.

For example, could the differences in recovery rates (Figure 3) be related to different biochemical interactions of the *RR*- and *SR*-isomers with the sensory neurons? If so, could this explain the lack of correspondence between behavioral and EAG responses of the nonnatural *SR*-isomer? Differences in EAG recovery times have been reported in other insects (Guerin and Visser, 1980; Averill et al., 1988; Fescemeyer and Hanson, 1990; Light et al., 1992) and several hypotheses have emerged from early attempts to explain these differences. Recovery rates may reflect the amount of time required for the impulse frequency of individual neurons to return to the nonstimulated state (Whitehead, 1986), the amount of time that a stimulant molecule spends bound to receptors (Roelofs and Comeau, 1971), the response latency and response onset and offset of the neuron (Baker and Roelofs, 1976), the time required for deactivation of the molecules (Kaissling, 1974), the specificity of PBPs, receptor proteins and enzymes (Dickens et al., 1993), and compartmentalization of odorant molecules in the aqueous sensillum lymph (Dickens et al., 1993). To our knowledge, none of these hypotheses have been formally tested. We speculate, like previous authors, that differences in EAG recovery rates reflect differences in the intrasensillar processing of odorants. We further suggest that for similar compounds that activate receptor neurons of the same type, disparate EAG recovery rates could explain the lack of correspondence between EAG and behavior. Others have also noted that EAG recovery rates may correlate better with behavior than with EAG amplitude (Averill et al., 1988; Fescemeyer and Hanson, 1990). Whereas the EAG is rarely used to study intrasensillar events, its ability to discriminate, as we speculate, between isomers that may interact with the same receptors but elicit different behavioral responses adds substantial power to this time-tested procedure. It highlights the importance of quantifying more than one EAG parameter, especially in studies of closely related odorants.

One factor that should not be overlooked in dose–response studies is the purity of the synthetic compounds, especially when used at relatively high doses with highly sensitive antennae, as in the present study. The high purity (i.e., high enantiomeric excess) of our samples ensured that artifacts were kept to a minimum. Nevertheless, the nonnatural *SS*-isomer has 4.4% *RR* so that the behavioral response observed at high doses of *SS* may be elicited by the *RR* impurity, provided that there is no antagonism between them. Impurities, however, do not explain the EAG and behavioral response elicited by the *SR*-stereoisomer, which is completely devoid of any diasteromers, i.e, it does not contain any detectable amounts of *RR* or *SS*. Because males do not respond to *RS* at any dose, *RS* impurity should have no effect on response to *SR*. Also, lack of any response to *RS*, even at high doses, indicates that its minor contamination with *SR* did not contribute to either behavioral or EAG responses. Regardless, these considerations do not explain the disparity between EAG and behavior observed with *SR*.

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ELECTROANTENNOGRAPHIC AND BEHAVIORAL RESPONSES OF THE SPHINX MOTH *Manduca sexta* TO HOST PLANT HEADSPACE VOLATILES

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Abstract—Coupled gas chromatography with electroantennographic detection (GC-EAD) using antennae of adult female *Manduca sexta* was employed to screen for olfactory stimulants present in headspace collections from four species of larval host plants belonging to two families: Solanaceae—*Lycopersicon esculentum* (tomato), *Capiscum annuum* (bell pepper), and *Datura wrightii*; and Martyniaceae—*Proboscidea parviflora*. Headspace volatiles were collected from undamaged foliage of potted, living plants. GC–EAD revealed 23 EAD-active compounds, of which 15 were identified by GC-mass spectrometry. Identified compounds included aliphatic, aromatic, and terpenoid compounds bearing a range of functional groups. Nine EAD-active compounds were common to all four host plant species: (*Z*)-3-hexenyl acetate, nonanal, decanal, phenylacetaldehyde, methyl salicylate, benzyl alcohol, geranyl acetone, (*E*)-nerolidol, and one unidentified compound. Behavioral responses of female moths to an eight-component synthetic blend of selected tomato headspace volatiles were tested in a laboratory wind tunnel. Females were attracted to the blend. A comparison of responses from antennae of males and females to bell pepper headspace volatiles revealed that males responded to the same suite of volatiles as females, except for (*Z*)-3-hexenyl benzoate. EAD responses of males also were lower for (*Z*)- and (*E*)-nerolidol and one unidentified compound. Electroantennogram EAG dose–response curves for the 15 identified EAD-active volatiles were recorded.

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At the higher test doses (10–100 μg), female antennae yielded larger EAG responses to terpenoids and to aliphatic and aromatic esters. Male antennae did respond to the higher doses of (*Z*)-3-hexenyl benzoate, indicating that they can detect this compound. On the basis of ubiquity of the EAD-active volatiles identified to date in host plant headspace collections, we suggest that *M. sexta* uses a suite of volatiles to locate and identify appropriate host plants.

Key Words—Lepidoptera, Sphingidae, *Manduca sexta*, antenna, electroantennography, EAG, gas chromatography, GC-EAD, host plant selection, olfaction, volatiles.

INTRODUCTION

Many phytophagous insects use odors as cues for orientation to food resources, either for their own nutrition or to deposit their offspring. Foliar, floral, and fruit odors comprise hundreds of compounds, but studies of insect olfaction reveal that only a minority of the components in the complex odor blend are detected by the antenna (Cossé and Baker, 1999; Jonsson and Anderson, 1999; Zhang et al., 1999; Huber et al., 2000). These commonly include an array of short-chain alcohols, aldehydes and esters, aromatic compounds, and various mono-and sesquiterpenoids (Bernays and Chapman, 1994; Schoonhoven et al., 1998).

Among the detected compounds, even fewer seem to be involved in eliciting behavioral responses from the insect (e.g., Thiéry and Visser, 1986; Baur et al., 1993; Cossé and Baker, 1999; Rojas, 1999; Zhang et al., 1999). Identifying the range of host-associated volatiles that insects can detect, therefore, is an important step toward understanding the role of olfaction in modulating insect behavior. Studies of this type also make important contributions to our general understanding of insect olfaction.

The sphinx moth *Manduca sexta* (Lepidoptera: Sphingidae) has been extensively investigated as an experimental model for studies of olfactory mechanisms (Hildebrand, 1996). Males use the volatile sex pheromone produced by conspecific females to locate sexually receptive mates (Tumlinson et al., 1989), while females rely primarily on olfactory cues to locate and identify appropriate host plants on which to lay their eggs (Yamamoto et al., 1969; Waldrop et al., 1986; Ramaswamy, 1988). Throughout the New World, adults oviposit and larvae feed on plants in the family Solanaceae, especially native and cultivated varieties of tobacco (*Nicotiana*), cultivated varieties of tomato (*Lycopersicon*), and native jimsonweed (*Datura*) (Mechaber and Hildebrand, 2000). In the southwestern United States, *M. sexta* also uses two native species of *Proboscidea* (family Martyniaceae) (Mechaber and Hildebrand, 2000). Adults of both sexes also use olfactory stimuli when seeking floral sources of nectar on which to feed (Raguso and Willis, 2002).

Despite the biological importance of plant odors to *M. sexta*, the bulk of our understanding of the organization and functioning of the olfactory system has come from investigations of the detection and processing of sex-pheromonal information

by males (e.g., Christensen et al., 1989; Kaissling et al., 1989; Hildebrand, 1996). Only recently have we begun to investigate the detection and processing of plant odors by females (King et al., 2000; Shields and Hildebrand, 2001).

We report here the results of studies of the responses of *M. sexta* to host plant odors. Specifically, we employed coupled gas chromatography with electroantennographic detection (GC-EAD; Struble and Arn, 1984) to screen antennae of adult female *M. sexta* for olfactory stimulants present in headspace collections from four species of host plants, belonging to two plant families. To determine whether antennal responses to individual compounds were sex-specific, we also compared GC-EAD responses of antennae from females and males to volatiles from one of the host plants, bell pepper (*Capsicum annuum*), and to dilution series of synthetic standards of plant compounds identified as stimulatory from the GC-EAD recordings. A synthetic blend of selected compounds identified as EAD-active was also prepared and tested for behavioral activity on *M. sexta* females in a wind tunnel.

METHODS AND MATERIALS

Insects. Moths were obtained from a laboratory culture that has been maintained for over 50 generations on artificial diet under a long-day photoperiod regimen (see Sanes and Hildebrand, 1976). Antennae for electrophysiological recordings were obtained from moths that had eclosed 1-3 d prior to testing. Individuals from either photophase or 1–3 hr into scotophase were used because light phase did not appear to influence antennal responses to volatiles. The behavioral bioassay was performed with female moths that had eclosed 2 d prior to testing and that had mated 1 d prior to testing. These individuals were tested 1–3 hr after the onset of scotophase. At no time prior to electrophysiological or behavioral testing were moths exposed to plant odor.

Plants and Collection of Headspace Volatiles. The plant species used in this study included two cultivated hosts, tomato (*Lycopersicon esculentum* var. Money Maker) and bell pepper (*Capsicum annuum* var. Yolo Gold and a second, unknown, variety), and two hosts native to the southwestern USA, sacred datura or jimsonweed (*Datura wrightii*) and devil's claw (*Proboscidea parviflora* var. hohokamiana). Tomato, bell pepper, and datura belong to the Solanaceae, whereas devil's claw is in the family Martyniaceae and is a native host used by *M. sexta* in southern Arizona (Mechaber and Hildebrand, 2000).

Leaf volatiles were collected from growing, potted plants. Flowers, floral buds, and fruits were removed from plants at least 24 hr before sampling, but otherwise plants were undamaged. Growing conditions and sampling techniques were as follows:

(a) Host plant species were grown in pots in an outdoor, screened enclosure under ambient conditions in Tucson, AZ (20–40°C; ca. 14.5:9.5 hr photoperiod

regimen). For each host plant species, 1–2 potted plants were placed in a 100 × 40 × 40 cm glass tank with a removable glass top fitted with openings for air exchange. Charcoal-filtered air was pumped into the tank at 3 l/min, and odor-laden air was withdrawn by vacuum at 1 l/min. Excess air escaped through a vent in the glass top. The vacuum line was split and connected to two glass collection cartridges, each containing 80 mg of Super Q polymer adsorbent (80/100 mesh, Alltech, Deerfield, IL, USA). Volatiles were collected for 24 hr in the dark and extracted from the trap using 400 μl of dichloromethane per cartridge. Extracted samples were stored in glass vials at −80°C until used. For each plant species, 2–4 collections were made and tested, each with different plants. Samples were used to compare EAD responses of females to different species of host plants.

(b) Five potted *C. annuum* plants (var. unknown), grown in a greenhouse, were placed in the 46 × 46 × 96 cm sealable glass chamber of an Analytical Research Systems (Gainesville, FL, USA) volatile collection system based on that described by Heath and Manukian (1994). Charcoal-filtered air was pumped into the chamber (8 l/min), and odor-laden air was withdrawn by vacuum (3 l/min). Excess air was vented from the chamber. Volatiles were collected for 24 hr (14:10 hr light:dark photoperiod regime) and extracted and stored as described in (a) above. A single collection was made and used repeatedly to compare EAD responses of males and females. Bell pepper was chosen as the test host plant because it evoked more responses from *M. sexta* antennae than did native host plants (*D. wrightii* and *P. parviflora*) and because it was readily available when these comparisons were conducted.

EAD responses of male and female antennae to compounds in the bell pepper headspace sample were compared using repeated measures analysis of variance. Compound was the repeated measure, and sex was a fixed factor. The Sex × Compound interaction term indicated whether males and females responded to the suite of compounds within the headspace sample in a similar manner. Data were square root transformed to equalize variances prior to analysis. Follow-up comparisons of male and female EAD responses to individual compounds within the headspace trace were conducted using Student's *t* tests, with an alpha significance level of 0.01 required for significance to adjust for nonindependence of these multiple comparisons.

GC-EAD. A 3-μl sample of collected headspace volatiles was injected (splitless, 1 min) into a Shimadzu model 14A GC (Columbia, MD, USA) equipped with a flame ionization detector (FID) and one of three columns: a DB-624 column (30 m × 0.25 mm ID, 1.4 μm film, J & W Scientific, Folson, CA, USA); a DB-WAX column (25 m × 0.32 mm ID, 0.5 μm film); or an Alltech EC-5 column (30 m × 0.32 mm ID, 1.0 μm film). Temperature programming was as follows: 38°C for 4 min; 10°C/min to 120°C, hold for 4 min; 8°C/min to 240°C, hold for 15 min (DB-624 and DB-WAX columns); 40°C for 4 min, 5°C/min to 250°C, hold for 8 min (EC-5 column). Helium was used as the carrier and makeup gas. Column

head pressure was set at 135 kPa for the DB-624 column and at 100 kPa for the DB-Wax and EC-5 columns.

Effluent was split 1:1 between the FID of the GC and the moth antenna (EAD) using a Gerstel Graphpack 3D-2® stainless steel splitter (Baltimore, MD, USA) on the DB624 column and a universal glass "Y" connector (J&W Scientific) on the DB-WAX and EC-5 columns. Deactivated, fused-silica capillary tubing of the same internal diameter as the separation column carried the effluent to each detector. Effluent to the antenna passed through a heated transfer line (Syntech, Hilversum, The Netherlands) set at 10°C above the maximum program temperature and entered a 16-mm diameter glass tube via a small hole in the wall of the glass tube. Effluent to the antenna was mixed with a stream of charcoal-filtered, humidified air that flowed through the glass delivery tube at a rate of 800 ml/min. Each EAD recording was made with an antenna excised at the scape and mounted between two glass capillary tubes filled with physiological saline solution (Hebets and Chapman, 2000). The distal annuli (subsegments of the flagellum) of the antenna were removed with a razor blade to provide contact between the hemolymph at the antennal tip and the saline. Electrical contact was made via silver wires running through the electrodes and connected to a portable EAG system (Syntech INR-2). A 5-sec baseline return time constant was applied to prevent the antennal signal from drifting off-scale. Each antenna was exposed to a single sample through the GC and then discarded. Data were recorded onto the hard drive of a computer using EZChrom Elite software (Scientific Software, Inc., Pleasanton, CA, USA). At least four individuals were tested with each plant sample, and traces were overlaid on the computer monitor to determine which GC peaks consistently yielded EAD responses.

GC-MS Analysis of Odor Samples and Verification of EAD Activity. A Shimadzu model 17A GC equipped with Shimadzu model QP5000 mass spectrometer system and a DB-624 column (30 m x 0.25 mm i.d. x 1.4 μm film), and also a Varian Saturn 2000 GC-MS system (Walnut Creek, CA, USA) equipped with an HP-5 column (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm film, Agilent Technologies, Palo Alto, CA, USA), were used to identify chemical compounds present in headspace samples. Temperature programs followed those of the GC-EAD system described above. Identities of EAD-active compounds were verified by matching retention times with purified synthetic standards injected onto these two columns. EAD activity of identified compounds was verified using 1 ng/μl samples of purified synthetic standards injected into the GC-EAD apparatus described above.

Bioassay for Behavioral Activity of EAD-Active Volatiles. An eight-component blend of odorants identified as EAD-active was prepared using purified synthetic standards and tested for behavioral activity on female moths in a wind tunnel. The blend was prepared by adding each component at a concentration that approximated that found in tomato headspace collections, with a total blend concentration of 100 ppm (Table 1). Synthetic blend aliquots of 20, 50, or

TABLE 1. COMPOSITION OF AN EIGHT-COMPONENT BLEND OF PURIFIED SYNTHETIC STANDARDS DESIGNED TO MIMIC A PORTION OF TOMATO FOLIAGE HEADSPACE VOLATILES AND USED IN A WIND TUNNEL BEHAVIORAL BIOASSAY ON FEMALE *M. sexta* MOTHS

Component	Purity (%)	Concentration (ppm)
$\alpha-$Terpinene	90	27
Methyl salicylate	99	22
(E)-β-ocimene	98	16
Benzyl alcohol	99	10.5
Decanal	97	10
Nonanal	97	8
Phenylacetaldehyde	95	6
Geranyl acetone	96	0.5
Total		100

100 μl were pipetted into conical glass inserts, sealed in capped glass vials, and chilled until use. Aliquots (20 μl) of tomato headspace collections were prepared in a similar manner. Dichloromethane was used as the solvent in headspace extracts and synthetic blends. Mineral oil (1 μl) was added to all aliquots to slow evaporation rates in the wind tunnel.

The efficacy with which the synthetic blend elicited oriented flight of *M. sexta* females to the upwind odor source was tested in a Plexiglas® wind tunnel (L $\times$ W $\times$ H $=$ 4 $\times$ 1.5 $\times$ 1.5 m). Flight responses were compared to those of females that were exposed to an extract of tomato headspace (positive control) and to no odor source (negative control). Air was forced into the upwind end of the tunnel through a carbon filter and exhausted at the downwind end through a duct vented into a laboratory fume hood. The wind speed was 54 cm/sec.

Females were tested individually by placing a moth, confined in a wire cage covered at both ends by plastic petri plates, on a platform located at the downwind end of the tunnel. The moth was permitted a 1-min acclimation period, after which a vial containing an odor stimulus (synthetic blend or tomato headspace collection) was placed on a stand located at the upwind end of the tunnel and at the same height as the caged moth. The vial was uncapped, and a filter paper wick was inserted into the conical vial. A stainless steel plate (L $\times$ W $=$ 30 $\times$ 15 cm) was situated 4 cm downwind of the odor source. This plate acted as a baffle to disperse the point source odor plume emanating from the vial over a larger area. End covers were immediately removed from the wire cage housing the moth, and the moth was gently prodded to initiate wing-fanning (required for flight muscle warm up). The same procedure was followed for the negative control except that an odor source was not introduced.

Flight behavior was observed from take-off until landing. A flight was deemed a positive response if the female approached and hovered within 10 cm of the

odor source. A response was recorded as negative if the female did not approach the baffle or the odor source. The number of females tested ranged from 4 to 16, depending on the treatment. Flight responses (approaches vs. nonapproaches) were compared among treatments in a 2×5 contingency table using a Chi-square test. A Fisher Exact Test was used to compare responses to the negative control (no odor) with those to the 50-μl aliquot of the synthetic blend. Sample sizes for other treatments were too small for rigorous pairwise statistical comparisons.

Dose–Response Relationships for EAD-Active Volatiles. Electroantennogram (EAG) dose–response studies for identified EAD-active volatiles were conducted using antennae from males and females. Dilutions of purified synthetic standards (purity = 95–99%, except 1,8-cineole = 88% and β-myrcene = 87%) were made in dichloromethane at decadic steps, from 10 μg/μl to 10^{-5} μg/μl. 1, 8-Cineole, (*E*)-nerolidol, and (*Z*)-3-hexenyl propionate were generously provided by Drs D. M. Light and R. A. Flath (USDA-ARS, Western Regional Center, Albany, CA, USA). All other compounds were obtained from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA). A standard aliquot (10 μl) of a dilution was pipetted onto a strip of filter paper (Whatman No. 1, $\sim 3 \times 40$ mm) exposed to the air for 20 sec to allow the solvent to evaporate, then inserted into a glass Pasteur pipette and capped at both ends until used. Solvent controls (10 μl dichloromethane on filter paper) were prepared in an identical fashion.

To present a stimulus, the wide end of a Pasteur pipette was attached by a small length of plastic tubing to a 20-cc plastic syringe mounted in a motor-driven syringe-olfactometer (Shields and Hildebrand, 2001). The pipette tip was inserted through a side hole in a glass tube (10-mm diameter) through which charcoal-filtered, humidified air flowed at 800 ml/min. Activating the motor-driven system caused 5-cc of air to be expelled from the syringe over a 200-msec time period. This pushed the odor-laden air from the Pasteur pipette into the air stream, where it was carried over a moth's excised antenna at the distal end of the glass airflow tube. The continuous flow of clean air through the airflow tube and over the antenna ensured that odors were removed immediately from the vicinity. EAG recordings were made using the Syntech INR-2 device described above for GC-EAD recordings and using EAG software supplied by Syntech.

For each compound, dilutions were presented in order of increasing dose. A solvent control (10 μl dichloromethane) was presented at the beginning and at the end of each dilution series. EAG responses to these two controls were averaged, and the mean subtracted from the responses to the corresponding dilution series to correct for solvent and mechanosensory responses. Successive stimuli were separated by at least 30 sec of clean air flow. EAG responses generally diminished over time. For a dilution series, however, the magnitude of the response to the solvent blank decreased by only 10%, on average, for both sexes. Each compound was tested on eight females and eight males, and no more than five compounds were tested on a single moth. For each compound, mean EAG response at each test dose

was compared between sexes using Student's *t* test, with an alpha significance level of 0.01 required for significance to adjust for nonindependence of these multiple comparisons.

RESULTS

EAD Responses to Different Host Plant Species. The GC-FID profiles of host plant volatiles varied among plant species (Figure 1). Within a plant species the differences in GC-FID profiles were mainly quantitative. GC-EAD profiles also varied with the plant species being tested (Figure 1), but for a given plant species, the regions of the GC-FID trace that elicited EAD responses were remarkably consistent among individuals and across plant samples. The magnitude of the EAD response varied slightly among individuals and with stimulus dose (approximated by the height of the FID peak).

For all plant species tested, the antenna responded to a minority of peaks visible in the FID trace (Figure 1). For example, the GC-FID profile of tomato-headspace volatiles consistently had more than 60 peaks. Only 20 of the peaks consistently elicited EAD activity (Figure 1, Table 2). Some areas within the FID profile consistently elicited EAD activity even though an FID peak was not clearly discernible (e.g., peaks l, m, r, u, v in Figure 1). Other, relatively large FID peaks (indicated by a number and an asterisk in Figure 1B) failed to elicit antennal responses.

The three solanaceous plants had approximately 26 FID peaks in common, but the most abundant volatile compounds were different for each species (Figure 1). In total, 23 areas in the GC-FID traces consistently elicited EAD activity (Figure 1, a–w). Nine of these were common to all four host species (g, i, k, m, o, p, r, s, and u; Figure 1, Table 2).

Identification of EAD-Active Peaks. Among the 23 regions in the GC-FID traces that elicited EAD-activity, 15 EAD-active compounds were identified (Table 2). These compounds belonged to a range of chemical classes (aliphatic, aromatic, and terpenoid compounds) and bore a variety of functional groups (alcohol, aldehyde, amine, ester, ether, and ketone groups) (Table 2). The nine EAD-active compounds common to all four host plant species were (*Z*)-3-hexenyl acetate (g), nonanal (i), decanal (k), phenylacetaldehyde (m), methyl salicylate (o), geranyl acetone (r), benzyl alcohol (s), (*E*)-nerolidol (u), and one unidentified compound (p). Eight of the 23 EAD-active compounds remain unidentified.

Injection of 1-ng standards of EAD-active volatiles indicated that female *M. sexta* antennae were most sensitive to (*E*)-β-ocimene, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl propionate, phenylacetaldehyde, methyl salicylate, geranyl acetone, and benzyl alcohol.

Although the number of EAD-active peaks obtained from *P. parviflora* was comparable to the numbers derived from the 3 solanaceous species, the amounts

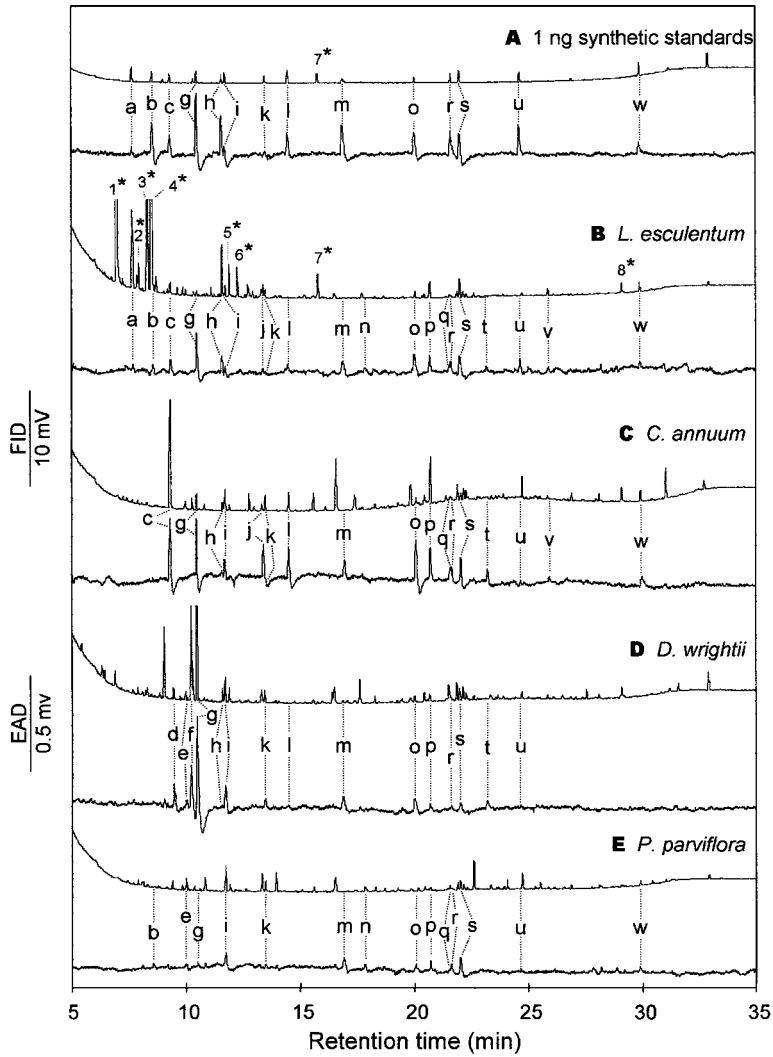

FIG. 1. Representative, GC-EAD recordings with a DB-WAX column (upper trace) and from a female *M. sexta* antenna (lower trace). Labels **A** through **E** above the FID trace refer to the identity of the sample injected into the GC. **A** is a mixture of synthetic standards, 1 ng per compound (see Table 2 for compound identities); **B** through **E** are headspace volatile collections of tomato, bell pepper, sacred datura, and devil's claw, respectively. Lowercase-lettered peaks indicate areas where an EAD response was detected. Chemical identities of these compounds are listed in Table 2. Numbered peaks followed by an asterisk indicate major peaks in tomato that did not elicit EAD responses: $1^* = 2$-carene, $2^* = \alpha$-terpinene, $3^* =$ limonene, $4^* =$ unidentified monoterpene, $7^* = (E)$-β-caryophyllene, 5*, 6* and $8^* =$ unidentified compounds. (E)-β-caryophyllene was also included in the synthetic standards mixture shown in 1**A**.

TABLE 2. VOLATILES ELICITING ELECTROPHYSIOLOGICAL ACTIVITY DURING GC-EAD RECORDING FROM FEMALE *M. sexta* ANTENNAE EXPOSED TO HEADSPACE COLLECTIONS OF FOUR HOST PLANT SPECIES

Peak no.[a]	Compound[b]	Class—functional groups	EAD response[c]			
			Tomato	Bell pepper	Sacred datura	Devil's claw
a	β-Myrcene	Monoterpene—hydrocarbon	+			
b	1,8-Cineole	Monoterpene—ether	+			+
c	(*E*)-β-ocimene	Monoterpene—hydrocarbon	+	+		
d	?				+	
e	?				+	+
f	?				+	
g	(*Z*)-3-hexenyl acetate	Aliphatic—ester	+	+	+	+
h	(*Z*)-3-hexenyl propionate	Aliphatic—ester	+	+	+	
i	Nonanal	Aliphatic—aldehyde	+	+	+	+
j	?		+	+		
k	Decanal	Aliphatic—aldehyde	+	+	+	+
l	Linalool	Monoterpene—alcohol	+	+	+	
m	Phenylacetaldehyde	Aromatic—aldehyde	+	+	+	+
n	?		+			+
o	Methyl salicylate	Aromatic—alcohol, ester	+	+	+	+
p	?		+	+	+	+
q	?		+	+	+	
r	Geranyl acetone	Irregular terpene—ketone	+	+	+	+
s	Benzyl alcohol	Aromatic—alcohol	+	+	+	+
t	?		+	+	+	
u	(*E*)-nerolidol	Sesquiterpene—alcohol	+	+	+	+
v	(*Z*)-3-hexenyl benzoate	Aromatic—ester	+	+		
w	Indole	Aromatic—amine	+	+		+
	Total		20	17	16	13

[a] Letters correspond to labeled peaks in Figure 1.
[b] ? = unidentified EAD-active compound.
[c] + = compound consistently elicited an EAD response.

collected from *P. parviflora* appeared to be far lower than those from the solanaceous host plants. It should be noted, however, that the doses of EAD-active compounds in headspace collections were not normalized to plant biomass for any of the plants sampled. Compared with the 1-ng/μl sample of synthetic standards, many were present at doses of 1 ng or less per 3-μl injection (compare Figure 1A with Figure 1B–E).

Compounds that were present in relatively high amounts in tomato headspace, but which did not elicit EAD activity (indicated by a number and an asterisk in Figure 1B), included 2-carene (1*), α-terpinene (2*), limonene (3*), an unidentified monoterpene (4*), (*E*)-β-caryophyllene (7*), and three other unidentified

compounds (peaks 5*, 6*, and 8*). A trial with a concentrated tomato sample, collected in the same manner as the bell pepper trace shown in Figure 2, did elicit small EAD responses to limonene, (*E*)-β-caryophyllene, and several unidentified compounds, but not to 2-carene or α-terpinene.

Bioassay for Behavioral Activity of EAD-Active Volatiles. Female flight response toward the baffle differed significantly among test treatments ($\chi^2 = 9.77$, $P = 0.04$, df = 4; Table 3). A significantly greater number of females approached the baffle when the 50-μl aliquot of synthetic blend was presented compared with the negative control of no odor ($\chi^2 = 7.50$, Fisher exact $P < 0.01$, df = 1; Table 3). Sample sizes for other treatments were too small to permit rigorous statistical testing. Thus, while we noted a trend of increasing attraction toward the odor source as we increased te volume of synthetic blend presented (Table 3), further testing is needed to determine if this effect is real. Most flights lasted less than 2 min, and females usually landed on the cloth screen at the upwind end of the tunnel.

At the time of behavioral testing, all eight compounds included in the blend were thought to be EAD-active, as determined by GC-EAD on a DB-624 column. Subsequently, all but one compound, α-terpinene, were verified as EAD-active at doses present in the headspace samples, and six of the eight compounds (excluding α-terpinene and (*E*) $-$ β-ocimene) were found to be present in all host plant species examined. We later discovered that α-terpinene obscured a co-eluting compound, (*Z*)-3-hexenyl acetate, in the GC traces in experiments using the DB-624 column and that (*Z*)-3-hexenyl acetate was responsible for the observed EAD response. Thus, the results presented here test the behavioral activity of selected compounds present in tomato headspace, only seven of which elicit EAD activity at doses collected from the headspace.

Comparison of Female and Male Antennal Responses to Host Plant Volatiles. A single more concentrated bell pepper sample was used to compare responses of female and male antennae to host plant volatiles. The GC-FID profile of this sample (Figure 2A) is not comparable to that shown in Figure 1C because the polarity of the columns differed (DB-624 vs. DB-WAX, respectively). Thus, EAD-active peaks in Figure 2 were labeled using numbers, rather than the letters used in Figure 1.

As in the headspace collections shown in Figure 1, antennae responded to a minority of peaks in the FID trace of bell pepper in Figure 2, and many large peaks failed to elicit an EAD response. Twenty-three FID peaks in the bell pepper headspace sample consistently elicited EAD responses from female antennae, whereas male antennae responded to 22 of these peaks (Figure 2A). Fourteen of the 23 EAD-active peaks were identified by GC-MS (Figure 2B). All of these except (*Z*)-nerolidol (peak 19) had been identified previously as EAD-active compounds (compare Table 2 and Figure 2B). Nine compounds remain unidentified.

Whereas antennae of males and females generally responded to the same suite of bell pepper volatiles (Figure 2A, and B), there was a significant Sex $\times$ Compound

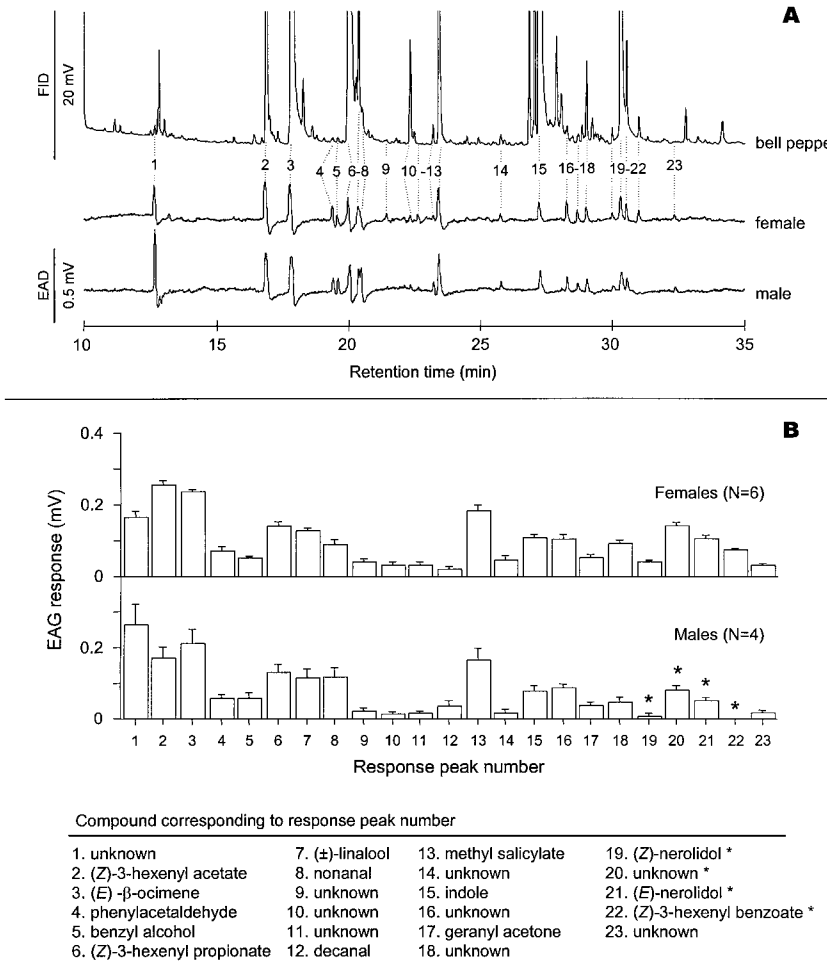

FIG. 2. Comparison of female and male *M. sexta* EAD responses to headspace volatiles from bell pepper (*C. annuum* unknown variety). **A**: Representative FID record (upper trace) and GC-EAD recordings from a female antenna (middle trace) and a male antenna (lower trace) using a DB-624 column. Numbered peaks indicate areas where EAD responses were detected. **B**: Mean (±SEM) EAD response to peaks numbered in Figure 2**A** ($N = 6$ female antennae, 4 male antennae). A significant difference between female and male antennal responses is indicated by an asterisk above the bar (t test, $P < 0.01$). Chemical identities of the numbered peaks are given at the bottom.

TABLE 3. FLIGHT RESPONSE OF FEMALE *M. sexta* MOTHS TO AN EIGHT-COMPONENT BLEND OF PURIFIED SYNTHETIC STANDARDS DESIGNED TO MIMIC A PORTION OF TOMATO FOLIAGE HEADSPACE[a]

Test treatment and aliquot	Percentage females responding positively (number responding/number tested)
No odor (negative control)	0% (0/13)
Synthetic blend, 100 ppm	
20 μl	25% (1/4)
50 μl	44% (7/16)
100 μl	50% (2/4)
Tomato headspace, 20 μl (positive control)	60% (3/5)

[a] See Table 1 for blend composition.

interaction (repeated measures ANOVA: $F_{22,176} = 5.21$, $P < 0.001$), indicating that the EAD response profiles differed between male and female antennae. Comparisons of male and female antennal responses to individual compounds indicated that female antennae produced larger responses to (*Z*)-nerolidol (peak 19, $P = 0.002$), one unidentified compound (peak 20, $P = 0.005$), (*E*)-nerolidol (peak 21, $P = 0.003$), and (*Z*)-3-hexenyl benzoate (peak 22, $P < 0.001$)(Figure 2B). There were no compounds to which male antennae produced larger EAD responses than did female antennae (Figure 2B).

Dose–Response Curves for EAD-Active Volatiles. Antennae of both sexes appeared to be most sensitive to (*E*)-β-ocimene, phenylacetaldehyde, nonanal, and (*Z*)-3-hexenyl acetate. Female antennae often yielded significantly larger EAG responses than those of males (indicated by an asterisk in Figure 3) whenever the stimulus response exceeded that of the solvent (normalized to zero in Figure 3). This difference became more pronounced with increasing dose for some but not all compounds.

Female antennae exhibited larger EAG responses to all of the terpenoids ((*E*)-β-ocimene, ($\pm$)-linalool, β-myrcene, and 1,8-cineole, geranyl acetone, and (*E*)-nerolidol) at the highest dose (100 μg) and at 10 μg for all terpenoids except β-myrcene (Figure 3). EAG responses to aromatic compounds were more varied. EAGs of females were significantly larger than those of males to the aromatic esters ((*Z*)-3-hexenyl benzoate and methyl salicylate) at a dose of 100 ng to 10 μg, and larger than male EAGs to phenylacetaldehyde at all except the lowest and highest test doses (Figure 3). EAG responses of both sexes to indole were small across all test doses and significantly greater in females only at the highest test dose (Figure 3). The two sexes showed no difference in their EAG response to benzyl alcohol (Figure 3).

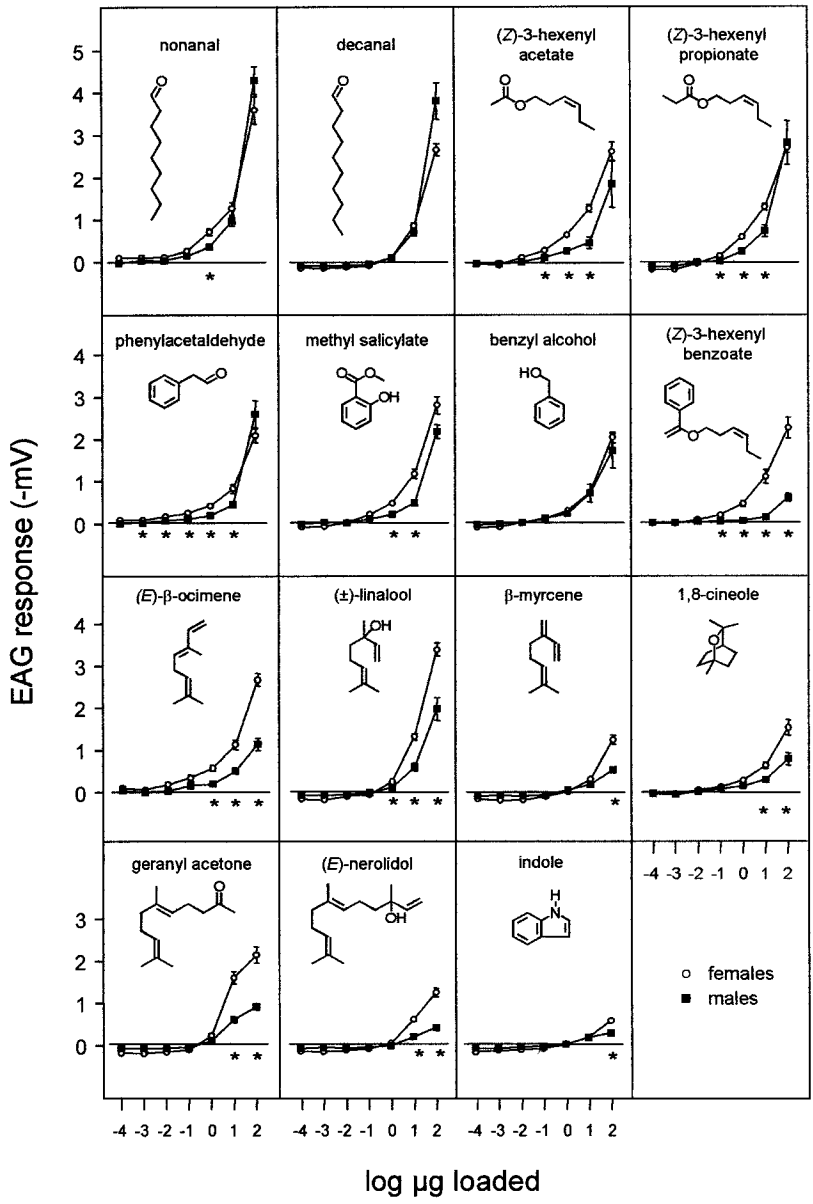

FIG. 3. Dose–response curves (mean ± SEM) of *M. sexta* antennae to dilution series of synthetic standards of 15 EAD-active compounds identified from headspace of *M. sexta* host plants ($N = 8$ antennae per sex tested at each dose). Antennal responses are indicated by open circles for females and by closed squares for males. An asterisk below the response curve indicates that the mean response differed significantly between the two sexes at that dose (t test, $P < 0.01$).

Among the aliphatic compounds, female antennae gave larger EAG responses than male antennae at intermediate doses of nonanal, (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenyl propionate, but these differences disappeared at the highest dose (Figure 3). Responses to decanal were identical for both sexes at all doses.

DISCUSSION

General Distribution of EAD-Active Compounds. Coupled GC-EAD revealed that antennae of female and male *M. sexta* moths responded selectively to the array of volatiles emitted by host plant foliage. The identified plant headspace compounds belong to a range of chemical classes (aliphatic, aromatic, and terpenoid compounds) and bear a variety of functional groups (alcohol, aldehyde, ester, ether, ketone, amine). All of the EAD-active compounds we identified are common foliar and floral odors (Gibbs, 1974; Harborne and Turner, 1984; Knudsen et al., 1993; Bernays and Chapman, 1994; Schoonhoven et al., 1998; Huber et al., 2000). The ubiquity of these odorants suggests that no single compound serves as a signature compound for host plant selection by *M. sexta*. Attraction to and recognition of appropriate host plants probably involves a suite of compounds released in particular ratios. For example, a blend of green-leaf volatiles elicits upwind orientation in another solanaceous feeder, the Colorado potato beetle (*Leptinotarsa decemlineata)* (Thiéry and Visser, 1986; Visser, 1988), and the apple maggot (*Rhagoletis pomonella*) is attracted to a particular blend of esters (Zhang et al., 1999).

M. sexta females exhibited directed upwind flight toward the eight-component synthetic blend we offered, with response appearing to increase with dose. This result indicates that information gathered from GC-EAD studies could be useful in elucidating which compounds are involved in host plant orientation in *M. sexta*. However, it is not known whether this response reflects female attraction for host plant selection for feeding or for oviposition.

Comparison of Female and Male Detection of Plant-Related Odors. Female and male *M. sexta* exhibited broad overlap in their EAG responses to individual plant odors. In terms of classes of compounds and functional groups, females appeared to be more sensitive than males to esters and terpenoids. When a range of doses of synthetic standards were presented, female antennae showed greater sensitivity to both mono- and sesquiterpenoids at higher doses. This is consistent with the suggestion by Bernays and Chapman (1994) that because of their relatively high volatility, monoterpenoids may be important in distance attraction for odor-guided insects.

There was no clear pattern of difference between responses of female and male antennae to different aromatic compounds. For example, female antennae responded more strongly to aromatic esters, especially (*Z*)-3-hexenyl benzoate,

but were identical to make antennae in their EAG response to benzyl alcohol. The functional group attached to the aromatic ring may therefore be important in odor recognition. Among the aliphatic compounds, females exhibited larger EAG responses than males to intermediate test doses of the two esters, (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenyl propionate. Nonanal and decanal elicited some of the largest EAG responses from both males and females, but response profiles were similar between the two sexes for most test doses.

Several hypotheses might explain why antennae of *M. sexta* females generally appear to be more sensitive to host odors than male antennae, at least at relatively high test doses. For example, Raguso et al. (1996) suggested that female antennae may bear larger populations of olfactory receptor cells (ORCs) that are responsive to plant volatiles, or may exhibit "greater chemosensory efficiency" owing to the smaller size of female vs. male trichoid sensilla. Alternatively, female antennae may have higher electrical resistance. Regardless of the underlying cause, the ability of females and males to detect the same range of volatiles, albeit females with greater sensitivity to certain compounds, appears to be a general phenomenon among phytophagous insects (e.g., Adler and Jacobson, 1972; van der Pers, 1981; Hansson et al., 1989; Ramachandran et al., 1990; Topazzini et al., 1990; Chinta et al., 1994; Raguso et al., 1996; Groot et al., 1999; Zhang et al., 2000). In the case of *M. sexta* at least, this similarity in EAG detection of plant volatiles by both sexes may stem from an overlap in odors from adult food resources, namely floral nectar (e.g., Loughrin et al., 1990; Knudsen et al., 1993), and the odors of foliage, fruit, or flowers (e.g., Buttery et al., 1987; Andersen et al., 1988; Luning, 1995) that are used as oviposition substrates by females.

Relationship between EAD Activity and the Olfactory System of M. sexta. The ability of *M. sexta* antennae to respond to a range of plant volatiles depends on the fact that olfactory sensilla bear a number of different types of ORCs that respond to those compounds. In females, some such ORCs reside in the type-A trichoid sensilla (Shields and Hildebrand, 2001). Thirteen of the 16 EAD-active compounds we identified were previously tested on type-A trichoid sensilla of female antennae, and 10 of the compounds stimulated ORCs in those sensilla (Shields and Hildebrand, 2001). Three EAD-active compounds ((*E*)-β-ocimene, nonanal, and decanal) did not stimulate ORCs in type-A trichoid sensilla, suggesting that other types of olfactory sensilla (see Shields and Hildebrand, 1999a,b) also are involved in the detection of host plant odors as in other species of moths (e.g., Pophof, 1997; Røstelien et al., 2000a,b). Less is known about the sensitivity and selectivity of male olfactory sensilla to plant odors. The long, type-I trichoid sensilla of males are specialized to detect components of the sex-pheromone blend produced by conspecific females (Sanes and Hildebrand, 1976; Kaissling et al., 1989), and plant odorants have not been tested systematically on these or other types of male sensilla (Lee and Strausfeld, 1990).

Some compounds that were present in our plant-headspace samples at relatively high levels (e.g., z-carene, α-terpinene, limonene, and (E)-β-caryophyllene) did not elicit EAD responses, or only when a very concentrated sample was tested. It is possible that there are no, or very few, antennal ORCs that are responsive to these compounds. In contrast, compounds such as (*Z*)-3-hexenyl acetate, phenylacetaldehyde, methyl salicylate, geranyl acetone, benzyl alcohol, and several unidentified compounds (peaks d, j, p, q, and t) elicited large EAD responses, even when these compounds were present in trace amounts (Figure 1). Among the identified compounds, all but (*Z*)-3-hexenyl acetate and phenylacetaldehyde were used as test stimuli in recordings from individual female type-A trichoid sensilla (Shields and Hildebrand, 2001). Only methyl salicylate elicited responses from a relatively large number of ORCs, but even in that case, individual ORC responses were only weak to moderate (Shields and Hildebrand, 2001). This too suggests that the large antennal responses recorded by GC-EAD to many compounds arise from other types of sensilla.

A Role for Information Processing. The volatiles from host plants of *M. sexta* that were found to be EAD-active also elicit electrophysiological activity in the olfactory systems of a variety of other insects. This includes members of the Homoptera (Visser et al., 1996), Hemiptera (Chinta et al., 1994; Groot et al., 1999; Weissbecker et al., 1999, 2000), Diptera (Zhang et al., 1999), Coleoptera (Weissbecker et al., 1997, 1999; Cossé and Baker, 1999; Dickens, 2000; Huber et al., 2000; Zhang et al., 2000), Hymenoptera (Henning and Teuber, 1992; Light et al., 1992; Röse et al., 1998), and Lepidoptera (Tichenor and Seigler, 1980; van der Pers, 1981; Hansson et al., 1989; Topazzini et al., 1990; van Loon et al., 1992; Baur et al., 1993; Raguso et al., 1996; Jonsson and Anderson, 1999; Rojas, 1999; Shields and Hildebrand, 2001). The broad overlap in the olfactory detection capabilities of insect species and in the range of volatiles emitted by host and nonhost plants suggests a crucial role for central olfactory information processing in the host selection process. Condensed neuropil structures called glomeruli, located in the antennal lobes of the insect brain, are the functional units responsible for first-order central processing of olfactory information (Hildebrand, 1995, 1996). *M. sexta* females possess three female-specific glomeruli (Rospars and Hildebrand, 2000), apparently homologous to the three glomeruli of the male-specific macroglomerular complex that processes components of the conspecific female's sex pheromone (Hildebrand, 1995, 1996). Neurons associated with the "lateral large female glomerulus" of *M. sexta* have been found to be consistently responsive to (±)-linalool in studies involving stimulation with a small panel of plant odorants (King et al., 2000), but it remains unclear whether the female-specific glomeruli of *M. sexta* function in host plant recognition. The findings from this GC-EAD study provide information about additional odorants that could be tested in further studies to gain further insight into the processing of olfactory information.

These compounds will also be useful in guiding odorant-selection for further behavioral tests. Our preliminary behavioral experiments using an eight-component blend were successful in eliciting behavioral activity in a wind tunnel and suggest that GC-EAD is a useful technique for identifying ecologically relevant odorants from the complex bouquet of plant volatile emissions, as it has been for discovery of sex-pheromone components.

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PHEROMONES OF MILKWEED BUGS (HETEROPTERA: LYGAEIDAE) ATTRACT WAYWARD PLANT BUGS: *Phytocoris* MIRID SEX PHEROMONE[1]

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Abstract—The synthetic aggregation pheromone of the large milkweed bug, *Oncopeltus fasciatus* (Dallas) (Lygaeinae), also attracted males of the plant bug, *Phytocoris difficilis* Knight (Miridae). Field testing partial blends against the six-component blend comprising the *Oncopeltus* pheromone showed that cross-attraction of *P. difficilis* males was due to synergism between (*E*)-2-octenyl acetate and (*E*,*E*)-2,4-hexadienyl acetate. Hexyl acetate was abundant in the metathoracic scent gland (MSG) secretion of *P. difficilis* males, but because female *P. difficilis* could not initially be found in the field, further combinatorial tests were guided by prior research on the pheromones of two *Phytocoris* species in the western United States. The combination of hexyl, (*E*)-2-hexenyl, and (*E*)-2-octenyl acetates was as attractive to *P. difficilis* males as the milkweed bug pheromone, yet no milkweed bugs were drawn to this blend. Gas chromatographic (GC)-electroantennographic detection (EAD) and GC-mass spectrometric (MS) analyses of female *P. difficilis* MSGs determined that their secretion contained predominantly hexyl, (*E*)-2-hexenyl, and (*E*)-2-octenyl acetates (all strongly EAD-active)—the latter two compounds found only in trace amounts from males—plus five minor female-specific compounds, three of which were EAD-active. (*E*,*E*)-2,4-Hexadienyl acetate was not detected from *P. difficilis* females or males. The blend of the three major components, hexyl, (*E*)-2-hexenyl, and (*E*)-2-octenyl acetates (2:1.5:1 by volume), was as attractive as the blend of all six EAD-active compounds identified from females, indicating that this ternary blend constitutes the sex pheromone of *P. difficilis*. Hexyl acetate with (*E*)-2-octenyl acetate also attracted males of another species, *P. breviusculus*

[1]Mention of commercial products does not constitute an endorsement by USDA.
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Reuter, but addition of (*E*)-2-hexenyl acetate and/or (*E*,*E*)-2,4-hexadienyl acetate inhibited attraction of *P. breviusculus* males. Attraction of *P. difficilis* males occurred mainly during the first half of scotophase. The possible neurophysiological basis for this asymmetrical cross-attraction is discussed.

Key Words—Miridae, *Phytocoris*, milkweed bug, sex pheromone, sex-attractant, metathoracic scent gland, hexyl acetate, (*E*)-2-hexenyl acetate, (*E*)-2-octenyl acetate, (*E*,*E*)-2,4-hexadienyl acetate, cross-attraction.

INTRODUCTION

In the course of testing synthetic pheromone blends for the large milkweed bug [*Oncopeltus fasciatus* (Dallas), Lygaeidae: Lygaeinae] (Aldrich et al., 1999), an interesting discovery was made: males of the plant bug *Phytocoris difficilis* Knight (Miridae) were also highly attracted. Most of these mirids were caught in traps near the edge of an oak-pine mixed forest in midsummer and early fall. This finding revealed an interesting cross-attraction between species in distantly related families (Schuh and Slater, 1995), and provided strong evidence for existence of a female-produced sex pheromone in *P. difficilis*.

The Miridae, known as plant bugs, comprise the largest family of the Heteroptera, with over 1200 genera, including about 10,000 described species (Schuh and Slater, 1995). In North America alone, 223 genera and 1930 species have been catalogued (Henry and Wheeler, 1988). Although some mirids are predacious (Stonedahl, 1988; Wheeler, 2001), most plant bugs are phytophagous, feeding on flowers, fruit, and meristematic tissue, causing plant deformation or fruit abscission. Because of their broad host range, many phytophagous mirids are serious pests of a wide variety of crops throughout the world. Yet, in spite of their importance as pests, methods for sampling and monitoring mirid populations are relatively primitive, relying on the time- and labor-intensive methods of beating-tray- or sweep-net sampling (Ho and Millar, 2002).

Pheromone-baited traps are an important tool for detection and monitoring of many agricultural and forest insect pests. The identification of pheromones for mirids would faciliate the scouting and timing of control measures required in crops attacked by these bugs (McBrien et al., 1994a,b). Mirid sex pheromones are also potentially useful for direct control through mating disruption or mass trapping (McBrien et al., 1996, 1997). Although repeated efforts have been made to identify the sex pheromones of various mirids, especially lygus bugs (Aldrich et al., 1988; Ho and Millar, 2002), sex pheromones have only been identified for six species thus far: *Campylomma verbasci* (Meyer) (Smith et al., 1991), *Phytocoris relativus* Knight (Millar et al., 1997), *P. californicus* Knight (Millar and Rice, 1998), *Trigonotylus caelestialium* (Kirkaldy) (Kakizaki and Sugie, 2001), *Distantiella theobroma* Distant, and *Sahlbergella singularis* Haglund (Downham et al., 2002, and personal communication).

The objectives of the work described here were 1) to determine which components from the milkweed bug pheromone were responsible for the cross-attraction of *P. difficilis*, 2) to collect live *Phytocoris* females for pheromone analyses, and 3) to monitor the diurnal flight activity of *P. difficilis* males.

METHODS AND MATERIALS

Adult Insects and Preparation of Extracts. Phytocoris difficilis males were collected from milkweed bug pheromone-baited bucket traps deployed in an oak-pine mixed forest, on the South Farm of the Beltsville Agricultural Research Center (BARC), Prince George's County, Maryland (Aldrich et al., 1999). Two female adults were collected in the same woods from a white sheet (1.5 m $\times$ 10 m) illuminated by a battery-driven black-light on September 9 and October 2, 2002. A few males of another mirid species, *Phytocoris breviusculus* Reuter, were also collected from the lighted sheet. All bugs were dissected within 24 hr of capture for extraction of the metathoracic scent gland (MSG) and for electrophysiological and chemical analyses. MSGs from both males and females of *P. difficilis* and from *P. breviusculus* males were excised from CO_2-anesthetized bugs submerged in tap water, and the glands were extracted individually in 30 μl of methyl-*tert*-butyl ether. No aerations of live insects were carried out because of the low number of females available.

Gas Chromatographic–Electroantennographic Detection (GC-EAD) Analysis. MSG extracts were analyzed using an Hewlett-Packard 6890 GC equipped with a DB-WaxETR column (30 m $\times$0.25 mm $\times$ 0.25 μm; splitless mode; J & W Scientific, Folsom, CA), and a 1:1 effluent splitter that allowed simultaneous flame ionization detection (FID) and EAD of the separated volatile compounds. Helium was used as the carrier gas, and the injector temperature was 220°C. The column temperature was 50°C/2 min, rising to 240°C at 10°C/min, then held for 10 min. The outlet for the EAD was held in a humidified airstream flowing at 0.5 m/sec over a male antennal preparation. A glass capillary indifferent electrode was filled with Beadle–Ephrussi Ringer (Zhang et al., 2000), grounded via a silver wire, and inserted into the open side of the severed mirid head. A similar recording electrode connected to a high-impedance DC amplifier with automatic baseline drift compensation was inserted over the distal ends of both antennae (the tip of each antenna was cut off). The antennal signals were stored and analyzed on a PC equipped with a serial IDAC interface box and the program EAD ver. 2.5 (Syntech, Hilversum, The Netherlands). In addition, EAD responses for males of each mirid species to a synthetic mixture (3 μl/injection), containing seven acetates and two butyrates (10 ng/μl each) were recorded. For each sample (both extracts and synthetic mixture), at least three pairs of antennae from each mirid species were tested. Only those that elicited electrophysiological responses

obviously different from background noise in at least two antennal preparations were considered as stimulatory to test antennae.

Compound Identification. The following authentic standards were obtained from commercial sources or were synthesized: 1-hexanol, (*E*)-2-hexenol, nonanal, decanal, hexyl acetate (HA), octyl acetate (OA), and octyl butyrate (Aldrich Chemical Co., Milwaukee, WI); (*E*)-2-octenyl acetate (2OA), hexyl butyrate (HB), (*E*)-2-hexenyl butyrate (2HB), (*E*)-2-heptenyl acetate (2HPA), (*E*)-2-hexenyl butyrate (2HB), and (*E*)-2-nonenol (Bedoukian Research, Inc., Danbury, CT); heptyl acetate (Eastman Organics); 3-hydroxy-2-butanone (TCI America) and (Z)-3-octenyl acetate (J. G. Millar, University of California, Riverside). Butyl butyrate, (*E*)-2-hexenyl acetate (2HA), (*E*,*E*)-2,4-hexadienyl acetate (2,4HA), (*E*)-2,5-hexadienyl acetate (2,5HA), and (*E*)-2,7-octadienyl acetate (2,7OA) were synthesized as previously described (Aldrich et al., 1997, 1999).

MSG samples were analyzed by gas chromatography-mass spectrometry (GC-MS) on an HP 6890 GC series coupled with an HP 5973 Mass Selective Detector by using the same type of GC column and conditions as described above. Compounds were identified by comparison of retention times with those of authentic standards and with mass spectra of standards.

Field Trapping. All field-trapping experiments were carried out in an oak-pine mixed forest on the BARC South Farm from early July through the fall of 2002, using Jackson Delta sticky traps (Agrisense, Fresno, CA) baited with 15–30 μl of individual or mixed neat test compounds loaded onto grey rubber septa (5 mm sleeve-type, The West Co., Lititz, PA). Rubber septa dispensers were replaced every week. Traps were hung on the trunk or branches of either oak or pine trees in lines at a height of 1.8–2.0 m, with a spacing of about 10 m between traps within each trap line, and about 15 m between trap lines. For each experiment, two sets of traps were deployed with their initial trap positions randomized. The trap positions were then systematically rotated within the same trap set after each replicate, based on a procedure of Latin-square design (Byers, 1991), so that each trap appeared in each location at least once. The bug collections and trap rotations were carried out when $\geq$1 mirid was caught in the best traps, to minimize positional effect. Each replicate lasted 1–3 days, depending on flight activity (i.e., numbers of bugs caught in the best traps). Experiments 1–3 were initiated before the *Phytocoris* females were analyzed, on the basis of earlier cross-attraction data (J. R. Aldrich, unpublished) and the published results on the pheromones of other *Phytocoris* species (Millar et al., 1997; Millar and Rice, 1998), whereas experiments 4–5 were carried out after analysis of the first female *P. difficilis* MSG extract.

Experiment 1 was conducted to determine in a subtractive manner which components in the milkweed bug pheromone blend, consisting of 2HA, 2,4HA, 2,5HA, 2HPA, 2OA, and 2,7OA, were responsible for the cross-family attraction. Experiment 2 tested the potential activity of hexyl acetate, (*E*)-2-hexenyl acetate, and (*E*)-2-octenyl acetates in a three-way factorial design, i.e., three individual

components and all their possible binary/ternary blends (in the same ratio). Hexyl acetate was used because it is an essential pheromone component of two *Phytocoris* spp. in the western United States, and is produced by both sexes of each species (Millar et al., 1997; Millar and Rice, 1998). (*E*)-2-Hexenyl acetate and (*E*)-2-octenyl acetate are female-specific compounds of the western *Phytocoris* spp. (Millar et al., 1997; Millar and Rice, 1998), and are also present in the milkweed bug pheromone (Aldrich et al., 1999). Experiment 3 was similar to experiment 2, but with one more milkweed bug pheromone compound, (*E*,*E*)-2,4-hexadienyl acetate, involved in the full factorial design. In addition, a milkweed bug pheromone blend (30 μl) was also added to this experiment as a positive control. Experiment 4 tested the complete reconstruction of the EAD-active female-specific MSG volatiles ("full blend"), subtraction of each EAD-active component from the full blend, and a blend of three major strongly EAD-active components, and the milkweed bug pheromone blend. In experiment 5, the diurnal flight activity of *P. difficilis* males was monitored with attractant-baited traps on three occasions in late September and the beginning of October 2002 by checking the traps each hour from late afternoon until midnight.

Statistical Analysis. Because of strong heterogeneity of variances among treatments, trap catch data (number of males caught /trap/replicate) were analyzed using the nonparametric Kruskal–Wallis ANOVA on rank test, followed by the Student–Newman–Kuels all pairwise comparison to separate means (Zar, 1984).

RESULTS

Field Experiments before Analysis of Female P. difficilis. In experiment 1, a total of 262 *P. difficilis* males were caught from July 10 to September 18, 2002, and no *P. breviusculus* were trapped. Traps baited with the six-component pheromone blend of milkweed bugs caught significantly more mirid males than the blank control ($P < 0.05$; Figure 1). Subtraction of (*E*)-2-hexenyl acetate, (*E*)-2,5-hexadienyl acetate, (*E*)-2-heptenyl acetate, or (*E*)-2,7-octadienyl acetate from the six-component blend had no effect on the trap catches. However, deletion of (*E*, *E*)-2,4-hexadienyl acetate or (*E*)-2-octenyl acetate from the blend eliminated attraction of *P. difficilis* males (Figure 1).

In experiment 2 conducted from July 20 to September 26, 2002, none of the three individual components tested alone (hexyl acetate, (*E*)-2-hexenyl acetate, and (*E*)-2-octenyl acetate) were attractive to *P. difficilis* males, nor were their binary combinations. However, the ternary blend attracted significant numbers of male bugs (Figure 2). A binary combination of hexyl acetate and (*E*)-2-octenyl acetate also attracted males of another mirid species, *P. breviusculus*, and addition of (*E*)-2-hexenyl acetate to this binary blend reduced the attraction of this mirid to a level not different from the blank (Figure 2).

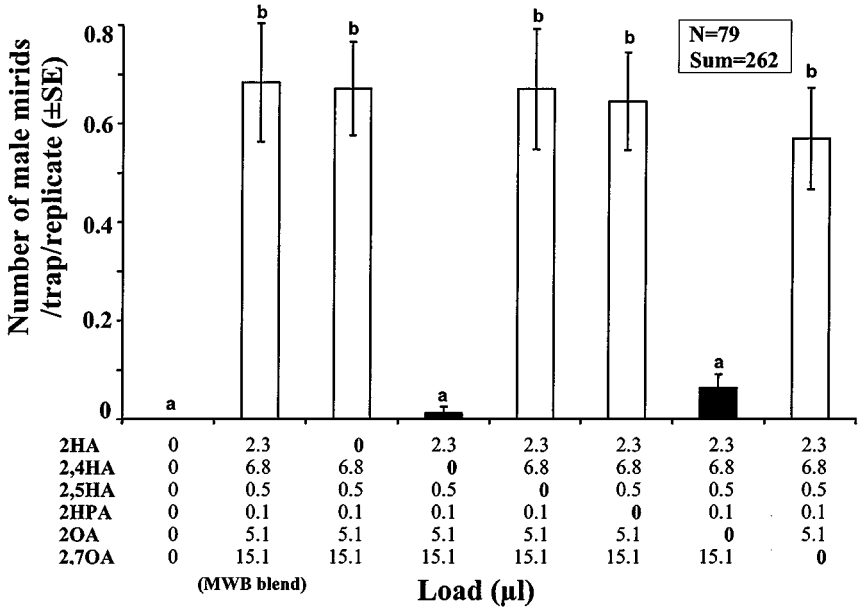

FIG. 1. Captures of male *Phytocoris difficilis* in traps baited with the synthetic pheromone of the large milkweed bug (MWB), *Oncopeltus fasciatus*, and deletion blends. Means followed by the same letter are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by Student–Newman–Keuls all pairwise comparison. 2HA = (*E*)-2-hexenyl acetate; 2,4HA = (*E*, *E*)-2,4-hexadienyl acetate; 2,5HA = (*E*)-2,5-hexadienyl acetate; 2HPA = (*E*)-2-heptenyl acetate; 2OA = (*E*)-2-octenyl acetate; 2,7OA = (*E*)-2,7-octadienyl acetate.

In experiment 3 (September 6–October 8, 2002), the combination of hexyl acetate, (*E*)-2-hexenyl acetate, and (*E*)-2-octenyl acetate again resulted in significant attraction to *P. difficilis* males, with trap catches similar to those baited with the milkweed bug pheromone blend (MWB blend) (Figure 3). Individual compounds and binary blends were not attractive except for the mixture of (*E*,*E*)-2,4-hexadienyl acetate and (*E*)-2-octenyl acetate. All three- or four-component blends containing (*E*)-2-octenyl acetate caught significantly more *P. difficilis* males than the blank control, and there were no differences in trap catches among these treatments. However, any blends that were missing (*E*)-2-octenyl acetate were not attractive. Interestingly, (*E*, *E*)-2,4-hexadienyl acetate, one of the two key components responsible for the cross-attraction in experiment 1, showed a strong synergism with (*E*)-2-octenyl acetate. In fact, (*E*, *E*)-2,4-hexadienyl acetate could substitute for either hexyl acetate or (*E*)-2-hexenyl acetate or both in the three-component blend to achieve the same level of attraction (Figure 3). As shown in

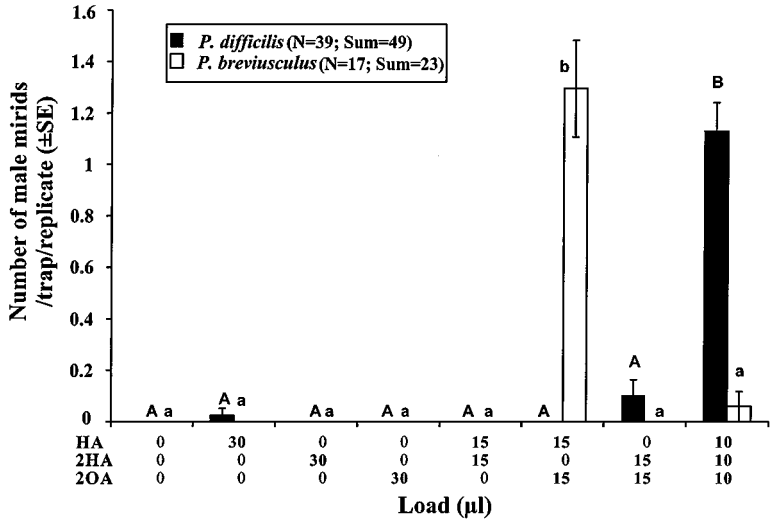

FIG. 2. Captures of male *Phytocoris difficilis* and *P. breviusculus* in traps baited with HA, 2HA, or 2OA, and their binary and ternary blends. Means followed by the same letter are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by Student–Newman–Keuls all pairwise comparison. Abbreviations as in Table 1 and Figure 1.

experiment 2, the binary blend of hexyl acetate and (*E*)-2-octenyl acetate was again attractive to *P. breviusculus* males, and addition of (*E*)-2-hexenyl acetate and/or (*E*, *E*)-2,4-hexadienyl acetate to this blend totally shut off attraction (Figure 3). Milkweed bugs were captured only in the traps baited with the milkweed bug pheromone.

GC-EAD and GC-MS Analyses. In general, antennal preparations of both mirid species remained active for at least 2 hr, which allowed time for 3–4 GC-EAD experiments per preparation. GC-EAD analyses of MSG extracts from two female *P. difficilis* showed an identical pattern in GC profiles and male EAD responses. Antennae of *P. difficilis* males responded strongly to three major components of female MSG extracts that were identified by GC-MS as hexyl acetate, (*E*)-2-hexenyl acetate, and (*E*)-2-octenyl acetate (with ratio of ca. 2:1.5:1) (Figure 4 and Table 1). Weak but reproducible responses to several minor or trace components (e.g., hexyl butyrate, (*E*)-2-heptenyl acetate, octyl acetate, and (*E*)-2,7-octadienyl acetate) were also detected (Figure 4). In male MSG extracts, there were only two major volatile components, hexyl acetate and hexyl butyrate, each of which elicited strong responses from male antennae (Figure 4, Table 1). Consistent EAD responses were also detected to three minor or barely FID-detectable components in extracts from males, (*E*)-2-hexenyl acetate, (*E*)-2-hexenyl butyrate, and (*E*)-2-octenyl acetate. Females of *P. breviusculus* were not available for comparison; however,

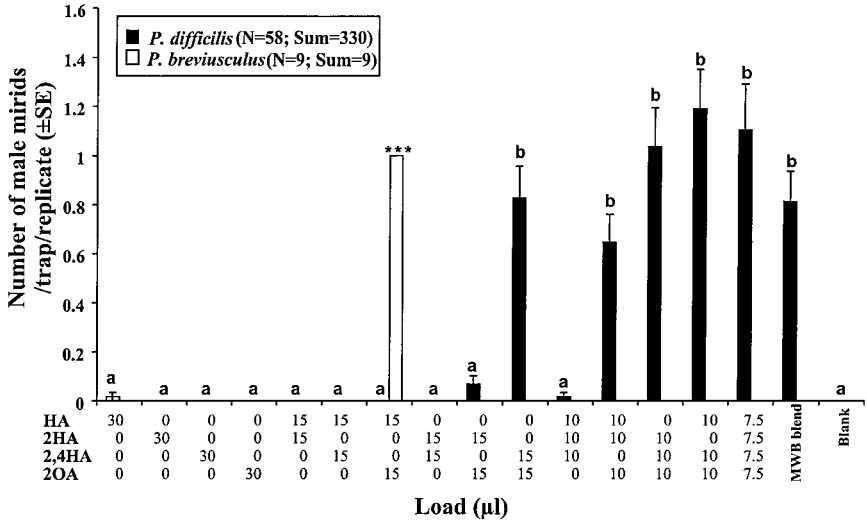

FIG. 3. Captures of male *Phytocoris difficilis* and *P. breviusculus* in traps baited with HA, 2HA, 2OA, or 2,4HA, and all possible two- to four-way combination blends. Milkweed bug pheromone (MWB) blend was included as a positive control. Means followed by the same letter are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by Student–Newman–Keuls all pairwise comparison. ***Significantly different from all other treatments. Abbreviations as in Table 1 and Figure 1.

GC-EAD/MS analyses of MSG extracts from males of this species showed a similar volatile profile to that of *P. difficilis* males, with hexyl acetate and hexyl butyrate being the major, strongly EAD-active volatile components (Table 1). No antennal responses to other minor components were detected. Analysis of a synthetic mixture of seven acetates and two butyrates indicated that male antennae of both mirid species responded strongly to all nine compounds at a dose of about 15 ng for each compound. This mixture included not only those compounds found in the mirid MSG (males or females or both), but also two milkweed bug pheromone components, (*E*, *E*)-2,4-hexadienyl acetate and (*E*)-2,5- hexadienyl acetate, which were not detected from these mirids (data not shown). Further recordings of this mixture coinjected with (*E*)-2-hexenyl (*E*)-2-hexenoate showed that antennae of both species strongly responded to acetates and butyrates, but not to the hexenoate ester.

Field Experiments after Analysis of Female P. difficilis. In experiment 4 (September 21–October 8, 2002), the reconstructed EAD-active six-component "full blend" of female MSG volatiles attracted significant numbers of *P. difficilis* males. However, the number of mirid males caught in traps baited with the full blend was not different from captures in traps baited with either the three-component blend of hexyl acetate, (*E*)-2-hexenyl acetate, and (*E*)-2-octenyl acetate, or the

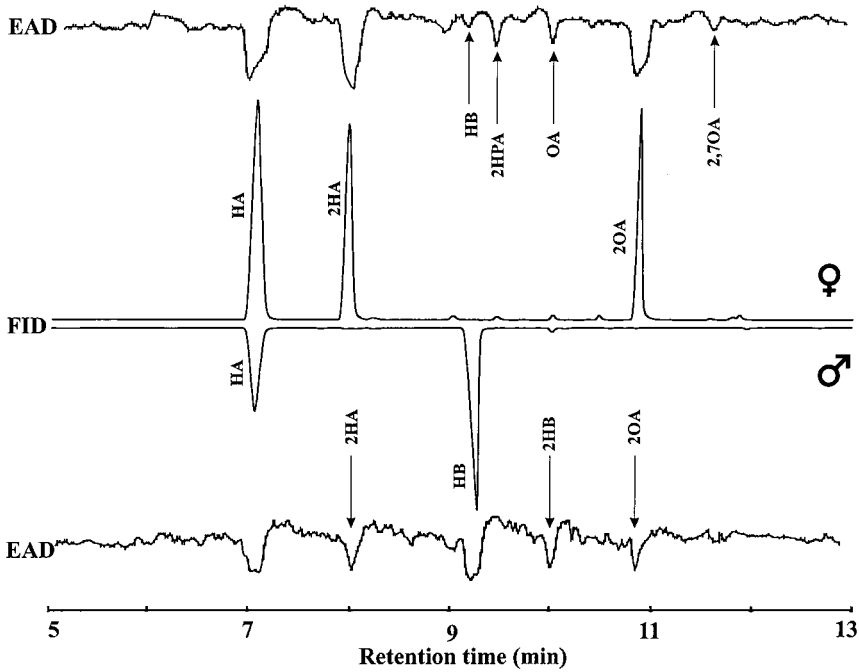

FIG. 4. GC-EAD responses of *Phytocoris difficilis* male antennae to metathoracic scent gland extracts of conspecific males and females. HB = hexyl butyrate, 2HB = (*E*)-2-hexenyl butyrate. Abbreviations of other chemical names as in Table 1 and Figure 1.

MWB blend (Figure 5). Deletion of any of the three major components, hexyl acetate, (*E*)-2-hexenyl acetate, or (*E*)-2-octenyl acetate, from this full blend resulted in reduction of trap catches to the level of the blank control, indicating that all three compounds are components of the *P. difficilis* sex pheromone. In contrast, subtraction of any or all of the minor components, (*E*)-2-heptenyl acetate, octyl acetate, or (*E*)-2,7-octadienyl acetate, had no effect on the attractiveness of baits (Figure 5). Only the blend from which (*E*)-2-hexenyl acetate had been deleted was attractive to *P. breviusculus* males (Figure 5). No milkweed bugs were caught in traps baited with partial, full, or three-component blends attractive to *Phytocoris* spp., while the MWB blend was still attractive to milkweed bugs.

Diurnal Flight Activity of P. difficilis. During late September and early October 2002, attractant-baited traps in the experimental area were checked at 1-hr intervals from late afternoon until midnight. Data from 3 days' observations were pooled to calculate the cumulative relative catch (Figure 6). Male flight of *P. difficilis* to sex-attractant traps started around 18:00 when sunset began, with peak flight occurring between 20:00 and 22:00, and flight stopped sometime after

TABLE 1. METATHORACIC SCENT GLAND COMPONENTS IDENTIFIED FROM *Phytocoris difficilis* AND *P. breviusculus*

Chemicals[a]	*P. difficilis* Relative amount (%) ± SE Females ($N = 2$)	*P. difficilis* Relative amount (%) ± SE Males ($N = 3$)	*P. difficilis* Male EAD activity[c]	*P. breviusculus males*[b] Relative amounts (%) ± SE	*P. breviusculus males*[b] Male EAD activity[c]
Butyl butyrate		0.04 ± 0.00			
Hexyl acetate (HA)	**43.05 ± 0.15**	**29.7 ± 1.48**	***	**27.8 ± 1.8**	***
3-Hydroxy-2-butanone				2.6 ± 0.1	
(*E*)-2-Hexenyl acetate (2HA)	**31.7 ± 1.604**	**0.23 ± 0.03**	***		
Hexyl propionate (?)		0.23 ± 0.03		0.9 ± 0.6	
1-Hexanol		0.60 ± 0.06		5.3 ± 1.2	
Heptyl acetate	0.10 ± 0.01	0.10 ± 0.03	*		
(*E*)-2-Hexenol	0.48 ± 0.03	0.04 ± 0.01	*		
Nonanal				1.5 ± 0.4	
Hexyl butyrate (HB)	**0.04 ± 0.01**	**68.0 ± 1.05**	***	**57.3 ± 2.2**	***
(*E*)-2-Heptenyl acetate (2HPA)	**0.35 ± 0.05**		**		
(*E*)-2-Hexenyl butyrate (2HB)		**1.03 ± 0.24**	**		
Octyl acetate (OA)	**0.45 ± 0.05**		**		
(*Z*)-3-Octenyl acetate	0.41 ± 0.01		*		
(*E*)-2-Octenyl acetate (2OA)	**22.35 ± 1.15**	**0.08 ± 0.01**	***		
(*E*)-2, 7-Octadienyl acetate (2,7OA)	**0.15 ± 0.05**		**		
Unidentified	0.45 ± 0.05				
Decanal				2.9 ± 0.5	
Octyl butyrate		0.13 ± 0.09		0.9 ± 0.3	

[a] (?) = Tentatively identified based on MS data; standard not obtained. Compounds in bold type are EAD-active.
[b] Females not found.
[c] *Occasionally (weak) active, not repeatable (0–0.02 mV); **Repeatably active at level of 0.05–0.14 mV; ***Strongly active (0.15–0.5 mV).

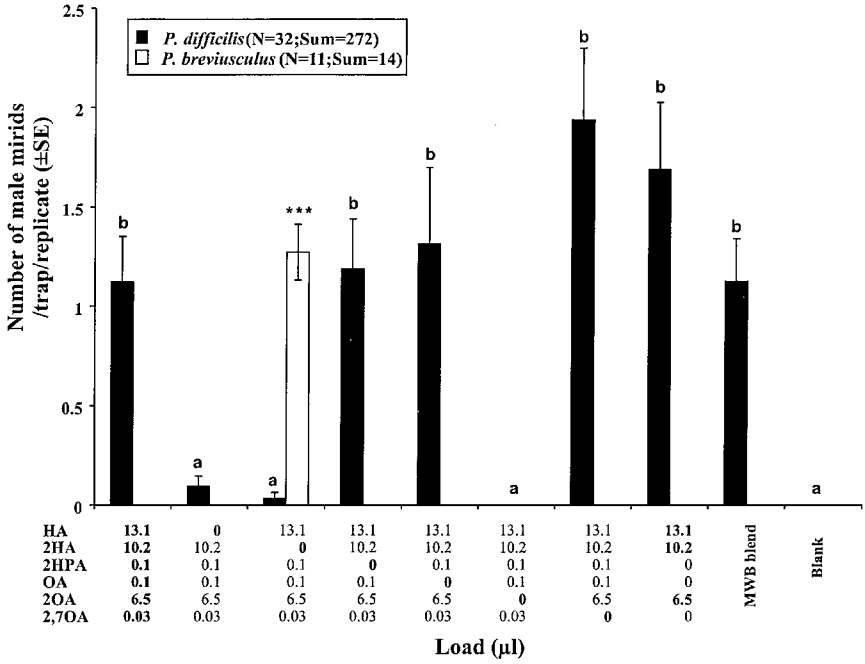

FIG. 5. Captures of male *Phytocoris difficilis* and *P. breviusculus* in traps baited with complete and partial blends of EAD-active female MSG volatiles. A three-component blend (HA, 2HA, and 2OA) and a milkweed bug pheromone (MWB) blend were included as positive controls. Means followed by the same letter are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by Student–Newman–Keuls all pairwise comparison. Abbreviations as in Table 1.

midnight (Figure 6). No flights to the traps were observed during daytime. Only two *P. difficilis* females were collected from the black-light trap in the same experimental forest; each was captured between 20:00 and 22:00. In addition, a few of the *P. breviusculus* males were caught in the traps baited with hexyl acetate and (*E*)-2-octenyl acetate during the same period.

DISCUSSION

The results indicate that the sex pheromone of *Phytocoris difficilis* consists of hexyl acetate, (*E*)-2-hexenyl acetate, and (*E*)-2-octenyl acetate. These compounds are produced by adult females in the metathoracic scent gland (MSG), the gland that in most heteropterans is responsible for chemical defense (Aldrich, 1988). Although females of *P. breviusculus* were not available for identification,

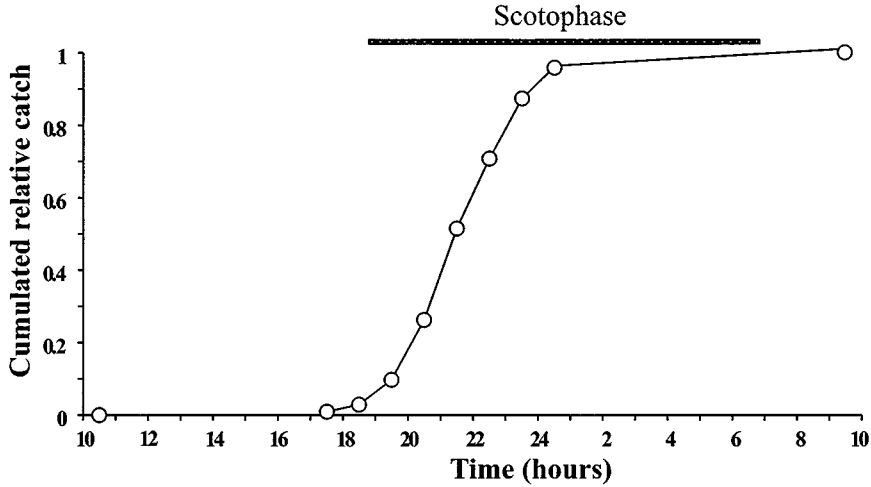

FIG. 6. Diurnal flight activity of *Phytocoris difficilis* males monitored by traps baited with attractive synthetic blends. Pooled data from 3 days' observation (with a total of 103 males captured) in late September and early October 2002 are presented as cumulative proportional catches.

our field trapping results strongly suggest that hexyl acetate and (*E*)-2-octenyl acetate are sex pheromone components for this species. Thistlewood et al. (1989) suspected the MSG as a possible pheromone gland in the mullein bug, *Campylomma verbasci*, and Millar et al. (1997) also found that the sex pheromone of *P. relativus* was produced in the thorax, but neither group definitively located the pheromone-producing glands. In fact, attractant pheromones are released from the MSG of either males or females (depending upon the species) in various Lygaeidae (Aldrich et al., 1997, 1999) and Alydidae (Leal et al., 1995; Aldrich et al., 2000). In the Miridae, all evidence suggests that females are the attractive sex (McBrien and Millar, 1999; Kakizaki and Sugie, 2001, and references therein). Including the two mirids reported here, identifications of sex pheromone or sex-attractant components have now been achieved for eight mirid species (Table 2). Their sex pheromones consist of blends of two or three simple esters (acetates, butyrates, or hexanoates), usually with one of the components being common to both sexes and one or two components being abundantly produced only by females.

Plant bugs in the genus *Phytocoris* have similar volatile profiles, and the chemical constituents are parsimoniously distributed among species (Table 2). Only four esters are involved in the pheromonal communication systems of the four species thus far investigated, with hexyl acetate (the only compound that is produced by both sexes in large amounts) being common to all species, and (*E*)-2-octenyl acetate being produced by females in at least three species (Table 2). (*E*)-2-Octenyl

TABLE 2. KNOWN PHEROMONE COMPONENTS (P), SEX ATTRACTANTS (SA), AND ATTRACTION INHIBITORS (I) OF MIRIDAE

Chemicals	*Campylomma verbasci*	*Phytocoris relativus*	*P. californicus*	*P. difficilis*	*P. breviusculus*	*Trigonotylus caelestianlium*	*Distantiella theobroma*	*Sahlbergella singularis*
Butyl butyrate	P							
(*E*)-2-Butenyl butyrate	P							
Hexyl acetate		P	P	P	SA			
(*E*)-2-Hexenyl acetate				P	I			
(*E*)-2-Octenyl acetate			P	P	SA			
Octyl butyrate						P		
(*E*)-2-Octenyl butyrate		P	I					
Hexyl hexanoate						P		
(*E*)-2-Hexenyl hexanoate						P		
Hexyl (3*R*)-hydroxybutyrate (HHB)							P	P
3-Ester with (*E*)-2-butenoic acid of HHB							P	P
References	1	2	3	4	4	5	6	6

Note: 1. Smith et al., 1991; 2. Millar et al., 1997; 3. Millar and Rice, 1998; 4. Current paper; 5. Kakizaki and Sugie, 2001; 6. Downham et al., 2002; and personal communication.

butyrate and (*E*)-2-hexenyl acetate, produced by females of *P. relativus* and *P. difficilis*, respectively, are antagonistic to their corresponding sympatric congeners, *P. californicus* (Millar et al., 1997; Millar and Rice, 1998) and *P. breviusculus* (herein). These antagonists appear to play an important role in maintaining reproductive isolation of the two pairs of sympatric species. In addition to the three pheromone components of *P. difficilis*, several other esters were also detected from the female MSG in minor or trace amounts, including (*E*)-2,7-octadienyl acetate (Figure 4, Table 1). This unsaturated acetate is a key aggregation pheromone component in many milkweed bugs (Lygaeinae), but had not been found previously in any other species outside of the Lygaeinae (Aldrich et al., 1997, 1999). The major constituents of male volatiles, whether from aeration or from MSG dissection, are almost identical among these four *Phytocoris* species, with hexyl butyrate being the dominant component, followed by hexyl acetate) (Millar et al., 1997; Millar and Rice, 1998) (Table 1). During the course of our study, only two *P. difficilis* females were collected, which made any attempts at aeration impractical. However, the GC profiles from our MSG extracts were similar to those obtained from aeration extracts of the two western *Phytocoris* spp. (Millar et al., 1997; Millar and Rice, 1998), suggesting that there is a direct correspondence between pheromone production in and release from the MSG in these mirids. What remains unknown is how the production of the pheromone is regulated by the MSG, which also produces defensive secretions.

Our GC-EAD analyses showed that antennae of males of both mirid species responded strongly to the acetates and butyrates from the MSG extracts, but not to the hexenoate ester tested. However, not all antennal stimulatory compounds were behaviorally active and, in fact, none of them were attractive when tested alone. The synergism among the three pheromone components of *P. difficilis* suggests that each component may target a different antennal receptor neuron (Todd and Baker, 1999). Integration of neuronal input, either in the antennal lobe or in the protocerebrum, is likely responsible for the synergism ultimately leading to the modulation of behavior (Hansson and Anton, 2000).

The cross-family attraction of *P. difficilis* males to the *Oncopeltus* pheromone blend resulted from the coexistence of (*E*)-2-octenyl acetate, one of its key pheromone components, and a nonpheromone compound, (*E*, *E*)-2,4-hexadienyl acetate. Thus, (*E*, *E*)-2,4-hexadienyl acetate could substitute for both hexyl acetate and (*E*)-2-hexenyl acetate in the *P. difficilis* pheromone blend. Furthermore, adding (*E*, *E*)-2,4-hexadienyl acetate to the three-component blend did not show any synergistic effect on trap catches of *P. difficilis* males as might have been expected if this compound were a true pheromone component. GC-EAD experiments revealed that (*E*, *E*)-2,4-hexadienyl acetate was highly stimulatory to the antennae of *P. difficilis* males. This may be due to either a high diversity or a low specificity of receptor neurons on the antennae of *P. difficilis*. It seems possible that (*E*, *E*)-2,4-hexadienyl acetate may activate one or both types of neurons (or

binding sites) that are specially tuned to hexyl acetate and (*E*)-2-hexenyl acetate. Further study of the diversity and specificity of receptor neurons to these behaviorally active compounds using the single-cell recording technique might clarify this situation.

Our strategy of using subtraction and factorial design methods for field screening proved effective. By these methods, we were able to pinpoint the two compounds responsible for the cross-family attraction and, guided by published data on the two western *Phytocoris* species (Millar et al., 1997; Millar and Rice, 1998), we arrived at the correct sex pheromone components of *P. difficilis* even before females were available for analysis. Once females of *P. difficilis* were collected and their MSGs were analyzed, a simple and clear picture of its sex pheromone system became obvious, verifying our deduction.

The two mirids studied are both conifer-associated species, with *P. difficilis* being reported to feed on pine trees (*Pinus virginana*) (Knight, 1927), whereas *P. breviusculus* is reportedly a predator of scale insects on cedar or juniper trees (Wheeler and Henry, 1977). Our observations indicate that both of these *Phytocoris* species are night fliers, with calling and mating events occurring mainly during the first half of scotophase. The economic importance of *P. difficilis* and *P. breviusculus* is unclear. Nevertheless, the results from this study enrich our knowledge of pheromone production and chemistry in the Miridae, and provide a simple model system for further study of this little-known but important group of insects.

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LARVAL EXPOSURE TO OVIPOSITION DETERRENTS ALTERS SUBSEQUENT OVIPOSITION BEHAVIOR IN GENERALIST, *Trichoplusia ni* AND SPECIALIST, *Plutella xylostella* MOTHS

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Abstract—The present study was undertaken to determine the effects of larval feeding experience on subsequent oviposition behavior of the resulting moths. Larvae of the cabbage looper (*Trichoplusia ni*, Noctuidae) and the diamondback moth (*Plutella xylostella*, Plutellidae) were exposed to the phenylpropanoid allelochemical *trans*-anethole (at 100 ppm fw in artificial diet) or the limonoid allelochemical toosendanin (10 ppm sprayed on cabbage leaves). Both compounds had been shown to deter oviposition in naïve moths in previous choice tests. Moths developing from "experienced" larvae (both sexes) showed a decrease in oviposition deterrence response when given a choice between control and treated leaves, unlike naïve moths. This phenomenon, analogous to habituation to feeding deterrents in lepidopteran larva, occurred irrespective of duration of feeding on the deterrent compound. We also observed that F_1larvae resulting from experienced moths (previously exposed to toosendanin as larvae) grew as well on toosendanin-treated foliage as on control foliage. In contrast, growth of F_1larvae from naïve moths was significantly impaired by toosendanin. These results demonstrate that host-selection behavior in cabbage looper (a generalist) and diamondback moth (a specialist) may be shaped by feeding experience according to Hopkins' Host Selection Principle in addition to chemical legacy.

Key Words—*Trichoplusia ni*, *Plutella xylostella*, *trans*-anethole, toosendanin, oviposition deterrents, larval experience.

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INTRODUCTION

Host plant acceptance by phytophagous insects can be conveniently divided into two complex traits; female oviposition preference and larval performance (Thompson, 1988). Oviposition preference refers to the sequence of behavioral traits involved in locating and evaluating a potential host plant that ultimately leads to the decision to oviposit or not. Thompson (1988) used the term oviposition preference as the "hierarchical ordering of plant species by ovipositing females when the plants are presented in equal abundance and availability." It is also defined by the number of eggs laid by a female on each of the plant species in a choice trial, in which plants of equal mass of several species are offered simultaneously (Wiklund, 1975; Thompson, 1986).

Oviposition preference is especially important in insects such as Lepidoptera because mobile adults must find host plants for their relatively immobile offspring (Tabashnik and Slansky, 1985). Neonate larvae of many species are incapable of locating a new host and are dependent on the host plant location "skills" of their mothers (Feeny et al., 1983). Therefore, the site of emergence is of such importance to the larvae of most (but not all) lepidopteran species that the choice of oviposition site by the female is considered in effect a choice of life history for her offspring (Resetarits, 1996), thereby influencing all aspects of larval performance.

The probability that an insect will feed or oviposit on a particular host individual will depend on the acceptability of the host to the insect (Singer, 1986). This will be influenced by both innate tendencies and prior experience; a number of such influences may operate synchronously, increasing or decreasing the probability of acceptance (Miller and Strickler, 1984).

For more than a century, entomologists suggested that the feeding and oviposition behavior of herbivorous insects was not only influenced by experience of the adult insect (Cassidy, 1978; Rausher, 1978; Prokopy et al., 1982; Jaenike, 1982, 1983; Hoffman, 1985) on a specific food, but also by experience obtained during the larval stage for that food. This phenomenon was called the "Hopkins Host Selection Principle" (HHSP) (Hopkins, 1917). Several authors claimed to have proven the HHSP in phytophagous insects (Walsh, 1864; cited by Szentesi and Jermy, 1989; Craighead, 1921; Kuznetzov, 1952; Hovanitz and Chang, 1963; Corbet, 1985; Jaenike, 1990; Futuyama et al., 1993; Barron, 2001; Rietdorf and Steidle, 2002), in *Drosophila* (Thorpe, 1939; Hershberger and Smith, 1967), and in parasitoids (Thorpe and Jones, 1937; Smith and Cornell, 1979). However, a number of studies have failed to show the effects of larval feeding experience upon subsequent oviposition behavior of the adult (Solarz and Newman, 2001). Jaenike (1983) proposed a "neo-Hopkins host selection principle," suggesting that the "exposure of adult insects to a particular type of host will often, though not always, increase the subsequent acceptability of that host as an oviposition site."

Since there are contradictory reports about the influence of larval feeding experience upon subsequent oviposition behavior of adult insects, this study aimed to look at the effects of larval feeding experience on subsequent oviposition behavior of adult moths. *Trichoplusia ni* and *Plutella xylostella* were chosen for several reasons. The cabbage looper, *T. ni*, a generalist, can be an important pest of cruciferous plants. This species also attacks several other crops including lettuce, beet, peas, celery, tomato, certain ornamentals, and many weedy plants. The diamondback moth, *Plutella xylostella*, a specialist on plants in the family Brassicaceae, is one of the most serious pests of cole crops worldwide. Both species have short life cycles (egg–adult, 24–33 days for *T. ni*, and 10–14 days for *P. xylostella*), and are easily reared in the laboratory. Both lay eggs singly, mostly on the lower surface of leaves.

The pure compounds used in the study were *trans*-anethole and toosendanin. *trans*-Anethole, a phenylpropanoid, is the main constituent of the essential oil from anise (*Pimpinella anisum*). It is a contact toxin and a feeding deterrent to the tobacco cutworm, *Spodoptera litura* (Hummelbrunner and Isman, 2001). Toosendanin, a limonoid from the bark of the tree *Melia azedarach*, has been used as a botanical insecticide since 1980. It possesses strong growth inhibitory and antifeedant properties against a number of insects (Zhang and Chiu, 1983; Chiu, 1989; Chen et al., 1995). Selection of the compounds used in our study was based on demonstrated oviposition deterrent properties against the test insects (*T. ni* and *P. xylostella*) (unpublished data). These compounds also acted as feeding deterrents against the respective larvae (Akhtar and Isman, unpublished data).

Using the model system described above, the main objectives were to determine: (1) if feeding experiences of larvae with a volatile (*trans*-anethole) or nonvolatile (toosendanin) oviposition deterrent compound can change oviposition preference of subsequent adults; (2) if response of an ovipositing female is affected by duration of larval exposure to the deterrent; (3) if there is a correlation between oviposition choice of an "experienced female" and growth performance of offspring on a preferred plant (treated cabbage foliage); and (4) if a specialist herbivore responds in the same manner as a generalist herbivore. We have measured oviposition preference by recording the distribution of eggs laid by captive females when given a choice between control and treated plants under controlled experimental conditions in the laboratory (Singer, 1986). The present study may be useful in predicting host-plant shifts in nature as a result of experience by phytophagous herbivores.

METHODS AND MATERIALS

Plant Material. Cabbage plants (*Brassica oleraceae* var. Stonehead) were routinely grown for bioassays (and to maintain the diamondback moth colony)

in plastic pots containing a mixture of sandy loam soil and peatmoss (4:1) in the greenhouse at the University of British Columbia, Vancouver, BC, Canada. Cabbage plants used in the bioassays were 5–6 weeks old.

Test Compounds. *Trans*-Anethole (99% purity) was provided by EcoSMART Technologies Inc. (Nashville, USA). Refined bark extracts of *Melia azedarach* (syn. *M. toosendan*) containing 60% toosendanin were provided by Dr Zhang (Northwest Agricultural University, Yangling, Peoples Republic of China).

Concentrations of Compounds Used for Larval Training (Exposure). Cabbage looper larvae were reared on artificial diet containing 100 ppm of *trans*-anethole or 50 ppm toosendanin. A 10 ppm methanolic solution of toosendanin was sprayed on cabbage leaves to condition diamondback larvae. Concentrations used were neither inhibitory to larval growth nor toxic to insects; there was no larval mortality attributable to the compounds during the training period.

Concentrations of Compounds Used for Testing. Concentrations of compounds used for testing were based roughly on their OD_{50} values (concentration of compound causing 50% oviposition deterrence, compared to control) determined in preliminary experiments (unpublished data). Methanolic solutions of 0.8% *trans*-anethole and 0.5% toosendanin were sprayed on cabbage foliage. Use of higher concentrations was avoided due to phytotoxic effects on cabbage foliage.

Test Insects. *T. ni* (Lepidoptera: Noctuidae) and *P. xylostella* (Lepidoptera: Plutellidae) were obtained from laboratory colonies maintained for >50 generations. Colonies were reared at room temperature (19–25°C) under a 16:8 LD photoperiod. *T. ni* larvae were fed on an artificial diet (No. 9795, Bioserv Inc., Frenchtown, NJ), supplemented with finely ground alfalfa to improve acceptability. All test diets were prepared according to Isman and Rodriguez (1983). Diamondback moths were reared on potted cabbage plants in plexiglass cages [60 × 60 × 40 cm (l × w × ht)]. Adult moths of both species were supplied with a 10% sucrose solution accessed by a cotton wick in a sealed 100-ml plastic container.

General Procedure. All experiments included two main groups, experienced and naïve. Experienced groups were reared on different concentrations of pure compounds, added to the diet or sprayed on cabbage plants, until pupation. To exclude the influence of early adult experience, pupae were removed from the diet or cabbage plants they developed on and placed in plastic containers. As we did not rinse the pupae, the possibility of the presence of chemical on the pupal cuticle modifying adult behavior cannot be excluded. Pupae of *T. ni* were sexed and kept in separate plastic containers until emergence. Each plastic container had 10 pupae with a 10% sucrose solution accessed by a cotton wick in a sealed 100-ml plastic container for the adult moths. Pairs of moths (one male and one female) were introduced into each cell of the cages with a control and a treated cabbage leaf. Each leaf (approximately 100–110 cm^2) was sprayed with 0.5 ml of MeOH or a methanolic solution of the test compound on each side. Eggs were counted on each cabbage leaf at intervals described below. Old leaves were replaced by

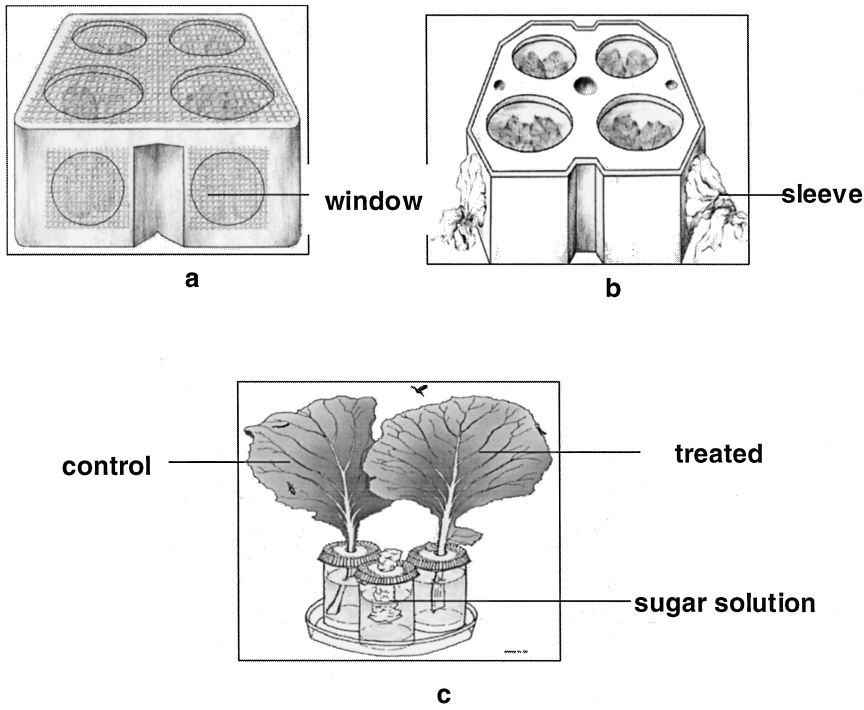

FIG. 1. Cage used for (a) cabbage looper, and (b) diamondback moths. Each cage consists of four cells with two leaves (control and treated) and sugar solution (c) per cell.

fresh ones after each observation. Two kinds of cages were used in the oviposition experiments (Figure 1a and b). Styrofoam containers used for packaging 4 × l MeOH bottles, were used as oviposition cages for *T. ni* females. Each styrofoam container provided four cages (cells) (24-cm length, 16.5-cm diameter). A circular window (9-cm diameter) was made on one side of each cell of the cage using a knife, and a plastic mesh (Quick Count, 7 mesh) was glued over it. A single plastic mesh was used to cover the top of the container and was secured by thumb-tacks to prevent moths from escaping.

Cages used for diamondback moths were similar to *T. ni* cages with a few modifications (Figure 1b). Styrofoam containers used for packaging solvent bottles (18-cm long, 15-cm diameter) were used. Windows were made in the same way as *T. ni* cages with the exception that gauze sleeves (25-cm long, 9-cm diameter) were attached to allow moths to be introduced without escaping. Tops of cages were covered with petri dishes (14.5-cm diameter) rather than mesh. Cages were placed in growth chambers under the following conditions: temperature ranging from 18 to 20°C, 50% RH, and 16:8 LD photoperiod.

Oviposition of T. ni Moths with Previous Exposure to Trans-Anethole from Neonates until Pupation. This experiment was designed to determine if larval experience of *T. ni* with a volatile deterrent compound, *trans*-anethole, changed the oviposition behavior of subsequent adults. Neonates <24 hr old were introduced onto diet containing 100 ppm fw *trans*-anethole in small styrofoam cups (360 ml), and allowed to feed until pupation. Naïve moths were reared on a diet containing MeOH only. A pair of moths was introduced into each cell of the cage containing a control (MeOH) and a treated leaf (0.8%*trans*-anethole) (Figure 1c). Plastic mesh was placed immediately over the cages to prevent moths from escaping. Plastic cups containing leaves were removed carefully from each cage, and the number of eggs was counted on each leaf after 48, 96, 144, and 192 hr.

Oviposition of T. ni Moths with Previous Exposure to trans-Anethole during the Last Instar Only. This experiment was conducted to determine the effect of the duration of larval experience with the oviposition deterrent on the subsequent ovipositing female. Experimental conditions were the same as above with the exception that freshly molted fifth instars were introduced onto diet containing 100 ppm of *trans*-anethole and allowed to feed until pupation. Naïve larvae were reared on diet containing MeOH only. Testing was done in the same manner as above, and the number of eggs was counted on each leaf as before.

Oviposition of T. ni Moths with Previous Exposure to Toosendanin from the Third Instar until Pupation. This experiment was conducted to determine if larval experience with a nonvolatile oviposition deterrent changed the oviposition behavior of subsequent moths. We intended to use moths with larval exposure to toosendanin either from neonates or the fifth instar as in our experiments with *trans*-anethole, but virus problems prevented us from doing so. Third instars of *T. ni* were introduced onto diet containing 50 ppm fw toosendanin and allowed to feed until pupation as in the previous experiments. Naïve moths were reared on diet containing MeOH only. Testing was done in the same manner as the previous experiments except that eggs were counted after 48 and 96 hr only.

Performance of the F_1 Larvae of T. ni (from Experienced and Naïve Moths) on Control and Treated Plants. This experiment was conducted to determine if there was a relationship between the choice of "experienced moths"(exposed to toosendanin as larvae) and the performance of their offsprings on preferred plants. Larval weights from the experienced and naïve moths on control and treated plants were compared. Experienced (50 ppm of toosendanin from third instar until pupation) and naive moths were released into separate cages containing a control and a treated plant (0.5% toosendanin) in each cage. Following egg laying (48 hr), each plant was placed in a separate cage. Eclosing larvae were allowed to feed. Larvae were weighed 8 days after eclosion.

Oviposition of Diamondback Moths with Previous Exposure to Toosendanin from Neonates until Pupation. This experiment was conducted to determine if a specialist herbivore acted in the same way as a generalist herbivore. Cabbage

leaves sprayed with a toosendanin solution (10 ppm) were placed into plexiglass cages with adult diamondback moths. The petioles of the cabbage leaves were immersed in 100-ml plastic containers with water to prevent desiccation. Neonates eclosing from eggs laid on the leaves were allowed to feed on cabbage *ad libitum* until pupation. Pupae were collected and placed in plastic containers (10 per container). Following emergence, six adult moths were introduced into each cell of the cage with the assumption that each group contained at least one female. Sexing pupae is difficult due to their small size. Cages remained covered with petri dishes and introduction of the moths and leaves was done through the gauze sleeves (Figure 1b). The naïve group was reared on cabbage foliage sprayed with MeOH only. Eggs were counted at 48 and 96 hr on the control and treated leaves for each group, using a binocular microscope.

Comparison of Oviposition Deterrence Indices (ODI) of the Experienced and Naive Groups. Both *trans*-anethole and toosendanin were oviposition deterrents for *T. ni* and *P. xylostella* (unpublished data). We wanted to see if there was a change in oviposition behavior of the experienced insects due to prior feeding experience (although we cannot exclude the possibility of chemical legacy affecting the choice of adult moths). ODI was calculated similar to FDI (Feeding deterrence index) (Isman et al., 1990) using the formula; ODI $= 100\ (C - T)/(C + T)$, where C and T are the number of eggs laid on the control and the treated leaves, respectively.

Data Analysis. Numbers of eggs laid on the control and treated leaves by the experienced and naïve groups were summed for all days. Data were analyzed on log transformed values by analysis of variance (ANOVA) using statistics software (Statistix 7, 2000). Where significant F values were found, Tukey's HSD, multiple comparison tests were conducted to test for significant differences between individual treatments (Zar, 1984). A mean oviposition deterrence index (ODI) was also calculated (as described in the previous section) and compared for experienced and naive groups in each experiment. Two-sample t-tests (on arcsine transformed data) were used to compare the oviposition deterrent responses of the experienced and naïve groups in each experiment. All experiments were replicated more than once except for the F_1 *T. ni* larvae performance experiment. Because there were no treatment effects between replicates, data were pooled. The alpha level used in all analyses was 0.05.

RESULTS

Oviposition of T. ni Moths with Previous Exposure to trans-Anethole from Neonates until Pupation. A two-factor ANOVA (group $\times$ treatment) on the number of eggs laid by experienced and naïve (groups) moths on the control and treated leaves (treatment) showed that there was no significant main effect of group. There was a main effect of treatment, $F(1, 116) = 14.68$, $P < 0.001$, with moths laying

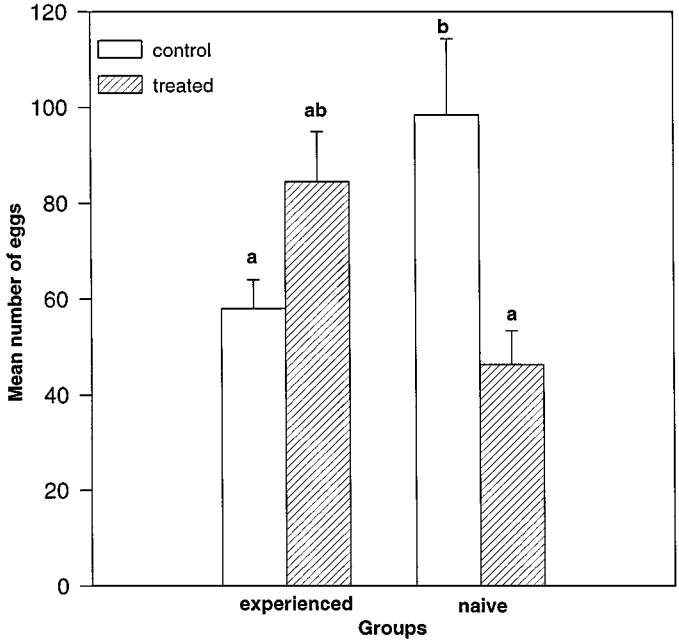

FIG. 2. Number of eggs laid by experienced (*trans*-anethole from neonates to pupation) and naïve (MeOH) *T. ni* moths when given a choice between control and treated (*trans*-anethole) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey's test, $P < 0.05$, $N = 3$ replications of 10 pairs of moths).

a greater number of eggs on the control leaves than the treated leaves (Tukey's test, $P < 0.05$). There was no group $\times$ treatment interaction, $F(1, 116) = 2.18$, $P < 0.142$ (Figure 2).

Comparison of the ODIs (oviposition deterrence indices) of the experienced and naïve groups showed that there was a decrease in oviposition deterrence to *trans*-anethole by the experienced moths when compared to the naive moths (two sample *t*-test, $df = 58$, $t = -6.43$, $P < 0.05$) (Table 1).

Oviposition of T. ni Moths with Previous Exposure to trans-Anethole from Fifth Instar until Pupation. A two-factor ANOVA (group $\times$ teatment) on the number of eggs laid by experienced and naïve groups of moths on the control and treated (*trans-anethole*) leaves showed that there was a main effect of group, $F(1, 116) = 16.02$, $P < 0.001$. Naive moths laid greater numbers of eggs than experienced moths (Tukey's test, $P < 0.05$). There was no main effect of treatment. There was a group $\times$ treatment interaction, $F(1, 116) = 18.49$, $P = 0.001$, with naïve moths laying greater numbers of eggs on control leaves than treated leaves (Tukey's test, $P < 0.05$) compared with the experienced moths (Figure 3).

TABLE 1. OVIPOSITION DETERRENCE INDICES OF EXPERIENCED AND NAÏVE CABBAGE LOOPER (*T. ni*) AND DIAMONDBACK MOTHS (*P. xylostella*)

Insects	Previous exposure (compounds)	Length of exposure	Oviposition deterrence index (%)	
			Experienced	Naïve
T. ni moth	*trans*-Anethole	Neonate to pupation	−16.3	34.58*
	trans-Anethole	Fifth instar to pupation	−8.55	20.71*
	Toosendanin	Third instar to pupation	−3.03	40.42*
P. xylostella moth	Toosendanin	Neonate to pupation	−21.91	10.93*

* Indicates significant difference between experienced and naïve groups (two-sample t-test, $P < 0.05$). Experienced moths showed a significant decrease in oviposition deterrence response compared with naïve moths. Negative values indicate that treated plants are preferred over control plants.

Comparison of the ODIs of experienced and naïve groups showed that there was a decrease in oviposition deterrence to *trans*-anethole by experienced moths when compared to naive moths (two sample t-test, $df = 58$, $t = -4.87$, $P < 0.05$) (Table 1).

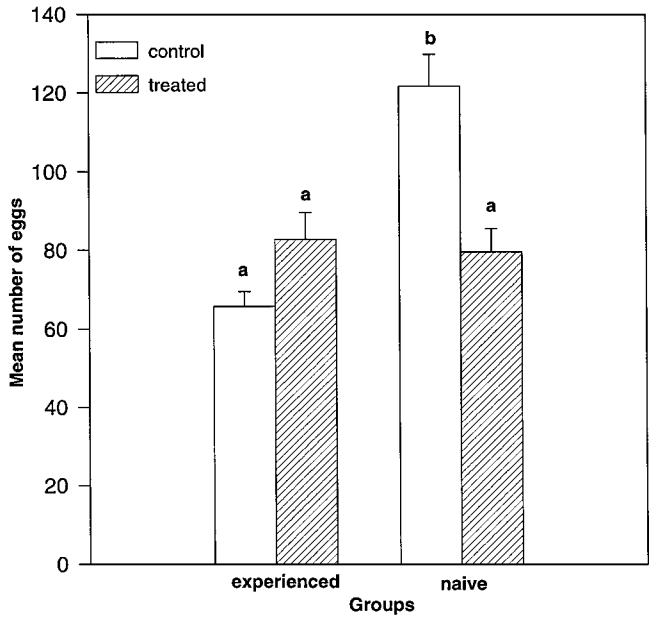

FIG. 3. Number of eggs laid by experienced (*trans*-anethole in last instar only) and naïve (MeOH) *T. ni* moths when given a choice between control and treated (*trans*-anethole) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey's test, $P < 0.05$, $N = 3$ replications of 10 pairs of moths).

Oviposition of T. ni Moths with Previous Exposure to Toosendanin, from Third Instar until Pupation. A two-factor ANOVA (group × teatment) on the number of eggs laid by experienced and naïve groups of moths on the control and treated (toosendanin) leaves showed that there was a main effect of group, $F(1, 156) = 10.49$, $P = 0.001$. Experienced moths laid greater numbers of eggs than naïve moths (Tukey's test, $P < 0.05$). There was a main effect of treatment, $F(1, 156) = 5.05$, $P = 0.026$, with greater numbers of eggs laid on the control leaves than treated leaves (Tukey's test, $P < 0.05$). There was a group × treatment interaction, $F(1, 156) = 9.56$, $P = 0.002$, with naïve moths laying significantly greater numbers of eggs on control leaves than treated leaves (Tukey's test, $P < 0.05$) compared with experienced moths (Figure 4).

Comparison of the ODIs of experienced and naïve groups showed that there was a decrease in oviposition deterrence to toosendanin by experienced moths when compared to naive moths (two sample t-test, $df = 72$, $t = -5.10$, $P < 0.05$) (Table 1).

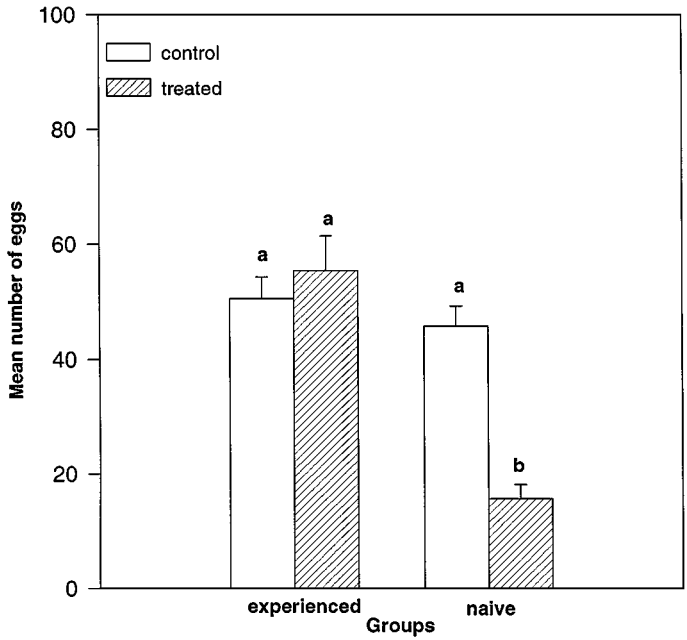

FIG. 4. Number of eggs laid by experienced (toosendanin from third instar to pupation) and naïve (MeOH) *T. ni* moths when given a choice between control and treated (toosendanin) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey's test, $P < 0.05$, $N = 4$ replications of 10 pairs of moths).

Performance of F_1 Larvae (from Experienced and Naïve Moths) on Control (MeOH) and Treated Plants (0.5% Toosendanin). A two-factor ANOVA (group × treatment) on the weight of experienced and naïve groups of larvae on control and treated (toosendanin) plants showed that there was no main effect of group. There was a main effect of treatment, $F(1, 196) = 41.95$, $P < 0.001$, with F_1 larvae weighing more on control plants than treated plants (Tukey's test, $P < 0.05$). There was a group × treatment interaction, $F(1, 196) = 33.77$, $P < 0.001$, with naïve larvae weighing more on control plants than treated plants (Tukey's test, $P < 0.05$) compared with experienced larvae, which showed no difference in weight on control and treated plants (Figure 5).

Oviposition of Diamondback Moths with Previous Exposure to Toosendanin from Neonates until Pupation. A two-factor ANOVA (group × treatment) on the number of eggs laid by experienced and naïve groups of moths on control and treated (toosendanin) leaves showed that there was a main effect of group, $F(1, 116) = 3.87$, $P = 0.05$. Experienced moths laid greater numbers of eggs than naïve moths (Tukey's test, $P < 0.05$). There was no main effect of treatment.

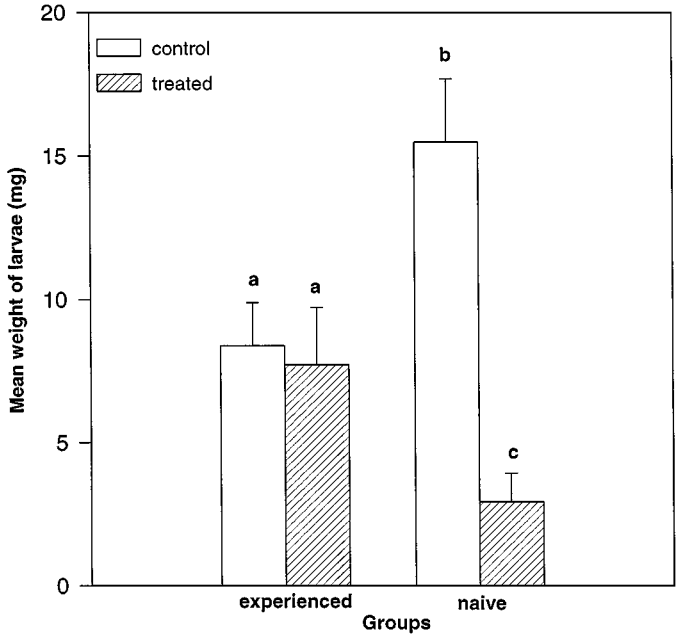

FIG. 5. Weight of F_1 larvae resulting from experienced (toosendanin from neonates to pupation) and naïve *T. ni* moths (with no exposure to toosendanin) on control and treated (toosendanin) cabbage plants. Bars marked by the same letters do not differ significantly (Tukey's test, $P < 0.05$, $N = 50$ larvae).

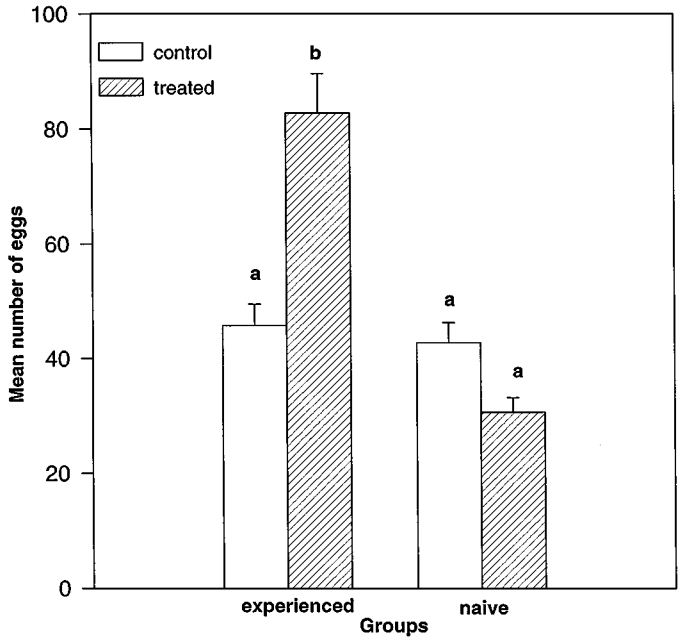

FIG. 6. Number of eggs laid by experienced (toosendanin from neonates to pupation) and naïve (MeOH) diamondback moths when given a choice between control and treated (toosendanin) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey's test, $P < 0.05$, $N = 3$ replications of 10 units of moths, each unit = 6 diamondback moths with the assumption that there is at least one female/unit).

There was a group $\times$ treatment interaction, $F(1, 116) = 6.12$, $P = 0.01$, with experienced moths laying greater numbers of eggs on treated leaves than control leaves (Tukey's test, $P < 0.05$) compared with naive moths with no difference in the number of eggs on control or treated leaves (Figure 6).

Comparison of the ODIs of the experienced and naïve groups showed that there was a decrease in oviposition deterrence to toosendanin by experienced moths when compared to naive moths (two sample t-test, $df = 58$, $t = -3.47$, $P < 0.05$) (Table 1).

DISCUSSION

The results demonstrate that feeding experiences of *T. ni* and *P. xylostella* larvae may have influenced the subsequent oviposition behavior of adult moths. "Experienced" female moths laid more eggs on plants treated with a deterrent compound, previously experienced as larvae. Although, in most cases there was

not a significant difference between the number of eggs laid on control and treated plants by experienced moths, naïve *T. ni* moths always laid greater numbers of eggs on control than treated plants. However, this trend was not seen in diamondback moths (Figure 6). Experienced diamondback moths laid more eggs on treated than control leaves. In contrast, naive diamondback moths did not discriminate between control and treated leaves, although preliminary experiments suggested that toosendanin was an oviposition deterrent at the concentration we used. Higher concentrations were avoided because of phytotoxic effects.

Comparison of ODIs of experienced and naïve moths showed that there was a decrease in oviposition deterrent response by experienced moths (Table 1). The fact that experienced moths in all cases exhibit a tendency towards decreasing oviposition deterrent response suggests that larval feeding experience with the oviposition deterrent compounds (or possibly chemical legacy) changed the oviposition choices of adult moths. This was true for both the generalist (*T. ni*) and the specialist (*P. xylostella*). It appears from our experiments that larval feeding experience or chemical legacy may be important in shaping host-selection choice of adult moths. We do not believe that selection played an important role in shaping behavior of our "experienced" groups of moths, because there was no appreciable mortality (or even evidence of any sublethal toxicity) of larvae during the training (exposure) period. The results may provide support for the "Hopkins' host selection principle" (Hopkins, 1917), although we cannot exclude the possibility of chemical legacy. Rietdorf and Steidle (2002) showed that when given the choice between seeds of maize and wheat in an olfactometer, adult granary weevils *Sitophilus granarius* preferred the odor of those plant seeds on which they had developed as larvae.

It is interesting that larvae of both the generalist (*T. ni*) and specialist (*P. xylostella*) herbivores showed a decrease in feeding deterrent response to toosendanin, following prolonged exposure (Akhtar, Rankin, and Isman unpublished data; Akhtar and Isman unpublished). Therefore, it is not surprising to see the failure of this compound to deter experienced moths that incurred no deleterious effects upon their growth as larvae. The information is apparently retained through metamorphosis from larva to adult, as is evident by the oviposition choice made by the experienced female moth. One possible explanation for this phenomenon is the storage of information obtained during larval development in the central nervous system (CNS) (Jermy et al., 1968). Alternatively, information about the larval environment can also be carried through to the adult stage by "chemical legacy" (Corbet, 1985). Traces of chemicals from the larval stage in the hemolymph of the insect or on the outside of the pupa might modify the perception and behavior of the adults.

In holometabolous insects such as some lepidopterans, the adult female must select a plant for oviposition that maximizes fitness of her offspring. Yet most adult lepidopterans are inequipped to obtain direct information on the quality of the food through feeding. The larva, in turn, while capable of making food choices

in an experimental situation, is often, in practice, without the opportunity to choose (Bernays and Chapman, 1994).

Female host plant choice is not only influenced by larval feeding capacities, but also by various ecological factors, such as host plant abundance (Rausher, 1980; Singer et al., 1989), search efficiency (Rausher, 1978; Courtney, 1983; Bernays, 1998) and predator effects (Camara, 1997). *Helicoverpa armigera* moths laid more eggs on the most abundant hosts in fields due to increased encounters (Cunningham et al., 1999). It would be interesting to know how larval feeding experience would affect the choice of an ovipositing female in the natural environment where there would be an interaction of a number of factors. A rigorously controlled laboratory test, although suitable for identifying particular behavioral mechanisms, does little to define the role such mechanisms play in shaping behavior in the field (Parmesan et al., 1995).

The results also show that duration of larval experience did not affect the oviposition behavior of adult moths. Other investigators have also sought to determine the developmental stage at which host plant preference is determined (Corbet, 1985; Anderson et al., 1995). It is believed that if an adult female does not experience any nutritional or noxious effect in relation to where she lays eggs (although, in our case she had experienced the compound as larvae), the adult may switch from using several hosts to using a single one, depending on plant abundance. She may also tend to lay eggs successively on the same plant type. If this pattern is repeated over generations in a region, larvae may be selected for fitness parameters on this one plant. If this is accompanied by reduced fitness on other plants, differential larval fitness may then place selection pressure on adult behavior. This dual process may hasten change (Bernays and Chapman, 1994). Host plant acceptance by an ovipositing female is mediated by a balance of sensory inputs from both positive and negative stimuli produced from potential host plants (Dethier, 1982; Huang and Renwick, 1993). The relative balance between these opposing cues can also be influenced by the internal physiological state of the insect, e.g., egg load, as well as other environmental factors (Dimock et al., 1991).

Our study also indicated that larvae from experienced female moths weighed almost the same when reared on the control and treated plants, suggesting that there was a positive correlation between larval growth performance and adult choice. Similar findings have been reported by others (Singer et al., 1994). There may be a flow of information from larvae to adult moths and then to F_1 larvae through the eggs, as indicated by larval performance on the chosen plant. It is possible that this transfer of information has been brought about through chemical trace compounds either within or on the egg (Corbet, 1985; Anderson et al., 1995).

The results demonstrate that there was a change in oviposition behavior of ovipositing females that resulted in the relative acceptability of previously unacceptable compounds. A hypothesis to explain the behavioral mechanism that may increase the acceptance of a host with which an individual has experience requires

some speculation as to the nature of learning involved (Cunningham et al., 1998). Some buttterflies such as *Battus philenor* (Papaj, 1986) and *Pieris rapae* (Traynier, 1984) learn through classical conditioning. These insects associated a chemical oviposition stimulus with a visual one from the oviposition substrate (leaf shape in *B. philenor* and color in *P. rapae*).

Other simple forms of learning can explain the observed change in host preference by an ovipositing female. Habituation, the waning of response to a stimulus upon repeated presentation (Szentesi and Jermy, 1989), can alter the response to oviposition deterrents in the host substrate, decreasing the net "inhibitory" stimuli, thus affecting acceptability and host preference (Cunningham et al., 1998). Although, learning may have been demonstrated in both generalist (*T. ni*) and the specialist (*P. xylostella*) herbivores in host selection behavior as a result of prior experience, we cannot establish the category of learning without specific testing. For example, if we propose that a decrease in response is a result of habituation, then a novel or noxious stimulus should dishabituate the response (Thompson and Spencer, 1966).

Anderson et al. (1995) showed that females of *S. littoralis* were no longer deterred by larval frass and potato extracts when they had spent their entire larval development on a potato-based diet, unlike naïve females. Three plausible explanations were selection, habituation, and induction of preference. An induction of preference (modification of feeding behavior leading to a change in host plant preference by prior feeding experience as described by Jermy et al., 1968) for oviposition deterrent compounds has also been shown in our studies. What the basis for this induction of preference is: selection, learning, chemical legacy, or a combination among these, we do not know at this point.

In summary, the response of the ovipositing female may be influenced by prior feeding experience of the larva (irrespective of the duration) or the chemical legacy. To rule out the possibility of chemical legacy, further experiments are planned (e.g., rinsing of pupae with water or other solvents to remove traces of chemical, or alternatively, administering chemicals to pupae from "naïve" larvae). The question remains whether or not it is possible to completely eliminate any traces of chemicals from the insect between the larval and adult stages. According to Corbet (1985), traces of chemicals obtained during the larval stage in the hemolymph of the insect or on the outside of the pupa might modify the perception and behavior of the adults. We can attempt to remove chemicals from the outside of pupae but it may not be possible to eliminate them from the hemolymph.

Both of the test compounds were larval feeding deterrents (Akhtar and Isman unpublished data) as well as oviposition deterrents for cabbage looper and diamondback moths . Larvae of both species showed a decrease in feeding deterrent response to toosendanin following prolonged exposure (Akhtar, Rankin and Isman unpublished data; Akhtar and Isman unpublished data). Data from the present study reveal that this decrease in feeding deterrent response may have been maintained

in F_1 larvae of cabbage loopers (as indicated by larval weight performance when reared on control and treated plants). The study also suggests a loss of oviposition deterrence as a result of larval experience. The positive correlation between female moth choice and larval growth performance indicates that female moths did not make a host choice deleterious to their offspring. This may be indicative of field situations when a generalist species like the cabbage looper and specialist species like the diamondback moth are forced to select a host plant that is out of their normal host range. This host plant switch can lead to acceptance of the plant as a host by the females in future generations as a result of larval experience (Anderson et al., 1995). This could be an important mechanism for extensions of host range through new feeding and oviposition preferences (Berenbaum and Zangerl, 1991; Bowers et al., 1992).

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MECHANISMS OF BEHAVIORAL ALTERATIONS OF PARASITOIDS REARED IN ARTIFICIAL SYSTEMS

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Abstract—A high quality of mass reared parasitoids is required for successful biological control of pest insects. Although the phenomenon of behavioral deterioration of parasitoids due to rearing in artificial conditions is well known, its significance is often underestimated, and the underlying mechanisms are poorly investigated. We quantified behavioral alterations of parasitoids reared in an artificial system vs. a natural system and elucidated some of the mechanisms involved. The model systems consisted of apple fruits (natural system) or an artificial diet devoid of apple (artificial system), the herbivore *Cydia pomonella*, and its larval parasitoid *Hyssopus pallidus*, a candidate biological control agent. Two parasitoid strains, one reared for 30 generations in the natural system and one in the artificial system, were compared by using the females' ability to respond to frass from codling moth caterpillars fed on apple fruits (apple-frass). The searching response of parasitoids reared in the artificial system compared to those reared in the natural system was reduced by an average of 53.2%. Gas chromatography–mass spectrometry (GC-MS) analyses of the two types of caterpillars' food and of the two corresponding types of frass showed that 15 compounds were present only in apple fruits and apple-frass, three compounds only in artificial diet and artificial-diet-frass, while four compounds were present in both frass types but not in the food sources. This suggests the presence of a food-derived and a host-derived component in the frass. Results from both bioassays and chemical analyses indicate that the kairomonal activity of the frass is due to both apple fruit and host components. The reduced response of parasitoids reared in artificial conditions might, therefore, be due to a lack of recognition of the apple fruit component. In a further experiment, the two parasitoid strains were reared in the opposite system for one generation. While the response to the host frass was significantly reduced in parasitoids that emerged from the artificial system, it was fully restored in parasitoids that emerged from

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the natural system. This indicates that the behavioral alteration was related to a learning process during ontogenesis rather than to a selection exhibited over generations.

Key Words—*Hyssopus pallidus*, parasitoid, *Cydia pomonella*, apple, frass, mass rearing, diet, artificial system, behavioral alteration, host searching.

INTRODUCTION

The success of inundative biological control with parasitoids depends, among other factors, on an efficient mass rearing of insects, with the purpose of producing large numbers of agents of high quality. Quality in terms of high growth, fecundity, reproduction, and survival has often been studied (Bigler, 1994; Thompson and Hagen, 1999). In contrast, little consideration has been given to quality in terms of behavioral performance, despite its significance for biological control (Noldus, 1989; van Lenteren, 1991). For a good performance after release in the field, parasitoids should maintain natural behavioral traits such as an efficient host searching and parasitization behavior. This implies an appropriate recognition and perception of relevant chemical cues.

It is well known that rearing parasitoids under artificial laboratory conditions might lead to the alteration of several behavioral traits (Bautista and Harris, 1997 and references therein), including the deterioration of host searching behavior (Lewis et al., 1990; Vet et al., 1990; Geden et al., 1992). The dramatic consequences of this quality decline have been described, and some attempts have been undertaken to improve the performance of parasitoids affected by behavioral deterioration with conditioning (Udayagiri, 1996, and references therein; Zaki et al., 1998), with priming to semiochemicals prior to release (Hare and Morgan, 1997), or with field manipulations (Lilley et al., 1994). However, in spite of the recurrence of behavioral alterations (Nordlund, 1998), the mechanisms underlying the phenomenon are still little explored.

The aim of this project was to quantify behavioral changes of insectary-reared parasitoids, and to elucidate the underlying mechanisms. As a study system, we used the parasitic wasp *Hyssopus pallidus* (Askew) (Hymenoptera: Eulophidae), a gregarious larval ectoparasitoid and candidate biological control agent of the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), a major pest of apple fruits, *Malus domestica* (Borkh.) (Rosaceae). *Hyssopus pallidus* has a high fecundity, a rapid development rate, and a strongly female-biased sex ratio (Zaviezo and Mills, 1999; Tschudi-Rein and Dorn, 2001). This parasitoid species has the special ability to enter infested apples through the calyx or the channel made by the host caterpillar, to irreversibly paralyze and then parasitize the host before it leaves the fruit (Mattiacci et al., 1999). As a consequence, *H. pallidus* has the potential to reduce codling moth population levels in orchards and could be a useful alternative to manual removal of infested fruits (Mattiacci et al., 1999). For successful host

location, adult wasps rely on a strong response to host frass traces on the surface and in the channels of the infested fruit.

In a first experiment, we compared two strains of parasitoids. One was reared on caterpillars fed on apple fruits, i.e., in a "natural system." The other was reared on caterpillars fed on an artificial diet, i.e., in an "artificial system," as a model for a commercial mass rearing. The host searching behavior of the two strains was compared in terms of response of adult females to chemical stimuli from the host frass (host feces and silk). Behavioral bioassays were coupled with the identification of the chemicals in both caterpillars' frass and food sources through solvent extraction and gas chromatography–mass spectrometry.

In a second experiment, we reared the two strains in the opposite system for one generation. The purpose was to study the mechanism of behavioral alteration and its reversibility. Parasitoids from the natural system were maintained during development in the artificial system to test whether behavioral alterations are due to a selection pressure over many generations or whether they occur at an ontogenetical level. *Vice versa*, parasitoids from the artificial system were maintained during development in the natural system to test whether the behavioral deterioration is reversible.

METHODS AND MATERIALS

Insect Rearing. Starting from a single colony of *H. pallidus* (Tschudi-Rein and Dorn, 2001), two strains were obtained by rearing parasitoids under different conditions. An "artificial diet strain" ("AD strain") was obtained in the artificial system and an "apple strain" in the natural system as detailed below. Bioassays were carried out with the 30th–32nd generation after split rearing.

AD strain parasitoids were reared on *C. pomonella* caterpillars fed on a wheat germ-based artificial diet ("AD") devoid of apple fruit material (Huber et al., 1972). Newly hatched first instar caterpillars from a laboratory colony (described by Mattiacci et al., 1999) were placed individually in a plastic box (18 $\times$ 18 $\times$ 10 mm) filled with 3.4 g artificial diet. Boxes were kept at 26 $\pm$ 1°C, 55 $\pm$ 10% RH, and 18:6 LD. After 16–21 days, 5th instars were offered to 4–7 days old mated female parasitoids in glass vials at a ratio of 1 parasitoid/host, with a droplet of honey as food. New adult parasitoids emerged 14–17 days later. Upon emergence, they were removed from the vials containing the dead host caterpillar, transferred into Plexiglas cages (25 $\times$ 25 $\times$ 25 cm), and maintained with water and undiluted honey. During parasitization and development, parasitoids were not exposed to plant cues.

Apple strain parasitoids were reared on *C. pomonella* larvae fed on organically grown apple fruits (cv "Bohnäpfel"). For infestation, first instars were placed on the surface of apples. Infested fruits were kept under the same conditions as infested

AD-boxes. After 16–21 days, apples were carefully dissected. Fifth instars were offered to the parasitoids as described for the AD strain. To provide parasitoids with chemical cues of the natural host habitat, a slice of apple fruit (approx. 30 mg) was added to the vials.

Bioassay. Bioassays were performed with 4–7 day old mated parasitoid females. Immediately prior to the bioassay, parasitoids were transferred individually from the emergence cages into glass vials (2 cm diam, 0.8 cm height). One half of a circular filter paper (Whatman, 1.3 cm, grade 1, CAT No. 1001013; Merck, Dietikon, Switzerland) was treated with 20 μl of solvent extracts, and left uncovered for 3 min to allow complete evaporation of the solvent. Subsequently, it was transferred onto a glass petri dish (5 cm diam). The open end of the glass vial with the wasp was positioned over the cue source. The observation started when the parasitoid began to move in the arena and ended 10 min later. The behavioral parameter *searching*, defined as intensive antennal examination on the filter paper, was recorded. The total searching time was measured with a stopwatch by accumulating periods of behavioral activity. Bioassays were conducted from 11:00 a.m. to 17:00 p.m. under natural daylight conditions (900–1800 lux), at 21 $\pm$ 1°C and 50 $\pm$ 5% RH.

Chemical Analyses. Chemical analyses were performed with a gas chromatograph (Hewlett-Packard 6890, Atlanta, USA), equipped with an HP-1 cross-link methyl silicone capillary column (30 m, 0.25 mm internal diam, 0.25 μm film thickness). This was coupled to a mass-spectrometer (Hewlett-Packard 5973, Atlanta, USA) with a mass selective FID-detector. Helium (purity $\geq$ 99.99%) at a constant pressure of 90 kPa was used as a carrier gas. Samples of 1 μl were injected manually in a splitless mode at an initial inlet temperature of 250°C. The oven temperature was held at 50°C for 2 min, then increased to 300°C at a rate of 8°C/min, and then held at 300°C for 15 min. A solvent delay of 4.5 min was programmed, and a purge flow of 50 ml/min was started after 2 min. Compounds were identified by comparison of the mass spectra with those in the mass spectra database NIST98 and in our own library. The identity of compounds was confirmed by coinjection of authentic samples. Synthetic compounds were obtained from Fluka Chemie AG (Buchs, Switzerland) and were $\geq$99.5–99.8% pure. For quantification, the internal standards *trans*-4-decen-1-al (IS1) and *cis*-11-hexadecen-1-yl acetate (IS2) were added to the sample at a ratio of 20 ng/μl. To improve detectability of compounds present in very small amounts, samples were concentrated tenfold and reinjected.

Frass Extracts. Preliminary experiments were conducted to obtain a bioactive frass extract. Frass material expelled from infested apples was air dried at 55 $\pm$ 10% RH and at room temperature for 5 days. Frass was subsequently extracted for 18 hr with solvents of increasing polarity: *n*-hexane, diethyl ether, acetone, and methanol (purity $\geq$99.5–99.8%). The four extracts were used for the bioassay described above to monitor the bioactivity. Additionally, dose-response tests were carried out for each extract. As a control, the bioactivity of fresh host frass expelled

by fifth instars feeding on apple fruits was tested. The most active extract was the one obtained with diethyl ether, followed by hexane.

Attempts to isolate the active component of the frass failed, as fractions made with mixtures of diethyl ether and hexane with increasing proportions of diethyl ether were all bioactive, though their chemical composition was different (data not shown). For all subsequent experiments the diethyl ether extract was used. The chemical composition of this extract was characterized by GC-MS as described above.

Comparison of the Two Parasitoid Strains. Cue sources. Parasitoids of both strains were offered diethyl ether extracts from "apple-frass" produced by caterpillars feeding on apple fruits, "AD-frass" produced by caterpillars feeding on artificial diet, and diethyl ether (purity $\geq$99.8%) as a solvent control. As differences were found between the response of the two strains to these cue sources, parasitoids were subsequently tested with diethyl ether extracts from apple fruit and artificial diet, and diethyl ether as solvent control. For both experimental sets, 20 female wasps were tested for each treatment and strain. Differences between strains and treatments were tested with two-way analysis of variance, followed by Student–Newman–Keuls pairwise multiple comparison (Zar, 1999).

Extractions. Both frass types were air dried for 2 days and extracted in diethyl ether (purity $\geq$99.8%) at a ratio of 0.13 g/ml solvent. Freshly prepared artificial diet was extracted at a ratio of 0.2 g/ml solvent, considering the fresh/dry ratio of 1/0.65 (w/w). For the preparation of the apple fruit extract, skin, pulp, and seeds were first extracted separately, as these organs are likely to have a different chemical composition (Guadagni et al., 1971). For the extracts, 0.2 g/ml of fresh skin and fresh pulp were grated directly in to diethyl ether, while seeds were cut in thin slices of 0.2–0.3 mm. The three apple organs were recombined to obtain an extraction with similar chemical composition to that of the apple component of the apple frass. The final extract was made from a mixture of 8.75% skin, 85% pulp, and 6.25% seeds at a ratio of 0.2 g/ml. On the basis of preliminary dose-response experiments, extracts from both food sources were concentrated tenfold in order to elicit a high response.

Mechanisms of Behavioral Alterations. Female parasitoids from the apple strain were transferred into the artificial system for parasitization, i.e., they were allowed to parasitize a caterpillar that had been feeding on artificial diet. The offspring was maintained in the artificial system throughout development. *Vice versa*, female parasitoids from the AD strain were transferred into the natural system, and their offspring was maintained in this system during development. The behavior of emerging adults was assessed with the bioassay described above, by testing the response of females to an extract of apple-frass. Both parasitoids from the apple strain and from the AD strain reared in their original system were used as control. For each treatment, 20 wasps were tested. Data were analyzed by two-way ANOVA followed by Bonferroni method for pairwise comparison (Zar, 1999).

RESULTS

The average amount of frass produced by one fifth-instar *C. pomonella* caterpillar during 24 hr was 60.5 ± 4.1 mg (mean ± SE, $N = 10$). Fresh frass and diethyl ether extracts of frass from caterpillars feeding on apple fruits were both attractive for *H. pallidus*. The highest response to frass extract was obtained with 20 μl of extract containing an amount of frass equivalent to that produced by a caterpillar in approx. 2 hr. Chemical analyses of the frass extract revealed 36 major compounds including fatty acids, *n*-alkanes, terpenes, sterols, aldehydes, fatty esters, and tocopherols (Table 1).

Comparison of the Two Parasitoid Strains. Extracts from the three apple fruit organs differed in their chemical composition (Table 2). The skin contained the majority of compounds, and in the highest quantity. The pulp contained only few compounds in relatively low quantities. Alpha-farnesene and esters were found only in the skin. Fatty acids and *n*-alkanes were detected mainly in the skin, only traces in other fruit parts. Sterols and tocopherols, on the contrary, were typical of seeds, except for β-sitosterol, which was mainly found in the skin. Squalene was found in all three apple fruit organs.

The apple-frass extract and the AD-frass extract differed in their chemical composition (Figure 1). Several compounds from apple-frass were not detected in AD-frass and *vice versa*. The terpene α-farnesene, the four aldehydes, 2-heptenal, nonanal, decanal, and 2,4-decadienal, and the five *n*-alkanes, *n*-heneicosane, *n*-tricosane, *n*-pentacosane, *n*-octacosane, and *n*-hentriacontane were found exclusively in apple-frass and apple fruits. Sorbic acid, methylparaben, and cholesterin were found only in AD-frass and artificial diet. Gamma-sitosterol, stigmastenone, and two unknown compounds were found in both frass types and not in the caterpillars' food sources. Both unknown compounds were also detected in diethyl ether extracts from silk of *C. pomonella* (data not shown).

Both types of caterpillar frass and both types of caterpillar food source elicited significant searching activity of parasitoids. However, the response of the two parasitoid strains differed depending on the frass type or food type offered as chemical stimuli (Figure 2). Apple strain parasitoids searched nearly twice as long (188%) on apple-frass as the AD strain (Mann–Whitney $t = 156.5$, $P < 0.01$, $N = 20$), while AD strain parasitoids searched as long on apple-frass as on AD-frass ($t = -0.241$, $P = 0.811$, $N = 20$) (Figure 2.1). Apple strain parasitoids showed a much stronger response to apple fruit cues than AD strain parasitoids (Kruskal–Wallis ANOVA $p = 3$, $q = 8.935$, $P < 0.05$) (Figure 2.2). *Vice versa*, AD strain parasitoids had a significantly higher response to artificial diet cues than to apple fruit cues (Kruskal–Wallis ANOVA $p = 2$, $q = 3.564$, $P < 0.05$).

Mechanisms of Behavioral Alterations. Parasitoids from the apple strain were reared for one generation in the artificial system. The response of emerged females to apple-frass was reduced on average by 49.2% (two-way ANOVA, Bonferroni,

TABLE 1. MAJOR COMPOUNDS (IN ng [a]) FOUND IN 1 μl DIETHYL ETHER APPLE-FRASS EXTRACT

Chemical class	Peak[b]	Compound	Apple-frass extract
Aldehydes	1	2-Heptenal	2.202 ± 1.123
	3	Nonanal	0.879 ± 0.511
	5	Decanal	0.303 ± 0.163
	6	2-Decenal	2.055 ± 1.142
	7	2.4-Decadienal	2.564 ± 1.702
Terpenes	10	α-Farnesene	48.801 ± 22.34
	32	Squalene	9.903 ± 6.335
n-alkanes	18	*n*-Heneicosane (*n*-C21)	0.558 ± 0.299
	22	*n*-Docosane (*n*-C22)	3.667 ± 1.997
	24	*n*-Tricosane (*n*-C23)	2.571 ± 1.327
	27	*n*-Tetracosane (*n*-C24)	3.885 ± 1.963
	28	*n*-Pentacosane (*n*-C25)	4.012 ± 2.331
	29	*n*-Hexacosane (*n*-C26)	7.115 ± 4.015
	30	*n*-Heptacosane (*n*-C27)	9.903 ± 4.982
	31	*n*-Octacosane (*n*-C28)	5.794 ± 3.258
	33	*n*-Nonacosane (*n*-C29)	50.036 ± 27.77
	34	*n*-Triacontane (*n*-C30)	4.030 ± 2.236
	36	*n*-Hentriacontane (*n*-C31)	4.709 ± 2.558
	39	*n*-Dotriacontane (*n*-C32)	1.952 ± 0.885
Fatty acids	2	Hexanoic acid	0.303 ± 0.201
	15	Palmitic acid	12.279 ± 7.159
	19	Linoleic acid	113.545 ± 61.52
	20	Elaidic acid	70.412 ± 38.26
	21	Stearic acid	22.861 ± 16.55
Fatty esters	8	Hexanoic acid hexyl ester	0.758 ± 0.410
	11	Fatty acid ester I	0.133 ± 0.088
	14	Palmitic acid methyl ester	0.030 ± 0.024
	17	Linoleic acid methyl ester	0.576 ± 0.287
	23	Fatty acid ester II	0.485 ± 0.256
	26	Fatty acid ester III	4.042 ± 2.203
Tocopherols	35	γ-Tocopherol	0.418 ± 0.258
	38	α-Tocopherol	1.036 ± 0.866
Unknown	40	Unknown I	53.515 ± 22.26
	41	Unknown II	89.103 ± 40.25
Sterols	42	γ-Sitosterol	15.448 ± 9.933
	45	Stigmastenone	0.303 ± 0.185

[a] All samples were analyzed in triplicate and are expressed as mean values (± SE).
[b] Peak number corresponds to that of Figure 1.

$t = 6.296$, $P < 0.05$, $N = 20$) (Figure 3). The response was as low as that of AD strain parasitoids reared for 32 generations in the artificial system (two-way ANOVA, Bonferroni, $t = -0.091$, ns, $N = 20$). *Vice versa*, parasitoids from the AD strain were reared for one generation in the natural system. The response of

TABLE 2. MAJOR COMPOUNDS (IN ng [a]) FOUND IN 1 μl DIETHYL ETHER EXTRACT OF APPLE FRUIT PREPARED FROM PULP, SEEDS, AND SKIN IN THE RATIO 0.85:0.0625:0.0875, AND CORRESPONDING VALUES FOR EACH OF THE THREE APPLE ORGANS

Chemical class	Peak[b]	Compound	Recomposed apple fruit extract	Apple organs		
				Pulp	Seeds	Skin
Aldehydes	1	2-Heptenal	0.098 ± 0.043	—	0.098 ± 0.043	—
	3	Nonanal	0.018 ± 0.005	—	—	0.018 ± 0.005
	5	Decanal	0.071 ± 0.026	—	—	0.071 ± 0.026
	7	2,4-Decadienal	0.034 ± 0.021	—	—	0.034 ± 0.021
Terpenes	10	α-Farnesene	1.999 ± 0.722	—	—	1.999 ± 0.722
	32	Squalene	3.875 ± 1.875	0.591 ± 0.302	0.819 ± 0.424	2.465 ± 1.246
n-alkanes	13	*n*-Nonadecane (*n*-C19)	0.029 ± 0.014	—	—	0.029 ± 0.014
	16	*n*-Eicosane (*n*-C20)	0.044 ± 0.019	—	—	0.044 ± 0.019
	18	*n*-Heneicosane (*n*-C21)	0.098 ± 0.058	—	—	0.098 ± 0.058
	24	*n*-Tricosane (*n*-C23)	0.216 ± 0.153	—	—	0.216 ± 0.153
	28	*n*-Pentacosane (*n*-C25)	0.461 ± 0.197	—	—	0.461 ± 0.197
	30	*n*-Heptacosane (*n*-C27)	9.056 ± 5.117	—	—	9.056 ± 5.117
	31	*n*-Octacosane (*n*-C28)	1.866 ± 0.887	—	—	1.866 ± 0.887
	33	*n*-Nonacosane (*n*-C29)	52.238 ± 31.41	0.178 ± 0.088	0.061 ± 0.032	51.999 ± 29.59
	36	*n*-Hentriacontane (*n*-C31)	0.551 ± 0.289	—	—	0.551 ± 0.289
Fatty acids	2	Hexanoic acid	0.004 ± 0.003	—	0.004 ± 0.003	—
	12	Myristic acid	0.074 ± 0.045	—	—	0.074 ± 0.045
	15	Palmitic acid	1.116 ± 0.785	—	0.018 ± 0.011	1.098 ± 0.569
	19	Linoleic acid	5.043 ± 3.214	—	—	5.043 ± 3.214
	20	Elaidic acid	1.817 ± 1.106	—	—	1.817 ± 1.106
	21	Stearic acid	1.751 ± 0.697	—	—	1.751 ± 0.697
	25	Eicosanoic acid	0.724 ± 0.385	—	—	0.724 ± 0.385
Fatty esters	8	Hexanoic acid hexyl ester	0.129 ± 0.078	—	—	0.129 ± 0.078
	14	Palmitic acid methyl ester	0.142 ± 0.098			0.142 ± 0.098
	17	Linoleic acid methyl ester	0.151 ± 0.076	—	—	0.151 ± 0.076

Tocopherols	35	γ-Tocopherol	0.134 ± 0.094	—	0.134 ± 0.094	—
	38	α-Tocopherol	0.148 ± 0.101	—	0.148 ± 0.101	—
Sterols	43	Stigmasterol	0.036 ± 0.021	—	0.036 ± 0.021	—
	44	β-Sitosterol	6.291 ± 2.789	—	0.696 ± 0.315	5.594 ± 2.222

[a] All samples were analyzed in triplicate and are expressed as mean values (± SE).
[b] Peak number corresponds to that of Figure 2.

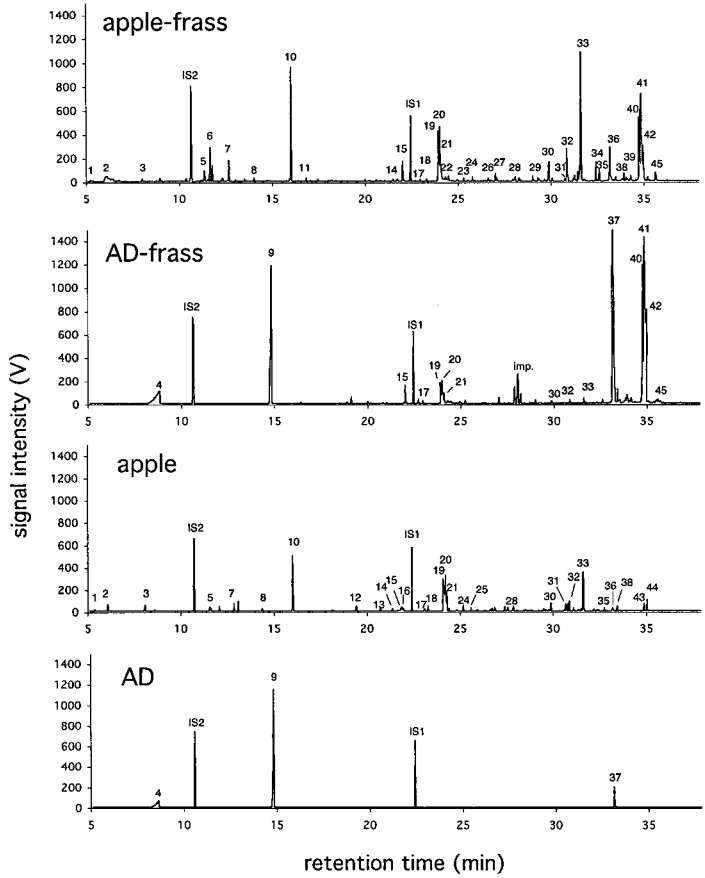

FIG. 1. Total ion chromatograms of diethyl ether extracts from apple-frass, artificial-diet-frass (AD-frass), apple fruit, and artificial diet (AD). Numbered peaks correspond to the following compounds: (1) 2-heptenal[a], (2) hexanoic acid[a], (3) nonanal[a], (4) sorbic acid[m], (5) decanal[a], (6) 2-decenal, (7) 2,4-decadienal[a], (8) hexanoic acid hexyl ester[a], (9) methylparaben[m], (10) α-farnesene[a], (11) unidentified fatty acid ester I, (12) myristic acid, (13) *n*-nonadecane (*n*-C19), (14) palmitic acid methyl ester[a], (15) palmitic acid, (16) *n*-eicosane (*n*-C20), (17) linoleic acid methyl ester, (18) *n*-heneicosane (*n*-C21)[a], (19) linoleic acid, (20) elaidic acid, (21) stearic acid, (22) *n*-docosane (*n*-C22), (23) unidentified fatty acid ester II, (24) *n*-tricosane (*n*-C23)[a], (25) eicosanoic acid, (26) unidentified fatty acid ester III, (27) *n*-tetracosane (*n*-C24), (28) *n*-pentacosane (*n*-C25)[a], (29) *n*-hexacosane (*n*-C26), (30) *n*-heptacosane (*n*-C27), (31) *n*-octacosane (*n*-C28)[a], (32) squalene, (33) *n*-nonacosane (*n*-C29), (34) *n*-triacontane (*n*-C30), (35) γ-tocopherol[a] (36) *n*-hentriacontane (*n*-C31)[a], (37) cholesterol[m], (38) α-tocopherol[a], (39) *n*-dotriacontane (*n*-C32), (40) unknown I[f], (41) unknown II[f], (42) γ-sitosterol[f], (43) stigmasterol, (44) β-sitosterol, (45) stigmastenone[f]. Compounds detected exclusively in apple fruit and apple-frass[a]; compounds detected exclusively in AD and AD-frass[m]; compounds detected exclusively in apple-frass and AD-frass[f]. IS1: *trans*-4-decen-1-al, IS2: *cis*-11-hexadecen-1-yl acetate.

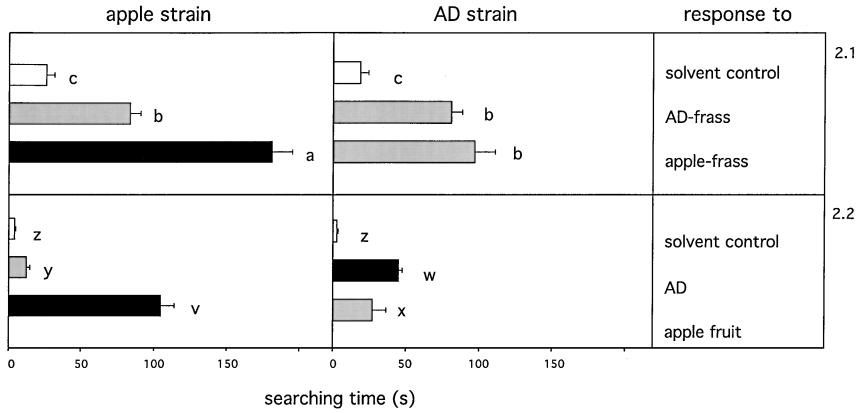

FIG. 2. Response of parasitoids from the apple strain and artificial diet (AD) strain to (2.1) diethyl ether extracts of both frass types, apple-frass and AD-frass, and (2.2) to diethyl ether extracts of both hosts' food types, apple fruit and AD. Bars indicate the average time (±SE) spent searching on each treatment in single-choice bioassays. Data from frass extracts and from food extracts were analyzed separately. Letters indicate significant differences among strains and treatments ($N = 20$).

emerged females to apple frass was increased on average by 235.3% (two-way ANOVA, Bonferroni, $t = 8.376$, $P < 0.05$, $N = 20$). This response reached the original level obtained with apple strain parasitoids reared for 32 generations in the natural system (two-way ANOVA, Bonferroni, $t = -2.170$, ns, $N = 20$). The response of parasitoids was affected only by the system in which they developed, and not by the strain of their mother (two-way ANOVA strain: $F = 2.56$, $P = 0.114$; treatment: $F = 107.63$, $P < 0.001$).

DISCUSSION

The ability of the parasitoid *H. pallidus* to find its host *C. pomonella* upon release in the field depends, among other factors, on an optimal response to host frass. In fact, females find caterpillars concealed in the fruit mainly by detecting frass on the surface of the fruit and by following frass traces inside the fruit (Mattiacci et al., 1999). The present study shows a strong response of *H. pallidus* to both fresh apple-frass and to frass extracts prepared with diethyl ether. This underlines the fundamental role of chemical stimuli in the frass for triggering a response of parasitoids. Unlike in other systems, where active mixtures elicited lower responses of parasitoids compared to the original cue sources (Turlings et al., 1990; Dutton et al., 2000), here, frass extracts maintained the bioactivity of fresh frass material.

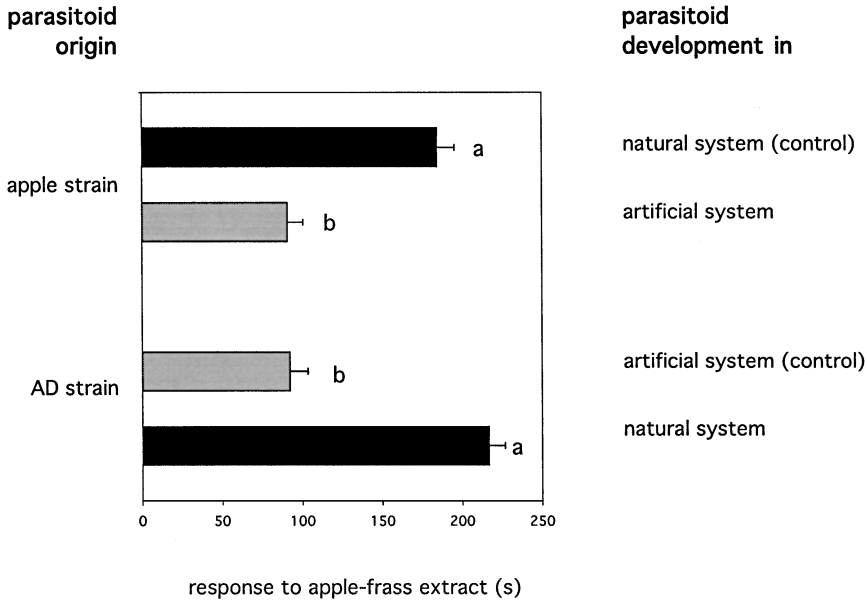

FIG. 3. Deterioration of the behavioral response of *H. pallidus* females to host frass cues during ontogenesis: response of parasitoids from the apple strain after developing in the artificial vs. the natural system. Reversibility of the behavioral deterioration during ontogenesis: response of parasitoids from the artificial diet strain (AD strain) after developing in the natural vs. the artificial system. In the natural system, apple fruits were used for rearing the host *C. pomonella*; in the artificial system, apple fruits were replaced by an artificial diet devoid of apple cues. Bars indicate the average time (±SE) spent searching on a diethyl ether apple-frass extract. Letters indicate significant differences among parasitoid groups ($N = 20$).

The bioactivity of frass for *H. pallidus* was affected by the caterpillar's food. Apple strain parasitoids preferred frass from apple-feeding hosts to frass from AD-feeding hosts. This indicates that apple-related compounds in the frass play an important role in eliciting a response of parasitoids. The significance of the host plant for the kairomonal activity of the herbivore's frass has been shown in different systems (Ramachandran et al., 1991; Eller et al., 1992; Thibout et al., 1995).

The present study demonstrates that the conditions in which parasitoids are reared are decisive for the behavioral performance of emerging adults. The comparison of the two parasitoid strains reveals a marked difference between the natural and the artificial system. Parasitoids reared in the natural system showed a high response to both apple and apple-frass cues. This is consistent with the results of many studies, providing ample evidence of the high innate response of parasitoids

to both the food plants of their hosts (Udayagiri and Jones, 1992; Cortesero et al., 1993; Dutton et al., 2002) and the plant-host complex (Vet and Dicke, 1992). Surprisingly, parasitoids reared in the artificial system without apple showed a dramatically reduced response to both apple and apple-frass cues. This may negatively affect their performance in biological control programs, in terms of habitat location and host location, respectively. The present investigation suggests that the response of this parasitoid species towards the plant–host complex is not entirely innate, but there is a phenotypic plasticity towards chemicals in the rearing environment. The behavioral response of the adult wasps becomes, therefore, an important parameter for assessing the quality of parasitoids, and a valuable tool for controlling the suitability of the rearing procedure. Chemical analyses of caterpillar frass and food sources revealed that many compounds in the apple-frass were present only in apple fruits, suggesting their plant origin. Similarly, some compounds in the AD-frass were present only in AD, suggesting their origin from the artificial diet. Other compounds were found in both types of frass, but not in the caterpillar food sources. These compounds are likely to be related to the host caterpillar. Most compounds identified in the apple-frass, such as terpenes and the *n*-alkanes, were also identified in apple skin. However, tocopherols and one sterol were only detected in apple seeds. The caterpillar feeds on the apple skin, then penetrates into the fruit, proceeds to its center, and eats the seeds. Our data indicate that initial and late feeding stages are of particular significance for the chemical composition of the caterpillar frass. The two terpenes and the three dominant *n*-alkanes that we identified in both apple fruit and apple-frass have previously been reported from apple plants and fruits (Dutton et al., 2000, 2002; Hern and Dorn, 2001). The two tocopherols that we identified in apple fruit and apple-frass have been detected recently in apple fruits (Schmitz and Noga, 2001). Unlike the two phytosterols β-sitosterol and stigmasterol, which we found only in the apple fruits, and likely to be metabolized and transformed by caterpillars (Svoboda and Lusby, 1994), γ-tocopherol and α-tocopherol may have been excreted by *C. pomonella* larvae directly. Further, the two unknown compounds that we identified exclusively in the two frass types were found to be dominant compounds of the silk of *C. pomonella* larvae (data not shown). These results indicate two categories of compounds in the frass: compounds derived from the caterpillar food (food components), ingested and released in the frass, and compounds derived from the caterpillar itself (host components). Behavioral experiments provide evidence that the kairomonal activity of the frass is due to the activity of both components. The low response of AD strain parasitoids to apple-frass is probably due to a recognition of host components only, but not of apple components. The high response of apple strain parasitoids is probably the result of a recognition of both apple and host components. Fruit cues are, therefore, not only important for host handling and oviposition behavior (Mattiacci et al., 2000), but also for promoting host finding in this species.

Further experiments were carried out to analyze whether the observed behavioral alterations are a direct consequence of the chemical composition of the rearing environment. In particular, we investigated whether the individual experience of parasitoids with fruit cues during development may be solely responsible for their response to host frass cues as adults. When parasitoids from the natural system were exposed to the artificial system during development, emerged adults showed a dramatic reduction in response. This indicates that behavioral deterioration was due to a lack of individual experience with apple cues during ontogenesis and not to a selection pressure. *Vice versa*, when parasitoids from the artificial system were exposed to the natural system during development, adult parasitoids showed the original high level of response. An experience with apple cues during ontogenesis was sufficient to revert completely the previous behavioral deterioration. The option to restore host searching ability within the developmental time of a single generation opens interesting perspectives for the quality management of *H. pallidus*.

In many parasitoid species, the response to host-related cues is considered to be genetically fixed. In contrast, the response to plant-related cues is considered flexible and subject to learning (Lewis and Tumlinson, 1988; Vet and Dicke, 1992). A remarkably high genetic component has been found for the response of the parasitoid *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) to *Pieris brassicae* (L.) (Lepidoptera: Pieridae) infested cabbage plants, i.e., to an unseparated host–plant complex (Gu and Dorn, 2000). The present study indicates that the response of *H. pallidus* to the host components of frass might be genetically fixed. The response to the apple components of frass, however, seems to depend on the mode of parasitoid rearing, and suggests a potential impact of learning. Parasitoids may have learned the apple components of the frass through association of apple cues with the presence of the host, a process that is expected to occur during emergence or in the early adult stage (Kester and Barbosa, 1991; Turlings et al., 1993; Storeck et al., 2000). However, unlike in many cases of classical conditioning (Kerguelen and Cardé, 1996), the association did not require a reward in terms of oviposition. Further, experimental evidence suggests a potential influence of chemical stimuli experienced in rearing on the degree of adult response to the same stimuli. Coincidently, parasitoids exposed to apple cues during rearing responded strongly to apple cues as adults, and parasitoids exposed to artificial diet cues responded strongly to artificial diet cues. This is reminiscent of a process of sensitization, in which continuous exposure to chemical cues may have enhanced and reinforced the adult response to these cues. However, sensitization *sensu* Papaj and Prokopy (1989) presumes an innate response to the stimulus that is not expected for artificial diet. Further work is in progress to define the learning mechanisms involved in detail and particularly to elucidate when the parasitoids learn the chemical stimuli that influence their behavior as adults.

In conclusion, the present study highlights the significance of plant cues in the rearing system for the behavioral quality of the emerging parasitoids. It further shows that, even after more than 30 generations of rearing under artificial conditions, the required response of parasitoids to important chemical cues from their hosts' habitat can be restored within a single generation, indicating that learning of plant-derived stimuli takes place during the parasitoid ontogenesis.

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INTRASPECIFIC VARIATION IN ODOR-MEDIATED HOST PREFERENCE OF THE MOSQUITO *Culex annulirostris*

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Abstract—The preference of two allopatric populations of *Culex annulirostris* mosquitoes in South Australia (SA) for guinea pigs and chickens was investigated using host-baited traps in the field and in a flight tunnel for laboratory assays. Mosquitoes from the Riverland of SA preferred guinea pigs, those from northeast SA preferred chickens. In the field, 2184 *Cx. annulirostris* were captured during four experiments in each region. The proportion choosing chicken was 0.5393 in northeast SA and 0.4348 in the Riverland. In the laboratory, replicate trials utilizing 15 mosquitoes at a time yielded results concordant with field assays. This study demonstrates variation in *Cx. annulirostris* host preference throughout this species geographic range. Semiochemical analysis of the two host species was performed on solvent extractions and volatile trapping samples from fur and feathers, respectively. This revealed differences that are likely to be the basis for host discrimination by *Cx. annulirostris*.

Key Words—Mosquitoes, *Culex annulirostris*, guinea pig, chicken, semiochemicals, host preference, intraspecific variation, South Australia.

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INTRODUCTION

Mosquitoes locate vertebrate hosts for blood meals by responding to olfactory and physical cues (Takken, 1996). While carbon dioxide is the major stimulus and attractant for many species (Hocking, 1963; Daykin et al., 1965; Snow, 1970; Gillies, 1980), other host semiochemicals may be used. These additional cues enable some species to discriminate among hosts or locate specific biting sites (Knols et al., 1994; De Jong and Knols, 1995; Dekker et al., 1998). The innate tendency to feed on particular host taxa, termed "host preference," is thought to be based on the differential responses of mosquitoes to host semiochemicals (Hii and Vun, 1987; Costantini et al., 1996, 1998; Clements, 1999).

Intraspecific variation in host preference has been documented for several species: *Anopheles arabiensis* Patton (Sharp and Le Sueur, 1991), *An. balabacensis* Baisas (Hii, 1985), *An. vestitipennis* Dyar and Knab (Arredondo-Jimenez et al., 1996), and *Culex tarsalis* Coquillett (Tempelis et al., 1967). This may be the result of selective processes creating genotypes that encode for specific host preferences (see Gillies, 1964; McCray and Schoof, 1970; Mukwaya, 1977; Angris, 1991).

Culex annulirostris Skuse is widespread throughout Australia, Melanesia, and Polynesia (Lee et al., 1989), and is a member of the *Culex sitiens* subgroup (Marks, 1982). It is morphologically indistinct from two other members of this subgroup: *Culex sitiens* Wiedemann and *Culex palpalis* (Taylor), making identification difficult in some parts of its range (Chapman et al., 2000).

This species transmits viruses that cause human illness: Ross River, Barmah Forest, Kunjin and Murray Valley Encephalitis virus (Russell, 1995; Mackenzie et al., 1998). Although *Cx. annulirostris* will take blood from a wide variety of endo- and ectothermic vertebrates (Kay et al., 1985), host preferences have been demonstrated. Preferences for mammals over birds (Kay et al., 1979; Muller et al., 1981; Kay et al., 1985) and vice versa (Russell, 1987a,b) have both been recorded.

In this study, we aimed to determine whether host-preference variability in *Cx. annulirostris* exists by studying host preferences of geographically isolated populations in the field and laboratory. Two vertebrate host species: guinea pigs (*Cavia porcella*) and chickens (*Gallus gallus domesticus*) were used. We also sought to determine whether laboratory host preference assays concord with results obtained from the field.

A further interest was to determine whether *Cx. annulirostris* discriminates between hosts on the basis of differences in semiochemicals (excluding carbon dioxide) or levels of carbon-dioxide emission. To this end, both types of emissions in chickens and guinea pigs were investigated.

METHODS AND MATERIALS

Mosquito Collection for Laboratory Assay. Collections from northeast South Australia (SA) were made at Embarka swamp, near the Tirrawarra oil and gas camp

(27°37′S, 140°10′E) from Jan. 12 to Apr. 1, 1999. In the Riverland of eastern SA (upper reaches of the River Murray in SA), collections were made from Kingston-on-Murray (34°13′S, 140°21′E) on Feb. 8, 1999 and Mar. 4, 1999, from Renmark (34°11′S, 140°45′E) on Sep. 30, 1999, and from Berri (34°17′S, 140°36′E) on Oct. 30, 1999. Observations from Riverland collections were pooled to be representative of *Cx. annulirostris* from that region.

Culex annulirostris were collected as eggs and larvae up to instar III. These were reared in 29 × 42 × 5 cm photographic developing trays, and were fed a mixture of autoclaved, finely ground dry dog food (Supercoat, Supercoat Pet Care, Yennora, NSW, Australia) and fish food (Wardley's Goldfish Foodflakes, Wardley Corp., Secaucus, NJ, USA) *ad libitum*. Pupae were placed in a 50 × 50 × 50 cm perspex cage humidified to 50–60% RH a temperature controlled room (24–26°C, 12:12 L:D) for emergence. Adult *Cx. annulirostris* were provided at all times with a 15% sucrose solution dispensed through cotton dental rolls.

As *Cx. annulirostris* raised from field collected larvae rarely mate in the laboratory (CRW personal observations, 1997–1999), only virgin nullipars were used. Mosquitoes used in host-seeking observations were aged from 3 to 8.5 days for Embarka Swamp specimens and 3 to 7.5 days for River Murray specimens.

Study Sites for Field Assays. In the Riverland region, animal baited trapping was conducted in low woodland and shrubland along Mundic Creek (34°11′S, 140°46′E), near Paringa SA, from Jan. 5 to 8 and 18 to 20, 2001. Two sites, 230 m apart, were used for experiments. In northeast SA, trapping was conducted at Mungerannie (27°59'S, 138°36′E) on the Birdsville Track, in tall, open woodland along the Derwent Creek, from Jan. 25 to 29, 2001. Two sites, 200 m apart, were used.

Identification of Specimens. Female mosquitoes collected by a variety of methods (human biting catch, larval dipping, carbon dioxide baited, and animal baited traps) from both northeast SA and the Riverland, were identified by morphological characters as *Cx. annulirostris*. Specimens were either stored at −80°C for allozyme electrophoresis or in 80% ethanol prior to DNA analysis. Allozyme electrophoresis of 25 loci was conducted by J. Kent (School of Pharmaceutical, Molecular and Biomedical Sciences, University of South Australia, Adelaide). A reverse transcriptase, polymerase chain reaction—restriction fragment length polymorphism (RT-PCR-RFLP) procedure was carried out by Dr N. Beebe (Department of Cell and Molecular Biology, University of Technology, Sydney) to distinguish *Cx. annulirostris* specimens from other members of the *Cx. sitiens* group. This procedure utilizes sequence variation in the ribosomal DNA ITS 1 region (Beebe et al., 2002). Similar procedures have been used in studies of other mosquito species groups (Beebe and Cooper, 2000).

Sources and Husbandry of Host Animals. Three English game bantam hens, *Gallus gallus domesticus*, purchased from a poultry supplier, were housed in a 2 × 1 m caged run, with sawdust bedding and perches in a constant laboratory

environment (22°C, 12:12 L:D). Wheat and drinking water were available continuously, while mixed vegetables were given every 2 days.

Six female laboratory guinea pigs [Institute of Medical and Veterinary Sciences (IMVS) tri-color strain], *Cavia porcella*, were housed separately in the same room as the chickens, in a 1.7 × 1 m run, with sawdust bedding and cardboard box refugia. Drinking water and rabbit pellets (Barastoc, Ridley AgriProducts, Murray Bridge, SA) were available continuously, while mixed vegetables were given every 2 days.

Carbon Dioxide Output of Hosts. Guinea pigs and chickens were weighed prior to each laboratory assay, using a Mettler PE 6 electronic balance (Mettler Instrumente AG, Zurich, Switzerland). Mass-dependent carbon dioxide outputs for guinea pigs and chickens were estimated from the literature (Barott and Pringle, 1946; Stupfel et al., 1983).

Analysis of Host Semiochemicals. Semiochemicals of the host species were analyzed by solvent extraction and volatile trapping. Host chemicals were extracted by soaking fur and feather samples in 20 ml of both hexane and methanol for 24 hr. Samples were stored in the dark at room temperature (20°C). Fur and feathers were taken from the dorsal surface of the head, back, breast, stomach, and rump. After 24 hr, the extracted fur/feathers was removed, and the extracts were concentrated to 1 ml by rotary evaporation. Methanol extracts were then methylated using boron trifluoride-methanol complex (BF_3). An equal volume of BF_3 was added to each extract, and the mixture was heated to 60°C in a shaking water bath for 10 min. Formed esters were extracted into hexane for analysis. Directly prior to analysis, all samples were concentrated to 20 μl under nitrogen. Analysis was undertaken on a Hewlett Packard 6890 gas chromatograph (GC) coupled to a 5972 Mass Selective Detector. The GC was fitted with a SGE-BPX5 silicone column (30 m × 0.25 mm × 0.25 μm). The flow for carrier gas was 1 ml/min He. Oven temperature was maintained at 50°C for 1 min and was then programmed at 8°C/min to 250°C. The injector temperature was held constant at 200°C. Ionization for mass analysis was by electron impact at 70 eV and 220°C; mass range 35–350 amu.

Additional samples of fur and feathers were sampled using headspace solid-phase microextraction (Zhang and Pawliszyn, 1993). Samples were placed in 4 ml glass sample vials and sampled using a commercially available 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco: Sigma-Aldrich Pty Ltd). The fiber was exposed to the headspace of the vial for 2 min at 40°C. Following sampling, the fibers were immediately introduced to the gas chromatograph's injector. A desorption time of 3 min was employed. Analysis was performed using the same column and GC conditions described above. All compounds were identified by mass spectral matching using the Wiley Registry of Mass Spectral Data, 7th edition (John Wiley & Sons, Chichester, UK) and in-house libraries.

Laboratory Flight Tunnel Assays—Olfactometer Design. A laminar flow flight tunnel based on the design of Keller (1990) was used for observation of odor-mediated host choice (Figure 1). The observation section was constructed of

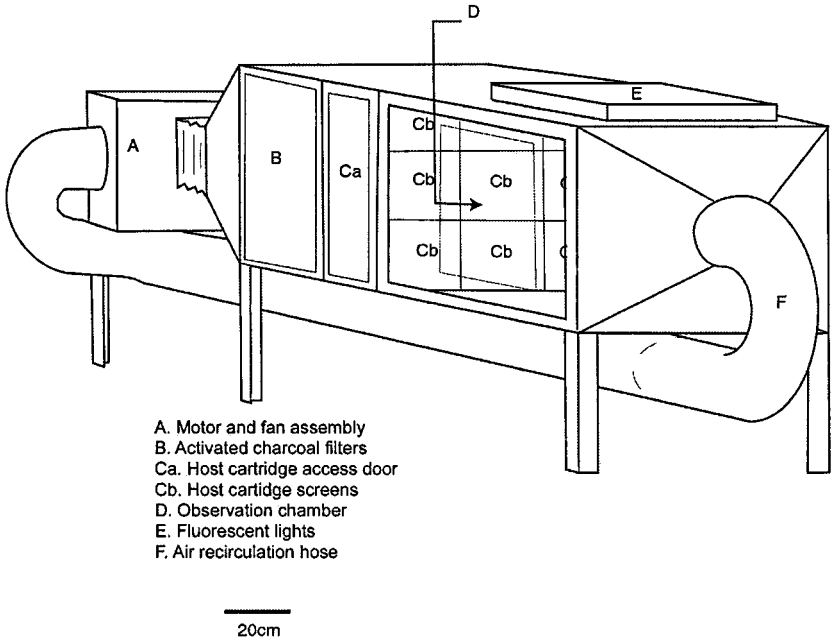

FIG. 1. Diagram of the flight tunnel used for assessing odor-mediated host preference in *Culex annulirostris.*

sheet metal except for one side that was transparent acrylic to allow observation. The main part of the chamber had internal measurements of 125 cm length × 52 cm width × 61 cm height. The downwind section tapered from 52 × 61 cm to 22 × 22 cm over a length of 39 cm. A flush mounted 35 × 35 cm door allowed the introduction and removal of mosquitoes.

Two fluorescent light tubes (Crompton F8T5/CW) behind semiopaque acrylic covered by two layers of red cellophane film illuminated the observation section. This "red" light had a mean intensity of 30 lux on the chamber floor and 50 lux 10-cm below the lights. The red screen was used because unfiltered light inhibited the flight behavior of field collected mosquitoes (CRW personal observations 1998–1999).

Air flow through the tunnel was provided by a ¾ horsepower DC electric centrifugal blower fan (SMT90878E, Warner Electric), which forced air through a dust filter (Rigasorb 25D particulate filter, Farr Co., Los Angeles, California, USA). Air then passed through a series of 12 filters in a zig-zag arrangement, filled with activated charcoal (RigaSorb 3CF Glide Pack holding frame, Farr Co., Los Angeles, California, USA) to remove organic volatile chemicals.

The cartridge consisted of nine stainless steel host restraint chambers in a 3 × 3 arrangement. These chambers measured 17 × 16 × 18 cm and consisted of

mesh on the windward and lee sides (hole size 1 cm diam) and solid sheets on the remaining sides. The top was a removable steel lid. The lee side of the host cartridge consisted of fine steel mesh (0.25 mm aperture), with the margins of the nine host restraint cages marked by brass edging. The lee side of the host cartridge formed the windward end of the observation chamber.

Air flowed through the observation chamber, exited via a porthole covered in fine mesh (0.25 mm diam), and was recycled along a flexible duct (20 cm diam) underneath the observation chamber to the inlet of the blower assembly.

Laboratory Assay Procedure. One hour prior to observations, both host cartridges and the access door were wiped with wet paper towel, followed by 70% ethanol on a paper towel. The flight tunnel fan was set at 0.3 m/sec to clean the air in the tunnel by the activated charcoal filters.

Immediately prior to observations, the fan speed was adjusted to 0.03 m/sec. One chicken and one guinea pig, selected at random, were introduced into freshly cleaned host restraint cages using gloved hands. Fresh gloves were worn when handling each host, which were then introduced into the host cartridge. Only the top row of the cartridge was used, following pilot studies demonstrating that response was greatest to hosts in this row. The position of chickens and guinea pigs in either the top left or top right positions was determined randomly for the first trial of a given mosquito population. Positions were alternated for each subsequent trial.

Fifteen virgin female *Cx. annulirostris* were aspirated from their cage immediately prior to introduction into the wind tunnel observation chamber. Following the introduction of mosquitoes, observations commenced from the start of the next minute. The number of mosquitoes probing through the screen at each of the respective hosts was counted each minute for 90 min. For each minute of observation, counts commenced at the 45 sec mark of the previous min, and continued until the 15 sec mark of that minute. One mosquito probing through the screen at a host constituted one count. Additional probing mosquitoes were extra counts. This 30 sec observation period allowed the true number of probing mosquitoes to be counted without the burden of observing the mosquitoes for each second of the trial.

For northeast SA specimens, 10 trials using 15 mosquitoes each were performed, while only nine trials were possible for River Murray mosquitoes. All trials were carried out within the first 2.5 hr of the scotophase, considered the time of maximum flight and host seeking activity for *Cx. annulirostris* (Russell, 1987a,b).

Statistical Analyses. Probing counts were totalled for each host for each 90 minute trial. These data were then used to calculate a preference quotient (PQ) for each trial according to the following formula:

$$\mathrm{PQ}_{\mathrm{host}} = \frac{N_{\mathrm{chicken}} - N_{\mathrm{guinea\ pig}}}{N_{\mathrm{chicken}} + N_{\mathrm{guinea\ pig}}}$$

N is the mean number of probing mosquitoes for the trial. PQ values generated using this formula range from 1 to −1, with positive values indicating bias for chicken odor, and negative values indicating bias for guinea pig.

PQs from the two populations were compared using the Mann–Whitney test employing the chi-square (χ^2) distribution (Zar, 1999). JMP IN® statistical software (SAS Institute, 1997) was used.

Field-Based Host-Baited Trapping—Trap Design. Carbon dioxide miniature light traps (Rohe and Fall, 1979) were modified so that chicken or guinea pig odor was used as bait. The "grain of rice" light and fan assembly were attached to the underside of a collapsible host restraint chamber. The chamber consisted of a plastic circular base (30 cm diam) with 48 holes (1 cm diam) allowing the passage of host odor. The underside was covered in nylon netting (1 mm hole size). The host was restrained by a transparent plastic cone (diam. at base 30 cm, height 40 cm) that sat atop the base. Stainless steel cords were joined to the perimeter of the base and joined at an eyelet above, providing a point for suspension of the trap and reinforcement for the cone. Chickens and guinea pigs were introduced individually into the traps. These traps allowed the odor of the bait hosts to act as attractants for host-seeking mosquitoes that were caught in the light and fan assembly. The hosts were not exposed to biting mosquitoes.

Field-Based Trapping Procedure. Four experiments were performed in both the Upper River Murray and northeast SA. Two experiments were performed at each of two sites in each region. Each experiment consisted of one pair of guinea pig and chicken baited traps operated over successive nights at the same site. Each pair of traps was suspended 1.5 m above the ground in a tree, approximately 2 m apart, positioned so that their plane was perpendicular to prevailing winds. The positioning of hosts along the plane was randomly selected on the first night and reversed on the second night of each experiment to account for spatial effects. Experiments were carried out in the Upper River Murray region from Jan. 5 to 7 and Jan. 18 to 20, 2001, and in northeast SA from Jan. 25 to 29, 2001.

Statistical Analyses. The number of female *Cx. annulirostris* captured at each host was totalled for the two nights of each experiment, for which PQ values were calculated. Experiments were replicated at each site, yielding four PQ values for each region.

The proportion of *Cx. annulirostris* caught in chicken-baited traps on each night was calculated. These proportions (p) were then subjected to a logit transformation as follows:

$$\pi = \log\left[\frac{p}{1-p}\right]$$

Transformed data (π) were then used to fit a linear regression model with nested factors to the data. This model contained terms for three experimental factors: region (northeast SA or Upper River Murray), trapping site within region

(sites 1 and 2 for each region), and positioning of hosts within sites within region, as well as an error term. Logistic regression (with ANOVA) was then used to determine the relative influence of each factor on the fit of the model to a $P = 0.05$ significance level. A normal quantiles plot was used to check that the assumption of normality for the linear regression model was satisfied.

RESULTS

Identification of Specimens. Positive identification of *Cx. annulirostris* by morphological characters was confirmed by molecular methods. RFLP analysis revealed that all such specimens displayed the markers defined for *Cx. annulirostris* (Beebe et al., 2002). Allozyme electrophoresis studies showed no difference in allozymes between the two populations studied (J. Kent, personal communication, 2001).

Carbon Dioxide Output of Host Animals. No statistically significant difference between the masses of guinea pigs (558.1 ± 13.9 g) and chickens (572.7 ± 12.9 g) was found using Student's t test ($t = 0.772$, $df = 52$, $P = 0.444$) (Zar, 1999). Previously published carbon dioxide output rates for chickens (14.3 ml/kg/min, Barott and Pringle, 1946) and guinea pigs (14.9 ml/kg/min, Stupfel et al., 1983), of similar size and age to those used in this study, indicate a similar rate for the two species. Given that the animals used in this study were of similar mass, it follows that their overall carbon dioxide output is also likely to be similar.

Host Semiochemicals. SPME and methylated methanol extraction showed differences in the chemical profile of chickens and guinea pigs (Figures 2 and 3). For solvent extractions, methanol proved more effective than hexane, which had a low extraction efficiency. Furthermore, hexane extracts were confounded by high molecular weight, nonpolar compounds unlikely to play a role in mosquito attraction.

SPME sampling showed that the monoterpenes α-pinene and β-myrcene were unique to chicken feathers, while 2,5-hexandione was unique to guinea pigs (Figure 2). Only compounds with a $>90\%$ match, however, have been reported here by name. Methylated methanol extractions revealed compounds unique to chickens (octanoic acid methyl ester, tetradecane, 3-eicosene) and another unique to guinea pigs (14-methyl hexadecanoic acid methyl ester) (Figure 3). Differences in the relative abundance of compounds common to both host species were also evident (Figures 2 and 3). In particular, caproic aldehyde was markedly more abundant in guinea pigs than chickens.

Laboratory Flight Tunnel Assays. Mosquitoes from both regions responded to host odors by probing at the screen where hosts were. Probing behavior consisted of insertion of the proboscis through the mesh, punctuated by periods of quick walking and short hovering flights. Probing only occurred at the screen where hosts were located.

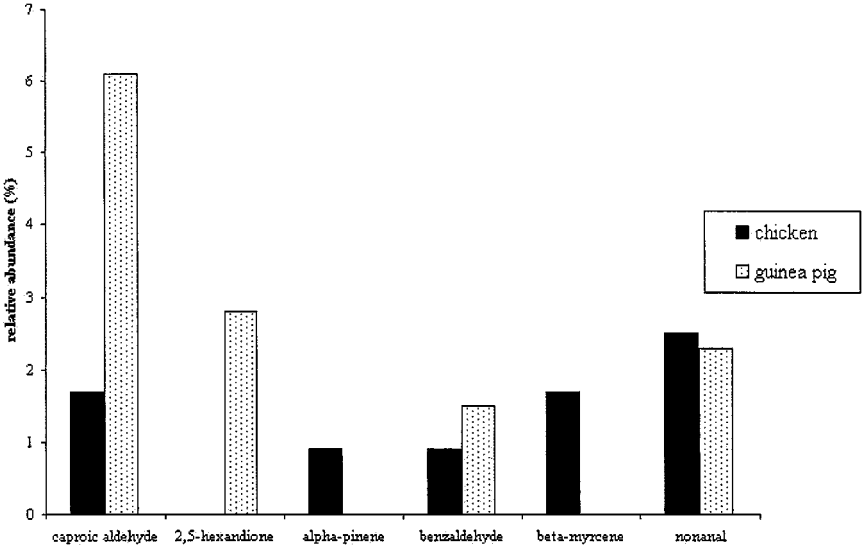

FIG. 2. Abundance of chemicals (%) relative to the total detected in each trace, preliminarily identified from chicken feathers and guinea pig fur using SPME volatile sampling. Only spectral library matches >90% are reported.

Mean PQ differed between the two populations. Northeast SA *Cx. annulirostris* preferred chicken odor, and yielded a positive PQ, in contrast to River Murray specimens that preferred guinea pig odor (negative PQ) (Figure 4). The mean PQs of the two populations were significantly different ($\chi^2 = 4.93$, $P = 0.026$).

Field-Based Host-Baited Trapping. A total of 2184 female *Cx. annulirostris* were captured in host-baited traps. PQs revealed a preference for chicken odors in three of the four experiments in northeast SA, and a preference for guinea pig in all four experiments in the Upper River Murray (Figure 4). The proportion of *Cx. annulirostris* choosing chicken was 0.5393 in northeast SA and 0.4348 in the Upper River Murray.

Logistic regression revealed that the effects of trapping site (within a region) ($df = 2$, $F = 3.704$, $P = 0.56$) and the position within each trapping site ($df = 4$, $F = 0.6335$, $P = 0.65$) did not affect the choice of hosts. This was determined by repeated regression of models in which factors found to be insignificant were removed before the model was re-fitted. The region in which trapping was conducted (Upper River Murray and northeast SA) was found to be a factor in the model ($df = 1$, $F = 10.202$, $P = 0.006$).

However, half of one experiment in northeast SA (the night of Jan. 7, 2001; catch: chicken 12, guinea pig 49) contrasted greatly with the overall preference for chicken in that region (Figure 4). This observation was found to be

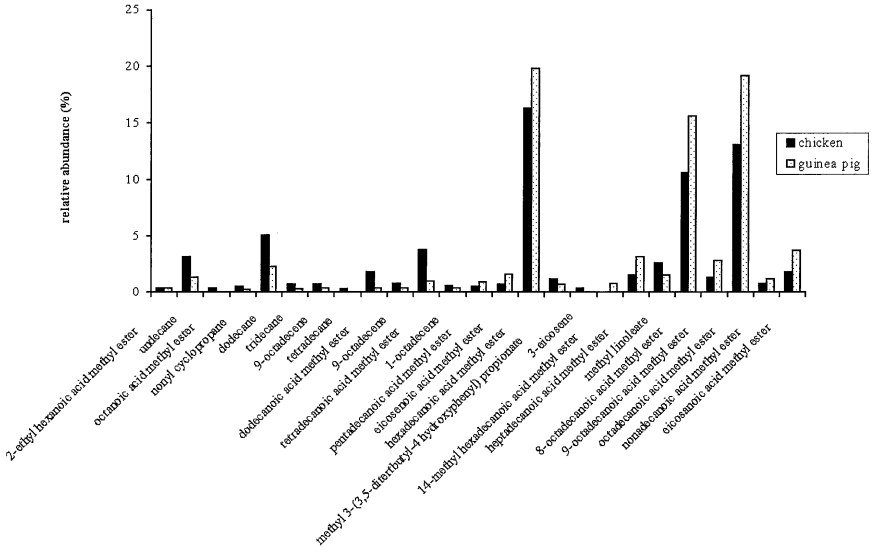

FIG. 3. Abundance of chemicals (%) relative to the total detected in each trace, preliminarily identified from chicken feathers and guinea pig fur subjected to methanol extraction and methylation. Only spectral library matches >90% are reported.

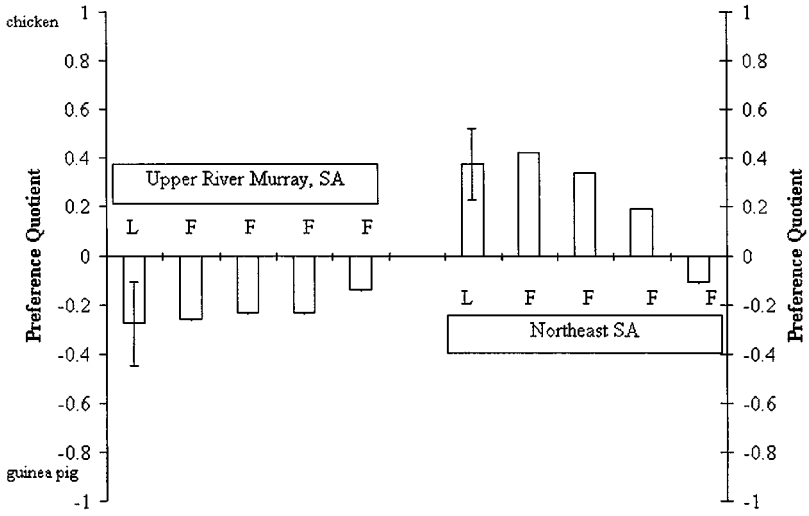

FIG. 4. Host preference of *Culex annulirostris* mosquitoes from two regions in South Australia given a choice of chicken and guinea pig odors. Preferences are expressed as mean PQs (±SEM) for laboratory assays (*L*), and as absolute values for field assays (*F*).

an outlier in a normal quantiles plot and Cook's distance plot, which describes the influence that each point has on the linear regression model fit (Cook and Weisberg, 1982). For this reason, a second analysis was performed with this datum removed.

Subsequently, significant effects were found for all three factors: region ($df = 1$, $F = 165.497$, $P < 0.001$), trapping site ($df = 2$, $F = 11.24$, $P = 0.006$) and position within site ($df = 1$, $F = 4.213$, $P = 0.047$). The fitted logit values from this latter model were then back-transformed, resulting in fitted proportions. For northeast SA, these were all greater than 0.5, indicating a preference for chicken (range 0.5237–0.5874), while for the Upper River Murray these were all below 0.5 (0.3855–0.4846).

DISCUSSION

Culex annulirostris from two regions in SA showed different odor-mediated host preferences, with mosquitoes in one region preferring guinea pigs, the other preferring chickens. These preferences were determined using laboratory and field-based investigations, which yielded concurring results. This validates the use of laboratory wind-tunnel assays in determining odor-mediated host preferences of *Cx. annulirostris*.

The preferences determined here are likely to be the result of discrimination between hosts based on differing arrays of chemicals, as host carbon-dioxide output rates are likely to be similar for both. Differences in the volatile chemicals emitted by the two host species and differences in their relative abundance are likely to be the basis for host discrimination.

The study of host chemicals here was not exhaustive, nor definitive in terms of identifying compounds. Nonetheless, differences in identified compounds were apparent in terms of their presence/absence and relative abundance. Studies of mosquitoes and other insects demonstrate that mixtures of the same compounds differ in their attractiveness and repellency, depending on their concentration ratios (Kawasaki, 1984; Ohmasa et al., 1991; Blackwell et al., 1996; Du and Millar, 1999; Boo et al., 2000; Kawazu et al., 2000). Intraspecific variation in moth sex pheromones occurs in geographically disparate populations, with only the ratio of compounds differing between regions (Kawazu et al., 2000). These studies illustrate the sensitivity of insects to slight differences in olfactory cue composition, and indicate that the differences seen here between the host taxa would be ample for mosquitoes to discriminate between them.

The presence of several fatty acids (indicated by the presence of their methyl esters, Figure 3) is consistent with the findings of other studies of these host taxa (Wheatley and James, 1957; Nicolaides et al., 1968). Of the identified compounds, octanoic acid (present in chickens, but not guinea pigs) is known to elicit an

electroanntennogram response in *An. gambiae s.s.* (Cork, 1996), whereas nonanal (in both hosts) elicits responses in *Cx. quinquefasciatus* and *Cx. tarsalis* (Du and Millar, 1999).

Further identification of the compounds present on chicken feathers and guinea pig fur and tests of their activity separately and in combination may reveal specific attractants for *Cx. annulirostris*. This may have applications in the monitoring and control of this species. Attractants such as 1-octen-3-ol and lactic acid have been used to augment carbon-dioxide baited traps to enhance the surveillance of particular species (Kline, 1994). It has been suggested that such compounds may be used in mosquito control through the diversion of biting mosquitoes away from hosts (Hocking, 1963; Knols, 1996; Knols and De Jong, 1996).

Intraspecific variation in odor-mediated host preference may be the result of adaptation of allopatric populations to different arrays of vertebrate hosts. Previous studies have demonstrated the response of mosquito host preference to selective breeding (Gillies, 1964; McCray and Schoof, 1970; Mukwaya, 1977; Angris, 1991), indicating the plasticity of this trait within a species. This said, the *Cx. annulirostris* populations studied here would not have adapted to guinea pigs and chickens. The results may reflect adaptation to other locally available hosts, or to a different relative abundance of hosts, which may emit some of the same chemicals as chickens or guinea pigs (Nicolaides et al., 1968).

Given its ability to freely feed on a wide variety of hosts (Kay et al., 1979, 1985), *Cx. annulirostris* should be regarded as an opportunistic feeder. Despite this, preferences for hosts have been demonstrated repeatedly (Kay et al., 1979; Wright, 1981; Russell, 1986, 1987a,b). By using the same assays on geographically isolated populations of *Cx. annulirostris*, this study has confirmed that odor-mediated host-preference varies geographically, and that opportunistic mosquito species are able to develop locally unique behavior.

This is the first study to demonstrate different odor-mediated host preferences in geographically isolated populations of a single mosquito species using the same group of bait-animals in the same month and year. It is also unique in being the first study to use the same group of hosts to conduct field and laboratory assays. This study confirms that differences in *Cx. annulirostris* host preference exist throughout this species' geographic range.

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DEFOLIATION-INDUCED RESPONSES IN PEROXIDASES, PHENOLICS, AND POLYAMINES IN SCOTS PINE (*Pinus sylvestris* L.) NEEDLES

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Abstract—Effects of artificial defoliation on defensive needle chemistry in Scots pine (*Pinus sylvestris* L.) were evaluated with particular emphasis on peroxidases, phenolic compounds, soluble sugars, polyamines, and foliar nitrogen levels. The study was carried out on a nutrient-poor Scots pine stand with 8- to 25-year-old trees. Defoliation treatment consisted of repeated defoliation in two successive years with respective control trees. Defoliation was done before needle flushing by removing all mature needles. Guaiacol peroxidase activity increased in the needles after the first defoliation. The difference between treatments diminished towards autumn, and disappeared before the second defoliation in the next summer. After the second defoliation, the activities showed a similar trend. Apparently, peroxidases are involved in inducible chemical changes and recovery reactions that occur in the intact needles shortly after defoliation. After the second defoliation, total nitrogen concentration in the current year needles was about 20% lower, and free putrescine (a polyamine)

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concentration was 40% lower in the defoliated trees than in control needles. These changes indicate a loss of nitrogen due to defoliation. Specific phenolic compounds such as quercitrin, (+)-catechin, and two catechin derivatives increased in current year needles in response to defoliation. Accumulation of starch and sucrose in the current year needles of repeatedly defoliated trees may imply decreased assimilate transport. The results are indicative that changes in needle phytochemistry in response to defoliation accompany changes in needle nitrogen metabolism.

Key Words—Defoliation, *Pinus sylvestris*, needles, peroxidases, phenolics, polyamines, carbohydrates, nitrogen.

INTRODUCTION

Physical damage to plants stimulates the expression of defensive chemistry including proteins and phytochemicals, which are involved in repair mechanisms, inducible chemical defenses against herbivores and pathogens, and adjusting plant metabolism to the imposed nutritional demands (e.g., Karban and Baldwin, 1997; Constabel, 1999; León et al., 2001). Among wound-induced proteins are proteinase inhibitors, oxidative enzymes, cell-wall proteins, enzymes in phenylpropanoid metabolism, lectins, carbohydrate-binding proteins and chitinases, and induced phytochemicals including phenolic compounds, terpenoids, and alkaloids (reviewed by Constabel, 1999). Jasmonic acid and its methyl ester are considered as important signalling molecules in wound-response pathways in plants (Wasternack et al., 1998). Application of exogenous jasmonic acid induces the activity of oxidative enzymes such as peroxidases and polyphenol oxidases (Thaler et al., 1996; Redman et al., 2001; Walters et al., 2002) and increases polyamine concentrations in plants (Walters et al., 2002). Mechanical wounding mimics insect feeding partially, but the wounding response can be modified by insect-derived chemicals or enzymes (Gatehouse, 2002).

Wounding and attack by herbivores enhance the formation of free radicals and other reactive oxygen species (ROS) in plant cells, which trigger defense reactions (Felton et al., 1994; Bi and Felton, 1995). Oxidative responses may decrease damage caused to plants by, e.g., herbivores, but enhance lipid peroxidation of cell membranes (Bi and Felton, 1995). Polyamines may directly or indirectly function as free radical scavengers (Boucherau et al., 1999). Phenolic compounds are suggested to mitigate the effects of oxidative stress by acting as direct radical scavengers and as reducing substrates for guaiacol peroxidase (Grace and Logan, 2000). The phenolic–peroxidase reaction system may function as a mechanism for hydrogen peroxide (H_2O_2) scavenging in plants (Takahama and Oniki, 1997; Yamasaki et al., 1997). Peroxidases and phenolics also are involved in reactions, that generate reactive oxygen species (Bestwick et al., 1998; Grace and Logan, 2000) and oxidation of polyamines generates H_2O_2 (Walters et al., 2002). Peroxidases, phenolic compounds, and polyamines that are induced in response to

wounding may act as defense compounds and be involved in lignification and modification of the plant cell-walls (Dixon and Paiva, 1995; Dowd and Lagrimini, 1997; Walters et al., 2002).

The carbon–nutrient balance hypothesis in plants predicts the allocation of resources to growth and carbon-based secondary metabolite production. A high C/N ratio is assumed to lead to the use of photosynthate for production of carbon-based metabolites (Bryant et al., 1983; Tuomi et al., 1984). Accumulation of foliar phenolics has been observed in nitrogen-deficient conditions (Bryant et al., 1983, 1993; Gershenzon, 1984; Tuomi et al., 1984, 1988). This especially concerns phenylpropanoids associated with the shikimate acid pathway, whereas hydrolyzable tannins with a distinct biosynthetic origin may respond differently to nitrogen deficiency (Haukioja et al., 1998). Addition of external carbon such as sucrose stimulates the biosynthesis of phenolic compounds in plants (Grace and Logan, 2000), and elevated CO_2 increases foliar catechin, total phenolics, and condensed tannin concentrations in *Pinus taeda* needles (Booker and Meier, 2001). The shikimate pathway involved in phenylpropanoid metabolism consumes carbon intermediates, but in addition it may represent an alternative route for consumption of metabolic reducing power, which prevents free radical production and oxidative damage in the chloroplasts (Grace and Logan, 2000).

In the present study, we evaluated the effects of severe defoliation on foliar chemistry in Scots pine (*Pinus sylvestris* L.). In addition to changes in foliar peroxidases, polyamines, and phenolics, we studied changes in foliar nitrogen and carbohydrate metabolism of the needles.

METHODS AND MATERIALS

Experimental Trees. Scots pine (*Pinus sylvestris* L.) trees were artificially defoliated in two successive years on the island of Hailuoto in northern Finland (65°03′N, 24°36′E). The study site was a dry Scots pine stand, and the age of experimental trees ranged from 8 to 25 years. Ground vegetation consisted of patches of dwarf shrubs (e.g., *Empetrum nigrum*, *Vaccinium uliginosum*, *Salix repens*), lichens (e.g., *Cladina arbuscula*, *Cladonia cornuta*, *C. botrytes*, *Stereocaulon* sp.), and mosses (e.g., *Racomitrium canescens*, *Polytrichum piliferum*, and *P. juniperinum*). The pH of the mineral soil (sand) was 4.8, and total nitrogen (N) concentration was below 0.01% of soil dry weight (Kuikka et al., in press). The soil organic layer was patchy and thin (0.5–1.0 cm) where present. Physical and chemical characters of soil and vegetation indicate that the growth site was nutrient-poor.

Fifteen trees were selected for the defoliation treatment with 15 respective control trees. Trees were approximately 2-m tall with a distance of 3 m between neighboring trees. Selected trees were randomly divided into two groups (control and defoliation), which showed no difference in age distribution or in height.

Trees were completely defoliated with a pair of scissors in two successive years between June 22–26, 1998, and June 21–26, 1999. At this time, the current shoot was fully elongated, but the current year needle growth had just started. Defoliation was done before needle flushing by cutting all mature, needles. Thus, the developing current year needles were left intact. The treatment simulates pine sawfly (*Neodiprion sertifer*) attack in that larvae attack mature, but avoid current year (C), needles (Niemelä et al., 1991). Approximately 10 needle pairs from previous year's growth were left intact on the middle part of the leader shoot for peroxidase analyses to study effects of defoliation on these 1-year-old previous year needles (C + 1).

Chemical Analyses. Current year's (C) and previous year's (C + 1) needles for guaiacol peroxidase acitivity analyses were collected on June 22 (C + 1), August 4 (C and C + 1), and September 28 (C) in 1998 and June 21 (C + 1), August 6 (C + 1), and September 24 (C) in 1999. Soluble polyamines were measured in the C needles collected from branches in the mid-crown on September 28, 1998, and September 24, 1999. Approximately 1 g (FW) of the current year needles was randomly collected from several 3rd to 4th branch whorl counted from the top of the tree on September 24, 1999. Needles were freeze-dried and homogenized to a fine powder. Two replicate samples were analyzed for phenolic compounds, total nitrogen, soluble sugars, and starch concentration. Needle growth was determined in 10 intact needle pairs per tree.

Guaiacol peroxidase activity was measured according to Polle et al. (1990) with slight modifications. A batch of 150 mg of needles was ground in liquid nitrogen and homogenized in 0.1 M potassium phosphate buffer (pH 7.8) in the presence of 5% PVPP (w/v). The homogenate was allowed to stand on ice for 45 min, then centrifuged at 22,000 *g* for 30 min. One hundred microliter of enzyme extract was added to total volume of 1 ml of 50 mM potassium phosphate buffer (pH 5.3), 40 mM guaiacol, and 10 mM H_2O_2. The change in absorbance was monitored at 470 nm for 5 min by spectrophotometer.

Soluble polyamine concentration was measured as described by Sarjala and Kaunisto (1993). A sample of 0.2 g needles was ground in liquid nitrogen and extracted with 5% $HClO_4$. The homogenate was centrifuged for 25 min at 22,000 *g*, and the supernatant was used for the analyses. Free polyamines were dansylated and separated by HPLC (Merck-Hitachi) with a fluorescence spectrophotometer (Merck-Hitachi F-1050).

Chlorophyll and carotenoids were extracted from fresh needle samples with DMSO as described by Barnes et al. (1992). Chlorophyll and carotenoid amounts were calculated according to Wellburn (1994). Soluble sugar (glucose, fructose, and sucrose) and starch were analyzed by enzymatic techniques (Beutler et al., 1978). Nitrogen concentration in the needles was determined with a CHN-analyzer (CE Instruments: Elemental Analyser EA1110).

Phenolics were measured as described by Julkunen-Tiitto et al. (1996). Five milligrams of freeze-dried and finely ground needles were extracted with 400-μl

methanol using an Ultra-Turrax homogenizer. Duplicate samples were prepared from each tree. Samples were centrifuged for 3 min at 13,000 rpm, and the extraction was repeated three times, after which the extracts were combined and methanol was evaporated under N_2. Samples were dissolved in methanol:water (1:1) and filtered with a syringe (0.2 μm). Phenolics were analyzed by HPLC/DAD by using 3-μm HP Hypersil ODS column (60 $\times$ 4.6 mm I.D.). Elution solvents were A (1.5% tetrahydrofuran + 0.25% *ortho*-phosphoric acid) and B (100% methanol). The following gradient was used: at 5 min 0% B, at 10 min 15% B, at 20 min 30% B, at 40 min 50% B. Identification of components was based on their retention times and spectral data. Secondary metabolites were quantified against commercial standards. (+)-Catechin was used for catechins, myricitrin for myricetin derivates, hyperin for quercetin derivatives, and kaempferol 3-glucoside for kaempferol glycosides.

Statistical Analyses. Peroxidase and polyamine data were analyzed by repeated measures analysis of variance, in which the trees were taken to be the subjects. Sampling year was considered as a within-subjects factor and treatment (control vs. defoliated) as a between-subjects factor. The following linear model was used: $Y = \mu + \text{Tree (Defoliation)} + \text{Defoliation} + \text{Year} + \text{Defoliation} \times \text{Year} + \varepsilon$ where Tree (nested within defoliation) is used as an error term for Defoliation, while Year and Defoliation $\times$ Year interaction are tested against ε (residual variation). The effects of defoliation on phenolics, soluble sugars, chlorophyll, and nitrogen were tested with Student's *t* test or with Mann–Whitney *U* test. The correlations were studied with Pearson's correlation coefficient within the treatment groups.

RESULTS

Defoliated trees contained higher peroxidase activity compared with control trees after the first defoliation in 1998 (Figure 1). The shift in activity was apparent in both needle age classes. The difference between the defoliation treatments diminished towards autumn, and had disappeared by the next summer before the second defoliation (Figure 1). After the second defoliation in 1999, the activities were similar to those in the 1st year. However, no statistically significant difference between the treatments was found in September 1999 (Figure 1). Foliar nitrogen concentration was reduced in the defoliated trees after the second defoliation compared to controls (Table 1). This resulted in an increased C/N ratio in the needles of the defoliated trees. Glucose and fructose concentrations decreased and starch and sucrose increased in the repeatedly defoliated trees (Table 1). Foliar starch concentration correlated negatively with nitrogen ($r = -0.68$, $P < 0.005$) and putrescine ($r = -0.72$, $P < 0.003$) in the defoliated trees, but less significantly in control trees (with nitrogen, $r = -0.46$, $P < 0.083$; and putrescine, $r = 0.2$, ns). Chlorophyll concentration was lower and carotenoids were slightly higher in

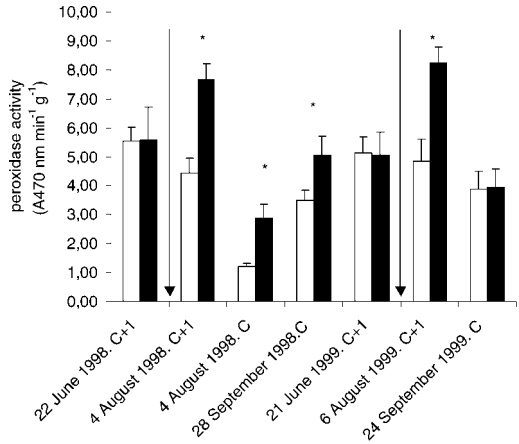

FIG. 1. Mean guaiacol peroxidase activity (±SE) in the current (C) and previous year's (C +1) needles. □ = control trees and ■ = defoliated trees. Values represent means of 5–15 trees. Arrows indicate the timing of the defoliation treatments in 1998 and 1999, respectively. The asterisk (*) indicates $P < 0.05$ according to t test. Results of repeated measures of ANOVA for 1998 flushed needles ar as follows: $F_{\text{treatment}} = 6.32$, $P < 0.05$; $F_{\text{time}} = 52.53$, $P < 0.001$; and $F_{\text{time}\times\text{treatment}} = 9.84$, $P < 0.001$.

TABLE 1. MEAN CONCENTRATION OF NITROGEN, CARBON, CHLOROPHYLL PIGMENTS, SOLUBLE SUGAR, AND STARCH CONCENTRATIONS IN THE CURRENT YEAR NEEDLES AFTER 2 YEARS OF DEFOLIATION[a]

	Defoliation treament			
	Control	Defoliated	t value	P value
Nitrogen (mg g^{-1})	9.0 ± 0.3	7.3 ± 0.3	4.41	0.000
C (%)	50.00 ± 0.14	49.62 ± 0.13	1.99	0.057
C/N	53.36 ± 1.73	69.01 ± 2.28	−4.41	0.000
Chlorophyll *a* (mg g^{-1})	0.85 ± 0.04	0.63 ± 0.03	4.13	0.000
Chlorophyll *b* (mg g^{-1})	0.27 ± 0.01	0.19 ± 0.007	4.25	0.000
Carotenoids (mg g^{-1})	0.25 ± 0.007	0.28 ± 0.009	−1.97	0.058
Glucose (mg g^{-1})	31.7 ± 2.4	22.9 ± 1.2	3.31	0.003
Fructose (mg g^{-1})	19.5 ± 1.3	16.3 ± 1.0	1.94	0.063
Sucrose (mg g^{-1})	14.8 ± 2.2	21.0 ± 1.6	−2.30	0.029
Starch (mg g^{-1})	22.3 ± 2.9	32.6 ± 3.6	−2.20	0.036
Needle length (mm)	28.3 ± 1.89	23.2 ± 1.0	2.37	0.025
Needle weight (mg)	7.29 ± 0.58	4.95 ± 0.53	3.28	0.006
DW/FW in the needles	0.46 ± 0.004	0.48 ± 0.006	−2.65	0.013

[a] Values are means of 15 trees (±SE). For each parameter, results of t test (t value and P value) are presented. Samples for analyses were collected on September 24 in second defoliation year 1999. Abbreviations: C/N = carbon/nitrogen ratio, DW/FW = dry/fresh weight ratio.

TABLE 2. MEAN CONCENTRATION OF PHENOLIC COMPOUNDS IN THE CURRENT YEAR NEEDLES AFTER THE 2ND YEAR OF DEFOLIATION[a]

	Control	Defoliated	*t* value	*P* value
(+)-Catechin	1.41 ± 0.11	1.76 ± 0.08	−2.618	0.014
Catechin derivative I	1.31 ± 0.13	1.79 ± 0.14	−2.484	0.019
Catechin derivative II	1.09 ± 0.09	1.49 ± 0.11	−2.797	0.009
Kaempferol-3-glucoside	0.18 ± 0.02	0.19 ± 0.01	−0.412	0.661
Quercetin-3-rhamnoside (=quercitrin)	0.60 ± 0.05	1.42 ± 0.08	−8.730	0.001
Quercetin-3-galactoside (=hyperin)	0.60 ± 0.05	0.58 ± 0.03	0.323	0.749
Mono-coumaroyl-isoquercitrin	1.11 ± 0.09	1.08 ± 0.09	0.195	0.847
3″,6″-Dicoumaroyl-astragalin	1.29 ± 0.07	1.01 ± 0.07	2.815	0.009
Myricetin-3-galactoside	0.21 ± 0.01	0.21 ± 0.01	−0.190	0.834
Taxifolin glucoside	5.91 ± 3.26	9.70 ± 4.63	81^{M-W}	0.858

[a] Values are means (mg g^{-1} DW) of 15 trees (±SE) Results of *t* test (*t* value and *P* value) or Mann–Whitney *U* $test^{M-W}$ are presented for each parameter. Current year needles for analyses were collected on September 24 in 1999.

the defoliated trees than in control trees (Table 1). Both needle weight and length decreased in the defoliated trees after the second defoliation (Table 1). Dry matter content (DW/FW ratio) was slightly higher in the defoliated trees after 2 years of defoliation (Table 1).

Specific phenolic compounds responded differently to defoliation. Defoliation induced the accumulation of quercitrin, (+)-catechin, and two catechin derivatives in the intact needles of defoliated trees, whereas 3″, 6″-dicoumaroyl-astragalin showed a decreasing trend in defoliated trees (Table 2). The concentration of (+)-catechin showed a negative relationship to chlorophyll *a* concentrations in the control ($r = -0.41$, ns) and defoliated trees ($r = -0.51$, $P < 0.054$). A similar trend was found with quercitrin and chlorophyll in defoliated trees ($r = -0.58$, $P < 0.022$), but was not found in control trees ($r = 0.026$, ns). The high variation in taxifolin glucoside concentration results from the presence of taxifolin glucoside rich trees (see Larsson et al., 1992, for the taxifolin chemotypes). Five trees out of a total of 30 trees belonged to this taxifolin glucoside-rich chemotype. Defoliation had no consistent effect on several identified compounds (Table 2).

After the first defoliation, putrescine concentration decreased in needles (Tables 3 and 4). Spermine and spermidine showed no significant changes in response to the treatment (Table 4). Similarly, after the second defoliation, putrescine concentration remained lower in the current year needles of the defoliated trees (Table 3). Foliar nitrogen correlated positively with the putrescine ($r = 0.63$, $P < 0.012$), spermidine ($r = 0.47$, $P < 0.078$), and spermine ($r = 0.49$, $P < 0.063$) in the defoliated trees, but not in control trees ($r = -0.077$, ns, for putrescine; $r = -0.11$, ns, for spermidine; and $r = -0.02$, ns, for spermine).

TABLE 3. MEAN CONCENTRATION OF FREE POLYAMINES IN THE CURRENT YEAR NEEDLES AFTER THE 1ST AND 2ND YEARS OF DEFOLIATION[a]

	The 1st year of defoliation		The 2nd year defoliation	
	Control (nmol g^{-1})	Defoliation (nmol g^{-1})	Control (nmol g^{-1})	Defoliation (nmol g^{-1})
Putrescine	229.55 (±37.21)	138.51 (±11.35)	252.86 (±20.39)	161.45 (±12.06)
Spermidine	154.33 (±6.09)	155.38 (±5.11)	183.38 (±12.32)	159.27 (±6.86)
Spermine	22.35 (±1.18)	26.47 (±1.68)	28.41 (±2.76)	31.67 (±2.84)

[a] Values are means of 15 trees (±SE). Soluble polyamines were measured in the C needles collected on September 28, 1998, and September 24, 1999.

DISCUSSION

Increased peroxidase and phenolics levels may be involved in the "oxidative burst" following leaf damage (Bestwick et al., 1998), modifications of cell-wall structure, (Dowd and Lagrimini, 1997), or chemical defense mechanisms (Felton and Duffey, 1991; Dowd and Vega, 1996; Dowd and Lagrimini, 1997). The formation of reactive oxygen species, and the following reactions, such as activation of the signalling system and quenching of the oxygen radicals represent rapid responses in plants (León et al., 2001). In the present study, peroxidase activity in intact needles of damaged trees remained at an elevated level for several months after the stimulus. Thus, the function of these enzymes is not limited to immediate reactions after the physical damage. Needle growth was also reduced and needle dry matter content slightly increased after the second defoliation (cf. Niemelä et al., 1991). Peroxidases and phenolics, in part, might be involved in these structural changes. Peroxidases promote the polymerization of monolignols into lignin, which strengthens cell walls (see Dowd and Lagrimini, 1997), and stress activation of oxidative linkage of phenolics to cell walls and cross-linking

TABLE 4. REPEATED MEASURES ANOVA FOR POLYAMINE CONCENTRATIONS IN THE CURRENT YEAR NEEDLES AFTER THE 1ST AND 2ND YEARS OF DEFOLIATION

		Putrescine		Spermidine		Spermine	
Source of variation	*df*	*F*	*P*	*F*	*P*	*F*	*P*
Between subjects							
Treatment	1	12.89	0.001	1.92	0.177	2.22	0.148
Error	28						
Within subjects							
Year	1	1.37	0.253	4.39	0.045	8.35	0.007
Year × treatment	1	0.00	0.993	2.56	0.121	0.049	0.827
Error	28						

of cell-wall polymers is expected to cause reduction in the capacity for expansion growth (Gaspar et al., 1991).

The observed accumulation of starch and sucrose in needles in late September may indicate source–sink imbalance. The starch concentration was 46% and sucrose concentration was 42% higher in the repeatedly defoliated trees than in controls. Sucrose is considered as an important transport form of photoassimilates, and regulation of source–sink relations is one role proposed for starch (Hansen et al., 1997). Although increased mobilization of starch reserves is regarded as a compensation mechanism subsequent to defoliation of Scots pine trees, export of carbohydrates from current year needles may be limited (Ericsson et al., 1980). In the present study, reduced phloem transport of carbohydrates might have taken place as a consequence of defoliation. Increased starch and sucrose concentrations in pine needles also may be an effect of disturbed hydrolysis of these components as proposed by Balsberg-Påhlsson (1989). Also, carbohydrate metabolism in conifers is affected by nitrogen supply (e.g., Pritchard et al., 1997). Nitrogen stress alters the source–sink balance of plants and less carbon is consumed and exported from source leaves for nitrogen assimilation and growth (Paul and Driscoll, 1997; Pritchard et al., 1997). Scots pine foliage acts as winter-storage for nitrogen and, thus, nitrogen remobilization for new growth may be restricted because of winter or spring time defoliation (Millard et al., 2001). Hence, the defoliated trees lost significant amounts of nitrogen with the removed needles, and nitrogen concentration in the current year needles was about 20% lower in the defoliated trees than in controls. Reduced nitrogen availability may have disturbed carbohydrate metabolism and resulted in starch accumulation in the current year needles of defoliated trees.

Defoliated trees had higher quercitrin, (+)-catechin, and catechin derivative concentrations in the needles. However, the concentrations of several phenolic compounds were not affected by defoliation. Consequently, specific phenolic compounds in pine needles can respond differently to defoliation as well as to reduced nitrogen availability. Concentrations of total phenolics increased in response to low nitrogen availability from the growth medium in *Pinus sylvestris* (Kainulainen et al., 1996), *Pinus palustris* (Pritchard et al., 1997), and *Pinus taeda* needles (Gebauer et al., 1998). However, Bongue-Bartelsman and Phillips (1995) showed that nitrogen stress has differential effects on foliar phenolic compounds.

Chlorophyll concentrations correlated negatively with (+)-catechin contents in needles. This is in agreement with Feucht et al. (1997), who reported an accumulation of flavanols in beech leaves with decreasing chlorophyll contents in an area of declining forests. They proposed (+)-catechin to have the potential to stabilize IAA and to promote growth. Richter et al. (1996) also observed increased catechin concentrations in *Picea abies* trees suffering needle loss in a forest damage area. Catechins serve as monomer units for condensed tannins, which are important

for the reinforcement of plant tissues (Koes et al., 1994). In the present study, 3″, 6″-dicoumaroyl-astragalin concentrations were decreased in defoliated trees. This compound has been shown to act as a UV-B-screening pigment and to be located almost exclusively within the epidermal layer in Scots pine needles (Schnitzler et al., 1996).

Nitrogen availability may affect the amount of putrescine in plant tissues (Altman and Levin, 1993), and decreased nitrogen levels may have resulted in reduced putrescine concentrations in defoliated trees. Accordingly, nitrogen fertilization has been found to increase foliar-free putrescine in *Pinus sylvestris* (Taulavuori et al., 1998) and *Pinus resinosa* needles (Minocha et al., 2000). However, reduced putrescine levels in damaged trees also could be due to degradation by oxidases (see Bagni and Tassoni, 2001).

Polyamines often occur as free-form cations in plant cells, but they can also be conjugated to small molecules like phenolic acids (conjugated forms) and also to various macromolecules like proteins (bound forms) (Galston and Sawhney, 1993; Boucherau et al., 1999). In the present study, only free polyamines were measured. However, the conjugated and bound forms may also play a significant role in stress responses. Walters et al. (2002) observed that treatment of first leaves of barley seedlings with methyl jasmonate did not affect free polyamines in the second leaves whereas soluble conjugates of putrescine and spermidine increased in the these untreated leaves.

Lower putrescine levels in defoliated trees in late September suggests differences in hardening processes between defoliated and control trees. A typical feature of seasonal fluctuation of putrescine in Scots pine needles is high concentration in the winter and low in summer (Sarjala and Savonen, 1994). Increased putrescine concentration in Scots pine needles has been attributed to an increased state of hardening, and in hardened Scots pines, putrescine concentration was positively correlated with frost resistance (Sarjala et al., 1997). However, the actual role of polyamines in the hardening processes is not known. Deposition of starch in needles in fall, as observed in this study, indicates disturbances in the frost hardening processes. The frost hardening mechanisms of conifers include carbohydrate pool shift from starch accumulation to soluble sugar formation, as soluble sugars act as cryoprotectants. Usually, starch content is high in summer and low in winter in aboveground parts of Scots pines, whereas the opposite is true for soluble sugars (Hansen et al., 1997).

In summary, the observed inducible changes in defensive chemistry may indicate increased oxidative stress due to the defoliation. While specific phenolic compounds can respond differently to previous defoliation, there seem to be predictable changes in the foliar chemistry of the Scots pine depending on whether defoliation affects leaf nitrogen or not. Because defoliation strongly reduced shoot growth and there were fewer sporocarps of mycorrhizal fungi associated with the present defoliated trees (Kuikka et al., in press), defoliation may have led to

imbalances in source–sink relationships as well as in the foliar nitrogen and carbohydrate contents. In contrast to Niemelä et al. (1991), foliar nitrogen decreased and dry matter content increased as a consequence of 2 years of successive defoliations. Although artificial defoliation may not mimic the response of Scots pine needles to actual herbivore damage, these results suggest that changes in needle phytochemistry in response to defoliation may accompany changes in needle nitrogen metabolism.

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LABORATORY ASSESSMENT OF THE ALLELOPATHIC EFFECTS OF FINE LEAF FESCUES

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Abstract—Laboratory screening studies were conducted to evaluate the allelopathic potential of fine leaf fescues. Of the seven accessions selected from prior field evaluations for weed-suppressive ability, all inhibited root growth of large crabgrass and curly cress in laboratory assays. Grown in agar as a growth medium and in the presence of living fescue seedlings for 14 or 21 days, test species were sensitive depending on the fescue cultivars. Growth inhibition increased when fescue was grown for increasing periods of time in agar. Seedling fescues produced significant quantities of bioactive root exudates, which were released into the agar medium. Bioactive root exudates were extracted from living fescue roots by using methylene chloride. Shoot tissue was extracted in water and the aqueous extract was partitioned against hexane, ethyl acetate, and methylene chloride. Extracts were tested for inhibitory activity on seedling growth as measured by inhibition of curly cress germination and radicle elongation. Root exudates were more toxic (70% inhibition) than shoot extracts (up 40% inhibition), when formulated at 0.25 mg/ml concentration. Light microscopy and transmission electron microscopy were utilized in an attempt to identify the cellular location of production of secondary products contained in bioactive root exudates. Ultrastructural analysis indicated that the exudate is produced in actively dividing tips of fibrous root cells. The mode of release of these exudates into the environment remains unknown.

Key Words—Allelopathy, fine leaf fescues, root exudates, weed suppressive ability, cultivar dependant.

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† Deceased Feb. 15, 2002. This paper is dedicated to his memory.

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INTRODUCTION

Members of the festucoids, often referred to as cool season grasses, include all fine leaf fescues and specifically slender and strong creeping red fescues (*Festuca rubra* L. spp. *rubra* and *Festuca rubra* L. spp. *trichophylla* Gaud. or spp. *littoralis* [Meyer] Auquiz), chewings fescues (*Festuca rubra* L. spp. *commutata* Gaud.), hard fescues (*Festuca longifolia* Thuill.), and sheep fescues (*Festuca ovina* L.). Fine fescues are the main turfgrass species currently used in the Northeastern United States, in addition to Kentucky bluegrass (*Poa pratensis* L.), perennial ryegrass (*Lolium perenne* L.), creeping bentgrass (*Agrostis stolonifera* L.), and tall fescue (*Festuca arundinacea* Schreb.) (Jauhar, 1993). These cool season grasses are currently used in golf course fairways, athletic fields, and private and public lawns throughout the temperate regions of North and South America and Europe. Currently, the hectarage of managed turf is on the increase in the United States, with approximately 10–12 million hectares of lawns, athletic fields, and golf courses.

The need for responsible environmental stewardship and the establishment of stricter pesticide usage regulations in many states, including New York and California, have led to a demand for biorational alternatives to herbicides for weed management in turf. Allelopathy, the study of biochemical interactions among plants, can be viewed as a possible biorational alternative. *Allelopathy* can be more specifically defined as the inhibition of growth or development of plants by secondary metabolites produced by surrounding plants (Einhellig, 1995). The selection and utilization of weed suppressive cultivars with enhanced allelopathic activity has only recently been attempted in such crops as rice (*Oryza sativa* L.) (Jensen-Bach et al., 2001; Rimando et al., 2001), wheat (*Triticum aestivum* L.) (Wu et al., 2001a,b), and winter rye (*Secale cereale* L.) (Reberg et al., 2001). Further development of other crops with enhanced weed-suppressive traits is feasible, using both traditional and molecular breeding approaches. Given the fact that few, if any, effective alternative weed management strategies have been developed for turfgrasses other than careful cultural management, allelopathic or weed-suppressive turfgrasses may offer an interesting possibility for the turf industry and the concerned public.

There are relatively few studies that have focused on fescue interference with other plant species. In laboratory studies, Hoveland (1964) reported little effect of extracts of dried tall fescue roots (*Festuca arundinaceae* Schreb.) on several clover (*Trifolium*) species including *Trifolium repens* L., *Trifolium nigrescens* Viv., *T. incarnatum* L., and *T. vesciculosum* Savi. However, other investigators, including Peters (1968), showed that aqueous extracts of dried tall fescue shoots and roots significantly reduced growth of rape (*Brassica nigra* L.) and negatively influenced the germination, seedling growth, and yield of birdsfoot trefoil (*Lotus corniculatus* L.) (Stephenson and Postler, 1988). Gilmore (1977) reported that fescue leachates significantly reduced the growth of sweet gum (*Liquidambar styraciflua* L.) seedlings.

Peters and Zam (1981) later reported a reduction of red clover (*Trifolium pratensis* L.) and bird's-foot trefoil germination by extracts of soaked fescue leaves. In forest ecology field studies, sweet gum, black alder (*Alnus glutinosa* (L.) Gaertn), black locust (*Robinia pseudoacacia* L.), and young black walnut (*Juglans nigra* L.) seedlings were found to be sensitive to the presence of established tall fescue (Peters and Luu, 1985; Van Sambeek, 1989). In other field studies, Weston (1990) showed that creeping red fescue cover crops were particularly weed suppressive to annual weeds in Kentucky, either as living covers or when killed with the herbicide glyphosate.

Fescue species are well known to be endophyte-infected, and some argue that the presence of this internal microorganism may influence the chemical interference or allelopathic potential of fine fescues. Past studies have shown that the presence of the endophyte organism, *Neotyphodium coenophialum* (Morgan-Jones and Gams) (Glenn et al., 1996) in tall fescue resulted in alkaloid production in fescue shoots and influenced the internal morphology of neighboring *Trifolium* species by reducing root length and density of clover. This indirect inhibition may have a direct impact on nutrient and water uptake and subsequent competitive ability (Malinowski et al., 1997, 1999). However, in other studies, the presence or absence of the endophyte did not influence the allelopathic potential of extracts of tall fescue seed on the germination and seedling growth of *Trifolium* species (Springer, 1997). Therefore, the presence or absence of endophytes and their influence on the allelopathic potential of fescues warrants further study.

Relatively few studies have been performed to assess the weed-suppressive or allelopathic effects of fine leaf fescues. Living or killed fescue sods were found to be highly weed suppressive over a 3-month period following establishment and then being killed with glyphosate, with red fescue being slightly more effective (95%) than tall fescue (90%) in suppressing weed growth (Weston, 1990). Fujii (2001) also recently demonstrated the allelopathic effect of several fescue species using a bioassay in sand. A 70% inhibition of radicle length of sensitive species in the presence of *Festuca rubra* grown in sand and 55% inhibition of seedlings in the presence of *Festuca arundinaceae* was reported (Fujii, 2001). A study evaluating the effect of fall-sown cover crops on weed control in the field concluded that meadow fescue (*Festuca eliator*) and tall fescue also were inhibitory to weed seedling establishment (Fujii, 2001).

Field studies conducted over the past year in Ithaca, NY, and Riverhead, NY, by Bertin and Senesac (unpublished) have shown considerable variation in the weed-suppressive ability of fine leaf fescue cultivars. These studies were performed in an attempt to select for those cultivars exhibiting superior weed-suppressive ability and growth (Bertin et al., 2002) and conducted following a recent National Turfgrass Evaluation Program (NTEP) trial established in Ithaca, NY, in 1998. After two seasons of field evaluations in 1999 and 2000, we selected several of the cultivars consistently exhibiting the greatest weed-suppressive potential in the

field (Bertin et al., 2002). Weed-suppressive cultivars included Treazure, Oxford, Intrigue, and Sandpiper. We also selected several cultivars (Columbra, Reliant II, and Rescue 911) with only moderate weed-suppressive ability.

The following experiments were designed to further investigate the possible allelopathic interference of selected cultivars (both more and less suppressive in field studies) of fine leaf fescue in the laboratory, and to specifically evaluate the phytotoxicity of both living root exudates and shoot extracts. In addition, anatomical studies were performed using electron microscopy to (1) evaluate the cellular site of production of fescue root exudates and (2) determine the presence or absence of fescue endophytes within the living root system.

METHODS AND MATERIALS

Plant Materials. Of the 78 fine leaf fescue cultivars evaluated in previous field studies (Bertin et al., 2002), 7 were selected for further laboratory evaluation. These included Oxford (hard fescue), Reliant II (hard fescue), Rescue 911 (hard fescue), Sandpiper (chewings fescue), Intrigue (chewings fescue), Columbra (chewings fescue), and Treazure (chewings fescue) (Table 1). Prior to each experiment, seeds of fine leaf fescue and indicator species (large crabgrass and cress (*Lepidium sativum* L.)) were surface-sterilized in a 20% (v/v) solution of sodium hypochlorite for 1 min, repeatedly rinsed in distilled water, dried, and stored under sterile conditions at ambient temperature (20°C).

Screening for Allelopathic Potential of Living Fescue Root Exudates. Under sterile conditions, 0.8% water agar (80 ml) was poured into plastic Magenta tissue culture boxes. Thirty sterilized fine leaf fescue seeds of each cultivar selected were seeded randomly on to the surface of the agar medium in individual boxes, which were then sealed. Control boxes contained 0.8% agar, but no fescue seedlings. All boxes were placed under artificial lighting with a 16-hr light (200 μmol/m^2/sec^1)

TABLE 1. FINE LEAF FESCUE CULTIVARS SELECTED FOR FURTHER LABORATORY EVALUATION

Cultivar name	Fescue type	Species	Weed-suppressive activity[a]
Oxford	Hard fescue	*Festuca longifolia* L. Thuill	High
Reliant II	Hard fescue	*Festuca longifolia* L. Thuill	Moderate
Rescue 911	Hard fescue	*Festuca longifolia* L. Thuill	Moderate
Sandpiper	Chewings fescue	*Festuca rubra* L. Subsp. *Commutata* Gaud.	High
Intrigue	Chewings fescue	*Festuca rubra* L. Subsp. *Commutata* Gaud.	High
Treazure	Chewings fescue	*Festuca rubra* L. Subsp. *Commutata* Gaud.	Moderate
Columbra	Chewings fescue	*Festuca rubra* L. Subsp. *Commutata* Gaud.	Moderate

[a] Weed-suppressive activity refers to levels of weed suppression visually evaluated once a month during the growing in replicated field trials from 1999 to 2000 in Ithaca, New York.

and 8-hr dark cycle at 20 ± 5°C. After 7 days, 10 large crabgrass or cress seeds were randomly placed within the fine leaf fescue seedlings, and the box was resealed and placed under light as described earlier for an additional period of 7 days. Experiments were arranged as completely randomized designs, with three replicates of each treatment. Percentage of germination of crabgrass and cress was recorded as well as root and shoot lengths of individual crabgrass and cress seedlings after 7 days. Experiments were repeated three times using identical methodology.

An additional bioassay to assess the allelopathic potential of fine leaf fescue over time was designed using the methodology described earlier. However, after letting the fine leaf fescue seedlings establish for 7 days, fescue seedlings were removed, and 10 curly cress (*Lepidium sativum* L.) seeds were placed randomly in the zone where fescue seedlings previously had been positioned. The box was resealed and placed under a 16-hr light and 8-hr dark cycle for 7 days. The control treatment contained no fescue seedlings, so none were removed. Treatments (fescue once present vs. fescue never present) were replicated three times, and the experiment was arranged as a randomized complete block design. Root and shoot lengths of individual cress seedlings, as well as their percentage seed germination, were recorded after 7 days. The experiment was repeated over time on three separate occasions.

Root Exudate Collection under Stressed and Nonstressed Conditions. Approximately 100 g of fine leaf fescue seed of each cultivar was surface-sterilized to reduce microbial contamination and then placed on to each of six screens of a capillary mat system used for seedling generation of large quantities of living root tissue (Czarnota, 2001). Seeds were allowed to germinate on the capillary mat system, and after 14 days, living roots were harvested by removal from the adjacent screen with a razor blade. Large quantities of healthy root tissue were harvested from each individual screen, and fresh weight was recorded. Collected root tissue was then dipped in 250 ml of methylene chloride for approximately 1 min. Roots were removed by filtration, and the methylene chloride extract, yellowish in color, was further filtered using a glass syringe fitted with a Gelman Acrodisc CR-PTFE 0.2-μm filter. The extract was evaporated to dryness using a rotary evaporator at ambient temperature. Dry weight of the methylene chloride extract was obtained separately for each of the six screen replicates for each cultivar studied. Samples were then subjected to chemical analysis and bioassayed to assess inhibitory activity on the seed germination of cress species. Root exudates were also collected for each of the seven cultivars evaluated when seedling roots were propagated under stress-simulated conditions. To stimulate drought stress, using a capillary mat system as described previously, water was available as normal for 7 days to allow sufficient germination. In contrast to standard moisture conditions utilized in the first experiment from day 0 to 14, water stress conditions were applied to young fescue seedlings from day 7 to 14 in the second experiment by limiting the

water supply at day 7, and thus drying the capillary mat progressively from day 7 through 14. This condition will be referred to as the dry growth conditions. The experiment was arranged as a completely randomized block and repeated three times.

Influence of Fine Fescue Root Exudates on Cress Growth. Experiments were designed to assess the toxicity of collected root exudates by using the cultivars Intrigue and Sandpiper because of their differential weed-suppressive activity in the field. Concentrations ranged from 0 to 1 mg/ml and also included 0.0625, 0.125, 0.25, and 0.5 mg/ml. Glass petri dishes were lined with filter paper (Whatman # 1), and 0.5 ml of each root exudate was dissolved in methylene chloride and added to the 5.5-cm filter paper, and methylene chloride was allowed to evaporate. After evaporation, 1 ml of distilled water and 10 cress seeds were placed into each dish. Petri dishes were maintained in an incubator for 72 hr at 26°C with relative humidity near saturation. Three replicates of each treatment were arranged in a completely randomized design. After 3 days, seedling root lengths were recorded. The control consisted of 0.5 ml of methylene chloride placed onto a filter paper, allowed to evaporate, and moistened with 1 ml of distilled water. Percentage inhibition of cress was expressed as a percentage decrease in radicle elongation in comparison to the control. Experiments were repeated on three separate occasions.

Influence of Fine Leaf Fescue Shoot Extracts on Cress Growth. Fine leaf fescue, cultivar Intrigue, was grown in a greenhouse under natural light conditions during the late summer of 2000. When shoots were 6 weeks old, they were harvested by trimming and placed into a drying oven at 35–40°C for 4 days. Dried tissue was then ground in a Wiley mill. After grinding, powdered shoot tissue (100 mg) was extracted for 24 hr with 3-l distilled water on a shaker in a 4°C cold room. After 24 hr, the mixture was filtered through four layers of cheesecloth to remove coarse particulates. Extracts were filtered through # 4-, # 1-, and # 42-grade filter paper (Whatman) by using a Buchner funnel. The clear extract was partitioned using a solvent series that included hexane, methylene chloride, and ethyl acetate. Extractions were performed three times for each solvent with 100 ml of solvent in a separatory funnel. Extracts were combined for each solvent and dried using rotoevaporation at ambient room temperature. Toxicity of leaf extracts was evaluated as percentage decrease in root elongation compared to the control. Extracts were evaluated over a range of concentrations (0.0125–0.5 mg/ml) as above, and cress radicle length was measured after 72 hr. The experiment was repeated three times.

HPLC. Fescue root exudates, cv Intrigue, were collected and compared using two separate production methods: (1) a capillary mat was utilized to collect large quantities of root exudates, and (2) root exudate was extracted from the water-based agar medium where fine leaf fescue had been grown for 5 weeks. Root exudate was extracted by homogenizing the agar medium in methylene chloride. This solution had been filtered through Whatman # 4 filter paper. The filtrate was dried using

rotoevaporation at room temperature. A comparison of these root exudates was performed by HPLC to evaluate the chemical composition of bioactive root exudates under two different growth conditions. Exudate separation was accomplished using an HPLC (Waters, Inc.) equipped with a photodiode array detection, evaluating at 230 nm. The column utilized was a Xterra RP_{18} (3.9×100 nm) (Waters, Inc.). Compounds were separated using a binary solvent system of acetonitrile and acidified water (2.5% acetic acid) over 30 min. The solvent composition was maintained at 20% acetonitrile for the first 20 min. At time 20 min, the solvent was stepped up to 100% acetonitrile at which it was maintained for 5 min before returning to the initial solvent conditions for subsequent analyses.

Data Analysis. All data collected were subjected to analysis of variance with repeated measures. Mean separations were performed using Fisher's protected LSD at the 5% significance level.

Microscopy. One fine leaf fescue, cv Intrigue, was chosen for further study on the basis of its potent allelopathic activity in prior laboratory assays. Hypochlorite-treated seed was placed onto a capillary mat system and allowed to germinate at room temperature (25°C) for 1 week, providing root tissue with ample surface area for examination under light and electron microscope.

Light Microscopy. Fresh roots were deposited on an Olympus SZX 12 (obj 90) on a wet paper towel, and root tips were observed.

Electron Microscopy. Fresh roots were fixed in glutaraldehyde, and washed by bathing several times in distilled water. Methanol was used to dehydrate roots to the critical point at which the root was completely dry. Dry roots were glued onto aluminum specimen stubs with conductive carbon disks. The specimen stub was coated with 30 nM of gold/palladium (60/40) in a sputter coater. Scanning electron micrographs were taken with a JEOL JSM 840 scanning electron microscope. For transmission electron microscopy (TEM), 2-mm cross-sections of fine leaf fescue roots were immersed in fixative buffer (4% glutaraldehyde in 0.1 M cacodylate buffer) overnight. The next day, samples were rinsed in 0.66 M cacodylic buffer for 30 min followed by a 1-hr postfixation in 1% OsO_4. Samples were rinsed in distilled water for resin. Thin sections, obtained with an ultramicrotome, were stained in 2% uranyl acetate and Reynolds lead citrate. Sections were observed using a Zeiss EM10CR transmission electron microscope.

RESULTS

Screening for Allelopathic Potential. Of the seven fescue cultivars evaluated, all reduced mean root and shoot lengths of large crabgrass and curly cress (Figure 1). Inhibition due to the presence of fescue ranged from 46% ("Treazure") to 80% ("Intrigue") for crabgrass and from 21% (Treazure) to 58% (Intrigue) when curly cress was the test species. Root length was generally inhibited more

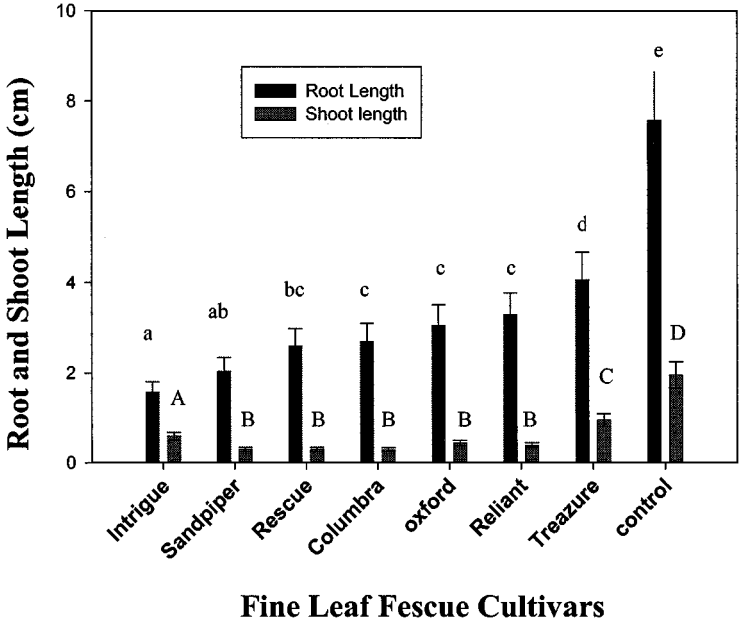

FIG. 1. Inhibition of seedling growth of *Digitaria sanguinalis* L. by the presence of fine leaf fescue seedlings after 2 weeks of establishment in an agar medium. Bar represents ± one standard error unit. $N = 90$. Values with the same letter are not significantly different with Fisher's protectd LSD test at the 0.05 level.

than shoot length for all fescue cultivars evaluated. For example, in the presence of cv "Intrigue," root length of curly cress was inhibited by 58%, while shoot length was inhibited by 37%. When fescue seedlings were removed from the agar before seeding with the indicator species, cress root length was still noted and inhibition increased with increasing residence time in the agar medium. Fescue seedlings established for 3 weeks resulted in a more suppressive growth media than those seeded for only 1 week when cress seedling growth was evaluated (Figure 2).

Root Exudate Collection under Stressed and Nonstressed Conditions. When produced using a capillary mat system, fresh root fescue cultivars yielded varying quantities of root exudates, ranging from 0.14 mg exudate/g of fresh root (Columbra and Rescue) to 0.83 mg/g (Sandpiper). Intrigue and Treazure yielded intermediate amounts exudates with 0.53 and 0.55 mg of exudate/g root tissue, respectively. Drier conditions during seedling growth on the capillary mat led to a considerable increase in production of root exudates (Table 2). Production of root exudates by 14-day-old seedlings was doubled under drier growth conditions.

Influence of Fine Leaf Fescue Root Exudates and Shoot Extracts on Cress Root Length. Methylene chloride and ethyl acetate extracts of fescue root exudates

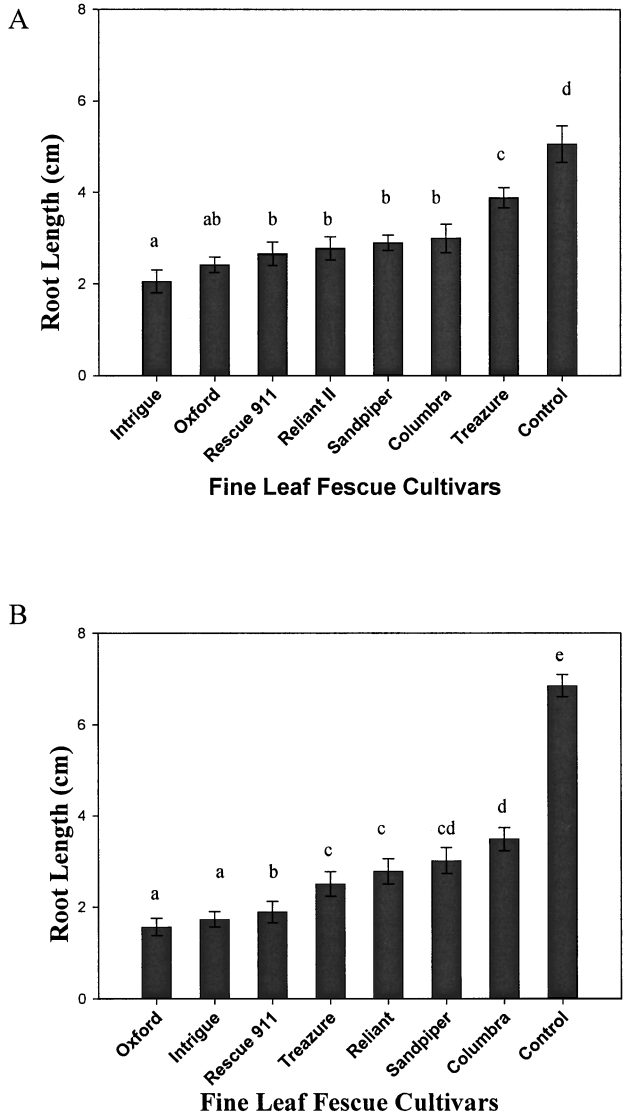

FIG. 2. Inhibition of seedling root elongation of curly cress (*Lepidium sativum* L.) by presence of fine leaf fescue seedlings established for 1 week (a) or 3 weeks (b), and removed from the growth medium before reseeding with cress. Bars represent ± one standard error unit. Values with the same letter are not significantly different with Fisher's protectd LSD test at the 0.05 level.

TABLE 2. PRODUCTION OF FINE LEAF FESCUE ROOT EXUDATE AMOUNT COLLECTED FROM SEVEN DIFFERENT CULTIVARS UNDER TWO GROWTH CONDITIONS

Fine leaf fescue cultivar	Root exudate production (mg/g)[a,b]	
	Normal conditions	Dry conditions
Intrigue	0.53 ± 0.03	1.43 ± 0.04
Sandpiper	0.83 ± 0.03	1.62 ± 0.06
Rescue	0.40 ± 0.03	0.90 ± 0.05
Columbra	0.55 ± 0.02	0.29 ± 0.06
Oxford	0.15 ± 0.02	0.31 ± 0.05
Reliant	0.57 ± 0.01	1.23 ± 0.03
Treazure	0.55 ± 0.02	1.20 ± 0.08

[a] Measurements of fine leaf fescue fresh root weight were obtained 2 weeks after seeding on a capillary mat system.
[b] Average exudate production was calculated on the basis of 18 total replicates, with 6 replicated values obtained from each of three separate experimental mat systems. Standard error was calculated.

caused significant decreases in root length of curly cress seedlings after 72-hr growth (Figure 3). Root length was a more sensitive indicator of growth inhibition than seed germination or shoot length (data not presented). Root exudates, at similar concentrations, were more inhibitory to root growth of the indicator species than were shoot extracts. The methylene chloride fraction collected from partitioning of the aqueous shoot extract was most inhibitory to curly cress radicle elongation compared with other solvent fractions, with 40% inhibition of root elongation at a concentration of 0.25 mg/ml. In comparison, the hexane fractions did not inhibit radicle elongation at all, whereas the ethyl acetate fractions resulted in 27% inhibition when the concentration reached 1 mg/ml (Figure 3).

HPLC. We observed 15–20 major components within each root exudate, using reverse phase HPLC for separation (Figure 4). This bioactive root exudate is apparently released naturally by living fine fescue seedlings in sufficient quantities within both agar growth assays, and also under field conditions, to result in inhibition of weed seed germination and growth. The exudates also can be collected in large quantities when roots are produced on a capillary mat system, and the resulting exudates cause inhibition of seed germination at very low concentrations. Using a methylene chloride extraction of the agar medium, where fine leaf fescue seedlings were grown for 14 days, thin layer chromatography and HPLC indicated that constituents present in the root exudates extracted directly from roots also were present in the agar medium (Figure 4).

Light and Electron Microscopy Studies. When grown on a capillary mat system, fine leaf fescue seedlings produced a large quantity of fibrous roots and tiny living root hairs, which originated from single cells attached to the main fibrous

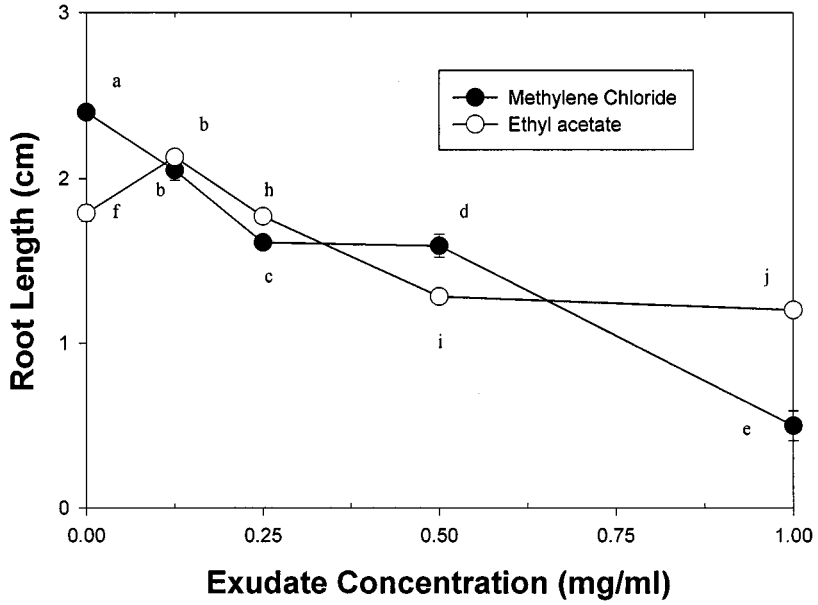

FIG. 3. Influence of ethyl acetate and methylene chloride extracts of fine leaf fescue shoots, cv "Intrigue," on root length of curly cress (*Lepidium sativum* L.) 72 hr after germination. Bars represent ± one standard error unit. Values with the same letter are not significantly different with Fisher's protectd LSD test at the 0.05 level.

root system (Figure 5). The root hairs themselves do not appear to be the origin of root production of the exudates. Their function may only be enhancement of the overall root absorbing surface area for water and nutrient resources. SEM images indicated that the root hairs were actually prolongations of individual epidermal root cells. No droplets of the root exudate were detected at the root hair tips. However, a smaller structure was observed at the tips of all roots when observed under the light microscope (Figure 6). This structure, observed under electron microscopy, showed that the epithelium cells located at the tips of living fibrous roots were cytoplasmically dense and appeared to serve as metabolic sites for the production of bioactive root exudates (Figure 7). The presence of numerous cellular organelles such as golgi bodies, endoplasmic reticulum, and mitochondria implies that the bioactive root exudates are synthesized within the epithelium cells and temporarily stored in small osmiophilic secretory vesicles located in the cytoplasm of these cells. In contrast, a similar study performed on lemna root tips was conducted for comparative purposes (data not shown). Using the same technique, lemna root tip cells possessed limited quantities of endoplasmic reticulum, golgi, and ribosomes and few, if any, dense osmiophilic inclusions, as seen in fescue tips.

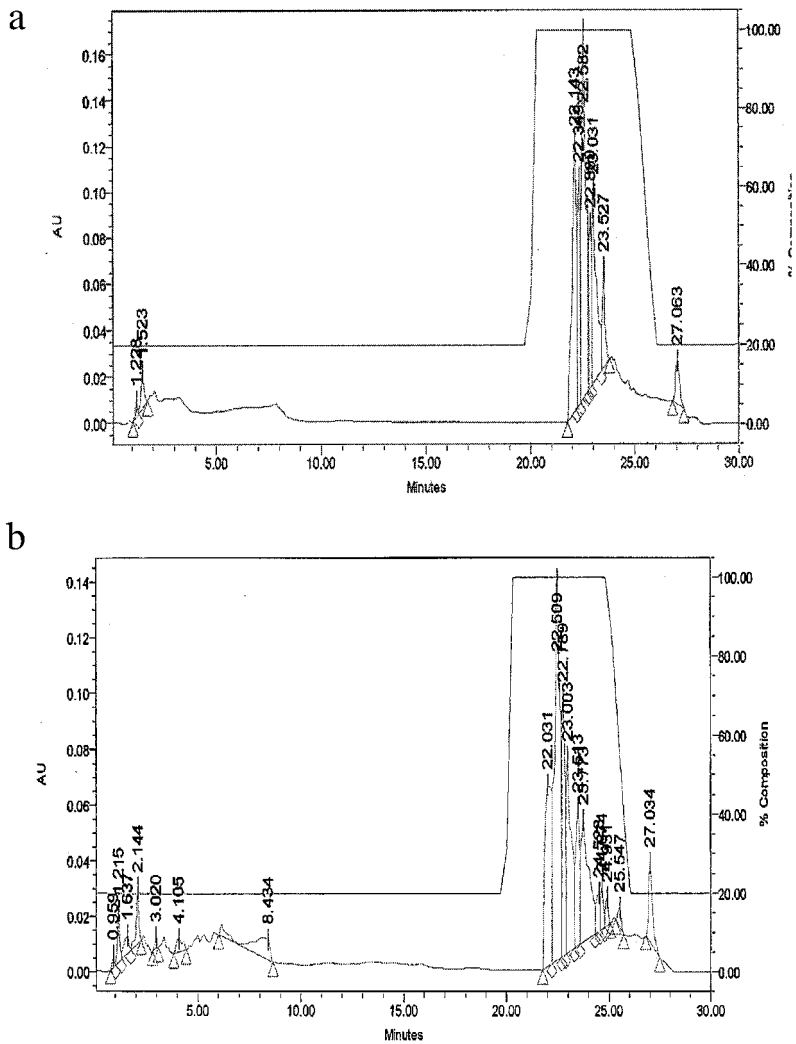

FIG. 4. Relationships between chemical composition of root exudates when roots are produced using agar medium (a), a capillary mat system (b).

DISCUSSION

Evidence for the allelopathic potential of any species may depend as much on the choice of method used to bioassay the activity, as on the choice of species (Stowe, 1979). Large crabgrass is a widespread weed in most U.S. turf setting, including golf courses and athletic fields. We used curly cress as another test species

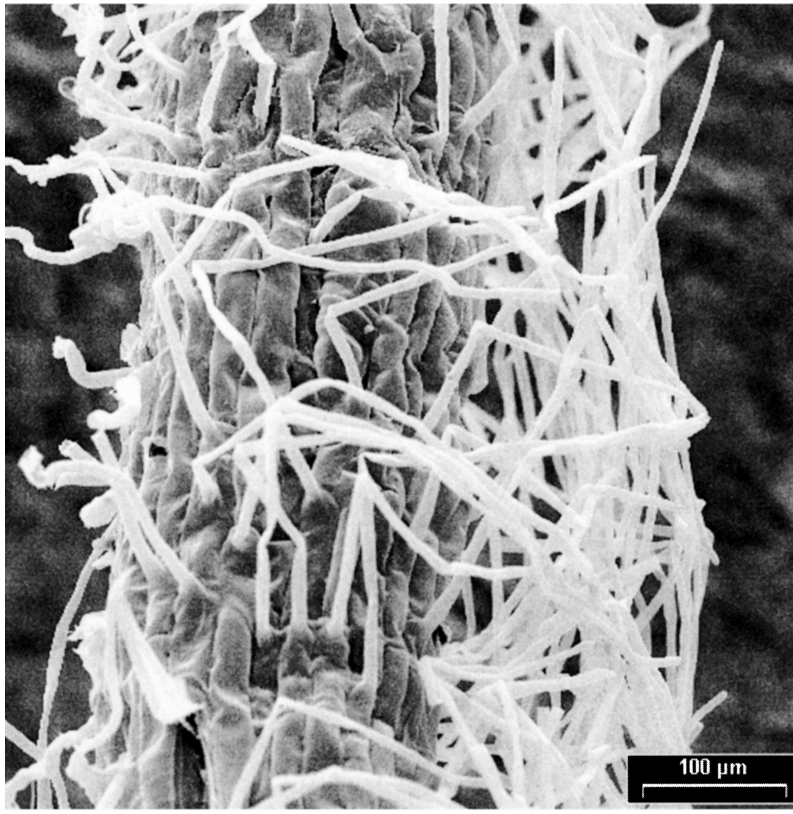

FIG. 5. SEM of root hairs present on fine leaf fescue root.

because of its uniform germination and the fact that it is a broadleaf. Obviously, success or failure in separating allelopathy from inhibition associated with resource competition can be influenced by a lack of rigorous methodology (Fuerst and Putnam, 1983). The choice of bioassays is, therefore, a major determining factor in screening programs seeking to draw conclusions about the allelopathic potential of the species in question. Utilization of intact plants and the development of experiments that reasonably simulate the natural conditions encountered during plant interference are two key criteria for developing convincing methods to assess allelopathic potential (Inderjit and Weston, 2000). This study attempted to address a number of these issues by designing experiments using 0.8% water agar, which ensured a stable support for plant growth and uniform moisture uptake in both fescue and the indicator species. The use of agar-containing magenta boxes also allowed us to later remove fescue seedlings from each container, leaving behind

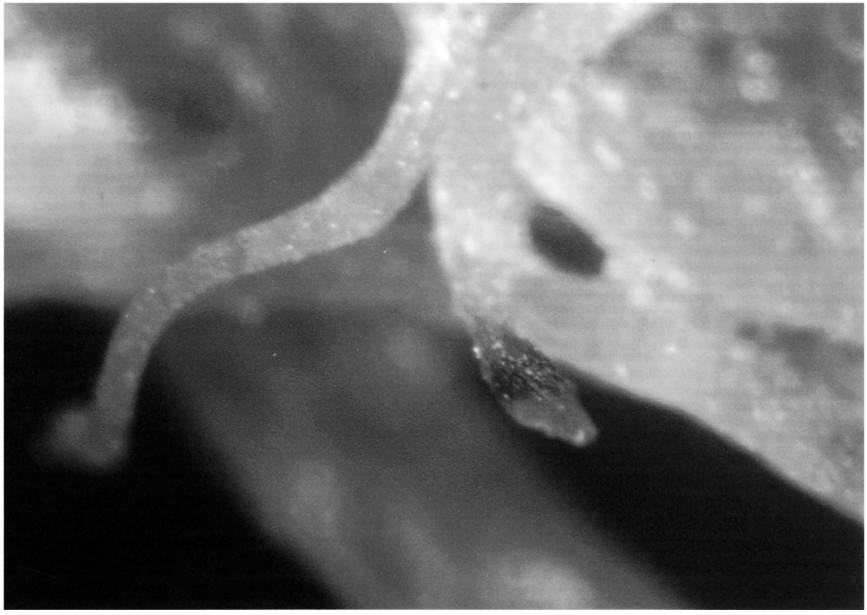

FIG. 6. Root tip of fine leaf fescue cv "Intrigue" secreting a brown exudate containing the allelochemicals assumed to be responsible for its allelopathic effect.

the growth media containing root exudates and potential allelochemicals. This created a more conclusive means to prevent competition for resources from impacting plant growth, while demonstrating allelopathic effects because of production of bioactive root exudates. Light supply was not limiting and was equally distributed within the magenta boxes because of the fine, upright canopy architecture of the fescue seedlings. Therefore, we do not believe that light was a limiting factor for test species development. Nutrient competition was also negated as an important factor in this study. Although nutrients were not added to the agar growth medium, experiments were conducted for such a short duration that resources providing for initial seedling growth were obtained from seed reserves and likely not limiting, judging by the healthy green appearance and growth of the control seedlings in the absence of living fescue. In addition, removal of the fine leaf fescue seedlings from the magenta boxes allowed us to later replant monocultures of our test species and clearly demonstrate the presence of bioactive inhibitors in the growth medium. Competition for space, moisture, or nutrients where living fescue was no longer present were essentially eliminated for test seedlings harvested shortly after planting.

These experiments provide strong evidence that fine leaf fescue seedlings are producing bioactive root exudates that reduce the growth of surrounding plants,

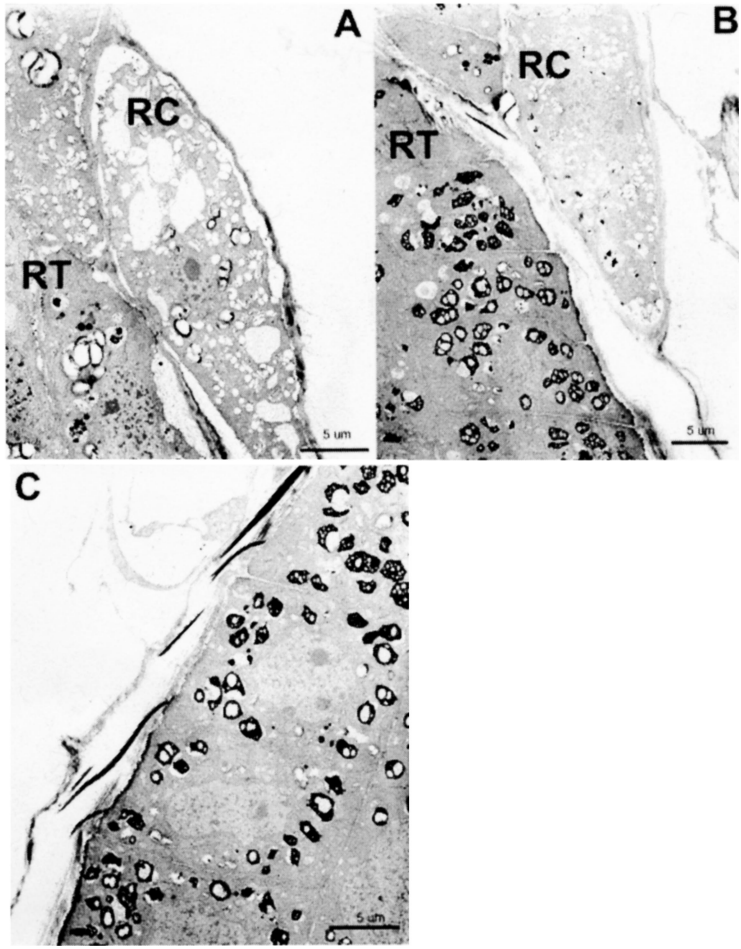

FIG. 7. A cross-section of a typical fine leaf fescue fibrous root (cv "Intrigue") showing the presence of numerous darkly stained inclusions in the cytoplasm of epithelium cells. RT: Root tip. RC: Root cap. A and C: Root tip cells. B: Root cap cells.

including large crabgrass, curly cress, and lettuce. All fescue accessions showed allelopathic potential either when fescue was present or later removed from the agar medium.

Lack of reproducibility is a common problem in allelopathy research (Latto and Wright, 1995); however, in both field experiments and laboratory assays, certain fescue cultivars were observed to be more suppressive. Veronneau et al. (1997) suggested that reproducible results from two or more bioassays systems

were required to demonstrate allelopathic potential in plants. In this case, cultivars Intrigue, Sandpiper, and Oxford were generally more suppressive than "Reliant II" and "Columbra," in both field and laboratory assays (Bertin et al., 2002). The cultivar "Treazure" was not suppressive in 2 years of field settings in two different locations. Also, it was not suppressive in a laboratory setting. Under field conditions, more factors must be taken into consideration than those under laboratory settings to understand the process of weed suppressivity. Differences observed in weed-suppressive ability of the same cultivar between in-field and in-laboratory might be due to the inherent complexity of the rhizosphere versus a gel medium. Soil moisture, texture and particle size, water content, buffering capacity, and microorganism populations may all interact with root exudates and influence the activity of chemicals released by plants. However, we observed many similar trends among field and laboratory assays in relative toxicity of cultivars and long-term suppressivity.

Even though results obtained from solvent extractions or from maceration and grinding of plant materials may result in the liberation of chemicals that would not normally be released in the field by living tissue (Lovett and Jessor, 1982), such studies have the potential to indicate allelopathic potential, both during the life of the plant and later during decomposition of plant residues (Inderjit and Weston, 2000). The somewhat inhibitory activity of the ethyl acetate and methylene chloride leaf extract of the fine leaf fescue accessions suggests the presence of phytotoxic constituants within these extracts. Leaf extracts of other crops and grassland forage species can also reduce the growth of certain test and weed species (Weston et al., 1989; Creamer et al., 1996; Wardle et al., 1996). Whether the presence of these secondary products in shoot tissues impacts the allelopathic activity of decomposing fescue residues in the field remains to be seen.

Root systems of various plants have been shown to be the site of production of root exudates with potent allelopathic potential, including sorghum (*Sorghum bicolor* L. Moensch) and black walnut (*Juglans nigra* L). These plants produce exudates containing mixtures of related hydroquinones, which are potent inhibitors of photosynthesis or respiration in higher plant systems (Einhellig et al., 1993, Hejl et al., 1993). Our agar bioassays have shown that the root system of fine fescue is responsible for producing allelochemicals, which have potent inhibitory activity upon developing weed seedlings. The agar extract that previously supported the growth of the living root systems of fine fescue seedlings contained identical chemical constituents, as did the root exudates we collected from living fescue root extraction. Fine leaf fescue accessions appear to exhibit marked differences in their ability to produce root exudates when grown under identical environmental conditions, suggesting that genetic diversity for exudate production exists within our collection of fine fescue cultivars. The exact role of the fescue root exudates in the rhizosphere is unknown. Exudate production may be logically associated with plant defense, which is particularly important during

seedling phase of growth. Differences in production of root exudates may be associated with the ability of fine leaf fescue cultivars to exhibit differential suppressivity in the field and the laboratory. Environmental factors also may influence both competition and allelopathic trait expression in fescue cultivars. Although further studies are required to elucidate bioactive constituents within the root exudates and determine their specific activity and mode of action in inhibiting weed seedling growth, strong evidence from both field and laboratory experiments suggests a role for allelopathic interference by certain cultivars of fine fescue.

Endophytes are widespread in the grasses (White, 1988; Clay and Leuchtmann, 1989) and influence turfgrass quality and pest resistance, but are also associated with mammalian toxicosis. All the fine leaf fescue cultivars were positively tested for the presence of endophyte by using Bacon's method. One could suggest that the presence of the endophyte might have a positive influence on the production and the toxicity of the root exudates. However, electron microscopy studies of the fine leaf fescue roots showed a complete absence of the endophyte, suggesting that, even though present in the seed and in the shoot of fine leaf fescue cultivars, the endophyte appears not to be associated with root exudate production or toxicity.

Past studies with a variety of plants have shown that root exudates generally contain a variety of organic constituents, which are released into the rhizosphere. Root exudates are often chemically diverse and include simple low-molecular-weight compounds such as organic acids, sugars, and amino acids as well as more complex substances, including vitamins, plant hormones, and secondary products (Rovira, 1969). The presence of cytoplasmically dense root tip cells of fine fescue containing large numbers of organelles including endoplasmic reticulum, Golgi bodies, and ribosomes, suggests that actively dividing fine fescue root cells are the site of production of the bioactive secondary products found within fescue root exudates. When examining the entire length of living fescue root systems, a differential pattern in production of secondary products was noted. Living roots originating near the growing point showed an absence of osmophilic compounds and organelles involved with synthesis, while living roots tips had an increased presence of metabolic organelles and vesicles containing dark staining chemical constituents. This observation suggests that fine leaf fescue root exudates are synthesized mainly within the tips or ends of actively dividing fibrous roots. The mechanism of excretion of these compounds from living roots is still unknown. One could hypothesize that during the natural process of suberization and autolysis of root cortical cells, the products of autolysis may enter the rhizosphere. Further studies to better understand the mechanism of root exudate excretion are under way, along with studies to chemically characterize all of the major constituents within these bioactive root exudates. The impact of environment on exudate production and weed suppression is also of interest. This information may allow us to

develop and utilize fine leaf fescue cultivars more effectively for their long-term weed-suppressive properties in the landscape.

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COMPARISON OF MEDITERRANEAN *Pistacia lentiscus* GENOTYPES BY RANDOM AMPLIFIED POLYMORPHIC DNA, CHEMICAL, AND MORPHOLOGICAL ANALYSES

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Abstract—Characterization of the genetic variability of Mediterranean *Pistacia lentiscus* genotypes by RAPD, composition of essential oils, and morphology is presented. High polymorphism in morphological parameters was found among accessions, with no significant differences in relation to geographical origin, or to gender. GC-MS analysis of leaves extracted by *t*-butyl methyl ether, showed 12 monoterpenes, seven sesquiterpenes, and one linear nonterpenic compound. Cluster analysis divided the accessions into two main groups according to the relative content of the major compounds, with no relation to their geographical origin. In contrast, a dendrogram based on RAPD analysis gave two main clusters according to their geographical origins. Low correlation was found between genetic and essential oil content matrices. High morphological and chemical variability on one hand, and genotypic polymorphism on the other, provide ecological advantages that might explain the distribution of *Pistacia lentiscus* over a wide range of habitats. The plants under study were grown together in the same climatic and environmental conditions, thus pointing to the plausible genetic basis of the observed phenotypic differences.

Key Words—Essential oil, genetic diversity, germplasm, morphology, *Pistacia lentiscus*, RAPD.

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INTRODUCTION

Pistacia lentiscus L. (Anacardiaceae), an evergreen dioecious shrub, is widely distributed along the Mediterranean basin shores (Zohary, 1952). It is a member of a heterogeneous family of eleven species. Zohary (1952), in his monographic study of the genus, included *P. lentiscus* in the section *EU Lentiscus* and disputed the varietal subdivision of this species, based mainly on size, shape, and number of leaflets.

P. lentiscus (mastic tree) is well known in Mediterranean countries for its resin, mastic gum, used since antiquity for incense, as a chewing gum for pleasant breath, for spicing liqueurs and jam, and in the cosmetic industry (Browicz, 1987). The therapeutic effect of mastic gum was demonstrated in healing of duodenal ulcers (Al-Habbal et al., 1984; Al-Said et al., 1986). Antimicrobial activity of the essential oil and resin was also reported (Magiatis et al., 1999). On the Greek island Chios, *P. lentiscus* (referred as *P. lentiscus* var. *chia* by Browicz, 1987) is cultivated and vegetatively propagated by the locals who collect the resin from the bark (Browicz, 1987).

Chemical analysis of the resin and/or essential oils of *P. lentiscus* was reported from different locations around the Mediterranean basin including: Spain (Boelens and Jimenez, 1991), Greece (Papageorgiou et al., 1991), Egypt (De Pooter et al., 1991), Israel (Fleisher and Fleisher, 1992), and Corsica (Castola et al., 2000). Comparison of these reports revealed variation in the chemical composition of the leaf essential oils. α-Pinene and myrcene were major components in the Spanish resin (Boelens and Jimenz, 1991). In Corsica, leaf oil was composed mainly of tepinene-4-ol and α-pinene (Castola et al., 2000). In Greece, α-pinene constituted 58.9–70% of the essential oil of mastic gum (Papageorgiou et al., 1991). Analysis of the essential oil from Mt. Carmel, Israel was characterized by a high relative content of α-terpineol (Fleisher and Fleisher, 1992). Cultivated *P. lentiscus* accessions grown in Egypt, had car-3-ene as the major component, constituting 65% of the essential oil (De Pooter et al., 1991).

P. lentiscus is a leading component of the low altitude Mediterranean maquis. Its distribution around the Mediterranean basin extends to North and Eastern Africa and Madeira Island (Zohary, 1996). It was suggested that ecotypic differentiation enabled Israeli *P. lentiscus* to grow in diverse habitats (Shaviv, 1978). At the J. Blaustein Institute for Desert Research (BIDR), located in the Negev desert, Sede Boker, Israel, a live germplasm collection of *Pistacia* spp. was established (Golan-Goldhirsh and Kostiukovsky, 1998). Accessions of *P. lentiscus* collected from different locations around the Mediterranean basin (Israel, Cyprus, Spain, and Tunisia) are maintained together under similar conditions. The germplasm, therefore, offers the possibility to assess the genetic basis for the phenotypic variation. We report the comparison of chemical and morphological variation among accessions, and the use of random amplified polymorphic DNA (RAPD) analysis,

to evaluate the genetic differences among genotypes of Mediterranean *P. lentiscus* growing in the germplasm collection.

METHODS AND MATERIALS

Plant Material. Leaves of *Pistacia lentiscus* were collected from the live germplasm collection at Blaustein Institute for Desert Research (BIDR) (Golan-Goldhirsh and Kostiukovsky, 1998). The accessions originated in Israel (Adoraim), Cyprus (Emba, Limasol area), Spain (Lleida Province) and from three locations in Tunisia (Aindrham, Takrouna, and Ain Sebaa) (Table 1). A total of 16 accessions were studied, both male and female trees.

Plants are grown under field conditions in a 3×4 m area in row and between rows, respectively (Golan-Goldhirsh and Kostiukovsky, 1998). The collection is located at the Negev desert highland (elevation 525 m); with average precipitation of ca. 90 mm. The average day and night temperatures in summer are 33°C and 15°C, respectively, with winter average temperatures of 15°C and 5°C, respectively, typical of a subtropical desert. During summer, plants are irrigated approx. once a week by a controlled drip irrigation system. In winter, run-off is supplemented with irrigation once every 2–3 wk.

RAPD Analysis. A total of 120 primers were pretested for the study of the genetic relations among *Pistacia* species and 39 revealed polymorphism (data not shown). Of the 39 primers, nine showed polymorphism among individuals of *P. lentiscus*. These were selected for further RAPD analysis with all 16 accessions

TABLE 1. *Pistacia lentiscus* GROWING IN THE GERMPLASM COLLECTION FIELD IN SEDE BOKER AND THEIR ORIGIN

No.	Country	Site of origin	Gender	Abbreviation
1	Israel	Adoraim	Male	Is-m
2	Israel	Adoraim	Female	Is-f
3	Cyprus	Limassol area	Male	Cy-m
4	Cyprus	Limassol area	Female	Cy-f
5	Tunisia	Aindrham	Male	Tn-m1
6	Tunisia	Takrouna	Male	Tn-m2
7	Tunisia	Takrouna	Female	Tn-f1
8	Tunisia	Ain Sebaa	Female	Tn-f2
9	Tunisia	Takrouna	Female	Tn-f3
10	Tunisia	Ain Sebaa	Female	Tn-f4
11	Spain	Lleida Province	Male	Sp-m1
12	Spain	Lleida Province	Male	Sp-m2
13	Spain	Lleida Province	Female	Sp-f1
14	Spain	Lleida Province	Female	Sp-f2
15	Spain	Lleida Province	Female	Sp-f3
16	Spain	Lleida Province	Female	Sp-f4

(Table 1). A DNA sample from *P. atlantica* was used in the RAPD analysis as an out-group species.

DNA extraction followed the protocol of Doyle and Doyle (1987) with modification by Hormaza et al. (1994). Optimization of reaction conditions for RAPD analysis was based on previous work (Khandka et al., 1997) and consisted of a 25-μl reaction mixture containing 10 mM Tris-HCl (pH 8.0), 20 ng of genomic DNA, 0.1 mM of each dNTP, 50 mM KCl, 2 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Bioline). Amplification was carried out in a BIO-RAD iCycler under the following conditions: 94°C for 4 min, 1 cycle; 94°C for 1 min, 35°C for 1 min, 72°C for 2 min, 45 cycles; 72°C for 7 min, 1 cycle. Amplified products were analyzed by electrophoresis in a 1.2% agarose gel after staining with ethidium bromide (0.5 μg/ml) and viewing under UV light. All reactions were repeated twice, and only reproducible bands were scored for statistical analysis.

Chemical Analysis. Following Lewinsohn et al. (1993), the essential oil composition of *Pistacia lentiscus* leaves was determined by GC-MS analysis of a *t*-butyl methyl ether (MTBE) extract of the leaves. Leaflets from each sample were cut and mixed. One gram of each sample was shaken in MTBE for 2 hr in a ratio of 1:5 (w/v), containing 10 μg/ml isobutylbenzene (Aldrich, USA) as an internal standard and kept in the solvent overnight. The extract was cleaned by passing it through a small column (Pasteur pipette) containing anhydrous Na_2SO_4 and silicic acid (Silicagel 60, 230–400 mesh, Merck, Germany), to dry the sample and to remove high molecular weight polar substances that interfere with the GC-analyses.

An Hewlett Packard G 1800 B GCD GC-MS system, with an electron ionization detector, was used for the essential oil analysis. Diluted MTBE extracts were injected on to the Rtx-5SIL MS column equilibrated with He gas at a flow rate of 1.0 ml/min. Temperature was programmed to start at 70°C for 2 min, followed by a gradient of 4°C/min from 70 to 200°C. The injector temperature was set to 250°C and the detector set to 280°C. The scanning range was recorded from 45 to 450 m/z, with an ionization energy of 70 eV. Identification of the main components was by coinjection of authentic standards and comparison of EI-MS spectra of authentic standards with computerized libraries. Quantification of the compounds was based on total ion chromatographic peak size, as related to the internal standard.

Morphological Measurements. Morphological traits, quantitative and qualitative, were measured and evaluated. For each accession, five measurements of leaf length, number of leaflets per leaf, leaflet length, and width were recorded. Qualitative traits included: leaflet shape, color, and texture and were assessed according to an evaluation scale, 1 (*light green*) to 5 (*dark green*) for color, 1 (*flexible*) to 5 (*leathery*) for leaf texture, and *lanceolate* (1) to *ovate* (5) for leaflet shape.

Data Analysis. RAPD data as well as the relative content of essential oil compounds were subjected to similarity matrix and cluster analyses, using the Numerical Taxonomy and Multivariate Analysis System program package for PC

(NTSYS-pc, version 2). The relative content of essential oil constituents was normalized by transformed values (arcsin), and the similarity index was constructed using similarity for interval data with Euclidean distances. For RAPD analysis, amplified bands on a gel were scored as 1 (*present*) or 0 (*absent*), and a similarity matrix was constructed using simple matching coefficient function. Both dendrograms were constructed by applying the unweighted pair group method with arithmetic averages (UPGMA) to the similarity matrix (Nei, 1987). A Mantel test, with 1000 permutations was conducted by NTSYS-pc to compare the chemical and genetic similarity matrices.

RESULTS

RAPD Analysis. A total of 77 bands were scored, ranging between 300 bp and 2 kb, an average of ca. 9 bands for each primer (Table 2). Seventy out of the 77 scored bands were polymorphic (Table 2). On the basis of these polymorphisms, similarity values were calculated, showing genetic relations among the accessions (Table 3). High similarity values were obtained among the Spanish accessions (0.76–0.89), Tunisian (0.81–0.91), Israeli (0.94), and Cyprian accessions (0.86). High similarity values were found between Tunisian and Spanish accessions, (0.68–0.86), indicating close relations between *P. lentiscus* from these two countries. The Israeli accessions showed higher similarity values to Tunisian accessions (0.69–0.80) than to the Spanish accessions (0.58–0.73). The Cyprian accessions showed lower similarity values to all other accessions (0.56–0.68). The similarity values between all 16 accessions and *P. atlantica*, as an out-group, was relatively low (0.35–0.50) (Table 3).

A dendrogram, divided the 16 accessions into two distinct genetic groups (Figure 1). One group included the Tunisian, Spanish, and Israeli accessions and

TABLE 2. PRIMERS USED FOR RAPD ANALYSIS AND THE NUMBER OF SCORED BANDS

Primer	5′-3′ Sequence	Total bands	Polymorphic bands
OPA -08	GTGACGTAGG	12	10
OPB-01	GTTTCGCTCC	3	2
OPC-02	GTGAGGCGTC	16	14
OPD-03	GTCGCCGTCA	12	12
OPD-05	TGAGCGGACA	10	9
OPD-07	TTGGCACGGG	8	8
OPD-13	GGGGTGACGA	8	8
OPD-20	ACCCGGTCAC	4	3
OPE-06	AAGACCCCTC	4	4
Total		77	70

TABLE 3. MATRIX OF SIMILARITY VALUES OBTAINED FROM RAPD ANALYSIS OF *Pistacia lentiscus* ACCESSIONS

Accession[a]	Is-m	Is-f	Cy-m	Cy-f	Tn-m1	Tn-m2	Tn-f1	Tn-f2	Tn-f3	Tn-f4	Sp-m1	Sp-m2	Sp-f1	Sp-f2	Sp-f3	Sp-f4	*P. atlantica*
Is-m	1.00																
Is-f	0.94	1.00															
Cy-m	0.68	0.73	1.00														
Cy-f	0.66	0.69	0.86	1.00													
Tn-m1	0.74	0.78	0.65	0.66	1.00												
Tn-m2	0.69	0.75	0.60	0.56	0.85	1.00											
Tn-f1	0.71	0.76	0.64	0.63	0.84	0.84	1.00										
Tn-f2	0.74	0.79	0.64	0.65	0.81	0.84	0.83	1.00									
Tn-f3	0.75	0.80	0.68	0.64	0.83	0.90	0.86	0.86	1.00								
Tn-f4	0.73	0.78	0.63	0.64	0.83	0.88	0.81	0.91	0.88	1.00							
Sp-m1	0.58	0.63	0.58	0.59	0.70	0.68	0.74	0.74	0.70	0.78	1.00						
Sp-m2	0.66	0.71	0.66	0.65	0.76	0.74	0.83	0.83	0.81	0.86	0.86	1.00					
Sp-f1	0.60	0.66	0.59	0.59	0.69	0.69	0.75	0.75	0.71	0.79	0.84	0.83	1.00				
Sp-f2	0.59	0.65	0.58	0.56	0.73	0.73	0.71	0.74	0.70	0.80	0.88	0.86	0.76	1.00			
Sp-f3	0.69	0.73	0.63	0.61	0.78	0.75	0.84	0.81	0.80	0.85	0.83	0.89	0.84	0.83	1.00		
Sp-f4	0.65	0.70	0.65	0.64	0.73	0.68	0.76	0.74	0.73	0.75	0.83	0.84	0.75	0.80	0.83	1.00	
P. atlantica	0.41	0.44	0.38	0.38	0.50	0.44	0.45	0.44	0.48	0.44	0.39	0.41	0.35	0.39	0.38	0.40	1.00

[a] For abbreviations see Table 1.

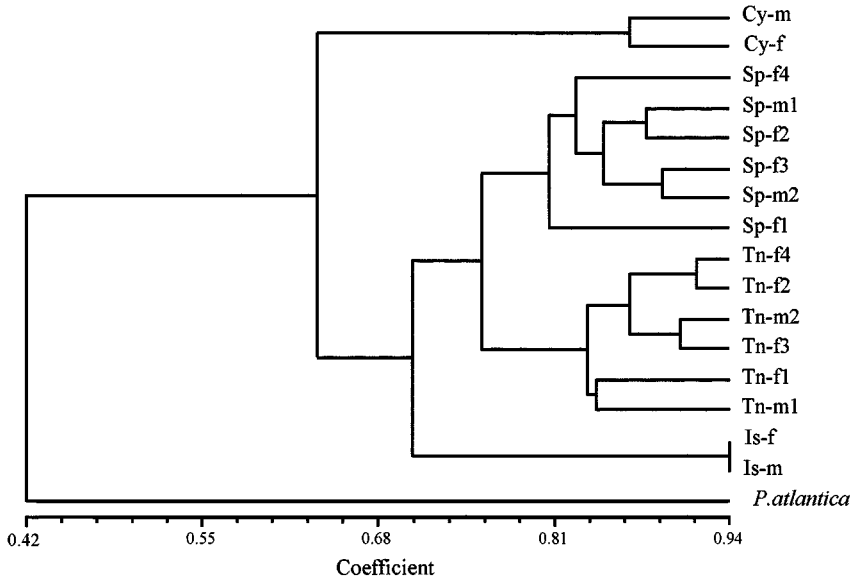

FIG. 1. Dendrogram of genetic RAPD analysis relations among *Pistacia lentiscus* accessions, obtained by applying unweighted pair group method with arithmetic averages (UPGMA). See Table 1 for abbreviations.

the second genetic group, the Cyprian. The Tunisian accessions were clustered together in the same phylogenic subgroup of the Spanish accessions (Figure 1). The Israeli accessions were genetically closer to the later two than to accessions from Cyprus. *P. atlantica* was in a separate outgroup, distinctly different from the rest (Figure 1).

Chemical Composition of Essential Oils. GC-MS analysis of MTBE extract from leaves of *Pistacia lentiscus* showed 20 main constituents, including 12 different monoterpene hydrocarbons, seven sesquiterpene hydrocarbons, and one linear nonterpenic compound (Table 4). Among them, α-pinene, sabinene, limonene, caryophyllene, and germacrene D were the major compounds (Table 4).

Cluster analysis (Figure 2) divided the 16 accessions into two main groups according to the relative content of one of the five main compounds. The first group was characterized by a high relative content of limonene (28.7–45.5%), and was further divided into two subgroups: (I) the limonene/α-pinene subgroup, included the Israeli and Cyprian male accessions and exhibited a high relative content of α-pinene (13.8–24.1%), and (II) the limonene/caryophyllene subgroup, included two Spanish (Sp-m2, Sp-f1) and Israeli female accessions and exhibited a high content of the sesquiterpene caryophyllene (13.2–22.4%). The second main group was divided into three subgroups: (I) the Cyprian female and two Tunisian

TABLE 4. ESSENTIAL OIL CONTENT OF *Pistacia lentiscus* LEAVES

Compound	RI[b]	Essential oil content (%)[a]															
		Is-m	Is-f	Cy-m	Cy-f	Tn-m1	Tn-m2	Tn-f1	Tn-f2	Tn-f3	Tn-f4	Sp-m1	Sp-m2	Sp-f1	Sp-f2	Sp-f3	Sp-f4
α-Thujene	923	0.0	0.0	0.3	0.0	0.5	0.0	0.0	0.5	0.0	0.0	11.1	0.0	0.0	0.0	12.3	0.0
α-Pinene	933	13.8	16.2	24.1	49.0	18.7	42.7	35.1	19.0	22.0	26.0	22.7	5.1	6.5	22.3	19.4	13.2
Camphene	950	0.5	1.0	0.0	1.2	0.0	0.9	0.0	0.4	0.0	0.7	2.8	0.0	0.0	0.9	2.7	1.3
Sabinene	972	0.7	0.7	1.2	12.2	32.8	12.6	19.9	24.1	42.3	45.8	24.3	11.0	10.2	9.6	28.6	21.7
β-Pinene	979	1.9	3.9	7.4	8.6	14.1	4.6	3.0	6.3	5.1	2.0	4.4	0.0	0.9	7.3	3.5	2.1
Myrcene	988	1.9	1.1	1.6	1.0	0.8	0.6	1.1	0.8	0.8	1.2	1.1	1.3	1.5	0.3	1.6	0.4
α-Phellandrene	1007	1.1	0.4	0.0	0.8	0.7	1.3	2.8	4.7	2.3	1.3	4.0	0.0	1.1	0.0	0.3	0.0
Limonene	1029	45.5	29.5	40.6	6.1	5.4	6.7	15.4	9.3	8.1	7.3	3.3	28.7	29.7	1.6	3.7	0.7
β-Phellandrene	1031	3.3	4.2	1.2	6.4	4.9	5.6	9.9	8.6	8.2	6.4	3.0	0.9	1.2	1.5	3.1	1.0
β-Ocimene Z	1034	0.0	0.2	0.0	0.0	0.0	1.7	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-Ocimene E	1044	0.0	0.6	0.0	0.0	0.6	5.2	5.3	1.9	0.0	1.8	0.0	0.0	1.4	0.7	0.0	0.0
γ-Terpinene	1057	0.0	0.0	0.0	0.2	1.0	0.0	0.2	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
2-Undecanone	1293	1.1	0.7	0.3	0.0	0.8	0.5	0.0	0.0	0.0	0.8	0.0	0.0	0.5	0.6	0.2	0.8
β-Elemene	1388	1.1	0.0	2.9	0.0	0.0	0.0	0.9	0.0	0.6	0.0	0.0	0.8	0.8	0.0	0.7	0.9
Caryophyllene	1418	5.4	13.2	0.0	3.7	3.7	7.1	1.1	2.8	0.8	3.1	10.7	22.4	20.7	6.0	9.0	6.8
α-Humulene	1454	1.3	2.4	1.2	0.7	1.4	0.0	0.0	0.5	0.4	0.0	1.8	3.2	2.9	3.0	1.8	2.7
γ-Muurolene	1476	0.0	0.0	0.4	0.0	1.3	0.0	0.0	1.0	0.5	0.0	0.0	1.1	1.0	2.2	1.7	1.0
Germacrene D	1480	22.3	26.0	18.8	10.0	11.8	10.4	3.7	18.7	8.3	3.4	10.2	23.7	20.3	41.1	9.7	45.9
α-Muurolene	1498	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.8	0.6	0.4
δ-Cadinene	1519	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.8	0.7	0.0	0.3	1.3	1.1	2.1	1.3	1.1
Total		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Total μg/g		974	545	1181	1084	1305	706	829	671	1347	683	793	904	762	749	1040	1514

[a] For abbreviations see Table 1.
[b] Retention indices.

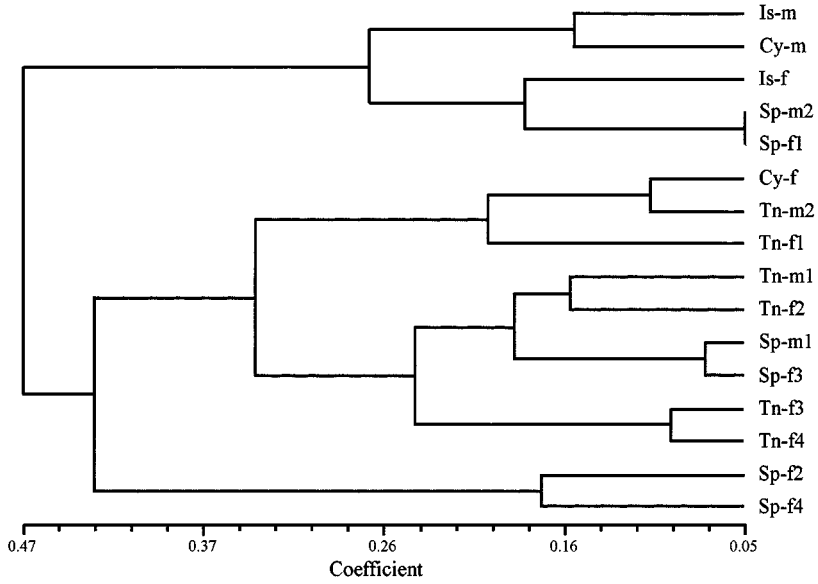

FIG. 2. Dendrogram of relations of essential oil content among *Pistacia lentiscus* accessions, obtained by applying unweighted pair group method with arithmetic averages (UPGMA). See Table 1 for abbreviations.

(Tn-f1, Tn-m2) accessions were clustered together in α-pinene group (35.1–49.0%); (II) Characterized by relatively high concentration of sabinene (24.1–45.8%), four Tunisian (Tn-m1, Tn-f2, Tn-f3, Tn-f4) and two Spanish (Sp-m1, Sp-f3) accessions were clustered together; and (III) Germacrene D group (41.1–45.9%), clustering two Spanish female accessions (Sp-f4, Sp-f2) (Figure 2).

Four of the six Spanish accessions were clustered in separate subgroups within each of the limonene dominant group and the sabinene subgroup (Figure 2). None of the six Tunisian accessions was clustered in the limonene group, nor did the Israeli or the Cyprian accessions clustered in the sabinene subgroup (Figure 2). In addition, α-muurolene was found in a low relative concentration (0.4–0.8%) just in four of the Spanish accessions; *cis*-β-ocimene was present in three accessions, one Israeli (0.2%) and two Tunisian (Tn-m2, Tn-f1) accessions (1.7–1.8%) (Table 4). High relative content of α-thujene (11.1–12.3%) was present in two Spanish accessions, Sp-m1 and Sp-f3 (Table 4).

Of the 1000 permutations, the Mantel test, conducted to analyze the relation between the genetic and chemical content matrices indicated low correlation ($R = -0.25$) with no significant relation between the matrices ($P = 0.02$).

Morphological Characteristics. The number of leaflet pairs ranged from 2 to 3 in Cyprian accessions, 3 to 4 in the Israeli, and 4 to 6 in both Spanish and Tunisian

TABLE 5. MORPHOLOGICAL CHARACTERISTICS OF MEDITERRANEAN ACCESSIONS OF *Pistacia lentiscus* GROWING AT BIDR

		Leaflets						Qualitative traits[b]		
Accession[a]	Leaf length (cm)	No.	Length (cm)	Width (cm)	Trunk diameter (cm)	Plant height (cm)	Canopy area (m^2)	Texture	Color	Shape
Is-m	3.2 (0.3)	4.0 (0.0)	2.0 (0.1)	0.8 (0.0)	3.7	79.0	1.7	3	4	3
Is-f	3.5 (0.3)	3.4 (0.5)	2.0 (0.0)	0.9 (0.1)	2.8	93.0	1.9	4	4	2
Cy-m	3.0 (0.0)	2.0 (0.0)	2.6 (0.3)	1.0 (0.0)	3.4	125.0	1.0	5	2	4
Cy-f	3.0 (0.7)	3.2 (0.8)	2.3 (0.7)	1.0 (0.0)	3.6	61.0	2.5	4	3	3
Tn-m1	6.9 (0.6)	5.8 (0.4)	2.2 (0.2)	0.8 (0.1)	2.3	80.0	0.9	2	1	2
Tn-m2	4.9 (0.4)	5.0 (0.0)	2.1 (0.1)	0.8 (0.0)	4.8	96.0	1.3	3	3	2
Tn-f1	4.2 (0.3)	5.6 (0.5)	2.0 (0.3)	0.6 (0.1)	3.6	75.0	1.7	3	2	1
Tn-f2	4.4 (0.4)	4.6 (0.5)	2.3 (0.1)	0.8 (0.1)	2.9	68.0	1.0	2	4	2
Tn-f3	4.5 (0.3)	4.0 (0.0)	2.7 (0.2)	0.8 (0.1)	3.1	77.0	1.4	2	3	1
Tn-f4	5.7 (0.5)	4.4 (0.5)	2.7 (0.2)	1.1 (0.1)	3.8	83.0	1.3	2	3	3
Sp-m1	4.2 (0.4)	5.6 (0.5)	1.9 (0.3)	0.6 (0.1)	3.5	93.0	1.4	2	4	1
Sp-m2	7.8 (1.1)	5.6 (0.5)	3.3 (0.2)	1.2 (0.1)	3.3	156.0	1.9	2	2	1
Sp-f1	3.6 (0.4)	4.0 (0.0)	2.2 (0.0)	0.9 (0.0)	3.3	135.0	2.3	2	3	2
Sp-f2	4.1 (0.2)	4.2 (0.4)	2.3 (0.3)	0.8 (0.1)	3.4	76.0	1.1	3	5	3
Sp-f3	5.7 (0.2)	4.0 (0.0)	2.8 (0.1)	0.9 (0.0)	5.6	82.0	1.8	2	2	2
Sp-f4	5.3 (0.6)	4.8 (0.4)	2.5 (0.2)	0.8 (0.1)	4.5	95.0	0.5	2	3	1

Note: Number in brackets indicates standard deviation of five measurements of each accession.

[a] For abbreviations see Table 1.

[b] Leaflet texture, color, and shape were evaluated by a scale of 1–5: 1 (*light green*) and 5 (*dark green*) for color, 1 (*flexible*) and 5 (*leathery*) for leaf texture, and from *lanceolate* (1) and *ovate* (5) for leaflet shape.

accessions (Table 5). In five accessions (Is-m, Cy-m, Tn-m2, Tn-f3, Sp-f1, and Sp-f3), the number of leaflet pairs was equal in all the counted leaves, in others, variability was found (Table 5). A terminal leaflet was observed in a few leaves of some of the accessions. In some accessions, e.g., Sp-m1, Tn-f2, and others, an uneven number of leaflets was also found. A "wing-like" rachis of ca. 1–2 mm was measured in all accessions.

Leaf length ranged from 3.0 cm (Cy-m, Cy-f) to 7.8 cm (Sp-m2), and leaflet length and width between 1.9 and 0.6 cm (Sp-m1) to 3.3 and 1.2 cm (Sp-m2), respectively (Table 5). Accession Tn-m1 had the thin trunk diameter (2.3 cm), while Sp-f3 was widest (5.6 cm). Plant height ranged from 61 cm (Cy-f) to 156 cm (Sp-m2), and canopy area from 0.5 m^2 (Sp-f4) to 2.5 m^2 (Cy-f).

ANOVA analysis conducted to compare all accessions, when sorted by gender, on the basis of geographic origin, or chemical composition, did not show significant differences in any of the quantitative morphological traits.

Leaflet shape ranged from almost lanceolate (e.g., Sp-m1) to ovate shape (Cy-m) (Table 5). Leaves from Cyprus and Israel accessions were characterized as having the most leathery leaflets, while others had a more flexible texture. Leaf color ranged from light green in the Tunisian accession Tn-m1, to dark green in the Spanish accession Sp-f2 (Table 5).

DISCUSSION

A phylogenetic tree of *P. lentiscus* analyzed by RAPD divided the accessions according to their geographic origin (Figure 1). Interestingly, the Israeli accessions were closer genetically to Tunisian and Spanish accessions than to the Cyprian accessions. The latter were clustered into a separate group, genetically distinct from the rest, suggesting the genetic isolation of *P. lentiscus* growing in Cyprus and the development of distinctive genotypes.

Phytochemical and morphological characters showed high phenotypic variability among accessions of *P. lentiscus*, and did not reflect differences by geographic location (Tables 4 and 5). On the basis of the relative content of the 20 constituents of the leaf essential oil, cluster analysis divided the different accessions according to what appears to be chemotypic groups rather than to their geographic origin or gender (Figure 2), thus indicating close resemblance in the chemical content of *P. lentiscus* around the Mediterranean. The Mantel test, used to compare essential oil content to the genetic matrices, indicated a low, nonsignificant relation between the matrices, suggesting that there was no chemotypic differentiation. However, it is possible that the dominant nature of RAPD markers and the limitation of the technique to detect polymorphism in cases of heterozygosity (Parker et al., 1998) may account for the low correlation between the matrices. Alternatively, genetic RAPD analysis was correlated with the volatile oil constituents

of accessions, as in the cases of *Ocimum gratissimum* (Vieira et al., 2001), and *Cymbopogon* sp. (Sangwan et al., 2001). Therefore, it is possible that using more accessions, and/or additional differential primers, could produce better correlation between the two matrices.

In comparison to other reports on *P. lentiscus* (e.g., Boelens and Jimenez, 1991; Fleisher and Fleisher, 1992; Castola et al., 2000), relatively high content of sabinene and germacrene D was found in leaf extracts, while oxygenated compounds were absent. It is possible that extraction with methyl *t*-butyl ether may be the cause for the observed differences. However, the effect of environmental conditions on phenotypic plasticity is likely. The effect of stress on the content of essential oil has been reported in several studies. Llusia and Penuelas (1998) reported that under drought conditions total terpene concentration in *P. lentiscus*, grown under irrigated conditions, was increased by 21.3% compared to control. Drought and nutrient deficiency (phosphorus and/or nitrogen) enhanced allocation of fixed carbon into the biosynthesis of essential oils in *Rosmarinus officinalis* and *Lavandula latifolia* (Ross and Sombrero, 1991). UV-B radiation enhanced the level of most of the major volatiles in *Ocimum basilicum* (Johnson et al., 1999). Therefore, it is possible that under the extreme desert conditions at our germplasm collection in the Negev desert, the concentration of specific compounds (e.g., sabinene and germacrene D) increased. However, to verify this assumption, the effect of environmental stress on the content of *P. lentiscus* essential oil should be further studied under controlled conditions.

More definite indication to the effect of the environmental desert conditions on *P. lentiscus* was supplemented by morphological characteristics. Asymmetry is an indicator of environmental stress in both plants and animals (Graham et al., 1993). Therefore, the uneven number of paired lateral leaflets and appearance of terminal leaflet, suggests that *P. lentiscus* is not well adapted to growth under desert conditions. Morphological variability was expressed in most of the analyzed parameters, e.g., leaflet characteristics, plant height, trunk diameter, and canopy area (Table 5). When comparisons were done after sorting the accessions by geographic origin or gender, statistical analysis did not show significant differences in any of the parameters, reflecting the high variability within accessions from each of the geographic regions. Similarly, morphological differences were not observed between genders of *P. lentiscus* in Portugal (Barradas and Correia, 1999).

The large morphological and chemical variability found among accessions of *P. lentiscus* on one hand, and the genotypic differentiation on the other hand, may contribute to the wide distribution of *P. lentiscus* around the Mediterranean basin. *P. lentiscus* was found to be one of the most drought tolerant plants among other evergreen species in the Mediterranean maquis (Gratani, 1995). Different ecophysiological characteristics such as drought resistance, fast regeneration after fires, and resistance to herbivores contribute to the potential of *P. lentiscus* for forestry in our region. This emphasizes the importance in preserving the genetic

variability of this species. The data reported in this work are based on accessions available in the live germplasm collection at BIDR and are not yet enough to extrapolate from these results to the genetic variability in nature. Further sampling is needed to determine the genetic variability and establishment of conservation management practices.

The fact that the plants were grown together under similar conditions reduced the effect of environmental variation on the measured differences. Thus, the *Pistacia* spp. live germplasm collection at BIDR enabled study of the genetic basis of observed and measured phenotypic polymorphisms. While genetic analysis separated the accessions according to their geographic origin, significant phenotypic differences, *i.e.*, content of essential oil and morphological parameters, were not observed. This points to the importance of DNA molecular marker techniques when ecotypic or chemotypic differentiation is discussed.

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ANNOUNCEMENT

The International Allelopathy Foundation is organizing the IV International Congress on Allelopathy—Allelopathy in Sustainable Terrestrial and Aquatic Ecosystems—to be held August 23–25,2004, at CCS Haryana Agricultural University, Hisar, India. There will be both oral and poster sessions. Fifteen symposia are being organized. Deadline for abstract submission is August 31, 2003.

The Conference aims to bring together active allelopathy scientists working worldwide to review progress, identify constraints, and project future goals for basic and applied allelopathy research in Terrestrial and Aquatic Ecosystems. Previous conferences were attended by approximately 200 scientists representing 17 countries. Accommodations are included with the registration fee and will be in the Faculty House at the University. Hisar is a small, ancient city about 165 km west of New Delhi.

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REVIEW PAPER

ARBUSCULAR MYCORRHIZA: BIOLOGICAL, CHEMICAL, AND MOLECULAR ASPECTS

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Abstract—Mycorrhizas are the most important mutualistic symbioses on earth. The most prevalent type are the arbuscular mycorrhizas (AMs) that develop between roots of most terrestrial plants and fungal species of the Zygomycota. The AM fungi are able to grow into the root cortex forming intercellular hyphae from which highly branched structures, arbuscules, originate within cortex cells. The arbuscules are responsible for nutrient exchange between the host and the symbiont, transporting carbohydrates from the plant to the fungus and mineral nutrients, especially phosphate, and water from the fungus to the plant. Plants adapt their phosphate uptake to the interaction with the AM fungus by synthesis of specific phosphate transporters. Colonization of root cells induces dramatic changes in the cytoplasmic organization: vacuole fragmentation, transformation of the plasma membrane to a periarbuscular membrane covering the arbuscule, increase of the cytoplasm volume and numbers of cell organelles, as well as movement of the nucleus into a central position. The plastids form a dense network covering the symbiotic interface. In some of these changes, microtubules are most likely involved. With regard to the molecular crosstalk between the two organisms, a number of phytohormones (cytokinins, abscisic acid, jasmonate) as well as various secondary metabolites have been examined: (i) Jasmonates occur at elevated level, which is accompanied by cell-specific expression of genes involved in jasmonate biosynthesis that might be linked to strong carbohydrate sink function of AM roots and induced defense reactions; (ii) apocarotenoids (derivatives of mycorradicin and glycosylated cyclohexenones) accumulate in most mycorrhizal

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roots examined so far. Their biosynthesis via the nonmevalonate methylerythritol phosphate (MEP) pathway has been studied resulting in new insights into AM-specific gene expression and biosynthesis of secondary isoprenoids.

Key Words—Arbuscular mycorrhiza, (apo)carotenoids, chemical interaction, ecology, MEP pathway, mutualism, phosphate transporters, secondary metabolism, symbiosis.

INTRODUCTION

More than 90% of terrestrial plants are associated with root-colonizing fungi, establishing a permanent and intimate mutualistic symbiosis, called mycorrhiza. Several types of mycorrhizas exist, defined by plant/fungus combination and the symbiotic structure. The endotrophic arbuscular mycorrhiza (AM) is the most common type, occurring in about 80% of plant species. The AM fungi are represented by more than 150 species of the Zygomycota included in the Glomales (Morton and Benny, 1990). Recent work on the phylogeny of AM fungi provided a basis for a new systematics (Schüßler et al., 2001; Schüßler, 2002), removing these fungi from the polyphyletic Zygomycota and placing them into a new monophyletic phylum, the Glomeromycota.

There is a disagreement about usage of the term "symbiosis," originally defined in 1869 by Heinrich Anton de Bary as an intimate, outcome-independent interaction between different species, ranging from parasitism to mutualism. Later, especially in Europe, the term "symbiosis" was used to mean only mutually beneficial association of organisms (=mutualism). In this review, the term "symbiosis" is used in its original meaning.

The term "mycorrhiza" was coined in 1885 by Bernhardt Frank by recognizing special structures in tree roots. The term means "fungus root," later characterized as ectomycorrhiza. Frank not only described its morphology but also inferred its physiological role (Frank, 1888). "Arbuscular mycorrhiza" (AM) replaced the earlier term "vesicular–arbuscular mycorrhiza" (VAM) because not all endomycorrhizas of this type develop vesicles, but all form arbuscules.

There are two major types of mycorrhizas, AMs and ectomycorrhizas. The latter evolved as a more recent symbiosis of woody trees and shrubs with ectomycorrhizal fungi. The plant hosts of AM fungi are mostly angiosperms, some gymnosperms, pteridophytes, lycopods, and mosses (Smith and Read, 1997). The physiological interactions of lower plant mycorrhizas, however, are poorly understood. With regard to the systematic distribution of AMs in higher plants, it is still an open question how some nonhost plants, e.g., members of the Brassicaceae, Caryophyllaceae, Chenopodiaceae, or Urticaceae, resist mycorrhizal colonization (Vierheilig et al., 1994, 1996). The nonmycorrhizal state of nonhost plants might be a derived trait. It might be the outcome of specialization regarding, e.g., the plant habitat (Fitter and Moyersoen, 1996).

According to the fossil record and molecular data, the origin of the AM symbiosis goes back at least to the Ordovician, 450–500 million years ago (Remy et al., 1994; Redecker et al., 2000). It is assumed that this symbiosis aided plants during their land colonization in the acquisition of water and minerals, especially phosphate (Simon et al., 1993). At the time of land colonization, the first bryophyte-like plants appeared in terrestrial environments. Today, a number of bryophytes and pteridophytes are still capable of forming AMs (Read et al., 2000; Schüßler, 2000).

In contrast to AMs, ectomycorrhizas (Tagu et al., 2002) and the more specialized ericoid mycorrhizas (Perotto et al., 2002) evolved later in evolution and are of polyphyletic origin (Fitter and Moyersoen, 1996). These symbioses are especially adapted to habitats rich in organic material that did not exist on earth when the AM symbiosis developed.

Interesting questions to be addressed are on the selective forces that led to the mutualistic coexistence of the two partners. Whereas the macrobiont (phytobiont) can live without AM fungi, although suffering in nutrient- and water-deficient soils, the microbiont (mycobiont) became dependent on plant roots and developed towards an obligatory biotrophic life cycle. Another challenging problem is the complexity of field situations, where many plant and fungal species coexist with many different soil organisms in different ecosystems.

ECOLOGY

In accordance with the evolutionary history, AM symbioses can be found in almost all ecosystems. They have been described from deserts (Corkidi and Rincön, 1997; Dalpé et al., 2000; Titus et al., 2002), tropical rainforests (Brundrett et al., 1999; Guadarrama and Álvarez-Sánchez, 1999; Siqueira and Saggin-Júnior, 2001; Zhao et al., 2001; Gaur and Adholeya, 2002), aquatic environments (Khan, 1993), as well as from ecosystems with strong saline (Carvalho et al., 2001; Sengupta and Chaudhuri, 2002), sodic or gypsum soils (Landwehr et al., 2002). The relatively low number of plants colonized by AM fungi in some arctic and antarctic habitats seems to be due to a lack of suitable vectors for fungal spores rather than to other causes (Allen, 1996).

In addition to the global distribution of AM symbioses, there is large functional diversity as well. Whereas most AM symbioses are mutualistic, a growing number of nonphotosynthetic plants are described, which are receiving a large portion of their nutrients from AM fungi (Imhof, 1999; Yamato, 2001), resembling the functioning of orchid mycorrhizas (Rasmussen, 2002). In some cases, these mycotrophic plants are living epiparasitically on other plants using the hyphae of their fungal partner for the transfer of nutrients (Bidartondo et al., 2002). On the other hand, AM fungi may become parasitic themselves in relation to their host plant under special circumstances (Allen, 1996).

Regarding plant communities, there is a large number of possible consequences of AM colonization (Francis and Read, 1994). Interactions between plant and fungal communities rely primarily on the fact that a given plant or fungus prefers some symbiotic partners and neglects others. In addition, the benefits obtained from the symbiotic partner are dependent on the actual partner and on various other conditions. Even the direct transfer of nutrients from one plant to another via fungal hyphae has been discussed (Francis et al., 1986). Within ecosystems, a network of feedback dynamics based on these interactions between fungal and plant populations has to be taken into account (Bever, 2002). These dynamics may be important to maintain diversity within plant communities. Sanders et al. (1996) have stressed the importance of fungal diversity for the ecological impact of the AM symbiosis, and van der Heijden et al. (1998) have shown in a case study that fungal and plant population diversity are directly correlated to each other. In general, the results from single ecological studies regarding ecology of AM symbiosis are highly dependent on the local situation (Hartnett and Wilson, 2002). As the main possible consequences, Koide and Dickie (2002) have singled out (i) increased plant reproduction, (ii) stable plant populations because positive mycorrhizal effects might inversely correlate with population density, (iii) favoring of the most robust individuals by AM fungi, and (iv) patchy distribution of mycorrhizal areas due to the spread of colonization starting from mycorrhizal plants.

Apart from supplying plants with phosphate and other nutrients, further beneficial effects have been described for AM fungi. The symbiosis has a positive effect regarding plant water potential especially for plants under drought stress (Augé, 2001). AM colonized plants show a significant degree of bioprotection against various pathogens (Cordier et al., 1996; Dugassa et al., 1996; Bødker et al., 1998; Vaast et al., 1998; Slezack et al., 2000; Elsen et al., 2001). In addition, positive effects of AM fungi on soil structure have been described. As a consequence, the AM symbiosis is regarded as a key component of sustainable agriculture (Bethlenfalvay and Lindermann, 1992; Jeffries and Barea, 2001), whereas under conventional agricultural conditions, AM fungi seem to be only of minor importance (Ryan and Graham, 2002). Mäder et al. (2000) have compared conventional and organic–biological agricultural systems directly. They found stronger mycorrhizal colonization for the organic–biological system and preliminary evidence for a partial compensation for the disadvantages of the organic–biological system by the AM fungi. Apart from agricultural systems (Kiers et al., 2002), the application of AM fungi is tested for the revegetation of desertified areas (Saito and Marumoto, 2002) and in cultivation of micropropagated plantlets (Yano-Melo et al., 1999). In this context, major technological problems are the form of application of the AM inoculum (Saito and Marumoto, 2002) and the combinations of AM inoculum with other microorganisms that are beneficial for plant growth (Vázquez et al., 2000, 2001; Vassilev et al., 2001).

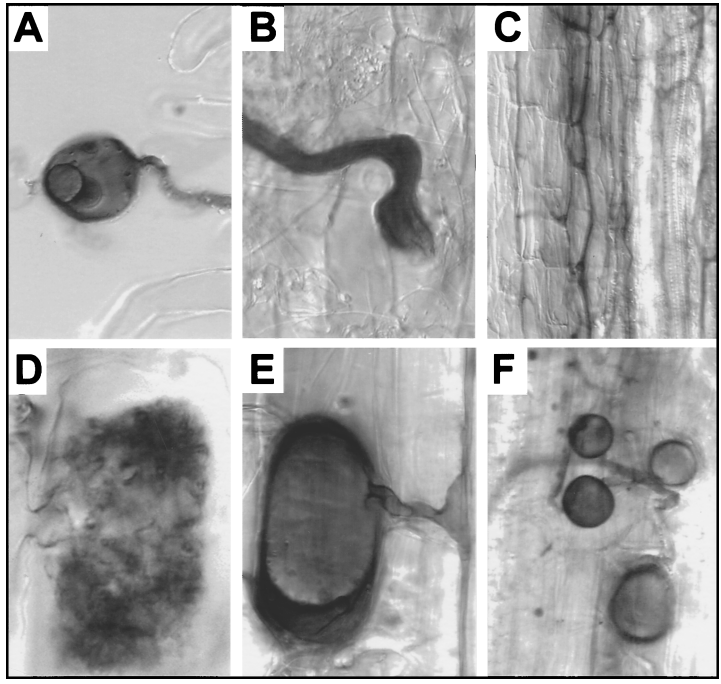


FIG. 1. Morphology of the life cycle stages of an AM fungus, *Glomus intraradices* in a maize root: A, germinating spore; B, appressorium; C, intercellular hyphae; D, arbuscule; E, intercellular vesicle; F, extraradical spores. The fungal structures are visible after staining with trypan blue (Phillips and Hayman, 1970).

COLONIZATION AND MORPHOLOGICAL CHANGES

Steps in root colonization by AM fungi are shown in Figure 1. The process starts with germination (hyphal growth) of fungal spores, followed by poorly understood events. Subsequently, appressoria are formed from which the fungus penetrates the root surface and colonizes the intercellular space of the root cortex. On the fungal side, nonaggressive cell wall-lytic enzymes become active, and both the plant root cells and the fungus change their gene expression pattern and morphology. The hyphae penetrate the cell walls and develop within the cortex cells tree-like structures, called arbuscules, by repeated dichotomous branching. In some cases, intercellular storage organs, lipid-rich vesicles, and finally extraradical spores are formed, which may enter another colonization process. Fungal root colonization is under control of the plant aiming at a morphological and functional compatibility of the two partners (Bonfante and Perotto, 1995).

The key feature of AMs is the arbuscule, a highly branched haustorium-like structure within root cortex cells, responsible for nutrient exchange. However, the arbuscules represent a dead-end in the growth of AM fungi (Bonfante and Perotto, 1995) and they finally senesce and collapse after 4–10 days of symbiosis (Sanders et al., 1977), possibly caused by the continuously stressful environment of the host cortex cell (Harley and Smith, 1983).

Colonization of root cortex cells by AM fungi has been shown to induce dramatic changes in the cytoplasmic organization and morphology of root cells. The fungal arbuscule occupies a major portion of the plant cell volume but is still separated from the cell protoplast by the host plasma membrane. This membrane completely surrounds the arbuscule and forms a periarbuscular membrane, leading to a two- to fourfold increase in the plasma membrane's surface. The resulting space between the plant protoplast and the fungus develops into an apoplastic compartment that represents the symbiotic interface (Bonfante and Perotto, 1995). Figure 2 shows some arbuscule branches surrounded by the plama membrane and the interfacial matrix in a colonized maize root cortex cell.

Formation of arbuscules is accompanied by alterations in morphology of the host cell: the central vacuole is fragmented, the volume of cytoplasm and number of cell organelles increase significantly, and the nucleus moves into a central position and undergoes hypertrophy (Balestrini et al., 1994). The nucleus of arbusculated cells (arbuscule-harboring cells) is characterized by enhanced fluorochrome accessibility, increased nuclease sensitivity, and chromatin dispersion, all together reflecting a greater transcriptional activity of the plant genome in the colonized cells (Gianinazzi-Pearson, 1996). The host cytoplasm and cell organelles proliferate around the branching hyphae. The number of plastids in colonized cortex cells increases (Bonfante and Perotto, 1995) and networks are formed covering the arbuscules (Fester et al., 2001; Hans, 2003). The plastids in these networks are connected to each other by so-called "stromules' (stroma-filled tubules) (Köhler and Hanson, 2000). In addition, the nucleus is surrounded by plastids forming "octopus-," "millipede"-like or ring-shaped structures. In cells that do not contain fungal structures, the plastids are distributed anisotropically throughout the cytoplasm, similar to cortex cells of nonmycorrhizal roots. The formation of those dense plastid network-covering arbuscules indicates a dramatically intensified metabolism in these host cells. Figure 3 shows confocal laser scanning micrographs of plastid networks in AM roots, labelled by green fluorescent protein (GFP) in transgenic tobacco roots (Fester et al., 2001) and by immunodetection of the plastid-located 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) in maize roots (Hans, 2003). This enzyme catalyzes the first committed step of the plastid-located methylerythritol phosphate (MEP) pathway (see below), stimulated in AM roots.

According to their role in a variety of cellular functions in plants, it has been shown that microtubules are involved in changes of host cell morphology and

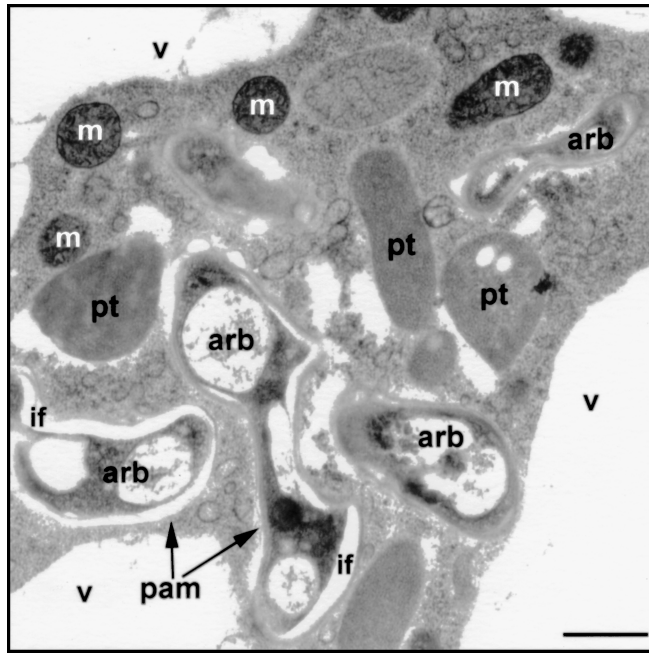


FIG. 2. Electron micrograph of an arbusculated cortex cell of a mycorrhizal maize root (bar, 0.5 μm). The graph shows sections of several arbuscule branches (arb) surrounded by the periarbuscular membrane (pam) and the interface (if). Note also some of the proliferated organelles (m, mitochondrion; pt, plastid) and the fragmented vacuole (v). The root was fixed by high-pressure freeze fixation and embedded in methacrylate. Ultrathin-sections (90 nm) were stained with uranyl acetate/lead citrate and observed with an EM 900 transmission electron microscope (Zeiss, Oberkochen, Germany). Micrograph courtesy of Diana Schmidt.

cytoplasmic architecture. Plant cytoskeletal components respond to the penetration of a symbiotic fungus with the reorganization of microtubules and microfilaments. However, an understanding of the role played by the cytoskeleton in formation and function of mycorrhizas has been hampered by technical difficulties in handling mycorrhizal tissues. Recently, application of improved labelling techniques, suitable for both the plant and the fungal symbiont, combined with either epifluorescence microscopy or laser scanning confocal microscopy, gave interesting results (Timonen and Peterson, 2002). They show extensive remodelling of the microtubular cytoskeleton from the early stages of arbuscule development until the arbuscule senesces and collapses (Genre and Bonfante, 1997, 1998). Four types of microtubule patterns were observed in arbusculated cells: (i) long bundles of microtubules crossing the cytoplasm among the arbuscule branches and passing

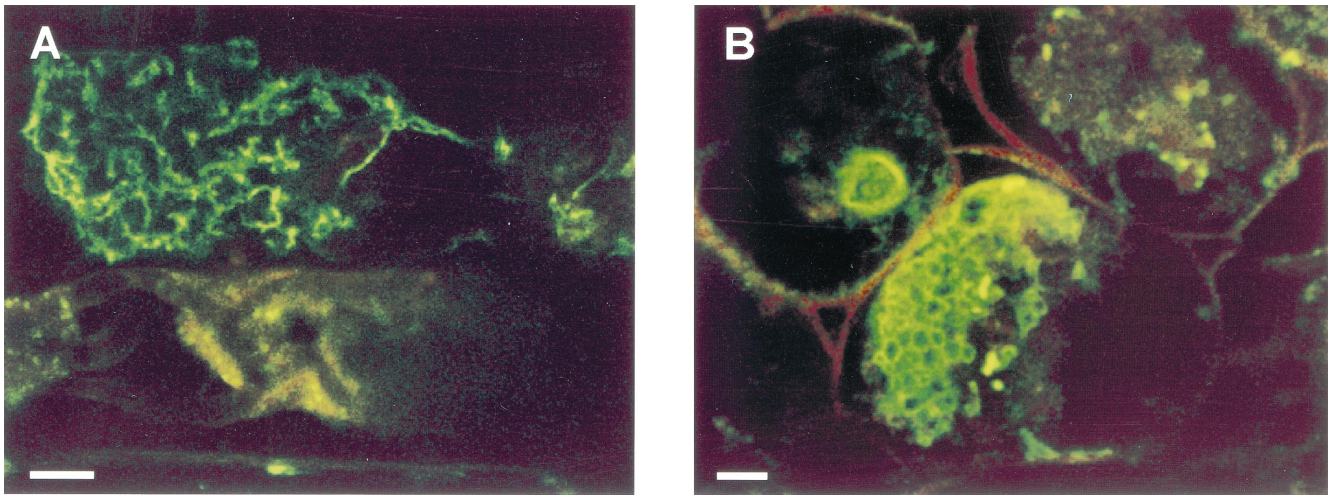


FIG. 3. Confocal laser scanning micrographs of plastid networks in AM roots, colonized by *Glomus intraradices*: A, fluorescence of the green-fluorescent protein targeted to the plastid compartment in transgenic tobacco roots; B, immunolocalization of DXR in arbusculated root cortex cells of maize. The plastid-located DXR protein is mainly detected by the green fluorescence around the nucleus (ring-shaped structure) and in the network around an active arbuscule. The presence of the arbuscule is indicated by autofluorescent signals (red) around and within the network structure. The lower cell in A and the upper right cell in B show disintegrating arbuscules (increased autofluorescence) with only few plastids left exhibiting green-fluorescent protein (A) or labelled DXR (B).

through the arbuscule; (ii) short microtubules connecting fine arbuscule branches or connecting arbuscule branches either to the cortical region of the cell or to the cell nucleus; (iii) bundles of microtubules in the periphery (cortical region) of the host cell and along the hyphal trunk; and (iv) perinuclear bundles of microtubules. In addition, it was found recently that cortex cells adjacent to the arbusculated cells or to the intercellular hyphae reorganize their microtubules as well (Blancaflor et al., 2001). This indicates a molecular dialogue between the symbionts prior to fungal penetration of the plant cell wall and an active role of the plant cytoskeleton in mycorrhization rather than passive reaction to the physical pressure created by the fungus invaginating the cell plasma membrane. The alterations of the microtubular network are consistent with the identification of a mycorrhiza-inducible α-tubulin gene in maize. Expression studies of corresponding *promoter::GUS* fusions in tobacco indicated that this gene is induced specifically in cells in which arbuscules are developing (Bonfante et al., 1996).

Besides the possible function of the cytoskeleton in reorganizing the cell for the accommodation of the arbuscule, the cytoskeleton might also be involved in developing the periarbuscular membrane. This membrane, although originating from the plant plasma membrane, shows differences in some of its properties relative to the membrane around the periphery of the cell. In particular, high activities of H^+-ATPases and phosphate transporters were shown to be located specifically in that membrane (Gianinazzi-Pearson et al., 1991; Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002). Recently, a plasma membrane H^+-ATPase gene from *Medicago truncatula* has been described for the first time that shows arbuscule-specific induced expression in mycorrhizal tissue (Krajinski et al., 2002).

The interface compartment that develops between the plant and the fungus is continuous with the peripheral plant cell wall (Bonfante and Perotto, 1995). Although the fibrillar interface differs from the peripheral plant cell wall in structure, its components reflect the composition of the wall of the host cell that is being invaded. By immunocytological approaches, the presence of pectins, xyloglucans, nonesterified polygalacturonans, arabinogalactans, and hydroxyproline-rich glycoproteins within the symbiotic interface was documented (Balestrini et al., 1994; Perotto et al., 1994; Bonfante and Perotto, 1995). This mixture of primary plant cell wall components indicates that the arbusculated plant cells have maintained their abilities to synthesize and secrete cell wall material. That this material does not assemble further to build up a secondary wall might be the result of lytic activities of the fungus (Peretto et al., 1995). When the arbuscule begins to senesce, the fibrillar material encapsulates the collapsed fungal structures that are then degraded completely by the plant cell. Subsequently, the cells regain their original morphology (Jacquelinet-Jeanmougin et al., 1987) and are able to allow another arbuscule formation.

Some processes of AM establishment are known to be mediated by phytohormones on the plant side, as suggested by application experiments (Barker and

Tagu, 2000). The levels of cytokinins are higher in shoots and roots of mycorrhizal plants compared to nonmycorrhizal ones (Allen et al., 1980). A possible role of abscisic acid was suggested from the fact that its level increases in AM roots (Danneberg et al., 1992; Bothe et al., 1994). Jasmonic acid applied exogenously promotes colonization and development of mycorrhizal structures (Regvar et al., 1996). The observed endogenous rise of jasmonates in barley roots correlating with myorrhization, however, is more indicative for a role in AM (Hause et al., 2002). The rise in jasmonates is accompanied by the expression of genes encoding for an enzyme involved in jasmonate biosynthesis, allene oxide synthase (AOS), and of a jasmonate-induced protein, JIP23. *In situ* hybridization and immunocytochemical analyses revealed that expression of the corresponding genes occurred cell-specifically within arbusculated root cells. Since jasmonate levels increased after the initial step of the plant–fungus interaction, the development of AM may cause expression of jasmonate biosynthetic genes and finally elevate jasmonate levels. The induction of jasmonate biosynthesis could be linked to the stronger carbohydrate sink function of mycorrhizal roots compared to nonmycorrhizal ones. Taking into account that jasmonate-induced genes are involved in various defense responses (Wasternack and Hause, 2002), higher endogenous jasmonate levels could help mycorrhizal roots to become more resistant to secondary infection and/or other stresses. It might also indicate plant control of the invading AM fungus.

CHEMISTRY

Despite increasing efforts in research on metabolic changes, virtually nothing is known about signalling compounds or about the role of secondary metabolites in the establishment and maintenance of a functioning AM. It is known that compounds from secondary metabolism play a significant role in the interaction between plants and their biotic and abiotic environment (Harborne, 1993), and it may be assumed that secondary metabolites play an important role in mycorrhizal symbioses as well. It has been shown that flavonoids occurring in plant roots promote spore germination of AM fungi (Gianinazzi-Pearson et al., 1989; Tsai and Phillips, 1991). Root exudates of phosphate-deficient white clover plants were more active in enhancing hyphal elongation of *Glomus fasciculatus* spores than exudates from phosphate-fertilized plants (Elias and Safir, 1987). The level of the phytoalexin medicarpin is reduced in mycorrhizal *Medicago truncatula* roots (Harrison and Dixon, 1993). Roots and root exudates from phosphate-deficient parsley contain additional compounds that are absent in phosphate-fertilized controls and mycorrhizal plants (Franken and Gnädinger, 1994), but the nature and function of these compounds is unknown. Mycorrhization of *Citrus jambhiri* led to an induced accumulation of leaf sesquiterpenoid volatiles (Nemec and Lund, 1990).

It was shown in 1924 by F. R. Jones that mycorrhizal roots of many plants develop a yellow coloration based on accumulation of the so-called "yellow pigment."

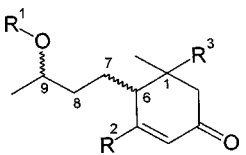
This phenomenon has been taken as an indicator to estimate the degree of mycorrhization by the naked eye (Daft and Nicholson, 1969; Fyson and Oaks, 1992) or by colorimetric measurement of root extracts (Becker and Gerdemann, 1977; Schmitz et al., 1991). The component containing the chromophore of the "yellow pigment" was isolated from mycorrhizal maize roots and identified (as its dimethyl derivative) by NMR spectroscopy and MS as all-*E*-4,9-dimethyldodeca-2,4,6,8,10-pentanedioic acid, named mycorradicin (Klingner et al., 1995a). Besides maize, other gramineous plants (wheat, barley, millet) show the same pattern of pigment formation (Klingner et al., 1995b). It was later shown in a screening of 58 species from 36 different plant families that mycorradicin as part of the "yellow pigment" is present in mycorrhizal roots of all Liliopsida analyzed and of a considerable number of Rosopsida (Fester et al., 2002a). In addition, chemical analysis of the "yellow pigment" indicated that mycorradicin is the core structure of a mixture of various oligo- or polyesters with glycosylated cyclohexenone derivatives. In mycorrhizal maize plants, these esters were localized in vacuolar hydrophobic droplets (Fester et al., 2002a).

Because of structural similarities of mycorradicin with crocetin (C_{20}-polyene) and azafrin (C_{27}-apocarotenoid), it was speculated that it derives from a C_{40}-carotenoid precursor by splitting off two C_{13}-units (Klingner et al., 1995a,b). At the same time, studies on changes in secondary metabolites in roots of cereals (wheat, barley, rye, oat) colonized by the AM fungus *Glomus intraradices* resulted in the structure elucidation of a mycorrhiza-induced glycosylated cyclohexenone derivative [blumenol C 9-*O*-(2′-*O*-β-glucuronosyl)-β-glucoside], called blumenin (**1**) (Maier et al., 1995). The level of blumenin was found to be directly correlated with the degree of root mycorrhization. Table 1 depicts the structure of blumenin and lists all other cyclohexenone derivatives identified so far from mycorrhizal roots (see below).

It was assumed (Walter et al., 2000) that the aglycone of blumenin, blumenol C, may be another carotenoid degradation product along with mycorradicin. Thus, cleavage at the 9,10(9′,10′)-positions of a C_{40}-carotenoid should lead to mycorradicin and the cyclohexenone derivative. Further detailed investigation of mycorrhizal barley roots revealed in addition to blumenin the presence of closely related cyclohexenone derivatives [7,8-dehydroblumenin (**2**) and 13-hydroxyblumenol 9-*O*-β-glucoside (**3**)], which showed an AM fungus-induced continuous accumulation, whereas putrescine and agmatine amides of 4-coumaric and ferulic acids increased only transiently in early stages of the root-fungus interaction (Peipp et al., 1997).

The occurrence of compounds **1**–**3** in cereal mycorrhizas initiated a study on their distribution within the Poaceae. After inoculation of various members of the Poaceae with *Glomus intraradices*, HPLC analyses of root extracts revealed marked changes in the patterns of UV-detectable metabolites along with accumulation of these cyclohexenone derivatives. The latter occur most often in the

TABLE 1. STRUCTURES OF GLYCOSYLATED CYCLOHEXENONE DERIVATIVES ISOLATED FROM MYCORRHIZAL ROOTS OF VARIOUS PLANTS

Compound	R^1	R^2	R^3	Occurrence	Reference	Structure scheme
1[a]	GlcUA(1″ → 2′)Glc-	CH_3	CH_3	Hv[b], Ta, Sc, As	Maier et al., 1995	
				Zm	Vierheilig et al., 2000	
2[c]	GlcUA(1″ → 2′)Glc-	CH_3	CH_3	Hv	Peipp et al., 1997	
3	Glc-	CH_2OH	CH_3	Hv	Peipp et al., 1997	
				Hv, Ta, Sc, As	Maier et al., 1997	
				Nt, Nr, Le	Maier et al., 2000	
4[d]	Glc(1″ → 6′)Glc-	CH_2OH	CH_3	Nt, Nr	Maier et al., 1999, 2000	
				Hv, Ta, Zm	Vierheilig et al., 2000	
					Walter et al., 2000	
5	Glc(1″ → 6′)Glc-	COOH	CH_3	Nt, Nr	Maier et al., 2000	
				Hv, Ta, Zm	Vierheilig et al., 2000	
6	Glc-	COOH	CH_3	Nt, Nr	Maier et al., 2000	
7	Glc(1″ → 6′)Glc-	CH_3	CH_3	Nt, Nr	Maier et al., 2000	
8	Glc(1″ → 6′)(Glc1‴ → 2′)Glc-	CH_3	CH_3	Nt, Nr	Maier et al., 2000	
9	Glc-	CH_3	COOH	Le	Maier et al., 2000	
10[e]	Glc(1″ → 4′)Glc-	CH_3	CH_3	Zm	Fester et al., 2002a	

[a] Blumenin (in all compounds listed: Glc = β-glucose; GlcUA = β-glucuronate).
[b] As = *Avena sativa*; Hv = *Hordeum vulgare*; Le = *Lycopersicon esculentum*; Nr = *Nicotiana rustica*; Nt = *Nicotiana tabacum*; Sc = *Secale cereale*; Ta = *Triticum aestivum*; Zm = *Zea mays*.
[c] 7,8-Dehydroblumenin; $\Delta^{7,8}$ present, absent in all other compounds listed.
[d] Nicoblumin.
[e] Hydrolysis product of the "yellow pigment."

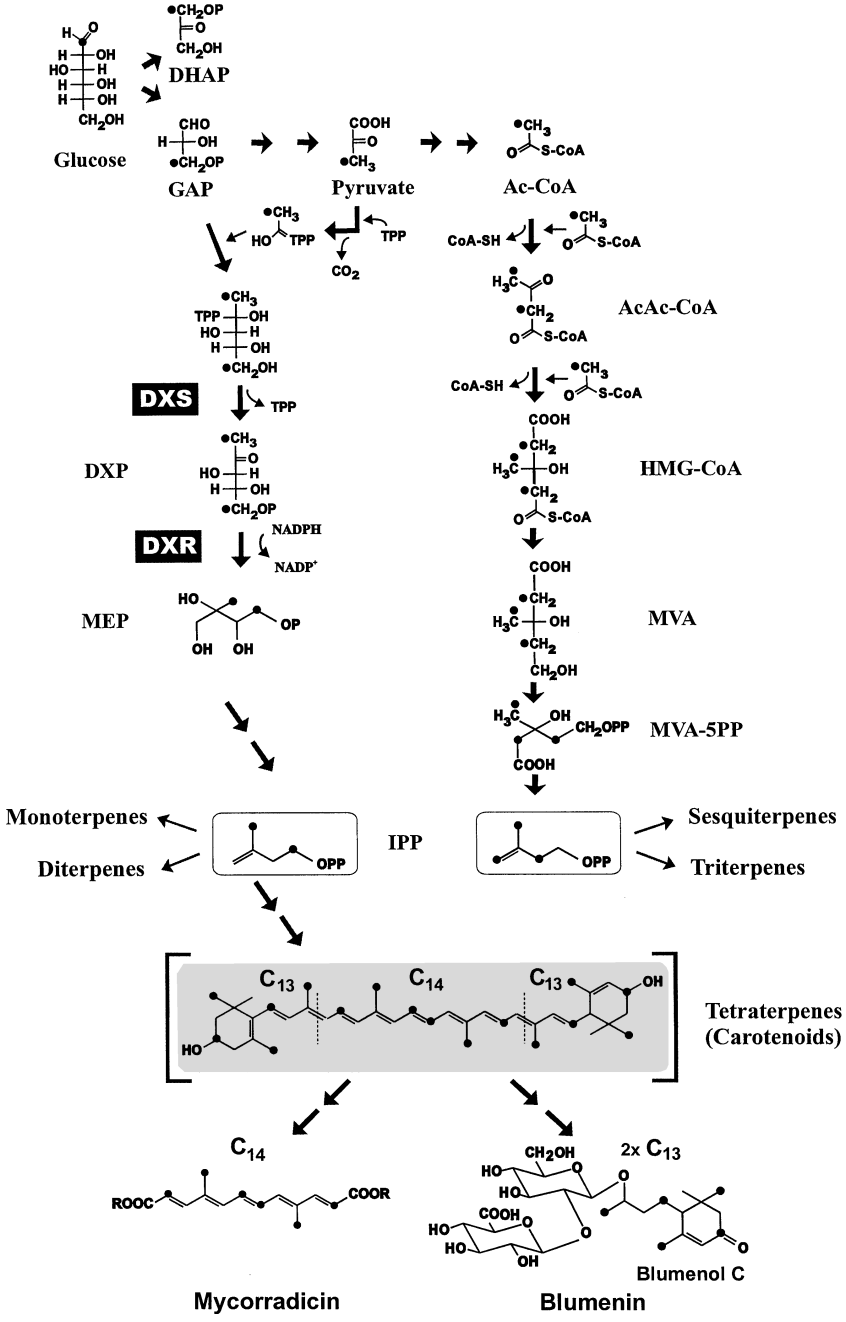
tribes Poeae, Triticeae, and Aveneae (Maier et al., 1997). To evaluate the AM-specific formation of cyclohexenone derivatives, some inoculations of barley roots with pathogens (*Gaeumannomyces graminis* and *Drechslera* sp.) or an endophyte (*Fusarium* sp.) were performed. These, as well as treatments with abiotic stressors (heat, cold, high intensities of light, heavy metals, and drought), did not induce the formation of cyclohexenone derivatives (Maier et al., 1997). Because of the occurrence of both C_{13} and C_{14} apocarotenoids (cyclohexenone derivatives and mycorradicin) in mycorrhizal roots, their formation might be similar to that of abscisic acid, involving a dioxygenase-catalyzed cleavage of a carotenoid precursor (Maier et al., 1998; Walter et al., 2000).

One of the first arguments for a carotenoid origin of cyclohexenone derivatives derived from NMR spectroscopic analysis of blumenin (**1**) after [^{13}C]glucose-tracing experiments, indicating a mevalonate-independent biosynthesis of the cyclohexenone derivative (Maier et al., 1998). Figure 4 shows the ^{13}C-labelling pattern of blumenol C, after feeding [1-^{13}C]glucose to barley roots. For comparison, potential labelling of mycorradicin via the MEP pathway and isopentenyldiphosphate (IPP) synthesized via the mevalonate pathway are shown.

The assumption that the AM-specific isoprenoids are apocarotenoids was recently supported by a study showing that carotenoid biosynthesis is strongly stimulated in AM roots, at least partially at the transcriptional level (Fester et al., 2002b). Tobacco plants transformed with a phytoene desaturase *promoter::GUS* construct showed a cell-specific induction of the phytoene desaturase promoter activity in arbusculated root cells.

The role of the cyclohexenone derivatives in mycorrhizal symbiosis is unknown, although there is some indication that they might be involved in control of mycorrhization. Exogenously applied blumenin strongly inhibits colonization and formation of arbuscules in the early stages of mycorrhiza formation in barley (Fester et al., 1999). Inoculation of barley, wheat, and maize with different AM fungi (*Glomus mosseae*, *G. intraradices*, and *Gigaspora rosea*) led to the accumulation of **1**, **4**, **5**, and another, yet unidentified, cyclohexenone derivative in all plant/fungi associations. The relation of all compounds to each other was quantitatively different, but qualitatively identical, indicating no fungus-specific induction of selected compounds (Vierheilig et al., 2000).

In addition to the well-characterized AM fungus-induced accumulation of cyclohexenone derivatives in gramineous plants, a set of similar compounds was found in roots of tobacco and tomato (Solanaceae) after colonization with *Glomus intraradices* (Maier et al., 1999, 2000). In the major compound, named nicoblumin (**4**), the aglycone of **3** (13-hydroxyblumenol C) is connected at the C-9 hydroxyl group with a 1,6-linked diglucose (gentiobiose). Both in mycorrhizal roots of two tobacco species (*Nicotiana tabacum*, *N. rustica*) and in tomato (*Lycopersicon esculentum*), **3** was found to accumulate. The aglycone of two further tobacco cyclohexenone derivatives is formed when the hydroxymethyl group at C-5 in **3** is


Glucose
DHAP
GAP
Pyruvate
Ac-CoA
AcAc-CoA
DXS
DXP
DXR
MEP
HMG-CoA
MVA
MVA-5PP
Monoterpenes
Diterpenes
IPP
Sesquiterpenes
Triterpenes
Tetraterpenes
(Carotenoids)
Mycorradicin
Blumenin
Blumenol C

oxidized to a carboxyl group. This structure accumulates as 9-*O*-gentiobioside (**5**) and 9-*O*-glucoside (**6**). Additional strongly induced major compounds in tobacco are the gentiobiosides of blumenol C occurring as *S*- and *R*-isomers at C-9 (**7**), whereas a minor component is a triglucosyl derivative of blumenol C (**8**). In tomato roots, an additional 9-*O*-glucoside (**9**) bearing a carboxyl group at C-1 occurs, but the relative configurations of the asymmetric sites (C-1, C-6, and C-9) could not be established from NMR spectroscopic data. Further comparative studies of cyclohexenone derivatives in mycorrhizal maize and wheat roots revealed the presence of six compounds from which only the 9-*R*,*S*-isomers of blumenin (**1),** nicoblumin (**4**), and **5** were tentatively identified by comparison of HPLC retention times with those of reference compounds (Walter et al., 2000). By alkaline hydrolysis of the "yellow pigment" purified from mycorrhizal maize roots, besides mycorradicin, an isomer pair of a cyclohexenone derivative was liberated, which was identified by NMR spectroscopy as blumenol C 9-*O*-β-cellobioside (**10**) (Fester et al., 2002a), assumed to form esterification products (oligo- or polyesters) with mycorradicin, establishing part of the "yellow pigment."

MOLECULAR BIOLOGY

Traditionally, levels of metabolites, proteins, or transcripts of known identity have been tested for being affected by a certain stimulus. In the case of the interaction of plant roots with a symbiotic partner, mineral nutrient transport proteins are obvious choices. Recently, nontargeted approaches, particularly at the transcript

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FIG. 4. Biosynthesis of isopentenyl diphosphate (IPP) via the MEP and mevalonate pathways leading to various terpenoids (Rohmer, 1999; Rodriguez-Concepcion and Boronat, 2002). The DXS- and DXR-catalyzed reactions are pointed out in this review. The pathways show the ^{13}C-labelling patterns of IPP and blumenol C, the aglycone of blumenin, that accumulated in AM fungus inoculated barley roots after feeding of [1-^{13}C]glucose (Maier et al., 1998). For a comparison, potential ^{13}C-labelling of mycorradicin via the MEP pathway and of IPP via the mevalonate pathway are shown (parts of the chart have been adapted from Lichtenthaler et al., 1997; Rohmer, 1999). The ^{13}C enrichment in blumenol C determined by ^{13}C NMR spectroscopy is indicated by dots. The precursor carotenoid (shaded in grey) is still elusive. Mycorradicin (R = H) is the core structure of the "yellow pigment" (R = glycosylated cyclohexenone derivatives) (Fester et al., 2002a). Abbreviations: Ac-CoA, acetyl coenzyme A; AcAc-CoA, acetoacetyl-CoA; DHAP, dihydroxyacetone phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GAP, glyceraldehyde 3-phosphate; HMG-CoA, β-hydroxy-β-methylglutaryl-CoA; IPP, isopentenyl diphosphate; MEP, 2-*C*-methyl-*D*-erythritol 4-phosphate; MVA-5PP, mevalonate 5-diphosphate; TPP, thiamine diphosphate.

level, have become popular and have now also been applied to mycorrhizal systems (Lapopin and Franken, 2000; Franken and Requena, 2001; Stougaard, 2001). Whole AM root transcriptomes (total mRNA transcripts at a specific stage of mycorrhization) can be captured in cDNA libraries. Partial sequencing of all or of a fraction of clones in the library then results in so-called expressed sequence tags (ESTs) (Journet et al., 2002). A biochemical function can often be attributed to specific ESTs or to the proteins they encode by finding sequences with high sequence similarity and a known function in DNA databases. If done on a sufficiently large scale, this approach can provide an, albeit still fragmentary, overview of metabolic activities during the mycorrhizal symbiosis. Analysis of changes in the levels of specific transcripts by RNA blot, real time PCR, or DNA array methods can identify transcripts and genes and, thus, areas of metabolic activity up- or downregulated during mycorrhization. Subtractive methods (subtraction of nonmycorrhizal transcripts from the population of mycorrhiza-regulated and mycorrhiza-unaffected transcripts) can reveal mycorrhiza-affected transcripts already at the cDNA cloning stage (Voiblet et al., 2001).

Diploid and autogamous plants with a small genome are particularly suitable for such approaches. Unfortunately, the prime model plant *Arabidopsis thaliana*, a member of the Brassicaceae, is not a host for AM fungi. However, extensive efforts of groups in the United States, France, and Germany on the model legume plant *Medicago truncatula*, which easily accommodates various symbiotic partners, have now led to the availability of more than 100,000 ESTs in databases from various *Medicago truncatula* tissues (Journet et al., 2002). These include control roots or roots colonized by AM fungi or other mutualistic and pathogenic microbes. This resource now even allows various "*in-silico*" (computer-based) analyses, e.g., to carry out "electronic" Northern experiments by comparing the frequency of specific ESTs (representing transcript levels) from defined tissues and environmental circumstances in the database. Unfortunately, fungal sequences with known identity are poorly or not at all represented in databases.

Another valuable resource are specific mutants of host plants, which are blocked at certain stages of the AM fungal colonization. In *Medicago truncatula*, many of these mutants are also disturbed in the accommodation of *Rhizobium* bacteria forming nodules for nitrogen fixation. A recent example is the identification of SYMRK, a receptor-like kinase required for both fungal and bacterial recognition (Stracke et al., 2002). Whereas this shows the existence of at least one common signalling component for the recognition of two very different symbionts, characterization of other mutants argues for additional SYM-independent signalling pathways (Kistner and Parniske, 2002).

One of the plant's main benefits from the AM symbiosis is improvement of phosphate uptake. Recent molecular studies of various phosphate transporters have demonstrated how plants can adapt their phosphate uptake to the interaction with the AM fungus. Specific phosphate transporters with different properties compared

to those known so far are expressed in the arbusculated root cells. It is assumed that they are located at the periarbuscular membrane where they are involved in the acquisition of phosphate supplied by the fungus (Gianinazzi-Pearson et al., 1991; Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002). Phosphate transporters active in nonmycorrhizal roots are downregulated. Interestingly, the AM-specific transporter OsPT11 from rice is not an ortholog of the potato transporter StPT3. Thus, this feature may have evolved several times independently (Paszkowski et al., 2002). The molecular mechanisms of phosphate transport in plants are described in a recent review (Rausch and Bucher, 2002).

Isotopic labelling and NMR spectroscopy contributed significantly to our understanding of the physiology of AM symbiosis (Douds et al., 2000; Pfeffer et al., 2001). It was shown that glucose and fructose are effectively taken up by the fungus within the root and are metabolized to yield mainly trehalose and lipids (Wright et al., 1998; Pfeffer et al., 1999). The lipids are then translocated to the extraradical mycelium, translocated within AM fungal colonies, and are recirculated throughout the fungus (Bago et al., 2002). Carbon flux and gene expression studies indicate that the glyoxylate cycle is central to the flow of carbon in the AM symbiosis (Lammers et al., 2001).

The first molecular studies on plant secondary metabolites in AM roots were carried out on phenylpropanoid metabolism in alfalfa and soybean. In alfalfa, increases in transcription levels of phenylalanine ammonia-lyase and chalcone isomerase were observed (Volpin et al., 1994). In soybean roots, however, the level of chalcone isomerase decreased (Lambais and Mehdy, 1993). In *Medicago truncatula* and *Phaseolus vulgaris*, transcript levels of phenylalanine ammonia-lyase and chalcone synthase increased (Harrison and Dixon, 1993; Blee and Anderson, 1996), whereas isoflavone reductase transcript formation was suppressed in *Medicago truncatula* (Harrison and Dixon, 1993). The latter correlated with a reduced accumulation of the phytoalexin medicarpin.

As discussed above, the biosynthesis of apocarotenoids is known to proceed via the plastid-located nonmevalonate route, which is now commonly called MEP pathway (Rodriguez-Concepcion and Boronat, 2002). Two key steps of the MEP pathway, catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (Figure 4), were investigated for changes in transcript levels upon AM formation. Levels of both *DXS* and *DXR* transcripts were considerably higher in AM fungus-colonized roots of various cereals compared to nonmycorrhizal roots (Walter et al., 2000). In a subsequent work with *Medicago truncatula*, two distantly related *DXS* transcripts were identified, which both specified functional DXS proteins (Walter et al., 2002). Only *DXS2* exhibited elevated transcript levels in mycorrhizal roots, whereas *DXS1* remained unaffected at a very low level during this interaction. Conversely, *DXS1* was highly expressed in aerial photosynthetic tissues, where *DXS2* transcripts were low. Additional experiments with nonmycorrhizal and mycorrhizal maize

and tomato plants corroborated these results. They indicate that there might be a division of labor between *DXS1*, expressed for primary functions, such as supply of chloroplast components, and *DXS2* whose expression profiles correlate with formation of secondary compounds, such as (apo)carotenoids in mycorrhizal roots (Walter et al., 2002).

The diversification of DXS enzymes and genes from the isopentenyl diphosphate-generating MEP pathway and their specific involvement in primary and secondary products came as a surprise. Previously, assignment of enzymes to primary and secondary isoprenoid formation was known only for a later step in this pathway, namely the one catalyzed by various terpene synthases (Trapp and Croteau, 2001). *DXS2* genes and their promoters are currently being isolated in our laboratory to identify regulatory sequences responsible for the differential, and particularly the mycorrhiza-dependent, expression. The subsequent step in the MEP pathway, catalyzed by DXR (Figure 4), does not appear to be diversified, since a single *DXR* transcript species accumulates in leaves and mycorrhizal roots (Hans, 2003).

Finally, production of the AM-specific apocarotenoids is catalyzed by dioxygenases. The first of these enzymes, characterized from the pathway leading to the C_{15} apocarotenoid abscisic acid, was the vp14 of maize (Tan et al., 1997). A related carotenoid-cleaving dioxygenase (CCD), generating C_{14} and C_{13} apocarotenoids, was identified later (Schwartz et al., 2001). This enzyme could be involved in the generation of AM-specific mycorradicin and cyclohexenone derivatives. Unpublished results from our work with maize and *Medicago truncatula* indicate that a CCD transcript is mycorrhiza-regulated in roots (Hans, 2003). Taken together, the AM-specific isoprenoid metabolism and apocarotenoid formation raise attractive questions to study both the evolution of plant secondary metabolism and its role in ecological interactions.

SUMMARY

Mutualistic symbioses of mycorrhizas are crucial in the ecology and physiology of terrestrial plants and are most effective in supporting plants to cope with various environmental stressors, such as nutrient- and water-deficient soils or pathogenic infection. With some recent success in mycorrhiza research on the metabolic and genetic levels, we are beginning to understand the complexity of the chemical dialogue of the two partners. We expect that the growing interest in mycorrhiza research and availability of new analytical techniques and molecular genetic approaches will lead in the near future to new insights into the strategies of plants and fungi to develop mutualistic symbiotic associations. In addition, understanding the mechanisms that prevent mycorrhizal colonization in nonhost species will help to elucidate the molecular interactions responsible for a successful establishment of mycorrhizal symbioses.

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DROUGHT STRESS ALTERS THE CONCENTRATION OF WOOD TERPENOIDS IN SCOTS PINE AND NORWAY SPRUCE SEEDLINGS

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Abstract—Drought is known to have an impact on the resistance of conifers to various pests, for example, by affecting resin flow in trees. Little is known, however, about the quantitative and qualitative changes in resin when trees are growing in low moisture conditions. We exposed Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) seedlings to medium and severe drought stress for two growing seasons and analyzed the monoterpenes and resin acids in the main stem wood after two years of treatment. In addition to secondary chemistry, we measured the level of nutrients in the needles and the growth response of seedlings. After the first year of treatment, drought stress did not affect the growth of seedlings, but in the second year, shoot growth was retarded, especially in Scots pine. In both conifer species, severe drought increased the concentrations of several individual monoterpenes and resin acids. Total monoterpenes and resin acids were 39 and 32% higher in severe drought-treated Scots pine seedlings than in the controls, and 35 and 45% higher in Norway spruce seedlings. In Scots pine needles, the concentrations of nitrogen and phosphorus increased, while magnesium and calcium decreased compared to controls. In Norway spruce needles, nutrient concentrations were not affected. The results suggest that drought stress substantially affects both the growth of

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conifers and the chemical quality of the wood. We discuss the potential trade-off in growth and defense of small conifer seedlings.

Key Words—Drought stress, monoterpenes, resin acids, wood, seedlings, *Pinus sylvestris* L., *Picea abies* (L.) Karst, conifers.

INTRODUCTION

The secretion of oleoresin, a complex mixture of terpenes, is an important part of the defensive mechanism of coniferous trees against phytophagous insects, fungi, and microbes (e.g., Croteau and Johnson, 1985; Eberhardt et al., 1994; Micales et al., 1994; Phillips and Croteau, 1999). Oleoresin accumulates in woody tissues as a result of normal physiological processes, or as a response to wounding (Croteau and Johnson, 1985). In most pine and spruce species, the main components of oleoresin are diterpenes (resin acids), but also minor amounts of volatile monoterpenes and sesquiterpenes (Croteau and Johnson, 1985).

Drought stress limits photosynthesis in plants, alters carbon allocation between roots and shoots (Teskey et al., 1987), and changes nutrient uptake ratios and nutrient circulation (Schulze, 1991). It also changes the levels of soluble sugars, inorganic ions, amino acids, and other osmolytes (Mattson and Haack, 1987). The susceptibility of host trees to attack by pathogens and herbivorous insects may be influenced by stress factors, such as adverse weather conditions and competition from limited resources (Lindberg and Johansson, 1992). The ability of trees to resist attack by bark beetles and their associated fungi is linked to overall vigor and to the amount of carbohydrates that can be used for defense reactions (e.g., Christiansen et al., 1987). Therefore, any environmental factor (e.g., drought stress) that restricts the size of the canopy or its photosynthetic efficiency can weaken the resistance of the tree (Christiansen et al., 1987).

Only a few studies have been published about the effects of drought stress on the quality of oleoresin in conifer wood. The changes detected in the wood of loblolly pine (*Pinus taeda* L.) have been an increase in concentration of all individual monoterpenes (Hodges and Lorio, 1975). In contrast, in the same tree species, Gilmore (1977) observed an increased concentration of α-pinene followed by a decrease in the β-pinene, myrcene, and limonene concentrations. In the needles, drought stress increased the concentration of monoterpenes in Norway spruce (*P. abies* (L.) Karst.) (Kainulainen et al., 1992), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) (Major, 1990), ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) (Johnson et al., 1997), and several Mediterranean woody plants, e.g., Aleppo pine (*Pinus halepensis* Mill.) (Llusia and Penuelas, 1998).

In many areas of the world, a trend toward warming of the climate has been observed in this century. According to climate-change scenarios, in Finland the average temperature is expected to increase and lead to changes in precipitation during

this century (Kuusisto et al., 1996). Differences in moisture at various sites might increase. In nature, there are great differences in soil moisture properties between different forest types, and some conifers are naturally exposed to drought. Finnish forests are dominated by two conifer species, Scots pine (*P. sylvestris* L.) and Norway spruce (*P. abies* (L.) Karst.), both of which are adapted to the disturbance regime of the particular forest type they occupy (Virkkala and Toivonen, 1999).

In this experiment we used small seedlings of Scots pine and Norway spruce to study the effect of drought on (a) quantitative and qualitative changes in wood monoterpenes and resin acids, and (b) growth of seedlings.

METHODS AND MATERIALS

Plant Material. To ensure genetic variation in seed material, we used a random sample of seeds collected from two orchards. The Scots pine seed orchard 249 was established in 1972 with grafts of 429 donor-tree genotypes. The Norway spruce seed orchard 111 was established in 1966 with 57 donor-tree genotypes. The seeds are the result of open pollination (a random mix of the pollen cloud from surrounding forests and the seed orchard). Although the clones in the seed orchards are the result of phenotypic selection (plus-trees), we expect this has no effect on the results.

For the drought experiment, nursery-grown potted 4-year-old Scots pine and 3-year-old Norway spruce seedlings were obtained from the forest nursery in Suonenjoki (62°38′N, 27°04′E), central Finland. At the beginning of June 1998, seedlings were planted individually in 7.5-l plastic pots, in a 2:1 (pine) or 1:1 (spruce) mixture of sand and fertilized peat (Finnpeat PP6, Kekkilä Co, Finland). Seedlings were transplanted to the experimental field of the Botanical Garden at the University of Kuopio. During the summer of 1998, seedlings were grown under natural rainfall, watered as needed, and fertilized once a week with a 0.1% solution of 5-Superex (Kekkilä Co, 11:25:4, N: P: K). Drought experiments were carried out during growing seasons 1999–2000. At that time, seedlings were fertilized with a slow-release fertilizer (Taimiston kestolannos, Kemira Co, Finland: 9% N, 3.5% P, 5% K, 4.8% Mg, 3.8% S, and $<1\%$ micronutrients). Treatment was 15 g/seedling in 1999 and 10 g/seedling in 2000. Fertilizer was mixed into the surface layer of the growth medium at the beginning of each growing season.

Drought Treatments. On May 17, 1999, seedlings were selected randomly for drought treatments, i.e., control (well watered), medium drought, and severe drought (40 seedlings per treatment per tree species). The growth medium in the pots was sheltered from natural rainfall with a plastic cover, and the pots were separated from the soil with a plastic sheet and a wooden lattice. Pots from different treatments were arranged randomly into two double rows with different conifer species located in different sections of the experimental field. Pots in double rows were next to each other, and the distance between the double rows was about half

a meter. The total area of the field containing both pine and spruce seedlings was about 60 m^2.

Pore space and dry weight of the growth medium for pine and spruce were used to determine the regimes for drought treatment. Control, medium-drought, and severe-drought treatment pots contained 60, 40, and 20%, respectively, of the amount of water in the pore space. Corresponding soil volumetric moisture contents (at control, medium drought, and severe drought) were calculated to be 26, 19, and 9% for pine and 27, 18, and 9% for spruce seedlings, respectively. On the basis of these data, target weights were calculated for each pot. In the first year, weighing the pots and adding enough water to reach the target weight of each pot controlled the soil volumetric moisture. Simultaneously, the soil volumetric water content was measured with a ThetaProbe. In August 1999, soil gravimetric moisture was measured by taking soil samples and weighing their fresh and dry weights. These results were compared with the simultaneous measurements of soil volumetric moisture taken with the ThetaProbe. The data showed a strong correlation between the soil gravimetric moisture content and the ThetaProbe measurements. Based on these measurements during 1999, it was possible to calculate the supplement of amounts of water; and in the year 2000, watering was based only on the results of the ThetaProbe measurements. In both experimental years, seedlings were watered 3–4 times a week according to need. Overall, in pine seedlings, the drought treatments were close to target levels, while in spruce seedlings it was harder to obtain severe drought stress, especially during the first treatment summer.

Sampling. In both years (1999–2000), the length of the current leader shoots and the diameter of the stem base were measured at the beginning of May. Several morphological parameters (length of the needles and shoots, total seedling length, and diameter of the stem base) and biomass of the roots and current-year shoots were measured during the final sampling at the end of August 2000. For monoterpene and resin acid analyses, wood disks (bark detached) were cut from the second annual growth of the main stem. For both pine and spruce, samples were collected from 12 trees of each treatment ($N = 12$). For analysis of secondary compounds, samples were immediately frozen in liquid nitrogen and stored at -80°C until analyzed. Wood samples from the second annual growth for measurements of water content were freeze-dried and weighed. Monoterpenes were extracted from frozen samples with *n*-hexane as described by Manninen et al. (2002). 1-Chlorooctane was used as an internal standard. Resin acids were extracted from freeze-dried and powdered samples with petroleum ether—diethyl ether (v: v) as described by Gref and Ericsson (1985). Terpene and resin acid extracts were analyzed by gas chromatography–mass spectrometry (Hewlett Packard GC type 6890, MSD 5973) using a 30-m long HP-5MS (0.25 mm I.D., 0.25 μm film thickness, Hewlett Packard) capillary column as described by Manninen et al. (2002).

Current-year needles were collected at the end of August 2000 and oven-dried at 60°C; the needle material was finely ground and wet-digested for N, K, Ca, Mg,

and P determinations (Allen, 1989). Total nitrogen was analyzed by the standard Kjeldahl method, K, Ca, and Mg by atomic absorption spectroscopy (Perkin Elmer 460) (Allen, 1989), and phosphorus with a spectrophotometer (Shimadzu UV-1201) (John, 1970).

Statistical Analyses. All data were analyzed using SPSS 9.0 for Windows (SPSS Inc., Chicago, Illinois). Differences between treatments were tested with a one-way ANOVA followed by Tukey's HSD test for multiple *post hoc* comparisons. To meet the requirements of ANOVA, the data of secondary compounds was $\log(x+1)$ transformed. Bivariate linear correlations were tested with Spearman's correlation test.

RESULTS

Concentrations of total monoterpenes and resin acids were considerably higher in the wood of Scots pine than in that of Norway spruce (Tables 1 and 2). Major monoterpenes in Scots pine were α-pinene and 3-carene (Table 1), while in Norway spruce, α-pinene and β-pinene dominated (Table 2). These monoterpenes constituted about 81% and 71% of the total monoterpenes in Scots pine and Norway spruce seedlings, respectively. In severe drought-stressed Scots pine seedlings, the concentrations of some individual monoterpenes were increased, and the total monoterpene concentration was almost doubled compared to controls (Table 1). Correspondingly, in Norway spruce wood, the concentrations of individual and total monoterpenes were not significantly affected (Table 2). With the medium drought treatment, the changes in the concentrations of monoterpenes were not significant in either tree species.

The main resin acids in the woody tissue of both tree species were abietic and levopimaric+palustric acids, constituting 77% of the total resin acids in Scots pine (Table 1) and 68% in Norway spruce (Table 2). In Scots pine, severe drought stress increased the concentrations of several individual resin acids (Table 1). In contrast, in Norway spruce seedlings, severe drought increased the concentrations of only three resin acids, which were mainly different compounds from those found in Scots pine (Tables 1 and 2). The total resin acid concentration was 32% higher in Scots pine seedlings and 45% higher in Norway spruce seedlings compared to controls. The only significant effect detected in the medium drought treatment was an increase in the concentration of dehydroabietic acid in Scots pine seedlings (Tables 1 and 2). In Scots pine, there was a negative correlation between the water content of the wood and the concentration of pimaric ($r = -0.333$, $P = 0.047$), sandaracopimaric ($r = -0.377$, $P = 0.024$), neoabietic ($r = -0.464$, $P = 0.004$), and levopimaric + palustric ($r = -0.391$, $P = 0.018$) acid.

In both conifer species, drought stress affected most of the growth parameters measured in the seedlings (Table 3). Severe drought–stressed Scots pine and Norway spruce seedlings had about 79 and 43% lower biomass of current

TABLE 1. CONCENTRATIONS [MEAN (MG/G DW) ± SE] OF INDIVIDUAL AND TOTAL MONOTERPENES AND RESIN ACIDS IN WOOD OF SCOTS PINE (*Pinus sylvestris L.*) SEEDLINGS[a]

	Treatments			ANOVA	
	Control	Medium drought	Severe drought	$F_{2, 33}$	P
Monoterpenes					
Tricyclene	+	+	+	2.775	0.077
α-Pinene	2.30 ± 0.27 a	3.49 ± 0.63 ab	4.88 ± 0.84 b	3.856	0.031
Camphene	0.03 ± 0.003	0.04 ± 0.01	0.05 ± 0.01	3.856	0.061
Sabinene	0.10 ± 0.02	0.07 ± 0.02	0.12 ± 0.02	1.167	0.324
β- Pinene	0.23 ± 0.09	0.23 ± 0.10	0.22 ± 0.05	0.402	0.672
Myrcene	0.10 ± 0.02 a	0.13 ± 0.02 ab	0.18 ± 0.02 b	5.657	0.008
3-Carene	1.66 ± 0.35	1.16 ± 0.32	2.28 ± 0.52	1.102	0.344
Limonene + β-Phellandrene[b]	0.59 ± 0.17	0.34 ± 0.08	0.58 ± 0.09	2.351	0.111
Terpinolene	0.15 ± 0.03	0.10 ± 0.03	0.18 ± 0.04	1.000	0.379
Bornyl acetate	+	+	+	0.840	0.441
Total monoterpenes	5.16 ± 0.59 a	5.57 ± 0.67 ab	8.52 ± 0.91 b	5.140	0.011

Resin acids					
Pimarane type resin acids					
Pimaric acid	0.49 ± 0.07 a	0.57 ± 0.06 ab	0.72 ± 0.06 b	3.907	0.030
Sandaracopimaric acid	0.25 ± 0.02 a	0.31 ± 0.04 ab	0.39 ± 0.03 b	6.903	0.003
Isopimaric acid	0.15 ± 0.04	0.30 ± 0.09	0.30 ± 0.05	2.159	0.131
Total	0.90 ± 0.11 a	1.18 ± 0.15 ab	1.41 ± 0.12 b	5.087	0.012
Abietane type resin acids					
Levopimaric + Palustric acid[c]	4.14 ± 0.30 a	4.78 ± 0.61 ab	6.12 ± 0.44 b	4.799	0.015
Dehydroabietic acid	0.81 ± 0.05 a	1.09 ± 0.10 b	1.09 ± 0.08 b	4.791	0.015
Abietic acid	7.23 ± 0.59	9.81 ± 1.75	10.23 ± 1.10	1.580	0.221
Neoabietic acid	1.48 ± 0.17 a	1.87 ± 0.27 ab	2.66 ± 0.29 b	5.910	0.006
Total	13.65 ± 1.01 a	17.54 ± 2.54 ab	20.10 ± 1.60 b	3.467	0.043
Total resin acids	14.56 ± 1.11 a	18.73 ± 2.68 ab	21.51 ± 1.70 b	3.655	0.037

[a] F and P values are from one-way ANOVA. Different letters in the row indicate significant differences between treatments (Tukey HSD, $P < 0.05$).
[b] Limonene and β-phellandrene were not separated from each other on HP-5MS column.
[c] Levopimaric and palustric acid were not separated from each other on HP-5MS column, + = trace amounts (<0.01 mg/g).

TABLE 2. CONCENTRATIONS [MEAN (MG/G DW) ± SE] OF INDIVIDUAL AND TOTAL MONOTERPENES AND RESIN ACIDS IN WOOD OF NORWAY SPRUCE *(Picea abies (L.) Karst.)* SEEDLINGS[a]

	Treatments			ANOVA	
	Control	Medium drought	Severe drought	$F_{2, 33}$	P
Monoterpenes					
Tricyclene	+	+	+	0.365	0.697
α-Pinene	0.46 ± 0.06	0.49 ± 0.05	0.69 ± 0.09	2.251	0.122
Camphene	0.01 ± 0.003	0.01 ± 0.003	0.02 ± 0.01	0.396	0.676
Sabinene	0.01 ± 0.001	0.01 ± 0.001	0.02 ± 0.01	1.872	0.170
β-Pinene	0.35 ± 0.07	0.42 ± 0.06	0.54 ± 0.12	0.184	0.833
Myrcene	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.179	0.837
3-Carene	0.02 ± 0.01	0.02 ± 0.01	0.12 ± 0.06	0.636	0.536
Limonene + β-Phellandrene[b]	0.25 ± 0.05	0.27 ± 0.05	0.29 ± 0.06	0.533	0.592
Terpinolene	+	+	+	2.032	0.147
Bornyl acetate	+	+	+	0.051	0.950
Total monoterpenes	1.14 ± 0.18	1.27 ± 0.14	1.75 ± 0.28	0.226	0.799

Resin acids					
Pimarane type resin acids					
Pimaric acid	0.04 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	1.138	0.333
Sandaracopimaric acid	0.17 ± 0.02	0.28 ± 0.07	0.28 ± 0.05	1.464	0.246
Isopimaric acid	0.25 ± 0.03 a	0.33 ± 0.05 ab	0.44 ± 0.07 b	3.765	0.034
Total	0.46 ± 0.06	0.67 ± 0.12	0.78 ± 0.12	2.747	0.079
Abietane type resin acids					
Levopimaric + Palustric acid[c]	1.11 ± 0.17	1.50 ± 0.24	1.91 ± 0.50	1.390	0.263
Dehydroabietic acid	0.25 ± 0.03 a	0.36 ± 0.05 ab	0.48 ± 0.08 b	4.537	0.018
Abietic acid	0.68 ± 0.10 a	1.07 ± 0.18 ab	1.41 ± 0.28 b	4.659	0.017
Neoabietic acid	0.15 ± 0.02	0.21 ± 0.04	0.28 ± 0.06	2.747	0.070
Total	2.19 ± 0.30	3.15 ± 0.48	4.07 ± 0.88	2.905	0.069
Total resin acids	2.65 ± 0.35	3.82 ± 0.59	4.86 ± 1.00	2.917	0.068

[a] F and P values are from one-way ANOVA. Different letters in the row indicate significant differences between treatments (Tukey HSD, $P < 0.05$).
[b] Limonene and β-phellandrene were not separated from each other on HP-5MS column.
[c] Levopimaric and Palustric acid were not separated from each other on HP-5MS column, + = trace amounts (<0.01 mg/g).

TABLE 3. SUMMARY OF GROWTH PARAMETERS (MEAN ± SE) IN SCOTS PINE AND NORWAY SPRUCE SEEDLINGS AT THE FINAL HARVEST 2000[a]

Parameter	Control	Medium drought	Severe drought	ANOVA $F_{2, 33}$	ANOVA P
Scots pine					
Biomass of current year shoot (g FW)	100.5 ± 4.2 c	60.7 ± 3.0 b	21.5 ± 2.9 a	134.398	<0.001
Root biomass (g DW)	38.4 ± 1.5 c	29.9 ± 1.4 b	19.7 ± 1.1 a	48.493	<0.001
Shoot length of current year (cm)	24.7 ± 1.2 c	14.2 ± 1.3 b	9.0 ± 0.9 a	52.130	<0.001
Shoot length of previous year (cm)	10.5 ± 0.5	10.9 ± 1.2	9.9 ± 0.7	0.326	0.724
Total shoot length (cm)	99.6 ± 1.8 c	91.8 ± 1.8 b	84.0 ± 1.6 a	20.814	<0.001
Stem base diameter (cm)	1.6 ± 0.0 c	1.4 ± 0.0 b	1.2 ± 0.0 a	69.857	<0.001
Needle length (current) (cm)	3.6 ± 0.1 b	3.4 ± 0.2 b	2.1 ± 0.2 a	26.181	<0.001
Norway spruce					
Biomass of current year shoot (g FW)	127.3 ± 5.5 b	111.6 ± 6.6 b	72.2 ± 3.7 a	27.607	<0.001
Root biomass (g DW)	33.1 ± 1.5 b	29.2 ± 1.5 b	18.1 ± 1.2 a	30.403	<0.001
Shoot length of current year (cm)	18.1 ± 1.8 b	13.5 ± 1.9 b	4.6 ± 0.9 a	18.619	<0.001
Shoot length of previous year (cm)	8.6 ± 2.3	8.4 ± 0.8	6.5 ± 0.8	0.670	0.519
Total shoot length (cm)	64.5 ± 2.3 b	60.1 ± 2.1 b	47.6 ± 1.9 a	17.222	<0.001
Stem base diameter (cm)	1.5 ± 0.0 c	1.4 ± 0.0 b	1.1 ± 0.0 a	30.023	<0.001
Needle length (current) (cm)	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.1	2.456	0.101

[a] F and P values are from one-way ANOVA. Different letters in the row indicate significant differences between treatments (Tukey HSD, $P < 0.05$).

TABLE 4. CONCENTRATIONS [MEAN (MG/G DW) ± SE] OF NUTRIENTS IN THE NEEDLES OF SCOTS PINE AND NORWAY SPRUCE SEEDLINGS AT FINAL HARVEST IN AUGUST 2000[a]

				ANOVA	
Nutrient	Control	Medium drought	Severe drought	$F_{2, 33}$	P
Scots pine					
N	7.9 ± 0.4 a	9.6 ± 0.4 b	12.7 ± 0.6 c	24.001	<0.001
Mg	0.9 ± 0.0 b	0.9 ± 0.0 b	0.7 ± 0.0 a	5.444	0.009
Ca	1.1 ± 0.1 b	1.0 ± 0.1 b	0.8 ± 0.0 a	8.734	0.001
K	6.5 ± 0.2	6.4 ± 0.2	7.0 ± 0.2	2.850	0.072
P	1.0 ± 0.0 a	1.1 ± 0.1 a	1.3 ± 0.1 b	8.451	0.001
Norway spruce					
N	7.5 ± 0.5	7.7 ± 0.5	8.9 ± 0.4	2.536	0.094
Mg	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	1.080	0.351
Ca	2.2 ± 0.2	2.1 ± 0.1	1.8 ± 0.2	1.402	0.260
K	6.3 ± 0.3	5.9 ± 0.2	6.2 ± 0.2	0.701	0.503
P	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.1	0.837	0.503

[a] F and P values are from one-way ANOVA. Different letters in the row indicate significant differences between treatments (Tukey HSD, $P < 0.05$).

year shoot, about 49 and 45% lower biomass of roots, and about 64 and 75% shorter current year shoots, respectively, than control seedlings (Table 3). In addition, the total length of the seedlings and diameter of the stem base were lower in drought-stressed seedlings. In Scots pine, the current-year needles were also shorter in the severe drought treatment (Table 3).

All nutrient concentrations, except potassium, in the needles of Scots pine were altered by severe drought treatment (Table 4). Concentrations of nitrogen and phosphorus increased, while magnesium and calcium concentrations decreased compared to control seedlings. Under conditions of medium drought, only the nitrogen concentration increased compared to control conditions. In the needles of Norway spruce seedlings, neither severe nor medium drought stress had a significant effect on nutrient concentrations (Table 4).

DISCUSSION

The concentrations of total monoterpenes and resin acids increased significantly in the woody tissue of Scots pine and Norway spruce seedlings exposed to severe drought stress. The results are in accordance with previous data, where the concentration of monoterpenes in drought-stressed conifer needles and wood increased (Hodges and Lorio, 1975; Major, 1990; Kainulainen et al., 1992; Johnson et al., 1997; Llusia and Penuelas, 1998). In *Pinus taeda* L., however, the concentration of resin acids decreased (Hodges and Lorio, 1975). According to Temnerud (1999), Norway spruce sawlogs, which had been grown in a dry geographic region,

had more resin pockets than sawlogs grown in a windy and humid region. It might be that dry growth conditions favor the formation of resin-producing structures in the stems of trees, thus enhancing the ability of the wood to produce resin.

Total concentrations of resin acids have been suggested to affect the decay resistance of conifers (see e.g. Harju et al., 2002), but the effect of specific resin acids seems to be especially important. In our experiment, drought increased the concentrations of abietane-type resin acids, which are known to be strongly toxic to white-rot fungi (Micales et al., 1994). We also found increased concentrations of pimarane-type resin acids, but the ecological effect of this is hard to interpret because little is known about the interaction of specific resin acids, e.g., stabilizing and relative proportion effects on resistance to decay (Eberhardt et al., 1994). It might be that the resin acids could partly explain the results of Lindberg and Johansson (1992) that found that growth of root rot (*Heterobasidion annosum* (Fr.) Bref.) decreased after drought.

Drought is known to be crucial promoter of outbreaks of many insects (Mattson and Haack, 1987). According to the plant quality hypothesis (e.g., Jones and Coleman, 1991; Larsson and Björkman, 1993), the reason for improved insect performance on stressed plants is the decreased host resistance and/or increased nutritional value of the plants. According to our results, in drought-stressed seedlings, the concentrations of secondary compounds in constitutive resin increased. The constitutive resin flow has been shown to increase in drought-stressed loblolly pines (see Lombardero et al., 2000). However, induced resin flow has been shown to decrease in drought-stressed Scots pines (Croise and Lieutier, 1993), pinyon pines (Cobb et al., 1997), and loblolly pines (Lombardero et al., 2000). It may be that drought-stressed seedlings tend to compensate for their decreased ability to defend (induced resin) by increasing the concentration of constitutive resin. According to Coley et al. (1985), trees exposed to drought stress have lower potential to compensate the lost resources eaten by herbivores by increasing their growth. Wagner (1986) also suggested that seedlings could probably not afford to lose any photosynthetic capacity and would, therefore, to avoid herbivore damage, evolve a more energy-expensive constitutive defense, e.g., resin compounds.

Drought stress usually decreases the growth of conifers (e.g., Christiansen, 1992; Björkman, 2000), as found in the present study in decreased length of shoots and diameter of the stem base, as well as decreased biomass of the shoots and roots. The carbon/nutrient balance (CNB) and growth differentiation balance (GDB) hypotheses predict a trade-off between growth and defense (Bryant et al., 1983; Herms and Mattson, 1992). Because growth of plants was decreased by drought stress, the carbon fixed in photosynthesis could be used for the formation of secondary compounds. Because of decreased growth, plants would use less nitrogen, resulting in the increased concentration of nitrogen in the needles. This increase was observed only in the seedlings of Scots pine. Drought stress also increased the concentration of P and decreased the concentrations of Ca and Mg in the Scots

pine needles. According to Schulze (1991), nutrient concentrations differ in their responses to drought stress, depending on the contribution of nutrient uptake versus intercirculation.

In summary, drought stress markedly increased concentrations of several individual and the total monoterpenes and resin acids in woody tissue, and also decreased the growth of Scots pine and Norway spruce seedlings. Apparently conifer seedlings that naturally grow on dry sites tend to compensate for the adverse effects of drought stress by producing more oleoresin compounds, which might influence the herbivore- and decay resistance of the trees.

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EFFECTS OF DROUGHT STRESSED COTTON, *Gossypium hirsutum* L., ON BEET ARMYWORM, *Spodoptera exigua* (HÜBNER), OVIPOSITION, AND LARVAL FEEDING PREFERENCES AND GROWTH

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Abstract—The beet armyworm, *Spodoptera exigua* (Hübner), has been anecdotally reported to oviposit more on drought stressed than on nonstressed cotton plants. Using potted cotton plants in cages, this study demonstrated that beet armyworms deposited 3.3, 4.6, and 2.3 times more ($P \leq 0.05$) eggs on cotton plants that were grown on 1500, 1000, and 750 ml water/wk, respectively, than on cotton plants grown in well watered (4000 ml water/wk) soil. Third instars, however, showed no preference for stressed cotton foliage over nonstressed foliage. Third instar beet armyworms raised on well watered cotton plants were 1.5, 2.3, and 2.6 times heavier than those reared on cotton grown in the 1500, 1000, and 750 ml water/wk plants ($P \leq 0.05$), respectively. Physiochemical analyses showed that drought stressed leaves had significantly greater accumulations of free amino acids that are essential for insect growth and development. Soluble protein and soluble carbohydrates were also more abundant in stressed leaves compared to nonstressed leaves. Despite the apparent increase in nutritional quality in drought stressed plants, larval survival was reduced, probably because the limiting factor became water. Greater amounts of cotton leaf area were consumed from drought stressed leaves ($P \leq 0.05$) than from nonstressed leaves, probably because the larvae had to metabolize greater portions of assimilated energy to supplement body water with metabolic water derived from

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respiration. The association of greater host plant nutritional quality to oviposition preference, and conversely, to reduced survivorship, is discussed.

Key Words—Beet armyworm, cotton, drought stress, *Gossypium hirsutum*, plant stress, *Spodoptera exigua*.

INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hübner), is native to southern Asia and was introduced to the United States in 1876 (Wilson, 1932). It is known to feed on >50 plant species from over 10 families worldwide (Wilson, 1932; Smits et al., 1987). In cotton, the beet armyworm can be economically important especially when pesticide applications disrupt populations of natural enemies (Eveleens et al., 1973).

Showler (2001) suggested that the nutritive value of host plants might play a role in the beet armyworm's selection of pigweed, *Amaranthus hybridus* L., over cotton, *Gossypium hirsutum* L., for oviposition and larval feeding. Drought stress has been associated with higher populations of herbivorous insects on plant foliage (White, 1984; McQuate and Connor, 1990), and could promote insect outbreaks in natural ecosystems (Mattson and Haack, 1991). Leaves on stressed plants often accumulate carbohydrates, as well as stress-related soluble proteins and free amino acids (FAAs) (White, 1984; Brodbeck and Strong, 1987; Shen et al., 1989). Host plant FAA accumulations have been associated with greater numbers of some phytophagous nematodes (Showler et al., 1990) and arthropods (Jayaraj and Seshadri, 1967; Blua et al., 1994). Water deficits often lead to oxidative stress in leaves with potentially damaging effects on membrane structures and protein complexes (Becana et al., 1998; Knight and Knight, 2001). A common response to water deficit and oxidation stress is the accumulation of antioxidants and FAAs (Becana et al., 1998). The former act to detoxify free radicals while the latter might act as osmoprotectants. Such changes in leaf chemistry in response to stress represent a significant shift in the nutritional quality of leaf tissue for herbivores (Jones, 1991).

Beet armyworm eggs have been reported to be more abundant on drought stressed cotton plants than on well watered plants in fields (Ruberson et al., 1994; Ruberson, 1996). Feeding behaviors of adult tobacco budworm, *Heliothis virescens* (F.), corn earworm, *Heliothis armigera* (Hübner), and *Spodoptera littoralis* (Boisdval) adults are correlated with the electrophysiological response of sensilla on the proboscis to amino acids and sugar (Blaney and Simmonds, 1988). Physical and chemical changes in drought stressed plants can alter both feeding and reproductive behaviors in multiple insect life stages (Mattson and Haack, 1991). The purpose of this study was to examine the relationships between drought stress on cotton plants and beet armyworm oviposition preference, larval feeding preference and growth, and the potential biochemical mechanisms for these interactions.

METHODS AND MATERIALS

This study was conducted at the U.S. Department of Agriculture – Agricultural Research Service's Kika de la Garza Subtropical Agricultural Research Center (SARC) in Weslaco, Texas, during fall 2001 and spring 2002. Cotton plants (var. C-208) were grown in a greenhouse in 7.6-l pots containing Sunshine™ mix no. 1 nursery potting soil (∼75% sphagnum peat moss, perlite, dolomitic limestone, and gypsum; Sungro Horticulture, Bellevue, Washington). Seedlings were thinned to three per pot at the cotyledon stage and 200 ml of Peters Professional® (Scotts-Sierra Horticultural Products Company, Marysville, Ohio) water-soluble general purpose 20-20-20 N-P-K fertilizer at 15.8 g/l of water was applied to each pot at the two true leaf stage. All pots were irrigated with 1 l of water once every week until the plants reached the four true leaf stage. Then, four groups of 20 pots were assigned separate irrigation regimes: 4000 ml water/wk (well watered), 1500 ml/wk, 1000 ml/wk, and 750 ml/wk, each delivered at one time per week. Pots were placed in 4-cm deep plastic dishes to retain any water that might have run through the soil and out of the holes in the bottoms.

Plant Phenology Measurements. After 8 weeks, when the cotton squares had reached the 7-mm diameter stage, measurements of stem diameters 6 cm above the soil surface, plant heights, numbers of nodes per stem, and numbers of leaves per plant were taken on 25 randomly selected plants in each treatment. Also, 30 upper leaves (from among the top four fully expanded leaves) on separate plants in each treatment were excised. Leaves were weighed immediately, then oven-dried at 60°C for 48 hr and weighed again. Twenty upper leaves from separate unsampled plants in each treatment were excised and their water potentials were measured with a Model 610™ (PMS Instrument Co., Corvallis, Oregon) pressure bomb.

Plant Physiochemical Measurements. At the same time as the phenology and water potential measurements were being taken, 15 other randomly selected upper leaves from each treatment were excised from remaining unsampled cotton plants. One gram (fresh weight) from the distal halves of each leaf was homogenized with 10-ml 0.1 N HCl using a Virtishear™ homogenizer (Virtis, Gardiner, New York). At least 5-g homogenate from each sample was placed into separate 10-ml tubes and centrifuged at 10,000 rpm for 30 min. Samples were stored at −80°C. One milliliter of supernatant from each sample was filtered through a 0.5-μl filter fitted to a 5-ml plastic syringe. Samples were placed in the autosampler of an Agilent 1100 Series™ (Agilent Technologies, Atlanta, Georgia) reversed-phase high performance liquid chromatograph (HPLC) with a binary pump delivering solvent "A" (1.36-g sodium acetate trihydrate + 500-ml purified HPLC grade water + 150-μl triethylamine [TEA] + sufficient acetic acid to bring pH to 7.2 ± 0.05) and solvent "B" (1.36-g sodium acetate trihydrate + 100-ml purified HPLC grade water [acetic acid added to this mixture to bring pH to 7.2 ± 0.05] + 200 ml acetonitrile + 200 ml methanol). Solvents A and B were pumped at 0.3 and

0.45 ml/min, respectively, on a concave gradient through an Eclipse™ XDB-C* 5-μ 4.6 $\times$ 150 mm C_{18} fluorescence column (Agilent Technologies, Atlanta, Georgia). The autosampler measured and mixed 6-μl sodium borate buffer (0.4 N, pH 10.2, in water), 1-μl 9-fluorenylmethyl chloroformate (FMOC-chloride) and 1-μl *o*-phthalaldehyde (OPA) derivatizing agents, and 2 μl of sample, then injected 2 μl for chromatographic separation of FAAs. Absorbances at 262 and 338 nm were monitored on a variable wavelength detector for 40 min per sample. Identification and quantification of derivatized FAAs was achieved by calibrating with a standard mixture of amino acids containing alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. Peak integration accuracy was enhanced by manual establishment of peak baselines using Agilent software.

Leaf soluble protein concentrations were determined according to the Bradford method (Bradford, 1976). Samples were taken and analyzed from the well watered controls, and plants grown in the 1500 and the 750 ml water/wk drought regimes ($N = 10$). Each sample was 0.3 g of frozen, ground upper cotton leaf tissue, derived from pools of three distal leaf halves excised from previously unsampled cotton plants.

Peroxidase activity was measured at 470 nm at 25°C using a 1.5-ml reaction mixture (150-μl protein extract and 0.025 M phosphate buffer, pH 7, containing 0.25% guaiacol (v/v) and 0.375% hydrogen peroxide). The reaction was monitored for 1 min, and the linear changes in absorbance determined as a measure of activity. Samples were taken and analyzed from the well watered controls, and cotton plants grown in the 1500 and the 750 ml water/wk drought regimes ($N = 10$).

Total soluble carbohydrates in upper cotton leaves were analyzed in 30 mg of homogenized lyophilized tissue using the anthrone-H_2SO_4 method (Center and Van, 1989). Reactions were quantified at 625 nm using the method of Helbert and Brown (1955). Samples were taken and analyzed from the well watered controls, and cotton plants grown in the 1500 and the 750 ml water/wk drought regimes ($N = 10$).

Oviposition Choice Assay. Beet armyworm larvae were obtained from a laboratory colony at the USDA-ARS SARC, and reared on a soybean–wheat germ diet (Shaver and Raulston, 1971). Insects were maintained in environmental chambers at 30°C, 85% relative humidity, and 13L:11D photoperiod. Pupae were sexed and placed in individual cups until adult emergence. On the day of emergence, females were paired individually with single males in 0.5-l cardboard containers. The next day, mated females were placed alone in a container lined with wax paper as oviposition substrate. Each container was monitored daily for the appearance of the first egg cluster, which signified the presence of a gravid female moth.

Gravid beet armyworm females were offered a choice of a well watered plant or a drought stressed plant in 1.8 $\times$ 1.8 $\times$ 1.1 m cages. Since maximum egg laying occurs during the first and second nights of oviposition activity (Fye and

McAda, 1972), only females that oviposited on the previous night were used in this assay. One gravid beet armyworm female was placed into each cage and allowed to oviposit for 48 hr. Each plant and the inner cage and outer pot surfaces were inspected for egg clusters. Each of the three sets of choices was replicated 35 times.

Larval Feeding Choice Assay. Cotton plants receiving 750 ml water/wk were placed 35 cm apart from a well watered plant. A 10-cm wide, 1.27-cm thick piece of wood, suspended at the height of the upper cotton leaves, was placed between the plants, and a 14-cm diameter Petri dish was placed on the wood. One upper leaf of each plant, still attached to the plant, was positioned in the Petri dish (slots were cut at opposite sides of the dish to accommodate the leaf petioles). Because the leaves overlapped to some extent, the overlap was randomized in terms of which leaf was on top. One third instar beet armyworm was placed 2 cm from the edge of either leaf. A ventilated Petri dish lid was taped to the dish so that the larva was unable to escape. The position of the larva (on the stressed or nonstressed leaves, or not on any of the leaves) was recorded at 4 hr after release into the Petri dish ($N = 30$). This assay was repeated using leaves from plants receiving 1500 ml water/wk. In a no-choice assay, a single leaf attached to a whole plant was inserted into a slotted Petri dish and a third instar beet armyworm was released into each dish. After 24 hr, the amount of leaf area consumed by the larva was estimated by overlaying a transparent 0.5×0.5 mm grid onto the leaves ($N = 20$). This was repeated using well watered and 1500 ml/wk cotton plants.

Larval Development Assay. Wax paper was used as a substrate for female beet armyworms to deposit eggs. The wax paper was cut with a razor blade so that there were 25–30 eggs on 3×3 cm pieces of wax paper. Thirty pots of cotton plants were grown on the four irrigation regimes. One square of wax paper with beet armyworm eggs was stapled to one upper cotton leaf on a cotton plant in each of the 30 pots. When the eggs hatched, the larvae were allowed to feed on the cotton plants until pupation. Surviving third instars were weighed and their lengths measured. Pupae were dug from the potting soil, weighed, and their lengths measured.

Statistical Analyses. Treatment effects were evaluated using one-way ANOVA for plant phenology, no-choice larval feeding and development assays, and biochemical measurements, and the means were separated using Tukey's HSD. The oviposition and larval preference choice assays were analyzed with Yates's corrected χ^2 test, but the numbers of eggs per cluster were analyzed using the two sample t test (Analytical Software, 1998).

RESULTS

Plant Phenology Measurements. Stem diameters, numbers of stem nodes, numbers of leaves, and heights of well watered cotton plants were ≥ 1.2 ($F = 24.67$; $df = 3, 96$; $P < 0.001$), ≥ 1.1 ($F = 7.60$; $df = 3, 96$; $P = 0.001$), ≥ 1.3

TABLE 1. MEAN (±SE) COTTON PLANT STEM DIAMETERS, NUMBERS OF STEM NODES AND LEAVES, HEIGHTS, GRAMS LEAF WATER/GRAMS LEAF DRY WEIGHT, AND LEAF WATER POTENTIALS OF COTTON PLANTS GROWN IN CONTINUOUSLY WELL WATERED (4000 ML/WK) POTTING SOIL, AND 1500, 1000, AND 750 ML WATER/WK

Treatment	Stem (diameter (cm))[a]	No. of stem nodes[b]	No. of leaves[b]	Plant (height (cm))[b]	Leaf water content (g)[c]	Water potential (barr)[d]
Well watered	$6.4 \pm 0.1_a$	$7.6 \pm 0.1_a$	$18.0 \pm 0.7_a$	$45.7 \pm 0.6_a$	2.4 ± 0.1	$10.1 \pm 0.1_d$
1500 ml/wk	$5.2 \pm 0.1_b$	$6.9 \pm 0.1_b$	$14.1 \pm 0.6_b$	$37.9 \pm 0.7_b$	2.5 ± 0.1	$11.8 \pm 0.2_c$
1000 ml/wk	$5.2 \pm 0.2_b$	$7.1 \pm 0.2_b$	$14.4 \pm 0.7_b$	$34.7 \pm 1.1_c$	2.6 ± 0.2	$29.4 \pm 0.5_b$
750 ml/wk	$4.9 \pm 0.1_b$	$6.5 \pm 0.2_c$	$11.1 \pm 0.5_c$	$32.6 \pm 0.8_c$	2.9 ± 0.2	$31.3 \pm 0.4_a$

Note: Means followed by different subscripts in the same column are significantly different ($P \leq 0.05$), one-way ANOVA, Tukey's HSD. Cotton plants were at the 7–10 mm square diameter square stage when the plants were 8-wk old.

[a] Stem measurements were taken 6 cm from the soil level, $N = 25$.

[b] $N = 25$.

[c] Grams water/g dry biomass. Leaves were taken from the top four fully expanded leaves, $N = 30$.

[d] Leaves taken from the top four fully expanded leaves, $N = 20$.

($F = 19.11$; $df = 3, 96$; $P < 0.001$), and ≥1.2 ($F = 50.07$; $df = 3, 96$; $P < 0.001$) times greater, respectively, than plants grown in any of the three water-deficit regimes (Table 1). The water content (g leaf water/g dry leaf weight) in each treatment was not statistically different ($F = 2.46$; $df = 3, 116$; $P = 0.066$) among the treatments, but the ratios increased progressively with greater water-deficit stress (Table 1). Leaf water potentials in the four treatments were different from one another ($F = 1025.74$; $df = 3, 76$; $P < 0.001$) (Table 1). The 750 ml water/wk leaf water potential was 1.1, 2.7, and 3.1 times higher than leaf water potentials in the 1000, 1500 ml water/wk and the well watered regimes, respectively.

Plant Physiochemical Measurements. Of the 17 detectable FAAs, 12 were found in cotton leaf extract: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, phenylalanine, proline, serine, threonine, and valine (Table 2). Of the 10 amino acids essential for insect growth and development (Vanderzant, 1958), 6 were detected in the leaf extracts: arginine, histidine, isoleucine, phenylalanine, threonine, and valine.

The well watered cotton plants had lower levels of foliar free alanine (≥32%; $F = 16.01$, $df = 3, 56$; $P < 0.001$), arginine (≥63%, $F = 6.27$, $P = 0.001$), glycine (≥51%, $F = 4.18$, $P = 0.01$), histidine (≥69%, $F = 17.01$, $P < 0.001$), isoleucine (≥38%, $F = 27.09$, $P < 0.001$), phenylalanine (≥35%, $F = 8.09$, $P = 0.001$), proline (≥91%, $F = 15.45$, $P < 0.001$), serine (≥39%, $F = 13.06$, $P < 0.001$), and valine (≥53%, $F = 18.26$, $P < 0.001$) than cotton plants grown in any of the three water-deficit regimes. In the cases of free glycine, isoleucine, and serine, accumulations detected in the 1500 ml water/wk cotton leaves were

TABLE 2. MEAN FREE AMINO ACID[a] (FAA) CONTENTS (±SE) OF UPPER COTTON LEAVES FROM PLANTS GROWN IN WELL WATERED (4000 ML WATER/WK) POTTING SOIL, AND 1500, 1000, AND 750 ML WATER/WK

FAA	Concentration (pmoles/μl extract)			
	Well watered	1500 ml/wk	1000 ml/wk	750 ml/wk
Alanine	$864.6 \pm 65.6_{b}$	$1262.9 \pm 50.9_{a}$	$1320.4 \pm 64.1_{a}$	$1456.5 \pm 71.8_{a}$
Arginine	$898.9 \pm 87.0_{b}$	$2649.1 \pm 510.3_{a}$	$2379.5 \pm 267.4_{a}$	$3229.9 \pm 400.2_{a}$
Aspartic acid	1879.7 ± 193.9	1551.2 ± 120.6	1803.1 ± 152.1	1810.7 ± 99.6
Glutamic acid	1807.1 ± 159.1	2044.3 ± 95.4	1695.6 ± 131.5	2058.9 ± 112.5
Glycine	$50.3 \pm 5.7_{b}$	$103.1 \pm 21.7_{a}$	$50.1 \pm 5.4_{b}$	$77.0 \pm 9.0_{a,b}$
Histidine	$35.6 \pm 13.2_{b}$	$128.2 \pm 35.9_{b}$	$115.0 \pm 31.9_{b}$	$335.9 \pm 37.2_{a}$
Isoleucine	$687.3 \pm 34.1_{c}$	$1805.9 \pm 109.0_{a}$	$939.5 \pm 100.2_{b,c}$	$1114.5 \pm 104.1_{b}$
Phenylalanine	$146.7 \pm 25.4_{b}$	$226.3 \pm 37.1_{b}$	$237.2 \pm 43.6_{b}$	$407.3 \pm 44.9_{a}$
Proline	$20.4 \pm 2.1_{d}$	$233.2 \pm 54.4_{c}$	$437.6 \pm 122.6_{b}$	$1181.0 \pm 219.1_{a}$
Serine	$400.8 \pm 33.3_{b}$	$660.6 \pm 33.3_{a}$	$366.0 \pm 43.6_{b}$	$442.0 \pm 35.6_{b}$
Threonine	449.6 ± 45.2	579.8 ± 32.5	520.4 ± 66.0	598.9 ± 26.9
Valine	$115.8 \pm 7.7_{c}$	$247.1 \pm 46.3_{b}$	$252.5 \pm 33.9_{b}$	$458.8 \pm 40.1_{a}$
Total free essential AAs	$2362.4 \pm 133.1_{c}$	$5636.4 \pm 576.8_{a,b}$	$4432.1 \pm 537.4_{b}$	$6145.3 \pm 485.6_{a}$
Total FAAs	$7152.2 \pm 538.0_{c}$	$12{,}204.2 \pm 1107.5_{a,b}$	$10{,}104.9 \pm 649.5_{b,c}$	$13{,}171.4 \pm 769.7_{a}$

Note: Means followed by different subscripts in the same row are significantly different ($P \leq 0.01$), $N = 15$, one-way ANOVA, Tukey's HSD.

[a] Free cysteine/cystine, leucine, lysine, methionine, and tyrosine were detectable but not present in the cotton extracts analyzed.

greatest. Accumulations of alanine, histidine, phenylalanine, proline, and valine were greatest in the 750 ml/wk leaves.

Total free essential amino acids in the control and 1500, 1000, and 750 ml water/wk regimes comprised 33, 46, 44, and 47% of the total FAA accumulations, respectively. Total free essential amino acids and total FAAs were least abundant in the well watered regime and most abundant in the 750 ml water/wk regime.

Significantly less soluble protein ($F = 10.18$; $df = 2, 29$; $P = 0.001$), peroxidase activity ($F = 20.38$; $df = 2, 29$; $P < 0.001$), and total available carbohydrates ($F = 32.32$; $df = 2, 29$; $P < 0.001$) were found in the control leaves than in the 1500 and 750 ml water/wk irrigation regimes (Table 3).

Oviposition Preference. Beet armyworm eggs on the 1500, 1000, and 750 ml water/wk cotton plants were 3.3, 4.6, and 2.3 times, respectively, more abundant ($P \leq 0.05$) than on the well watered cotton plants (Table 4). Also, egg clusters on the cotton plants in the three water-deficit regimes were 2.5, 5.8, and 1.6 times, respectively, more numerous ($P \leq 0.05$) than on the well watered cotton plants, but treatment effects on the numbers of eggs per cluster were not detected (Table 4). Differences in the numbers of egg clusters found on the cage walls and plant pots were also not detected.

TABLE 3. SOLUBLE PROTEIN, PEROXIDASE ACTIVITY, AND CARBOHYDRATE CONTENTS OF COTTON LEAVES GROWN IN WELL WATERED (4000 ML WATER/WK) SOIL, AND LEAVES OF PLANTS GROWN IN TWO DIFFERENT WATER DEFICIT REGIMES

Irrigation treatment	Soluble protein (mg/g)[a]	Peroxidase (mg/g)[a]	Soluble carbohydrates (mg/g)[b]
Well watered	$6.0 \pm 1.1_{b}$	$3.0 \pm 0.3_{b}$	$7.17 \pm 0.29_{b}$
1500 ml/wk	$13.1 \pm 0.6_{a}$	$10.3 \pm 0.7_{a}$	$16.11 \pm 0.76_{a}$
750 ml/wk	$11.7 \pm 1.7_{a}$	$10.6 \pm 1.4_{a}$	$18.02 \pm 1.57_{a}$

Note: Means followed by different subscripts in the same column are significantly different ($P \leq 0.05$), $N = 10$, one-way ANOVA, Tukey's HSD.
[a] mg/g fresh weight.
[b] mg/g dry weight.

Larval Feeding Preference. At 4 hr after third instars were introduced into the Petri dishes, 57 and 50% ($P > 0.05$) of the larvae were found on the 1500 and 750 ml water/wk cotton leaves, respectively, as compared to the corresponding well watered controls. After 24 hr in the no-choice feeding assay, each larva had

TABLE 4. MEAN NUMBERS (±SE) OF EGGS, CLUSTERS, AND EGGS/CLUSTER OVIPOSITED BY BEET ARMYWORMS ON COTTON PLANTS GROWN IN CONTINUOUSLY WELL WATERED (4000 ML WATER/WK) POTTING SOIL, AND 1500, 1000, AND 750 ML WATER/WK

Choice	Eggs[a]	Clusters[a]	Eggs/cluster[b]
Well watered	41.3 ± 10.4	0.8 ± 0.2	59.0 ± 5.9
1500 ml/wk	137.2 ± 16.6	2.0 ± 0.3	77.7 ± 7.9
χ^2	4057.8	65.16	
t			1.89
P	0.001	<0.001	0.064
Well watered	37.5 ± 9.9	0.5 ± 0.1	66.9 ± 4.2
1000 ml/wk	170.9 ± 23.6	2.9 ± 0.4	75.1 ± 9.8
χ^2	3189.0	49.51	
t			0.77
P	<0.001	0.049	0.445
Well watered	52.5 ± 11.3	1.0 ± 0.2	58.8 ± 4.1
750 ml/wk	122.2 ± 25.0	1.6 ± 0.3	95.1 ± 20.9
χ^2	4741.8	64.37	
t			1.71
P	<0.001	0.013	0.094

[a] $N = 35$, Yates corrected χ^2 test.
[b] $N = 25$, two-sample t test.

TABLE 5. THIRD INSTAR AND PUPAL BEET ARMYWORM MEAN WEIGHTS AND LENGTHS (±SE) AFTER BEING RAISED FROM EGGS ON COTTON GROWN ON CONTINUOUSLY WELL WATERED (4000 ML WATER/WK) POTTING SOIL, AND 1500, 1000, AND 750 ML WATER/WK

Treatment	Larval weight (g)	Larval length (mm)	Pupal weight (g)	Pupal length (mm)
Well watered	$0.02 \pm 0.0005_a$	$10.7 \pm 0.1_a$	$0.11 \pm 0.004_a$	11.6 ± 0.3
1500 ml/wk	$0.01 \pm 0.0003_b$	$8.5 \pm 0.4_b$	$0.08 \pm 0.003_b$	11.0 ± 0.2
1000 ml/wk	$0.009 \pm 0.0002_c$	$6.6 \pm 0.2_c$	$0.07 \pm 0.003_{b,c}$	11.4 ± 0.2
750 ml/wk	$0.008 \pm 0.0003_c$	$5.6 \pm 0.1_d$	$0.06 \pm 0.003_c$	10.8 ± 0.3

Note: Means followed by different subscripts in the same column are significantly different ($P \leq 0.05$); means not followed by different letters are not significantly different, larvae $N = 26$, pupae $N = 25$, one-way ANOVA, Tukey's HSD.

consumed 2.01 ± 0.22, 2.88 ± 0.21, and 1.27 ± 0.16 mm^2 of leaf area from the 1500 and 750 ml water/wk leaves and the well watered leaves, respectively ($F = 16.21$; $df = 2, 59$; $P < 0.001$), and each mean was significantly different than were the other two ($P \leq 0.05$).

Larval Development. Third instar beet armyworms raised on the well watered cotton plants were 2.0, 2.2, and 2.6 times ($F = 251.92$; $df = 3, 100$; $P < 0.001$) heavier than third instars raised on the 1500, 1000, and 750 ml water/wk cotton plants, respectively, and the larvae were 1.3, 1.6, and 1.9 times ($F = 101.62$; $df = 3, 100$; $P < 0.001$) longer, respectively (Table 5). Pupae in the well watered regimes were 1.4, 1.6, and 1.8 times ($F = 27.46$; $df = 3, 96$; $P < 0.001$) heavier than the pupae in the 1500, 1000, and 750 ml water/wk regimes, respectively, but significant differences between pupal lengths were not detected (Table 5). Larval mortalities, mostly resulting from cannibalism, occurred at rates that were not significantly different between treatments.

DISCUSSION

Leaf water potential increased with greater drought stress, and this was reflected by the greater accumulations of free proline, which is an indicator of drought stress in cotton (Showler, 2002). Drought stress resulted in physiological as well as biochemical changes that might have influenced the behavior and development of different life stages of the beet armyworm. Although moderate water-deficit stress in cotton does not reduce photosynthetic activity (Genty et al., 1987), drought stressed cotton plants have fewer main stem nodes, and lower CO_2 uptake (Bradford and Hsiao, 1982), as well as reduced transpiration (Showler, 2002), aboveground dry biomass, leaf area, leaf biomass, and lint yield (Earl, 2000). Also, FAAs

accumulate in stressed cotton leaves, especially free proline (Showler, 2002), which indicates water-deficit stress in many plant species and could act as an osmoregulator (Labanauskas et al., 1981; Golan-Goldhirsch et al., 1989; Ranieri et al., 1989; Bussis and Heineke, 1998). Gadallah (1995) demonstrated that proline sprayed on cotton plants counteracted the effects of water-deficit stress, and significantly increased chlorophyll content and stability to heat, and leaf relative water and dry matter contents. Heat shock pretreatment of cotton elevated levels of free proline and several other FAAs, and improved drought tolerance (Kuznetsov et al., 1999). Leaf water contents relative to dry biomass were greater in plants subjected to drought stress regimes. This result, in combination with the increased protein and FAA contents and reduction in plant growth, suggests lower rates of protein synthesis and assimilation into structural matter, possibly accompanied by synthesis of stress-induced proteins (White, 1984; Dubey, 1999), although some studies have reported decreases in protein in drought stressed plants (Ramanjulu et al., 1999; Garg et al., 2001). Declines in protein content may be features of drought stress in plants in which drought reduces photosynthesis.

The greater peroxidase content in the cotton plants grown in the drought regimes suggests general oxidative stress (Becana et al., 1998). Peroxidases reduce hydrogen peroxide concentrations and promote oxidation of proteins and other molecules. This activity may improve plant tolerance to drought stress by removal of active oxygen species that accumulate under stress (Knight and Knight, 2001; van Breusegam et al., 2001). In tomatoes, *Lycopersicon esculentum* Mill., and barley, *Hordeum vulgare* L., peroxidase activity does not increase under drought stress (English-Loeb et al., 1997; Acar et al., 2001), while both soluble proteins and peroxidase activity increase in oats, *Avena sativa* L. (Chandra et al., 1998), suggesting different potential tolerance mechanisms in different plants. The observed drought-induced increases in soluble carbohydrates, in contrast, are a reliable marker of stress in many plants, including lupin, *Lupinus albus* L. (Pinheiro et al., 2001), moth bean, *Vigna acontifolia* (Jacq.) Marechal (Garg et al., 2001), soybean, *Glycine max* (L.) Merr. (Streeter et al., 2001), and tobacco, *Nicotiana tabacum* L. (Schellenbaum et al., 1999). Increases may be a consequence of reduced growth if photosynthesis is not affected by water-deficit stress (Mattson and Haack, 1991) or catabolism of polysaccharides into monomers that may serve as osmoprotectants (Bray et al., 2000).

The earlier reports of greater numbers of beet armyworm eggs on drought stressed cotton than on nonstressed plants (Ruberson et al., 1994; Ruberson, 1996) are supported by the findings of this study. Infestations of some herbivorous species of pest arthropods are associated with crop plants growing under some degree of drought stress (White, 1984). Foliar FAAs, especially those that are essential for insect growth and development are readily available for utilization by phytophagous insects, being unbound to more complex molecules such as proteins and presumably saving energy costs associated with proteolysis (Helms et al., 1971; Brodbeck

and Strong, 1987). Responses of proboscis sensilla to amino acids and sugar were correlated to feeding behavior in three noctuid species (Blaney and Simmonds, 1988). Assuming that adult female beet armyworms can similarly detect amino acids and sugars, and that such detection has a role in determination of host plants for oviposition, greater levels of essential unbound amino acids and available carbohydrates help to explain the beet armyworm's oviposition preference for drought stressed cotton. In the absence of stress, beet armyworm moths preferred pigweed, *Amaranthus hybridus* L., which has greater quantities and diversity of FAAs than cotton (Showler, 2001). Severe drought stress can reduce the nutritional quality of the host plant (Rhoades, 1983), and this could influence oviposition by lepidopterans. Reports indicate that improved host plant quality and altered crop environment, as when irrigation is used during conditions of drought, may influence oviposition behavior by some insects (Wolfson, 1982; Myers, 1985). Unlike the adults, the larvae in our study showed no feeding preference for the stressed over the nonstressed leaves, suggesting that beet armyworm larvae do not necessarily move from plant to plant in response to differences in nutritional quality (Showler, 2001).

Infestations of some herbivorous species of pest arthropods are associated with crop plants growing under drought stress (White, 1984). Foliar FAAs, especially those that are essential for insect growth and development, are more readily available for utilization by phytophagous insects in drought stressed plants. FAAs not bound to complex molecules such as proteins, presumably save energy costs associated with proteolysis (Helms et al., 1971; Brodbeck and Strong, 1987). Increases in total soluble protein and total carbohydrates may also be beneficial for insect nutrition (Mattson and Haack, 1991), but these effects may be counteracted by increased oxidative enzyme activity in plants, of which peroxidase activity is a good indicator. Oxidation leads to the formation of complexes of proteins and other nutrients and with other metabolites, such as phenolics and tough lignified tissues, potentially making digestion more difficult (Bi et al., 1997). Overexpression of transgenic peroxidases in sweetgum, *Liquidambar styraciflua* L., increases resistance to gypsy moth, *Porthetria dispar* (L.) (Dowd et al., 1998). However, direct associations between enhanced peroxidase activity and decreased insect feeding and growth have not been found in some plant–insect systems (Stout et al., 1998). The beet armyworm larvae in our study fed more on drought-stressed than on control cotton leaves, but they developed poorly and mortality was greater on the water-deficit stressed cotton plants than on the controls. Beet armyworm larvae showed similar decreases in growth when fed dried, ground material from water-deficit stressed tomato plants incorporated into a nonnutritive diet (English-Loeb et al., 1997). Cecropia moth, *Hyalophora cecropia* L., larvae reared on drought stressed wild cherry, *Prunus serotina* Ehrh., leaves grew more slowly and were less efficient at utilizing plant biomass, energy, and nitrogen than larvae fed on plants fully supplemented with water (Scriber, 1977). Cecropia larvae were more desiccated, and metabolized greater portions of assimilated energy, perhaps to

supplement body water with metabolic water derived from respiration. This finding helps to explain why the increased consumption rate of cecropia larvae and increased leaf area removal by beet armyworms did not increase their growth rates on drought stressed leaves. Fall armyworm, *Spodoptera frugiperda* (J. E. Smith), soybean looper, *Pseudoplusia includens* (Walker), and beet armyworm larval survivorships are higher in irrigated than dryland soybean, and development is faster (Huffman and Mueller, 1983).

This study demonstrates that when given a choice between drought stressed and nonstressed cotton plants, adult beet armyworms prefer stressed plants for oviposition, but the larvae develop poorly on the stressed plants. In many insects, associations between oviposition choices and larval performance are variable (Bernays and Chapman, 1994). Berdegué et al. (1998) showed that beet armyworm choice of oviposition host is not necessarily governed by the nutritional quality of host plants. The incongruity between some of the beet armyworm's choices of host plants for its progeny can be explained by the absence of evolutionary time to select against detrimental behavior (Thompson, 1988). Both cotton and the beet armyworm were introduced to the United States (Wilson, 1932; Reynolds et al., 1975), and despite cotton's chemical defenses (e.g., gossypol) against herbivorous arthropods (Hedin et al., 1991), the beet armyworm will oviposit on it even in the presence of a nutritionally superior and less chemically toxic host plant, such as pigweed (Ali and Gaylor, 1982; Showler, 2001). Also, field scouting for the presence of beet armyworm eggs alone might not necessarily be a reliable way to predict levels of larval infestation or damage to foliage, especially if scouts focus their searching primarily on drought stressed cotton plants.

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DISTRIBUTION OF NORDITERPENE ALKALOIDS IN TALL LARKSPUR PLANT PARTS THROUGH THE GROWING SEASON

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Abstract—Previous research showed that toxic and total alkaloid pools in tall larkspur (*Delphinium barbeyi*) increased during early growth, then declined precipitously during the late flower and pod stage of growth. The objective of this study was to measure the concentration and pools of toxic and total alkaloids in tall larkspur plant parts, including roots, and to evaluate the changes in these pools over the growing season as an estimate of diterpenoid alkaloid kinetics in tall larkspur. Twenty entire plants were harvested at each phenological stage: beginning of growth in the spring, early flower, early pod, late pod, and senescence. The plants were separated into their respective parts, freeze-dried, extracted, and analyzed for toxic and total alkaloid concentration, and alkaloid pools were calculated. Concentration of toxic and total alkaloids in leaves and stems declined as the plants matured, while concentration in flowers and pods increased ($P < 0.004$). Concentration of alkaloids in the root declined in the early growth, then increased at the end of the season ($P = 0.002$). Alkaloid pools in the root decreased during early growth, with a corresponding increase of pools in foliar parts. In the late flower and pod stage, alkaloid pools in the leaves and stems declined rapidly, while the pool in the crown and roots tended to increase.

Key Words—Tall larkspur, *Delphinium barbeyi*, poisonous plant, diterpenoid alkaloids, methyllycaconitine, alkaloid pools.

INTRODUCTION

Tall larkspur (*Delphinium barbeyi*) is an important poisonous plant on mountain rangelands, causing acute poisoning and rapid death in cattle. There are many diterpenoid alkaloids in larkspur species, but those that contain the *N*-(methylsuccinimido)-anthranilic ester group (MSAL) are most toxic (Manners

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et al., 1995). Concentration of total alkaloids in tall larkspur, as well as the toxic MSAL alkaloids, decline as the plant matures (Williams and Cronin, 1966; Pfister et al., 1994; Ralphs et al., 1997), rendering the plant relatively nontoxic toward the end of the growing season.

Ralphs et al. (2000) proposed the hypothesis that larkspur alkaloids are synthesized in early growth. As the plant continues to grow, the alkaloids are diluted by the increasing biomass, thus accounting for the linear decrease in toxic and total alkaloid concentrations over the growing season. In contrast, alkaloid pools (the total quantity of alkaloids in the stalks) increase in the first 3–4 weeks of growth, level out from weeks 4 to 6 during the flower stage, and then decline precipitously from late flower through the pod stage when the plant has reached its maximum growth. The objective of this study was to measure the concentration and pools of toxic and total alkaloids in larkspur plant parts, including roots, and evaluate the changes in these pools over the growing season as an estimate of diterpenoid alkaloid kinetics in tall larkspur.

METHODS AND MATERIALS

Tall larkspur plants were harvested from the mountains of central Utah 46 km west of Ferron at an elevation of 3150 m. The site was in the subalpine zone and the vegetation consisted of scattered subalpine fir stands interspersed in the tall forb plant community dominated by tall larkspur. Tall larkspur is a large, robust native perennial plant growing up to 1–2 m tall. The plant consists of many stalks (10–150) growing from the root crown each year.

There is a large amount of variation in concentration of toxic and total alkaloids among tall larkspur plants in a population. Manners and Pfister (1996) suggested 20 tall larkspur plants must be sampled to obtain an accuracy of 10% of the mean at 95% probability. The variability in MSAL alkaloids in tall larkspur plants on our site would require 12–34 plants to be sampled to estimate the population at that level of accuracy and precision (Ralphs et al., 2002). Therefore, we selected 20 plants to dig up at each of the following growth stages: new growth, early flower, early pod, late pod, and senescence. The root mass consists of a caudex of intertwining and connecting tap roots that descend about 25 cm, and then give way to fibrous adventitious roots. The soil was shaken off, and the roots were later washed. Stalks were cut from the root crown at the soil level. Flowering racemes (flower and pod) were removed from the entire plant and placed in a plastic bag. The large biomass of the foliage was unwieldy, and therefore it was subsampled. The wet weight of all the stalks on a plant was recorded, and then three representative stalks were selected and the leaves separated from the stems. Both fresh and dry weights of the leaves and stems were measured and then backcalculated to estimate the dry weight of all the stalks from the entire plant.

The parts (root, stem, leaf, flower, pod) were placed in separate plastic bags and frozen.

At the end of the growing season in late September following senescence, 20 larger plants were dug and the roots harvested. The new growth buds that form next years stalks were cut out from the remainder of the root material to determine if the alkaloid concentration was higher in this new meristematic tissue than the surrounding root tissue. The new buds were white, 1–2 cm in length, and there were 10–40 on each plant. Larger plants were selected to increase the amount of bud tissue for analysis.

Parts from each plant were placed in separate plastic bags, frozen in the field, and freeze-dried. The plants were weighed to obtain dry weight, and then ground through a cyclone grinder to pulverize the material. Alkaloids were extracted and concentrations of toxic and total alkaloids were measured by Fourier-transform infrared spectroscopy (FTIR) (Gardner et al., 1997).

The quantity of toxic and total alkaloid pools in each part was calculated by multiplying the alkaloid concentration by the dry weight of that part. Alkaloid pools are used here to express the amount of alkaloids in a plant part, which better describes the dynamics than does alkaloid concentration.

The concentration and pools of MSAL alkaloids were compared in a repeated-measures mixed model analysis of variance, comparing plant parts and dates of collection using the compound structure as a covariate. There was a significant part-by-date interaction; therefore, the model was reduced, and alkaloids in each part were compared over time. Orthogonal contrasts were used to describe the response of each part over time.

RESULTS

Alkaloid Concentration. Toxic MSAL alkaloid concentrations differed among all the larkspur plant parts ($P < 0.001$). MSAL concentration was highest in the early leaves at the beginning of the study and declined linearly over the season ($P < 0.001$) (Figure 1). Concentrations of MSAL alkaloids in stems were similar to the leaves and declined linearly ($P < 0.001$). The floral parts increased slightly at the end of the season ($P = 0.004$). Concentrations of MSAL alkaloids in the roots declined during the flower stage and then increased toward the end of the season in a quadratic manner ($P = 0.002$).

Toxic alkaloid concentration in the new growth root buds at the end of the season was lower than in the remainder of the root tissue (5.7 ± 0.33 vs. 14.9 ± 1.3 mg/g, $P < 0.001$). This was contrary to plant defense theory, which states that new meristematic tissue should be higher in defense compounds (McKey, 1974). However, as these new shoots begin rapid aerial growth in the spring, they have high concentrations of toxic and total alkaloids (Figure 1).

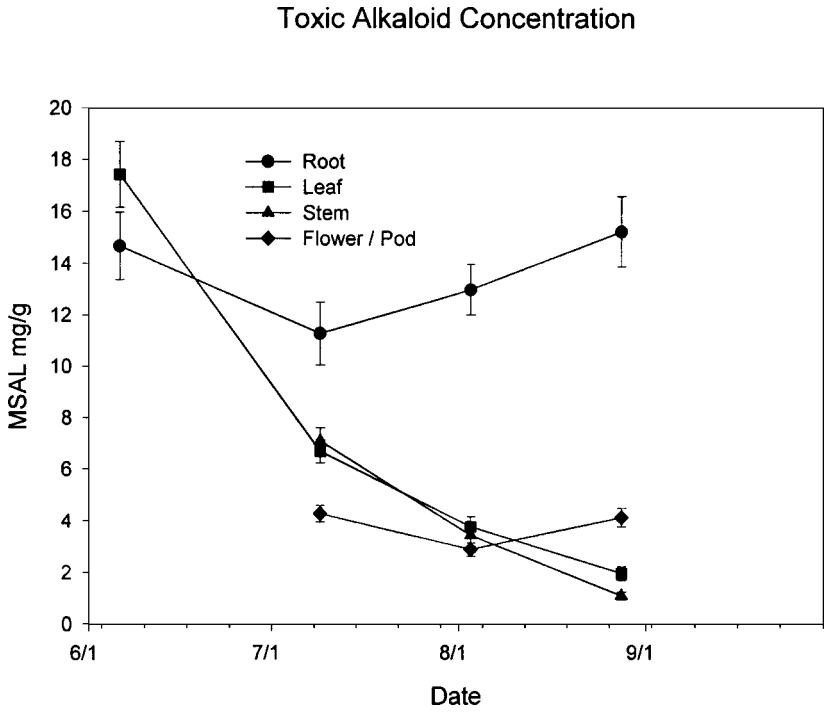

FIG. 1. Toxic alkaloid concentration in larkspur plant parts. Bars are standard error of the mean.

Alkaloid Pools. MSAL alkaloid pools in roots were greater than in the leaf and stem ($P < 0.001$; Figure 2). Although the concentration of toxic alkaloids in the roots differed over the season ($P = 0.002$), the alkaloid pools in roots were not significantly different ($P = 0.37$), due to the large variation in root size (see wide standard error bars of roots in Figure 2). However, the direction of change indicates that alkaloid pools declined in the flower stage, and then increased in the pod stage (Figure 2).

The change in the toxic alkaloid pool in leaves and stems was opposite to that of the roots. There was a significant difference ($P < 0.001$) over the season, and the concave quadratic response ($P < 0.001$) indicates the pool in leaves and stems increased from the vegetative to the flower stage, and then declined during the pod stage (Figure 2). The small pools in the floral parts (due to the relatively small biomass) increased from the flower to the pods ($P = 0.002$).

The quantitative change in MSAL alkaloid pools in larkspur plant parts (Table 1) shows that the relative increase in larkspur leaves and stems ($+273$ mg) is greater than the decline in roots (-172 mg) during the vegetative stage of growth.

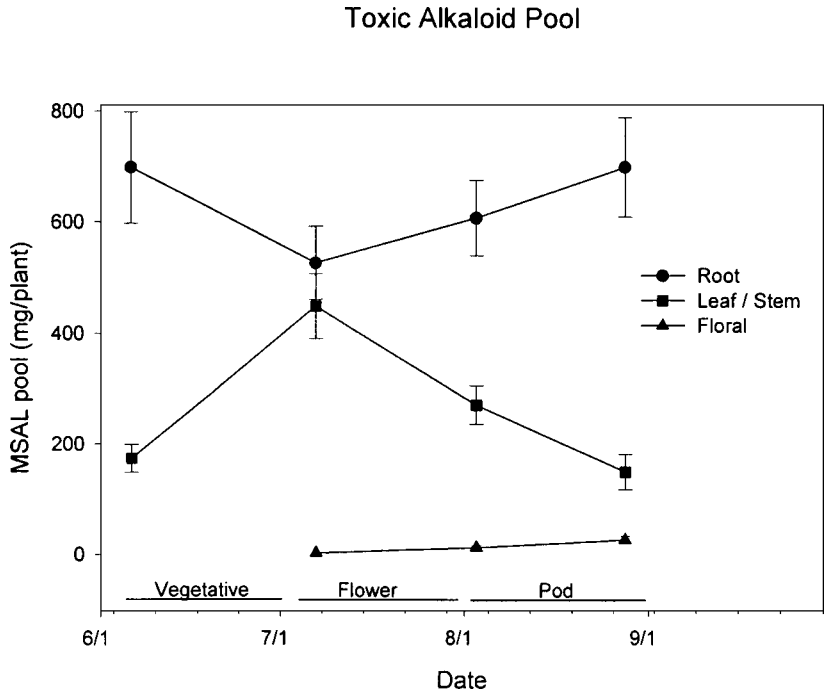

FIG. 2. Toxic alkaloid pools in larkspur plant parts. Bars are standard error of the mean.

The difference in magnitude may be due to continued alkaloid synthesis in roots in early growth (Ralphs et al., 2000). The loss of alkaloids from the leaf/stem (−292 mg) during the flower and pod stages was greater than the gain in the roots (+173 mg) at the end of the season. This suggests there may be some catabolism of the alkaloids. The seed pool is relatively small (26 mg), and would not account for much loss of alkaloid as the seed is shed. It is unlikely that toxic alkaloids are

TABLE 1. CHANGE IN MSAL ALKALOID POOLS IN LARKSPUR PLANT PARTS OVER THE GROWING SEASON

		Root		Leaf/stem		Flower/pod	
Date	Stage	Pool	Change	Pool	Change	Pool	Change
				mg			
6/6	Veg	698	−172	174	+273		
7/9	Flower	526	+81	447	−177	2	+8
8/3	Pod	607	+92	270	−121	12	+14
8/29	Seed ripe	699		149		26	

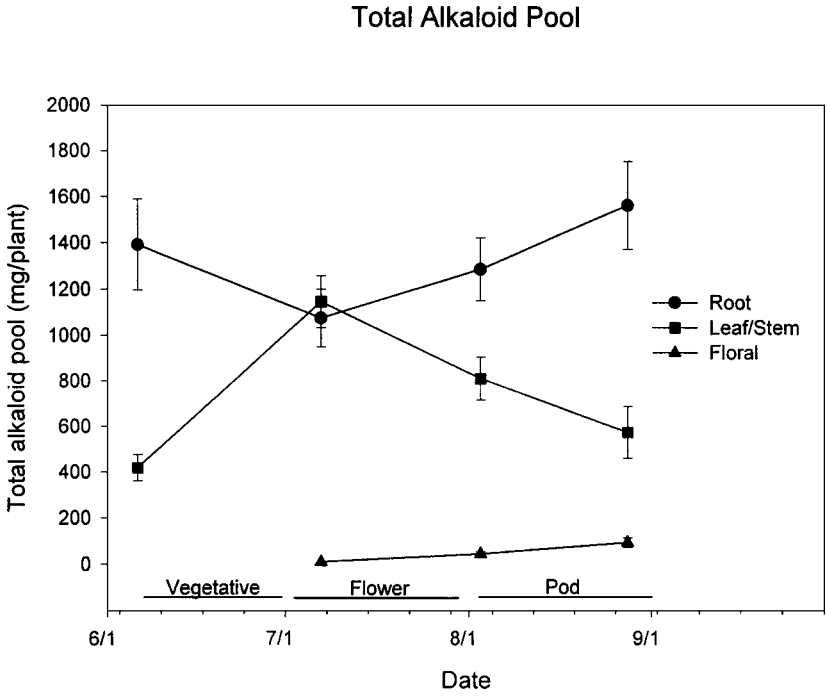

FIG. 3. Total alkaloid pools in larkspur plant parts. Bars are standard error of the mean.

metabolized to other alkaloids since the total alkaloid pools responded in a similar fashion (Figure 3).

DISCUSSION

Alkaloids are classified according to their biosynthetic origin. Diterpenoid alkaloids are characterized by the structural fragment derived from the C_{20} terpenoid. Norditerpenoid alkaloids are partially degraded structures. Biosynthetic pathways for norditerpenoid alkaloids have been postulated; however, experimental evidence is lacking (Joshi and Pelletier, 1999). It is assumed that norditerpenoid larkspur alkaloids are derived from the mevalonate pathway of isoprenoid synthesis (Cordell, 1981). However, another pathway for terpenoid synthesis has recently been proposed, starting from pyruvate and glyceraldehyde-3-phosphate (Lichtenthaler et al., 1997).

The biosynthesis of monoterpenes in peppermint (*Mentha piperita*) has been described by Gershenzon (1994) and presents a model similar to what we have observed in larkspur. Monoterpenes were produced only during the first 2 weeks

of growth when enzymes for synthesis were present in the plant. The amount of monoterpenes remained constant for the remainder of the growth stage, but 50–75% of the monoterpenes were catabolized after the plants began to flower (Croteau, 1988). The principal monoterpene, menthone, was reduced to equal amounts of menthol and neomenthol. Neomenthol was transported via its glycoside to the rhizome where it was sequentially hydrolyzed and oxidized to reform menthone (Croteau et al., 1984).

Hartman et al. (1989) reported that pyrrolizidine alkaloids (PA) in *Senecio vulgaris* were synthesized in roots, and then translocated up to the shoot through the phloem and stored mostly in the inflorescence. The alkaloids were synthesized and translocated as the N-oxide, which is a polar, salt-like compound that is soluble in water and suited for transport. However, almost 70% of the total amount of alkaloid was lost during the period of flowering to senescence, and most of that remaining in the plant was in the seed (Hartman and Zimmer, 1986).

The kinetics of alkaloid pools in tall larkspur in our study is similar to that of monoterpenes in peppermint and PA in Senecio. Benn and May (1964) suggested that biosynthesis of diterpenoid alkaloids occurs in larkspur roots. Strzelecka (1968) also reported that methyllycaconitine (MLA), the major MSAL alkaloid, was synthesized in root cultures of *D. elatum*. Environmental stresses, such as light, temperature, and photosynthesis inhibition, did not affect toxic or total alkaloid pools in tall larkspur (Ralphs et al., 1998). Since these major factors that control plant metabolism did not affect alkaloid pools, we speculate that alkaloids are not synthesized in the leaves. The decline of alkaloid pools in tall larkspur roots and the corresponding increase in the foliar parts suggests the alkaloids are being translocated upward in the early growth.

The decline in tall larkspur alkaloid pools in the above ground parts following flowering is consistent with other species, such as peppermint and *Senecio*. Kreps (1969) reported that concentration of total alkaloids in the root crown of duncecap larkspur increased slightly in late summer when the buds on the crown began to enlarge. Strzelecka (1967) reported that MLA accumulated in roots and rhizomes of *D. elatum* during the flower stage and as new buds form on rhizomes. However, it is not known if the alkaloids are translocated back to the root, or if they are catabolized in the shoots, and synthesis in the root increases at this time. It is peculiar that the concentration of alkaloids in the root buds in our study were substantially lower than in the surrounding root tissue in the fall.

In summary, alkaloid pools in tall larkspur declined in roots during early growth, while pools in foliar parts increased. We speculate that the alkaloids are synthesized in the roots and are translocated upward to the leaves and stems. During the late flower and pod stage, pools in the leaves and stems declined rapidly, while pools in roots increased. However, we do not know if the alkaloids are translocated back to the roots, or if they are catabolized in the shoots and synthesis in roots began anew. A relatively small amount of alkaloid is lost in the seeds.

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CHEMICAL STIMULANTS OF LEAF-TRENCHING BY CABBAGE LOOPERS: NATURAL PRODUCTS, NEUROTRANSMITTERS, INSECTICIDES, AND DRUGS

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Abstract—Larvae of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae), often transect leaves with a narrow trench before eating the distal section. The trench reduces larval exposure to exudates, such as latex, during feeding. Plant species that do not emit exudate, such as *Plantago lanceolata,* are not trenched. However, if exudate is applied to a looper's mouth during feeding on *P. lanceolata*, the larva will often stop and cut a trench. Dissolved chemicals can be similarly applied and tested for effectiveness at triggering trenching. With this assay, I have documented that lactucin from lettuce latex *(Lactuca sativa*), myristicin from parsley oil (*Petroselinum crispum*), and lobeline from cardinal flower (*Lobelia cardinalis*) elicit trenching. These compounds are the first trenching stimulants reported. Several other constituents of lettuce and parsley, including some phenylpropanoids, monoterpenes, and furanocoumarins had little or no activity. Cucurbitacin E glycoside found in cucurbits, another plant family trenched by cabbage loopers, also was inactive. Lactucin, myristicin, and lobeline all affect the nervous system of mammals, with lobeline acting specifically as an antagonist of nicotinic acetylcholine receptors. To determine if cabbage loopers respond selectively to compounds active at acetylcholine synapses, I tested several neurotransmitters, insecticides, and drugs with known neurological activity, many of which triggered trenching. Active compounds included dopamine, serotonin, the insecticide imidacloprid, and various drugs such as ipratropium, apomorphine, buspirone, and metoclopramide. These results document that noxious plant chemicals trigger trenching, that loopers respond to different trenching stimulants in different plants, that diverse neuroactive chemicals elicit the behavior, and that feeding deterrents are not all trenching stimulants. The trenching assay offers a novel approach for identifying defensive plant compounds with potential uses in agriculture or medicine. Cabbage loopers in the lab and field routinely trench and feed on plants in the Asteraceae and

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Apiaceae. However, first and third instar larvae enclosed on *Lobelia cardinalis* (Campanulaceae) failed to develop, even though the third instar larvae attempted to trench. Trenching ability does not guarantee effective feeding on plants with canal-borne exudates. Cabbage loopers must not only recognize and respond to trenching stimulants, they must also tolerate exudates during the trenching procedure to disable canalicular defenses.

Key Words—*Trichoplusia ni*, lactucin, myristicin, lobeline, *Lactuca sativa*, *Petroselinum crispum*, *Lobelia cardinalis*, plant–insect interactions, plant defense, laticifer, insect behavior.

INTRODUCTION

The cabbage looper, *Trichoplusia ni* Hübner (Noctuidae), has a broad host range that includes over 20 families of plants (Eichlin and Cunningham, 1978; Sutherland and Greene, 1984). Its catholic diet includes numerous crop plants, particularly crucifers and lettuce, on which it is an important pest (Flint, 1987). Loopers are able to feed on such diverse hosts, in part, because of their ability to disable defensive plant canals by cutting a trench. A larva nibbles back and forth across a leaf, thus creating a continuous line of bites that isolates a portion of the leaf on which it feeds. Cabbage loopers are known to cut trenches in composites with latex canals such as lettuce (Asteraceae: Lactuceae), umbellifers with oil ducts such as parsley (Apiaceae), and cucurbits such as cucumber (Cucurbitaceae), which exude drops of sticky phloem sap from damaged tissues (Dussourd and Denno, 1994). Cabbage loopers placed on cardinal flower, *Lobelia cardinalis* (Campanulaceae), also cut trenches in this latex-bearing plant (Figure 1). Trenches sever secretory canals and, thus, reduce the amount of exudate emitted distal to the trench at the looper's feeding site (Dussourd, 1999). Diverse plant species that lack exudates are not trenched (Dussourd and Denno, 1994).

The goal of this study was to identify cues that cabbage loopers use to determine which plants to trench. Lettuce (*Lactuca sativa* L.), parsley (*Petroselinum crispum*), cucumber (*Cucumis sativus* L.), and cardinal flower (*Lobelia cardinalis* L.) are classified in three different orders (Angiosperm Phylogeny Group, 1998). They share the presence of canals that emit exudate, but the canals (laticifers, oil ducts, and phloem) differ greatly in anatomy and chemical composition (Fahn, 1979; Metcalfe and Chalk, 1983; Dussourd and Denno, 1991). *Lactuca* and *Lobelia* latex and *Cucumis* sap all become sticky upon exposure to air. Their adhesiveness should be easily detected by feeding caterpillars; Lepidoptera larvae have both mechanoreceptors and chemoreceptors on their maxillae and other mouthparts (Grimes and Neunzig, 1986; Chapman, 1995). However, cabbage loopers exposed to cucumber sap prevented from gelling still trenched, suggesting that another factor triggers the behavior (Dussourd, 1997).

In this paper, I describe a test of the alternative hypothesis that noxious chemicals in exudates elicit trenching. My approach was to place drops of dissolved

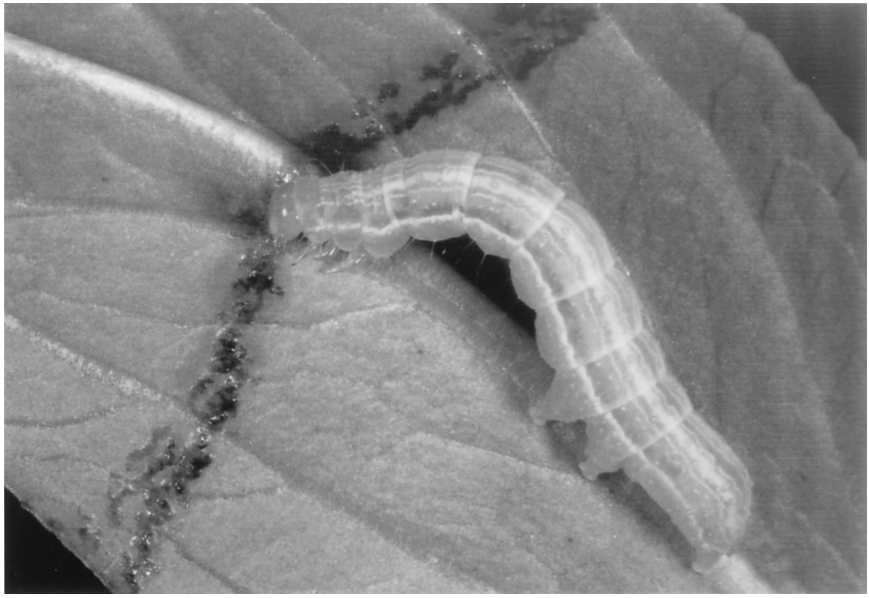

FIG. 1. Final instar cabbage looper cutting a trench across a leaf of *Lobelia cardinalis* before feeding on the distal tip to the left.

chemicals on the mouthparts of loopers feeding on *Plantago lanceolata* L. (Plantaginaceae), a plant that normally is not trenched. Loopers that receive drops of lettuce latex (*Lactuca serriola*) or cucumber sap often stop feeding and cut a trench, responding as though the exudate is emitted by the plantain leaf (Dussourd, 1997). I tested if single chemicals similarly trigger trenching, and if different plants have the same or different trenching stimulants. Three natural products that elicit trenching were identified; all three have been reported to affect the nervous system of mammals. One of the compounds, lobeline, acts specifically as an antagonist at nicotinic acetylcholine receptors (Dwoskin and Crooks, 2001, 2002). Another of the trenching stimulants, myristicin, is thought to have anticholinergic activity (Lavy, 1987; Pytte and Rygnestad, 1998). To determine if loopers respond selectively to compounds that affect acetylcholine synapses, I assayed a series of neurotransmitters, insecticides, and drugs. These trials test if cabbage loopers respond only to a few specific trenching stimulants from plant exudates, more generally to various compounds acting on acetylcholine receptors, or if diverse neuroactive chemicals elicit trenching. Cabbage loopers routinely feed on plants in the Asteraceae and Apiaceae (Dussourd and Denno, 1994, and references therein), but members of the Campanulaceae, such as *Lobelia cardinalis,* have not been previously reported as host plants (Sutherland and Greene, 1984). The final objective was to determine how frequently cabbage loopers trench *L. cardinalis* and to test if the larvae can develop on this species.

METHODS AND MATERIALS

Trenching Assay. Cabbage loopers from a laboratory culture were reared to the final instar on potted plantain, *Plantago lanceolata*. Plantain does not emit exudate and is not trenched. Thus, the loopers used in this assay had no prior experience with exudates or trenching. Early final-instars were deprived of food for 1–1.5 hr at 24–27°C, then were allowed to walk onto the underside of an excised plantain leaf held in a water pic to maintain turgidity. For consistency between trials, only flat mature leaves with a maximum width of ∼1.2 to 2.2 cm were used. When a larva initiated feeding, a drop of solution was carefully applied directly to the side of its mouth with a 5-μl Wiretrol micropipette. Each larva received a total of 1.5-μl solution dispensed in a sequence of four to six drops, with the exception that larvae tested with 60% acetone solutions typically received only three drops because of rapid evaporation of solvent. Multiple droplets were applied to simulate the natural outflow of exudate that occurs when larvae sever canals while attempting to feed on plants with exudates. With innocuous solutions, such as water controls, larvae consumed the drops while continuing to feed without showing any signs of disturbance. Drops were sucked into the mouth and swallowed during normal feeding. With deterrent solutions, larvae usually stopped feeding immediately, wiped their mouth vigorously on the leaf, opened and closed their mandibles repeatedly, and then moved to a new feeding site. Each additional drop of solution was applied only after the larva recommenced feeding. I recorded larval response to each droplet of solution and whether larvae interrupted feeding to cut a trench. For this study, a trench was defined as a continuous line of bites extending at least 5 mm partially or completely across the leaf. For consistency between trials, all assays were performed by the author. Each caterpillar was tested only once.

Whenever possible, chemicals were tested in water. Compounds with inadequate solubility were dissolved in aqueous solutions of either 10% Tween 80 (a surfactant) or the minimal concentration of acetone required (10–60%). Both Tween 80 and acetone at these high concentrations deterred feeding by cabbage loopers and occasionally triggered trenching. The solutions were each tested with 10 naive larvae, except for the two pesticides methomyl and methamidophos, which were extremely toxic to the cabbage loopers and were tested with only five larvae each. Chemicals were tested at a standard dose of 50 nmol in 1.5 μl (=33.3 mM). The only exceptions were lactucin and bergapten, which had poor solubility in water and required unacceptably high concentrations of solvent to dissolve at 33.3 mM. Instead, both were tested at 10 nmol in 1.5 μl (=6.7 mM).

Bioassay of Natural Products, Neurotransmitters, Insecticides, and Drugs. Natural products tested with the trenching assay included the sesquiterpene lactone, lactucin, found in the latex of wild and cultivated lettuce (Tamaki et al., 1995; Sessa et al., 2000). Lactucin was isolated from the latex of *Lactuca virosa* L. (generously

supplied by M. H. Bennett, University of London) and tested at both 5 and 10 nmol per 1.5 μl to match concentrations reported in the latex of cultivated lettuce (Sessa et al., 2000). I also tested two phenylpropanoids, caffeic acid and chlorogenic acid, both of which reportedly occur in lettuce latex (Tibbitts and Read, 1976) and have a widespread distribution in plants (Harborne and Baxter, 1993).

Parsley, *Petroselinum crispum* (Mill.) Mansf., produces a complex mixture of phenylpropanoids, coumarins, and terpenoids (MacLeod et al., 1985; Knogge et al., 1987; Porter, 1989). These compounds occur at least partly within the oil ducts (Hegnauer, 1971; Stahl-Biskup and Wichtmann, 1991; Wu and Hahlbrock, 1992; Reinold and Hahlbrock, 1997). I tested a representative series that included two phenylpropanoids, myristicin and parsley apiole; three linear furanocoumarins, xanthotoxin, imperatorin, and bergapten; and two monoterpenes, α-pinene and γ-terpinene.

Lobelia cardinalis L. and the medicinal plant *L. inflata* L. (Indian tobacco) both contain the piperidine alkaloid, lobeline (Krochmal et al., 1972). These plants were used by Native Americans and settlers in the United States for diverse medicinal purposes, including as an emetic and respiratory stimulant; lobeline is believed to be the principal active compound (Stephens, 1980; Fodor and Colasanti, 1985; Kindscher, 1992). I tested if lobeline deters feeding by cabbage loopers and if it triggers trenching.

Cucurbits are well-known for the presence of bitter triterpenes named cucurbitacins. The compounds occur throughout the plant including the leaves (Rehm et al., 1957; Guha and Sen, 1975), but are apparently absent from sap exudate (Tallamy, unpublished data, cited in McCloud et al., 1995). Early studies with squash beetles suggested that trenching functions to prevent cucurbits from increasing cucurbitacin levels in response to damage (Carroll and Hoffman, 1980; Tallamy, 1985). Subsequent work revealed that cucurbitacins actually stimulate feeding by squash beetles and that trenching serves instead to reduce beetle exposure to sap exudate (McCloud et al., 1995). I used the cabbage looper bioassay to test if cucurbitacin E glycoside triggers trenching. The cucurbitacin sample was kindly provided by D. W. Tallamy, University of Delaware. The aglycone or glycoside of cucurbitacin E occurs in diverse cucurbits including watermelon, *Citrullus vulgaris* (Rehm et al., 1957; Guha and Sen, 1975), a plant that cabbage loopers trench (Dussourd, unpublished observation).

Each natural product was tested independently in a randomized series with a solvent control (except for chlorogenic acid and γ-terpinene, which were tested alone). Fisher exact tests were used to compare the number of larvae cutting trenches in response to the test chemical and control.

The survey of natural products described earlier identified three compounds with significant trenching activity. To verify activity, each of the compounds was retested with a solvent control using a new cohort of cabbage loopers. Myristicin and lobeline were each tested with 20 larvae, whereas lactucin was tested with 10.

To determine if trenching is triggered specifically by compounds acting on acetylcholine receptors, I used the cabbage looper assay to test the activity of acetylcholine and several drugs that affect acetylcholine synapses. These compounds comprised an agonist for nicotinic cholinergic receptors, (−)-nicotine; an agonist for muscarinic cholinergic receptors, (+)-muscarine chloride; three antagonists for nicotinic cholinergic receptors, decamethonium bromide, succinylcholine chloride, and D-tubocurarine chloride; an antagonist for muscarinic cholinergic receptors, ipratropium bromide; and two anticholinesterases, pyridostigmine bromide and edrophonium chloride. The compounds were tested in random order together with an aqueous control.

Insecticides acting on acetylcholine synapses were also tested. They included the chloronicotinyl compound, imidacloprid, and the nereistoxin analog, cartap. Imidacloprid is currently the largest-selling insecticide worldwide (Nauen et al., 2001); it acts as an agonist of nicotinic acetylcholine receptors (Matsuda et al., 2001) and is commonly used to control sucking insects on lettuce and other crops attacked by cabbage loopers (Palumbo et al., 1997; Thomson, 1998). Cartap also targets the nicotinic acetylcholine receptor, but at a different site (Casida and Quistad, 1998). I also tested the carbamate insecticide, methomyl, and the organophosphorous insecticide, methamidophos. Both compounds are used to control cabbage looper populations on lettuce and other vegetable crops (Anonymous, 1989; Thomson, 1998). Carbamate and organophosphorous insecticides inhibit acetylcholinesterase (Cremlyn, 1991).

In insects, acetylcholine is a major neurotransmitter in the central nervous system (Benson, 1993) and most sensory information is delivered to the CNS through the release of acetylcholine (Trimmer, 1995). To determine if cabbage loopers respond only to chemicals that bind to acetylcholine receptors, I tested four additional compounds that act as neurotransmitters, neuromodulators, and/or neurohormones: dopamine, serotonin, octopamine, and GABA. To test if loopers respond differently to stimulants and inhibitors, as documented with other insects (Long and Murdock, 1983; Mullin et al., 1994; Cohen et al., 2002), I also assayed the following drugs: R(−) apomorphine hydrochloride and fluphenazine dihydrochloride, agonist and antagonist of dopamine receptors; buspirone hydrochloride and WAY-100635 maleate, agonist and antagonist of serotonin receptors; clonidine and metoclopramide, agonist and antagonist of octopamine receptors; muscimol hydrobromide and (-) bicuculline methobromide, agonist and antagonist of γ-aminobutyric acid (GABA) receptors. Testing the 12 compounds required several days, a sufficiently long time that decomposition could occur if the compounds were tested in a random sequence. Therefore, the 12 chemicals were tested one at a time.

Drug activities are based on studies with either insect (Benson, 1993; Roeder, 1994; Burrows, 1996; Osborne, 1996; Aydar and Beadle, 1999) or mammalian models (Hardman and Limbird, 1996; Watling, 1998). Where information is

available, most compounds are active in both groups. A notable exception is bicuculline, a diagnostic $GABA_A$ antagonist in vertebrates that is largely inactive against GABA responses in insects (Benson, 1993; Aydar and Beadle, 1999). Sources of chemicals are indicated in Tables 1 and 2 and in Figure 3.

Survival of Cabbage Loopers on Lobelia. To test if cabbage loopers can develop on *Lobelia cardinalis*, 20 newly eclosed first instar larvae were placed in clip cages on newly mature leaves of potted *L. cardinalis* that had not yet bolted. The clip cages were padded to provide a seal over the leaf surface without compressing the latex canals (laticifers). Each larva was enclosed on a separate plant. As a control, 40 newly emerged first instars were offered excised leaves of *L. cardinalis* cut in half to deplete the laticifers. Fresh leaves were provided as needed, typically daily.

Cabbage loopers begin trenching in the second instar (Dussourd, 1993). To test if trenching allows cabbage loopers to feed on *L. cardinalis*, 20 early third instars and 10 early fifth instars were each sleeved on separate nonbolted plants. The larvae were reared to the third or fifth instar on excised leaves of *L. cardinalis*. Larvae were checked daily for trenching and feeding.

In nature, newly eclosed larvae are not isolated in clip cages; they can roam freely to find suitable food. To test if unrestrained larvae can develop on *L. cardinalis*, I placed 20 newly eclosed first instars each on a separate nonbolted plant surrounded by a water moat to prevent dispersal. As a control, 40 newly eclosed larvae were again offered excised *L. cardinalis* leaves. Larvae in all *Lobelia* experiments were held indoors at $\sim 25^{\circ}$C.

RESULTS

Trenching Bioassays. Of the 12 natural products tested from potential host plants of cabbage loopers, only 3 triggered significant trenching: the sesquiterpene lactone, lactucin, from lettuce latex; the phenylpropanoid, myristicin, from parsley oil; and the alkaloid, lobeline, from *Lobelia* (Table 1). Retesting the three compounds with new cohorts of larvae confirmed their activity (Figure 2). Compounds with little or no activity included the two phenylpropanoids from lettuce (caffeic acid and chlorogenic acid), plus a phenylpropanoid (parsley apiole), three furanocoumarins, and two monoterpenes from parsley (Table 1). Cucurbitacin E glycoside from cucumber also did not trigger trenching at 50 nmol/larva (Table 1), nor at four lower concentrations tested in water (0.02, 0.2, 2, 20 nmol/larva; 10 larvae/concentration; Dussourd, unpublished data). None of the plant compounds in Table 1 caused substantial mortality; with most chemicals, 100% of the larvae survived to produce normal moths.

Acetylcholine did not affect looper behavior at the dose tested, but four of the eight drugs active at acetylcholine synapses elicited trenching (Table 2A).

TABLE 1. CABBAGE LOOPER TRENCHING IN RESPONSE TO NATURAL PRODUCTS

Solution[a]	% Trenching[b]
Asteraceae: *Lactuca*	
1. Lactucin (10 nmol/larva in 50% acetone)	60*
Lactucin (5 nmol/larva in 50% acetone)	60*
50% Acetone control	10
2. Caffeic acid[c] (50 nmol/larva in 30% acetone)	0
30% Acetone control	0
3. Chlorogenic acid[c] (50 nmol/larva in water)	0
Apiaceae: *Petroselinum*	
4. Myristicin[c] (50 nmol/larva in 10% Tween 80)	70**
10% Tween 80[c] control	0
5. Parsley apiole[d] (50 nmol/larva in 10% Tween 80)	0
10% Tween 80[c] control	0
6. Xanthotoxin[c] (50 nmol/larva in 60% acetone)	0
60% Acetone control	0
7. Imperatorin[e] (50 nmol/larva in 60% acetone)	20
60% Acetone control	0
8. Bergapten[f] (10 nmol/larva in 60% acetone)	0
60% Acetone control	20
9. (1R)-(+)-α-Pinene[f] (50 nmol/larva in 10% Tween 80)	20
10% Tween 80[c] control	0
10. γ-Terpinene[f] (50 nmol/larva in 10% Tween 80)	0
Campanulaceae: *Lobelia*	
11. (-)-Lobeline hydrochloride[f] (50 nmol/larva in water)	70**
Water control	0
Cucurbitaceae	
12. Cucurbitacin E glycoside (50 nmol/larva in 10% Tween 80)	0
10% Tween 80[c] control	10

[a] Numbers on left indicate solutions tested together in a randomized sequence; $N = 10$ larvae/solution.
[b] Fisher's exact tests compare the number of larvae cutting a trench in *Plantago lanceolata* in response to chemicals and solvent controls.
[c] Source of chemical: Sigma.
[d] Source of chemical: Indofine.
[e] Source of chemical: ICN.
[f] Source of chemical: Aldrich
* $0.05 > P > 0.01$; ** $0.01 > P > 0.001$.

Only two of these showed statistically greater activity than the water control; however, even low levels of trenching suggest biological activity for these water-soluble compounds since none of >100 cabbage loopers tested with water controls in different experiments have ever cut trenches (Dussourd, 1997, unpublished data). Furthermore, none of the thousands of cabbage loopers that I have raised on *Plantago lanceolata* have ever been observed to trench during rearing. The four compounds that triggered trenching included both agonists and antagonists

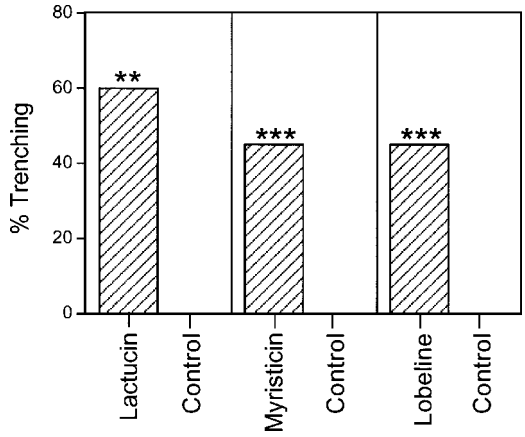

FIG. 2. Percentage of cabbage loopers cutting a trench in *Plantago lanceolata* after receiving 10 nmol lactucin in a 50% acetone solution, 50 nmol myristicin in 10% Tween 80, or 50 nmol lobeline in water. Each chemical was tested independently with a control, which was the same solution used to dissolve the test chemical. Lactucin and its control were each tested with 10 larvae, whereas the other solutions were all tested with 20 larvae. Asterisks indicate statistically greater trenching response than the control (Fisher's exact tests, $**0.01 > P > 0.001$, $*** P < 0.001$).

of acetylcholine receptors. The two anticholinesterases tested were both inactive as trenching stimulants (as were the anticholinesterase insecticides, methomyl and methamidophos, Table 2B). Nicotine caused one larva to regurgitate onto the leaf; none of the other compounds caused overt poisoning. Larvae survived their exposure to all compounds; 90–100% of the larvae produced moths for all compounds tested.

Of the four insecticides tested, only imidacloprid triggered significant trenching (Table 2B). Two larvae showed clear signs of poisoning; one trenched half way across the leaf before falling off, regurgitating, and quivering with spasms. One of the loopers tested with cartap likewise completed only a partial trench before becoming immobilized and unresponsive. A second larva had just begun trenching when it too was immobilized by cartap. All of the larvae tested with imidacloprid and cartap eventually resumed feeding and developed into adults of normal appearance. In contrast, both methomyl and methamidophos were fatal. Surprisingly, the larvae scarcely responded to drops of these insecticides. Either the loopers were unable to detect methomyl and methamidophos or the compounds were not deterrent. Shortly after ingesting the entire dose, the larvae regurgitated, convulsed, fell from the leaf, and eventually became immobilized. The lack of larval response even to a lethal dose undoubtedly increases mortality when the insecticides are used against cabbage loopers on crops.

TABLE 2. CABBAGE LOOPER TRENCHING IN RESPONSE TO DRUGS AND INSECTICIDES THAT AFFECT ACETYLCHOLINE SYNAPSES

Solution[a]	% Trenching[b]
A. Drugs	
1. Acetylcholine chloride[c]	0
(−)-Nicotine[c]	30
(+)-Muscarine chloride[c]	0
Decamethonium bromide[d]	40*
Succinylcholine chloride[c]	0
D-Tubocurarine chloride[d]	20
Ipratropium bromide[c]	70**
Pyridostigmine bromide[d]	0
Edrophonium chloride[d]	0
Water control	0
B. Insecticides	
2. Imidacloprid[e] in 30% acetone	40*
30% Acetone control	0
3. Cartap hydrochloride[e]	10
4. Methomyl[e]	0
5. Methamidophos[e]	0
6. Water control	0

[a] Numbers on left indicate solutions tested together in a randomized sequence. All larvae received 50 nmol test chemical dissolved in 1.5-μl solvent. Unless indicated otherwise, all chemicals were dissolved in water. $N = 10$ larvae/solution, except $N = 5$ for methomyl and for methamidophos.

[b] Fisher's exact tests compare the number of larvae cutting a trench in *Plantago lanceolata* in response to chemicals and solvent controls.

[c] Source of chemical: Sigma.

[d] Source of chemical: ICN.

[e] Source of chemical: Chem Service.

* $0.05 > P > 0.01$; ** $0.01 > P > 0.001$.

Cabbage loopers also responded to chemicals that bind to other neuroreceptors (Figure 3). Dopamine and serotonin both deterred feeding and triggered trenching. Octopamine was mildly deterrent to some larvae, but was inactive as a trenching stimulant. GABA had little affect on looper behavior at the dosage tested. The low activity of GABA is not surprising since GABA is known to stimulate feeding by diverse herbivores (Mullin et al., 1994). All drugs tested that affect dopamine, serotonin, and octopamine receptors elicited trenching, whereas only the antagonist of GABA receptors was active (Figure 3). No toxic responses were noted; 90–100% of the larvae developed into moths in all treatments.

Many of the chemicals tested were deterrent to the loopers. Larvae often wiped solution off their mouthparts onto the leaf, sometimes repeatedly with each application. The larvae frequently withdrew their head away from the leaf, then

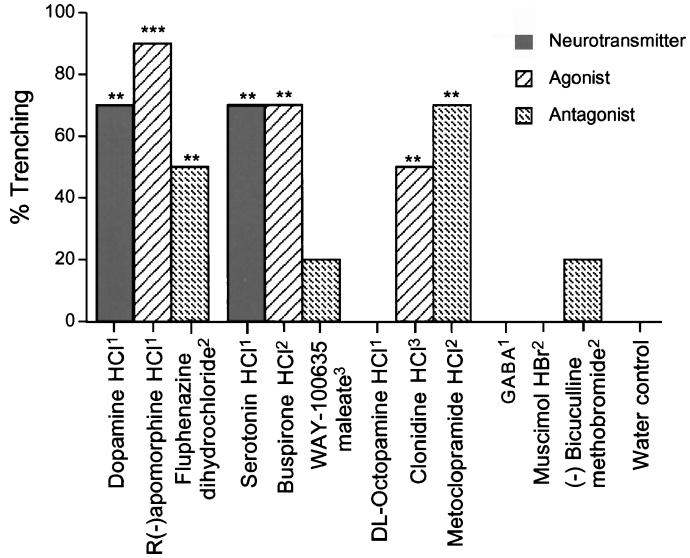

FIG. 3. Percentage of cabbage loopers that cut trenches in *Plantago lanceolata* leaves after receiving 50 nmol of the test chemical dissolved in 1.5-μl water. Each chemical was tested with 10 final instar larvae. The chemicals included four neurotransmitters (dopamine, serotonin, octopamine, GABA), plus an agonist and antagonist of receptors stimulated by each neurotransmitter. The three biogenic amines may act partly or primarily as neuromodulators or neurohormones in insects (Burrows, 1996). Asterisks indicate statistically greater trenching response than the aqueous control (Fisher's exact tests, $**0.01 > P > 0.001$, $*** P < 0.001$). Superscripts after the chemical names indicate the source of the chemical: [1]ICN, [2]Sigma, [3]RBI.

moved their mandibles together and apart repeatedly as though chewing. Some larvae lowered their head to their legs to groom legs and mouthparts. Larvae often abandoned their feeding site and moved to another location on the leaf before continuing to feed.

Compounds that triggered trenching were especially deterrent. For example, of the nine chemicals tested that affect acetylcholine synapses (Table 2A), the four that triggered trenching were more deterrent than the five that did not. Larvae exposed to the trenching stimulants were more likely to wipe their mouth on the leaf (9.5 ± 0.3 larvae/chemical; mean ± 1 SE) than larvae tested with the other five chemicals (1.8 ± 0.7, $0.05 > P > 0.01$, Mann–Whitney U test). Likewise, larvae tested with the four trenching stimulants were more likely to withdraw and exhibit chewing motions (9.0 ± 0.7 versus 1.8 ± 0.8 larvae, $0.05 > P > 0.01$), to groom (2.5 ± 0.9 vs. 0.2 ± 0.2, $0.05 > P > 0.01$), and to abandon feeding sites (6.8 ± 0.6 vs. 0.6 ± 0.4, $0.05 > P > 0.01$, Mann–Whitney U). Nearly all

of the 132 loopers that trenched in this study exhibited signs of deterrence before trenching: 95% showed mouth-wiping, 98% chewing motions, 13% grooming, and 67% abandoning feeding sites (not including when larvae stopped feeding to trench). For chemicals tested in water, deterrency can be attributed directly to the test chemical. Lobeline, for example, triggered mouth-wiping in 100% of the 30 larvae tested (vs. 0% for water controls), 100% chewing motions (vs. 3%), 17% grooming (vs. 0%), and 83% abandoning feeding sites (vs. 0% for water). Furthermore, 10 of the 30 larvae walked off the leaf after receiving lobeline.

Although solutions that elicited trenching were all deterrent, not all deterrent solutions caused trenching. Xanthotoxin elicited vigorous repetitive mouth-wiping; 7 of 10 larvae left the leaf after receiving just one or two drops, but none cut a trench. Excluding data for xanthotoxin, only 29 of 650 larvae tested in this study walked off the leaf; larvae were reluctant to move onto the water pic and usually resumed feeding nearby. Xanthotoxin retested at a range of concentrations again was highly deterrent. Fewer larvae abandoned the leaf at lower xanthotoxin doses, but none trenched (Figure 4). Other deterrent solutions also had little or no activity as trenching stimulants. For example, bergapten in 60% acetone and terpinene in 10% Tween 80 caused mouth-wiping, respectively, in 90 and 100% of the larvae, chewing movements in 100 and 90%, grooming in 80 and 30%, and abandoning feeding sites in 50 and 60%; neither solution triggered trenching.

The naive larvae in this study required repeated contact with solutions before they initiated trenching. Only 6% started trenching after receiving the first drop,

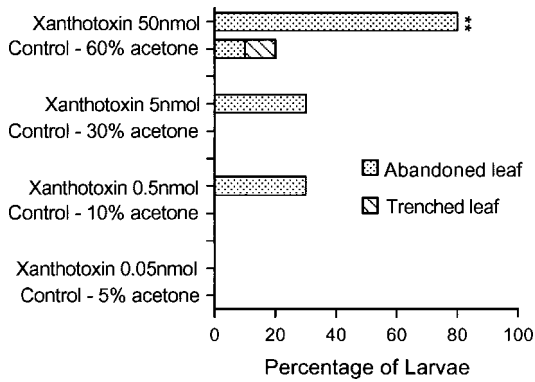

FIG. 4. Effect of xanthotoxin on cabbage loopers. Each concentration of xanthotoxin was tested independently with a control, which was the solvent alone. Ten loopers were tested with each solution; each received 1.5 μl. Asterisks indicate a statistically significant difference in the number of loopers abandoning the leaf comparing the xanthotoxin solution and control (Fisher's exact tests, $^{**}0.01 > P > 0.001$). Xanthotoxin was highly deterrent, but did not trigger trenching at any of the concentrations tested.

32% after the second, 31% after the third, and 31% after the fourth to sixth drops. Even then, larvae often cut one or more veins first (9% of 132 trenchers) or cut only a short trench (52%) before continuing to feed. After encountering an additional drop(s) of the test chemical, the larvae resumed trenching, as though their first attempt to disrupt leaf canals had been inadequate and further trenching was necessary. Only 39% of the larvae cut a continuous trench across the entire leaf the first time that they trenched. This indicates that trenching is not a fixed action pattern that is invariably carried through to completion on first attempt. On the contrary, larvae appear to require continued stimulation to persevere at trenching. On plants with laticifers, trenching causes latex to flow onto the trench. Larvae presumably encounter trenching stimulants in latex continuously until all laticifers crossing the trench have been severed, at which point the larvae stop trenching and begin to feed.

Cabbage Looper Survival on Lobelia. First instar cabbage loopers enclosed on potted *Lobelia cardinalis* leaves all died within 2–6 days (Figure 5). Only one reached the second instar and it died shortly thereafter. Larvae ate small pits in the leaves, often causing latex to flow into the pits. Two larvae died with their heads glued to the leaf by latex. Third instar larvae sleeved on *L. cardinalis* also perished. Eighteen of the 20 larvae cut one or more trenches, mostly across leaf tips; however, the larvae did not feed beyond 40% of the trenches. When these intact leaf tips were severed with scissors, latex oozed readily from all of them documenting that the trenches failed to sever all laticifers crossing the trench. Third instars also ate small pits in the leaves and fed on the midrib. The larvae survived up to 11 days (mean = 5 days), but none molted to the fourth instar.

In contrast, most final instar cabbage loopers enclosed on *L. cardinalis* cut trenches readily and fed beyond the trenches. The 10 larvae trenched 68 leaves and

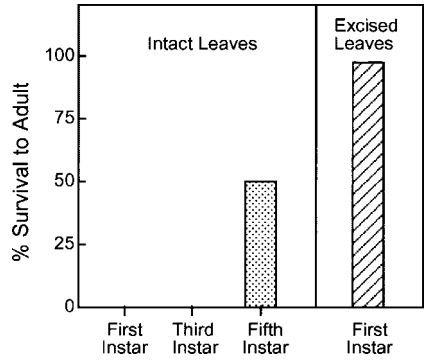

FIG. 5. Survival of cabbage loopers enclosed with either intact or excised leaves of *Lobelia cardinalis*. Twenty larvae in the first instar, 20 in the third instar, and 10 in the fifth instar were tested on intact leaves, whereas 40 first instar larvae were offered excised leaves.

completely consumed the distal tips in 81% of them. Seven larvae pupated, but only five adults emerged from the cocoons. The three larvae that failed to pupate fed beyond only 10 of their 20 trenches. The uneaten tips readily released latex when severed indicating that the larvae were often unsuccessful in deactivating the latex system.

Excised leaves of *L. cardinalis* were highly acceptable for cabbage loopers. Fully 97.5% of the 40 loopers pupated in just 12–14 days from egg hatch (mean = 12.4 days) and all pupae produced an adult (Figure 5).

The newly emerged cabbage loopers released on entire *L. cardinalis* plants also perished. However, four of the larvae reached the second instar by feeding extensively on senescent or damaged leaves, as well as by pit-feeding. The four larvae cut trenches across 18 leaves, but fed successfully on only four of them. The remaining 14 leaves all released latex readily when their tips were severed documenting that some latex canals remained intact. As previously, the larvae on excised leaves fed readily; 90% survived to pupate and 87.5% produced an adult moth.

DISCUSSION

Isolated chemicals sufficed to elicit trenching by cabbage loopers. These compounds were active in the absence of other exudate components; notably, solutions did not have to be sticky to stimulate trenching. Effective stimulants included a diversity of structures (Figure 6) suggesting that either multiple receptors are involved in their detection or the receptors are broadly tuned. Three constituents of potential food plants caused significant trenching: lactucin from lettuce, myristicin from parsley, and lobeline from *Lobelia*. As described below, these compounds are known or suspected to have toxic properties, suggesting that loopers cut trenches specifically to reduce their exposure to noxious substances during feeding.

Lactucin is classified as a sesquiterpene lactone, a diverse group of almost 3500 reported structures known for cytotoxic, antibacterial, antifungal, and allelopathic properties (Picman, 1986). Several sesquiterpene lactones have documented deterrency or toxicity to insect herbivores (Picman, 1986; Gershenzon and Croteau, 1991; Mullin et al., 1991). To my knowledge, lactucin has not been tested previously for its effects on insects. However, two other sesquiterpene lactones reported from lettuce latex, lactucopicrin and 8-deoxylactucin (Price et al., 1990), are known to deter feeding by locusts (Rees and Harborne, 1985).

The sesquiterpene lactones in the latex of cultivated lettuce occur as a complex mixture that includes oxalate and sulfate conjugates (Sessa et al., 2000). Lactucin is only a minor component. It reaches a maximum level of $\sim$2.8 mg/ml latex in bolted plants, whereas the total sesquiterpene lactone titer of these plants averages 147.1 mg/ml latex (Sessa et al., 2000). Titers of sesquiterpene lactones in the latex

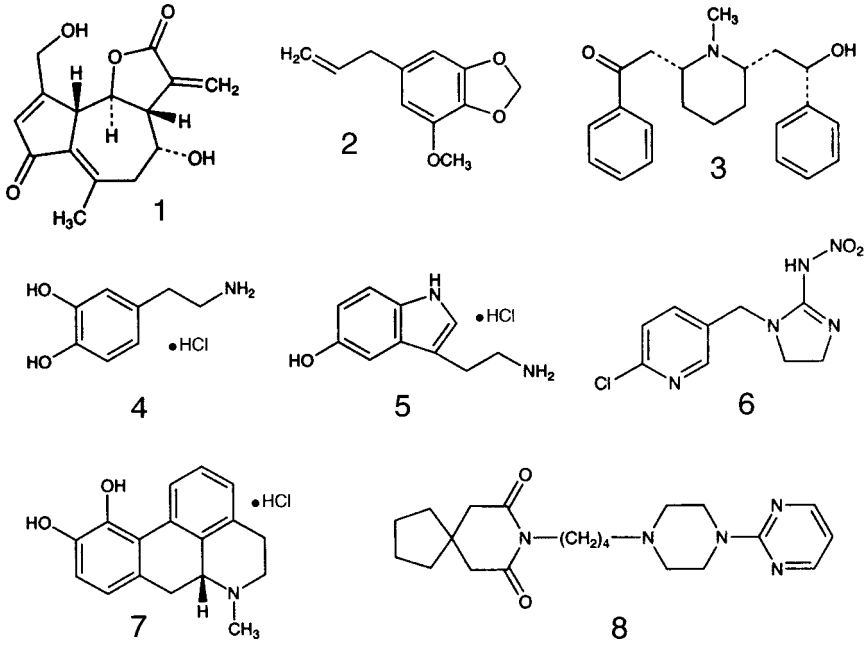

FIG. 6. Chemicals that trigger trenching include the sesquiterpene lactone, lactucin (**1**), from lettuce latex; the phenylpropanoid, myristicin (**2**), from parsley oil; the alkaloid, lobeline (**3**) from *Lobelia*; the neurotransmitters, dopamine hydrochloride (**4**) and serotonin hydrochloride (**5**); the insecticide, imidacloprid (**6**); and several drugs that stimulate or inhibit neural receptors, such as R(−) apomorphine hydrochloride (**7**) and buspirone hydrochloride (**8**).

of wild *Lactuca* species, notably *L. virosa*, can be much higher (Tamaki et al., 1995). In this study, lactucin triggered trenching at 0.92 mg/ml (=5 nmol/1.5 μl), documenting that the compound is active at natural lactucin levels. Presumably the abundant conjugates and derivatives of lactucin contribute to the trenching activity of *Lactuca* latex. Cabbage loopers feed extensively on composites in the field, including cultivated and wild lettuce (*L. sativa*, *L. serriola*, *L. canadensis)* and other latex-bearing composites such as *Sonchus asper, Taraxacum officinale*, and *Pyrrhopappus carolinianus* (Dussourd and Denno, 1991, 1994). I have found as many as 20 cabbage loopers on individual plants of *L. serriola* and *S. asper*, which can be completely stripped of leaves. The loopers routinely cut trenches in these species, apparently due specifically to the sesquiterpene lactones that they must encounter often in these important food plants.

Cabbage loopers also cut trenches in multiple species of Apiaceae, including parsley, carrot, and parsnip (Dussourd and Denno, 1991; Zangerl and Bazzaz, 1992). All three species produce myristicin (Lichtenstein and Casida, 1963;

Kubeczka and Stahl, 1977; Porter, 1989; Hallstrom and Thuvander, 1997). Myristicin inhibits detoxification enzymes (mixed function oxidases) in caterpillars and thereby synergizes the activity of insecticides and natural toxins such as furanocoumarins (Lichtenstein and Casida, 1963; Berenbaum and Neal, 1985; Neal, 1989). When tested by itself, myristicin is poisonous to some fly, aphid, beetle, and caterpillar species (Lichtenstein and Casida, 1963; Lichtenstein et al., 1974; Marston et al., 1995; Srivastava et al., 2001), but does not increase mortality of two generalist caterpillars, the corn earworm and southern armyworm (Lichtenstein and Casida, 1963; Berenbaum and Neal, 1985). Myristicin levels vary greatly in different parsley accessions, ranging from 0.1% to over 60% of the essential oils extracted from whole plants (Simon and Quinn, 1988; Porter, 1989). When present at high concentration, myristicin is undoubtedly an important trenching stimulant. Whether additional oil components such as the nonpolar sesquiterpene caryophyllene also trigger trenching remains to be determined.

Surprisingly, furanocoumarins were ineffective trenching stimulants, even though these compounds occur in the oil ducts (Camm et al., 1976; Wu and Hahlbrock, 1992; Reinold and Hahlbrock, 1997) and are toxic to diverse herbivores (Berenbaum, 1990; Reitz and Trumble, 1996). Xanthotoxin mixed in artificial diet at 0.24% fresh mass prevents growth of cabbage loopers (Zangerl, 1990). Nevertheless, xanthotoxin did not elicit trenching, although it was strongly deterrent (Figure 4). Parsley apiole also was inactive at the dosage tested, despite its structural similarity to myristicin. Cabbage loopers clearly do not trench indiscriminately in response to all allelochemicals. A further example is provided by cardenolides, which are extremely deterrent and toxic to cabbage loopers, but did not cause trenching when tested at 25 μg/larva (=33 nmol/larva for digitoxin; Dussourd and Hoyle, 2000).

Little information is available on how lobeline affects insects, although it has been reported to deter feeding by a ctenuchid caterpillar and honeybees (Wink and Schneider, 1990; Detzel and Wink, 1993). Mammalian herbivores (cows, sheep, goats) that feed on various *Lobelia* species suffer negative effects that can include sluggishness, hemorrhage, and coma; overdoses of lobeline produce similar effects (Dollahite and Allen, 1962; Kingsbury, 1964). *Lobelia cardinalis* contains 7 mg lobeline/g dry weight, which is comparable to levels in the medicinal plant, *L. inflata* (Krochmal et al., 1972). Overdoses of lobeline or *L. inflata* in humans cause dizziness, stupor, tremors, paralysis, convulsions, coma, and death (Arena, 1970; Duke, 1985; Dwoskin and Crooks, 2002).

Cucurbitacin E glycoside was the only compound characteristic of the Cucurbitaceae that I tested. When dissolved in water, it elicited minimal reaction from the loopers even though cucurbitacins are intensely bitter to humans (Metcalf et al., 1980) and have deterrent or toxic effects on many herbivores (Tallamy et al., 1997). However, cucurbitacin B applied to leaf disks had no significant effects on cabbage looper feeding (Tallamy et al., 1997). Trenching activity in cucumber sap

has been traced not to cucurbitacins, but to a polar fraction (Dussourd, 1997) that contains at least two active components. One of the components has been partially characterized as an *N*-alkyl pyridinium salt (Capron, Wiemer, and Dussourd, unpublished data). Lettuce, parsley, *Lobelia*, and cucumber thus contain strikingly different trenching stimulants: a sesquiterpene lactone, phenylpropanoid, and two alkaloids; there is no common releaser for trenching in these diverse plants. The solvents used to dissolve less polar compounds in this study sometimes also elicited trenching, but only at much higher concentrations than the natural products (e.g., 60% acetone vs. 6.7 mM lactucin).

Even with the most active stimulants, less than 100% of the larvae trenched, whereas final-instar cabbage loopers isolated on intact plants of prickly lettuce (*Lactuca serriola*) or on other laticiferous composites invariably trench (Dussourd and Denno, 1994). However, loopers tested with the trenching assay had not previously experienced exudates. In addition, the larvae received only 4–6 droplets of solution, unlike loopers on intact plants which contact exudates repeatedly during trenching (Dussourd, 1999). It is also unlikely that lactucin, myristicin, and lobeline are the only trenching stimulants in lettuce, parsley, and *Lobelia*. *L. cardinalis*, for example, contains multiple piperidine alkaloids (Gupta and Spenser, 1971; Krochmal et al., 1972), in addition to lobeline. Although not all allelochemicals were tested, this study documents that isolated chemicals at biologically relevant concentrations suffice to elicit trenching and provides a method for screening additional compounds for trenching activity.

Of the four insecticides tested, imidacloprid was the least harmful for the loopers and the most effective at triggering trenching. Imidacloprid is commonly used to control sucking insects, fleas, and beetles; it is generally less effective against caterpillars (Thomson, 1998). Methomyl and methamidophos were both lethal at the dosage used, whereas cartap caused temporary immobility in all larvae. Cartap is converted *in vivo* to nereistoxin, which blocks cholinergic synaptic transmission and rapidly immobilizes insects (Sattelle, 1985).

Of the 21 neurotransmitters and drugs tested, 13 caused trenching (Table 2A, Figure 3). Many of the active compounds are potent neurotoxins such as tubocurarine, a constituent of curare arrow poison (Hardman and Limbird, 1996), and nicotine, a contact insecticide from tobacco used to kill insect pests for over 300 years (Cremlyn, 1991). Both agonists and antagonists of cholinergic, dopaminergic, and serotonergic receptors were deterrent and caused the loopers to trench. Other insects are more specific in their responses. For example, in blowflies and cockroaches, octopamine and octopamine agonists delivered by injection or by ingestion increase feeding, but octopamine antagonists decrease feeding (Long and Murdock, 1983; Cohen et al., 2002). Likewise, applying agonists for GABA/glycine receptors to flower disks stimulates feeding by western corn rootworms, whereas GABA/glycine antagonists deter feeding (Mullin et al., 1994). Cabbage loopers, in contrast, often responded the same to agonists and antagonists,

and sometimes reacted differently to compounds with similar modes of action. For example, octopamine was inactive at the dosage tested, whereas the octopamine agonist, clonidine, elicited trenching in 5 of 10 larvae (Figure 3). The similar activity of agonists and antagonists suggests that neurotransmitters and drugs trigger trenching by stimulating peripheral chemoreceptors and not through direct contact with the CNS following absorption from the gut. This conclusion is supported by the rapid response of some larvae; for example, one looper initiated trenching after receiving just a single drop of dopamine.

The three natural trenching stimulants, lactucin, myristicin, and lobeline, all have been reported to affect the nervous system of mammals. Latex from wild *Lactuca* species (lactucarium) has been used medicinally as a sedative and an analgesic for hundreds of years (Stevens, 1948; Crellin and Philpott, 1990). Laboratory studies with mice suggest that lactucin is at least partly responsible for its activity (Schenck, 1966; Gromek et al., 1992). Myristicin injected in various mammals causes behavioral depression, ataxia, and disorientation (Truitt et al., 1961; Florio et al., 1972; Truitt, 1979). Higher doses in monkeys lead to respiratory arrest (Truitt et al., 1961). Lobeline has multiple pharmacological effects. It acts as a potent antagonist at $\alpha 3\beta 2$ and $\alpha 4\beta 2$ neuronal nicotinic receptor subtypes (Dwoskin and Crooks, 2001). Lobeline also alters dopamine storage and utilization apparently by inhibiting dopamine uptake by synaptic vesicles and by stimulating the release of dopamine from the vesicles into the cytosol (Dwoskin and Crooks, 2002). Interestingly, with all three trenching stimulants, plant species containing the compounds are smoked or consumed for their purported hallucinogenic, narcotic, or euphoric effects (Truitt et al., 1961; Huang et al., 1982; Duke, 1985; Foster and Tyler, 1999). Lobeline is currently being investigated as a treatment for methamphetamine abuse (Harrod et al., 2001; Dwoskin and Crooks, 2002) and has been marketed as a treatment for tobacco smoking (Fodor and Colasanti, 1985). Presumably the three trenching stimulants also affect the nervous system of cabbage loopers, although none have been tested for neurotoxicity to insects.

Neurotoxins, including many alkaloids, are abundant in plants (Murdock et al., 1985; Hartmann, 1991; Wink, 1998). The toxins are sometimes sequestered in latex canals, such as opium alkaloids found in poppy latex (Bernath, 1998) and heart poisons (cardenolides) in milkweed latex (Dussourd and Hoyle, 2000). Neurotransmitters, including acetylcholine, dopamine, and serotonin, also have a widespread distribution in plants (Roshchina, 2001), and sometimes occur at high concentration (Steelink et al., 1967; Oliver et al., 1991). In poppy latex (Roberts et al., 1983), dopamine concentrations reach 2.66 mg/ml (=17.4 mM), close to the levels that elicited trenching in this study (33.3 mM).

Despite their ability to trench, cabbage loopers were unable to develop on intact *Lobelia cardinalis*. Early instar larvae ate pits in the leaves, but failed to develop on potted plants, except on senescent or damaged leaves. Third and fifth instar loopers cut trenches, but only the larger larvae were able to feed effectively.

Excised leaves, in contrast, were highly acceptable, presumably because excision depressurizes latex canals, thereby reducing larval ingestion of latex chemicals. These results illustrate the difficulty of defining an insect's host range. Plant suitability depends not just on allelochemical content, but also on diverse factors that can include larval stage (Zalucki et al., 2002) and prior damage (Lewis, 1984).

In summary, the results reported herein support the hypothesis that cabbage loopers cut trenches specifically to deactivate secretory canals in host plants and thereby reduce their ingestion of poisons that may often include neurotoxins. However, trenching ability alone is inadequate to permit feeding. Trenchers must also be able to tolerate high levels of exudate chemicals during trenching and lower levels during subsequent feeding. The known toxicity of the three identified trenching stimulants suggests that cabbage loopers use host toxins specifically as cues for trenching.

Many questions remain. Are lactucin, myristicin, and lobeline poisonous to cabbage loopers, and do these compounds affect their nervous system? Lactucin is known to occur in the latex of *Lactuca* (Sessa et al., 2000). Is lobeline similarly sequestered in *Lobelia* latex, and is myristicin stored in the oil ducts of *Petroselinum*? How much of these compounds do cabbage loopers ingest during trenching and during subsequent feeding on distal tissues with depleted canals? Why do some feeding deterrents trigger trenching, whereas others do not? Do the diverse compounds that elicit trenching all stimulate the same taste receptor(s)? Cabbage loopers are classified in the plusiine tribe Argyrogrammatini (LaFontaine and Poole, 1991) together with other trenching species that include *Enigmogramma basigera* and the soybean looper, *Pseudoplusia includens*. These three species differ substantially in host range. Cabbage loopers, for example, cannot develop on undamaged *Lobelia cardinalis*, but *E. basigera* cut trenches and develop rapidly to pupation on intact plants in both lab and field (Dussourd, unpublished observation). Likewise, soybean loopers, *Pseudoplusia includens*, develop poorly on *Lactuca serriola*, a highly acceptable host for cabbage loopers (Tune and Dussourd, 2000). Do these three plusiine species differ in their tolerance of trenching stimulants, resulting in the observed variation in host range?

Finally, why do cabbage loopers fail to trench plants lacking exudates? *Lactuca*, *Petroselinum*, and *Lobelia* all have canal systems that deliver exudates with concentrated allelochemicals directly to the mouthparts of biting herbivores. Do plants not trenched lack effective trenching stimulants or are stimulants present, but available at too low a concentration or counterbalanced by phagostimulants?

The cabbage looper is just one of over 100 insect species known to sever veins or cut trenches before feeding (Dussourd and Denno, 1991; Dussourd, 1993 and references therein; Becerra, 1994; McCloud et al., 1995; Jolivet, 1998; Clarke and Zalucki, 2000; Becerra et al., 2001). Given that over 20,000 species of plants contain latex or resin canals (Farrell et al., 1991; Lewinsohn, 1991), that up to 7 species of vein cutters have been reported per plant species (Dussourd and

Denno, 1991), and that some latex-bearing species are attacked by over 125 species of leaf-feeding insects (Basset and Novotny, 1999), the true number of insects exhibiting these behaviors undoubtedly numbers in the thousands. Plants manipulated with vein cuts or trenches include members of over a dozen families (Dussourd, 1993). The insect behaviors provide a convenient assay for identifying compounds in these plants that are especially significant for insect herbivores, indeed of such importance to have selected for sophisticated behavioral countermeasures. One wonders, for example, what compounds in mulberry stimulate trenching by soybean loopers (*Pseudoplusia includens*), what chemicals in papaya and cassava elicit trenching by the cassava hornworm (*Erinnyis ello*), and what substances in milkweed latex trigger vein severance by monarchs (*Danaus plexippus*). The results reported here suggest that trenching stimulants are likely to be important chemical defenses for plants, not just harmless cues. Vein cutting and trenching assays, thus, have the potential to direct the isolation of novel structures with insecticidal properties and possible applications in agriculture or medicine.

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IDENTIFICATION AND QUANTITATION OF ALLELOCHEMICALS FROM THE LICHEN *Lethariella canariensis*: PHYTOTOXICITY AND ANTIOXIDATIVE ACTIVITY[1]

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Abstract—Phytotoxicity-based extraction and fractionation were employed to separate allelochemicals contained in an extract of *Lethariella canariensis*. Twelve phenolic substances were isolated from the phytotoxic fraction "Letharal" of the thalli. These were identified by spectroscopic methods, physicochemical constants, and HPLC chemical correlation, and determined to be atranol (**2**), chloroatranol (**3**), hematommic acid (**4**), chlorohematommic acid (**5**), methyl hematommate (**6**), methyl chlorohematommate (**7**) (new compound), ethyl hematommate (**8**), ethyl chlorohematommate (**9**), methyl β-orsellinate (**10**), atranorin (**11**), chloroatranorin (**12**), and (+)-usnic acid (**13**). Further identification and quantification of these allelochemicals in the environment were conducted by HPLC. Several phenolic compounds showed moderate antimicrobial activity. The cytostatic activity of the polyphenols was investigated on U937 and HL-60 cells. All compounds were assayed, with the exception of **10**. The "Letharal" mixture decreased cell viability in both cell lines. Protection against lipid peroxidation was investigated using brain homogenates. Compounds **2**, **3**, **6**, **8**, **11**, and Letharal decreased H_2O_2/Fe^{+2} induced lipid peroxidation in a

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[1]This article is dedicated to the memory of Professor Antonio G. González.

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concentration-dependent manner, while **10** and **13** were unable to protect tissue against oxidative stress.

Key Words—Lichen, Parmeliaceae, *Lethariella canariensis*, allelopathy, allelochemical, phytotoxicity, phenolics, HPLC, cytostatic activity, U937 and HL-60 cells, lipid peroxidation.

INTRODUCTION

In researching compounds with biological activity, we have focused our attention on metabolites of lichens from the Canary Island flora. *Lethariella canariensis* (Ach.) Krog grows on volcanic terrain in the Canary Islands (La Caldera de Los Pinos in Gáldar, island of Gran Canaria, and Cumbre Vieja in El Paso, island of La Palma) and was formerly called *Usnea canariensis* until it was included in the genus *Lethariella*, family Parmeliaceae (Krog, 1976). This species belongs to the usneoid lichens. It is interesting to note that the land upon which *L. canariensis* grows has few other species (lichenic, pinaceous, or herbaceous) competing for available nutrients. This suggests a possible allelopathic interaction in addition to biological and geological factors (such as the immaturity of the volcanic terrain) that may be intervening. Allelopathy occurs by the release of chemicals from one plant species that affects other species in the vicinity, usually to their detriment (Harborne, 1982; Barnes and Putnam, 1985, 1987; Putnam and Tang, 1986; Barnes et al., 1987; Weston et al., 1987, 1989; An et al., 2000). Any allelopathic activity of lichenic compounds may involve inhibitory action against seed germination and/or plant growth. Earlier work in this field by other authors includes reports on the inhibitory effect of several lichenic compounds on germination and subsequent development of seeds from diverse plant species (Follman and Nakagava, 1963; Dalvi et al., 1972; Hüneck and Schreiber, 1972; Ramaut and Thonar, 1972a,b; Dauriac and Rondon, 1976; Garcia et al., 1982; Lawrey, 1986; Nishitoba et al., 1987). It had also been suggested that the growth of vascular plants is affected by lichenic inhibition of the fungi of the associated mycorrhiza, responsible for fixing phosphorus (Rundel, 1978).

Fernández and Pizarroso (1958) described the isolation of atranorin **11**, chloroatranorin **12** (Figure 1), and canarionic acid, the structure of which was not resolved, from *Lethariella canariensis*. Follmann and Hüneck (1970) continued the study without determining the structure of canarionic acid, once again finding the depsides atranorin **11** and chloroatranorin **12**. Finally, canarione (**1**) was isolated and characterized (Hüneck et al., 1977). Since the literature contained reports on several water-soluble phenols from higher plants (Harborne, 1982), including related allelochemicals, we believed that the phenolic fraction of our lichen "Letharal" could be responsible for an analogous phenomenon. The background literature suggested that both depsides, atranorin and chloroatranorin, could act as "parent metabolites" for the enzymatic and/or chemical transformation of a series of compounds that are released into the environment during rainy weather

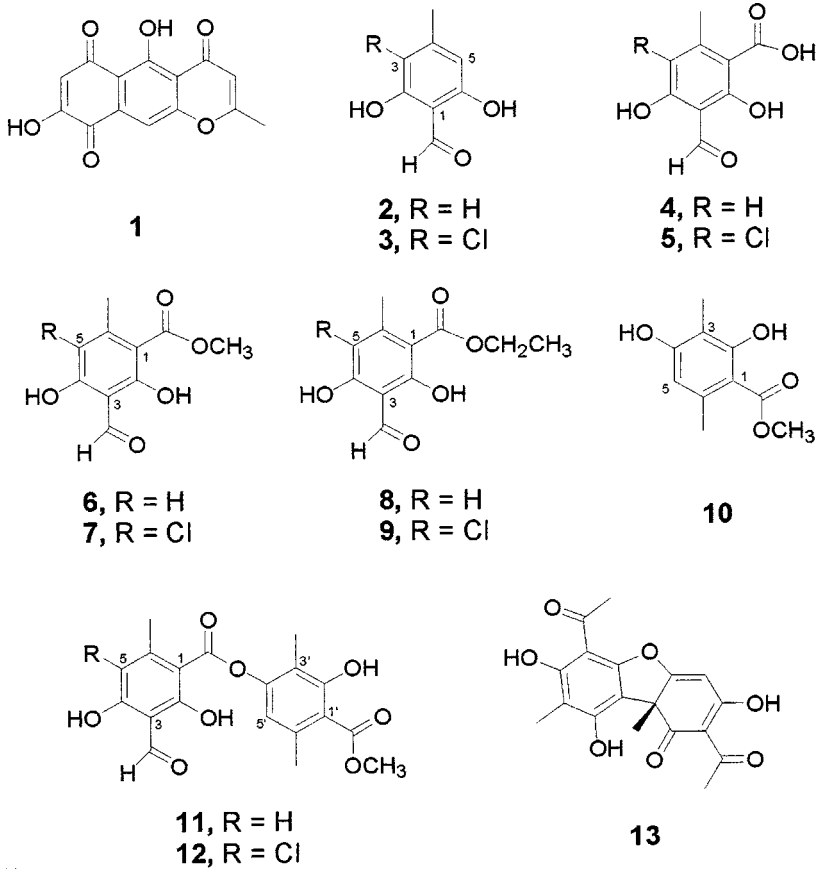

FIG. 1. Chemical structures of phenolic compounds isolated from *Lethariella canariensis*. Compounds **2**–**13** were identified as constituents in the herbicide *Letharal*.

(conditions of hydrolysis), precisely when the lichen needs to combat germination and growth of herbaceous and pinaceous plants.

$$\text{DEPSIDE} + \text{WATER} \xrightarrow{\textit{Depside-hydrolase}} \text{HYDROSOLUBLE MONOCYCLIC PHENOLS}$$

In times of drought, the aforementioned processes would be inhibited, thus allowing survival over time of competing species. Previously, and favoring this hypothesis, a preexisting, partially inactive hydrolase was isolated from *Evernia prunastri*. This enzyme is activated after rehydration, and hydrolyzes the depside evernic acid to produce equimolar amounts of two monocyclic phenols (González et al., 1984). Moreover, lichens undergoing extreme conditions of

change in temperature, humidity, and photoillumination respond to such changes by biosynthesizing large amounts of stress metabolites. One interesting example is *Pertusaria alaianta*, which responds to the arid–warm climate of the Cape Verde islands by biosynthesizing a mixture of chloroxanthones that constitute over 20% of its dry weight (Huneck and Höfle, 1978).

In this work, the chemical composition of the phytotoxic phenolic-fraction "Letharal" of *L. canariensis* is described. Evidence is presented to indicate that this phytotoxic mixture of compounds is disseminated into the environment via lixiviation by rainwater, where such compounds accumulate by resisting microbiological degradation, penetrating the cell walls of seeds to act as allelochemicals, and bring about an adaptative advantage.

Several of these letharal components are antioxidants. Reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radicals are constantly produced in all aerobically living cells and are potentially toxic (Fridovic, 1978; Cerutti, 1985). Oxidative stress is believed to be an important contributing factor in several human chronic diseases, such as atherosclerosis and cardiovascular diseases, mutagenesis and cancer, several neurodegenerative disorders, as well as in aging (Frei, 1999). Lipid peroxidation is a well-established mechanism of cellular injury that occurs in both plants and animals. Malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA), formed from the breakdown of polyunsaturated fatty acids, are representative of the degree of lipid peroxidation. Peroxidation of brain homogenates with hydrogen peroxide (H_2O_2) is a sensitive assay for determining oxidant ability, and added metal ions such as ferrous ion (Fe^{+2}) largely stimulate peroxidation by decomposing peroxides to radicals capable of abstracting hydrogen and continuing the chain reaction.

METHODS AND MATERIALS

General. IR spectra were recorded in $CHCl_3$. ^{1}H-NMR were run at 200 MHz in $CDCl_3$, at room temperature and with TMS as internal standard. ^{13}C-NMR-DEPT spectra were run at 50 MHz from $CDCl_3$ solutions. EIMS were recorded at 70 eV. Scharlau silica gel (0.04–0.06 mm) was used for low pressure and medium pressure column chromatography (carried out in a Buchi 684/688 system for liquid chromatography). TLC analysis was performed with silica gel G with fluorescent (254 nm) plates using EtOAc/*n*-hexane (x:10 − x) as solvent. HPLC analysis was carried out on a Varian 9010/9050 apparatus. The injected samples were dissolved in acetone or methanol (200 ppm) after filtration through C_{18}-Seppak. The results of the quantitative analyses were determined with a μ-Bondapak-C_{18} column, with AcOH/H_2O/MeOH (0.4:19.6:80.0 v/v/v) or AcOH/H_2O/MeOH (0.8:39.2:60.0 v/v/v) as mobile phase at a flow rate of 1.4 ml/min (pressure 94 atm) or 0.8 ml/min (pressure 80 atm), $T = 25^{\circ}$C, and a UV detector (254 nm) with

methyl ß-orsellinate (1.02 mmol/l) as external standard. The relative retention times (RRt) are given for the eluant AcOH/H_2O/MeOH (0.4:19.6:80.0 v/v/v) used at a flow of 1.4 ml/min.

The semimicrochemical assays were performed with an ethanolic solution of 3% ferric chloride. The treated acetone (freed of water, acids, and alcohols) was obtained from acetone for analysis by refluxing in excess of $KMnO_4$, distillation over anhydrous K_2CO_3, filtration, and distillation from P_2O_5.

Collection and Extraction of Lethariella. L. canariensis was collected in La Caldera de Los Pinos, in Gáldar (Gran Canaria, Canary Islands, Spain) and identified by Dr José Naranjo, Jardín Botánico de Gran Canaria. A voucher specimen is filed in the herbarium of the Jardín Botánico Canario "Viera y Clavijo" (LPA:18542). Dried, powdered thalli (3.5 kg) were extracted with methanol (5 l) at room temperature. The extract was concentrated under reduced pressure, and the semicrystalline concentrate (256 g) was percolated through silica gel (1 kg) eluted with acetone to give a $FeCl_3$-positive fraction that was chromatographed through 1.5 kg of silica gel with a gradient series of *n*-hexane, *n*-hexane: acetone, acetone, acetone/methanol and, finally, methanol. The phytotoxic fraction "Letharal" (63 g) was obtained by eluting in the earlier silica gel chromatography with *n*-hexane to *n*-hexane/acetone (1:1). This fraction also contained the antioxidative activity.

Isolation and Identification of Allelochemicals. Compounds making up the phenolic fraction "Letharal" were separated by column chromatography and preparative TLC. Twelve phenolic compounds were isolated (2–13, Figure 1) and identified by spectroscopic methods [(IR, UV, ^{1}H-NMR, ^{13}C-NMR (Table 1) MS, HRMS, DEPT)], physical–chemical constants (m.p., TLC R_f values), and HPLC chemical correlation and included: atranol **2** (12.02 g), chloroatranol **3** (0.50 g), hematommic acid **4** (1.20 g), chlorohematommic acid **5** (0.50 g) (compounds **2**–**5**; Sánchez et al., 1997), methyl hematommate **6** (0.65 g) (Cordell et al., 1977), methyl chlorohematommate **7** (0.16 g) (new compound), ethyl hematommate **8** (1.30 g) (Macmillan and Browne, 1977), ethyl chlorohematommate **9** (0.72 g) (Terajima et al., 1988), methyl β-orsellinate **10** (8.72 g) (Hylands and Ingolfsdottir, 1985), atranorin **11** (5.20 g), chloroatranorin **12** (0.90 g), and (+)-usnic acid **13** (0.27 g) (compounds **11**–**13**; Culberson, 1969).

The new compound, methyl chlorohematommate **7**, was detected by HPLC as part of a mixture of **6** (5.19%), **7** (17.20%), **8** (1.16%), and **9** (76.45%). To confirm the existence of methyl chlorohematommate **7** in this mixture, a chemical and chromatographic correlation was carried out. Thus, by transesterification with MeOH and glacial AcOH (9:1) a homogenization of the mixture was observed by HPLC, which transformed four components into two, namely **6** and **7**. Removal of the solvent and hydrolysis of one part with 20-ml H_2SO_4 (5%) at room temperature gave and acid mixture (**4**, RRt = 1.18 + **5**, RRt = 1.38). Compounds **4** and **5** slowly decarboxylated at 40°C to give a stable mixture (**2**, RRt = 0.83 + **3**, RRt = 0.96). Consequently, the third peak at RRt = 1.48 was assigned to methyl chlorohematommate

TABLE 1. ^{13}C-NMR SPECTRAL DATA FOR SEVERAL COMPOUNDS FROM "LETHARAL" IN $CDCL_3$

	2	**3**	**6**	**7**	**8**	**9**	**10**	**11**
C_1	107.34	108.63	104.20	105.43	103.94	105.43		103.00
C_2	150.73	159.72	168.26	165.54	168.34	165.54		169.07
C_3	108.28	109.01	108.46	108.56	108.44	108.56		108.54
C_4	150.65	156.96	166.64	162.42	166.50	162.42		167.47
C_5	107.34	111.02	112.11	130.88	112.04	130.88		116.01
C_6	161.90	147.56	152.26	149.04	152.42	149.04		152.41
C_7	22.09	21.02	25.09	20.80	25.29	20.80		23.99
C_8			172.10	171.02	171.58	171.02		169.68
C_9	193.72	193.72	193.65	193.56	193.91	193.75		193.62
C'_1							105.19	110.20
C'_2							158.05	162.86
C'_3							108.54	116.77
C'_4							163.10	169.68
C'_5							110.55	112.84
C'_6							140.14	139.85
C'_7							24.03	25.54
C'_8							172.61	172.40
C'_9							7.62	9.34
$-CO_2-CH_3$			52.32	52.75			51.79	52.31
$-CO_2-CH_2CH_3$					61.87	62.45		
$-CO_2CH_2-CH_3$					14.15	14.11		

(**7**). The other part of the homogenized mixture of **6** and **7** was separated by preparative TLC to give a sample of methyl chlorohematommate **7**: IR ν_{max} (cm^{-1}): 3035, 1722, 1642, 1296; EIMS, 70 eV *m/z* (rel. int.): 244 (5.1), 216 (2.04), 213 (12), 212 (20.4), 184 (100), 156 (6.7), 128 (4.0); HRMS *m/z* 246.010901 ($C_{10}H_9O_5^{37}Cl$ requires 246.011497), 244.015316 ($C_{10}H_9O_5^{35}Cl$ requires 244.013851), 218.015987, 216.018937, 213.984686, 212.995462, 211.987636, 186.997597, 185.989772, 185.000547, 183.992722, 157.994857, 155.997807, 129.999942, 128.002893; ^{1}H-NMR ($CDCl_3$) δ 2.70 (3H, s, CH_3), 4.00 (3H, s, CO_2CH_3), 10.35 (1H, s, CHO), 12.68 (1H, s, OH), 12.90 (1H, s, OH); ^{13}C-NMR (Table 1).

Germination Inhibition Assays. Assays were performed at constant and/or alternate temperature, simulating the temperature changes that occur in nature as a consequence of diurnal temperature cycle. Prior blank controls were carried out (0% of "Letharal" on diatomaceous earth (Celite), previously treated with acetone and taken to dryness). The LD_{50} was graphically measured for 50% inhibition of germination (with respect to the blank assay for each seed).

1. *At Constant Temperature.* The herbicide "Letharal," with the composition specified in Table 1 (winter extract) was applied at variable concentration

(0.0–1.1% w/w) onto powdered diatomaceous earth (the herbicide was dissolved in 100 ml of acetone, the Celite was added, and the mixture was taken to dryness in a rotavapor). The mixtures thus obtained were extended in rows of pleated filter paper on glass trays (20 × 25 × 1 cm) on which were sown seeds of cabbage, lettuce, pepper, and tomato (100 seeds per tray, 10 per furrow), and stored in a temperature-controlled room (20 ± 2°C). Evaluation of seed germination was carried out up to 48 hr after the last observed germination.

2. *At Alternate Temperature.* The effect of nocturnal frost was simulated by maintaining the seeded trays (with concentrations similar to those described) on a 12L:12D photoperiod, with 12 hr of light at 20 ± 2°C and 12 hr of darkness at 5 ± 1°C.

Allelochemicals from the Lichenic Thallus and the Soil. The chemical composition of the allelochemical mixture "Letharal" was quantitatively determined, and the chromatographic data (HPLC) of its components were as follows: RRt × 100 64.3 ± 5 ((+)-Usnic acid, **13**), 83.3 ± 0.9 (Atranol, **2**), 96.3 ± 0.8 (Chloroatranol, **3**), 100.0 ± 0.0 (Methyl ß-orsellinate, **10**), 118.0 ± 1.5 (Hematommic, acid **4**), 127.5 ± 1.7 (Methyl hematommate, **6**), 138.1 ± 0.3 (Chlorohematommic acid, **5**), 148.4 ± 2.4 (Methyl chlorohematommate, **7**), 164.1 ± 6.2 (Ethyl hematommate, **8**), 186.6 ± 5.4 (Ethyl chlorohematommate, **9**), 250.9 ± 13.7 (Atranorin, **11**), 306.6 ± 18.4 (Chloroatranorin, **12**).

Three samples, lichenic thalli (2 g dry wt.) were extracted with 50 ml of MeOH, 50-ml acetone and 50-ml acetone treated as described above, for 5 hr each with constant shaking at room temperature. After filtration and concentration in a rotavapor at 40°C, the samples were each redissolved in acetone for HPLC (conc. = 1000 ppm). The resulting solutions were qualitatively identical in their behavior in different thin-layer chromatograms on silica gel using *n*-hexane/EtOAc (9:1) or benzene/EtOAc (8.5:1.5), as well as by HPLC. Small quantitative differences were observed, with regard to the extract obtained with treated acetone (Table 2).

Soil phenols were extracted by washing the previously dried earth (*in vacuo*, 40°C, 72 hr) with treated acetone, filtered through Whatman No. 1 paper, evaporating the filtrate to dryness in a rotavapor (40°C), and redissolving the residue in acetone for HPLC (conc. = 1000 ppm). The chemical composition of these extracts proved to be comparable qualitatively to that of the lichenic extract (Table 3).

Extraction of the Allelochemicals by Lixiviation with Rainwater. Lichenic thallus was packed into a glass column (36 × 3 cm. diam.) and rainwater was percolated through at a rate of 2.5 ± 0.5 ml/min. Fractions (30 ml) were collected and filtered through a filtration crucible and extracted with $CHCl_3$. A chromatographic study (TLC and HPLC) of these $CHCl_3$ extracts confirmed the presence of phenols that constitute the herbicidal fraction "Letharal" (Table 4).

TABLE 2. COMPOSITION OF THE ACETONE EXTRACTS OF *Lethariella canariensis*[a]

Phenolic compounds	*L. canariensis**	
	Summer	Winter
(+)-Usnic acid (**13**)	0.01	0.06
Atranol (**2**)	ND	68.28
Chloroatranol (**3**)	ND	3.18
Methyl ß-orsellinate (**10**)	0.71	10.71
Hematommic acid (**4**)	0.08	1.25
Methyl hematommate (**6**)	0.09	0.00
Chlorohematommic acid (**5**)	0.24	2.52
Methyl chlorohematommate (**7**)	0.35	0.00
Ethyl hematommate (**8**)	1.52	1.67
Ethyl chlorohematommate (**9**)	0.70	1.53
Atranorin (**11**)	50.62	6.35
Chloroatranorin (**12**)	45.36	4.00
Others	0.28	0.22

Note: ND = not detected.
[a]% $w/w_{extract}$.

Penetration of Letharal into Seeds. A fixed amount (10 g) of seeds of pepper, cabbage, tomato, and lettuce was placed in to 500-ml Erlenmeyer flasks and covered with 100 g of diatomaceous earth spiked with "Letharal" (0.4%, w/w). Each mixture was covered with a thin layer of sand, 100 ml of rainwater were

TABLE 3. COMPOSITION OF THE ACETONE SOIL EXTRACT[a]

Phenolic compounds	*L. canariensis**	
	Summer	Winter
(+)-Usnic acid (**13**)	0.74	0.15
Atranol (**2**)	ND	4.92
Chloroatranol (**3**)	ND	49.69
Methyl ß-orsellinate (**10**)	2.11	24.63
Hematommic acid (**4**)	0.33	0.14
Methyl hematommate (**6**)	0.00	0.43
Chlorohematommic acid (**5**)	2.40	1.53
Methyl chlorohematommate (**7**)	ND	1.96
Ethyl hematommate (**8**)	3.95	11.41
Ethyl chlorohematommate (**9**)	0.97	0.19
Atranorin (**11**)	87.58	3.95
Chloroatranorin (**12**)	1.35	1.00
Others	0.58	0.00

Note: ND = not detected.
[a]% $w/w_{extract}$.

TABLE 4. COMPOSITION OF THE AQUEOUS EXTRACT OBTAINED BY LIXIVIATION OF *Lethariella canariensis*

Phenolic compounds	% $w/w_{extract}$
(+)-Usnic acid (**13**)	0.04
Atranol (**2**)	83.34
Methyl ß-orsellinate (**10**)	9.91
Hematommic acid (**4**)	0.93
Methyl hematommate (**6**)	3.26
Ethyl hematommate (**8**)	1.15
Atranorin (**11**)	0.68
Chloroatranorin (**12**)	0.51
Others	0.18

added, and the mixture was left to stand for the lethal time (27 days for pepper, 15 days for remaining seeds). The seeds were then separated, washed, dried (*in vacuo*, 40°C), extracted with acetone in a Soxhlet, and quantitatively analyzed by HPLC (Table 5).

Water Solubility of "Letharal". Three hundred milligram (11.914 mmoles for averaged molecular weight, 251.8 mg/mmol) of the phenolic fraction of *L. canariensis* "Letharal" was shaken for 48 hr at ambient temperature with 100 ml of rainwater. The resulting saturated solution was then analyzed by HPLC, which afforded a solubility of 3.0620×10^{-3} mmol/ml for the phenolic mixture. The

TABLE 5. ESTIMATES OF PENETRATION OF *Lethariella canariensis* METABOLITES INTO SEED EXPOSED TO "LETHARAL" (AT LETHAL CONCENTRATION AND TIMES)

Phenolic compounds	p.p.m. (μg of metabolite/g of seed)			
	Cabbage	Lettuce	Pepper	Tomato
(+)-Usnic acid (**13**)	Traces	Traces	Traces	Traces
Atranol (**2**)	97.68	4.22	235.62	6.68
Chloroatranol (**3**)	0.00	0.00	92.08	26.48
Methyl ß-orsellinate (**10**)	49.34	58.44	433.68	58.81
Hematommic acid (**4**)	0.00	0.44	Traces	2.72
Methyl hematommate (**6**)	3.95	0.80	Traces	1.77
Chlorohematommic acid (**5**)	8.38	107.69	47.24	29.79
Methyl chlorohematommate (**7**)	0.00	1.45	0.00	0.00
Ethyl hematommate (**8**)	2.81	15.11	0.71	8.94
Ethyl chlorohematommate (**9**)	1.37	0.00	10.87	0.00
Atranorin (**11**)	Traces	0.00	0.00	0.00
Chloroatranorin (**12**)	0.89	0.00	0.00	0.00
Others	0.57	0.00	0.00	0.00
Totals	164.99	188.15	820.20	135.19

TABLE 6. ESTIMATED WATER SOLUBILITY OF THE COMPONENTS OF THE HERBICIDE "LETHARAL"

Phenolic compounds	(Moles/l $\times 10^6$	% (w/w)
(+)-Usnic acid (**13**)	0.82	0.05
Atranol (**2**)	746.40	20.04
Chloroatranol (**3**)	725.39	23.83
Methyl ß-orsellinate (**10**)	1505.49	52.11
Hematommic acid (**4**)	16.57	0.57
Methyl hematommate (**6**)	1.45	0.05
Chlorohematommic acid (**5**)	6.72	0.27
Methyl chlorohematommate (**7**)	0.93	0.04
Ethyl hematommate (**8**)	1.15	0.05
Ethyl chlorohematommate (**9**)	5.03	0.23
Atranorin (**11**)	1.57	0.10
Chloroatranorin (**12**)	2.82	0.20
Others	47.86	2.25
Total	3062.18	99.79

exact composition of this dissolved fraction as determined by HPLC is shown in Table 6. Remaining undissolved solids ($\approx$ 0.8852 mmol) were recovered by filtration.

Antibacterial Activity. Assays on individual phenolic compounds (Table 7) were done by the agar dilution method as described by Ericsson and Sherris (1971) and the National Committee for Clinical Laboratory Standards (1990). Concentrations of samples tested ranged from 0.25 to 512 μg/ml. Test compounds were added to sterile Mueller–Hinton agar medium as a solution in DMSO. Solvent blanks were included as controls. Antibacterial activities were measured after 24-hr incubation at 37°C. The minimal inhibitory concentration (MIC) (i.e., the lowest concentration of compound that completely prevented growth of the test organisms after incubation for 24 hr at 37°C was determined.

Cell Culture. Human monoblastic leukemia cell line U937 was derived from a patient with diffuse histiocytic lymphoma (Sundstrom and Nilsson, 1976). The human promyelocytic leukemic cell line HL-60 was derived from a patient with

TABLE 7. ANTIBACTERIAL ACTIVITY IN VITRO (MIC; μg/ml)

Test compound	*Staphylococcus aureus* (ATCC 25923)	*Enterococcus faecalis* (ATCC 29212)	*Escherichia coli* (ATCC 25922)	*Pseudomonas aeruginosa* (ATCC 25619)
Atranol (**2**)	256	512	512	>512
Methyl β-orsellinate (**10**)	256	ND*	ND	ND
Atranorin (**11**)	512	ND	ND	ND

* *Note*: ND: not determined.

acute promyelocytic leukemia (Collins et al., 1977; Gallagher et al., 1979). Both cell lines were grown in RPMI 1640 medium (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum supplemented with L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator containing 5% CO_2 at 37°C. Cell numbers were determined by trypan blue exclusion and counting with a hemacytometer, and were kept at 1.5×10^5 cells/ml U937, and HL-60 cultures were passed twice weekly. Over 95% of the cells excluded trypan blue in all experiments. Leukemia cells were washed and resuspended in the above medium to a concentration of 1×10^4 cells/ml. Polyphenols were dissolved in dimethylsulfoxide (DMSO) and stored at −20°C. Further dilutions were made in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 1% (v/v), a concentration that was nontoxic to the cells.

Growth Inhibition. Growth inhibitory activity of the polyphenols was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay (Mosmann, 1983). Cells (1×10^4 per well) were cultured with increasing concentrations (0.1–100 μM) of the different polyphenols in 96-well plates for 72 hr at 37°C. Cells were stained with MTT for 4 hr and incubated with lysis buffer (20% SDS, 0.02 M HCl) for a further 6 hr. Optical density at 570 nm was used as a measure of cell viability. A dose–response curve was plotted for each sample and the concentration giving 50% inhibition (IC_{50}) was calculated using the curve-fitting routine of Prism 2.0 (GraphPad™ CA, USA).

Brain Homogenates. Adult Sprague–Dawley rats were killed by cervical dislocation. The brains were quickly removed, frozen in liquid N_2, and stored at −80°C until homogenate preparation. On the day of the assay, whole brains were homogenized in ice-cold Tris-buffer (50 mM Tris-HCl, pH 7.4) with a Polytron-like stirrer to produce a 1/10 homogenate. Protein concentration was measured (Bradford, 1976) using BSA as the standard.

Lipid Peroxidation. Malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA) are terminal lipid peroxidation chain reaction products, and can be used as an estimate of the degree of lipid peroxidation. Briefly, 400 μl of brain homogenates were incubated for 60 min at 37°C with 150 μM H_2O_2/15μM $FeSO_4$ (final concentration) to induce lipid peroxidation, in absence (control) or presence of the natural phenolic compounds (100–250 μM). Control groups received the same amount of DMSO as those containing the natural products, and the concentration was always below 0.25%. After incubation, the reaction was stopped by placing the homogenates into ice-cold water for 10 min, and then samples were centrifuged at 10,000 × *g* for 10 min at 4°C. The amount of MDA plus 4-HDA in the supernatants was determined by using a lipid peroxidation kit (Calbiochem, La Jolla, CA) that contained a chromogenic reagent (*N*-methyl-2-phenylindole) that reacts with MDA and 4-HDA at 45°C yielding a stable chromophore with a maximal absorbance at the 586-nm wavelength.

RESULTS

Phytotoxicity. The composition of the "Letharal" fraction is specified in Table 2. Seed germination of cabbage, lettuce, pepper, and tomato is plotted in Figures 2, 4, 5, and 6. The three seeds most resistant to the herbicide mixture (cabbage, pepper, and tomato) were sown and grown under simulated, alternating nights/days (12 hr of darkness at 5°C, 12 hr of light at 20°C) on Celite spiked with the same concentrations of the phenolic fraction described earlier, affording the percentage of germination presented in Figures 3–6.

Although the seeds of some plants are more resistant than others, at concentrations of 1.1% of the phenolic fraction "Letharal," inhibition of germination is total at the constant temperature of 20 ± 2°C. Germination observed at concentrations lower than 1.1% generated a diseased population that withered after a few days. The LD_{50} (in % w/w) was 0.84 for tomato, 0.83 for pepper, 0.72 for cabbage, and 0.24 for lettuce, values that are greater than the total phenolic concentration

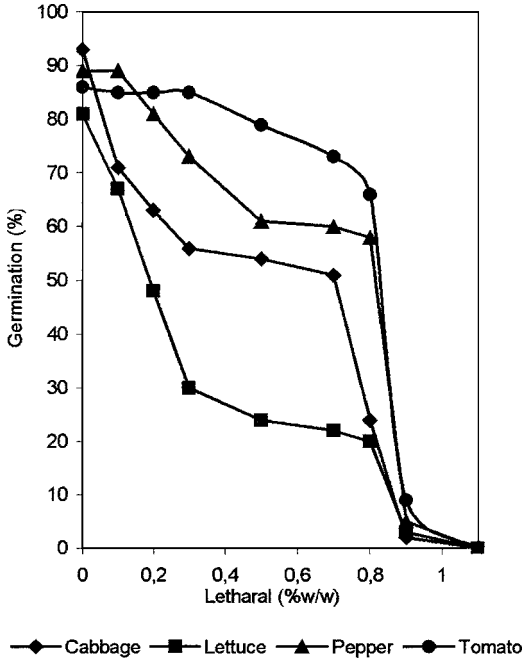

FIG. 2. Phytotoxicity of "Letharal" at constant temperature (20 ± 2°C). The "Letharal" fraction was applied to diatomaceous earth at different concentrations, seed germination was measured, and plotted as percentage of germination. The "Letharal" composition corresponds to the mixture in Table 1 (winter).

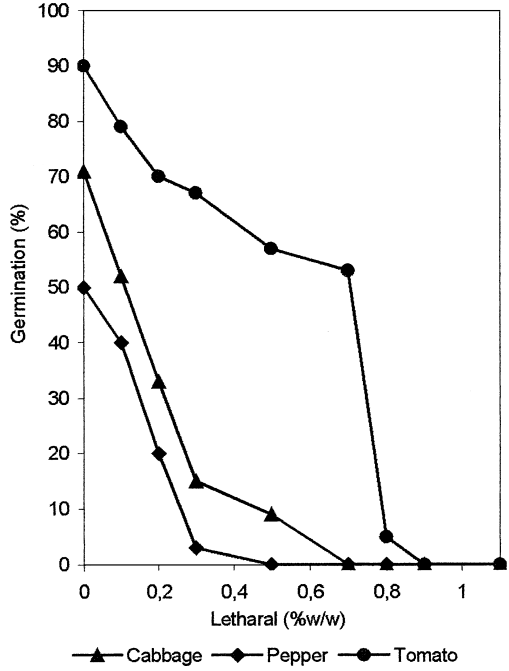

FIG. 3. Phytotoxicity of "Letharal" at alternate temperatures. The "Letharal" fraction was applied to diatomaceous earth at different concentrations and exposed to simulated day and night temperatures (12 hr light at $20 \pm 2^{\circ}$C and 12 hr darkness at $5 \pm 1^{\circ}$C). Seed germination is plotted as a percentage of germination. The "Letharal" composition corresponds to the mixture in Table 1 (winter).

found in land colonized by *L. canariensis* (~0.2%, w/w). However, the dramatic decrease in temperature that takes place periodically in nature during nocturnal frosts increased the phytotoxicity of this mixture by some 17–75%, reaching total inhibition of germination at concentrations of 0.9, 0.7, and 0.5% for tomato, cabbage, and pepper, respectively (Figure 3):

$$\%I = \frac{G_1 - G_2}{G_1} \times 100$$

where $I =$ Inhibition of germination attributable to nocturnal frosts, $G_1 =$ Germination at $\text{Temp}_{\text{constant}}$ ($20 \pm 2^{\circ}$C) and concentration of "Letharal," and $G_2 =$ Germination at $\text{Temp}_{\text{variable}}$ (simulating nocturnal cooling at the same concentration of "Letharal").

The LD_{50} (in % w/w) descended to 0.73 for tomato, 0.19 for cabbage, and 0.17 for pepper. Note that these last two seeds present LD_{50} below the phenolic concentration found in the habitat of *L. canariensis* (~0.2% w/w).

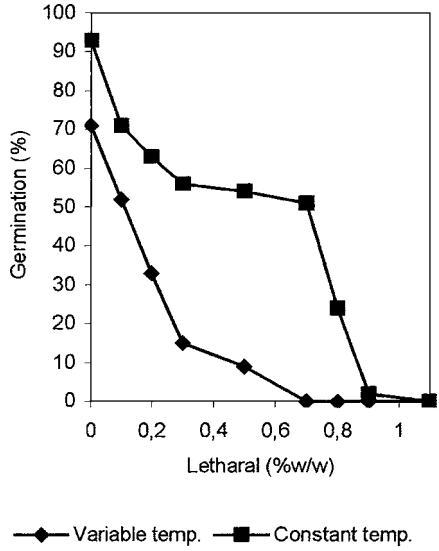

FIG. 4. Phytotoxicity of "Letharal" on a cabbage seed bed at constant ($20 \pm 2^{\circ}$C) and variable (12 hr light at $20 \pm 2^{\circ}$C and 12 hr darkness at $5 \pm 1^{\circ}$C) temperature. The "Letharal" composition corresponds to the mixture in Table 1 (winter).

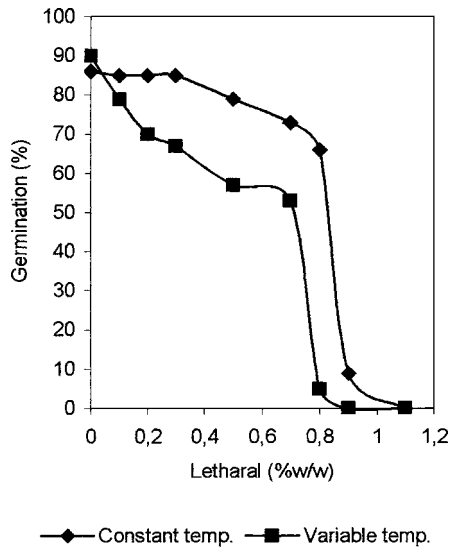

FIG. 5. Phytotoxicity of "Letharal" on a tomato seed bed at constant ($20 \pm 2^{\circ}$C) and variable (12 hr light at $20 \pm 2^{\circ}$C and 12 hr of darkness at $5 \pm 1^{\circ}$C) temperature. The "Letharal" composition corresponds to the mixture in Table 1 (winter).

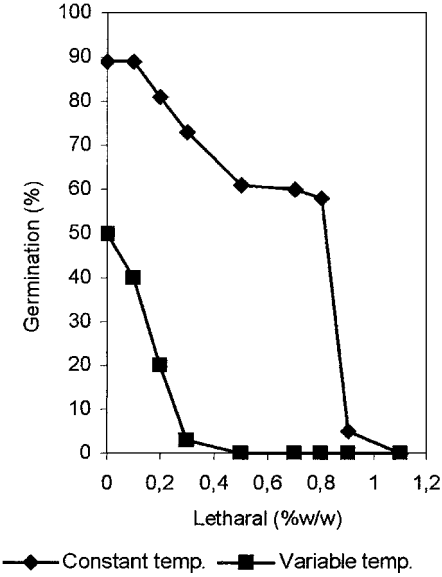

FIG. 6. Effect of "Letharal" on pepper seed germination at constant ($20 \pm 2^{\circ}$C) and variable (12 hr light at $20 \pm 2^{\circ}$C and 12 hr of darkness at $5 \pm 1^{\circ}$C) temperature. The "Letharal" composition corresponds to the mixture in Table 1 (winter).

Origin of "Letharal". Since the extracting solvents generally bear variable amounts of weak nucleophiles, the question arose as to whether or not compounds **2**–**10** were artifacts formed by alcoholysis–hydrolysis–decarboxylation of the authentic metabolites, the depsides **11** and **12**. Although these reactions are documented (Hylands and Ingolfsdottir, 1985), the following experiments were carried out to further investigate the origin of "Letharal."

Cold extraction of the lichen with acetone for analysis (0.01% MeOH, 0.01% EtOH, 0.2% H_2O) gave an extract that was analyzed by HPLC every 2 weeks. Slow chemical transformations were observed over 3 months. The rate of transformation of both depsides **11** and **12** in the acetone solution proved to be very low (3.20×10^{-6} and 2.05×10^{-6} moles/(1 day), respectively). These transformations were also studied in a sample of the extract spiked with catalytic amounts of *p*-toluenesulfonic acid, but no acceleration of the process was noted. However, when the acetone was replaced with methanol, transformation processes proceeded at a greater rate (Table 8).

Reextraction of the previously dried lichen with treated acetone (alcohol-delivered, acid-delivered, and water-delivered), under an N_2 atmosphere, gave an extract with variable amounts of all the compounds described being found (Table 2). This cannot be explained by a simple process of alcoholysis–hydrolysis–decarboxylation of depsides **11** and **12** during the extraction, which should fulfil

TABLE 8. MEAN RATE OF *Lethariella canariensis* METABOLITES DECOMPOSITION, mol/l/day ($\times 10^6$)

	Compound					
	11	**12**	**4**	**5**	**8**	**9**
Autocatalyzed in acetone	3.20	2.05	—	—	—	—
Catalyzed with *p*-TsOH in acetone	3.85	2.21	—	—	—	—
Autocatalyzed in methanol	5.38	6.23	4.44	4.44	2.00	2.12

the following balance of masses:

$$\text{A-C} \longrightarrow A_1 + A_2 + A_3 + A_4 + \text{C}$$

$$\text{B-C} \longrightarrow B_1 + B_2 + B_3 + B_4 + \text{C}$$

$$[A_1] + [A_2] + [A_3] + [A_4] + [B_1] + [B_2] + [B_3] + [B_4] = [\text{C}](\text{mol/l})$$

A-C = Atranorin **11**, B-C = Chloroatranorin **12**, A_1 = Methyl hematommate **6**, A_2 = Ethyl hematommate **8**, A_3 = Atranol **2**, A_4 = Hematommic acid **4**, B_1 = Methyl chlorohematommate **7**, B_2 = Ethyl Chlorohematommate **9**, B_3 = Chloroatranol **3**, B_4 = Chlorohematommic acid **5**, C = Methyl ß-orsellinate **10**.

The following amounts were actually observed (in mol/l ($\times 10^6$)) and it was deduced, therefore, that the monocyclic phenols **2**–**10** are produced enzymatically within the organism and are authentic metabolites of *Lethariella canariensis*.

In the summer extract: 3.8227 (A_1) + 12.5303 (B_1) + 59.7114 (A_2) + 23.8862 (B_2) + 0.0000 (A_3) + 0.0000 (B_3) + 3.4097 (A_4) + 9.0738 (B_4) = 112.4341, C = 31.7966

In the winter extract: 0.0000 (A_1) + 0.0000 (B_1) + 39.5326 (A_2) + 31.3634 (B_2) + 2380.9705 (A_3) + 90.5104 (B_3) + 33.6735 (A_4) + 58.1826 (B_4)= 2634.2330, C = 289.5828

Acid hydrolysis with heating of a pure sample of atranorin **11** yielded methyl ß-orsellinate **8** and atranol **2** in a 1:1 ratio (also with traces of hematommic acid **4**).

These experiments led to the following conclusions. First, the *heated* extraction with unpurified solvents by weak nucleophiles (H_2O, R—OH) is unsuitable because it gives way to multiple transformation products, quickly reaching a situation of thermodynamic equilibrium that over time is irreversibly displaced (—CO_2) towards atranol **2**, chloroatranol **3**, and methyl ß-orsellinate **10**, hematommic acids **4** and **5** being intermediates in the said process. Second, that despite atranorin **11** and chloroatranorin **12** undergoing a chemical process of hydrolysis and transesterification with alcohols and with heating, the monocyclic molecular fragments already existed in varying amounts in the thallus of *L. canariensis* (Table 2), and,

thus, compounds **2**–**13** should be considered as true metabolites, whose concentrations fluctuate with climatic conditions. As environmental moisture increases (February), production of hydrosoluble molecular fragments increases (mainly methyl ß-orsellinate **10**, atranol **2**, and chloroatranol **3**) and the depsides atranorin **11** and chloroatranorin **12** are reduced in concentration since they are extremely insoluble (Table 6). These results indicate that enzymatic activity in the lichen is different in periods of drought (enzymes accumulate in the thallus and mainly free the two depsides **11** and **12** into the environment) and rain (when the hydrosoluble monocyclic fragments **2**–**10** are mainly produced).

Diffusion of Phenolics. To provide supporting evidence for our model, we carried out a study of the diffusion of these metabolites into the environment. A simulation was carried out by washing the thallus of the lichen with rainwater, followed by verification of the washings contents by HPLC (Figure 7), thereby demonstrating that water does indeed flush the phenolic metabolites from the thallus of *L. canariensis* by lixiviation (Table 4).

The acetone soil extract (from 0.02 to 0.20% of total organic matter) proved to be rich in the same phenols (Table 3) and revealed two important facts. First, that one of the soil components is (+)-usnic acid (0.15–0.74% of the total organic matter), a metabolite having known phytotoxic activity (Dalvi et al., 1972). Second, that in times of drought (July), depsides abound in the soil (88.93% of the total organic matter), while their monocyclic molecular fragments are greater during rainy weather (February) (94.90% of total organic matter).

Solubility of these metabolites in water was also affected (Table 6). Scant solubilization of the depsides atranorin and chloroatranorin ($0.1 + 0.2 = 0.3\%$) can be seen versus that of their monocyclic fragments atranol, chloroatranol, and methyl ß-orsellinate ($20.0 + 23.8 + 52.1 = 95.9\%$).

The penetration of phenolic metabolites of "Letharal" into seeds exposed to lethal concentration also over lethal times was also studied (Table 5). Although all the components of the herbicide mixture are capable of crossing the cell walls of seeds, the small hydrosoluble monocyclic fragments are the most efficient. Thus, the depsides **11** and **12** were only detected in cabbage seeds, where they constituted only 0.54% of the total "Letharal" contaminating them. In contrast, the monocyclic phenols **2**–**10** were detected in all the seeds assayed and constituted 99.46–100% of the total "Letharal" that penetrated them.

Antibacterial Activity. Antimicrobial assays (Table 7) indicated that different components of "Letharal" had moderate antibacterial activity (minimal inhibitory concentration, MIC, ≤ 512 μg/ml), which accounts for the fact that these lichenic residues persist in the medium by overcoming natural microbial degradation. Atranol, methyl β-orsellinate, and atranorin were assayed against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Atranol and methyl β-orsellinate were more active against *Staphylococcus aureus*. However, in general, they were weakly active against the tested bacteria.

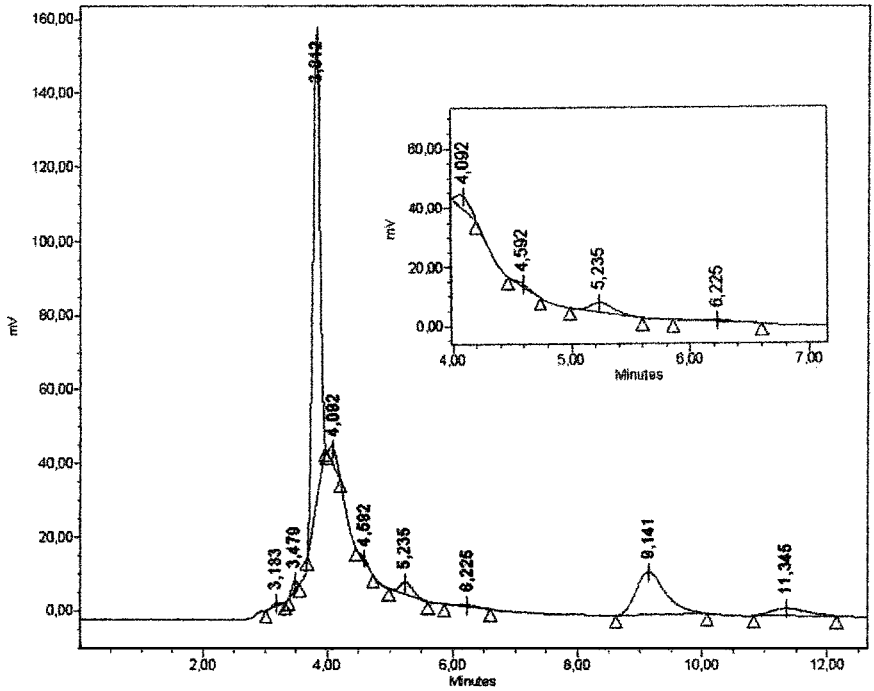

FIG. 7. HPLC chromatogram of "Letharal." (+)-Usnic acid (3.183), atranol (3.479), chloroatranol and methyl β-orsellinate (3.183), hematommic acid (4.092), chlorohematommic acid (4.592), ethyl hematommate (5.235), ethyl chlorohematommate (6.225), atranorin (9.141), and chloroatranorin (11.245). μ-Bondapack-C18, AcOH/H_2O/MeOH (0.4:19.6:80.0 v/v/v), 0.8 ml/min., 80 atm., 25°C, UV (254 nm). Chloroatranol and methyl β-orsellinate were resolved with AcOH:H_2O:MeOH (0.8:39.2:60.0 v/v/v) as mobile phase.

Cytostatic Activity. Cytostatic properties of the isolated polyphenols were evaluated on human monocytic leukemia cell lines U937 and HL-60. The cells were continuously treated with each sample for 72 hr, and cell growth was measured by an MTT assay procedure. Most polyphenols inhibited the proliferation of U937 and HL-60 cells, with the exceptions of **10** and "Letharal." Their cytotoxic potencies expressed as IC_{50} values are shown in Table 9. The U937 cells proved to be more sensitive than HL-60 cells to the cytotoxic action of the polyphenols tested and compound **3** was almost 10 times more potent in U937 cells than HL-60 cells (IC_{50} 8.8 μM vs. 78.7 μM). This was not the case of **13** whose IC_{50} was identical (14.3 μM) in both cell lines.

Antioxidative Activity. When brain homogenates were incubated with 150 μM H_2O_2 plus 15 μM $FeSO_4$, a sevenfold increase in MDA + 4-HDA levels was obtained as compared with control groups (Figure 8). The effects of polyphenols

TABLE 9. ANALYSIS OF CELL VIABILITY IN POLYPHENOLS-TREATED U937 AND HL-60 CELLS BY MTT ASSAY

Compound	IC_{50} (μM)	
	U937 cells	HL-60 cells
2	37.6 ± 1.6	>100
3	8.8 ± 3.7	78.7 ± 5.0
6	26.2 ± 6.7	39.8 ± 5.0
8	74.1 ± 13.9	100.0 ± 10.0
6(29%) + 8(71%)	31.7 ± 3.5	47.6 ± 13.2
10	ND	ND
11	>100	83.4 ± 6.7
13	14.3 ± 0.1	14.3 ± 0.1
Letharal	ND	ND

Note: Cells were placed in 96-well plates with increasing concentrations (0.1–100 μM) of the indicated compounds for 72 hr. MTT was added to the medium for an additional 4 hr. The viability of cells was detected by measuring the absorbance at a wavelength of 570 nm and the concentration giving 50% inhibition (IC_{50}) was calculated with computer software. Results are the mean ± SEM of three different experiments. ND- not determined, i.e., cell viability was not affected even at the highest used dose.

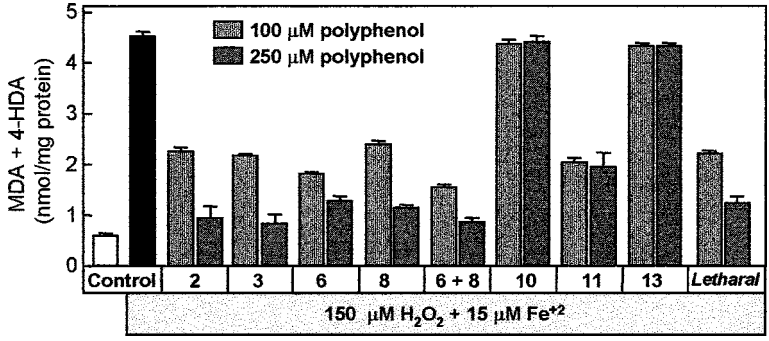

FIG. 8. Effect of polyphenols on the level of lipid peroxidation induced by H_2O_2/Fe^{+2} in brain homogenates. Samples were incubated with 150 μM H_2O_2/15 μM Fe^{+2} for 60 min at 37°C in absence (control) or presence of the indicated concentration of isolated polyphenols. The amount of MDA plus 4-HDA was determined by a colorimetric method as described in "Methods and Materials." The basal level of MDA + 4-HDA (control) was determined from samples incubated at 4°C in the absence of any stimulus. Results are shown as mean ± SE of a representative experiment with four replicates. "**6** + **8**" corresponds to the mixture of **6** (29%) and **8** (71%) and "Letharal" corresponds to the composition of the mixture in Table 1 (winter).

under study on the H_2O_2/Fe^{+2} induced lipid peroxidation was tested using two different concentrations, 100 and 250 μM. Compounds **2**, **3**, **6**, **8**, (**6** + **8**), **11**, and "Letharal" exhibited antioxidant activity that was dose-dependent (Figure 8). At the lower dose used (100 μM), compounds **6** and **6** + **8** were the most active with 66 and 60% inhibition in the MDA + 4-HDA formation, respectively. Percentages of inhibition in the presence of **2**, **3**, **8**, **11**, and "Letharal" was about 50%. At the higher concentration (250 μM), the percentages of inhibition on lipid peroxidation were about 70% with **6** and **8**, and 80% in the presence of **2**, **3**, and **6** + **8**. At this concentration, compound **11** was the least active (57% inhibition), while compounds **10** and **13**, even at the higher dose used, were unable to prevent lipid peroxidation.

DISCUSSION

Despite the fact that the depsides atranorin and chloroatranorin undergo hydrolysis/transesterification, the monocyclic molecular fragments resulting from these processes are present in varying amounts in the thallus of *L. canariensis* and should be considered as true stress metabolites whose concentrations fluctuate with the climatic conditions. In particular, the depsides accumulate in summer, whereas environmental humidity increases in winter and enhances the production of the molecular fragments (principally methyl-ß-orsellinate, atranol, and chloroatranol). Rainwater carries the phenolic metabolites by lixiviation into the soil, where they accumulate, making up 88.93–94.90% of the total organic matter (0.20%, w/w). There, they are redissolved in water and penetrate seeds, where they can interfere with the germination process. A soil concentration of 1.1% (w/w) of the above-mentioned phenolic fraction "Letharal" is sufficient to cause total inhibition of germination at a constant temperature (20°C), while the germination observed at lower concentrations produces a diseased population that withers within a few days. The LD50s (in % w/w) are presented for the seeds assayed in the interval 0.24–0.84, which values are greater than the total phenolic concentration present in the soil colonized by *L. canariensis* ($\sim$0.20%). However, the drastic periodic decrease in temperature (simulating the nocturnal frosts in nature) increased the allelopathic activity by 17–75%, which resulted in total inhibition of germination being observed at concentrations of 0.50–0.90% (w/w), with LD_{50} even below the environmental phenolic concentration (0.19% for cabbage and 0.17% for pepper).

Oxidative stress can damage many biological molecules such as proteins, DNA, cell membrane lipids, etc., that are significant targets of cellular injury (free radical attack). Plant polyphenols are well recognized for their antioxidative activities. They scavenge free radicals, thus breaking the free radical chain reaction of lipid peroxidation. The antioxidant activity of phenolics has been measured using a lipid peroxidation assay. The isolated metabolites have strong activity

against lipid peroxidation in the rat brain homogenate test. At least one type of resistance against oxidative stress, as well as ultraviolet (UV) radiation, could be associated with the polyphenolic compounds present in "Letharal," protecting the lichen from oxidative damage. Reactive oxygen species, ROS (H_2O_2, $HO^{\cdot}$, $HOO^{\cdot}$, $O_2^{-\cdot}$, etc.) are generated by UV light, and are initiators of the free radical chain process. Therefore, these polyphenols present in *L. canariensis* afford an inhibition of both ROS and free radicals. This fact is important from an ecological point of view because sunlight irradiation is intensive in the Canary Islands (12 hr/day). In addition, the majority of the compounds was found to exhibit low cytotoxicity against U937 and HL-60 cells.

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EVALUATION OF ROOT EXUDATES OF SEVEN SORGHUM ACCESSIONS

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Abstract—Seven sorghum accessions were evaluated quantitatively and qualitatively for the composition of their root exudates. Utilizing a unique capillary mat growing system, root exudates were collected from all sorghum accessions. Exudates were subjected to TLC and HPLC analysis to evaluate their chemical composition. Within each sorghum accession, variation existed in the amount of exudate produced and the chemical constituents of each exudate. Sorgoleone was the predominant constituent identified in each accession's exudate. Other closely related compounds, including 5-ethoxysorgoleone, 2,5-dimethoxysorgoleone, three other minor components (MW = 364, 388, and 402), and one unidentified component comprised the minor constituents of the root exudate. Our past work has shown that several of these compounds have potent phytotoxic activity as photosystem II inhibitors, thereby lending further support to the concept that *Sorghum* spp. are allelopathic and weed suppressive.

Key Words—Allelopathy, forage sorghum, grain sorghum, johnsongrass, shattercane, sorgoleone, 5-ethoxysorgoleone, 2,5-dimethoxysorgoleone.

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INTRODUCTION

The allelopathic properties of sorghum have been known for some time, and its ability to suppress weeds is well documented (Guenzi and McCalla, 1966; Guneyli et al., 1969; Forney et al., 1985; Einhellig and Rasmussen, 1989; Weston et al., 1989). Residues of sorghum–Sudangrass hybrid cover crops provide short-term weed suppression (Forney et al., 1985; Geneve and Weston, 1988), which appears to be due to phenolic compounds released from decomposing sorghum shoot residues (Guenzi and McCalla, 1966; Weston et al., 1989). Most of these phenolic compounds are relatively simple in structure and are quickly metabolized, once in the soil rhizosphere. Netzly and Butler (1986) discovered that sorghum produced root exudate containing phytotoxic chemicals. The exudate was reported to contain two major components, which were identified as 2-hydroxy-5-methoxy-3-[(8′Z,11′Z)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone (sorgoleone) and its reduced *p*-hydroquinone form also called xenognosin (Chang et al., 1986). Seeds from the parasitic witchweed (*Striga asiatica*) can be stimulated to germinate with xenognosin rates as low as 2×10^{-7} M (Fate et al., 1990). Since that period, much work has been done to evaluate the phytotoxic activity of sorgoleone (Einhellig and Souza, 1992; Einhellig et al., 1993; Nimbal et al., 1996b; Gonzalez et al., 1997). With the exception of the identification of 5-ethoxysorgoleone (Rimando et al., 1998) in *Sorghum bicolor* root exudates, little is known about the other minor chemical components of sorghum root exudates, or about the exudate production abilities of related sorghum species. Nimbal et al. (1996a) evaluated the root exudates of 25 accessions of *Sorghum bicolor* and sorghum–Sudangrass hybrids, and found differences in the production levels of root exudates among sorghum accessions. However, large quantities of exudate were not available for further evaluation. A capillary mat system for the collection of large quantities of root exudate was subsequently developed (Czarnota et al., unpublished data), which allowed an accurate comparison of the root exudation over a diverse collection of sorghum accessions. With large quantities of exudates being generated, it is also possible to investigate the minor chemical constituents in the exudates. This study was designed to evaluate root exudate composition and production ability of seven genetically diverse sorghum accessions.

METHODS AND MATERIALS

Plant Material. Seven genetically diverse sorghum accessions were chosen and included the following: two weed species, shattercane (*Sorghum bicolor* (L.) Moench, Azlin Seed Service, Leland, MS) and johnsongrass (*Sorghum halepense* (L.) Pres., Azlin Seed Service, Leland, MS); two cultivated forage type sorghum–sudangrass hybrids, SX-15 and SX-17 (*Sorghum bicolor* × *sudanense*, Monsanto Company, St. Louis, MO); two cultivated grain sorghum species, 8446 and 855F

(*Sorghum bicolor* (L.) Moench, Pioneer Hi-Bred International, Inc., Des Moines, IA); and a cultivated sweet sorghum, Della (*Sorghum bicolor* cv. Della, Southern States Cooperative, Inc., Richmond, VA); Root exudate from each was collected utilizing the capillary mat production system described by Czarnota et al. (unpublished data). Two separate but identical collection systems were constructed at Cornell University. Each system contained six individual screens (28 × 43 cm), and each screen was considered a replication. Exudates were collected for each accession using 12 screens (six from each separate exudate collection system).

Seed Processing. With the exception of johnsongrass, approximately 1000 g of seed from each sorghum accession were surface sterilized. Seed treatment involved wrapping each seed lot in a double layer of cheesecloth (50 × 50 cm), and soaking in a 20% sodium hypochlorite solution for 20 min. Seeds were removed from the solution and placed under a continuous flow of cold tap water for 10 min. After removal from the cheesecloth, seeds were spread on a piece of Versi-Dry super absorbent underpad (Fisher Scientific, Suwanee, GA) and allowed to dry at room temperature for 24–48 hr. Seed lots were refrigerated at 4°C until use. In preliminary studies, johnsongrass seed had a much lower germination percentage than other sorghum accessions. A specific protocol was, therefore, designed to attain greater and more uniform germination. Approximately 2000 g of johnsongrass seed were placed into a 18 l bucket containing cold tap water. Seeds were hand-mixed to ensure complete and uniform wetness, and allowed to sit for 5 min. Two distinct seed layers formed. Floating seeds were skimmed off with a strainer and discarded. This process is commonly practiced with horticultural crops to remove nonviable seed. The bottom layer of seeds, which was found to have a higher germination percentage, was collected by sieving through two layers of cheesecloth (50 × 50 cm), and subjected to same seed treatment and drying procedures used for the other accessions.

Root Exudate Collection. For each accession, with the exception of johnsongrass, 300 g of seed were placed on each of the 12 screens. With the contamination problem of johnsongrass, only 100 g of seed were required per screen for uniform screen coverage. Seeds were allowed to germinate on the capillary mat system for 5 days. During this time, the root systems developed and penetrated the screens. At 5 days, roots were harvested. The slower-growing Johnsongrass required a 10–14-day growth period to obtain a harvestable root system. At harvesting, roots were shaved from the bottom of the screen with a paint scraper containing a single-edged razor blade. Once harvested, they were placed in a 1000-ml beaker containing 500 ml of methylene chloride (acidified with 0.25% glacial acetic acid) for approximately 2 min. Using forceps, roots were removed from the methylene chloride solution, allowed to dry for approximately 5 min at room temperature, and weighed to determine fresh weights. The methylene chloride extract was stored under refrigeration at 4°C. This procedure was repeated for all 12 replicates of each accession. The solvent extract, generally a golden brown color, was filtered

using a glass syringe fitted with a Gelman Acrodisc CR-PTFE 0.2-μm filter and evaporated to dryness using a Büchi® rotary evaporator model RE-111 with water bath at 40°C. Dry weight of the methylene chloride extract was obtained separately for each replicate sample. The solvent extract can turn from golden brown to violet purple (Netzly and Butler, 1986) and this color shift begins to occur at pH 5.7–5.8. At the completion of the study, exudate replicates from each collection system were combined, yielding two samples per sorghum accession, one from each collection system. Both samples were subjected to chemical analyses.

Thin Layer Chromatography Analysis (*TLC Analysis*). TLC analysis of root exudate of the seven sorghum accessions was performed on an aluminum-backed silica gel 60 F_{254} plate, 0.2 mm thickness, using 96% hexane/4% isopropyl alcohol as the developing solvent. Dried exudates were dissolved in methylene chloride (1 mg/100 μl), and 1 μl of each sample was applied to the plate. Samples of sorgoleone, 4,6-dimethoxy-2-[(8′Z,11′Z)-8′,11′,14′-pentadecatriene] resorcinol, and the other minor constituents were also spotted on the same plate for qualitative analysis. The plate was visualized under UV light at 254 nm (Figure 1).

High Pressure Liquid Chromatography (*HPLC Analysis*). A general survey of the sorghum root exudates (two per accession) was performed by HPLC (see Figure 2). A 1 mg/ml solution of each sample was prepared in 65% acetonitrile/35% water acidified with 2.5% acetic acid. Johnsongrass samples, which were not completely soluble in this solvent mixture, were subsequently dissolved in 75% acetonitrile/25% methylene chloride. Samples were subjected to HPLC analysis with a Waters 2690 Alliance HPLC equipped with a reverse phase C18 column (Novapak, 3.9 × 150 mm). The mobile phase was 75% acetonitrile and 25% water acidified with 2.5% acetic acid with a flow rate of 2 ml/min. Sorgoleone and related compounds in the root exudate were detected using a Waters Photodiode array detector. Analysis of the constituents of the exudates was also performed by HPLC, independently, using an Hewlett Packard 1050 system (see Figure 3 for representative chromatograms). Samples were dissolved in acetonitrile (1 mg sample/1 ml of acetonitrile), and 20 μl were injected. Components of the root exudates were separated using a 3 μ Alltech EPS C18 column 100 Å (150 mm length × 4.5 mm internal diam.). The mobile phase was eluted at a flow rate of 2 ml/min as follows (solvent A is 2.5% acetic acid in water, solvent B is acetonitrile): 0–15 min 45% solvent A/55% solvent B isocratic; 15–22 min linear gradient from 55 to 100% solvent B; 22–25 min 100% solvent B; 25–26 min 100–55% solvent B; 26–30 min 45% solvent A/55% solvent B isocratic. The array detectors on both systems were set at 280 nm.

RESULTS AND DISCUSSION

Root Exudate Collection. The cultivated sorghum accessions yielded similar quantities of exudate, approximately 2 mg of root exudate per gram of fresh root weight (Table 1). The two weedy species produced markedly different

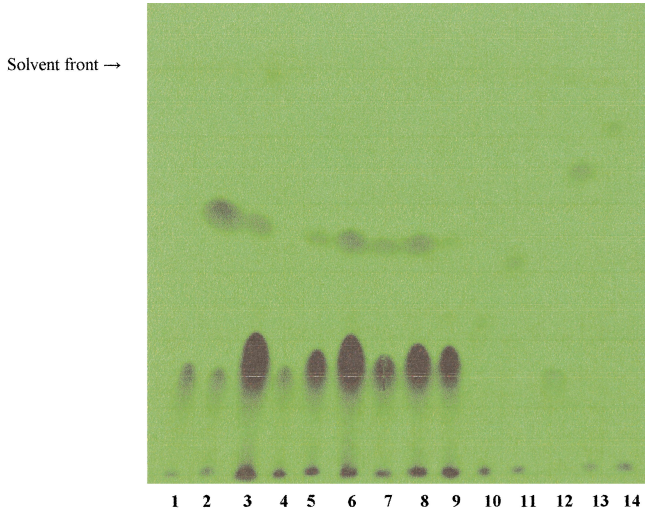


FIG. 1. Thin layer chromatogram of root exudate of sorghum accessions and compounds isolated from the root exudate. (1) sorgoleone, R_f 0.24, (2) 4,6-dimethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol, R_f 0.63, showing sorgoleone as a contaminant, (3) shattercane, (4) Johnsongrass, (5) SX-17, (6) SX-15, (7) 8446, (8) 855F, (9) Della, (10) ethoxysorgoleone, R_f 0.38, (11) Dimethoxysorgoleone, R_f 0.52, (12) 2-hydroxy-5-methoxy-3-pentadecyl-*p*-benzoquinone, R_f 0.24, (13) 4-methoxy-6-ethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol, R_f 0.73, and (14) 4-hydroxy-6-ethoxy-2-[(10′*Z*,13′*Z*)-10′,13′,16′-heptadecatriene]resorcinol, R_f 0.84.

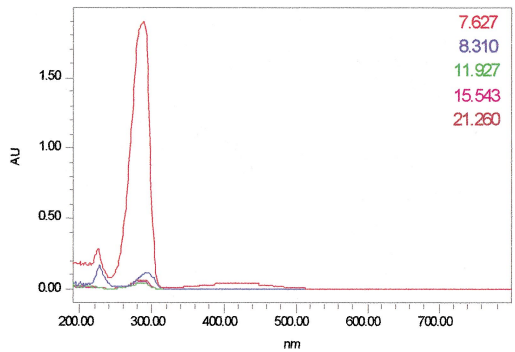


FIG. 2. UV absorption spectra overlays showing the similarities in UV absorption among the constituents in the root exudates of accession 8446 (λ 280 nm): 7.627 min, 4,6-dimethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol; 8.310 min, sorgoleone; 11.927 min, ethoxysorgoleone; 15.548 min, dimethoxysorgoleone; 21.260 min, 2-hydroxy-5-methoxy-3-pentadecyl-*p*-benzoquinone.

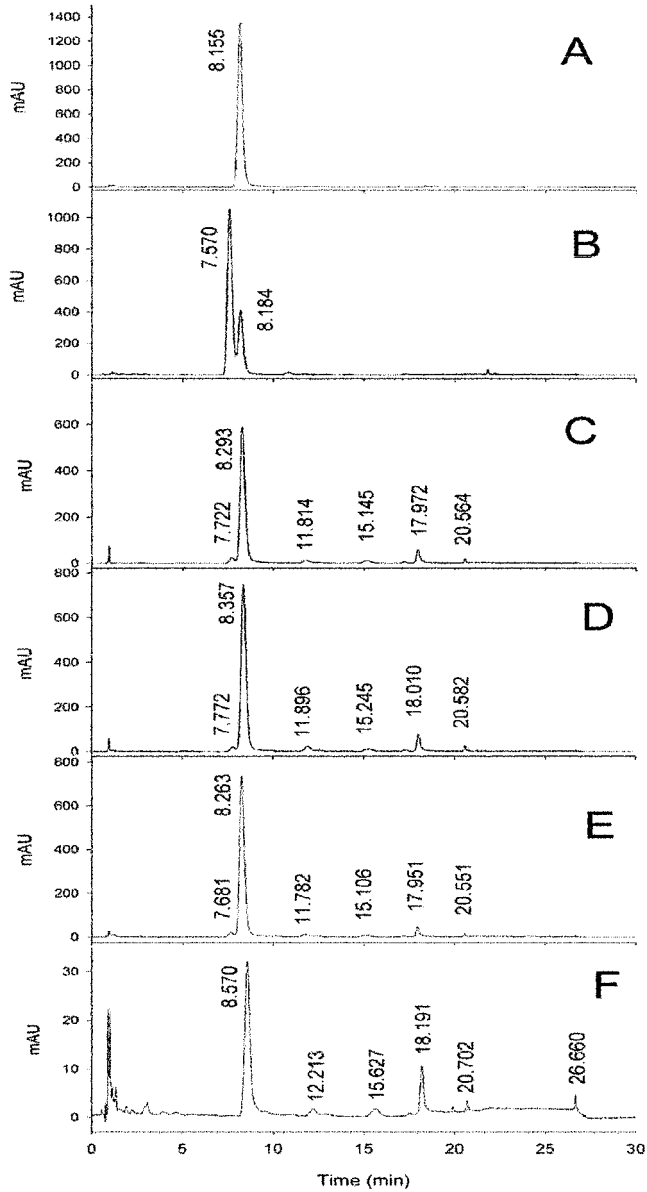


FIG. 3. HPLC chromatogram of (A) sorgoleone, (B) 4,6-dimethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol showing minor peak of sorgoleone, (C) 8446, (D) SX-17, (E) Shattercane, and (F) Johnsongrass (Hewlett Packard 1050 system, Alltech EPS C18 column 100 Å, 150 × 4.5-mm i.d.; see text for full chromatographic method).

TABLE 1. ROOT EXUDATE COLLECTED FROM SEVEN SORGHUM ACCESSIONS

Sorghum accession	Average exudate production	
	Exudate (mg/g root fresh weight)[a]	Standard deviation
Shattercane	0.50	±0.35
Johnsongrass	14.75	±6.56
SX-15	1.33	±0.74
SX-17	1.55	±0.19
8446	1.62	±0.18
855F	1.81	±0.38
Dellacane	1.85	±1.29

[a] Average is based on 12 replicates, six collected from each of the two systems.

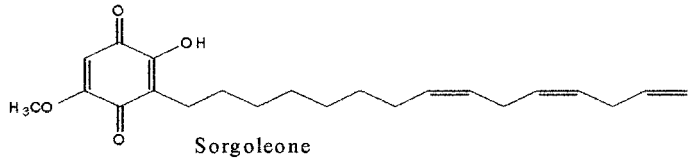
levels of exudate. Johnsongrass yielded 14.8 mg root exudate per gram of fresh root weight, while shattercane yielded 0.5 mg root exudate per gram of fresh root weight. Although the cultivated accessions had similar exudate-producing potential, johnsongrass produced far more when grown under identical environmental conditions. Using a different root exudate collection process, Nimbal et al. (1996b) also indicated differences in root exudate production among different sorghum accessions grown under the same conditions. The difference in exudate production among sorghum accessions indicates that genetic diversity for exudate production exists, especially among wild ancestors of cultivated sorghum. Preliminary studies have shown that selected populations of rhizobiontic bacteria selectively utilize sorgoleone as a carbon source (Henry, 2000). Although the exact roles of sorghum root exudates in the rhizospere have not been elucidated, they may play roles in plant defense against other plants, soil microflora, or microfauna. Root exudates have been reported to foster nutrient uptake directly or indirectly through the enhancement of soil microflora growth (Bar-Yosef, 1996; Marschner and Romheld, 1996). Further studies with sorghum are required to validate these hypotheses. Differences in production of root exudate levels may be related to a species' ability to exert allelopathic effects in field conditions. Cultivated sorghums are currently utilized as cover crops or green manures for weed suppression (Weston, 1996). Johnsongrass typically establishes over time in dense patches or monocultures, and is also reported to possess strong allelopathic activity (Abdul-Wahab and Rice, 1967; Holm et al., 1991).

TLC Analysis. The exudates of the seven sorghum accessions displayed similar TLC profiles, showing two distinct spots with R_f values of 0.33 and 0.66, corresponding to sorgoleone (Lane 1) and 4,6-dimethoxy-2-[8′Z,11′Z]-8′,11′,14′-pentadecatriene]resorcinol (Lane 2), respectively (Figure 1). The root exudate of Johnsongrass (Lane 4) showed only a faint spot of sorgoleone compared to the other accessions, and none of the resorcinol. Of the minor constituents, ethoxysorgoleone

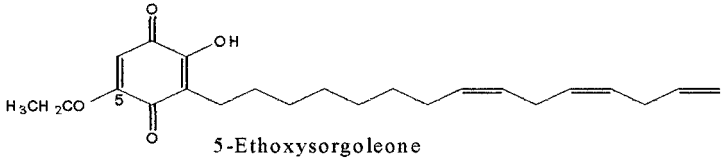
(Lane 10) was faintly detectable in most of the accession exudates. It did not appear in Lanes 4 (Johnsongrass), 5 (SX-17), and 7 (8446); however, 5-ethoxysorgoleone was detectable in HPLC chromatograms. The other constituents were present in small quantities and were not visible on the TLC plates. As mentioned, 5-ethoxysorgoleone (Lane 10) and 2,5-dimethoxysorgoleone (Lane 11) have been previously identified in sorghum exudate (Rimando et al., 1998). Structural characterization by spectroscopic means and activities of the other minor constituents [2-hydroxy-5-methoxy-3-pentadecyl-*p*-benzoquinone, MW 364 (Lane 12); 4-methoxy-6-ethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol, MW 388 (Lane 13); and 4-hydroxy-6-ethoxy-2-[(10′*Z*,13′*Z*)-10′,13′,16′-heptadecatriene] resorcinol, MW 402 (Lane 14)] have been reported recently (Rimando et al., 2001, 2003). Minor constituent MW 364 had the same R_f value as sorgoleone. However, these two compounds were separated on a C18 reverse phase column by HPLC (sorgoleone, ret. time 8.2 min, MW 364 ret. time 20.5 min; see Figure 3).

HPLC Analysis. HPLC analyses of the sorghum root exudates showed similar chromatographic profiles. The chromatograms showed that each root exudate contained primarily sorgoleone and five other minor but closely related constituents. Retention times of the major components were observed at approximately 7.6, 8.2, 11.7, 15.5, 17.9, and 20.5 min. Of these components, sorgoleone at 8.2 min (Figure 3) accounted for the largest percentage of the exudate, based on total peak area. This peak was identified by analysis of its UV absorbance spectrum and by comparison of its retention time with a purified standard. The concentration within the exudates ranged in quantity from 80 to 90% of the total exudate, depending on the accession. Photodiode array analysis has shown that the other components absorbed at a similar UV spectral range (275–285 nm) and are likely structural analogues of sorgoleone (the UV spectral data for accession 8446 is presented in Figure 2 as an example).

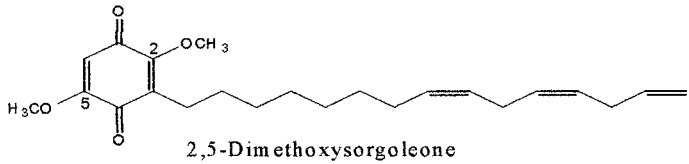
The minor constituents of the root exudates were identified by comparing HPLC retention times to purified standards of these compounds previously isolated from sorghum roots. In an independent analysis, sorgoleone and the resorcinol were shown to have similar retention times, at around 8.2 and 7.6 min, respectively (Figure 3B). The exudates, except for johnsongrass, showed a small peak of resorcinol occurring almost as a shoulder fronting the sorgoleone peak. Except for Johnsongrass, the chromatograms of the six accessions were similar, showing small peaks at around 11.7, 15.1, 17.9, and 20.5 min (8446, SX-17, Della, and shattercane shown as examples, Figure 3). The peaks at 11.7, 15.1, and 20.5 min were identified as 5-ethoxysorgoleone, 2,5-dimethoxysorgoleone, and 2-hydroxy-5-methoxy-3-pentadecyl-*p*-benzoquinone, respectively. The peak at 17.9 min has not been identified, as it did not match the retention times of the other minor constituents (4-methoxy-6-ethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol and 4-hydroxy-6-ethoxy-2-[(10′*Z*,13′*Z*)-10′,13′,16′-heptadecatriene]resorcinol) that had been isolated and analyzed by TLC (Figure 1). Further studies are required



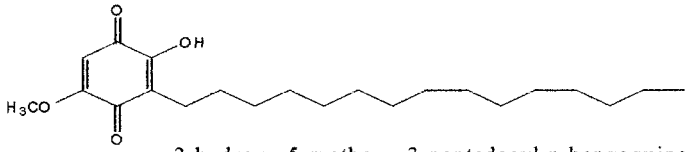
Sorgoleone



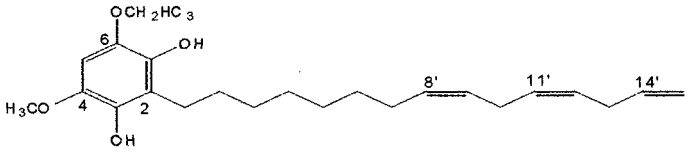
5-Ethoxysorgoleone



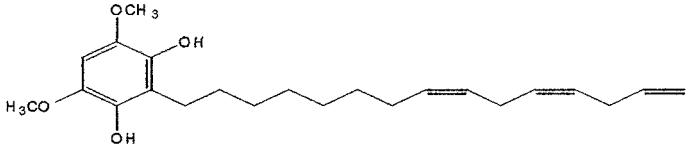
2,5-Dimethoxysorgoleone



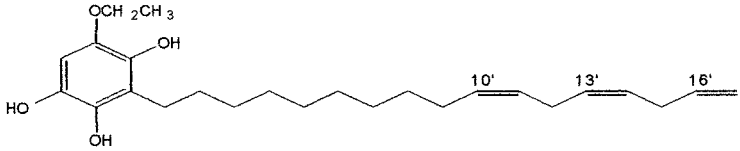
2-hydroxy-5-methoxy-3-pentadecyl-p-benzoquinone



4,6-Dimethoxy-2-[(8'Z,11'Z)-8',11',14'-pentadecatriene]resorcinol



4-methoxy-6-ethoxy-2-[(8'Z,11'Z)-8',11',14'-pentadecatriene]resorcinol



4-hydroxy-6-ethoxy-2-[(10'Z,13'Z)-10',13',16'-heptadecatriene] resorcinol

FIG. 4. Compounds identified in sorghum root exudates.

for identification of this component. Johnsongrass exudate contained another major unidentified component which exhibited maximum absorption at a λ_{max} of 180 nm. Given the lack of similar spectral characteristics to other sorghum constituents, we are not certain that this component contributes to the allelopathic activity of johnsongrass. Root exudate constituents whose molecular structures have been identified are listed in Figure 4. Shattercane, like johnsongrass, is a weedy species. However, its HPLC chromatogram looked remarkably similar to those of the cultivated accessions, indicating a closer chemical relationship to the cultivated sorghums evaluated. Shattercane did differ in the quantity of total exudate produced, and amounts of constituent(s) in the exudate, producing less total exudate per gram fresh root weight. These results suggest that such variations have a genetic basis, since all environmental conditions during exudate production were identical for each sorghum accession.

Allelopathic crops offer potential for the development of model herbicides, as well as providing a source of germplasm that could be manipulated to enhance weed suppression in an environmentally compatible way (Weston, 1996). As we learn more about the chemical nature of bioactive root exudates, we may be able to successfully manipulate our germplasm sources to select for enhanced levels of bioactive allelochemicals and enhanced weed suppression (Duke et al., 2001). The strong weed suppressive ability of sorghum cover crops, and the phytotoxicity of sorgoleone and other minor root exudate constituents offer interesting possibilities for effective biorational approaches to weed management.

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CHEMICAL AND OLFACTORY CHARACTERIZATION OF ODOROUS COMPOUNDS AND THEIR PRECURSORS IN THE PAROTOID GLAND SECRETION OF THE GREEN TREE FROG, *Litoria caerulea*

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Abstract—When stressed or challenged by a predator, the Australian green tree frog, *Litoria caerulea*, emits a characteristic nutty odor from its parotoid glands. This study identifies the source of the odor as the cyclic amide 2-pyrrolidone (2-PyrO). In addition, we demonstrate the presence of 2-PyrO's straight chain form, γ-aminobutyric acid or GABA, in the frog's glandular secretion and propose an odorant–precursor relationship. What role both compounds play in the frog's defensive strategy remains unknown. Prolonged exposure to the odor is shown to result in adverse effects that may be attributed to a GABAergic mechanism. It is our hypothesis, however, that the odor acts as an aposematic signal, indicating the toxicity of the frog's nonvolatile secretion.

Key Words—*Litoria caerulea*, odorous/volatile secretion, nutty odor, 2-pyrrolidone, GABA.

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INTRODUCTION

The skin chemistry of the Australian green tree frog, *Litoria caerulea* (White, 1790), has received considerable attention because of the presence of enlarged parotoid (granular) glands on the dorsal surface of its head. Although no life history role has been demonstrated for the secretion, it seems likely, on the basis of behavioral observations and the pharmacological action of some of its components, that it could serve a defensive function. If unable to escape a predator, *L. caerulea* will adopt a defensive position whereby the body is inflated and raised off the ground on all four legs, the rear of the body being held higher than the head. The head and consequently its glands are then directed towards, and sometimes "butted" against the threat. At the same time large quantities of the secretion are released (Williams et al., 2000). Compounds isolated from this secretion include 5-hydroxytryptamine (serotonin), 5-hydroxyindoleacetic acid, 5-hydroxytryptophol (Roseghini et al., 1976; Erspamer, 1994), histamine and *N*-acetylhistamine—which, because of their well-known irritant properties on buccal tissue, would serve well in chemical defense (Daly, 1995)—and the bioactive peptides bombesin (vasoactive), caerulein (vasoactive and myoactive), neuromedin U-23 (myoactive), the caerins and caeridins (antimicrobial) (Anastasi et al., 1968; Stone et al., 1992; Waugh et al., 1993; Erspamer, 1994; Salmon et al., 2000). In addition to these components, odorous volatiles are usually released; often well in advance of the nonvolatile secretion and even well before defensive posturing is exhibited (personal observation). Williams et al. (2000) described this odor as nut-like and raised the question as to the role odor may play in the frog's defense strategy.

In reviewing the literature it appears many frog species emit charactistic odors when stressed. For example, Noble (1931) noted that the odors of *Rana septentrionalis* and *Gastrotheca monticola* are like mink whereas *Bufo vulgaris* and *Hydromantes italicus* both smell like vanilla, and *Hydromantes genei* liberates a ". . .sweetish, penetrating. . ." odor. Boulenger (1911) reports that some pelobatids smell like onions, Martin and Littlejohn (1966) state that *Litoria jervisiensis* smells like curry, Tyler (1968, 1976) described the odor of *Litoria infrafrenata* from New Britain and *L. aurea* from New South Wales as similar to that of dried thyme, whereas Waye and Shewchuk (1995) note that *Scaphiopus intermontanus* skin secretions smell like peanuts. Furthermore, the odors of many frog secretions have been reported to be disagreeable to humans; for example, *Rana septentrionalis*, *Scaphiopus holbrooki*, *Rana galamensis*, *Pelobates fuscus*, *Chiromantis xerampelina*, *Edalorhina perezi*, and various species of *Phyllomedusa* (Duellman and Trueb, 1986; Daly et al., 1987) and may cause sneezing, nasal discharge, congestion, and other generally irritating effects (Nussbaum et al., 1983; Stebbins and Cohen, 1995).

Despite interest as to the role these odors may play in a frog's life and the effect that they may have on other organisms, few studies have sought to identify the

volatile compounds present in frog skin secretions. A primary reason for this deficiency lies in the difficulty of sampling volatile compounds and their subsequent chemical identification. Here, we provide an in-depth description of *L. caerulea's* odor, along with its effect on human (heterospecific) subjects, and isolate and identify the compounds responsible by using chemical and organoleptic techniques. Furthermore, we identify key odorant precursors and provide an explanation for the presence of the nutty odor released by *L. caerulea*.

METHODS AND MATERIALS

Animals. Twenty-two frogs were collected from Nutwood Downs Station, Northern Territory, Australia. Sixteen were retained alive and transported back to the laboratory for chemical (6) and olfactory (10) analysis. Six were euthanized by placing them in a -4°C freezer. Prior to being placed in the freezer, frogs were held for 1 hr at 5°C. The parotoid glands were then excised and placed in liquid nitrogen for transport back to the laboratory.

An additional 10 frogs were obtained from other localities for olfactory analysis to determine whether intraspecific variation in odors occurred: Normanton, Queensland (4); Moomba, South Australia (3); Derby, Western Australia (1); and Darwin, Northern Territory (2). To determine whether the odor was synthesized by the animal or was reliant on environmental sources, five captive-bred animals were assessed.

Olfactory Characterization. One hundred and forty-eight naive volunteers (i.e., volunteers untrained in olfactory analysis and with no prior experience of the frog's odor) were asked to smell the secretion released by a stressed frog and describe, in five words or less, the odor they perceived. Volunteers were also asked to rate the hedonic nature of the odor (i.e., whether they liked or disliked the sensation) and comment on any physiological effects caused by the secretion. Frogs were restrained and caused to secrete by applying mild electrical stimulation to the parotoid glands following the technique described by Tyler et al. (1992). All handlers wore latex gloves to avoid contact with the secretion and prevent contamination with human odor. Immediately prior to exposure, volunteers were asked to smell the latex gloves for 1 min so as to familiarize themselves with that odor.

Animals were held approximately 15 cm below the nose of a volunteer, and the volunteer was instructed to breathe normally. If after 5 sec no odor was perceived, the subject was asked to inhale deeply through the nose and to repeat this procedure three times. If, after three attempts, an odor was still not recognized, the stressed animals were removed. Not all animals stimulated in this way produced an odor. Furthermore, odor release was, at times, intermittent, with some animals having periods where they did not release an odor on stimulation. If the handler was unable to perceive an odor, no result was recorded for that animal or volunteer.

From the 148 volunteers, 20 were randomly selected and asked to evaluate the odor against a set of reference odorants. Subjects were asked to indicate the qualitative similarity on a scale of 1–9 (1 being *dissimilar* and 9 *similar*), between the frog's odor and 13 references: Balsamic (Benzoin), Fruity (Ethyl caproate), Minty (Peppermint oil), Green (*cis*-3-hexen-1-ol), Mossy (Oak moss absolute), Herbaceous (Oreganum), Camphoraceous (Camphor), Aldehydic (Lauric aldehyde), Animal (Civet), Unpleasant (Valeric acid), Earthy (Patchouli oil), Ethereal (1,2-dichloromethane) and Nutty (2-acetylpyrazine). Standards (obtained from J. H. Lever & Co. Pty Ltd., Adelaide, SA, Australia) were diluted to 10% (wt/wt) concentrations in ethanol. Subjects were presented with the odor on strips of soaked blotting paper after evaporation of the solvent (Toyota et al., 1978). Odor categories were selected to cover a wide range of olfactory stimuli and include the odor descriptors used by Williams et al. (2000) to classify the scent associated with the defense secretions of Australian frogs.

The same group of volunteers was also used to assess the odor differences of captive bred and wild caught frogs from different localities. Wild caught animals were assessed within 1 week of capture. Frogs were marked by electrocauterization of the epidermis (Clark, 1971) and randomly placed in pairs. Volunteers were asked to smell both frogs and rate their similarity.

Chemical Identification of Odorants. Dissected glands (of four frogs) were extracted by soaking them in a range of solvents of varying polarity: methanol, ethanol, acetonitrile, chloroform, ethyl acetate, and hexane. Sections of glands (1×1 cm) were placed in 2 ml of solvent and allowed to soak for 4 days. Extracts were stored in the dark at room temperature (25°C). Extracted tissue was removed and the odor quality of each solution noted. Extracted odor was assessed by smelling samples of each extract presented on blotting paper strips, after evaporation of the solvent. Both methanol and ethanol extracts retained the characteristic nutty odor of the frog and were used for subsequent analysis. An added advantage of both solvents was their low affinity for fats, thus minimizing the extraction of unwanted compounds associated with the glandular tissue. Prior to analysis, extracts were concentrated under nitrogen and gentle heat (30°C) to 20 μl. Analysis was undertaken on an Agilent 6890 GC coupled to 5972 Mass Selective Detector (GC-MS). The gas chromatograph was fitted with a 30 m $\times$ 0.25 mm I.D. HP-5MS fused silica capillary column with a 0.25-μm film thickness (Agilent, Palo Alto, CA, USA). The carrier gas was helium with a flow rate of 1 ml/min. Oven temperature was maintained at 50°C for 1 min and then programmed at 8°C/min to 250°C. The injector temperature was held constant at 210°C and operated in splitless mode. Ionization for mass analysis was by electron impact at 70 eV and 220°C; mass range 35–350 amu.

Six live frogs and additional gland sections were sampled using headspace solid phase microextraction (SPME). Gland sections were placed in 4-ml glass sample vials and sampled using a polydimethylsiloxane/divinylbenzene

(PDMS/ DVB) fiber (SUPELCO, Bellefonte, PA, USA). The fiber was exposed to the headspace of the vial for 1 min at room temperature (25°C). Live frogs were sampled by placing them in 500-ml glass sample chambers. Prior to sampling, frogs were voided of urine by applying gentle pressure to the bladder. Frogs were stimulated to secrete by using electrical stimulation then placed immediately in the sample chamber. The headspace of the vial was sampled at 25°C for 10 min using the PDMS/DVB fiber. Following sampling, the fibers were immediately introduced to the gas chromatograph's injector. Analysis was performed using the same column and GC conditions described earlier. To achieve sharp SPME injection bands, the inlet liner was replaced with a narrow bore (0.75-mm I.D.) liner. A desorption time of 3 min was employed. Volatile compounds were identified on the basis of their retention times and mass spectra in comparison with those of reference compounds (Sigma-Aldrich Bellefonte, PA, USA) under the same GC-MS conditions. Mass spectra from NIST92, WILEY, and in-house libraries were also used.

To facilitate identification of the compound(s) responsible for the odor, concurrent studies were undertaken using gas chromatography–olfactory port (GC-OP) techniques. This enabled the human nose to be used as a detector and evaluate the organoleptic properties of the GC effluent. Studies were conducted on a Varian 3400C gas chromatograph fitted with a BPX-5 capillary column (30 m $\times$ 0.25 mm $\times$ 0.53 μm; SGE, Ringwood, Victoria, Australia). The effluent was split with approximately 80% entering the nasal port (where it was mixed with humidified air) and 20% sent to a flame ionization detector. The carrier gas was helium, and an identical temperature program to that of the GC-MS was employed.

Because of the strong association of odor with the ethanol extract, it was used for olfactory studies. A 3-μl extract was injected, the temperature program started, and the individual whose nose was being used gave a running report to a second person. This investigator recorded the time and odor description of eluted compounds. Frog volatiles were assessed four times by trained people. Volunteers were trained by monitoring their response to a series of standard odor compounds. These standards were also used randomly throughout the testing protocol to control for fatigue and variation in odor perception.

Chemical Identification of Odorant Precursors. Following identification of the primary odorant as a cylic amide, attention focussed on the presence of amines and amino acids in the frog's secretion. Sections of glands (2 $\times$ 1 cm), obtained from two frogs previously used for olfactory analysis and two newly collected frogs, were extracted (individually) by soaking in 10 ml of a (1:1) water/methanol mix. In addition, the secretions of two live frogs were collected using surface electrical stimulation. The secretion was washed from the skin with 20 ml of water (Milli-Q). An equivalent amount of methanol was added to this aqueous extract. All samples were continuously shaken in a water bath held at room temperature (25°C) for 1 hour. The glandular tissue was removed, the extracts centrifuged, and

the supernatant liquid filtered through a Millipore 0.45-μm filter. The collected solution was stored at -80°C until analyzed.

Initial studies were conducted on the GC-MS system described earlier. To detect non-volatile amines, the extract was acetylated under Schotten–Baumann conditions: 4 ml of the extract was treated with acetic anhydride (0.3 ml). A slight molar excess of sodium bicarbonate was added. The reacted solution was extracted with dichloromethane. A further 4 ml was evaporated (under pressure) to dryness. The remaining material was heated, for 30 min at 70°C, with 50 μl of trimethylchlorosilane (TMCS). Authentic samples of γ-aminobutyric acid (GABA) (Sigma-Aldrich, Bellefonte, PA, USA) and 2-pyrrolidone (2-PyrO) were treated the same way (total dilution 1:100).

Subsequent analysis was performed using liquid chromatography–electrospray mass spectrometry (LC-ESMS), the method adapted from Mesmer and Satzger (1997). The sample was loaded using a flow injector (Rheodyne model 8125, Cotati, CA, USA) fitted with a 5-μl loop onto a C18 reverse-phase HPLC column (1 $\times$ 250 mm, μBondapak from Waters Chromatography Division, Millipore Corporation, Bedford, MA) fitted with a Novapak C18 guard column (1 $\times$ 20 mm). The mobile phase consisted of 85% acetate buffer (25 mM ammonium acetate, pH adjusted to 4 with acetic acid), 15% methanol delivered at a flow rate of 10 μl/min by a dual syringe pump (140B Solvent Delivery System, Applied Biosystems, Perkin Elmer, USA). The column was connected directly to an electrospray ion source (ES) of a triple quadrapole mass spectrometer (API-300, PE Sciex, Thornhill, Ontario, Canada). All connections between the column and ion source consisted of PEEK tubing (0.005″ I.D.). The mass spectrometer was operated in positive ion mode and scanned from m/z 30 to m/z 110 with a step size of 0.1 amu and a dwell time of 0.3 msec. The ES needle, orifice, and ring potentials were set at +4500, +60 and +300 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 10 units, respectively. For tandem mass spectrometry (MS/MS) experiments, nitrogen gas was used as collision gas setting at two units. The collision energy varied between the experiments and, therefore, was optimized as appropriate. The instrument was tuned using polypropylene glycol, and resolution was set at a peak width of about 0.7 amu at 50% peak intensity.

RESULTS

Olfactory Characterization. Table 1 shows the classifications used to describe the frogs' odor. No obvious differences were observed between frogs from different localities or between captive bred and wild caught frogs, indicating that the odor is produced by the animal and not obtained from environmental sources. Of the 148 descriptions, the term "nutty" was the most common (54.73% of answers including "peanut butter" and "peanut brittle"). This corresponds with the odor matching study (Figure 1) and the observations by Williams et al. (2000). In

TABLE 1. DESCRIPTIVE TERMS USED TO CLASSIFY THE ODOR OF *Litoria caerulea* SECRETIONS

Descriptive terms	Number of classifications
Acrid/Bad/Distasteful/Foul/Noxious/ Offensive/Unpleasant	14
Asian food	2
Biscuit	4
Boiled meat	1
Bread	6
Butter	1
Buttered popcorn	2
Corn chips	3
Dirty socks	7
Earthy/dusty	2
Fungal	2
Grassy	1
Hay	4
Herbs	1
Leaves	1
Metallic	1
Musty	1
Nutty	77
"Burnt"	(7)
Peanut	(16)
Cashew	(5)
Almonds	(2)
Walnut	(1)
Pine nut	(1)
General	(45)
Peanut brittle	1
Peanut butter	3
Rice plant	1
Satay	3
Savoury	3
Sesame oil	1
Shellfish (cooked)	2
Spermous	1
Urine	1
Woody	1
Yeast	1

regards to the hedonic nature of the secretion, on short (<5 sec) exposure, 73% of volunteers stated that the odor was "not too bad" or "quite nice." Three percent were indifferent to the odor, and 24% found it disagreeable and reported nasal irritation and lacrimation. Continued exposure (10–60 sec), however, resulted in a significant shift in the sensation characteristics, with all registering discomfort, ranging from mild irritation to light-headedness, nausea, and headache.

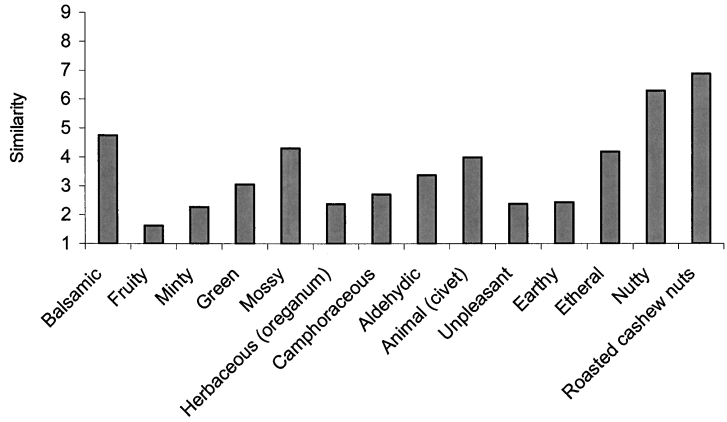

FIG. 1. Matching of the odor of *Litoria caerulea* secretions to industry standards using a naive panel of 20 volunteers. Columns represent the mean similarity score on an arbitrary scale of 1 (dissimilar)–9 (similar).

Chemical Identification of Odorants. Chemical analysis of the volatiles emitted identified a single compound, 2-PyrO, as the major component responsible for the nutty aroma. The nutty property of pure 2-PyrO was confirmed by olfactory analysis. Pure 2-PyrO was presented to five volunteers as described. All volunteers described the odor as roasted nuts. Interestingly, the nutty smell of 2-PyrO was observed to intensify on standing in air. This increase in odor was accompanied by the formation of soapy-colored crystals, later identified by infrared spectroscopy as 2-PyrO monohydrate (distinguished from anhydrous 2-PyrO on the basis of a strong, broad peak at 3700–3300 range/cm^{-1} corresponding to a hydrogen bonded O—H stretch).

Despite variation in the volatiles of the tree frogs, 2-PyrO was a major component (S/N > 10) present in all frogs tested with both SPME and solvent extraction. Even in animals where the odor intensity was low or nonexistent (to the nose), 2-PyrO could be found, albeit in trace amounts. Identification was obtained by comparing retention time, mass spectral data, and odor with that of the reference compound (Sigma-Aldrich, Bellefonte, PA, USA) under the same chromatographic conditions.[4] To interpret the mass spectrum, the work of Budzikiewicz et al. (1967) was used. 2-PyrO (molecular ion $M^{+\cdot} = m/z$ 85) gave a prominent peak M—1 peak (loss of a H-atom possibly from the hydroxy group). In addition, ring cleavages gave rise to a typical fragment at m/z 56 (loss of $CH_2{=}NH_2$) and to fragments at m/z 42 and 41, because of release of H_2NCO and HNCO, respectively (Figure 2).

[4]Key reference spectra and chromatograms are available on request.

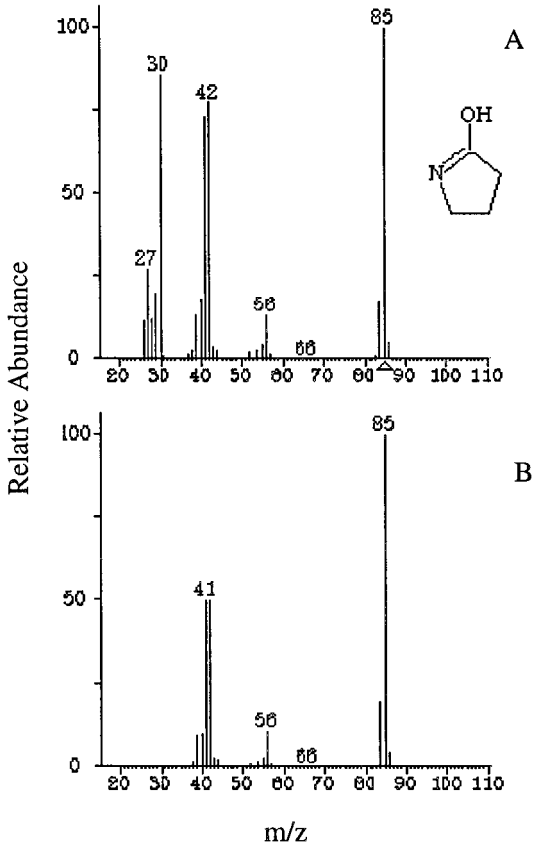

FIG. 2. Mass spectra of 2-pyrrolidone: (A) reference spectrum from NIST92 mass spectral library and (B) isolated from the ethanol extract of a *Litoria caerulea* parotoid gland.

Other compounds identified as "likely" to contribute to the overall nuttiness of the odor included butyrolactone (fatty, caramel), benzaldehyde (almond), β-caryophyllene (terpene odor, woody, spicy) and cinnamic acid ethyl ester (sweet, honey, balsamic, cinnamon, plum; commonly used in commercial nut flavors). Identification in most cases was by coinjection of standards and mass spectral matching (>70% match to the Wiley mass spectral library). All compounds, except caryophyllene, were found in both long-term captive and wild caught frogs. Caryophyllene was absent in captive animals, only being isolated from wild caught specimens.

Chemical Identification of Odorant Precursors. Because of the close relationship of 2-PyrO with its straight chain form, GABA, we proposed that GABA

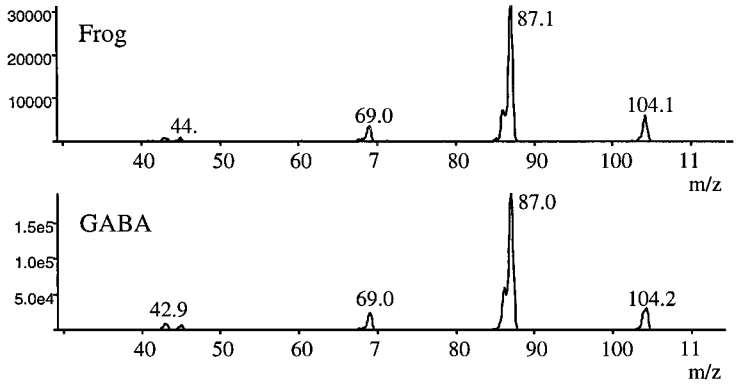

FIG. 3. Daughter ion spectrum for m/z 103 for a secretion extract from *Litoria caerulea* and a pure sample of GABA.

was also present in the frog's secretion and, consequently, linked to the production of odor. This hypothesis was strengthened by the presence of butyrolactone in the odorous secretion. Butyrolactone (GBL) is formed via the cyclization of γ-hydroxybutyric acid (GHB), a naturally occurring metabolite of GABA found in the central nervous system (CNS) and peripheral tissues. (Bessman and Fishbein, 1963; Roth and Giarman, 1969; LeBeau et al., 2000). This reaction requires minimal energy, and aqueous solutions of GHB will occur as equilibrium mixtures of the acid and lactone (Albert, 1968). The conversion of GABA to 2-PyrO requires greater energy and most likely proceeds via an enzyme-catalyzed reaction.

Attempts to derivatize the acid and confirm its presence proved unsuccessful. GABA and 2-PyrO produce identical mass spectra even after acetylation (acetylated pyrrolidone). An $M^{+\cdot}$ ion (127) is fragmented to m/z 99 and 98 (ring cleavage); loss of the acetyl group (m/z 43) leads to the pyrrolidonyl residue (m/z 86, 85). Derivatization of GABA by heating with silylanizing agent also led to the formation of the trimethylsilyl derivative of PyrO (as observed by Bergström and Gürtler, 1971).

Comparison of ES mass spectra against a standard solution (0.1 mg/ml GABA), however, showed all extracts contained significant levels of GABA (taking into account the dilution effect of the solvent). Figure 3 shows the daughter ion mass spectrum (m/z 103) for the frog extract and pure GABA using positive ion mode. Note the characteristic ion at m/z 104 (protonated GABA) and m/z 87 (corresponding pyrrolidonyl residue).

DISCUSSION

Although most odors are the result of the presence of mixtures of several, sometimes many, different compounds; single compounds can have a significant

impact on an aroma. This is the case with 2-PyrO, which appears to be primarily responsible for the nutty note of *L. caerulea*'s secretion. In fact, two colleagues familiar with the frog's odor mistook a blind presentation of 2-PyrO (aqueous solution 10% v/v) for an extract of the secretion.

What role the nutty odor, and consequently 2-PyrO, plays in the frog's defensive strategy remains to be shown. It is clear that exposure to the volatile secretion can cause adverse effects in humans, and the olfactory and gustatory perception of humans have often, in the past, suggested chemically protected organisms (e.g., Wassersug, 1971; Brodie, 1983; Weldon and Rappole, 1997). Despite its relatively low (acute) toxicity—rat oral LD50 and maximum tolerable dose 6.5–7.3 and 4 g/kg, respectively, (Aleshin, 1974)—2-PyrO may be responsible for some of the effects observed by experimenters. The substance is listed as a general skin, eye, and respiratory tract irritant (IPCS International Programme on Chemical Safety, ICSC—International Chemical Safety card—0562, April 1997), and, because of its close relationship with GABA, has been implicated in the promotion of lethargy and sleep (Juhasz et al., 1989), the depression of food intake and food avoidance (Tews et al., 1986, 1987), and is known to possess psychotropic properties resembling those of tranquillizing drugs in mice, rabbits, cats, and pigeons (CRCS, Inc., 1985).

It seems unlikely, although the analysis was qualitative, that under natural conditions, the intensity of the odor (and concentration of 2-PyrO) can reach levels high enough to act as a primary repellent. Rather the unique odor may serve in the formation of a conditioned reflex (i.e., as an aposematic odor signalling toxicity or unpalatability). Factors that would promote the formation of a conditioned aversion to the nutty odor are (1) the observed bioactivity of *L. caerulea*'s secretion (Salmon et al., 2000), (2) the fact that the odor is released during the act of defense posturing (Williams et al., 2000), and (3) the unique nature of the signal. For optimal efficiency of a warning the signal should be source-specific, because other undefended organisms emitting the same odor would function as Batesian mimics. The greater the proportion of mimics to models, the larger is the time required for predator learning, and the greater the potential cost to the model (Pianka, 1978).

Other frog species known to produce a nutty odor are the closely related and sympatric magnificent tree frog, *Litoria splendida* (Williams et al., 2000), and the allopatric red spotted toad, *Bufo punctatus* (personal observation), the western spadefoot toad, *Scaphiopus hammondii* (Stebbins, 1985) and the Great Basin spadefoot toad, *S. intermontanus* (Waye and Shewchuk, 1995). Whether or not the secretion of these frogs contains 2-PyrO needs to be determined, but sensory studies, conducted by BPCS, demonstrate significant similarity in the odor characteristics of each frog's secretion (unpublished data). In general, few data are available concerning the presence of 2-PyrO in the environment, making it a likely choice for a warning signal. As a curiosity, it has been found to be abundant in the glue of cobwebs (Schildknecht et al., 1972) and insect larvae (Deml and Dettner,

1995; Deml, 2001). It also occurs in white bread (Luning et al., 1991) and was recently identified by GC/MS in raw and roasted cocoa (Ziegleder, 1991) and a large number of dried foods (Mende et al., 1994).

Of primary interest is the relationship of 2-PyrO with its straight chain form GABA, the principal inhibitory neurotransmitter in the vertebrate CNS. The presence of both compounds in the frogs' secretion suggests an odor precursor–odorant relationship. Despite an abundance of studies on the chemistry of frog skin secretions (for a detailed review see Erspamer, 1994), along with GABAs widespread use as a neurotransmitter and neuromodulator in many animals (including frogs), and its presence in many nonneural organs such as the ovaries, adenohypophysis, liver, digestive tract, and pancreas (Takeuchi and Takeuchi, 1965; Gilon et al., 1990), the presence of GABA in amphibian skin secretions is scarcely documented. To date, GABA has only been recorded in the secretion of three frogs, the yellow unk, *Bombina variegata* (Michl and Bachmayer, 1964; Habermehl, 1981), in high concentration in the secretion of the African rhacophorid frog, *Hylambates maculatus*[5] (Yasuhara et al., 1981), and now in the parotoid gland secretion of *L. caerulea*.

GABA secreted onto the skin may have a defensive function. Ingestion of GABA (by humans) has been implicated in gastrointestinal disorders, insomnia, headache, and pyrexia (Reynolds and Prasad, 1982). Perry et al. (1974) noted severe peripheral vascular collapse after ingestion of 8 g of chromatographically pure aminobutyric acid. Fisher et al. (1974), however, showed doses of 1–2 g administered daily and 40 g given over 48 hrs produced no serious side effects. The most potent and rapid effects of GABA, and GABA agonists, in mammals are the inhibition of skeletal muscle reflex (systemic effect). Thus, some possible effects *on* a predator might be to inhibit movement, decrease the speed of muscle contraction, cause tetany or decrease the capability for coordinated activity (personal communication A. Blackshaw, Nerve–Gut laboratory, Royal Adelaide Hospital, Sept. 2001). However, uptake of GABA across the enteric mucosa is relatively slow and poor. The chief site of GABA absorption is the small intestine (Guyton and Hall, 1996). Realistically, the release of GABA is unlikely to provide a direct benefit for the frog. Perhaps the only significant possibility is that the predator may learn to associate muscular events with the consumption of the frog. Such an effect may be strengthened by the presence of odor, particularly if the odorant enhances the physiological effect.

Alternatively, the presence of GABA and 2-PyrO may be secondary, being an artifact of the embryological origin of the glands and/or a by-product of nonessential amino acid synthesis. The early evolution of amphibian skin secretions probably involved glands containing endogenous control compounds such as

[5]Now recognized as *Kassina maculata* and placed in the Family Hyperoliidae Laurent, 1943 (Duellman and Trueb, 1986).

monoamine and peptide neurotransmitters and neuromodulators. The skin glands that synthesize these secretions seem to have arisen from the same neural ectoderm that forms the embryonic neural tube, neural crest, and the ectodermal placodes (Pearse, 1976, 1977; Pearse and Takor, 1976; Mueller et al., 1980; Barthalmus, 1994). Recognized for being unique to vertebrate chordates and for the diversity of tissues/organs for which they anlage, the neural crest and the above adjacent embryonic tissues give rise to (1) much of the nervous system (sensory nerves, autonomic nervous system) and its (2) sensory placodes; (3) pigment cells, (4) ultimobranchial bodies that secrete calcitonin; (5) adrenal chromaffin cells that secrete adrenaline, noradrenaline, dopamine, and probably serotonin; (6) the visceral skeleton and its many cartilaginous and bony derivatives; (7) a variety of orofacial muscles; (8) neuromast organs of the lateral line system of amphibians and fishes; and (9) the dermal glands of amphibians (Gans and Northcutt, 1983; Le Douarin, 1986). Considering the many biological control substances that have arisen via neural ectoderm, it is not surprising that the amphibian poison glands, which arise from larval cells during metamorphosis (Delfinio et al., 1985; Seki et al., 1989), seem to be extensions of the nervous and endocrine systems. In fact, the melanophores of amphibians are highly modified catecholaminergic neurons in which the amino acid substrate tyrosine is converted into DOPA (a precursor for dopamine, adrenaline, and noradrenaline synthesis) and eventually to melanin pigment. Thus, the migration to the skin of neural crest and adjacent embryonic cells results in pigment cells, sensory cells and organs, and anuran skin glands that contain natural control substances found in all vertebrate bodies (Barthalmus, 1994).

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SEMIOCHEMICALS FROM THE PREDATORY STINK BUG *Eocanthecona furcellata* (WOLFF): COMPONENTS OF METATHORACIC GLAND, DORSAL ABDOMINAL GLAND, AND STERNAL GLAND SECRETIONS

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Abstract—Volatile compounds from the metathoracic glands (MTG), dorsal abdominal glands (DAG), and sternal setae of the generalist predatory stink bug *Eocanthecona furcellata* (Wolff) were studied by gas chromatography–mass spectrometry. MTGs of both male and female bugs contained (*E*)-2-decenal as the major component, with lesser amounts of (*E*, *E*)-2,4-decadienal, (*E*, *Z*)-2,4-decadienal, and (*E*)-2,9-decadienal. The relative amount of (*E*)-2,9-decadienal in the MTGs of female bugs was higher than in male bugs. There was no dimorphism in the morphology of the DAGs of adults. However, the DAG contents of adult bugs were different between the sexes. Geraniol was found only in the DAGs of males, and limonene and α-terpineol were found only in the DAGs of females, whereas linalool oxide isomers, linalool, neral, and geranial were found in DAGs of both sexes. The DAG contents of nymphs were also analyzed, with 4-oxo-(*E*)-2-hexenal, 4-oxo-(*E*)-2-octenal, tridecane, and tetradecanal being major components. Male bugs but not females possess sternal setae, and 6,10,13-trimethyltetradecyl isovalerate was detected from extracts of the sternal setae of males. The possible biological significance of dimorphism in the contents of the glands in adults is discussed.

Key Words—*Eocanthecona furcellata*, decadienal, limonene, α-terpineol, geraniol, 6,10,13-trimethyltetradecyl isovalerate, Pentatomidae, metathoracic gland, dorsal abdominal gland, sternal gland.

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INTRODUCTION

Pheromones of predatory bugs have been developed for augmentative biological control purposes (Aldrich, 1999). The best-known asopine pheromone system is that of the spined soldier bug, *Podisus maculiventris* (Say), which is composed of (*E*)-2-hexenal, α-terpineol, and benzyl alcohol (Aldrich et al., 1984). These were the three major compounds found in the enlarged III–IV dorsal abdominal glands (DAGs) of male *P. maculiventris* (Aldrich et al., 1978). A commercial Spined Soldier Bug Attractor™ composed of (*E*)-2-hexenal and α–terpineol has been developed (Anonymous, 1992). Sant'Ana et al. (1997) further indicated that blends of (*E*)-2-hexenal, α–terpineol, and benzaldehyde can be used to attract *P. maculiventris* nymphs from points of augmentation.

Sexual dimorphism of the DAGs has been found in some asopine bugs including *Podisus*, *Zicrona*, and *Alcaeorrhynchus* species (Aldrich et al., 1986), with much larger DAGs in males than in females. However, in *Oechalia schellennbergii* (Guérin-Ménevelle), DAGs of males are not always bigger than those of female bugs (Aldrich et al., 1996). Males of specialist predators such as *Perillus*, *Stiretrus*, *Mineus*, and *Oplomus* species also have sternal glands (SG) (Aldrich, 1998). In contrast, some asopines have neither sexually dimorphic DAGs nor SGs (e.g., *Picromerus*, *Dinorhynchus*, *Cermatulus*, and *Euthyrhynchus spp.*) (Aldrich, 1998).

With this wide variation in the morphology and chemical compositions of the gland contents in asopine bugs, we were interested in investigating the semiochemistry of the predatory stink bug, *Eocanthecona furcellata* (Wolff) (Heteroptera: Pentatomidae: Asopinae). This bug is a generalist predator that feeds on larvae of Lepidoptera, Coleoptera, and Heteroptera. Its range includes India, Southeast Asia, the southern part of China, Taiwan, and Okinawa (Chu and Chu, 1975a,b; De Clercq, 2000; Yasuda, 2000). In Taiwan, it is used as a biological control agent to control pests in agricultural crops. Our specific objectives were to study sexual dimorphism of the glands in these bugs, and analyze volatile components in the glands to identify possible sex pheromones and/or aggregation pheromones that could be exploited for manipulation of bug populations.

METHODS AND MATERIALS

Insects. A colony of *E. fucellata* was started from bugs provided by H. K. Tseng. The bugs were reared on mealworm, *Alphitobius sp.* (Coleoptera: Tenebrionidae) and blowfly larvae, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) in the laboratory under a 14:10 L:D regime with temperature controlled at $23 \pm 3^{\circ}$C and relative humidity at 70%. Mealworms were bought from a pet shop, and blowfly larvae were from a fishing supply store. The colony was maintained as described in the literature (Chu and Chu, 1975a,b).

Extraction of Metathoracic Gland Contents. The procedure for extracting volatiles from MTG was similar to those previously described (Ho and Millar, 2001). To prevent the premature discharge of gland contents, mature bugs were anesthetized with CO_2 and then killed by freezing. Legs and wings of freshly killed bugs were clipped off with scissors, and the carcass was pinned through the head, dorsal side up. The outer edges of the abdomen were cut open with a pair of fine iris scissors. The dorsal cuticle was flipped open, exposing the contents of the abdomen. Tissues inside the body cavity were carefully removed to expose the orange-colored metathoracic glands. Gland contents were sampled by piercing the gland with a drawn-out glass microcapillary tube. The tube was broken in 200 μl of pentane in a conical glass insert (250 μl) inside a screw-capped vial to release the extracted gland contents. The extract was kept at -20°C until GC and GC/MS analysis. Compounds were tentatively identified by GC/MS, and identifications were confirmed by comparisons of the retention times and mass spectra of the unknowns with those of authentic samples.

Isolation of Components. Compounds in the MTG of adults were separated by reverse phase HPLC using a single pump system with a Hyperprep HS C18 column (150 $\times$ 4.6 mm, 5 μ particle size) (Thermo Hypersil, Cheshire, England), a Rheodyne Model 7125 sample injector, and a UV-visible variable wavelength detector (Shimadzu SPD-6AV). The combined extracts of MTGs of 20 female bugs were dissolved in 500 μl of CH_2Cl_2, and 100 μl aliquots were injected, followed by elution with 0.1 M $AgNO_3$ in methanol:water (2:1) for 50 min at a flow rate of 0.7 ml/min (Phelan and Miller, 1981; Millar, 1998), monitoring the effluent at 254 nm, and collecting 2 min fractions. Each fraction was extracted with pentane with vortexing, and the pentane layer was filtered through anhydrous $MgSO_4$ to remove water. The pentane extract was analyzed by GC. Fractions of interest were analyzed further by derivatization and NMR analysis.

Derivatization of Decadienal. Because derivatization of the conjugated aldehyde with dimethyl disulfide (DMDS) was not successful, the unknown was first reduced to the alcohol with $LiAlH_4$ in dry THF and then was derivatized by DMDS to form the dimethyl disulfide adduct (Vincenti et al., 1987; Ho et al., 1996).

Extraction of Dorsal Abdominal Gland Contents. The extraction procedure was similar to the extraction of MTG contents described above. The III–IV DAGs of both male and female bugs were small. Contents were collected in a drawn-out glass microcapillary tube, the tube was broken in 100 μl of pentane, and the extract was concentrated under a stream of nitrogen for GC/MS analysis. Each bug was extracted and analyzed individually.

The dorsal abdominal gland contents of last instar nymphs were extracted and analyzed in similar fashion. The 2nd and 3rd pairs of DAGs appeared as orange lobes attached to the cuticle. When one of the DAGs was pierced by the microcapillary tube, the other one collapsed, and so the contents of both glands

were collected together. The amount of each component in individual bugs was quantified vs. benzophenone as internal standard.

Extraction of Sternal Gland Contents. There was a pubescent area on the sternites of male bugs, similar to *P. bioculatus* (Aldrich et al., 1986). Because no obvious macroscopic glands or reservoirs were found after dissection, the pubescent area was simply rinsed with solvent as described by Aldrich et al. (1986) for *P. bioculatus* and *O. severus*. Virgins of different ages of both sexes, and adults from the colony were anesthetized with CO_2, and the ventral surface was rinsed with 40 μl of heptane. Extracts were subjected to GC and GC–MS analysis. The amount of trimethyltetradecyl isovalerate was quantified using benzophenone as internal standard. Each bug was extracted and analyzed individually.

Analysis of Extracts. Extracts were analyzed by splitless coupled gas chromatography-mass spectrometry (GC–MS) with a Thermo Quest Trace GC, interfaced with Finnigan Trace Mass spectrometer (electron impact ionization, 70 eV). The GC was held at 40°C for 10 min, then programmed at 10°C/min to 250°C, with injector and transfer line temperatures of 200 and 250°C, respectively. A DB-5ms column (30 m × 0.25 mm ID, J & W Scientific, Folsom, CA) was used, with helium carrier gas. GC analyses were performed on a Varian 3400 instrument (Palo Alto, CA) with a DB-5ms column and flame ionization detector, with chromatographic conditions as described above. ^{1}H NMR spectra were obtained on a Bruker 300 spectrometer at 300.135 MHz. $CDCl_3$ was used as solvent.

Chemical Standards. (*E*)-2-Decenal, (*E*,*E*)-2,4-decadienal, tridecane, tetradecanal, linalool, geraniol, limonene, α-terpineol, (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-nonenal, and (*Z*)-4-decenal were purchased from Sigma-Aldrich Co. (Milwaukee, WI). Linalool oxide as a mixture of isomers was from Fluka Chemie AG, Switzerland. (*E*)-4-Oxo-2-hexenal and (*E*)-4-oxo-2-octenal were gifts from Dr J. G. Millar of University of California, Riverside, CA. 6,10,13-Trimethyltetradecyl isovalerate was provided by Dr J. R. Aldrich of the U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD.

Geranial and neral were available as a mixture (citral) from TCI (Tokyo Kasei Kogyo Co., Tokyo, Japan). Geranial and neral were identified by NMR spectral data, with the proton of the aldehyde group having different chemical shifts (9.85 ppm for neral and 9.93 ppm for geranial; Joseph-Nathan and Manjarrez, 1967). From the integration of the protons of the aldehyde peak, the percentage of neral and geranial in the commercial mixture was estimated as 1:1.6. This allowed assignment of the neral and geranial peaks in GC and GC–MS traces.

Quantification. GC or GC–MS was used for the quantification of the components in the glands when applicable. Instrument responses to chemical standards and the internal standard, benzophenone, were determined by coinjecting equal amounts (10 ng) of the compounds into the GC and GC–MS. Ratios of the peak areas in the GC chromatogram or the GC–MS total ion chromatogram were used to calibrate the responses of each instrument to each compound. If pure standard

compounds were not available, instrument responses to chemicals with similar structures were used for calculation purposes.

Isolation of (E,Z)-2,4-Decadienal from Commercial (E,E)-2,4-Decadienal. Commercial (*E*,*E*)-2,4-decadienal contains ~5% of (*E*,*Z*)-2,4-decadienal according to the NMR spectrum of the sample. The minor component in the commercial standard of (*E*,*E*)-2,4-decadienal was purified by preparative reverse phase HPLC as described above, with the exception that the compounds were eluted with 0.1 M $AgNO_3$ in methanol: water (2:1) for 120 min at flow rate of 1 ml/min, and a Hyperprep HS C18 column was used (250 × 10 mm, 8 μ particle size) (Thermo Hypersil, Cheshire, England). Decadienal was dissolved in CH_2Cl_2 to a concentration of 1 mg/ml, and 0.2 ml aliquots were injected per run. The major component, (*E*,*E*)-2,4-decadienal, eluted at about 96 min, and the minor component eluted at about 89 min. ^{1}H NMR of (*E*,*Z*)-2,4-decadienal ($CDCl_3$): δ 0.92 (3H, t, $J = 6$ Hz), 2.37 (2H, dt, $J = 6, 8$ Hz), 6.04 (1H, dt, $J = 11, 8$ Hz), 6.09 (1H, dd, $J = 8, 15$ Hz), 6.3 (1H, dd, $J = 11$ Hz), 7.5(1H, dd, $J = 11$, 15 Hz), 9.63 (1H, d, $J = 8$ Hz). These data matched that reported by Naf and Decorzant (1974).

RESULTS

Components in MTG of Adults. A typical gas chromatogram of the metathoracic gland contents of an adult male *E. furcellata* is shown in Figure 1. The volatile gland constituents consisted primarily of unsaturated aldehydes for both males and females. Table 1 lists the identities of the components and their amounts relative to (*E*)-2-decenal, the most abundant component in extracts from both sexes. Mass spectral data suggested that peaks A, B, C, D, and F were (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-nonenal, (*Z*)-4-decenal, and (*E*)-2-decenal, respectively. These identifications were confirmed by comparison of retention times and mass spectra with those of authentic standards. The relative ratios of peaks E and G to the most abundant peak F were different for female and male bugs (Table 1).

Identification of Isomers of Decadienal (Peaks E, G, and H). Mass spectral data of peaks E, G, and H, all with molecular weights of m/z 152 and base peaks at m/z 81, suggested that these compounds were isomers of decadienal by comparison of mass spectra with those from the National Bureau of Standards GC–MS database. Comparison of the mass spectrum and retention time of commercially available (*E*,*E*)-2,4-decadienal with the peaks from gland extracts determined that authentic (*E*,*E*)-2,4-decadienal corresponded to peak H of the components of the gland. Similarly, the retention time and mass spectrum of peak G matched those of (*E*,*Z*)-2,4-decadienal, a minor component in commercial (*E*,*E*)-2,4-decadienal that was isolated by reverse phase HPLC.

Peak E in the MTG extract was isolated from female MTG extracts by HPLC (ret. time 6.1 min), producing about 10 μg of the compound from 20 female

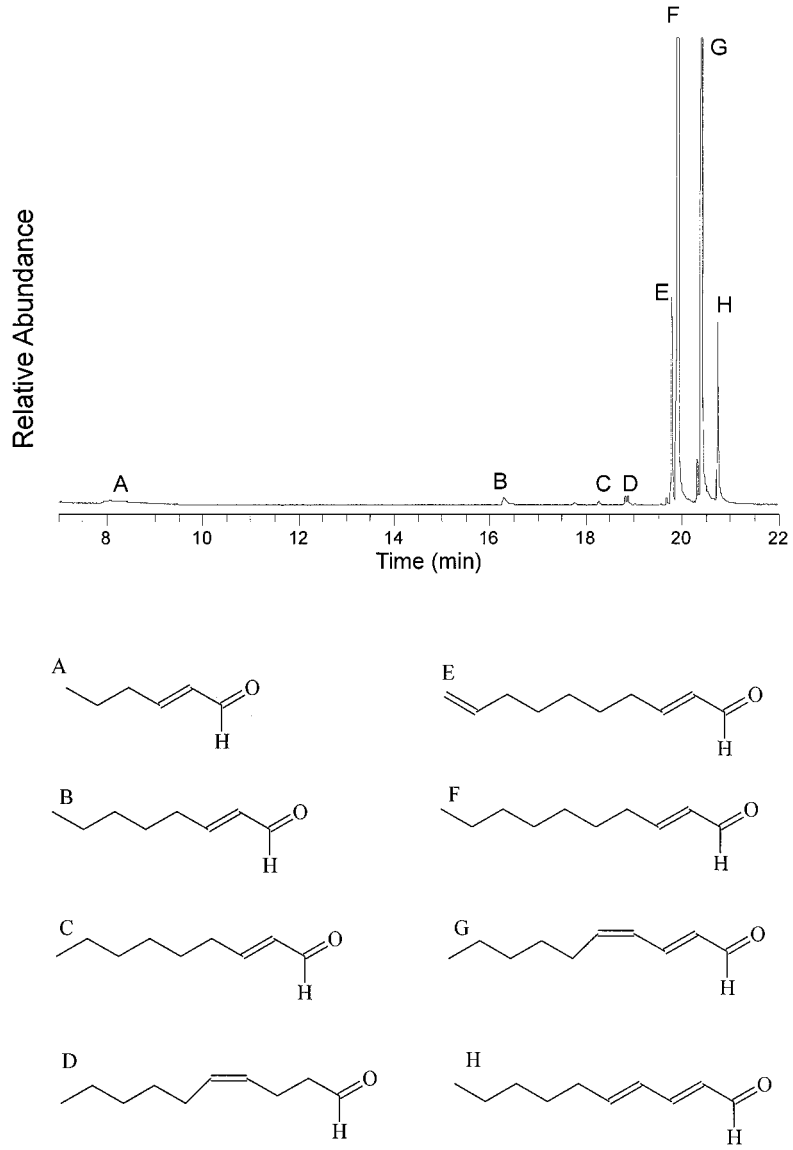


FIG. 1. GC profile and structures of the compounds in metathoracic glands of adult male *Eocanthecona furcellata*. Compounds: A: (*E*)-2-hexenal, B: (*E*)-2-octenal, C: (*E*)-2-nonenal, D: (*Z*)-4-decenal, E: (*E*)-2,9-decadienal, F: (*E*)-2-decenal, G: (*E*,*Z*)-2,4-decadienal, H: (*E*,*E*)-2,4-decadienal. Column: DB5-ms (30 m × 0.25 mm I.D.).

TABLE 1. PERCENTAGE OF COMPONENTS IN THE METATHORACIC GLANDS OF FEMALE AND MALE BUGS (MEAN ± SE) RELATIVE TO THE MOST ABUNDANT COMPOUND (PEAK F), (*E*)-2-DECENAL ($N = 24$ FOR PEAKS E, F, AND H; $N = 5$ FOR PEAKS A, B, C, AND D)

Peak	Compound	Relative ratio[a]	
		Female	Male
A	(*E*)-2-Hexenal	1.5 ± 0.4	1.8 ± 0.4
B	(*E*)-2-Octenal	0.8 ± 0.1	0.5 ± 0.1
C	(*E*)-2-Nonenal	0.6 ± 0.1	0.4 ± 0.1
D	(*Z*)-4-Decenal	0.4 ± 0.1	0.2 ± 0.1
E	(*E*)-2,9-Decadienal[b]	41.9 ± 3.5	8.0 ± 0.6
F	(*E*)-2-Decenal	100	100
G	(*E*,*Z*)-2,4-Decadienal[b]	41.6 ± 2.5	64.5 ± 3.6
H	(*E*,*E*)-2,4-Decadienal	5.1 ± 0.7	6.4 ± 0.6

[a] Peak areas were calculated from the reconstructed total ion current from GC–MS analysis and corrected for instrument response of each standard.
[b] The relative ratio of these components between female and male is significantly different by *t*-test comparison ($P < 0.05$).

metathoracic glands. The compound was identified as (*E*)-2,9-decadienal from its NMR spectrum: δ 1.3–1.6 (4H, m), 2.07 (2H, dt, $J = 7, 7$ Hz), 2.33 (2H, dt, $J = 7, 7$ Hz), 4.95 (1H, m), 5.0 (1H, ddt, $J = 17, 2, 1$ Hz), 5.78 (1H, ddt, $J = 17, 10, 7$ Hz), 6.11 (1H, dd, $J = 15, 8$ Hz), 6.8 (1H, dt, $J = 15, 7$ Hz), 9.5 (1H, d, $J = 8$ Hz). No NMR data were available in the literature for (*E*)-2,9-decadienal, but the corresponding data for the homolog (*E*)-2,8-nonadienal (Takano and Kawaminami, 1987) were a good match to those of the insect-produced compound. Further confirmation of the structure was obtained from derivatization experiments. Because direct derivatization of peak E with dimethyldisulfide (DMDS) was not successful, the aldehyde was first reduced to the corresponding alcohol with $LiAlH_4$ [EI MS; m/z (relative abundance)]: 41 (83), 55 (74), 57 (79), 67 (100), 81 (49), 95 (20), 121 (8), 136 (0.6; $M^+ - 18$). The mass spectral data of the DMDS adduct of this decadienol were as follows: 41 (17), 55 (23), 61 (100), 67 (23), 87 (20), 91 (17), 107 (29), 119 (16), 155 (37), 195 (9), 247 (15), 251 (9), 281 (1), 294 (1), 342 (1; M^+). The diagnostic fragment ion pairs of m/z 61 and 281, and 91 and 251, corresponding to cleavages between C2 and C3, and between C10 and C9, respectively, of the DMDS adduct, confirmed the double bond positions in the parent compound (Ho et al., 1996).

Components in DAGs of Adults. There was no apparent dimorphism in the morphology of the DAGs of females and males, and the III/IV DAGs were small in both sexes. Components of DAG extracts were identified by comparison of the mass spectral data and retention times of the unknowns with authentic samples (Figure 2). Limonene (peak 1) and α-terpineol (peak 5) were only found in DAGs

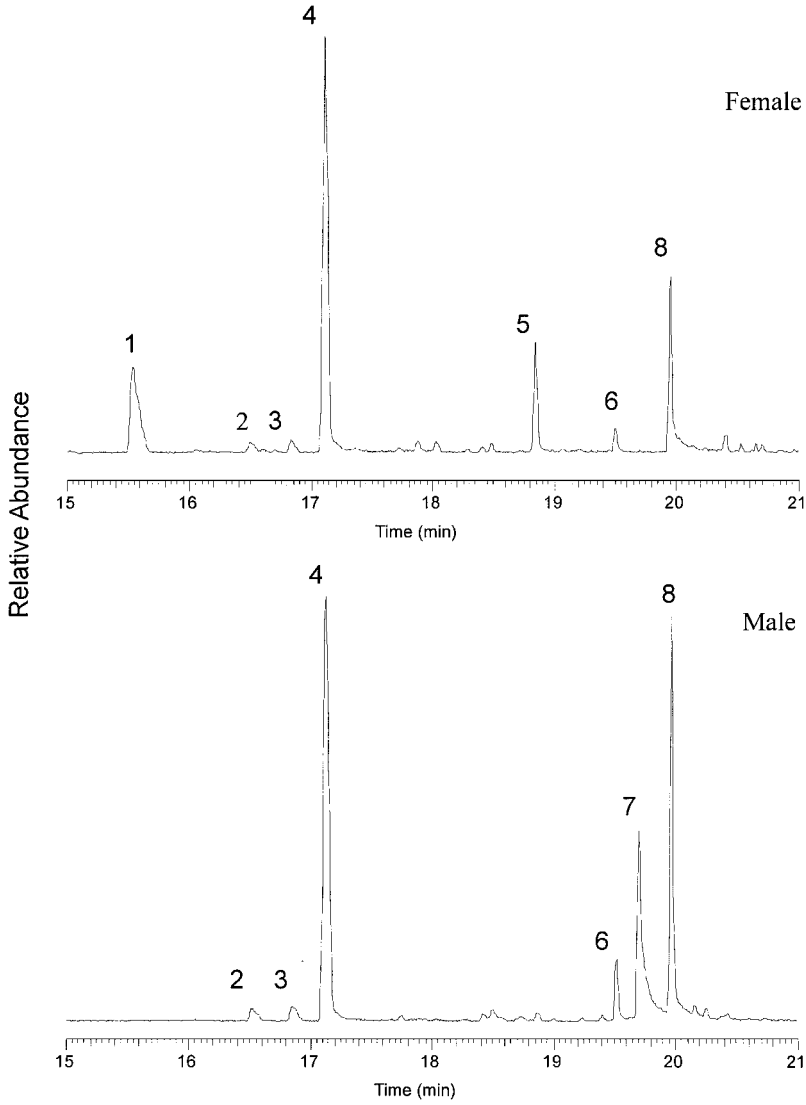
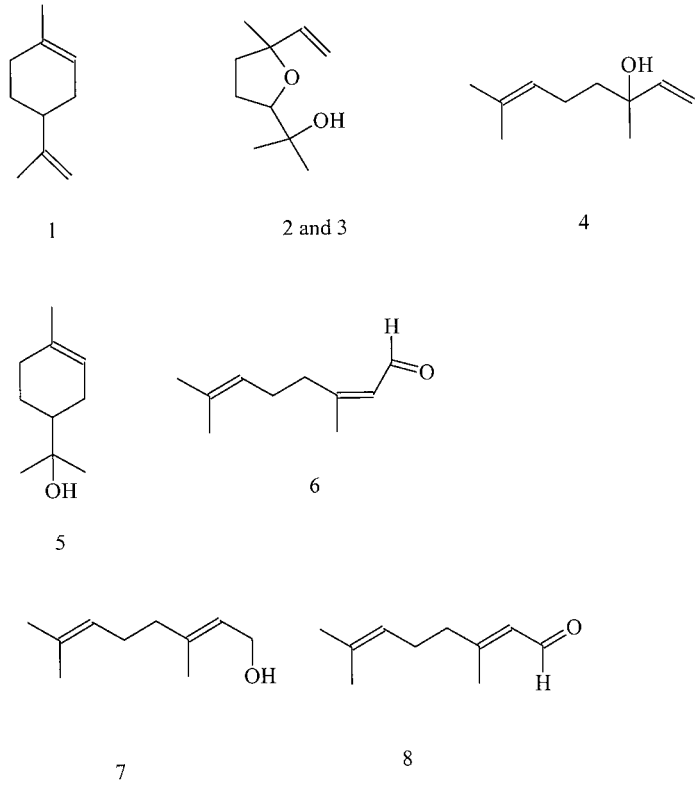


FIG. 2. Comparison of GC profiles of the dorsal abdominal gland contents of male and female *E. furcellata* and structures of the compounds. Compounds: 1: limonene, 2 and 3: linalool oxide isomers, 4: linalool, 5: α-terpineol, 6: neral, 7: geraniol, 8: geranial. Column: DB5-ms.



1 2 and 3 4

5 6

7 8

FIG. 2. CONTINUED.

from females, and geraniol (peak 7) was only found in DAGs from males. *Cis*- and *trans*-linalool oxides (peaks 2 and 3), linalool (peak 4), neral (peak 6), and geranial (peak 8) were found in extracts from both sexes.

Components in DAGs of Nymphs. The compounds found in the 2nd and 3rd pairs of DAGs of nymphs were identified by comparison with spectra and retention times of authentic compounds as described above, and the amount of each component is shown in Table 2.

Components in Heptane Washes of Adult Sternites. Morphologically, male but not female *E. furcellata* possess sternal setae. The mass spectrum of the major peak in the GC trace of heptane washes of the male sternites and setae exhibited major fragment ions of m/z 97, 103, 182, and 283. By comparison with the GC/MS spectral database, the compound was tentatively identified as 6,10,13-trimethyltetradecyl isovalerate. The identification was confirmed by comparison of the mass spectrum and the retention time with an authentic sample (as a mixture

TABLE 2. MAJOR COMPONENTS AND THE AMOUNT OF EACH COMPONENT IN THE DORSAL ABDOMINAL GLANDS OF NYMPHS

Peak	Compound	Amount (μg)[a] ($N = 3$) (mean $\pm$ stdev.)
A	(*E*)-4-Oxo-2-hexenal	42 ± 26
B	(*E*)-4-Oxo-2-octenal	4 ± 2
C	Tridecane	6 ± 1
D	Tetradecanal	2 ± 1

[a] Amounts were calculated relative to benzophenone internal standard and corrected based on instrument response to each compound.

of stereoisomers) provided by Dr J. R. Aldrich. The bug-produced stereoisomer was not determined. The amount of 6,10,13-trimethyltetradecyl isovalerate found in extracts from individual bugs varied greatly, and it was not detected in some extracts. Of the male bugs sampled, extracts from 3 out of 15 bugs taken from the lab rearing colony and 6 out of 90 virgin bugs contained more than 50 ng of 6,10,13-trimethyltetradecyl isovalerate (range 55–1400 ng/bug). For the three bugs from the colony, the average was 308 ± 197 ng/bug (mean $\pm$ SE) and for the six virgins, the average was 688 ± 236 ng/bug (mean $\pm$ SE). The rest of the extracts from male bugs either produced no detectable isovalerate, or the amount was less than 10 ng. A small amount (ranging from 0.4 to 1 ng) of isovalerate was observed from heptane washes of the sternites of female bugs from the colony. None of the compound was detected in sternite washes from virgin females.

DISCUSSION

MTG Content. Typical compounds found in MTGs of pentatomid bugs include alkanes, 2-alkenals, 2-alkenyl acetates, and 4-oxo-2-alkenals (Kou et al., 1989; Aldrich, 1995; Krall et al., 1999; Ho and Millar, 2001; McBrien et al., 2002). We report here that major compounds in MTG extracts of this asopine bug are the commonly occurring 2-alkenals, and some less common decadienal isomers.

Decadienal and 2-alkenals have been found in the secretion from the ventral glands of sawfly larvae (Boevé et al., 1997) and were suggested to be a defense against predators like ants (Boevé and Pasteels, 1985). These compounds were also found in the volatiles in the fruit of olive *Olea europaea* L. (Oleaceae) and exhibited a broad antimicrobial spectrum (Kubo et al., 1995; Bisignano et al., 2001).

MTG dimorphism has been observed for other Heteroptera, including alydid (Aldrich et al., 1993, 2000) and lygaeid bugs (Aldrich et al., 1997, 1999). For the alydid bugs *Riptorius serripes* (Fabricius) and *Mirperus scutellaris*, the MTGs are sexually dimorphic both morphologically and chemically (Aldrich et al., 1993). Females of the broad-headed bug *Alydus eurinus* (Say) release female-specific compounds from their MTGs, which attract conspecific males and to a lesser extent, females and nymphs (Aldrich et al., 2000). For the lygaeid bug *Tropidothoras cruciger* (Motschulsky), two compounds attractive to both male and female bugs are found more abundantly in the male MTG than in the female MTG (Aldrich et al., 1997). The MTG contents of the large milkweed bug *Oncopeltus fasciatus* (Dallas), are different between the sexes, but the significance of the difference remains unclear (Aldrich et al., 1999).

No dimorphism in structure and content of MTGs has been reported for pentatomid bugs before. Here, we report that the proportions of decadienal isomers is different in MTGs of male and female bugs, with the amount of (*E*)-2,9-decadienal being much higher in females than in males, and conversely, the relative amount of (*E*,*Z*)-2,4-decadienal being higher in males. (*E*)-2,9-Decadienal has been reported as an attractant for male anthocorids, *Orius insidiosus* (Say), in Y-tube olfactometer bioassays (Aldrich and Puapoomchareon, 1996). A homologous dienal, 4,8-nonadienal, also has been suggested as a possible pheromone of the West African shield bug, *Sphaerocoris annulus* (Heteroptera: Pentatomidae: Scutellerinae) (Gough et al., 1986). Thus, the different distribution of (*E*,*Z*)-2,4-decadienal and (*E*)-2,9-decadienal in male and female *E. furcellata* suggests that these compounds may be involved in communication between the sexes.

DAG Contents. Compounds found in the male DAGs of *Podisus spp.* include (*E*)-2-hexenal and benzyl alcohol, with terpineol for *P. maculiventris* and linalool for *P. neglectus* (Aldrich, 1998). For the Australasian predaceous bug, *Oechalia schellenbergii*, only during part of its life cycle, the DAGs of males are larger than those of females, and the compounds found in male DAGs were 3-methylenehexyl acetate and 9-hydroxygeranyl diacetate (Aldrich et al., 1996).

Although the DAGs in male *E. furcellata* were not enlarged, sexual dimorphism in the contents of DAGs was found. Of the major components in the DAGs, geraniol was only observed in the DAGs of males, and limonene and α-terpineol were only found in the DAGs of females. Linalool oxide isomers, linalool, neral, and geranial were found in DAGs of both female and male bugs. These compounds are similar to those found in the enlarged DAGs of adult *Podisus spp.* It is possible that the compounds found in the DAG of adult *E. furcellatta* also serve as attractants, similar to the case of *Podisus spp*. This remains to be tested in bioassays.

Sternite Extracts. Previously, sternal glands have been reported only in stink bug species that are considered specialist predators (Aldrich, 1998). Aldrich et al.

(1986) also reported that predatory bugs that have sternal glands do not have hypertrophied DAGs. Although *E. furcellata* is a generalist rather than a specialist predator, it fits this pattern, because it appears to have SGs that exude trimethyltetradecyl isovalerate onto the sternal setae of males. This compound had been reported previously only from sternal setae of males of several predaceous stink bugs native to the Americas (*Perrillus bioculatus*, *Oplomus severus*, *O. dichrous*, and *Mineus strigipes;* Aldrich et al., 1986; Aldrich and Lusby, 1986). Thus, this represents the first report of this compound from an Asian true bug species. For reasons unknown, this compound was only detected in significant amounts from about 5% of virgin or colony males, similar to what had been reported for *O. dichrous* and *M. strigipes* (Aldrich and Lusby, 1986).

DAG Contents of Nymphs. The DAG contents of nymphs were typical of pentatomids, with 4-oxo-(*E*)-2-alkenals and alkanes as major components (Pavis et al., 1994; Ho and Millar, 2001).

In summary, the results of chemical analyses of the gland contents of the generalist stink bug, *E. furcellatta* are presented. We found dimorphism in the contents of DAGs and MTGs of adults and a male specific compound in extracts of sternal setae. With these dimorphisms between the sexes, it is likely that some of the chemicals identified play a role in intersexual communication. The biological significance of these chemicals is currently being studied.

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DISRUPTION BY CONOPHTHORIN OF THE KAIROMONAL RESPONSE OF SAWYER BEETLES TO BARK BEETLE PHEROMONES

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Abstract—Antennally active nonhost angiosperm bark volatiles were tested for their ability to reduce the response of three common species of coniferophagous wood-boring Cerambycidae to attractant-baited multiple funnel traps in the southern interior of British Columbia. Of the nonhost volatiles tested, only conophthorin was behaviorally active, disrupting the attraction of sawyer beetles, *Monochamus* spp., to traps baited with the host volatiles α-pinene and ethanol and the bark beetle pheromones ipsenol and ipsdienol. Conophthorin did not affect the attraction of sawyer beetles to the host kairomones α-pinene and ethanol in the absence of bark beetle pheromones, nor did it have any behavioral effect on adults of *Xylotrechus longitarsis*, which were not attracted to bark beetle pheromones. These results indicate that conophthorin does not act as a general repellent for coniferophagous Cerambycidae, as it seems to do for many species of Scolytidae, but has the specific activity of disrupting the kairomonal response of sawyer beetles to bark beetle pheromones.

Key Words—Coleoptera, Cerambycidae, *Monochamus*, *Xylotrechus longitarsis*, Scolytidae, kairomones, nonhost volatiles, disruptant, conophthorin, bark beetle.

INTRODUCTION

Wood-boring insects aid in decomposition of coarse woody debris, but this ecological role also leads to economic damage of timber destined for lumber production.

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Most damaging in British Columbia, Canada, are the coniferophagous ambrosia beetles (Coleoptera: Scolytidae), for which semiochemical-based management has become standard practice (Borden, 1995; Shore and McLean, 1995). Larger wood-boring beetles in the families Cerambycidae and Buprestidae also cause considerable damage to the softwood lumber industry (Phero Tech Inc., 1997), but relatively little is known about their chemical ecology (Borden, 1995).

In British Columbia, the sawyer beetles, *Monochamus* spp., and *Xylotrechus longitarsis* Casey (both cerambycids) are widely distributed (Linsley, 1964; Linsley and Chemsak, 1984) and are abundant pests in log storage facilities (McIntosh et al., 2001; Morewood et al., 2002a). Moreover, *Monochamus scutellatus* (Say), is known to vector the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Linit, 1988). Studies on host selection by these species have led to improved trap designs (McIntosh et al., 2001; Morewood et al., 2002a) and attractant lures (Allison et al., 2001, 2003; Morewood et al., 2002b). Knowledge of how beetles recognize and avoid unsuitable hosts or nonhosts would allow for a greater range of management options, possibly including "push-pull" tactics (Lindgren and Borden, 1993).

Many coniferophagous Scolytidae detect and respond to volatile chemicals from nonhost angiosperm trees. Various six carbon alcohols and aldehydes, known as green leaf volatiles, disrupt responses to attractive semiochemicals in at least 11 species of these beetles (Dickens et al., 1992; Wilson et al., 1996; Borden et al., 1997; Deglow and Borden, 1998a,b; Poland et al., 1998; Zhang et al., 1999; Poland and Haack, 2000). Similarly, nonhost angiosperm bark volatiles, often including green leaf volatiles, produce antennal responses (Tømmerås and Mustaparta, 1989; Borden et al., 1998; Huber et al., 2000b; Zhang et al., 2000) and disrupt behavioral responses to attractive semiochemicals (Schroeder, 1992; Borden et al., 1998; Byers et al., 1998; Huber et al., 2000a, 2001; Huber and Borden, 2001a,b; Zhang et al., 2001) in various species of bark and ambrosia beetles. Avoidance responses to nonhost volatiles may help these beetles to maximize the efficiency of their search for suitable hosts and, thus, conserve energy and minimize their exposure to adversities such as predators and environmental extremes (Huber et al., 1999).

In conjunction with investigations of the activity of nonhost angiosperm bark volatiles on coniferophagous bark beetles, we tested the hypotheses that volatiles would elicit antennal activity in the cerambycids *Monochamus clamator* LeConte, *M. scutellatus*, and *X. longitarsis*, and that antennally-active nonhost volatiles would disrupt the process of host selection.

METHODS AND MATERIALS

Adult *M. clamator*, *M. scutellatus*, and *X. longitarsis* were collected in southern British Columbia from log decks and from multiple funnel traps baited

with α-pinene and ethanol in the Lillooet Forest District and in millyards at Westbank and Okanagan Falls during the summers of 1998 and 1999 to initiate laboratory colonies. Beetles were transported to the laboratory in glass jars with fresh branches of lodgepole pine, *Pinus contorta* Douglas ex Loudon var. *latifolia* Engelmann, and ponderosa pine, *Pinus ponderosa* P. & C. Lawson, in portable coolers with freezer packs. In the laboratory, each species was held separately in ca. $1 \times 1 \times 1$ m cages with freshly cut lodgepole pine and ponderosa pine bolts (20–30 cm diam, 50–75 cm length), the cut ends of which were sealed with paraffin wax, for oviposition. Caged adults fed on the bark and foliage of lodgepole pine and ponderosa pine branches, which included the current and previous years' growth and were changed weekly, and were offered cotton wicks dipped in water. Lab-reared adults were used to infest fresh host logs to maintain the colony, and subsequently were used for coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses.

Bark volatiles were collected as described by Huber et al. (2000b) from six angiosperm trees native to British Columbia, black cottonwood, *Populus trichocarpa* Torrey & A. Gray (Salicaceae), trembling aspen, *Populus tremuloides* Michaux (Salicaceae), paper birch, *Betula papyrifera* Marshall (Betulaceae), bigleaf maple, *Acer macrophyllum* Pursh (Aceraceae), red alder, *Alnus rubra* von Bongard (Betulaceae), and Sitka alder, *Alnus viridis* ssp. *sinuata* (Regel) Á. Löve & D. Löve (Betulaceae). In the GC-EAD analyses (Arn et al., 1975; Gries, 1995), 2 μl of a blend of the aerations from these nonhost angiosperm trees were tested against male or female antennae of *M. scutellatus*, *M. clamator*, and *X. longitarsis* ($N = 3$–5 in each case) using a Hewlett-Packard 5890A gas chromatograph equipped with a fused silica column (30 m $\times$ 0.25 mm ID for *Monochamus* spp., 30 m $\times$ 0.32 mm ID for *Xylotrechus*) coated with DB-5 (J & W Scientific, Folsom, California).

Behavioral activity of selected antennally-active nonhost volatiles (Table 1) was evaluated in field trapping experiments conducted July 20–October 2, 2000, and July 19–September 26, 2001, at the Riverside Forest Products Ltd. 4-Mile Log Yard on the Bear Forest Service Road west of Kelowna, British Columbia. Twelve-unit multiple funnel traps (Phero Tech Inc., Delta, British Columbia) were spaced $\geq$10 m apart and suspended from metal poles such that the top funnel was ca. 1.5 m above ground. A small block of Vapona No-Pest Strip (Green Cross, Fisons Horticulture Inc., Mississauga, Ontario) was placed in each collecting cup to minimize predation and cannibalism. Traps were either unbaited (negative controls) or baited with attractive kairomone lures (positive controls) alone or combined with one or more nonhost volatiles.

In 2000, two trapping experiments were conducted concurrently, one directed at *Monochamus* spp. and one at *X. longitarsis*. In the *Monochamus* experiment, the attractive kairomone lures (Table 1) consisted of α-pinene [99% chemically pure, 96% (−) enantiomer] plus ethanol plus four bark beetle pheromones [all as racemic mixtures] that were collectively known to act as attractive kairomones for

TABLE 1. COMPOUNDS USED IN FIELD TRAPPING EXPERIMENTS, THEIR SOURCES, CHEMICAL PURITIES, AND RELEASE RATES

Compound[a]	Chemical purity (%)	Source[b–e]	Release device and rate (mg/24 hr)[f]	
Attractant kairomones				
(−)-α-Pinene	99	Phero Tech[b]	Blue plastic sleeve	2200
Ethanol (2000)	95	Phero Tech	Black plastic sleeve	1800
Ethanol (2001)	95	Phero Tech	White plastic sleeve	40
(±)-Frontalin	99	Phero Tech	Polyethylene vial	2.6
(±)MCH	99	Phero Tech	Bubble cap	4.5
(±)-Ipsenol	98	Phero Tech	Bubble cap	0.2
(±)-Ipsdienol	95	Phero Tech	Bubble cap	0.1
Nonhost volatiles				
Nonanal	95	Aldrich[c]	Polyethylene bottle	22
Decanal	95	Aldrich	Polyethylene bottle	34
(*Z*)-3-Hexen-1-ol	98	Bedoukian[d]	Polyethylene bottle	2
Guaiacol	98	Aldrich	Polyethylene bottle	11
4-Allylanisol	98	Aldrich	Polyethylene bottle	76
(−)-Bornyl acetate	97	Sigma[c]	Polyethylene bottle	11
(±)-(*E*)-Conophthorin	86	S.F.U.[e]	Polyethylene vial	5

[a] I.U.P.A.C. names if different from names used in the table: (−)-α-pinene is (1*S*, 5*S*)-2, 6, 6-trimethylbicyclo[3.1.1]hept-2-ene (optical purity 96%); frontalin is 1,5-dimethyl-6,8-dioxabicyclo [3.2.1]octane; MCH is 3-methyl-2-cyclohexen-1-one; ipsenol is 2-methyl-6-methylene-7-octen-4-ol; ipsdienol is 2-methyl-6-methylene-2,7-octadien-4-ol; guaiacol is 2-methoxyphenol; 4-allylanisol is 1-methoxy-4-(2-propenyl)benzene; (*E*)-conophthorin is (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane. Optical purity of (−)-bornyl acetate is unknown. Frontalin and MCH are listed as attractant kairomones because they were components of our original kairomonal blend of bark beetle pheromones (Allison et al., 2001); subsequently, we showed that all of the bioactivity resides in ipsenol and ipsdienol (Allison et al., 2003).

[b] Phero Tech Inc., 7572 Progress Way, Delta, British Columbia, V4G 1E9 Canada.

[c] Sigma-Aldrich Canada Ltd., 2149 Winston Park Drive, Oakville, Ontario, L6H 6J8 Canada.

[d] Bedoukian Research, Inc., 21 Finance Drive, Danbury, Connecticut, 06810 USA.

[e] Synthesized by H. D. Pierce, Jr., Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia, V5A 1S6 Canada.

[f] All release devices provided by Phero Tech; release rates of the ethanol lures used in 2001 and of frontalin, MCH, ipsenol, and ipsdienol determined at 20–23°C by Phero Tech; all other release rates determined at 28–30°C at S.F.U.

Monochamus spp. (Allison et al., 2001). Seven nonhost volatiles that elicited antennal responses in *M. scutellatus* and *M. clamator* were tested for their ability to reduce attraction to the kairomone lures. In the *Xylotrechus* experiment, the attractive kairomone lures consisted of α-pinene plus ethanol, an optimal combination of attractive host kairomones for this species (Morewood et al., 2002b). Three nonhost volatiles that elicited antennal responses in *X. longitarsis* were tested for their ability to reduce attraction to the kairomone lures.

In 2001, a single trapping experiment was conducted to confirm and clarify the results obtained with *Monochamus* spp. and conophthorin in 2000. Three combinations of attractive kairomones were used, including those from the two

experiments conducted in 2000. The third combination of kairomones consisted of α-pinene plus ethanol plus ipsenol and ipsdienol because these two compounds were thought to be the bioactive components of the four-component bark beetle pheromone blend used in 2000 (Allison et al., 2003). A racemic mixture of predominantly (*E*)-conophthorin (*E*/*Z* ratio 23:1) was tested for its ability to reduce attraction to each of the three combinations of kairomones. Conophthorin from natural sources occurs as 65–100% (−) enantiomer, with *E*/*Z* ratios from 2:1 to 40:1; the (+) enantiomer is generally inactive and the *Z* stereoisomer is thermodynamically unstable (Zhang et al., 2002).

Treatments were tested in eight (2000) or 15 (2001) randomized complete blocks. Insects were collected from the traps, and the treatments rerandomized within blocks, weekly (2000) or biweekly (2001), and captured insects were frozen until they could be sorted and counted. Data were transformed by $\log(x + 1)$ to correct for non-normality and heteroscedasticity (Thöni, 1967; Zar, 1999), and then subjected to analysis of variance (PROC ANOVA) for randomized complete blocks (Kvanli, 1988; SAS Institute Inc., 1999). Means from the 2000 experiments were compared using the Ryan-Einot-Gabriel-Welsch (REGWQ) multiple range test (Day and Quinn, 1989; SAS Institute Inc., 1999).

Planned comparisons were used to evaluate differences among certain sets of means from the 2001 conophthorin experiment. One-tailed paired-sample *t* tests were used to test for significant reductions in catches in traps with conophthorin compared to the corresponding treatments without conophthorin. Two-tailed paired-sample *t* tests were used to determine whether catches of *Monochamus* spp. differed significantly in traps baited with only two or all four of the bark beetle pheromones. Sheffé's test was used to determine whether catches of *Monochamus* spp. in traps with conophthorin differed significantly from those in unbaited traps and whether catches of *X. longitarsis* in traps with bark beetle pheromones differed significantly from those in traps with host volatiles alone. These planned comparisons were conducted as outlined by Zar (1999).

RESULTS

Antennae of *Monochamus* spp. consistently showed strong responses to a number of volatile compounds from the bark of nonhost angiosperm trees, including (*Z*)-3-hexen-1-ol, conophthorin, guaiacol, nonanal, 4-allylanisol, decanal, and bornyl acetate (Figure 1). Antennae of *X. longitarsis* consistently showed strong responses to many of the same compounds, with the obvious exception of bornyl acetate (Figure 2). Antennae of both *Monochamus* spp. and *X. longitarsis* showed strong responses to a number of additional compounds, such as α-pinene and β-phellandrene, that were also found in host conifers (Morewood et al., 2002b) and were ignored for the purposes of this study.

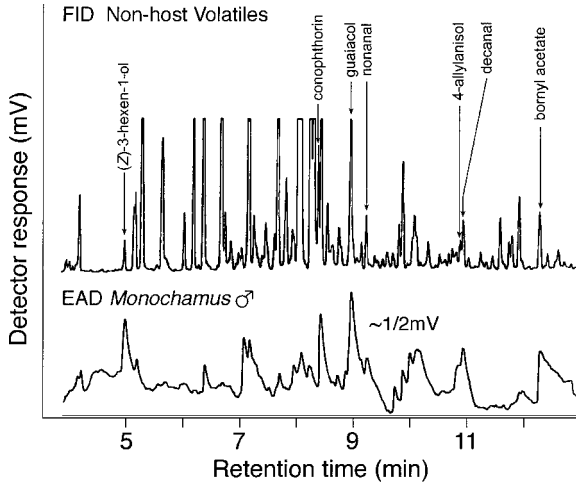

FIG. 1. Flame-ionization detector (FID) and electroantennographic detector (EAD: male *Monochamus clamator* antenna) responses to a blend of bark volatiles from six nonhost angiosperm tree species native to British Columbia. Chromatography: splitless injection; injector and FID detector: 250°C; DB-5 column; temperature program: 50°C (1 min) then 10°C/min to 240°C.

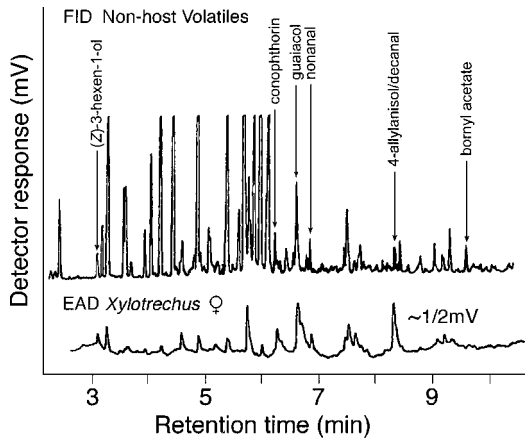

FIG. 2. Flame-ionization detector (FID) and electroantennographic detector (EAD: female *Xylotrechus longitarsis* antenna) responses to a blend of bark volatiles from six nonhost angiosperm tree species native to British Columbia. Chromatography: splitless injection; injector and FID detector: 250°C; DB-5 column; temperature program: 50°C (1 min) then 10°C/min to 240°C.

In the 2000 *Monochamus* experiment (Figure 3), greater numbers of both sexes of *M. scutellatus*, *M. clamator*, and *X. longitarsis* were captured in traps baited with attractive kairomones (host volatiles plus four bark beetle pheromones) than in unbaited control traps. Smaller numbers of both sexes of both *M. scutellatus* and *M. clamator* were captured in traps with conophthorin than in traps with attractant kairomones only; traps with conophthorin captured consistently, and in most cases significantly, smaller numbers of both sexes of both *Monochamus* spp. than any other traps except the unbaited controls. In contrast, catches of *X. longitarsis* showed no differences in traps with nonhost volatiles compared to traps with attractant kairomones only.

In the 2000 *Xylotrechus* experiment (Figure 4), greater numbers of both sexes of *M. scutellatus*, female *M. clamator*, and both sexes of *X. longitarsis* were captured in traps baited with host volatiles than in unbaited control traps. There were no differences in catches of any species among any of the treatments other than the unbaited traps. In particular, none of the treatments with nonhost volatiles reduced trap catches of any species compared to host volatiles alone.

In the 2001 conophthorin experiment (Figure 5), catches of both sexes of both *M. scutellatus* and *M. clamator* in traps baited with host volatiles plus bark beetle pheromones were reduced with the addition of conophthorin ($P < 0.001$ in all cases). In contrast, catches of *Monochamus* spp. in traps baited only with host volatiles did not differ with the presence or absence of conophthorin ($P > 0.25$ for each sex of each species). Catches of *Monochamus* spp. in traps with conophthorin did not differ from those in unbaited traps ($P > 0.05$). In addition, catches of *Monochamus* spp. did not differ among traps baited with only two or all four of the bark beetle pheromones ($P > 0.50$ for each sex of each species). Catches of *X. longitarsis* in traps with conophthorin were not reduced compared to traps with attractive kairomones only ($P > 0.50$ for each sex in each comparison). Finally, catches of *X. longitarsis* did not differ in traps baited with host volatiles plus bark beetle pheromones compared to traps baited with host volatiles alone ($P > 0.05$).

DISCUSSION

The only behavioral effect of the nonhost volatiles tested in this study was that the response to traps baited with α-pinene, ethanol, ipsenol, and ipsdienol by *M. scutellatus* and *M. clamator* was disrupted by conophthorin (Figures 3 and 5), although the response to α-pinene and ethanol without ipsenol and ipsdienol was unaffected (Figures 4 and 5). Because of strong location effects, evidenced by the differences in catches in unbaited traps (compare Figures 3 and 4), this pattern of responses could only be confirmed through the direct comparisons made in the 2001 conophthorin experiment. α-Pinene plus ethanol was attractive to *Monochamus* spp. in the absence of bark beetle pheromones (Figure 4), but traps

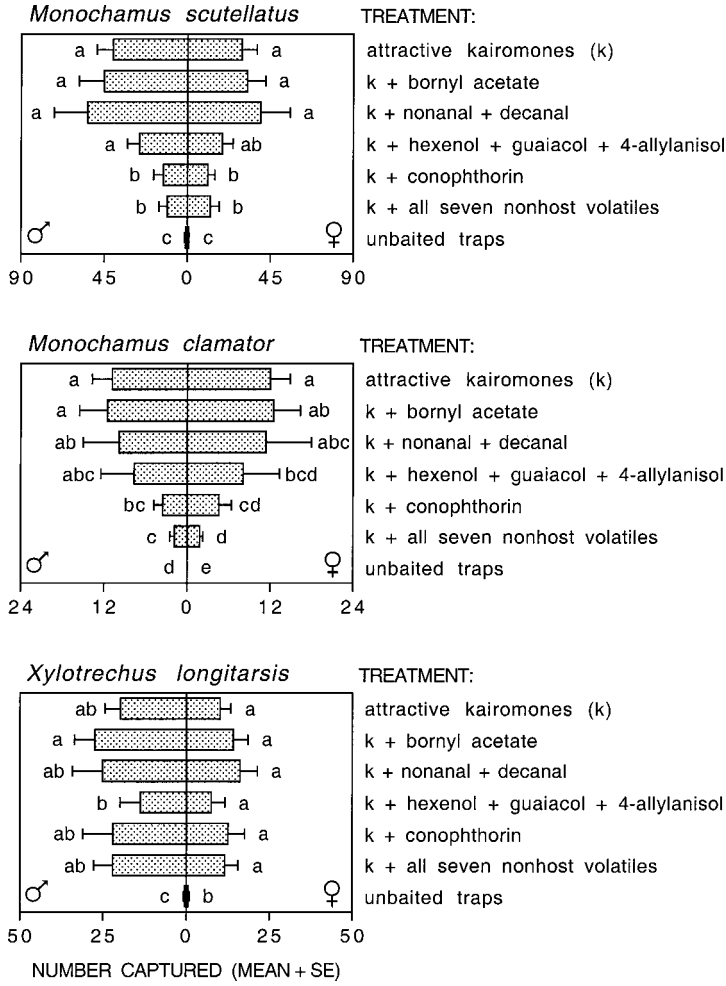

FIG. 3. Numbers of *Monochamus scutellatus*, *Monochamus clamator*, and *Xylotrechus longitarsis* captured July 20–October 2, 2000, around the Riverside Forest Products Ltd. 4-Mile log yard on the Bear Forest Service Road west of Kelowna, British Columbia, in traps baited with different combinations of attractive kairomones and nonhost volatiles tailored for *Monochamus* spp. The "attractive kairomones" are the host volatiles α-pinene and ethanol, and the bark beetle pheromones frontalin, MCH, ipsenol, and ipsdienol; "hexenol" is (*Z*)-3-hexen-1-ol. For a given species and sex, differences among bars with the same letter are not statistically significant (REGWQ, $P > 0.05$).

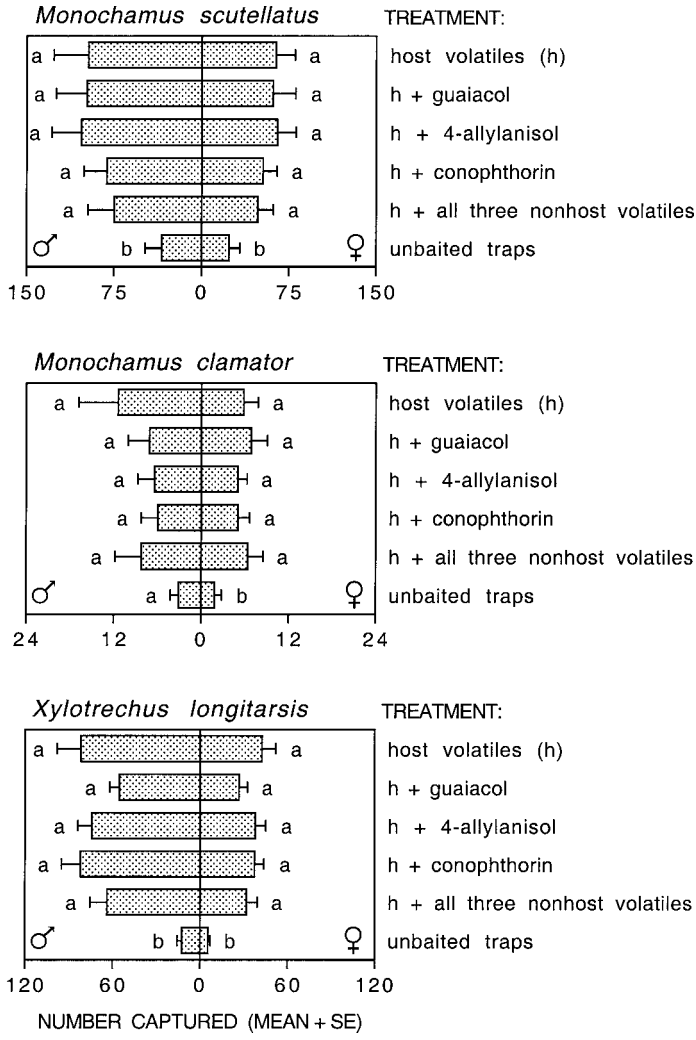

FIG. 4. Numbers of *Monochamus scutellatus*, *Monochamus clamator*, and *Xylotrechus longitarsis* captured July 20–October 2, 2000, around the Riverside Forest Products Ltd. 4-Mile log yard on the Bear Forest Service Road west of Kelowna, British Columbia, in traps baited with different combinations of host volatiles (α-pinene and ethanol) and nonhost volatiles tailored for *X. longitarsis*. For a given species and sex, differences among bars with the same letter are not statistically significant (REGWQ, $P > 0.05$).

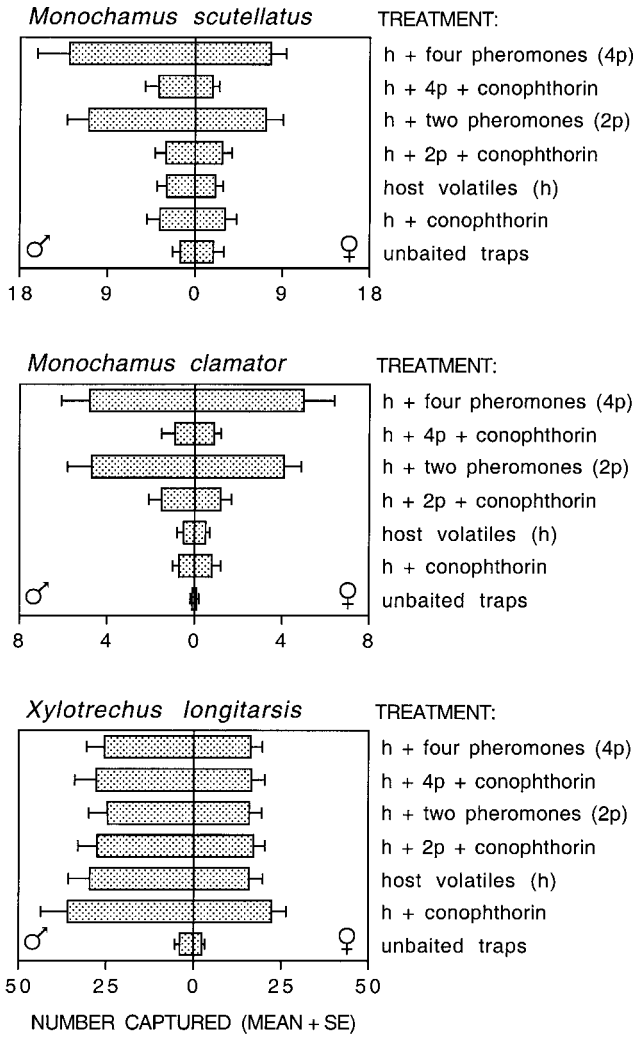

FIG. 5. Numbers of *Monochamus scutellatus*, *Monochamus clamator*, and *Xylotrechus longitarsis* captured July 19–September 26, 2001, at the Riverside Forest Products Ltd. 4-Mile log yard on the Bear Forest Service Road west of Kelowna, British Columbia, in traps baited with different combinations of attractive kairomones (host volatiles and bark beetle pheromones) and the nonhost volatile conophthorin. The "four pheromones" are frontalin, MCH, ipsenol, and ipsdienol, the "two pheromones" are ipsenol and ipsdienol, and the "host volatiles" are α-pinene and ethanol. Statistical analyses are described in the text.

baited only with α-pinene plus ethanol were outcompeted by traps with bark beetle pheromones added (Figure 5). These results are consistent with those of Allison et al. (2001), and further confirm that ipsenol and ipsdienol are the bark beetle pheromones that act as attractive kairomones for sawyer beetles (Allison et al., 2003). None of the nonhost volatiles, not even conophthorin, showed any general repellent activity in combination with host volatiles (α-pinene and ethanol) for any of the three species. Furthermore, *X. longitarsis* showed no attraction to bark beetle pheromones and no inhibition by the presence of conophthorin.

Conophthorin was first found in workers of *Paravespula vulgaris* (Hymenoptera: Vespidae) and was determined to be the major component of species-specific "bouquets" of spiroacetals in that species and in *Paravespula germanica* and *Dolichovespula saxonica* (Hymenoptera: Vespidae) (Francke et al., 1979). In these social wasps, conophthorin was thought to reduce aggression among workers, a role that would likely be related to species recognition, considering its occurrence in species-specific combinations of volatiles. Conophthorin is also produced by various Scolytidae, namely male *Leperisinus varius* and adult *Cryphalus piceae* (Francke et al., 1979; Kohnle et al., 1992), male and female *Conophthorus coniperda* (Birgersson et al., 1995), and male *Conophthorus resinosae* (Pierce et al., 1995), *Conophthorus ponderosae* (Miller et al., 2000), *Pityophthorus carmeli*, and *Pityophthorus nitidulus* (Dallara et al., 2000). Recently, conophthorin has been found among the bark volatiles of various angiosperm trees, including species of birch (*Betula*), aspen and poplar (*Populus*), and maple (*Acer*) (Byers et al., 1998; Huber et al., 1999, 2000b; Zhang et al., 2000, 2001).

With the exception of *P. carmeli*, for which conophthorin, in combination with pityol [(2*R*, 5*S*)-(+)-2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran], was reported to be attractive (Dallara et al., 2000), conophthorin inhibits attraction to aggregation pheromones or host kairomones in every species of Scolytidae known to produce it (Kohnle et al., 1992; Birgersson et al., 1995; Pierce et al., 1995; de Groot et al., 1998; Dallara et al., 2000; de Groot and DeBarr, 2000; Rappaport et al., 2000), as well as the related species *Conophthorus conicolens* and *Conophthorus teocotum* (Rappaport et al., 2000), *Pityophthorus cariniceps* (de Groot and DeBarr, 2000), and *Pityophthorus setosus* (Dallara et al., 2000) that are not known to produce it. In all of these species, conophthorin is thought to act as an antiaggregation pheromone or kairomone, reducing competition among the beetles by providing a signal indicating that a given resource is occupied.

Conophthorin also acts as a repellent or disruptant for species of bark and ambrosia beetles that are not known to produce the compound, including *Xylosandrus germanus* (Kohnle et al., 1992), *Pityogenes chalcographus* and *Ips typographus* (Byers et al., 1998; Zhang et al., 2000), *Dendroctonus ponderosae* (Huber et al., 1999; Huber and Borden, 2001b), *Dendroctonus pseudotsugae* (Huber et al., 1999, 2000a; Huber and Borden, 2001a), *Ips pini* (Huber et al., 2000a, 2001), and *Ips duplicatus* (Zhang et al., 2001). In these species, conophthorin is thought to act as

a nonhost kairomone, aiding in host location by providing a signal representing nonhost angiosperm trees for the beetles to avoid in their search for conifer hosts.

The role of conophthorin in the chemical ecology of sawyer beetles is not yet entirely clear, although it may simply act as a nonhost kairomone that helps focus the host-location behavior of the beetles once they have detected a particularly suitable host for reproduction (Huber and Borden, 2001b). If these beetles disperse randomly in search of host material, it would not be adaptive to respond to negative stimuli from nonhost material until there is a positive alternative available. Responding to negative stimuli in the absence of a positive alternative would not aid in the location of suitable resources. Thus, the beetles should become responsive to conophthorin, which is produced by a number of nonhost species, only after detecting appropriate host stimuli. α-Pinene and ethanol may be used to help locate hosts for adult feeding, but may not be a critical cue for recognition of suitable breeding material (Morewood et al., 2002a) and would not trigger the response to conophthorin. Ipsenol and ipsdienol, on the other hand, provide cues representing host material favorable for reproduction of sawyer beetles (Allison et al., 2001, 2003). It would be adaptive for adult sawyer beetles to use conophthorin as a cue to avoid nonhost material once they have been alerted to the presence of suitable host material by ipsenol or ipsdienol, thus ensuring that they select the correct host to provide their progeny with the greatest chance for survival.

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FACTORS ACCOUNTING FOR THE VARIABILITY IN THE BEHAVIORAL RESPONSE OF THE ONION FLY (*Delia antiqua*) TO *n*-DIPROPYL DISULFIDE

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Abstract—The onion fly *Delia antiqua* is a specialist herbivore attacking only onions and closely related *Allium* species. *n*-Dipropyl disulfide (Pr_2S_2) has long been known to be attractive to onion flies in the laboratory and in the field. However, the insect's response is highly variable. Using behavioral bioassays we found that Pr_2S_2 was highly attractive to gravid, mated female onion flies, but did not stimulate oviposition. The response of female onion flies was concentration dependent. The physiological state of the flies (i.e., age, mating status, egg load/oviposition experience) also affected their responsiveness. The response of both sexes of onion flies varied with age, but females were always more strongly attracted than males. Responsiveness of females increased during the first 10 days after emergence. It stayed at a high level until 21 days after which the experiment was terminated. The responsiveness of males reached a maximum at 6–7 days after which it declined. Mated, gravid females responded more strongly to Pr_2S_2 than unmated, gravid females. Females deprived of the opportunity to oviposit were more attracted compared to females that had oviposited on cut onions prior to the experiment. Electroantennograms (EAG) of females revealed a higher response to stimulation compared to males. The EAG-response of females was not affected by mating status.

Key Words—*Delia antiqua*, onion fly, dipropyl disulfide, electroantennogram, host location, oviposition, olfaction.

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INTRODUCTION

The onion fly *Delia antiqua* (Meigen) (Diptera: Anthomyiidae) is a specialist herbivore attacking only onions (*Allium cepa* L.) and closely related *Allium* species (Loosjes, 1976). There is evidence that positive anemotaxis at the patch level of resource distribution is mediated by volatile onion compounds (Judd and Borden, 1988, 1992). In contrast to earlier reports (Boelens et al., 1971), dipropyl disulfides do not appear to be actual headspace volatiles of *Allium* species, but rather are analytical artifacts (Auger et al., 1990; Ferary et al., 1996). However, there is evidence that dipropyl disulfides, such as *n*-dipropyl disulfide (Pr_2S_2), are emitted from bacterial metabolism of onion root exudates and decomposition of plant material (Ikeshoji, 1984). Onion material that is infected with decomposing microorganisms is especially attractive to onion flies (Dindonis and Miller, 1980; Hough et al., 1981; Hausmann and Miller, 1989).

Pr_2S_2 is often deployed in traps to monitor onion fly population dynamics. However, a number of field studies have shown that the attractiveness of Pr_2S_2 baited traps is highly variable (Eckenrode et al., 1975; Loosjes, 1976; Ishikawa et al., 1981; Miller and Haarer, 1981; Judd and Borden, 1988). This variation is partly due to two reasons: (a) the physiological status (age, mating status, ovarian development, and egg load), and (b) the sex ratio of the onion fly population. There are controversial results on whether males or females are more responsive to Pr_2S_2 (Judd and Borden, 1988, 1991, 1992; Judd and Whitfield, 1997). While in a number of studies, mainly females were caught in Pr_2S_2 loaded traps (Matsumoto, 1970; Dindonis and Miller, 1981a,b; Miller and Haarer, 1981), others report that males were more strongly attracted (Eckenrode et al., 1975; Loosjes, 1976; Vernon et al., 1981; Judd and Borden, 1992; Judd and Whitfield, 1997).

Within a host patch, Pr_2S_2 was found to be only marginally attractive (Miller et al., 1984; Judd and Borden, 1992). However, it still plays an important role during later steps in the host finding/selection process, as it increases the flies' overall activity and frequency of alighting, and mediates the host examination behavior (Harris et al., 1987; Harris and Miller, 1988, 1991; Judd and Borden, 1991). Studies also have reported an oviposition stimulating effect of Pr_2S_2 (Matsumoto and Thorsteinson, 1968; Vernon et al., 1977; Ishikawa et al., 1978; Pierce et al., 1978; Harris et al., 1987). There is strong evidence, however, that within a host patch complex chemical signals (Dindonis and Miller, 1981a; Vernon et al., 1981; Judd and Borden, 1992), tactile (Harris and Miller, 1988; Mowry et al., 1989), and visual stimuli (Harris and Miller, 1988; Judd and Borden, 1991) are needed for successful host-finding and host-selection.

This study was conducted to further clarify the role of Pr_2S_2 in the searching and oviposition behavior of onion flies and to better understand the variability of the flies' response to this host plant kairomone. Physiological parameters that were tested included the age of flies, female mating status, and effect of oviposition

deprivation. In addition, electroantennograms were recorded to test for differences in the receptor sensitivity to Pr_2S_2 between the sexes and between mated and unmated females.

METHODS AND MATERIALS

Insect Material. Onion fly pupae were collected from a laboratory culture continuously reared since 1973 on an artificial diet based on onions, beans, and yeast. After emergence, flies were kept in cages (34 × 34 × 39 cm) in the greenhouse at 25 × 1°C and 30–50% RH. Prior to use, adult flies were provided with water, a mixture of yeast and sugar (1:1, w:w), and a water–malt solution. If not otherwise stated, 10–15-day-old females were used to ensure that they were gravid and mated (Theunisson, 1976; McDonald and Borden, 1995; Spencer and Miller, 2002).

Chemicals. n-Dipropyl disulfide (purity of 98%) was purchased from Aldrich. Pr_2S_2 was dissolved in paraffin oil that acts as a slow release formulation. An amount of 5 ml of test solution was used in the experiments.

Bioassay Conditions. Bioassays were conducted in cages (30 × 25 × 28 cm) in the greenhouse at 25 × 1°C and 30–50% RH. All experiments were started between 09:00 and 10:00 hr and terminated after 7 hr.

Trapping Bioassays. The test material was placed in a 100-ml conical flask covered with a glass funnel (diam. 5 cm; entry hole diam. 5 mm). This set-up prevented the escape of flies that had been attracted and entered the flask. We tested two flasks in a choice set-up, one containing the test material, the other one serving as the control ($N = 80$ females). In the alternative no-choice set-up, only one flask containing a Pr_2S_2 solution was offered to females belonging to two different treatment groups ($N = 40$ females for each of the two treatment groups).

The numbers of flies caught were square root transformed to stabilize variance. Comparisons were made between the number of flies caught in the two flasks (choice experiments) or the number of flies belonging to the two treatment groups (no-choice experiments) using the paired *t* tests.

Oviposition Station Bioassay. This bioassay was adapted from Vernon et al. (1977). The oviposition station consisted of an inverted lid from a petri-dish (diam. 9 cm) which contained filter papers (Schleicher and Schuell, 8 cm diam.) moistened with distilled water. A 100-ml plastic cup (diam. 6 cm) with 10 regularly cut triangular nicks around the lip was inverted onto the filter paper. The test material was offered in small petri-dishes (3.2 cm diam.) placed inside the plastic cup. This set-up ensured that test insects were not able to contact the odor source and that their reaction depended solely on olfactory cues. In a choice test, 20 female onion flies were used. The number of eggs laid per treatment were square root transformed to stabilize variance and compared using the paired *t* test.

Kairomone Concentration. The effect of Pr_2S_2 concentration on the behavioral response of female flies was measured in choice experiments. Six concentrations of Pr_2S_2 dissolved in paraffin oil were tested (0.0001, 0.001, 0.01, 0.1, 1, and 10%; v/v) against pure paraffin oil as the control. In parallel, the flies' response to 6 g chopped onions was tested against wet cotton wool as the control. On each experimental day, flies from the same rearing batch were tested in the trapping bioassay (one replication each) and in the oviposition station bioassay (two replications each). In total, the trapping bioassay was replicated 10 times and the oviposition bioassay was replicated 20 times.

Age and Sex of Flies. Females and males (80 each) of varying age (1–2, 3–4, 6–7, 10–11, 15–16, 20–21 days old), but from the same rearing batch, were placed into a cage together with a conical flask containing a 0.01% Pr_2S_2 solution. The number of females and males caught was recorded. The experiment was replicated twice on each of three days, leading to a total of six replications.

Female Mating Status. Some females were removed from the rearing cage $<$24 hr after emergence and kept separately to ensure that they were unmated (McDonald and Borden, 1995). When the flies were 10–15 days old, 40 unmated and 40 mated flies from the same rearing batch were released together into a test cage. Females belonging to one of the groups were marked with a small dot of water soluble color on the thorax. One conical flask containing a 0.01% Pr_2S_2 solution was placed into the cage, and later the number of mated and unmated females caught in the flask was counted. The experiment was replicated 15 times.

Oviposition Experience/Egg Load. Mated females were offered chopped onions 1 day prior to the start of the experiment for oviposition. Forty of these females (with a reduced egg load) were placed into a cage together with 40 deprived, inexperienced females (with a relatively high egg load) from the same rearing batch. Females belonging to one of the groups were marked. One conical flask containing a 0.01% Pr_2S_2 solution was placed into the cage and later the number of experienced and deprived females caught in the flask was counted. The experiment was replicated 17 times.

Electroantennograms (EAG). Recordings from whole antennae (still attached to the head which was separated from the rest of the body) were made using stainless steel-wire electrodes connected to an EAG amplifier (10^{15} Ω Gain 10x; Syntech INR 01, Hilversum, The Netherlands). A spot of electrically conductive gel (Spectra 360, Parker Laboratories, Orange, USA) ensured a good contact between the two electrodes, the antenna, and head. Preliminary experiments had shown that thus prepared, the antennae gives reliable EAG's for a minimum of 10 min.

Pr_2S_2 was diluted in paraffin oil in decadic steps between 10^{-5} and 10%. The solutions (20 μl) were pipetted onto 100 mm^2 filter papers that were immediately inserted into a glass Pasteur pipette. A pipette containing a filter paper with pure paraffin oil acted as control. After 1 min, a puff of air (0.5 sec, 30 l/hr) was delivered through the pipette into the room air. The atmosphere inside the pipette

was allowed to equilibrate for 1 min before the antennal preparation was exposed to the chemical. Olfactory stimulations were delivered by a 0.5 sec puff (30 l/hr) of air into a continuous airflow (30 l/hr) via the pipette inserted through a port in the side of the air delivery tube about 7 cm upstream from the antenna using a stimulus control system (Syntech, CS-33). Thirty seconds elapsed between consecutive stimulations. Odor cartridges were replaced after every four stimulations (two insects from the two groups that were compared). Pure paraffin oil was tested as a control stimulus at the beginning and the end of the experiment. EAG signals were analyzed using the software program Electroantennography™ (Syntech). Means $\pm$ 95% confidence intervals were calculated from the electroantennograms. Responses were considered significantly different when their means $\pm$ 95% confidence intervals did not overlap (Visser and Piron, 1997). The following comparisons were made: females vs. males ($N = 20$), and mated females vs. unmated females ($N = 32$). Insects were between 10 and 15 days old.

RESULTS

Kairomone Concentration. Female onion flies were attracted to Pr_2S_2 and chopped onions when offered in the conical flask (Figure 1). The flies' response was concentration dependent with most females caught at the concentrations of 0.001 and 0.01%. At these concentrations, Pr_2S_2 was as attractive as the fresh onion material. At the highest concentration tested (10%), females were repelled. Depending on concentration, few eggs were laid on the oviposition stations, while control stations never received any eggs (Figure 1). However, this difference was not significant for any of the concentrations tested. In comparison, the flies responded significantly to fresh onion material.

Age and Sex of Flies. The response of both sexes of onion flies to Pr_2S_2 varied with age but females were always more attracted than males (Figure 2). The responsiveness of males reached a maximum at an age of 6–7 days after which it declined. The responsiveness of females increased during the first 10 days after emergence and remained high for 21 days after which the experiment was terminated.

Female Mating Status. Mated females were more strongly attracted to Pr_2S_2 than unmated females ($t = 9.17$, $df = 14$, $P < 0.001$) (Figure 3). While an average of 42.7% of the mated flies released were captured, only 13.7% of the unmated flies were found in the conical flask.

Oviposition Experience/Egg Load. Females that had the opportunity to oviposit during the 24 hr prior to the experiment (experienced females) were significantly less attracted to Pr_2S_2 than females lacking this oviposition experience (deprived females) ($t = 5.24$, $df = 16$, $P < 0.001$) (Figure 4). While an average of 17.8% of the experienced females with a relatively low egg load responded, 36.2% of the deprived females were caught.

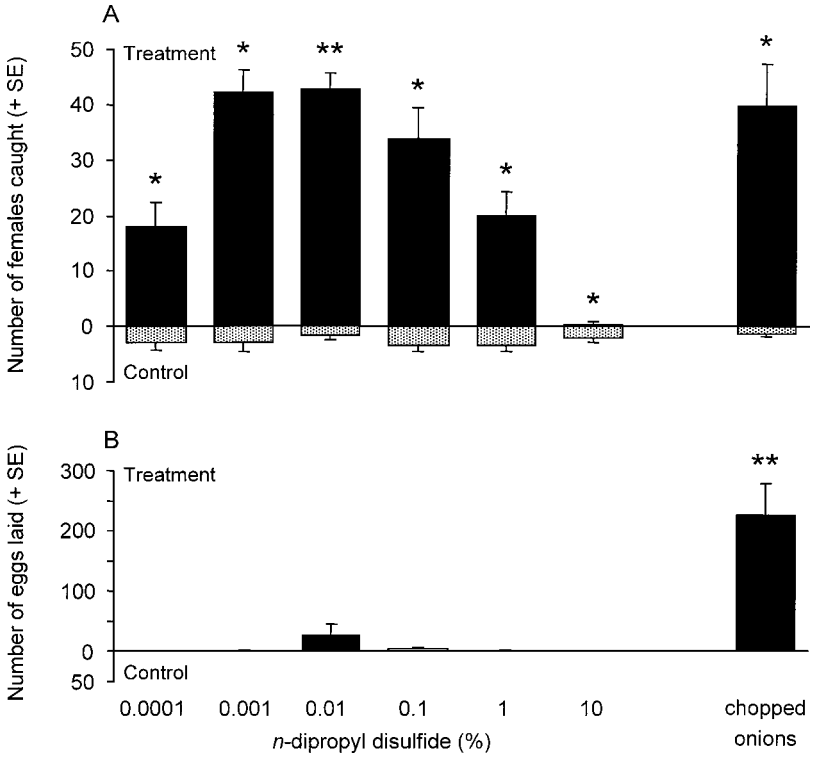

FIG. 1. Behavioral response of female onion flies to different concentrations of *n*-dipropyl disulfide (dissolved in 5 ml paraffin oil) or to chopped onions (6 g) in the (A) trapping ($N = 5$; 80 females per cage, 10–15 days old) and (B) oviposition bioassay ($N = 10$; 20 females per cage, 10–15 days old). Asterisks indicate significant differences between treatment and control ($^{*}P < 0.01$, $^{**}P < 0.001$; paired t test). All other differences were not significant ($P > 0.05$).

Electroantennograms (EAG). There was a significant difference in the EAG response to pure paraffin oil by female (mean ±95% confidence intervals; 0.43 ± 0.078 mV) and by male (0.27 ± 0.085 mV) onion flies ($t = 2.76$, $df = 38$, $P = 0.009$). Therefore, the response to paraffin oil was subtracted from the response to the different Pr_2S_2 concentrations. The response by male flies to Pr_2S_2 was lower when compared to female flies (Figure 5). However, the difference was only significant at the higher concentrations, i.e., 1 and 10%. There was no significant difference in the antennal sensitivity between mated (0.43 ± 0.065 mV) and unmated females (0.38 ± 0.062 mV) to pure paraffin oil ($t = 1.07$, $df = 62$, $P = 0.29$) or to any of the Pr_2S_2 concentrations tested.

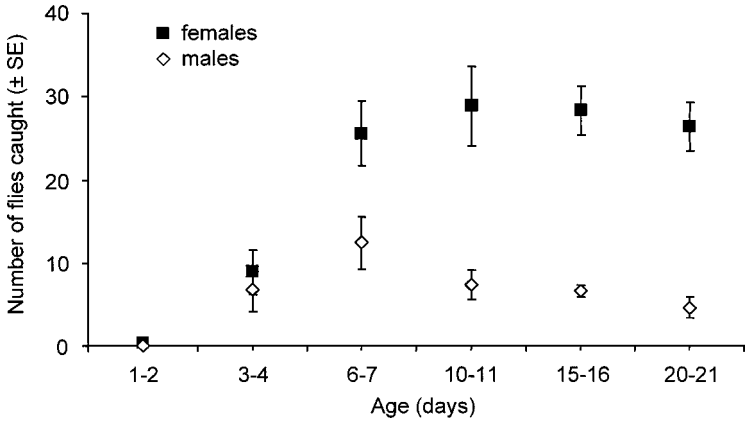

FIG. 2. Response of female and male onion flies of varying age to a 0.01% solution of *n*-dipropyl disulfide dissolved in 5 ml paraffin oil ($N = 8$); 80 flies of each sex per cage.

DISCUSSION

Pr_2S_2 was highly attractive to female onion flies, but the flies' response was strongly concentration dependent. This finding agrees with earlier studies (Matsumoto and Thorsteinson 1968; Dindonis and Miller 1981b; Harris et al., 1987; Harris and Miller 1988). The fact that at the optimum concentration, Pr_2S_2 is as attractive as onion material, has already been shown by Judd and Borden (1992) in field trials where both odor sources were offered outside host patches in fallow fields. However, Pr_2S_2 did not stimulate oviposition. Using the same bioassay, Vernon et al. (1977) and Pierce et al. (1978) observed a significant stimulation

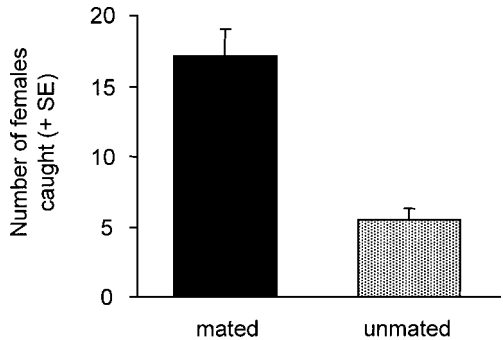

FIG. 3. Mean number of mated and unmated female onion flies caught in a conical flask containing a 0.01% solution of *n*-dipropyl disulfide dissolved in 5 ml paraffin oil ($N = 15$) ($P < 0.001$; paired *t* test); 40 flies of each group per cage, 10–15 days old.

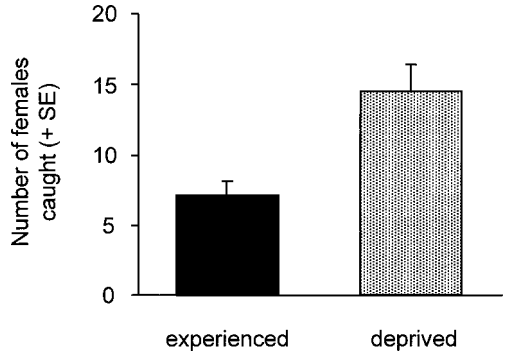

FIG. 4. Mean number of deprived or experienced female onion flies caught in a conical flask containing a 0.01% solution of *n*-dipropyl disulfide dissolved in 5 ml paraffin oil ($N = 17$) ($P < 0.001$; paired t test); 40 flies of each group per cage, 10–15 days old.

of oviposition by Pr_2S_2 dissolved in pentane (1%, v:v), but the stimulation was low compared to that caused by onion material. Matsumoto and Thorsteinson (1968), Ishikawa et al. (1978), and Harris et al. (1987) also suggested that Pr_2S_2 acts as an oviposition stimulant. In these experiments, Pr_2S_2 was provided together with moistened sand or glass beads. Tactile stimuli also elicit oviposition in the onion fly (Harris and Miller, 1988; Mowry et al., 1989) and it is possible that physical contact with the oviposition substrate together with the olfactory stimulation elicited the oviposition response.

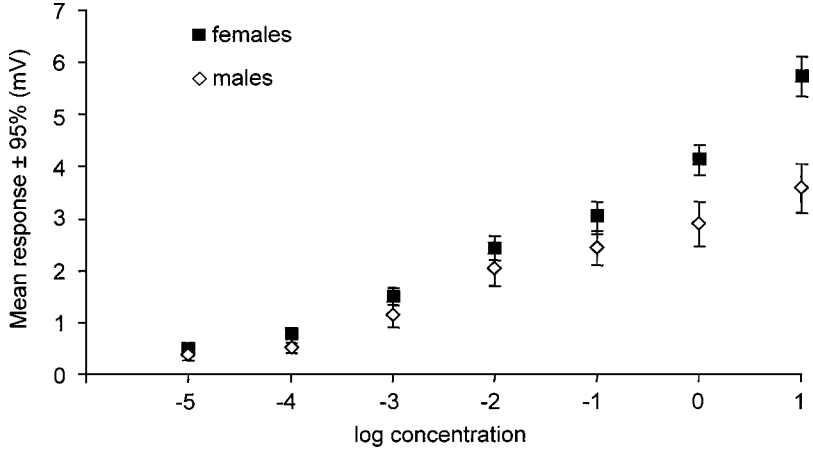

FIG. 5. Mean EAG response of female and male onion flies (10–15 days old) to stimulations with graded doses of *n*-dipropyl disulfide dissolved in paraffin oil ($N = 20$).

There was a clear correlation between insect age and the behavioral response to Pr_2S_2 for both female and male onion flies. However, independent of age, females were always more responsive than males. Thus, the sexes differ in their responsiveness to olfactory stimuli as for visual stimuli (Judd and Borden, 1991; Judd and Whitfield, 1997). Our results are in accordance with field data by Judd and Borden (1988). The age at which the two sexes start responding to Pr_2S_2 parallels the onset of sexual activity, when ovaries are at an early stage of vitellogenesis (Theunisson, 1976; Harris and Miller, 1988; McDonald and Borden, 1995; Spencer et al., 1995). This led Judd and Borden (1988) and McDonald and Borden (1997) to postulate that the response to onion volatiles, including Pr_2S_2, enhances the probability of mating on or near the host plant.

Judd and Borden (1988) and McDonald and Borden (1997) showed that the response to Pr_2S_2 increases in unmated female and male onion flies up to an age of 12 days. On the basis of our data, the mating status and egg load also partly affect the insect's response to Pr_2S_2 just as reported for visual orientation responses (Judd and Borden, 1991). When directly compared, 10–15-day-old gravid, mated females responded more strongly to Pr_2S_2 than gravid, unmated flies. In wind tunnel experiments McDonald and Borden (1997) found that both gravid, mated females and gravid, unmated females were attracted by a fresh onion shoot. However, similar to our results, the proportion of females that showed a response was significantly higher for mated females. Laboratory studies revealed that mating increases steadily in flies older than 3–4 days (McDonald and Borden, 1995). It is possible that, at least for females, an increase in the proportion of mated flies partly caused the increase in their behavioral response. In contrast, studies by Judd and Borden (1988, 1992) showed that in fallow fields, Pr_2S_2 was similarly attractive to 10–14-day-old mated and unmated females, while within an onion field, mated females were less responsive. The differences between our laboratory results and the results from these field experiments could have been caused by the fact that in the laboratory the larger number of mated flies caught in the flasks was due to an increase in overall activity or an arrestment response rather than an attraction.

The fact that the response of females towards Pr_2S_2 remained high until an age of 21 days suggesting that this compound acts as a kairomone during the final stage of the host-finding process. This is also indicated by the result that mated, gravid females that were host deprived showed a significantly stronger response to Pr_2S_2 than females that had had an oviposition experience (and, therefore, a lower egg load) one day prior to the experiment. That internal states such as satiation or egg load affect the searching behavior and host acceptance in herbivorous insects has often been reported (Dethier, 1982; Minkenberg et al., 1992; Bernays and Chapman, 1994). Similar to our results, McDonald and Borden (1997) observed that both gravid, mated females and previtellogenic, mated females preferred for a fresh onion shoot in wind tunnel choice experiments with the latter showing a

much lower preference. Also, field studies by Judd and Borden (1988) confirmed that immature females were less responsive to Pr_2S_2 than gravid females. Unmated females were far less inclined to oviposit than mated females (Theunisson, 1976; Spencer et al., 1992; Weston et al., 1992; Spencer and Miller, 2002). This suggests that the mating status does not directly affect insect behavior, but acts indirectly through the females' egg load.

The EAG response of both female and male antennae to Pr_2S_2 increased with increasing concentration. A similar dose-response has been found by Honda et al. (1987) for single sensillum recordings. In contrast to an earlier study by Ishikawa (1988), we found a response difference in female and male antennae to Pr_2S_2. This sexual difference in the EAG response could be due to differences in the antennal sensitivity caused by a higher density of olfactory sensilla on female antennae (Ross, 1992), or to the fact that chemoreceptors on male antennae are adapted to lower concentrations. Alternatively, it could simply be due to aging or senescence effects, as males live much shorter lives than females (Noordink, 1971). The result agrees with the data from behavioral bioassays in which males responded less to Pr_2S_2 than did females. The fact that there was no difference in the EAG response between mated and unmated females, while there was a significant difference in the flies' behavioral response, indicates that the central nervous system rather than the peripheral sensors influences the different behavioral responses in the onion fly (Ikeshoji et al., 1981; Guerin and Städler, 1983).

Our results confirm that Pr_2S_2 plays an important role in the host patch and host plant location by onion flies. This plant volatile, however, seems to elicit an ovipositional response only in connection with additional (tactile and/or visual) stimuli. The behavioral response towards Pr_2S_2 was not only conditional upon age-related events, but also affected by the mating and/or egg load status of the female flies. This together with the different response of the sexes is likely to be an important factor accounting for the variable catches of Pr_2S_2 baited traps in the field. These factors must be considered when such traps are used in monitoring programs.

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ELECTROPHYSIOLOGICAL RESPONSES OF FEMALE AND MALE *Hypsipyla grandella* (ZELLER) TO *Swietenia macrophylla* ESSENTIAL OILS

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Abstract—The mahogany shoot borer, *Hypsipyla grandella*, is one of the most economically important Neotropical forest pest insects because it attacks valuable timber of the Meliaceae. Despite detailed information on the life cycle of *H. grandella*, factors influencing attack by the shoot borer are not well known. Electroantennogram studies of borer antennae indicate that both female and male moths elicited significant antennal responses to essential oil of the terminal shoots, mature and senescent leaves, in three concentrations. γ-Himachalene, germacrene D, germacrene A, cadina-1,4-diene, hexadecanoic acid, and ethyl hexadecanoate appeared in all essential oils from *Swietenia macrophylla*. Germacrene-D and γ-himachalene were also present in the active essential oil of *Cedrela odorata* and *Toona ciliata.* These findings suggest that these compounds play a role in attracting *H. grandella* to oviposit on the leaves of these plants. The GC-EAD of essential oil from *S. macrophylla* mature leaves to female *H. grandella* antennae led to the identification of β-caryophyllene as the main constituent responsible for the antennae response.

Key Words—*Hypsipyla grandella, Swietenia macrophylla*, essential oils, electrophysiological responses.

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INTRODUCTION

Because it attacks plants of the Meliaceae family, the mahogany shoot borer, *Hypsipyla grandella* (Zeller, 1848) (Lepidoptera: Pyralidae), is one of the most economically significant Neotropical forest pest insects (Schabel et al., 1999). It is a key pest of mahogany, *Swietenia macrophylla* (Kim).

S. macrophylla (Meliaceae) is well-known for its fast growth, adaptability, good shape, and high value, which are desirable properties in Latin America and Caribbean timbers (Grijpma and Ramalho, 1969). However, efforts to establish large-scale homogenous plantations of this native tree have almost invariably failed because of attack by mahogany shoot borer larvae. The damage consists of killing young terminal shoots, retarding growth, and the formation of numerous secondary shoots that develop into twisted and unprofitable trees.

Various strategies have been developed and considerable research efforts made to control this pest, but no practical method of control has been devised. Insecticidal trials have failed because of the effects of rainfall and the short period during which larvae are exposed prior to boring into shoots. In larval and pupal stages, the insects are protected in galleries inside the tree. Chemical treatment is still limited to forest nurseries. In plantations, only silvicultural measures have been employed (Grijpma, 1973; Whitmore, 1976; Newton et al., 1993; Speight, 1997).

Despite detailed information on the life cycle of *H. grandella*, the factors influencing attack by shoot borers are not well known. An understanding of these is essential if practical control methods are to be developed. The aim of the present work was to determine the selectivity and sensitivity of antennal receptors of both sexes of *H. grandella* to volatile compounds identified from the essential oil of the terminal shoots, and mature and senescent leaves of the *S. macrophylla*. Comparative studies among these tree essential oils were made to determine similarities in olfactory receptors, using the electroantennogram (EAG) technique.

METHODS AND MATERIALS

Essential Oil Extraction and Analysis. Fresh terminal shoots and mature and senescent leaves of *S. macrophylla* were collected in the town of Igarapé-Açu, located 136 km from Belém in the state of Pará, Brazil, at Eidai do Brasil Madeiras S.A.'s experimental forest station.

The fresh aerial parts of each tissue were steam-distilled for 4 hr, using a Clevenger apparatus. The essential oils were dried over anhydrous Na_2SO_4 and kept in a freezer. Analyses were carried out on a Shimadzu GC-17A gas chromatograph fitted with a fused silica DB-5 (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness) capillary column, using helium as the carrier gas at a flow rate of 1.6 ml $\cdot$ min^{-1}. The temperature was programmed initially at 60°C for 2 min and then increased

at a rate of $3^{\circ}C \cdot min^{-1}$ to 240°C. The injection was split and its temperature was 225°C, while the interface temperature was 250°C. The chromatograph was coupled to a Shimadzu QP5000 mass selective detector at 70 eV; EIMS. Identification of the components was made by determining their retention indices relative to those of a homologous series of *n*-alkanes (Dool and Kratz, 1963), by comparison with (a) coinjection with authentic samples, and (b) fragmentation patterns in mass spectra with those stored on the spectrometer database and reported in the literature (Adams, 1995).

Insects. Pupae of *H. grandella* used in our experiments originated from the Faculdade de Ciências Agrárias do Pará's Laboratory of Entomology in Belém, PA, Brazil. The insects were reared in this laboratory on a diet of mahogany foliage. Pupae were established in the Federal University of São Carlos's Insect Bioassay Laboratory. They were sexed (Parra, 1986) and placed individually in to plastic vials (6×6 cm diameter) for emergence of adults. Male and female pupae were maintained in a growth chamber at $25 \pm 1^{\circ}C$, $60 \pm 5\%$ RH and 12L:12D.

Electroantennograms. Antennae of 1 to 2-day-old male and female moths were used for electroantennographic experiments (EAG) and electroantennographic detection (EAD).

Each antenna was excised (pulled out of the head) using forceps, and a few segments were cut off at the base and the tip (Bjostad, 1998). The antenna was then fixed between two stainless steel electrodes by pushing the base and tip into droplets of an electrically conductive gel (Spectra 360® electrode gel, Parker, Orange, NJ) applied onto the metal electrodes. The specimen with the antennal preparation was placed in such a way that the humidified air directed the compounds eluted from the gas chromatograph (GC) over the antenna. The antennal responses were amplified and recorded with a data acquisition controller and software EAG (Syntech).

EAGs experiments were performed to elucidate the selectivity of the antennal receptors of *H. grandella*. The EAG response was evaluated as follows. The compounds were released from Pasteur pipettes containing a piece of filter paper (about 0.8 cm^2) impregnated with 5 μl of each freshly prepared solution of the test essential oil and passed over the antennae. In addition to the pipettes with the test preparations, one Pasteur pipette containing a filter paper impregnated with 5 μl of the hexane solvent was used as a control. The puff containing the test essential oil (from terminal shoots, mature, or senescent leaves of *S. macrophylla*) was delivered into a continuously humidified and purified air stream of $1.2\ l \cdot min^{-1}$ by purified air, passing for 0.3 sec through the impregnated filter paper in the pipettes. Control stimulation was made at the beginning and at the end of every series of experiments. The test essential oils were applied randomly at intervals of 30 sec. EAG amplitudes in response to the essential oils were expressed in relation to the responses to the control (hexane), because of the large differences in overall sensitivity between individual antennae, and to compensate the decline

in antennal sensitivity during a measuring session. In this normalization procedure, responses to the control were defined as 100%. Values obtained between two references (controls) were corrected according to the values of the references by linear interpolation. The Syntech EAG software calculated the normalized values automatically. Essential oils were tested using 10 antennae each of female and male *H. grandella*. The mean normalized responses of the different compounds were analyzed by ANOVA and compared using the Tukey test ($P < 0.05$).

Coupled Gas Cromatography–Electroantennographic Detection (GC-EAD). The GC-EAD equipment was used to identify individual compounds in the oil that could elicit an electroantennographic response. Five replicates were obtained for females, using essential oils from the terminal shoot. The GC-EAD instrument consisted of a Shimadzu 17-A chromatograph equipped with a flame ionization detector coupled to an electroantennography system (Syntech, Hilversum, The Netherlands) and a DB-5 column (30 m $\times$ 0.25 mm I.D., 0.25 μm film thickness, J and W Scientific), using a temperature program of 60°C/min, 3°C/min to 150°C/10 min, 1°C/min, and finally to 280°C held for 16 min. The column effluent (carrier gas hydrogen) was mixed with a nitrogen make-up gas flow (12 ml $\cdot$ min^{-1}) before it was split to the flame ionization detector (FID) and to the heated transfer capillary leading to the antennal preparation. Compounds in the transfer capillary were eluted into a humidified and purified air stream (1.2 l $\cdot$ min^{-1}), which led them directly to the antennal preparation. The same chromatographic conditions as above were used for the chromatographic separation. The FID was kept at 280°C, whereas the temperature of the transfer capillary was maintained at 290°C to prevent condensation.

RESULTS AND DISCUSSION

Essential Oils. The composition of the essential oils is given in Table 1. Component concentrations were calculated from GC peak areas and they were arranged in order of GC (DB-5) elution. Table 1 shows that the oils consisted largely of sesquiterpenes. Oils from the terminal shoots (212 mg, 0.053% w/w), mature leaves (193 mg, 0.048% w/w), and senescent (188 mg, 0.047%) leaves of *S. macrophylla* contained 19, 16, and 13 components, respectively. Of these, 84% of the compounds in the terminal shoots were identified compared with identification of 63 and 69% of the components in the mature and scenescent leaves, respectively.

The chemical composition of the volatile oils of terminal shoots, mature, and senescent leaves of *S. macrophylla* were dissimilar (Table 1), except the main constituent and five other compounds. The major constituent in all samples (58.5–66.5%) was germacrene D, while all the oils contained γ-himachalene, germacrene A, cadina-1,4-diene, hexadecanoic acid, and ethyl hexadecanoate, though in different proportions. The most similarity was found in the oils from mature and senescent leaves, both of which contained 10 similar compounds.

TABLE 1. COMPOSITION OF ESSENTIAL OILS (%) OF S. *macrophylla* (MAHOGANY) TERMINAL SHOOTS (S), MATURE (ML), AND SENESCENT LEAVES (SL); OBSERVED HERE AND REPORTED IN THE LITERATURE, KI ADAMS (ADAMS, 1995).

Compound	KI Adams	S (%) (KI observed)	ML (%) (KI observed)	SL (%) (KI observed)
α-Copaene	1376	3.31(1379)		
β-Bourbonene	1384		2.03 (1386)	1.13 (1382)
β-Cubebene	1390	0.56 (1393)		2.10 (1386)
β-Elemene	1391	0.66 (1395)		
β-Caryophyllene	1418	0.80 (1421)	6.49 (1421)	
β-Gurjunene	1432	0.47 (1431)		
NI			2.61 (1436)	
Allo-Aromadendrene	1461	0.27 (1463)		
γ-Himachalene	1476	0.93 (1482)	Tr (1479)	0.79 (1480)
Germacrene D	1480	66.54 (1487)	59.99 (1485)	58.50 (1485)
β-Ionone	1485		1.19 (1486)	
Bicyclogermacrene	1493	0.26 (1495)		
Germacrene A	1503	6.89 (1502)	5.29 (1499)	4.68 (1502)
α-Bisabolene	1504	1.18 (1504)		
β-Bisabolene	1509	0.59 (1509)		
γ-Bisabolene	1515		1.56 (1514)	
7-*epi*-α-Selinene	1517	0.71 (1519)		0.66 (1519)
Cadina-1,4-diene	1532	3.43 (1529)	2.00 (1529)	2.28 (1529)
NI			1.60 (1568)	
NI			2.93 (1582)	
NI Sesquiterpene MW 204	1641	1.78 (1645)		
NI Sesquiterpene MW 204	1652	0.43 (1651)		
NI Sesquiterpene MW 204	1658	1.74 (1659)		1.62 (1659)
NI			2.40 (1721)	2.28 (1721)
NI			0.61 (1897)	2.25 (1897)
NI			1.82 (1902)	3.87 (1902)
Hexadecanoic acid		5.69 (1973)	1.76 (1973)	13.70 (1973)
Ethyl hexadecanoate	1993	3.76 (2003)	7.72 (2003)	6.14 (2003)

Note: NI-not identified.

Ecological Significance. The essential oil of the terminal shoots and mature and senescent leaves, in the three concentrations, elicited a significant EAG response in both female and male moths (Figures 1 and 2). As expected, the responses of the different EAG preparations increased along with the concentration, although not all the differences between concentrations were significant.

Figure 3 compares the effect of the three essential oils, at 100 $mg \cdot ml^{-1}$, on females and males. Females tend to show greater depolarization of the antennae when exposed to the essential oils. Male antennae responded most strongly to the oil of mature leaves, whereas female antennae responded most noticeably to the shoot oil. The responses were different only for the essential oil of terminal shoots,

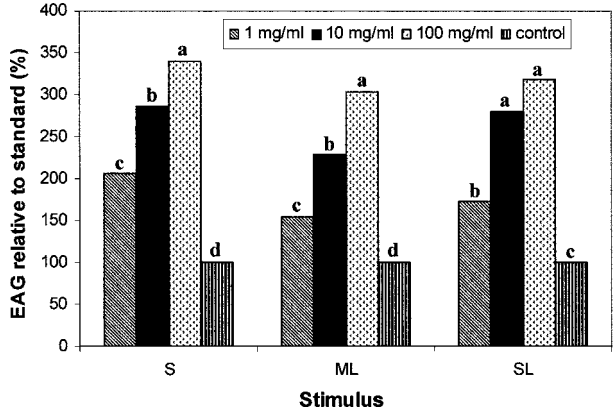


FIG. 1. Mean EAG in percentage elicited from *Hypsipyla grandella* females with different concentrations of *S. macrophylla* essential oil. Mean values marked with the same letter are not significantly different at $P < 0.05$ on the basis of the Tukey test. S = shoots, ML = mature leaves, and SL = senescent leaves.

which elicited higher antennal response from females. However, since both sexes responded to all the essential oils and the difference in response between male and female antennae were slight, these results suggest that the oils are general attractants rather than specific oviposition or host plant selection attractants. Maia et al. (2000) observed that the high selectivity of *H. grandella* female antenna to

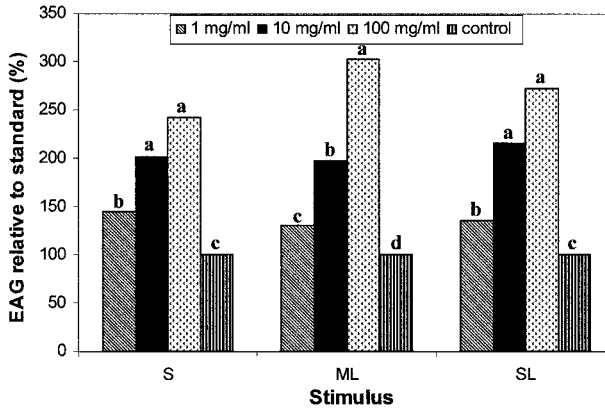


FIG. 2. Mean EAG in percentage elicited from *Hypsipyla grandella* males with different concentrations of *S. macrophylla* essential oil. Mean values marked with the same letter are not significantly different at $P < 0.05$ on the basis of the Tukey test. S = shoots, ML = mature leaves, and SL = senescent leaves.

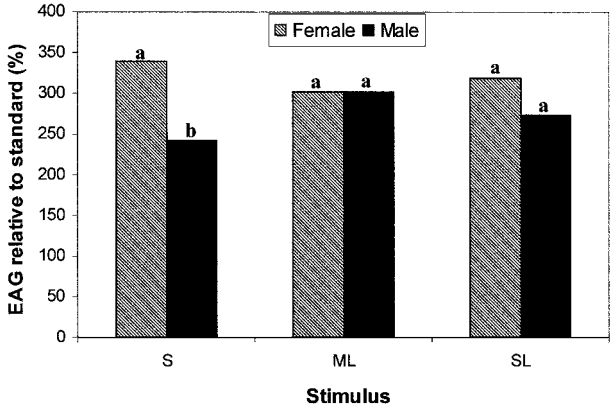


FIG. 3. Mean EAG in percentage elicited from *Hypsipyla grandella* females and males at a concentration of 100 mg · ml^{-1} of *S. macrophylla* essential oil. Within each sex, mean values marked with the same letter are not significantly different at $P < 0.05$, on the basis of the Tukey test. S = shoots, ML = mature leaves, and SL = senescent leaves.

the essential oils from the leaves of *Cedrela odorata* and *Toona ciliata* indicate its potential role as a chemical messenger for habitat location and oviposition.

For phytophagous insects, certain factors are important in host plant selection, such as color, shape, and odor. In some cases, specific odors facilitate the location and identification of suitable hosts. In the case of *H. grandella*, many authors have suggested that plant secondary compounds are largely responsible for this selection. Grijpma and Gara (1970) reported a direct relationship between the ratio of new leaves (shoots) and the number of attacks by *H. grandella* in mahogany.

Sanchez et al. (1976) observed that, in Costa Rican trials, *S. macrophylla* proved more susceptible to *H. grandella* than *Cedrela odorata*. Differences in susceptibility to attack may reflect variations in the production of chemical attractants detected by ovipositing females, although differences in shoot growth and morphology may also be influential factors.

γ-Himachalene, germacrene D, germacrene A, cadina-1,4-diene, hexadecanoic acid, and ethyl hexadecanoate appeared in all essential oils tested from *S. macrophylla.* Germacrene-D and γ-himachalene were also present in the active essential oils of *C. odorata* and *T. ciliata.* This observation suggests that these compounds play a role in attracting *H. grandella* to oviposit in the leaves of these plants. However, numerous attempts to identify active constituents in leaf oils by coupled GC-EAD have failed, probably because the responses of female antennae to each eluted component were weak and below the limits of detection of the apparatus, or because stimulation requires a mixture of certain constituents. The GC-EAD (Figure 4) of the essential oil (0.1 mg · ml^{-1}) from *S. macrophylla*

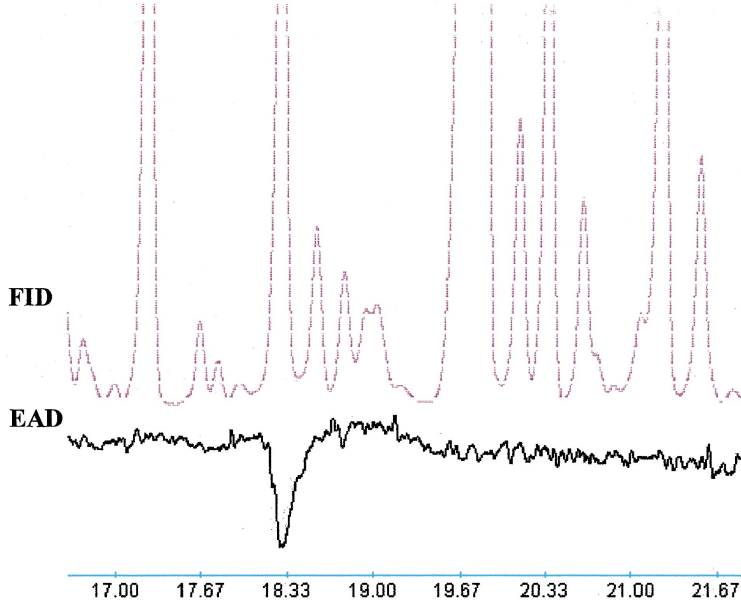
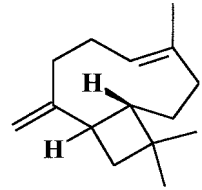


FIG. 4. GC-EAD of essential oil (0.1 mg · ml^{-1}) from *S. macrophylla* mature leaves over *H. grandella* female antennae.

mature leaves to female *H. grandella* antennae suggests β-caryophyllene (**1**, TR, 18.33 min and KI 1418) is the main constituent responsible for antennal response (scheme 1). This compound appears in lower concentrations in the terminal shoots than in mature leaves (Table 1); the same result was not observed in the GC-EAD of the essential oil from terminal shoots. Therefore, either the concentration of β-caryophyllene is response-dependent or there are other important compounds for the insect's response to the oils.



1 β-Caryophyllene

SCHEME 1.

The short lifetime of antennal preparations (30–60 min) pose practical problems; however, the EAG of essential oils before GC-EAD displayed a strong response. Field studies, optimized GC-EADs, and/or EAG experiments with pure compounds are required to clarify the role of volatile constituents in *H. grandella* host selection and ovipositing behavior.

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SEX PHEROMONE OF THE CRANBERRY BLOSSOM WORM, *Epiglaea apiata*

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Abstract—The cranberry blossom worm, *Epiglaea apiata* (Grote) (Lepidoptera: Noctuidae), is a major pest of cranberries in New Jersey. The female sex pheromone of this moth was identified as a blend of (*Z*)-9-hexadecenyl acetate (*Z*9-16:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac), and tetradecyl acetate (14:Ac) by gas chromatographic–electroantennographic detection and gas chromatography–mass spectrometry. The ratio of the components in extracts of the female pheromone gland was determined to be 65 : 2 : 33 of the *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac, respectively. The double bond positions of the pheromone components were confirmed by dimethyl disulfide derivatization. In addition to the above three components, a mixture of C_4–C_{10} aliphatic acids was present in both gland extracts and effluvia collections, and the acids elicited significant EAD responses from male moth antennae. However, addition of the C_4–C_{10} aliphatic acids to the pheromone blend did not significantly increase trap captures. Three-hundred- and 1000-μg doses of a synthetic blend containing *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac (65 : 2 : 33), on a rubber septum were more attractive to males than lower doses.

Key Words—Cranberry blossom worm, *Epiglaea apiata*, gas chromatographic–electroantennographic detection, aliphatic acids, (*Z*)-9-hexadecenyl acetate, (*Z*)-9-tetradecenyl acetate, tetradecyl acetate, sex pheromone.

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INTRODUCTION

The cranberry blossom worm, *Epiglaea apiata* (Grote) (Lepidoptera: Noctuidae), is one of the three most important pests of cranberry, *Vaccinium macrocarpon* (Aitan), in New Jersey (Beckwith, 1929), although it is considered a relatively minor pest of cranberries in Massachusetts and is not known to infest cranberries elsewhere. In addition to cranberries, this insect also feeds on blueberries and leatherleaf (Ericaceae) (Beckwith, 1929; Franklin, 1948) throughout the northeastern United States from Maine to Illinois (Franklin, 1948).

The cranberry blossom worm lays eggs from September to October, singly on fallen leaves and pieces of dead vines littering the bottom of the cranberry bog, where they overwinter. Eggs in New Jersey typically hatch over a 4-week period beginning the 2nd week of May. The newly hatched larvae first nibble the leaves or bore into terminal buds causing great loss in fruiting capacity. As the larvae mature, they become nocturnal (Beckwith, 1929), feeding voraciously on entire leaves and flowers, and often cut off many more blossoms than they eat. Beckwith (1929) reported that each larva could destroy as many as 100 blossoms over a 3-week period. Pupation takes place within an earthen cell just below the soil surface in late July and August. Adults emerge in September and are active until late October. The adult is a large moth with a wingspan of 3.8–4.0 cm. There is only one generation per season.

Failure to recognize and delineate infestations is a major factor contributing to severe crop losses due to cranberry blossom worm. The nocturnal habit and highly localized pattern of infestations are factors limiting timely detection of *E. apiata* infestations. Adult captures in pheromone traps could provide information on population levels and help delineate infestations for timely pest management interventions. The objective of this study was to identify the sex pheromone components of *E. apiata* and develop a pheromone lure for population monitoring. The identification of the sex pheromone will also enable the future development of mass trapping and mating disruption technology for managing this major cranberry pest.

METHODS AND MATERIALS

Insects. E. apiata larvae were collected from cranberry bogs in New Jersey during late May and early June. These larvae were individually reared in Chatsworth, NJ, in screen top plastic containers (10 cm high and 4 cm diam.) on 8–10 cm long cranberry twigs with leaves and blossoms in an incubator at 25°C and 16L:8D photoperiod. Plastic containers were filled with sand to 3–4 cm deep and five to six cranberry twigs were stuck in the sand in each container. Water was added to the sand to maintain a moisture level near saturation. Twigs were

replaced with fresh ones as necessary. Upon reaching the prepupal stage, larvae burrowed into the sand and formed cells constructed of sand, silk, and debris. Moths emerging from these cells were sexed and maintained at 15°C and 16L:8D photoperiod until used.

Pheromone Gland Extraction. Pheromone gland extracts were obtained from three groups of 1- to 5-day-old virgin females (3, 5, and 9 females per group) in photophase. A female abdomen was compressed gently until the ovipositor everted from the abdominal tip. The ovipositor was then excised with small scissors into a conical glass vial containing ~100 μl methylene chloride/methanol (3 : 1). Glands were soaked for at least 30 min at room temperature. Extracts were removed, and the glands were reextracted with 100 μl methylene chloride. The combined solution was concentrated to ~20 μl under nitrogen and kept at −30°C in a freezer for future analysis.

Collection of Volatiles. Volatiles were collected using three groups of 1-day-old females (3, 6, and 7 females per group). The moths were separately introduced into three 1-l, 4-necked glass containers (Zhang et al., 1994) upon their emergence. Air was drawn into the container through 6–14 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA), and out of the container through two traps (15 × 1.5 cm o.d.) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL) by vacuum (~1 l/min). Female moths were fed with 10% sugar solution on cotton balls and aerated continuously for 7 days at room temperature and 16L:8D photoperiod. Adsorbent traps were changed every 24 hr. Adsorbents were eluted with methylene chloride (4 × 0.5 ml); the eluates (2 ml/each sample) were concentrated to ~20 μl under nitrogen and stored at −30°C for future analysis.

Instrumentation. The coupled gas chromatographic–electroantennographic detection (GC-EAD) system used was as previously described (Zhang et al., 1997, 1999). A Hewlett–Packard 6890 gas chromatograph equipped with a 60 m × 0.25 mm i.d., 0.25-μm film-thickness DB-WAXETR capillary column (J&W Scientific, Inc., Folsom, CA) was used for GC-EAD analysis in the splitless mode with hydrogen as carrier (1.4 ml/min) (80°C for 2 min, then programmed to 250°C at 15°C/min and held for 15 min). The capillary column effluent and nitrogen makeup gas (10 ml/min) were split (~1 : 1) by a fixed outlet splitter (SGE, Inc. Austin, TX) to a flame ionization detector (FID) and EAD. After both antennae were excised from a moth and the tips were cut off, they were positioned between two gold wire electrodes, which were immersed in saline-filled (0.9% NaCl) wells (1.25 mm diam; about 3 mm apart) in a small acrylic plastic holder (8 cm long × 0.8 cm wide × 0.6 cm thick). The output recording electrodes were connected to a high-impedance (1 : 100) amplifier with automatic baseline drift compensation. The airstream flowing over the antennae (about 500 ml/min) was humidified by bubbling through distilled water before entering the EAD interface. The antennal preparation was cooled to ~5°C inside a condenser by circulating near 0°C water from a benchtop refrigeration unit (RTE-100, NESLAB

instruments, Inc., Portsmouth, NH) through the insulation layer of the modified condenser containing the acrylic plastic antenna holder mounted on top of the GC. The flame ionization and electrophysiological output signals were recorded using Hewlett-Packard ChemStation software.

Electron impact (EI, 70 eV) gas chromatography–mass spectrometry (GC-MS) was conducted on a Hewlett-Packard 6890 GC coupled to a HP 5973 Mass Selective Detector using an identical DB-WAXETR capillary column (50°C for 2 min, then programmed to 230°C at 15°C/min and held for 15 min for regular analysis; 180°C for 2 min, then programmed to 230°C at 15°C/min and held for 50 min for analysis of DMDS adducts) or a DB-5 capillary column (60 m × 0.25 mm ID, 0.25-μm film-thickness, J&W Scientific, Inc.; 50°C for 2 min, then programmed to 300°C at 15°C/min and held for 50 min) but with helium as carrier gas.

Chemicals. (*Z*)-9-hexadecenyl acetate (*Z*9-16:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac), and tetradecyl acetate (14:Ac) were obtained from Dr J. Klun of the USDA-Chemicals Affecting Insect Behavior Laboratory, Beltsville, Maryland. These pheromone components had previously been synthesized and purified in this laboratory (Lynch et al., 1984), and stored in a −30°C freezer. Purities of chemicals were checked on a 60 m polar DB-WAXETR GC capillary column before preparing the lures for the field study. All of the C_4–C_{10} straight-chain aliphatic acids were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Derivatization. Dimethyl disulfide (DMDS) derivatives of extracts and synthetic standards were prepared according to standard procedures (Buser et al., 1983; Dunkelblum et al., 1985). Methylene chloride/methanol (3 : 1) solutions of female gland extracts or hexane solutions of synthetic monounsaturated acetates (10 μl, 20 ng/μl) were treated with 50 μl of DMDS (Aldrich Chem. Co., 99 + %) and one drop of an iodine solution (60 mg/ml in diethyl ether). The mixtures were kept at 60°C overnight. After cooling to room temperature, one drop of 5% aqueous sodium thiosulfate ($Na_2S_2O_3$) was added, and the solutions were shaken vigorously to reduce the iodine. The organic phase was removed, and the aqueous phase was extracted with 100 μl hexane. The combined extracts were dried over anhydrous Na_2SO_4 and concentrated to ∼20 μl under nitrogen for GC-MS analysis.

Field Tests. Gray rubber septa (5 mm, The West Company, NE) loaded with the desired rates of candidate pheromone components in ∼40 μl of hexane solution were used for the field trails. The same amount of solvent (hexane) was loaded on a septum for the blank control. After loading, the solvent was allowed to evaporate in a fume hood for 30 min. Lures were then wrapped in aluminum foil, stored in 20 ml plastic vials, and shipped by express carrier on the same day. Upon arrival, lures were kept in a freezer at −30°C until use.

In all experiments, Pherocon 1C traps (Trécé, Salinas, CA) were used. Traps were arranged in a randomized complete block design with five replicates and 30 m intertrap distance within replicates. In most cases, a single row of traps (replicate)

was placed in each cranberry bog. Replicate trap lines were placed in adjoining cranberry bogs with 50–300 m between replicates. Traps were hung at crop canopy level ~45–60 cm above ground level, checked and moved by one position every 1–3 days in different tests, and the bottoms were replaced when catches exceeded 25 moths per trap.

Statistical Analysis. Data on trap catches from each test were square-root-transformed $[(x + 0.5)^{1/2}]$ to normalize the variance before analysis. Means were compared by one-way analysis of variance (ANOVA) followed by the least significant differences (LSD) multiple comparisons test (SPSS 10.0 for Windows) for significance at $\alpha = 0.05$.

RESULTS

Identification of the Sex Pheromone Components. Coupled GC-EAD analyses of effluvia of females demonstrated that male moth antennae consistently responded to a series of compounds (Figure 1A), the mass spectra and GC retention times of which closely matched those of C_4–C_{10} straight-chain aliphatic acids. Authentic standards elicited EAD responses from antennae of male moths. (Figure 1B, Response 2 was due to an impurity in the C_5 acid). The same series of acids (compounds **1**–**7**, C_4–C_{10}) were also found in the extracts of female pheromone glands and elicited significant male antennal responses when those extracts were analyzed by GC-EAD (Figure 1C). Besides these acids, male moth antennae also responded strongly to three other compounds (compounds **8**–**10**) found in both types of extracts. Therefore, they were considered likely to be the female sex pheromone components.

The mass spectrum of the active component corresponding to the greatest EAD response (compound **10**) in the gland extracts exhibited a comparatively strong ion at *m*/*z* 222 (20%) as the highest mass fragment, and it closely matched a spectrum of a monounsaturated 16:Ac retrieved from the Wiley 275 mass spectral database. In addition, the mass spectrum of compound **9** exhibited similar fragments, but with the largest ion at *m*/*z* 194 (20%), 28 amu less than compound **10**. This is consistent with a monounsaturated 14 carbon acetate analog. The mass spectrum of the third component (compound **8**) showed the largest ion at *m*/*z* 196 (5%), 2 amu more than compound **9**, indicating that it could be the saturated 14:Ac.

The double bond locations in the two monounsaturated acetates were determined by GC-MS analysis of DMDS derivatives of the pheromone gland extracts. Pairs of diagnostic fragments appeared at *m*/*z*117 (80%) and 231 (100%), with molecular ions at *m*/*z* 348 (20%), and *m*/*z* 145 (90%) and 231 (100%) with molecular ion at *m*/*z* 376 (18%), indicating that Δ9-14:Ac and Δ9-16:Ac were likely candidates for the pheromone components. To confirm the above conclusion, and to determine the double bond geometries, synthetic standards of the (*E*)- and (*Z*)-isomers of Δ9-14:Ac and Δ9-16:Ac were subjected to the corresponding analysis.

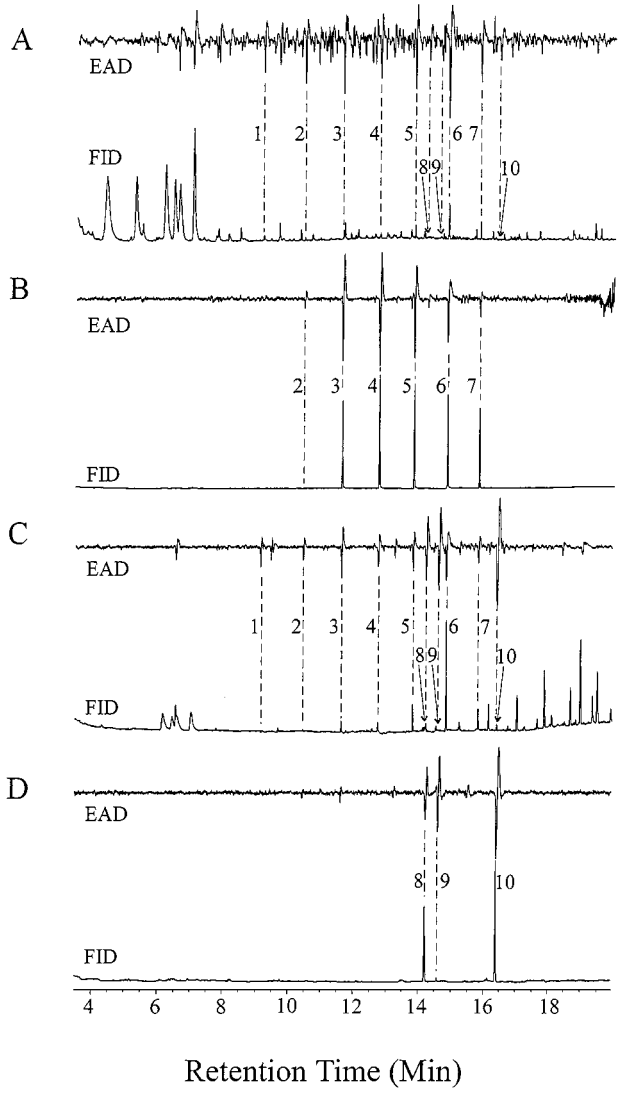

FIG. 1. Simultaneous FID and EAD responses of an adult male *E. apiata* antenna to (A) effluvia from five female *E. apiata*; (B) a mixture of acids (C_6–C_{10}, 20 ng each acid; (C) extracts of three female *E. apiata* pheromone glands; and (D) a mixture of synthetic *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac (10 ng, 65 : 2 : 33 ratio, v/v). Identifications of peaks: **1**–**7**, C_4–C_{10} aliphatic organic acids; **8**, 14:Ac; **9**, *Z*9-14:Ac; and **10**, *Z*9-16:Ac.

TABLE 1. COMPOSITION AND RATIO OF FEMALE SEX PHEROMONE COMPONENTS OF *E. apiata* IN GLAND EXTRACTS[a]

Compound	Mean (%)[b]	SD	Range
*Z*9-16:Ac	64.5	9.5	51.8–69.9
*Z*9-14:Ac	2.0	0.7	1.2–2.6
14:Ac	33.5	10.1	28.0–47.0

[a] Gland extracts were obtained from 17 virgin females.
[b] $N = 3$.

The mass spectra and GC retention times of the adducts of synthetic *Z*9-14:Ac, *Z*9-16:Ac were indistinguishable from those of the natural products on the DB-WAXETR column. The pheromone components in each case corresponded to the earlier-eluting (*Z*)- isomers.

The relative GC peak areas of these three components (compounds **8**, **9**, and **10**) could not be determined by FID from single female gland extracts due to the trace amounts of the materials. Accordingly, female glands were combined to increase the concentration. Extracts obtained from three groups (3, 5, and 9 females, respectively) of female glands were subjected to GC analysis, and the mean ratio of *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac was determined to be 65 : 2 : 33 (Table 1). Estimates of the total amounts of *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac present in a single female gland extract ranged from 0.2 to 5 ng, with a mean of 2 ng. The activities of these three acetates were confirmed by strong antennal responses elicited by 10 ng of a synthetic blend in the ratio of 65 : 2 : 33 (Figure 1D), consistent with the responses obtained from female effluvia and gland extracts (Figure 1A and C). Thus, *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac were identified as electrophysiologically active components of *E. apiata* extracts.

The aliphatic acids (C_4–C_{10}) were consistently found in both female effluvia and gland extracts (Figure 1A and C). The ratios of these acids from the two sources were similar, and C_9 acid was the most abundant in both samples (Table 2). These acids elicited significant GC-EAD responses from both male antennae (Figure 1B) and female antennae (EAD responses not shown). The ratio of total acids to the sum of components **8**–**10** in the female gland extracts was estimated to be 23 : 1.

Field Tests. In the first field test, we compared *Z*9-16:Ac alone and in combination with *Z*9-14:Ac and 14:Ac (Figure 2). Moth catches were significantly higher in traps baited with *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac (hereafter referred to as 3-component blend) compared to captures in traps baited with *Z*9-16:Ac alone, or blends containing *Z*9-16:Ac plus *Z*9-14:Ac or *Z*9-16:Ac plus 14:Ac. The blend of *Z*9-14:Ac plus *Z*9-16:Ac (hereafter referred to as 2-component blend) was more attractive than *Z*9-16:Ac alone (Figure 2).

TABLE 2. COMPOSITION AND RATIO OF ALIPHATIC ACIDS IN FEMALE GLAND AND EFFLUVIA EXTRACTS[a]

Compound	Glands			Effluvia		
	Mean (%)[b]	SD	Range	Mean (%)[b]	SD	Range
C_4	1.0	0.5	0.6–1.5	5.8	1.6	4.0–7.1
C_5	1.0	0.5	0.6–1.5	6.8	2.4	4.1–8.4
C_6	9.7	4.8	6.1–15.2	13.7	5.8	10.2–20.4
C_7	7.3	4.2	4.3–12.1	6.1	0.4	5.6–6.5
C_8	16.2	3.2	13.6–19.7	14.4	1.7	12.7–16.1
C_9	53.0	13.1	37.9–61.0	34.7	9.1	24.2–40.3
C_{10}	11.8	0.5	11.2–12.2	18.6	3.7	14.5–21.6

[a] Gland extracts were obtained from 17 virgin females and effluvia extracts were obtained from 16 virgin females.
[b] $N = 3$.

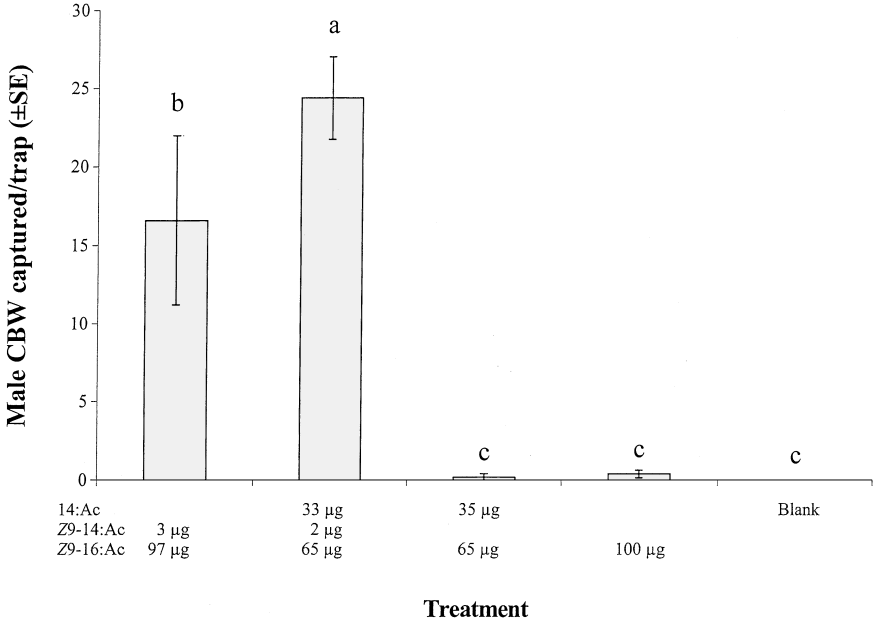

FIG. 2. Captures of male *E. apiata* moths in traps baited with *Z*9-16:Ac alone, various test blends, and a blank. The trial was conducted from September 25 to 29, 2002. Total number of male *E. apiata* captured was 208 in five replicates. Bars superscripted by different letters are statistically different (LSD, $P < 0.05$).

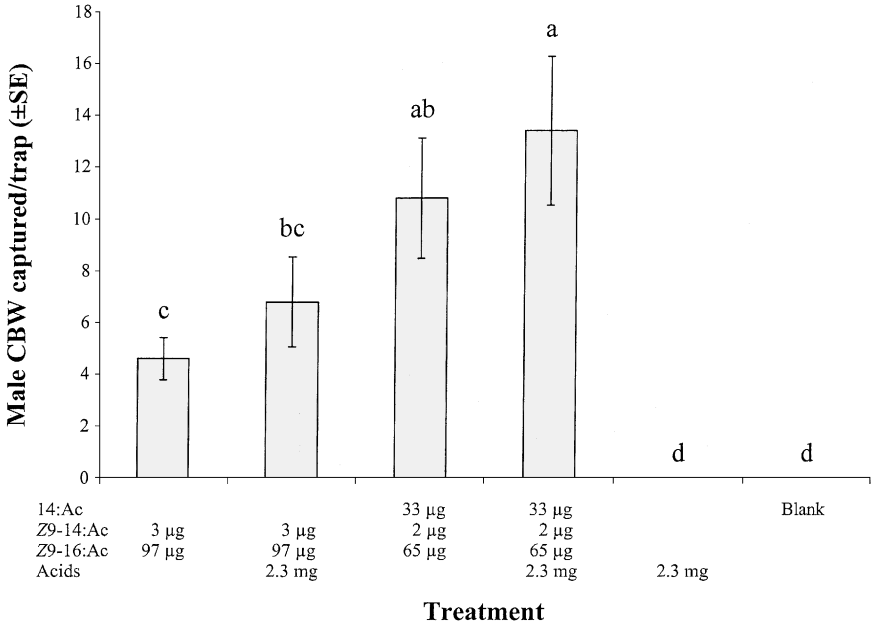

FIG. 3. Effect of addition of C_4–C_{10} organic acids to 2- and 3-component blends on captures of *E. apiata* male moths. The trial was conducted from October 4 to 14, 2002. Total number of male *E. apiata* captured was 188 in five replicates. Bars superscripted by different letters are statistically different (LSD, $P < 0.05$).

In the second trial we determined that addition of aliphatic organic acids to the 2- or 3-component blends did not significantly increase male moth catches, nor were females attracted to traps (Figure 3). As in the first field test, the 3-component blend was more attractive than the 2-component blend. Traps baited with the organic acids alone were not attractive.

In the final field test, rubber septa containing *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac in a 65 : 2 : 33 ratio at 30–1000-μg doses were evaluated to determine the optimum loading of pheromonal components. Moth captures were not significantly different in traps baited with 300- or 1000-μg doses (Figure 4), whereas traps baited with 30- and 100-μg doses captured significantly fewer moths.

DISCUSSION

Although very small amounts of the pheromone components, *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac, were present in the effluvia compared to their presence in gland extracts, they consistently elicited male antennal responses (Figure 1A, compounds

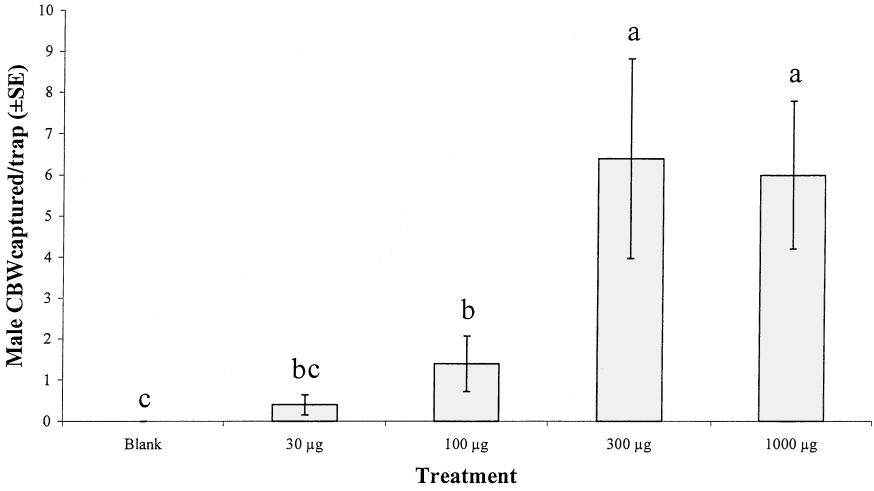

FIG. 4. Results of *E. apiata* pheromone dose response field trials (*Z*9-16:Ac : *Z*9-14:Ac : 14:Ac = 65 : 2 : 33). The trial was conducted from October 11 to 21, 2002. Total number of male *E. apiata* captured was 71 in five replicates. Bars superscripted by different letters are statistically different (LSD, $P < 0.05$).

8, **9**, and **10**), confirming that the pheromone components identified in female gland extracts are actually released by calling females. Attempts to determine the ratio of pheromone components from combined 7-day effluvia collections failed because of accumulated background impurities and the complexity of the GC trace. Therefore, the pheromone component ratio in the effluvia remains to be determined, and the ratio obtained from analysis of gland extracts was employed in all field experiments. Of the three acetate components, *Z*9-16:Ac was present in the greatest amount, and, therefore, was considered to be the major sex pheromone component of *E. apiata*.

The field studies indicated that *Z*9-16:Ac and *Z*9-14:Ac are essential components in the sex pheromone. Traps baited with *Z*9-16:Ac alone or in combination with 14:Ac failed to attract moths. However, the blend of *Z*9-14:Ac with *Z*9-16:Ac was attractive. The three-component blend containing *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac at approximately the same ratio as found in the female gland extracts (65 : 2 : 33) was the most effective lure in trapping *E. apiata.* The optimal loading of the pheromone appears to be in the range of 300–1000-μg/septum. Considering the long half-lives of acetate pheromone components (McDonough, 1991), 300–1000-μg loading may provide an effective lure for the entire adult moth flight period (Heath et al., 1986).

Aliphatic acids (C_4–C_{10}) were found in both female effluvia and gland extracts, and they elicited antennal responses from both sexes. However, at the same

rate as found in the gland extracts, the acids did not significantly increase male moth catches in the field. The lack of significance may be due to the fact that the experiment was conducted at the end of the flight season when the adult insect population was declining. Further research is needed to define the precise role played by these compounds in the communication system of *E. apiata*. For example, these compounds might play a role in close range courtship behaviors.

This is the first sex pheromone identified for an insect in the genus *Epiglaea*. However, *Z*9-16:Ac, *Z*9-14:Ac, and 14:Ac are relatively common sex pheromone components of many Noctuid moth species (Mayer and McLaughlin, 1991). Nevertheless, no other moth species were trapped during the course of this study with any of the synthetic pheromone combinations.

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AGGREGATION PHEROMONE OF THE CEREAL LEAF BEETLE: FIELD EVALUATION AND EMISSION FROM MALES IN THE LABORATORY

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Abstract—The previously identified, male-specific compound of the cereal leaf beetle (CLB, Chrysomelidae; *Oulema melanopus*), (*E*)-8-hydroxy-6-methyl-6-octen-3-one, was studied further with respect to field activity and emission rate from male beetles. In a 5-week field experiment in Oregon, the compound was shown to function as an aggregation pheromone in attracting male and female CLBs migrating from overwintering sites in spring. Traps baited with the synthetic compound (500 μg per rubber septum) caught 3.3 times more CLBs than control traps. Lower doses of the pheromone (50 and 150 μg) were less attractive than the 500 μg dose. One relatively abundant, volatile compound from the host plant (oats), (*Z*)-3-hexenyl acetate, that elicited responses from beetle antennae was not attractive, either by itself or as a synergist of the pheromone. Both sexes were captured about equally for all treatments. We also measured daily pheromone emission by male beetles in the laboratory. Individual males feeding on oat seedlings under greenhouse conditions emitted as much as 6 μg per day, which is about 500 times higher than had been previously observed under incubator conditions. The pheromone emission rate was at least five times higher during the day than at night, and in one male, emission spanned a period of 28 d. The release rate of synthetic pheromone from the 500 μg septa was very similar to the maximum from single males; thus, future experiments should evaluate even higher doses. The field results indicate that the pheromone has potential as a monitoring tool for early detection of CLBs

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as they move from their overwintering sites into newly planted cereal crops in spring.

Key Words—Cereal leaf beetle, *Oulema melanopus*, Coleoptera, Chrysomelidae, aggregation pheromone, (*E*)-8-hydroxy-6-methyl-6-octen-3-one, field evaluation, emission rates, (*Z*)-3-hexenyl acetate.

INTRODUCTION

The cereal leaf beetle (CLB), *Oulema melanopus* (L.) (Coleoptera: Chrysomelidae), is a new pest in cereals in the western U.S. (Rao et al., 2002). The beetle has been in eastern and midwestern regions since 1962 (Castro et al., 1965). It was first detected in the west coast states of Oregon and Washington in 1999, and it continues to spread. There are currently quarantine restrictions on movement of hay and forage from infested counties in Oregon and Washington into neighboring California and Canada. Annual surveys are now required in all counties in Oregon and Washington, and these presently involve labor intensive sweep-net sampling and visual examination of plants. An effective attractant for CLBs would facilitate the early detection of the pest in new areas during these surveys.

The CLB has one generation per year and overwinters in the adult stage. In spring, adults migrate from overwintering sites into cereal grain fields with succulent new foliage. The adults feed, mate, and lay eggs for a period of 6 weeks. Larvae also feed on the foliage. Mature larvae drop to the ground, pupate, and emerge as adults 2–3 weeks later. These emerging adults, present in the field in late summer, feed for 3 weeks prior to migration to overwintering sites (Royce and Simko, 2000). Adults are reproductively active only during the spring. Those emerging from pupation in summer feed to build up fat reserves for overwintering, but they do not mate. Hence, traps baited with an attractant would be especially beneficial for monitoring or managing CLBs during spring, as they move into the newly emerging cereals.

In an earlier study, a putative male-derived aggregation pheromone (*E*)-8-hydroxy-6-methyl-6-octen-3-one, was identified from CLB (Cossé et al., 2002). The compound stimulated antennae (GC-EAD) of both males and females. The total amount of male-specific compound emitted per male per day was observed to be 6.3 ± 7.9 ng when adults were placed in incubators and exposed to artificial light. In addition, male and female antennae responded to four host-derived compounds present in volatiles collected from feeding beetles, namely benzaldehyde, nonanal, methyl salicylate, and (*Z*)-3-hexenyl acetate (HA) (Cossé et al., 2002). Of these, HA was the most abundant in the volatile collections.

Certain chrysomelid beetles, such as rootworms (Metcalf and Metcalf, 1992) and flea beetles (Pivnick et al., 1992) are known to be strongly attracted to specific host compounds, but such information is not known for *O. melanopus*. In

beetle groups with aggregation pheromones, pheromone emission occurs at the host/breeding site, and some host-derived volatiles are potent synergists of the pheromone. Examples include yeast-fermentation volatiles for the *Carpophilus* sap beetles (family Nitidulidae; Bartelt, 1999) and conifer terpenes for *Dendroctonus* bark beetles (family Scolytidae; Borden, 1985). Synergistic interactions of host compounds and pheromones have not been published for chrysomelid beetles but could be crucial to the development of effective attractants for monitoring cereal leaf beetle.

The present study was conducted to establish whether (*E*)-8-hydroxy-6-methyl-6-octen-3-one functions as an aggregation pheromone under field conditions. In addition, we tested whether HA was attractive to CLB or was synergistic with the hydroxy ketone pheromone. Finally, additional information was obtained in the laboratory on the rate and timing of pheromone emission from male CLBs under greenhouse conditions for optimizing release rates of the synthetic pheromone.

METHODS AND MATERIALS

Chemicals, Instrumentation, and General Procedure for Collection of Volatiles. (*E*)-8-Hydroxy-6-methyl-6-octen-3-one used in the field trial was synthesized as described previously (Cossé et al., 2002). The compound was 95% pure (GC, impurities were not identified) after chromatographic purification on silica gel. The oat volatile HA was purchased from Lancaster Synthesis (Windham, New Hampshire).

Gas chromatography analyses were conducted on a Hewlett-Packard 5890 instrument equipped with flame-ionization detection. Samples were injected in splitless mode using 30 m Econocap (EC-1 and EC-5) capillary columns (0.25 mm ID, 0.25 μm film thickness, Alltech Associates, Deerfield, IL). The oven temperature was programmed from 50 to 275°C at 10°C per min. GC-MS was performed using a Hewlett-Packard 5973 instrument (EI, 70 eV) interfaced to an HP 6890 GC.

For volatile collections, the source material was contained in a glass tube (5 cm diam $\times$ 20 cm long) equipped with glass inlet and outlet adapters; all parts had 24/40 joints. A volatile trap was fitted to each adapter. Traps were made from 5-mm diam glass tubing and contained a 5–8 mm plug of Super-Q porous polymer (80–100 mesh, Alltech). The Super-Q was held in place by fine stainless steel screen (which was fused into the trap wall) and glass wool. A gentle vacuum was drawn on one of the traps so that air flowed through the assembled apparatus. Emitted volatiles were collected in the downstream trap, and the other trap served to clean the incoming air. All collection glassware was silanized to reduce sample adsorption. Volatiles were rinsed from the traps into vials with 300 μl hexane (HPLC grade). Solvent desorbtion efficiency was checked by a second 300 μl hexane rinse. Trap breakthrough was checked by placing a second Super-Q trap

in series with the first. The additional hexane rinse only showed trace amounts of pheromone, and no pheromone was detected in the breakthrough trap.

Lures for Fields Study. Synthetic (*E*)-8-hydroxy-6-methyl-6-octen-3-one was appropriately diluted with hexane and 10 μl aliquots were applied to rubber septa (20 $\times$ 11 mm diam, red rubber, Aldrich Chemical Co., Milwaukee, WI) at rates of 500, 150, and 50 μg per septum followed by 300 μl of methylene chloride. Once the solvent had soaked into the septa, septa were aired in a fume hood for 1 hr and stored at -70°C in tightly closed bottles until needed. Lures containing HA were prepared in a similar manner. The synthetic ester (4 mg in 50 μl hexane) was applied to a rubber septum, and the septum was then placed in a flat-top polypropylene microcentrifuge tube (1.5 ml, Bio Plas, San Francisco, CA). A single pin hole was made in the lid to slowly release the compound. Punctured tubes were stored at -70°C in tightly closed bottles until needed.

Prior to field tests, emission rates were measured from freshly prepared 500 μg septa, and from 500 μg septa that had been aired in a chemical hood for 1 week. The septa were placed in volatiles collectors at 25°C with an air-flow rate of 100 ml/min. Volatiles were collected for 24 hr. The amount of pheromone released was quantified by GC using hexadecan-1-ol as internal standard. The released amounts of HA were not measured.

Field Study. The experiment was conducted at the Oregon State University Agricultural Experiment Station at Union in a newly-seeded 2.4-hectare field of oats, May 9 to June 13, 2002. Normal cereal production practices were adopted at the experimental site, but no insecticides were applied to the crop. The study commenced about 10 d before seedling oats emerged from the soil.

There were six experimental treatments. Four of these tested for individual and synergistic activity of the 500 μg dose of the pheromone (P500) and HA; these treatments were P500, HA, P500 + HA, and unbaited control. It was considered likely, *a priori*, that a synergistic response to the combination would occur and, therefore, a sensitive way to test for a dose response to the pheromone would be in combination with the synergist. Thus, the final two treatments were the 50 and 150 μg doses of synthetic pheromone in combination with HA (P50 + HA and P150 + HA, respectively). Resources did not allow testing all possible combinations.

The experiment was set up as a randomized block design with 10 replicates. In each block, traps were set up in a line, 15 m apart. P and HA lures were attached with separate clips on the downwind side of yellow sticky traps (Pherocon® AM, Trécé Inc.) attached to bamboo stakes for trapping CLB adults. The trap height was initially 60 cm and later was raised to 90 cm above ground level.

Traps were checked, and lures and sticky traps were replaced weekly for 5 weeks. Captured CLB adults were counted, removed from the sticky traps, and preserved in 70% alcohol. Samples of adults collected from P500 traps on May 18 (190 CLBs) and May 30 (144 CLBs) were sexed using characters described

by Myser and Schultz (1967) to estimate the sex ratio of adults trapped in the experiment.

Statistical Analyses. Counts of CLB adults on traps were transformed using $\log(X + 1)$ to stabilize variance and then submitted to a two-way analysis of variance, using Statistix for Windows software (Analytical Software, 1998). In this analysis, the blocking factor was the replicate-by-week interaction (a total of 50 levels); thus, any week-to-week shifts in CLB population density in 1 or more of the 10 replicate areas would not inflate residual error and obscure treatment difference. Such shifts would occur, for example, if the field margins were infested first, and the central areas became infested later. Treatment effects were assessed with three linear contrasts involving the four means, P500 + HA, P500, HA, and control: (1) effect of P500, (2) effect of HA, and (3) interaction of P500 and HA. In addition, a linear contrast was fitted for P500 + HA, P150 + HA, and P50 + HA, using equal spacing of levels (which is approximately true for the logarithms of the doses). Finally, individual means were compared to the control, using the least significant difference (LSD) test at $\alpha = 0.05$.

Laboratory Study of Pheromone Emission from Male CLB. The CLB adults used in this study were field collected during late summer and early fall of 2001 from CLB-infested oat fields at Lake County, MT, Spokane Co., WY, and Multonomah Co., OR, and shipped overnight to Peoria, IL. Upon arrival, beetles were placed in cages containing oat seedlings and moist cardboard rolls for shelter, and cooled from 15 to 4°C over a 10-d period for winter storage in total darkness (Ruthann Berry, USDA/APHIS/PPQ, Niles, MI, unpublished 2000). Beetles were kept under these conditions for at least 4 months. After this period, beetles were removed from the cardboard rolls and sexed using characters described in Myser and Schultz (1967). Beetles were placed individually in 35-ml plastic cups with fresh pieces of oat seedlings as food sources in the greenhouse (21–30°C, 14L:10D). Food was replaced every morning.

A previous study indicated a positive correlation between pheromone production and visible feeding damage (Cossé et al., 2002). Therefore, only those males that showed feeding activity after spending several days in the cups were chosen for the subsequent pheromone emission study.

Volatiles collections were first made from seven individual males under greenhouse conditions using glass collectors as described earlier. To accurately detect (>0.5 ng/hr) and analyze the daily pheromone emission patterns, only those beetles that showed relatively high and consistent amounts of pheromone (relative abundance estimated by GC/MS) during collections preceding the day and night study were selected (3 out of 7 beetles). The day–night collection apparatus was essentially the same as the standard collector, and each contained one male CLB and an oat seeding (10–15 cm tall) with the roots in a vial of water. The difference between the two systems was that the upper adaptor on the day–night apparatus had two ports, and a trap and vacuum line were attached to each. A

switching system using a solenoid valve on an electric timer controlled which of the two vacuum lines and traps was in use at any moment. Daily pheromone emissions were collected for 30 d in two portions: a 16-hr collection that included the entire photophase and the 2 hr of the scotophase during which morning and evening twilight occurred, and a second 8-hr collection that included the remainder of the scotophase. The air-flow rate was 120 ml/min. Collectors were generally changed early each morning, and the oat seedlings were replaced every other day. Because of the laborious nature of the experiments and the limited amount of apparatus, volatile collections were limited to three individual beetles.

RESULTS

Field Study. Beetles were significantly more attracted to some lures than to others (Figure 1, $F = 26.01$, df $= 5, 245$, $P < 0.001$). Overall, traps with the 500 μg dose of synthetic pheromone (P500 + HA and P500) caught 3.29 times as many CLBs as those that did not (HA and control) (linear contrast: $t = 11.1$, df $= 245$, $P < 0.001$). (Z)-3-hexenyl acetate did not have a detectable effect either alone or in combination with the pheromone. Overall, the traps with HA (P500 + HA and HA) caught 0.97 times as many CLBs as the comparable ones that did not include HA (P500 and control) (linear contrast: $t = -1.34$, df $= 245$, $P = 0.18$).

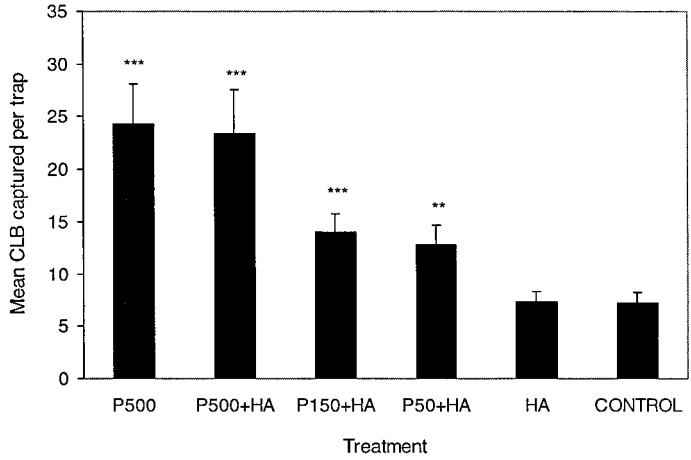

FIG. 1. Mean number (+SE) of overwintering CLB adults captured in 5 weeks on yellow sticky traps, baited with 500 μg (P500), 150 μg (P150), or 50 μg (P50) synthetic CLB aggregation pheromone (P) and 4 mg of the plant-related compound (Z)-3-hexenyl acetate (HA), placed in an oat field in Oregon. Means significantly different from the control at the $\alpha = 0.001$ and 0.01 levels are denoted by *** and **, respectively.

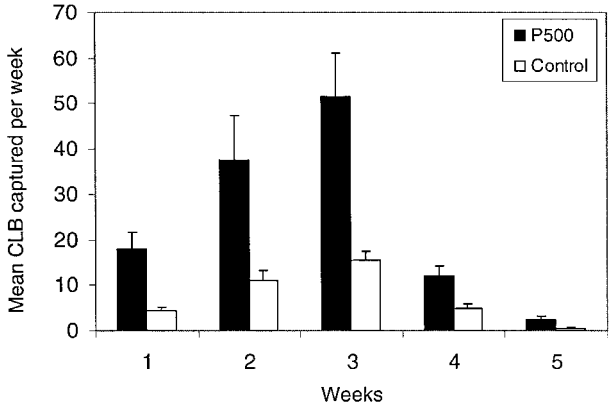

FIG. 2. Comparison of mean numbers (+SE) of overwintering CLB adults captured each week on yellow sticky traps baited with 500 μg synthetic CLB aggregation pheromone (P500) and unbaited control traps placed in an oat field in Oregon.

Furthermore, the effect of P500 in the presence of HA was not different from the effect of P500 in the absence of HA (i.e., there was no interaction; linear contrast: $t = 1.01$, df $= 245$, $P = 0.32$). There was a significant increase in trap catch with increasing dose of pheromone (for the linear contrast including P50 + HA, P150 + HA, and P500 + HA, $t = 3.95$, df $= 245$, $P < 0.001$). Considering individual means, those for all treatments except HA were significantly higher than the control ($P < 0.003$).

Numbers of beetles captured on a single trap in a week ranged from 0 to 140. Random samples from P500 traps showed that males and females were captured about equally; on May 18, the female:male ratio was 1.0 ($N = 190$), and on May 30, it was 1.1 ($N = 114$). The numbers of adults trapped across all treatments increased from an average of 12.5 beetles per trap in the first week to 27.5 per trap in the third week. Subsequently there was a decline and the weekly average dropped to 1.08 per trap in the fifth week. The pattern of change over weeks was similar for all treatments (Figure 2).

The release rate test conducted prior to the field experiment indicated that the synthetic pheromone was emitted at 5.6 ± 0.9 μg/d ($N = 3$) from freshly prepared rubber septa (P500), and 3.6 ± 1.5 μg/d ($N = 3$) from septa that had been aired for 1 week in a fumehood.

Pheromone Emission from Males. Traces of pheromone (GC/MS) were detected 3–10 d after removal of the beetles from cold storage (7 out of 7 beetles). The daily mean pheromone release rates ($\pm$ SD) from individual male beetles (prior to greenhouse equipment failure) was 3.4 (± 1.7) μg/d ($N = 27$), and the maximum was 6.0 μg/d. The periodicity of daily pheromone release by three individual

male cereal leaf beetles is presented in Figure 3. For each male, pheromone release rate was at least five times higher during the photophase than during the scotophase. Pheromone secretion ceased completely when the greenhouse temperature reached 45°C because of equipment failure, but it started again when the normal temperature range (21–30°C) was reestablished.

DISCUSSION

In an earlier study, Cossé et al. (2002) demonstrated that the compound (*E*)-8-hydroxy-6-methyl-6-octen-3-one isolated from male CLB adults elicited strong responses from antennae of both male and female CLBs in the laboratory. In the present study, we demonstrated that the compound attracted both sexes under field conditions, functioning as an aggregation pheromone.

Aggregation pheromones are well known in the Coleoptera (Borden, 1985), but they were not known in the Chrysomelidae until recently. The first chrysomelid pheromone to be identified was the female-produced sex pheromone of the western corn rootworm (Guss et al., 1982), which attracts only males. Chemically related pheromones were subsequently defined for seven other *Diabrotica* species and subspecies (Krysan et al., 1989, and references therein). It is now known, however, that males in other chrysomelid species produce long-range pheromones to which both sexes respond. Examples include the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Dickens et al., 2002), and the crucifer flea beetle, *Phyllotreta cruciferae* (Goeze) (Peng et al., 1999; Bartelt et al., 2001). The present study provides further evidence for the latter pattern.

The release rate from freshly prepared rubber septa with the highest dose of synthetic pheromone (P500) used in the field study was similar to the maximum daily rate released by a single male under greenhouse conditions, and the rate for 1-week-old septa was similar to the average rate for single males under greenhouse conditions. Furthermore, if the daytime release from live males (Figure 3) is concentrated in just a few hours, then the peak natural emission rate from males may be higher than that from the rubber septa. It is possible that all lures in the field experiment emitted the pheromone at a lower rate than the natural maximum from males. Using incubators and artificial light, Cossé et al. (2002) showed a pheromone release rate of 6 ng/beetle/d. In the current study, the daily pheromone release rates from individual male beetles were about 500 times higher in the greenhouse than they were in the incubator with fluorescent lighting. Furthermore, pheromone collections in the previous study were made from groups of up to five beetles. The grouping of beetles may have negatively affected the pheromone release rate. However, the original intent was to deploy lures that released in the range of 10–100 male equivalents, based on the initial emission data (Cossé et al., 2002). The present information on emissions from males indicates that the initial

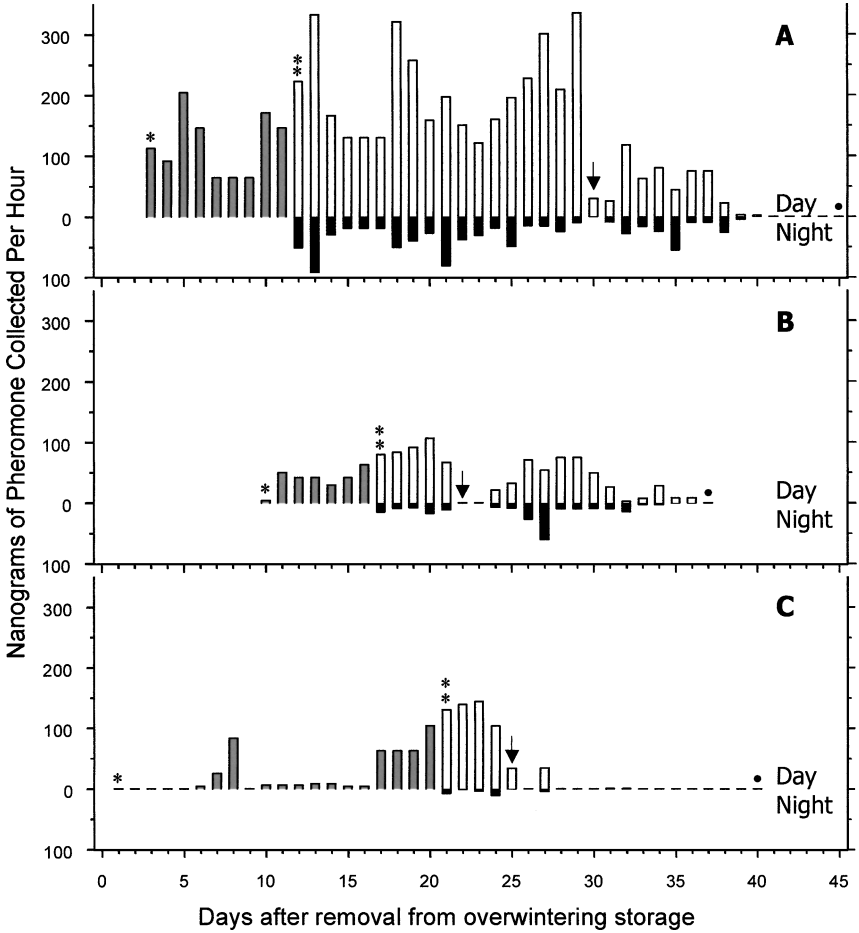

FIG. 3. Periodicity of pheromone emissions from three individual male *Oulema melanopus* adults (A, B, C) feeding on oat seedlings in a greenhouse environment. Gray bars represent 24-h collections, white bars represent daytime collections, and black bars represent nighttime collections. * Denotes the first collection, ** denotes the first day/night collection, • denotes the final collection, and ↓ denotes overheating of the green house (45°C).

target was too low and suggests that considerably higher doses of the compound should be tested in the field, to determine if stronger responses are possible.

The results of the present study differ from those of a preliminary study conducted in late summer 2001 (Rao et al., 2002). During the earlier study, compared with control traps, there were apparently more beetles captured on traps baited

with CLB aggregation pheromone, but the differences were not significant. In the earlier study, the beetles were newly emerged, nonreproductive adults that were feeding prior to entering overwintering diapause. In the present study, the captured adult CLBs had already overwintered and were moving into the oat field to begin reproduction. It is likely that the effect of the pheromone on CLB behavior depends on their physiological state (reproductive or diapause) with diapausing beetles being less likely to respond. In this study, numbers of trapped CLB adults increased for the first 3 weeks and then declined. By the fifth week there were few captures. This pattern likely reflects, first, the immigration of beetles into the field, and, eventually, the cessation of reproductive activity.

Many studies have documented that host volatiles enhance responses of insects to aggregation pheromones (Borden, 1985; Bartelt, 1999). Cossé et al. (2002) observed that a number of volatiles from damaged oat seedlings elicited strong antennal responses from male and female CLBs. However, the most abundant of these, HA, was neither an attractant nor a pheromone synergist. Pheromone synergism by host volatiles may still occur in the CLB, using blends of host compounds or adjusting the emission rate.

Finally, further attention must be given to trap design. In an earlier attempt to develop a survey technique for CLB, sticky boards of various colors were tested (Wilson and Shade, 1967). Of the various colors tested, yellow attracted the greatest numbers of adults. Whereas the yellow sticky traps used in the present study were effective in trapping CLBs, our observations indicated that after capture, some beetles were dislodged from the trap.

The present study suggests that the CLB aggregation pheromone may be useful for monitoring and managing CLB, especially in the spring when new fields are infested by overwintered beetles. One particular benefit with the CLB pheromone is that it attracts both sexes.

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OVEREXPRESSION OF GLUTAMATE DECARBOXYLASE IN TRANSGENIC TOBACCO PLANTS DETERS FEEDING BY PHYTOPHAGOUS INSECT LARVAE

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Abstract—Gamma-aminobutyrate (GABA) is a ubiquitous four-carbon, non-protein amino acid synthesized by glutamate decarboxylase. Previous research suggests that the endogenous synthesis of GABA, a naturally occurring inhibitory neurotransmitter at neuromuscular junctions, serves as a plant resistance mechanism against invertebrate pests. In this study, two homozygous transgenic tobacco lines constitutively overexpressing a single copy of a full-length chimeric glutamate decarboxylase cDNA and possessing enhanced capacity for GABA accumulation (*GAD* plants), a homozygous transgenic line lacking the gene insert, and wild-type tobacco were employed. Tobacco budworm larvae were presented with plantattached wild type and transgenic leaves for 4 hr in a feeding preference study. Larvae consumed six to twelve times more leaf tissue from wild-type plants than from GAD plants. These results suggest that leaf GABA accumulation, which is known to occur in response to insect larval walking and feeding, represents a rapidly deployed localresistance mechanism.

Key Words—Gamma-aminobutyrate, GABA, glutamate decarboxylase, phytophagous, plant resistance, insect larvae, transgenic plants, invertebrate pests.

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INTRODUCTION

GABA is a four-carbon, non-protein amino acid found in virtually all prokaryotic and eukaryotic organisms. GABA is synthesized via the alpha-decarboxylation of glutamate in an irreversible reaction catalyzed by the cytosolic enzyme glutamate decarboxylase (GAD; EC 4.1.1.15). It is metabolized in the mitochondrion to succinic semialdehyde by GABA transaminase (EC 2.6.11.9), and then to succinate by succinate semialdehyde dehydrogenase (EC 1.2.1.16). These three enzymecatalyzed reactions constitute the GABA shunt pathway of glutamate metabolism (Bown and Shelp, 1997). In plants, diverse abiotic stresses including mechanical stimulation, mechanical damage, cold shock, heat shock, hypoxia, cytosolic acidification, and water stress stimulate rapid and large GABA accumulation (Bown and Shelp, 1997 and references therein).

A variety of roles for stress-induced GABA accumulation has been suggested (Bown and Shelp, 1997). In invertebrates, GABA is an inhibitory neuromuscular transmitter acting at GABA-gated Cl^- channels. GABA accumulation as a plant resistance mechanism against phytophagous insect larvae was first suggested when mechanical stimulation or damage of leaf tissue was shown to result in rapid GABA synthesis (Wallace et al., 1984). In insect larvae, neuromuscular junctions are not protected by a covering of glial cells, and injection of physiological concentrations of neurotransmitters into the hemolymph of *Lucilia sericata* larvae causes reversible paralysis (Irving et al., 1979). Thus, larval neuromuscular junctions are exposed to ingested GABA if it is absorbed into the hemolymph. Many commercial insecticides are agonists or antagonists of the GABA-gated Cl^- current and disrupt normal neuromuscular activity (Casida, 1993; Hosie et al., 1997). High levels of ingested GABA may have a similar disruptive effect. Significant GABA accumulation in tobacco and soybean leaves occurs within 10 min in response to insect larval crawling (Bown et al., 2002).

Recently, McLean et al. (2003) demonstrated that constitutive transgenic expression of GAD in tobacco confers resistance against the northern root-knot nematode, probably via a GABA-based mechanism. In this paper, we used two of the transgenic lines derived in that study to demonstrate that GAD overexpression also confers resistance against tobacco budworm larvae. We suggest that GABA accumulation in responseto insect larval crawling or herbivory deters feeding.

METHODS AND MATERIALS

Two phenotypically normal, independently transformed, homozygous transgenic lines of tobacco (*Nicotiana tabacum* L. cv. Delgold) overexpressing a single copy of a full-length, chimeric native GAD (designated as *GAD* 2 and *GAD* 5 plants), and a "no insert" homozygous transgenic line expressing only the empty vector were employed in this study (McLean et al., 2003). For assay of GAD

activity, seeds were germinated and grown under greenhouse conditions (McLean et al., 2003). At four weeks of age, the leaves were removed from each plant ($N = 3$) and crude activity in leaf extracts was assayed at pH 5.8 after desalting on a G-25 Sephadex column, as described previously (Serraj et al., 1998).

For feeding preference trials, tobacco seeds were germinated and grown, leaf GABA was determined, and tobacco budworm (*Heliothis virescens* Fabricius) egg masses were reared according to Bown et al. (2002). Plants were used at the seven-leaf stage, with leaf number five being placed within the feeding chamber. Fourth and fifth instars weighing 140–150 mg each were employed. One tobacco budworm larva was placed in a feeding chamber containing two leaves, one attached to a wildtype plant and the other attached to a transgenic plant. Thus, each experiment was internally controlled through the use of one larva given equal access to two different leaves. The feeding chamber was constructed from a 100 × 15 mm Petri dish with slots cut on opposite sides to allow the insertion of the two plant-attached leaves. After 4 hr, leaf area consumed was determined using an Analytical Imaging Station (Imaging Research Inc., St. Catharines, ON). The wild type and transgenic lines did not differ significantly when dry weight to leaf area ratios were measured. Thus, leaf are consumed is a valid measure of tissue consumed. For each plant comparison, a minimum of six trials were conducted, and P was calculated using the 1-tailed Wilcoxon Signed-Rank Test.

RESULTS

Assays of total GAD activity demonstrated that the twotransgenic lines, *GAD* 2 and *GAD* 5, have twiceas much activity as either the wild-type or the transgenic line lacking the gene insert (Table 1), confirming that both *GAD*2 and *GAD*5 overexpress GAD.

The mechanical manipulation required to place two plant-attached leaves into a feeding chamber might influence GABA levels and subsequent feeding activity. To investigate this possibility leaves were placed in a feeding chamber without larvae for 2 hr prior to GABA determinations. No significant differences in GABA levels were found between leaves from the chamber and from resting, non-manipulated leaves (data not shown).

TABLE 1. GAD ACTIVITY IN LEAVES OF FOUR-WEEK-OLD WILD-TYPE AND TRANSGENIC TOBACCO LINES

Genotype	GAD activity (μmol · g^{-1} FW · h^{-1}, mean ± s.e.)
Wild-type	24.5 ± 1.3
No-insert control	21.9 ± 2.9
*GAD*2	40.4 ± 5.3
*GAD*5	39.1 ± 1.0

TABLE 2. LEAF AREA CONSUMED DURING FEEDING PREFERENCE ASSAYS BETWEEN WILD-TYPE AND TRANSGENIC TOBACCO LINES

Leaf pair	Leaf tissue consumed (mm^2, mean ± s.e.)	
(1) Wild-type	13.8 ± 3.2	$P = 0.28$
No-insert control	11.6 ± 3.8	
(2) Wild-type	24.9 ± 11.0	$P = 0.047$
*GAD*2	4.4 ± 1.1	
(3) Wild-type	117.3 ± 81.3	$P = 0.013$
*GAD*5	9.2 ± 4.8	

Feeding preference assays were performed in order to determine whether tobacco budworm larvae prefer wild-type tissue to tissue from *GAD* 2 or *GAD* 5 plants. One tobacco budworm larva was placed in a feeding chamber with one plantattached wild-type leaf and one plant-attached transgenic leaf. After 4 hr of feeding, larvae had consumed 6–12 times more leaf tissue from wild-type plants than from *GAD* 2 or *GAD* 5 plants, indicating that they have a significant feeding preference for wild-type tissue (Table 2). Compared with leaf pairs 2 and 3, a lower value of wild type tissue consumed in leaf pair 1 was expected if no deterrencewas associated with the no-insert control leaf. Despite large variations in the total leaf area consumed by individual larvae, the preference for wild-type tissue was consistently observed. Furthermore, comparison of wild-type plants with transgenic plants lacking the gene revealed that this feeding preference was not caused by the transformation process.

DISCUSSION

Several experimental approaches indicate that the transgenic tobacco lines *GAD* 2 and *GAD* 5 overexpress a functional GAD. 1. The resting GABA levels of intact 2-wk-old seedlings grownin the dark were 150–180% of those in the wild-type (McLean et al., 2003). 2. The GABA-producing potential was determined using a freeze-thaw cycle, which stimulates GAD by destroying cellular compartmentation (see below). Leaf GABA levels of 12 wk-old plants were 170–240% of those in the wild-type (McLean et al., 2003). 3. Assays of total activity in GAD2 and GAD5 plants revealed twice as muchGAD compared to wild-type material (Table 1).

In vitro and *in vivo* data demonstrate that GAD activity is stimulated by increases in the cytosolic levels of H^+ or Ca^{2+} (Bown and Shelp, 1997). Mechanical stimulation results in rapid increases in plant cell cytosolic Ca^{2+} levels, and mechanical damage disrupts cellular compartmentation releasing vacuolar stores of Ca^{2+} and H^+ to the cytosol (Bown and Shelp, 1997 and references therein).

Mechanical stimulation and damage both result in GABA accumulation (Wallace et al., 1984), as does larval crawling on leaf tissue (Bown et al., 2002). Similarly, phytophagous activity will disrupt cellular compartmentation and activate GAD.

Converging data now support the hypothesis that mechanically-induced GABA accumulation functions as a resistance mechanism against invertebrate pests. 1. The introduction of GABA levels found in mechanically-stimulated leaf tissue into a synthetic diet, reduces rates of growth, development and survival of the phytophagous larvae of the oblique-banded leaf roller (*Choristoneura rosaceana*) (Ramputh and Bown, 1996). 2. Tobacco budworm larvae crawling on tobacco or soybean leaves stimulate 5- or 10-fold increases in GABA levels within 10 min (Bown et al., 2002). 3. Overexpression of GAD in transgenic tobacco plants confers resistance to the northern root-knot nematode (McLean et al., 2003), as indicated by a significant reduction in egg masses on the root surface. 4. Overexpression of GAD in transgenic tobacco plants reduces feeding of tobacco budworm larvae (Table 2). It is hypothesized that GABA accumulation in response to pest activity represents a rapidly deployed local resistance mechanism. It may function prior to the deployment of local and systemic resistance mechanisms, which are dependant on changes in gene expression of proteinase inhibitors or other defense products (Kessler and Baldwin, 2002).

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COMPARISON OF OLIGOSACCHARIDE COMPOSITIONS IN MALE NUPTIAL SECRETIONS OF THREE COCKROACH SPECIES OF THE GENUS *Blattella*

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Abstract—Male cockroaches of the genus *Blattella* secrete courtship pheromones from abdominal tergal glands. Conspecific females mount courting males and feed on their tergal exudates. In the German cockroach, *B. germanica*, a complex mixture of oligosaccharides was previously identified as the major phagostimulants in the male tergal secretion. We compared the oligosaccharide compositions of three *Blattella* species from different habitats, *B. germanica* (house), *B. lituricollis* (field), and *B. nipponica* (forest), by gas chromatographic (GC) analysis of their trifluoroacetylated derivatives. The three possessed a series of maltooligosaccharides and oligoglucosyl trehaloses with $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 1$ linkages in common. However, *B. germanica* was conspicuous for its accumulation of a oligoglucosyl trehalose with a $\alpha 1 \rightarrow 6$ linkage [*O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl α-D-glucopyranoside] as the most prominent component of the secretion. Such diversification of the male pheromonal compositions within the genus might be reflected by biosynthetic systems in males and chemosensory properties in females suited for their natural habitat.

Key Words—German cockroach, *Blattella germanica*, *Blattella lituricollis*, *Blattella nipponica*, tergal gland, male courtship pheromone, phagostimulant, oligosaccharides.

INTRODUCTION

Males of most cockroach species in the Blattellidae have abdominal tergal glands of diverse shape, position, and number (Roth, 1969). The gland secretions function

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as male courtship pheromones during the sequential courtship behavior. In the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae), the male secretion elicits female nuptial feeding, an important step that arrests her in a precopulatory position and leads to copulation. The major phagostimulants in the male nuptial secretion are composed of a complex mixture of synergistically acting oligosaccharides and phospholipids (Nojima et al., 1999a; Kugimiya et al., 2002). The oligosaccharides consist of at least nine compounds in three groups: maltooligosaccharides, oligoglucosyl trehaloses, and pseudosaccharides (Nojima et al., 2002). Besides *B. germanica*, a household pest widely distributed in urban areas throughout the world, there are several closely related species in Japan, including *B. nipponica* in the forest habitats, and *B. lituricollis* in fields, often in cultivated areas. We examined the oligosaccharide compositions of the male nuptial secretions to compare the chemical profiles among closely related species that occupy different habitats.

METHODS AND MATERIALS

Insects. Adults of *B. germanica* were obtained from a laboratory colony maintained for more than 10 years under the conditions described previously (Kugimiya et al., 2002). Sexually mature virgin males 10 days after emergence were used for chemical analysis. Nymphs of *B. nipponica* were captured in forests in Kyoto, and adults of *B. lituricollis* were caught in fields in Okinawa. They were raised in the laboratory under the same conditions as above, and male adults of the next generation were used for chemical analyses.

Chemical Analyses. A series of oligosaccharides spectroscopically characterized (Nojima et al., 2002) were trifluoroacetylated for GC analysis (Selosse and Reilly, 1985). The eighth abdominal terga, which contain tergal glands (TG-8), were removed from males of *B. germanica*, *B. nipponica*, and *B. lituricollis*. TG-8 of each species was extracted for 1 min with methanol (100 μl/gland). After paper filtration of the extract, the filtrate was partitioned between chloroform (50 μl/gland) and water (40 μl/gland). An internal standard, phenyl α-D-glucopyranoside (5 μg), was added to the upper aqueous phase containing the extract of 5 males, and the sample was evaporated under vacuum at 60°C. The residue was dissolved in pyridine (20 μl) and *N*-methylbis(trifluoroacetamide) (80 μl), and held at 75°C for 10 min. After cooling to room temperature, 2 μl of the reaction mixture were analyzed with an Hewlett Packard 4890 GC system equipped with a flame-ionization detector and an HP-1 capillary column (25 m $\times$ 0.2 mm i.d.; 0.33 μm film thickness, Hewlett Packard), in splitless mode, under programmed temperature from 100°C (0.5 min) to 300°C (9.5 min) at 10°C/min. Three sets of extracts were subjected to the GC-quantification for each species.

RESULTS AND DISCUSSION

The tergal glands from males of *B. germanica*, *B. lituricollis*, and *B. nipponica* are similar morphologically. They have two glandular openings on the 7th and 8th abdominal tergites. Preliminary observations indicated that females preferred to lick the TG-8 of conspecific males during his wing-raising courtship behavior.

Oligosaccharides in the tergal glands of males of the three *Blattella* species were detected as trifluoroacetylated derivatives in GC analyses. Although di- and trisaccharides could be analyzed quantitatively with great precision, larger saccharides could not be quantified reliably because of their low detection threshold under the GC conditions. Males of the three species shared several saccharide components in their TG-8 (Figure 1). Maltooligosaccharides [e.g., maltose (M2) and maltotriose (M3)], trehalose (T), and oligoglucosyl trehaloses [e.g., *O*-α-D-glucopyranosyl-(1$\rightarrow$4)-α-D-glucopyranosyl α-D-glucopyranoside (G1T)] were detected as common constituents in TG-8 of the three species. While the oligosaccharide compositions of *B. lituricollis* and *B. nipponica* were similar to each other, much more

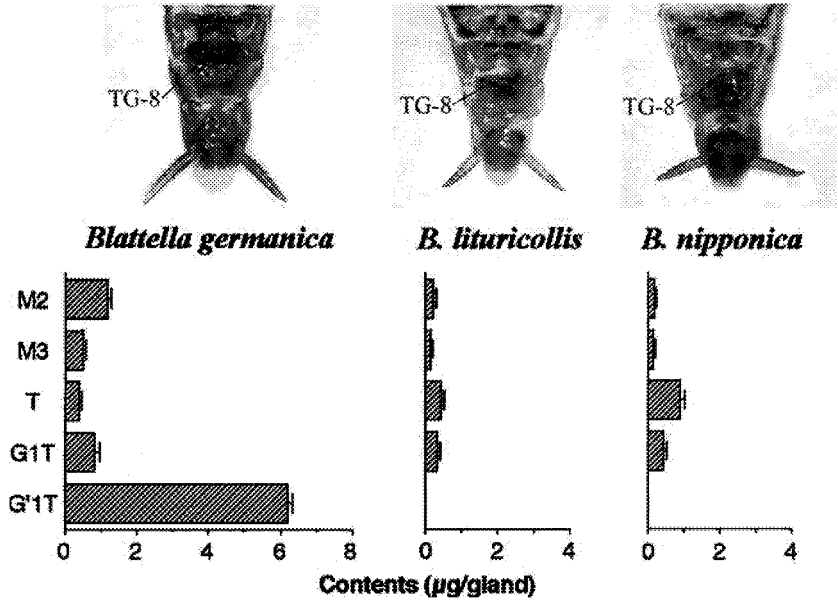

FIG. 1. Oligosaccharide compositions from 8th tergal glands (TG-8) of three male cockroach species of genus *Blattella* (mean $\pm$ SEM, $N = 3$). M2: Maltose, M3: Maltotriose, T: Trehalose, G1T: *O*-α-D-glucopyranosyl- (1$\rightarrow$4)-α-D-glucopyranosyl α-D-glucopyranoside, G′1T: *O*-α-D-glucopyranosyl-(1$\rightarrow$6)-α-D-glucopyranosyl α-Dglucopyranoside.

O-α-Dglucopyranosyl-(1→6)-α-D-glucopyranosyl α-D-glucopyranoside (G′1T) was found in *B. germanica* (6.2 ± 0.1 μg/gland, mean ± SEM, $N = 3$) than in the other two species. G′1T was found to be a prominent oligosacharide that synergizes the phagostimulant activity of TG-8 phospholipids in *B. germanica* (Nojima et al., 2002).

The nuptial secretions are likely to be orally perceived through the same gustatory mechanisms as is food (Nojima et al., 1999b). The remarkable difference in the quantity of G′1T between the household pest, *B. germanica*, and the other two outdoor species, *B. lituricollis* and *B. nipponica*, might reflect intrinsic preferences or demands of females for the sugars in their dietary environment. We have a little knowledge about the chemical basis of taste preferences in these cockroaches. It is uncertain whether the difference could be associated with different selection in terms of "domestication" or "laboratory segregation" over generations in *B. germanica*. Oligoglucosyl trehaloses, including G′1T, have been found as intermediate metabolites of trehalose synthesis in microorganisms (e.g., *Arthrobacter* sp. Q36) (Nakada et al., 1995). In *Blattella* species, the biosynthesis of these oligosaccharides in tergal glands or possible mediation of symbiotic bacteria in their production remains to be elucidated.

Saccharides in the tergal glands, because of the diversity of constituent sugar units and linkages, might be used for chemotaxonomy, together with molecular phylogeny of *Blattella* and other cockroaches (Mukha et al., 2002). Further exploration of pheromones and enzymes, as well as DNA sequences, involved with their biosyntheses are needed to infer the evolution of the courtship pheromone systems and mating behavior in the Blattaria.

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BIOACTIVITY, SYNTHESIS, AND CHIRALITY OF THE SEX PHEROMONE OF CURRANT STEM GIRDLER, *Janus integer*

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Abstract—It was previously reported that females of the currant stem girdler, *Janus integer* Norton (Hymenoptera: Cephidae), produce a compound, (Z)-9-octadecen-4-olide (**1**), that is sensitively detected by the antennae of males only. These characteristics suggested a pheromonal function, and this has now been confirmed with behavioral tests. Field tests conducted during two seasons in a commercial red currant field in Washington State showed that synthetic racemic **1** is attractive to male *J. integer* under natural conditions. A clear dose-response was evident, with greatest numbers of girdlers caught in sticky traps baited with 10 mg of the pheromone (in rubber septa) and least in traps baited with 1 mg or less. During May 2002, 10, 5, 3, and 1 mg baited traps caught means of 41.4, 26.6, 6.7, and 2.7 males/trap/visit (3–5 day intervals), respectively, with a maximum of 229 males caught in a single trap baited with 5 mg. A new synthetic method for racemic **1** is presented. The absolute configuration of natural **1** from the male sawflies was determined to be (*R*). The potential for using the sex pheromone of *J. integer* to improve management of this currant and gooseberry pest is discussed.

Key Words—*Janus integer*, sawfly, sex pheromone, (*R*, *Z*)-9-octadecen-4-olide, (*R*,*Z*)-dihydro-5-(5-tetradecenyl)-2-(3*H*)-furanone, chiral synthesis, field tests, trapping.

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INTRODUCTION

The currant stem girdler, *Janus integer* Norton (Hymenoptera: Cephidae), is a sawfly pest of currants and gooseberries in North America. Its range extends from Quebec south to Maryland and west to Iowa, Washington State, and Manitoba (Middlekauf, 1969). The pest status of *J. integer* is greatest in commercial fields of red currants, black currants, and gooseberries in New York, New England, Wisconsin, and Washington State. In Washington State, recent adoption of mating disruption for the clearwing moth, *Synanthedon tipuliformis* Clerck (Lepidoptera: Sesiidae) (James, 2002), leaves *J. integer* as the only pest of red currants for which insecticides are required.

Damage from *J. integer* is caused by tunneling of larvae in canes and also by the girdling habit of the adult female, which results in the death of young shoots (Britton, 1921). In Washington, there is one generation annually with adults normally emerging in early May. Currant growers are not usually aware of the presence of *J. integer* until damage to young shoots becomes visible. Females puncture shoots with their ovipositor and place a single egg in the pith. Above the egg, the shoot is girdled with the stem partially cut, causing wilting and eventual death of the shoot. The larva feeds on the pith during the summer, excavating a tunnel and pupating in early spring.

Cossé et al. (2001) reported the occurrence of a female-specific compound from *J. integer*, which they identified as the chiral lactone, (*Z*)-9-octadecen-4-olide (**1**). The structure was proven by synthesis of the racemic compound. By chiral GC analysis, only one enantiomer was detected from the sawflies, but the absolute configuration was not determined. By GC-EAD, the lactone was sensitively detected by the antennae of male *J. integer*, but not by females. The sex-specific production and detection of the compound suggested that it functions as a sex pheromone. In this report, we present data from field tests demonstrating attraction of male *J. integer* to the racemic lactone, and we determine the absolute configuration of the natural enantiomer.

METHODS AND MATERIALS

*Bioactivity of **1** in the Field.* Experiments on attraction of male *J. integer* to (±)-**1** were conducted in a commercial red currant field at Prosser, Washington in 2001 and 2002. Synthetic racemic lactone was prepared at NCAUR in Peoria, as described below, and appropriate volumes of hexane solutions were applied to rubber septa (11 by 20 mm, red rubber, Aldrich, Milwaukee, WI). Septa were kept in tightly closed bottles and stored at −20°C prior to deployment in traps.

The experiment in 2001 was conducted from 25 April until 5 June and assessed the attraction of *J. integer* males to Pherocon IC sticky traps (Trécé, Salinas,

CA) baited with rubber septa with 0.01, 0.1, or 1 mg of (±)-**1**. These were deployed (singly) by placing in the center of the sticky area in each trap. Septa were renewed on 15 May. Unbaited traps served as controls. Treatments were replicated five times. A randomized complete block design was used with traps separated by 20 m. Traps were inspected for sawflies at 7-day intervals, and sawflies were sexed, counted, and removed with forceps. No insecticides were applied to the field until late May when the grower applied sprays of fenpropathin on 18 and 22 May for *J. integer*.

The experiment in 2002 was conducted from 3 May until 7 June and compared the attraction of *J. integer* males to Pherocon IC sticky traps (Trece, Salinas, CA) baited with 0.1, 0.3, 1.0, 3.0, 5.0, or 10.0 mg of (±)-**1**. The four lower rate treatments were compared in a complete randomized block design with traps separated by 20 m. Treatments were replicated five times, and unbaited traps served as controls. An additional treatment (live virgin female *J. integer* in muslin covered plastic cups) was incorporated during 3–16 May but was abandoned due to problems in maintaining viability of the insects. Septa were not renewed during the experiment.

The two high rate treatments (5.0, 10.0 mg) were examined in another section of the field. Traps were placed at 20-m intervals in a single row of red currants in the center of the field. The treatment and unbaited control traps were assigned alternately with three replicates of each. Multiples of 1-mg septa were used to bait these traps and renewed on 28 May. All traps were inspected at 3–5 day intervals, and the sawflies were sexed, counted, and removed with forceps. Fenpropathin was applied to alternate rows in the field (after trap catches recorded) on 20 and 25 May and on 2 June.

In both experiments, trapping data were transformed to the log $(x + 1)$ scale to stabilize variance and subjected to analysis of variance and Fisher's least significant difference procedure.

*Synthesis of Racemic Lactone **1**.* 1,4-Butanediol (**2**, 88 mmol) was monoprotected as tetrahydropyranyl (THP) ether **3**, using a biphasic mixture of 3,4-dihydro-2*H*-pyran (DHP, 80 mmol), CH_2Cl_2 (240 ml), a catalytic amount of 0.1 N aqueous HCL (8 ml), and stirring at room temperature (rt) for ca. 96 hr (Figure 1). Using the biphasic system almost completely prevented formation of the THP diether. The reaction was terminated by the addition of saturated $NaHCO_3$ (until aqueous layer basic to pH paper, ca. 1 ml). Layers were separated; the aqueous layer was extracted twice with 30 ml CH_2Cl_2, and combined organic layers were dried (Na_2SO_4) and filtered. Solvent removal by rotary evaporation gave **3** (73% yield from DHP, purity 97% by GC; in this report, percent yields are based on amounts of actual starting materials and desired products, correcting for purity). The mass spectrum indicated the expected molecular weight [*m*/*z* (%): 173 ($M\text{-}1^+$, 0.11), 85 (100)], and the proton and carbon NMR spectra were consistent with those reported by Johns et al. (1989).

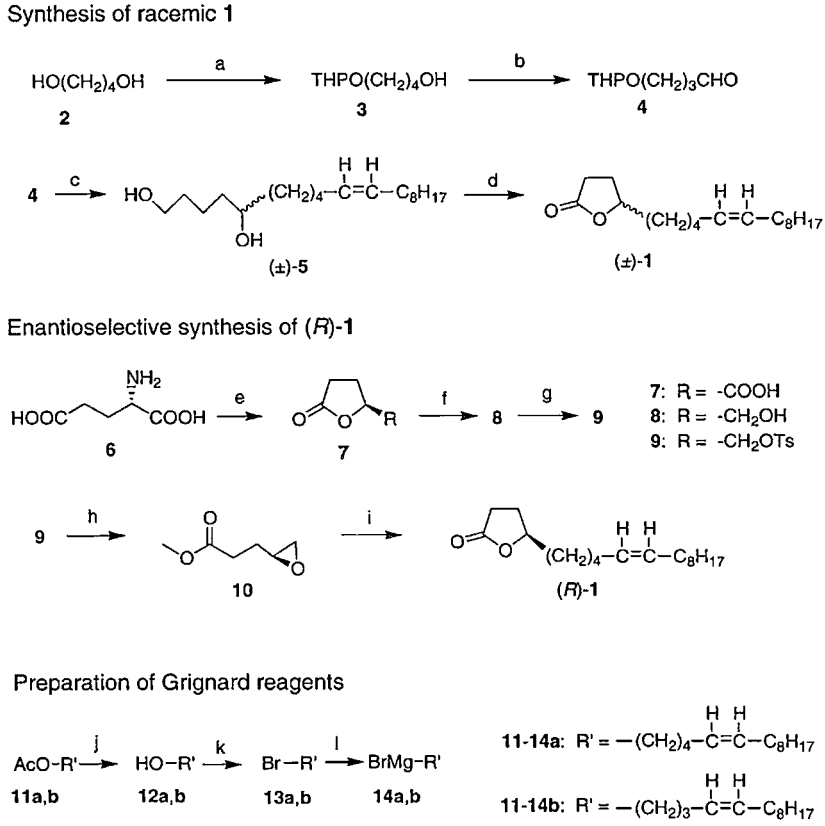

FIG. 1. Syntheses of lactone **1**. Reaction conditions: (a) DHP, CH_2Cl_2, 0.1 N aqueous HCl, rt; (b) PDC, CH_2Cl_2, $MgSO_4$, rt; (c) **14a**, THF, 0°, then NH_4Cl, MeOH, reflux; (d) TPAP, NMO, CH_2Cl_2, rt; (e) HONO; (f) BH_3-Me_2S; (g) TsCl, $Me_2N(CH_2)_3NMe_2$, 0–5°; (h) NaOMe, anhydrous MeOH, rt; (i) **14b**, THF, 0°; (j) KOH in MeOH, rt; (k) PBr_3; (c) Mg, THF, reflux.

The free hydroxyl function of **3** was oxidized with pyridinium dichromate (PDC) in CH_2Cl_2 (Corey and Schmidt, 1979; 3 mmol PDC per 10 ml, 1.5:1 molar ratio of PDC:**3**) and with addition of anhydrous $MgSO_4$ (0.6 g for 10-ml reaction volume) as a water scavenger, to give the aldehyde **4.** After the usual workup (Corey and Schmidt, 1979), the product (in hexane) was dried (Na_2SO_4) and filtered. Rotary evaporation (35°C at ca. 15 Torr) removed the solvent and residual pyridine to afford **4**, which was used without further purification in the next reaction (50% yield from **3**, purity 97% by GC). The mass spectrum indicated the proper molecular weight [*m/z* (%): 171 ($M\text{-}1^+$, 0.07), 85 (100)], and the proton and carbon NMR spectra were consistent with Johns et al. (1989).

Grignard reagent **14a** was obtained from ester **11a**, first by hydrolysis to alcohol **12a** with methanolic KOH (Hijfte et al., 1987), then by conversion to bromide **13a** with PBr_3 (Lin et al., 1987), and treatment of the bromide with Mg metal in refluxing, dry THF (distilled from Na and benzophenone). The intermediate products had appropriate mass spectra [*m*/*z* (%) for **12a**: 212 (M^+, 0.4), 194 (M-H_2O^+, 13), 82 (100); for **13a**: 276 (M^+, 13), 274 (M^+, 13), 55 (100)]. Aldehyde **4** (3.6 mmol) was added dropwise to the stirred Grignard reagent **14a** (3.6 mmol in 20 ml THF) at -20°C. Stirring continued for 1 hr as the reaction mixture warmed slowly to room temperature. The mixture was diluted with hexane (100 ml) and quenched with saturated NH_4Cl solution (40 ml). The aqueous phase was extracted with hexane (2×50 ml), and the combined hexane phases were washed with brine (20 ml), dried (Na_2SO_4), and filtered. The solvent was removed by rotary evaporation, and the product was used directly in the next reaction.

The THP protecting group was removed with NH_4Cl in refluxing methanol (Yadav et al., 1998). After workup, the crude product was purified by column chromatography on silica gel (10 g for 1.0 g of crude diol ***5***). The product was eluted with ethyl acetate (75 ml), after impurities were eluted with hexane (60 ml) and 10% ethyl acetate in hexane (60 ml). Yield was 47% from **13a**, and the final purity was 88% by GC. The following spectra were obtained for **5**: MS *m*/*z* (%) 284 (M^+, 1.4), 266 (16), 223 (4), 207 (5), 180 (4), 166 (12), 135 (18), 121 (34), 87 (91), 82 (100). ^{1}H NMR δ 0.91 (3H, *t*, $J = 6.9$), 1.27–1.30 (16H, m), 1.49 (4H, m), 1.72 (2H, m), 2.07 (4H, m), 3.68 (1H, m), 3.72 (2H, m), 5.36–5.40 (2H, m). ^{13}C NMR δ 14.0, 22.5, 25.2, 27.0, 27.1, 29.0–29.6 (5 carbons), 31.8, 34.2, 37.4, 62.9, 71.7, 129.3, 130.1. IR cm^{-1} 723 (*cis* alkene C—H stretch), 1655 (*cis* alkene C—C stretch), 3320 (broad O—H stretch).

The terminal OH of **5** (1.4 mmol) was selectively oxidized with tetrapropylammonium perruthenate (TPAP, 0.16 mmol) and 4-methylmorpholine-*N*-oxide (4.8 mmol) as co-oxidant (Bloch and Brillet, 1991). The solvent was CH_2Cl_2 (25 ml), and 4Å molecular sieves (800 mg) were added to absorb water; the reaction was stirred for 2 hr at rt. Cyclization occurred during the reaction, so the final product was racemic **1**. The mixture was filtered to remove molecular sieves, and the dark green filtrate was passed through a column of silica gel (10 g). The product remaining on the column was eluted with CH_2Cl_2 (100 ml); **1** elutes before the dark green TPAP. After further purification by HPLC (8% acetone in hexane, isocratic), the yield of **1** was 43% (calculated from **5**), and the purity was >99% by GC. The overall yield of **1** from **11a** was 11%. The following spectra were acquired for **1**: MS *m*/*z* (%) 280 (M^+, 27), 220 (13), 168 (9), 150 (23), 136 (56), 122 (36), 109 (44), 95 (82), 81 (100), 67 (96) (consistent with Cossé et al., 2001). ^{1}H NMR δ 0.91 (3H, *t*, $J = 7.0$), 1.29–1.35 (12 H, m), 1.42 (4H, m), 1.63 (1H, m), 1.78 (1H, m), 1.86 (1H, m), 2.35 (1H, m), 2.56 (2H, br *t*, $J = 7.3$), 4.51 (1H, m), 5.34–5.42 (2H, m). ^{13}C NMR δ 14.0, 22.5, 24.7, 26.8, 27.1, 27.9–29.6 (6 carbons), 31.8, 35.4, 80.8, 129.0, 130.3. IR, neat, 723, 1655, 1778 (γ-lactone carbonyl stretch).

*Enantioselective Synthesis of (R)-**1**.* As shown in Figure 1, (*S*)-(+)-glutamic acid (**6**) was oxidized to the lactone carboxylic acid **7** with $NaNO_2$ and HCl (Doolittle et al., 1980) (79% yield from **6**, purity 95% by GC). The mass spectrum of **7** supported the conversion: MS *m*/*z* (%) 130 (M^+, 1), 129 (M^+-1, 2), 115 (1), 85 (100), 73 (4), 57 (12), 45 (7). Reduction of **7** to alcohol **8** was done with borane-methyl sulfide complex (Cohen et al., 1979) (89% yield from **7**, purity 96% by GC); MS *m*/*z* (%) 116 (M^+, 0.7), 98 (1.4), 85 (100), 57 (11). Tosylation of **8** with TsCl in the presence of $Me_2N(CH_2)_3NMe_2$ at 0–5° (Yoshida et al., 1999) gave ester **9** (56% yield from **8**, purity 97% by GC); MS *m*/*z* (%) 270 (M^+, 3), 206 (19), 155 (11), 107 (8), 99 (9), 85 (100), 91 (31). Tosylate **9** was converted to chiral epoxide **10** with NaOMe in anhydrous MeOH at room temperature (Ho and Davies, 1983) (99% yield from **9**, purity 97% by GC). The following spectra were obtained: MS *m*/*z* (%) 130 (M^+, 0.8), 115 (19), 100 (26), 99 (22), 98 (26), 85 (35), 71 (100). ^{1}H NMR δ 1.76 (1H, m), 1.95 (1H, m), 2.46 (2H, *t*, $J = 7.3$), 2.50 (1H, dd, $J = 2.7, 4.9$), 2.76 (1H, dd, $J = 3.9, 4.9$), 2.97 (1H, m), 3.68 (3H, s). ^{13}C NMR δ 27.7, 30.3, 47.1, 51.3, 51.8, 173.4.

The final reaction was a Grignard cross-coupling between chiral epoxide **10** (0.4 mmol) and alkenyl magnesium bromide **14b** (prepared from 0.32 mmol **13b**). The Grignard reagent was obtained from ester **11b** as described for **14a**. Mass spectra supported the desired conversions: *m*/*z* (%) for **12b**, 198 (M^+, 0.03), 180 (M-18$^+$, 7), 68 (100); for **13b**, 262 (M^+, 9), 260 (M^+, 9), 69 (100). The coupling reaction was run in THF (15 ml), starting at 0°C, warming gradually to rt, and stirring overnight. The mixture was diluted with hexane (50 ml) and shaken with saturated NH_4Cl (10 ml). Cyclization to the lactone occurred during workup. After separation of layers, the aqueous phase was extracted with hexane (2 × 25 ml), dried (Na_2SO_4), filtered, and the solvent was removed by rotary evaporation. The crude product was purified first on an open column of silica gel (1 g, elution with 10% ether in hexane) and then by HPLC, as above. The desired lactone (*R*-**1**) was obtained, but the yield was low (ca. 1%); purity was >99% by GC after HPLC. Mass spectrum was as for racemic **1**.

Instrumentation for Synthesis. Reactions in the syntheses of lactone **1** were monitored by GC. The Hewlett-Packard (HP) 5890 Series II GC was equipped with flame ionization detector and interfaced to an HP ChemStation data system. The oven temperature was programmed from 50 to 250°C at 10°C/min, the cool on-column inlet tracked the oven temperature, and the detector temperature was 250°C. A DB-1 capillary column (15 m × 0.25 mm, 0.25-μm film thickness, J&W Scientific, Folsom, CA) was used.

Electron impact mass spectra (70 eV) were obtained with an HP 5973 MSD instrument, interfaced to an HP 6890 GC equipped with a splitless inlet. Achiral analyses were done on an EC-1 capillary GC column (30 m × 0.25 mm, 0.25-μm film thickness, Alltech, Deerfield, IL). Oven temperature program was as above;

the inlet and transfer line temperatures were 250°C. Chiral analyses were done as before (Cossé et al., 2001) on the GC–MS system. The column was β-DEX 225 (30-m length, 0.25-mm ID, 0.25-μm film thickness; Supelco, Bellefonte, PA) operated at 200°C.

^{1}H-NMR, ^{13}C-NMR, and 2D NMR spectra ($CDCl_3$) were obtained on a Bruker (Bellerica, MA) Avance 400 instrument. (^{1}H and COSY at 400 MHz, ^{13}C and DEPT at 100 MHz). Chemical shifts are referenced to tetramethylsilane; coupling constants (J) are in Hertz. Infrared spectra were obtained on a Nicolet Impact 410 mid-range infrared spectrometer.

HPLC purification of synthetic products was conducted with a Waters 515 pump,Waters R401 differential refractometer detector, and a Dynamax Macro silica HPLC column (Rainin Instrument Co., Woburn, MA).

RESULTS

*Bioactivity of (±)-**1** in the Field.* In 2001, low numbers of male *J. integer* were trapped during 1 May–5 June with a peak around mid-May. On most visits, 0–4 males were recorded from pheromone-baited traps except for one trap on 15 May that had 29 individuals. Only 3 males were found in unbaited traps. No females were trapped. Greatest numbers of males were caught in traps baited with 1 mg of the pheromone, 2.5 ± 1.4 (mean/trap/visit ± SE), which was significantly greater than traps baited with 0.1 mg (0.55 ± 0.16), 0.01 mg (0.50 ± 0.18), or unbaited traps (0.15 ± 0.10) ($F = 2.90$, $df = 3, 76$, $P = 0.04$).

In 2002, very few males were trapped prior to 16 May. Large numbers (maximum of 229 in a single 5-mg trap during a 4-day period) were recorded in traps baited with 3.0, 5.0, and 10.0 mg of pheromone on 16 and 20 May. Insecticide was applied on the latter date and numbers were much smaller thereafter (Figure 2). In the low dose range experiment, traps baited with 3.0 mg of pheromone captured more males 6.75 ± 2.9 (mean/trap/visit ± SE) than 0.3 mg (0.52 ± 0.20), 0.1 mg (0.17 ± 0.08)) or unbaited traps (0.2 ± 0.2) ($F = 4.96$, $df = 4, 195$, $P < 0.001$). Traps baited with 1 mg of pheromone captured a mean of 2.7 ± 1.5 males. In the high dose range experiment, traps baited with 5.0 mg of pheromone caught 26.6 ± 18.1 compared to 41.4 ± 17.3 in 10-mg baited traps and 0.2 ± 0.2 in unbaited traps ($F = 4.62$, $df = 2, 33$, $P = 0.01$). A positive response by male *J. integer* to increasing pheromone dose was evident in these experiments (Figure 3).

Synthetic Lactones. The synthesis of racemic **1** afforded sufficient material for field testing. The present method was more efficient than the one initially used to prove the structure (Cossé et al., 2001), giving an overall yield of 11% from the commercially available ester **11a**. By GC retention and mass spectrum, the lactone products from the previous and present methods were identical. When the (*R*)-**1** from the enantioselective synthesis was compared to racemic **1**

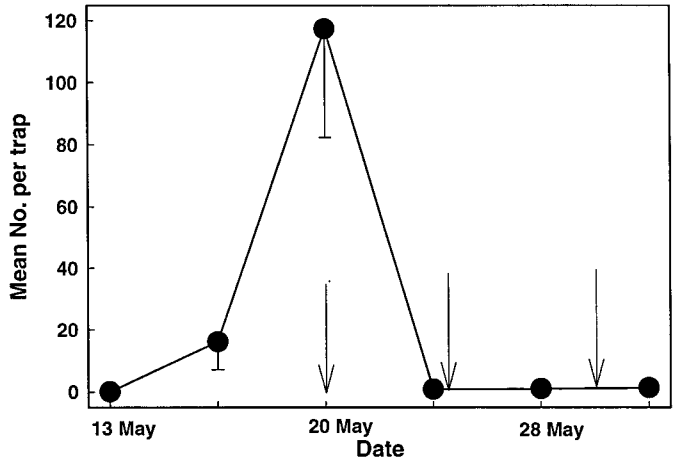

FIG. 2. Mean incidence of male *Janus integer* in high dose (5 and 10 mg) pheromone traps in a Prosser, Washington red currant field during May 2002. Arrows indicate spray applications of fenpropathin.

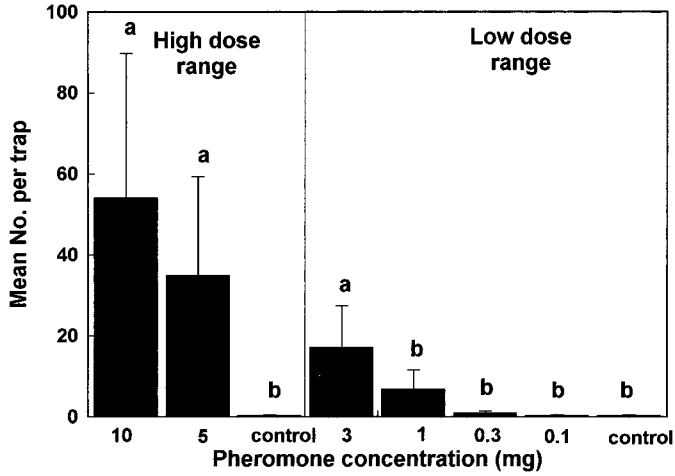

FIG. 3. Mean incidence of male *Janus integer* in sticky traps baited with different concentrations of racemic (*Z*)-9-octadecen-4-olide in a Prosser, Washington red currant field from 3 May to 7 June 2002. Data derived from two separate experiments (low dose range, high dose range). Bars represent standard errors, without taking blocking into account. Columns marked with different letters are significantly different ($P < 0.05$, ANOVA) (within experiments).

on the chiral GC column, the (*R*) enantiomer had the same GC retention time as the earlier of the two peaks from (±)-**1** (which occurred at 64.3 and 65.2 min). Only one enantiomer was detected from the enantiomeric synthesis. In all of these analyses, the proper mass spectrum was observed for the GC peaks. It was already known that the natural lactone from *J. integer* was the earlier eluting enantiomer (Cossé et al., 2001). Therefore, the lactone from the sawfly has the (*R*) configuration. There was not enough synthetic product to allow field testing.

DISCUSSION

Field tests in two seasons confirmed that the putative pheromone of *J. integer* (Cossé et al., 2001) was attractive to males in the field and functions as a sex pheromone. It was further determined that the natural pheromone has the (*R*) configuration. Apparently the (*S*) enantiomer in the racemic synthetic lactone did not prevent attraction. 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). Further field testing will be required to determine whether deployment of the pure (*R*) enantiomer alone would increase field attraction to male sawflies.

Greatest field attraction occurred with the highest doses of the compound tested (5, 10 mg), and it is possible that still higher rates could increase attraction further. The lowest concentration eliciting significant attraction of males appeared to be around 1 mg. Cossé et al. (2001) reported a pheromone release rate of ~10 ng/day for *J. integer* in the laboratory. Release rates of synthetic **1** from rubber septa have yet to be measured.

The trapping experiments were characterized by substantial variability. For example, in 2001, most counts of trapped males were in the range of 0 to 4, apart from a single trap on one occasion that captured 29 individuals. Similarly, in 2002, there was often great variation among numbers trapped in the five replicates of the same concentration. For instance, on 20 May, the numbers for 3.0-mg baited traps were 49, 3, 82, 2, 77. This type of variability in response to pheromones has been noticed with other sawfly species (personal observations) and may be a result of patchy population distribution or subtle microhabitat trap placement effects. There was no evidence in our experiments of individual female *J. integer* being captured in traps, thus enhancing attraction.

In spite of female-produced pheromones having been well studied in several sawfly families (Anderbrant, 1999, and references therein), relatively little is known of the pheromone systems of sawflies in the Cephidae. Only one other cephid pheromone has been studied, that of the wheat stem sawfly, *Cephus*

cinctus Norton (Bartelt et al., 2002; Cossé et al., 2002). In *C. cinctus*, products from the natural oxidation of cuticular lipids and alkenediol diacetates served as an aggregation pheromone, attracting both sexes. In contrast, the currant stem girdler pheromone is clearly a female-produced sex pheromone. With only two cephid pheromone systems studied so far, it is too early to tell whether the diverse nature and action of the identified compounds is characteristic of the pheromones of the 100 known Cephidae species (Anderbrant, 1999).

Availability of a synthetic sex pheromone for *J. integer* may substantially improve chemical management of this cryptic sawfly pest by providing an effective means of monitoring emergence. Currently, gooseberry and currant growers apply insecticides for *J. integer* after visible damage (girdling of shoots) by females has already occurred. Pheromone-baited sticky traps attracting males (which appear to exhibit protandry, emerging prior to females) have the potential to provide an "early warning" to growers, allowing targeted application of an insecticide prior to crop damage. The possibility of using the sex pheromone of *J. integer* for direct control (e.g., as an attracticide or in mating disruption) should also be explored. The behavioral response of males to this pheromone in the field provides some optimism for the potential of these approaches.

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ENANTIOMERS OF (*Z*,*Z*)-6,9-HENEICOSADIEN-11-OL: SEX PHEROMONE COMPONENTS OF *Orgyia detrita*

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Abstract—(6*Z*,9*Z*,11*S*)-6,9-Heneicosadien-11-ol (*Z*6*Z*9-11*S*-ol-C21) and (6*Z*,9*Z*,11*R*)-6,9-heneicosadien-11-ol (*Z*6*Z*9-11*R*-ol-C21) were identified as major sex pheromone components of female tussock moths, *Orgyia detrita* Guérin-Méneville (Lepidoptera: Lymantriidae), on the basis of (1) analyses of pheromone gland extracts of female *O. detrita* by coupled gas chromatographic-electroantennographic detection (GC-EAD) and GC mass spectrometry, and (2) field trapping experiments with synthetic standards. *Z*6*Z*9-11*S*-ol-C21 and *Z*6*Z*9-11*R*-ol-C21 in combination, but not singly, attracted significant numbers of male moths. Racemic *Z*6*Z*9-11-ol-C21 was more attractive than the 1:3.5 (*R*:*S*) blend ratio found in pheromone gland extracts from female moths. Lower and higher homologues of *Z*6*Z*9-11-ol-C21 were also detected in GC-EAD recordings of pheromone extracts, and the racemic compounds enhanced attractiveness of *Z*6*Z*9-11-ol-C21 in field experiments. Because of trace amounts of these homologues in extracts, their enantiomeric composition could not be determined. This is the first report of secondary alcohols as pheromone components in the ditrysian (advanced) Lepidoptera.

Key Words—Ditrysian Lepidoptera, Lymantriidae, *Orgyia detrita*, sex pheromone, (6*Z*,9*Z*,11*S*)-6,9-heneicosadien-11-ol, (6*Z*,9*Z*,11*R*)-6,9-heneicosadien-11-ol, (6*Z*,9*Z*)-6,9-eicosadien-11-ol, (6*Z*,9*Z*)-6,9-docosadien-11-ol.

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[4] Dedicated to my Aunt Martha Dittmar in recognition of her contribution to my upbringing.

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INTRODUCTION

The tussock moth, *Orgyia detrita* Guérin-Méneville (Lepidoptera: Lymantriidae), occurs in the southeastern United States, from New York south along the coast to Florida and west into Texas (Ferguson, 1978). This coastal distribution matches that of live oak, *Quercus virginiana* Mill., (Little, 1971), which together with bald cypress, *Taxodium distichum* (L.) Rich., serve as main food plants for *O. detrita* larvae (Ferguson, 1978). During an outbreak in coastal North Carolina, larvae also fed on sweetbay, *Magnolia virginiana* L., and cultivated azalea, *Rhododendron* spp., and a laboratory generation was successfully reared on red maple, *Acer rubrum* L. (Drooz et al., 1986).

Male *O. detrita* have never been captured in traps baited with synthetic pheromone components of congeners, suggesting that *O. detrita* employ a different pheromone blend. Moderate population densities of *O. detrita* around Gainesville (Florida) in the last several years provided the opportunity to collect egg masses for laboratory rearing of this tussock moth, and for studying its sex pheromone. We report identification and field testing of sex pheromone components of *O. detrita*.

METHODS AND MATERIALS

Experimental Insects and Pheromone Extraction. O. detrita eggs were collected in Gainesville, Florida, in January 1999, and sent to the USDA Beneficial Insect Introduction Research quarantine facilities, Newark, Delaware. There, hatching larvae were reared on gypsy moth diet (Bell et al., 1981) at 25°C, 60–80% RH, and a 14L:10D photoperiod. Pupae were extricated from their cocoons, and isolated by sex (Winter, 2000). Abdominal tips with pheromone glands of calling, 1–2-day-old females were removed and extracted for 15–30 min in high-performance liquid chromatography (HPLC) grade hexane. Ampoules with the supernatant of pheromone extracts and male pupae were sent by courier to Simon Fraser University.

Analyses of Pheromone Extracts. Aliquots of one female equivalent (FE) of pheromone gland extract, or synthetic standards, were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975) with a Hewlett-Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm i.d.) coated with either DB-5, DB-23, DB-210, or Cyclodex-B (J&W Scientific, Folsom, California, USA). The most antennal stimulatory and abundant candidate pheromone component was isolated by HPLC with a Waters LC 626 high-performance liquid chromatograph equipped with a Waters 486 UV-Visible detector set to 210 nm, and a reverse phase Nova-Pak C-18 column (300 × 3.9 mm i.d.) eluted with acetonitrile (1 ml/min). The (*R*)- and (*S*)-enantiomers of insect-produced (6*Z*,9*Z*)-6,9-heneicosadien-11-ol

(*Z*6*Z*9-11-ol-C21) were separated by GC on a custom-made chiral fused silica column coated with a 1:1 mixture of heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin and OV-1701 (König et al., 1992; Pietruszka et al., 1992).

For field experiments, the (*R*)- and (*S*)-enantiomers of synthetic racemic *Z*6*Z*9-11-ol-C21 were separated by HPLC (50–100 μg per injection) on a Chiralpak AD column (250 $\times$ 4.6 mm i.d.; Chiral Technologies Inc., Exton, Pennsylvania, USA) eluting with 0.9 ml/min of hexane: 2-propanol (99.9:0.1). Enantiomers eluted several minutes apart, resulting in >99% enantiomeric excess (ee) and >98% chemical purity of field tested chemicals. Mass spectra of synthetic or insect-produced compounds were obtained in full-scan electron impact ionization mode from a Varian Saturn II Ion Trap GC-MS (70 eV) fitted with a DB-5 column.

Synthesis of Racemic and Enantiomerically Pure 1-Tridecyn-3-ols and Corresponding Acetates. (Figure 1). Racemic 1-tridecyn-3-ol was obtained by stirring 10 g of lithium acetylide–ethylenediamine complex (**2**, 90% pure, 97.8 mmol) (Aldrich Chem. Co.) with freshly prepared undecanal (**1**, 8.36 g, 48.9 mmol) in DMSO-THF (1:1) at 65°C overnight under argon. Silica gel flash chromatography of the worked-up reaction mixture afforded alcohol **3** (3.22 g, 33.6% yield). Alcohol **3** (2.94 g, 15.0 mmol) was stirred with 2.12 ml (23.0 mmol) of vinyl acetate in 10 ml of hexane with 0.5 g of immobilized lipase Novozym 435 (Sigma Chemical Co.) (Xiao and Kitazume, 1997) at 40°C for 4 hr. Novozym-containing resin and solvents were removed by filtration and evaporation *in vacuo*, respectively. Separation of the reaction mixture by flash chromatography (25 g of SiO_2; 10% ether in hexane as eluent) gave alcohol **4** (1.26 g) and acetate **5** (1.97 g). Alcohol **4** (10 mg) was acetylated to **6** overnight at room temperature (rt) with acetic anhydride and pyridine. GC analyses of **6** and **5** on the Cyclodex-B column (150°C isothermal) determined that their ee's were 99.9 and 98.4%, respectively.

The absolute configurations of **4** and **5** were confirmed through an alternative synthesis, as follows: Sharpless asymmetric epoxidation of (*E*)-2-tridecenol (**7**) with L-(+)-diisopropyl tartrate (0.15 equivalent), titanium (IV) isopropoxide (0.15 equivalent), and *t*-butyl hydroperoxide (2.2 equivalent) in dichloromethane at -25°C for 3 days afforded the epoxy-alcohol **8**. Replacement of the hydroxy group of **8** with chlorine by refluxing **8** for 48 hr with an equivalent amount of triphenylphosphine and catalytic amounts of sodium bicarbonate in carbon tetrachloride, and treatment of the resulting epoxy-chloride **9** with a three-fold excess of lithium diisopropylamide (LDA) (Takano et al., 1989; Rodriguez and Spur, 2001, and references cited therein) gave the alcohol **10**. Acetylation of **10** with acetic anhydride and pyridine overnight produced (*S*)-1-tridecyn-3-yl acetate (**5**, overall yield 16%).

*Synthesis of (6Z, 9Z, 11R)-6,9-Heneicosadien-11-ol [(**R**)-**16**]* (Figure 1). (*R*)-1-Tridecyn-3-ol (**4**, 1.25 g, 6.42 mmol) was protected by overnight reaction with

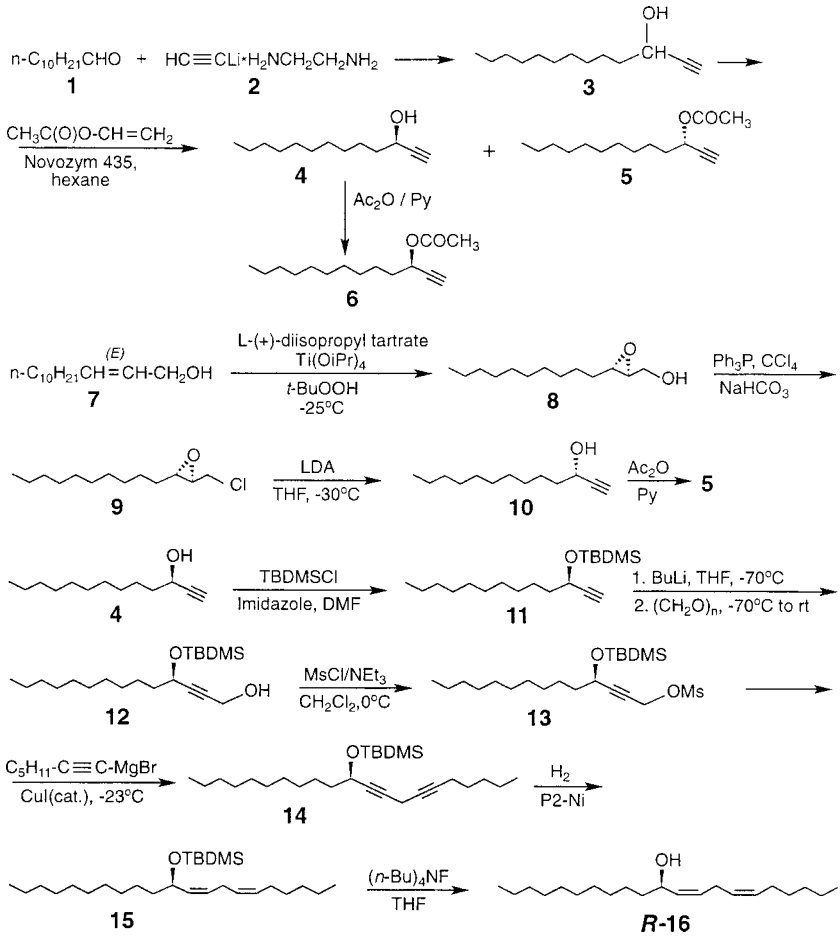

FIG. 1. Syntheses of (*R*)- and (*S*)-1-tridecyn-3-yl acetates and (6*Z*,9*Z*,11*R*)-6,9-heneicosadien-11-ol.

t-butyldimethylsilyl chloride (1.05 g, 7.00 mmol) and imidazole (7.00 mmol) in DMF at rt to afford after work-up ether **11** (1.79 g, 90% yield). Ether **11** (1.79 g, 5.77 mmol) in THF was extended by one carbon by treating it consecutively with butyllithium at −70°C and paraformaldehyde, warming the reaction mixture to rt, and stirring for 24 hr to form after work-up the mono-protected diol **12** (1.65 g, 84% yield). Mesylation of **12** (1.65 g, 4.85 mmol) with an equivalent amount of methanesulfonyl chloride and 1.5 excess of triethylamine in dichloromethane (0°C) for 30 min gave (*R*)-4-(*tert*-butyldimethylsilyloxy)-2-tetradecyn-1-yl

methanesulfonate (**13**). Immediate reaction of **13** in THF at −23°C with a two-fold excess of freshly prepared heptynylmagnesium bromide (from 1-heptyne and EtMgBr; 2 hr reflux in THF) with CuI catalyst (180 mg) gave 1.67 g (4.00 mmol, 82% yield) of the *tert*-butyldimethylsilyl ether of (11*R*)-6,9-heneicosadiyn-11-ol (**14**). Hydrogenation of **14** (0.89 g, 2.00 mmol) with P2-nickel catalyst in ethanol (Brown and Ahuja, 1973), and removal of the silyl protecting group by treatment with excess tetrabutylammonium fluoride in THF (rt, 12 hr) gave (6*Z*,9*Z*,11*R*)-6,9-heneicosadien-11-ol [**(*R*)-16**] [0.495 g, 1.61 mmol, 81% yield; >95% *ee* (conservative estimate considering that enantiomers were not completely baseline resolved)]. The overall yield of **(*R*)-16** (based on **4**) was 50%. The optical rotation of **(*R*)-16** was $[\alpha]_D^{22}+1.9^\circ$ (c 6.8, $CHCl_3$), and NMR data and retention characteristics were consistent with those reported for racemic (*Z*,*Z*)-6,9-heneicosadien-11-ol (Gries et al., 1997). The retention time of **(*R*)-16** on the custom-made chiral column (König et al., 1992; Pietruszka et al., 1992) was identical to that of the first eluting enantiomer of racemic (*Z*,*Z*)-6,9-heneicosadien-11-ol.

Field Experiments. Field experiments were conducted in a wooded area of Gainesville, Florida, latitude 29.688°N, longitude 82.388°W. Host trees in the area included water oak, *Quercus nigra* L., laurel oak, *Q. laurifolia* Michx., live oak, *Q. virginiana* Mill., and Shumard oak, *Q. shumardii* Buckl. Delta-type traps made from 2-l milk cartons (Gray et al., 1984) were coated with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan, USA) and suspended from trees at a height of 2 m and spacings of 20–25 m in complete randomized blocks. Traps were baited with folded filter papers (Whatman # 1; Whatman International Ltd, Maidstone, England) or gray rubber septa (The West Company, Lionville, Pennsylvania, USA) impregnated with candidate pheromone components just prior to the field experiment.

Treatments for all experiments are listed in Table 1. Experiment 1 tested attractiveness of *Z*6*Z*9-11-ol-C21 versus that of unbaited controls. Experiment 2 tested the effect of dispenser types (rubber septum or filter paper) on the attractiveness of *Z*6*Z*9-11-ol-C21. Experiments 3 and 4 tested whether additional candidate pheromone components, including (*Z*,*Z*)-6,9-eicosadien-11-ol (*Z*6*Z*9-11-ol-C20), (*Z*,*Z*)-6,9-docosadien-11-ol (*Z*6*Z*9-11-ol-C22), and (*Z*)-6-heneicosen-11-one (*Z*6-11-one-C21), enhanced attractiveness of *Z*6*Z*9-11-ol-C21. Experiment 5 tested *Z*6*Z*9-11*S*-ol-C21 and *Z*6*Z*9-11*R*-ol-C21 (isolated from the racemate by chiral HPLC) singly and in combination. Experiment 6 tested the effect of ratio between *Z*6*Z*9-11*R*-ol-C21 and *Z*6*Z*9-11*S*-ol-C21 on blend attractiveness, and Experiment 7 compared the most attractive blend ratio (1:1) from Experiment 6 versus the blend ratio (1:3.5) found in pheromone gland extracts from female *O. detrita*.

Trap catch data were analyzed by nonparametric analyses of variance (Friedman's test) followed by comparison of means by Bonferroni (Dunn) *t* test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

TABLE 1. NUMBER OF ($\bar{x} + SE$) OF MALE *Orgyia detrita* CAPTURED IN EXPERIMENTS 1–8 IN TRAPS BAITED WITH CANDIDATE PHEROMONE COMPONENTS ALL EXPERIMENTS CONDUCTED NEAR GAINESVILLE, FLORIDA, USA

Exp. no.	Treatment[a]	Number ($\bar{x} + SE$) of males captured[b]	*N*	Dispenser type	Time period
1	Unbaited control	0 a	10	Filter paper	18–20 April 2000
	*Z*6*Z*9-11-ol-C21 (50)	3.3 + 0.45 b	10	Filter paper	
2	Unbaited control	0.2 + 0.2 c	5	Rubber septum	10–11 May 2000
	Unbaited control	0.2 + 0.5 c	5	Filter paper	
	*Z*6*Z*9-11-ol-C21 (25)	0.8 + 0.4 b	5	Rubber septum	
	*Z*6*Z*9-11-ol-C21 (25)	9.8 + 1.8 a	5	Filter paper	
3	Unbaited control	0.1 + 0.1 d	8	Filter paper	2–3 May 2000
	*Z*6*Z*9-11-ol-C21 (50)	7.6 + 1.9 c	8	Filter paper	
	*Z*6*Z*9-11-ol-C21 (50) + *Z*6*Z*9-11-ol-C20 (5)	12.4 + 3.3 c	8	Filter paper	
	*Z*6*Z*9-11-ol-C21 (50) + *Z*6*Z*9-11-ol-C22 (5)	15.6 + 2.2 b	8	Filter paper	
	*Z*6*Z*9-11-ol-C21 (50) + *Z*6*Z*9-11-ol-C20 (5) + *Z*6*Z*9-11-ol-C22 (5)	20.3 + 2.1 a	8	Filter paper	
4	Unbaited control	0 b	8	Filter paper	3–4 May 2000
	*Z*6*Z*9-11-ol-C21 (50)	19.3 + 6.8 a	8	Filter paper	
	*Z*6*Z*9-11-ol-C21 (50) + *Z*6-11-one-C21 (5)	19.6 + 2.8 a	8	Filter paper	
5	Unbaited control	2.8 + 0.9 b	5	Filter paper	29 April–1 May 2000
	*Z*6*Z*9-11*S*-ol-C21 (10)	3.6 + 1.3 b	5	Filter paper	
	*Z*6*Z*9-11*R*-ol-C21 (10)	3.2 + 0.8 b	5	Filter paper	
	Racemic *Z*6*Z*9-11-ol-C21 (20)	11.4 + 2.0 a	5	Filter paper	
6	Unbaited control	0 b	5	Filter paper	
	*Z*6*Z*9-11*S*-ol-C21 (25)	0.2 + 0.2 b	5	Filter paper	13–14 May 2000
	*Z*6*Z*9-11*R*-ol-C21 (5) + *Z*6*Z*9-11*S*-ol-C21 (25)	0 b	5	Filter paper	
	*Z*6*Z*9-11*R*-ol-C21 (25) + *Z*6*Z*9-11*S*-ol-C21 (25)	3.0 + 0.7 a	5	Filter paper	
7	*Z*6*Z*9-11*R*-ol-C21 (8.5) + *Z*6*Z*9-11*S*-ol-C21 (30)	4.2 + 0.6 b	10	Filter paper	27–28 April 2002
	*Z*6*Z*9-11*R*-ol-C21 (30) + *Z*6*Z*9-11*S*-ol-C21 (30)	10.3 + 1.5 a	10	Filter paper	

[a] Numbers in brackets refer to micrograms; all control treatments received the equivalent amount of solvent.
[b] In each experiment, numbers followed by different letters are significantly different; $P < 0.05$.

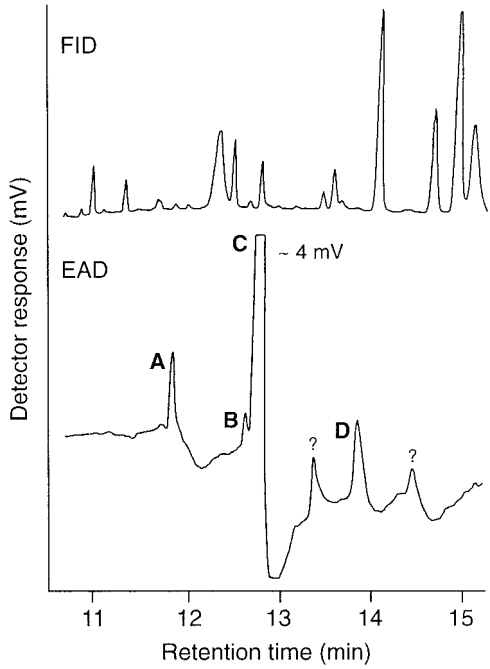

FIG. 2. Flame-ionization detector (FID) and electroantennographic detector (EAD: male *Orgyia detrita* antenna) responses to one female equivalent (FE) of pheromone extract from female *O. detrita*. Chromatography: DB-23 column; splitless injection; injector and FID detector: 240°C; temperature program; 100°C (1 min), then 10°C/min to 200°C (5 min). **A** = (*Z*,*Z*)-6,9-eicosadien-11-ol; **B** = (*Z*)-6-heneicosen-11-one; **C** = (*Z*,*Z*)-6,9-heneicosadien-11-ol; **D** = (*Z*,*Z*)-6,9-docosadien-11-ol; and **?** = unknown.

RESULTS

GC-EAD analyses of pheromone gland extracts from female *O. detrita* revealed four compounds (**A–D** in Figure 2) that consistently elicited responses from male antennae. Retention indices (RI) (van den Dool and Kratz, 1963) of the most antennal stimulatory and abundant component **C** [RI (versus alkane standards): 2240 (DB-5); RI: 2432 (DB-210); RI: 2727 (DB-23)] were consistent with those of previously synthesized *Z*6*Z*9-11-ol-C21 (Gries et al., 1997). The diagnostic fragment ion *m*/*z*171 in identical mass spectra [*m*/*z* (relative intensity): 41 (36), 55 (59), 69 (54), 83 (59), 97 (100), 111 (35), 152 (2), 171 (11), 312 (1)] of authentic heneicosan-11-ol and of HPLC-isolated and hydrogenated **C** confirmed that the hydroxy-group was on C11. With no mass spectra obtainable for components **A**, **B**, and **D**, their structural assignments were based on retention

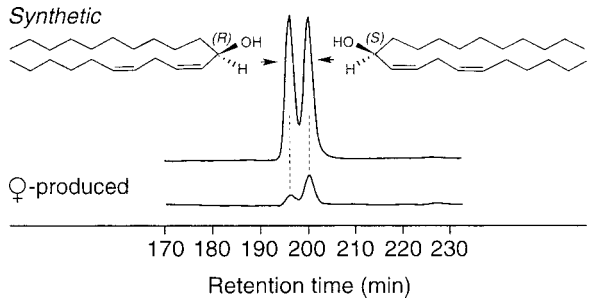

FIG. 3. *Top*: Chromatogram of synthetic (*Z*,*Z*)-6,9-heneicosadien-11-ol with (almost) baseline resolution of the enantiomers of (*Z*,*Z*)-6,9-heneicosadien-11-ol. *Bottom*: Chromatogram of insect-produced (*Z*,*Z*)-6,9-heneicosadien-11-ol (123 female equivalents). Chromatography: custom-made chiral column; splitless injection; injection port and FID: 240°C; temperature program: 150°C isothermal. Note: (*R*)- and (*S*)-enantiomers of (*Z*,*Z*)-6,9-heneicosadien-11-ol occurred in a 1:3.5 ratio in pheromone gland extracts from female *O. detrita*.

indices. For **A** and **D**, the retention indices on all three columns were 100 units lower and higher, respectively, than those of *Z*6*Z*9-11-ol-C21, suggesting that **A** and **D** were lower and higher homologues of *Z*6*Z*9-11-ol-C21. Retention indices of compound **B** were consistent with those of (*Z*)-6-heneicosen-11-one (*Z*6-11-one-C21), a pheromone component of *Orgyia pseudotsugata* (Smith et al., 1975). Comparative analyses of pheromone extract and authentic standards by GC-EAD for components **A–D**, and by GC-MS for component **C**, confirmed these structural assignments.

Chromatography of HPLC-isolated component **C** on a chiral stationary phase column revealed that female *O. detrita* produced *Z*6*Z*9-11*R*-ol-C21 and *Z*6*Z*9-11*S*-ol-C21 in a 1:3.5 ratio (Figure 3). Both the lower and higher homologues of **C** (compounds **A** and **D**) occurred in quantities too low to allow their isolation by HPLC and determination of their enantiomeric composition.

In field experiment 1 (Table 1), traps baited with racemic Z6*Z*9-11-ol-C21 captured significant numbers of male *O. detrita*. For unknown reasons, filter paper was a more suitable dispenser than rubber septa for release of *Z*6*Z*9-11-ol-C21 (when tested at a 25 μg dose) (Table 1; experiment 2). Lower and higher homologues of racemic *Z*6*Z*9-11-ol-C21 enhanced attractiveness of *Z*6*Z*9-11-ol-C21 (Table 1; experiment 3), whereas *Z*6-11-one-C21 had no effect (Table 1; experiment 4). Racemic *Z*6*Z*9-11-ol-C21, but neither enantiomer alone, attracted significant numbers of males (Table 1; experiment 5). Surprisingly, racemic *Z*6*Z*9-11-ol-C21 was also more attractive than other blend ratios (Table 1; experiments 6, 7), including the 1:3.5 (*R*:*S*) ratio found in pheromone gland extracts from female *O. detrita* (Figure 3).

DISCUSSION

(6*Z*,9*Z*,11*S*)-6,9-Heneicosadienol (*Z*6*Z*9-11*S*-ol-C21) and (6*Z*,9*Z*,11*R*)-6,9-heneicosadien-11-ol (*Z*6*Z*9-11*R*-ol-C21) are major sex pheromone components of female *O. detrita*. This conclusion is based on the following evidence: (1) *Z*6*Z*9-11*S*-ol-C21 plus *Z*6*Z*9-11*R*-ol-C21 were the most abundant and antennal stimulatory compounds in GC-EAD recordings from pheromone gland extracts (Figure 2); (2) identifications were based on comparative GC, GC-MS, and GC-EAD analyses of insect-produced and authentic standards; and (3) blends of the synthetic compounds attracted significant numbers of males in field experiments (Table 1).

Lower and higher homologues of racemic *Z*6*Z*9-11-ol-C21 seem to be part of the pheromone blend. They were detected in GC-EAD analyses of pheromone extract (Figure 2), and enhanced attractiveness of *Z*6*Z*9-11-ol-C21 in field experiments (Table 1). Whether their enantiomeric composition is equivalent to that of *Z*6*Z*9-11-ol-C21, and whether they were tested at the most appropriate blend ratio could not be determined because of their trace occurrence in pheromone gland extracts. Two as yet unknown compounds also elicited responses from male antennae (Figure 2). Although they may be part of the pheromone blend, their presence does not appear to be essential for significant attraction of male moths (Table 1).

Synergism between pheromonal enantiomers has been reported only rarely in the Lepidoptera. A racemate of *cis*-3,4-epoxy-(*Z*,*Z*)-6,9-heptadecadiene attracted three times more males of the geometrid moth *Epelis trunctaria* than did either enantiomer alone (Millar et al., 1990). Similarly, a 1:1 and more strongly 1:4 ratio of (6*R*,7*S*)- and (6S,7R)-*cis*-6,7-epoxy-(*Z*,*Z*)-3,9-nonadecadiene attracted males of the noctuid moth *Bleptina caradrinalis* (Millar et al., 1991). Finally, attraction of male pink gypsy moths, *Lymantria mathura*, in Japan not only required the presence but also the same ratio (1:4) of (9*S*,10*R*)- and (9*R*,10*S*)-*cis*-9,10-epoxy-(*Z*,*Z*)-3,6-nonadecadiene as produced by conspecific females (Gries et al., 1999b). Conversely, male *O. detrita* appeared to be more strongly attracted to the unnatural 1:1 ratio of *Z*6*Z*9-11*R*-ol-C21 and *Z*6*Z*9-11*S*-ol-C21 than to the 1:3.5 blend ratio found in pheromone gland extracts of conspecific females (Figure 3). Such an unexpected result was also obtained in pheromone studies of the Japanese giant looper, *Ascotis selenaria cretacea* (Ando et al., 1997), in which females produced nearly the racemate of (*Z*,*Z*)-6,9-*cis*-3,4-epoxy-nonadecadiene, whereas males responded most strongly to the pure (3*R*,4*S*)-enantiomer in a field experiment.

Secondary alcohol pheromone components, as produced by female *O. detrita*, contrast with the ketone pheromone components produced by most congeners. These ketones include (*Z*)-6-heneicosen-11-one (Smith et al., 1975), (*Z*,*E*)-6,8-heneicosadien-11-one (Gries et al., 1997), (*Z*)-6-heneicosen-9-one (Gries et al., 1999a), and (*Z*,*Z*)-6,9-heneicosadien-11-one (Liu, 1999). Production of *trans*-11,12-epoxy-(*Z*,*Z*)-6,9-heneicosadiene by female *O. postica* (Chow et al.,

2001; Wakamura et al., 2001) as a pheromone component seems to suggest a greater diversity of pheromone components in *Orgyia* spp. than previously realized.

Secondary alcohols as pheromone components are reported here for the first time in a species of the ditrysian (advanced) Lepidoptera. Secondary alcohols have previously been identified as pheromone components in several species of the nonditrysian Lepidoptera, including *Stigmella malella* [(*S*)-(*E*)-6,8-nonadien-2-ol and (*S*)-(*Z*)-6,8-nonadien-2-ol (Tóth et al., 1995)], *Eriocrania cicatricella* [(2*R*)-heptan-2-ol (Zhu et al., 1995)], *E. sangii* [(2*S*,6*Z*)-nonen-2-ol (Kozlov et al., 1996)], and *E. semipurpurella* [(2*S*,6*Z*)-nonen-2-ol and (2*R*,6*Z*)-nonen-2-ol (Kozlov et al., 1996)]. The short-chain pheromones of these primitive moth species, however, resemble more closely those of caddisflies than those of ditrysian Lepidoptera (Löfstedt et al., 1994; Zhu et al., 1995) such as *O. detrita.*

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IDENTIFICATION OF A SEX PHEROMONE COMPONENT OF *Pseudococcus cryptus*[4]

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Abstract—A sex pheromone component of *Pseudococcus cryptus* has been isolated and identified. The crude pheromone extract obtained by airborne collection was fractionated by liquid chromatography (LC) on Florisil, and further purified by high performance liquid chromatography and preparative Gas Chromatography (GC). The pheromone component was shown to be an ester, the alcohol part of which was identical to the known alcohol moiety of the pheromone of *Planococcus citri*. The chemical structure was determined to be 3-isopropenyl-2,2-dimethylcyclobutylmethyl 3-methyl-3-butenoate by MS and ^{1}H NMR analyses. The absolute configuration of the pheromone was assigned as (1*R*,3*R*) by comparison of the retention time of the alcohol derived from the *P. cryptus* pheromone with those of the alcohol derived from *P. citri* pheromone, and a synthetic sample of alcohol enriched in the (1*R*,3*R*)-enantiomer, using a chiral GC stationary phase. The structure of the pheromone was confirmed by synthesis, and by bioassays in a glasshouse.

Key Words—Identification, sex pheromone, *Pseudococcus cryptus*, (1*R*,3*R*)-3-isopropenyl-2,2-dimethylcyclobutylmethyl 3-methyl-3-butenoate.

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INTRODUCTION

Pseudococcus cryptus Hempel (Homoptera: Pseudococcidae) is one of the major mealybugs infesting citrus in Japan. It often occurs in outbreaks and causes serious problems to high-value citrus cultivated in greenhouses. In cultivating citrus in greenhouses, it is necessary to spray insecticides to control the pest. The developmental rate of this mealybug depends on temperature, and with good monitoring of infestation levels it is possible to predict the emergence of the first and second instar nymphs of the mealybug that are most susceptible to insecticides (Arai, 1996). However, the mealybugs tend to inhabit the shady parts of the tree, where it is difficult to monitor them by visual inspection, particularly when densities are low. In addition, it is difficult to discriminate the developmental stages of *P. cryptus* because the change of morphological characteristics during the immature stages is small. Therefore, control of this pest is often conducted only after its damage becomes noticeable. The emergence period of the first instar nymphs could be predicted from adult male flight phenology in combination with seasonal degree-day accumulations of 302 DD above a base of 10°C (Arai, 2002). *Pseudococcus cryptus* was verified to use sex pheromones like other mealybugs (Arai, 2000), and it was anticipated that the sex pheromone of *P. cryptus* would be useful in developing a more efficient and exact monitoring system for the mealybug, analogous to that for California red scale, *Aonidiella aurantii* (Gardner et al., 1983). This paper reports the isolation and identification of the sex pheromone of *P. cryptus*, and the attractiveness of the synthesized sex pheromone to adult males.

METHODS AND MATERIALS

Insects. P. cryptus and *P. Planococcus citri* used in the experiments were collected in glasshouses of the Department of Citrus Research, Okitsu, National Institute of Fruit Tree Science in Shizuoka Prefecture, and were reared at 20–25°C under a photoperiod of 16L:8D.

Chemicals. Chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise mentioned.

Collection of Volatiles. Virgin females and adult males of *P. cryptus* and *P. citri* were prepared by the method of Arai (2000). Volatiles of a total of 600,000 female day equivalents (hereinafter FDE) of the sex pheromone of *P. cryptus* and 50,000 FDE of *P. citri* were collected by the previously described mass collection method (Arai, 2000). Mealybug volatiles trapped on Tenax GC (30/60 mesh, GL Science, Tokyo, Japan) were eluted with pentane, and extracts were concentrated with a rotary evaporator at room temperature, and kept at 5°C in a refrigerator until use.

Liquid Chromatography (LC). Column chromatography was carried out on Florisil (30 g, containing 7% water, 100/200 mesh) by the class-separating method

for lipids (Carroll, 1961). The column was successively eluted with 50 ml hexane, 120 ml 5% ether in hexane, and 150 ml each of 15, 25, and 50% ether in hexane. High performance liquid chromatography (HPLC) was carried out with a Hewlett-Packard (HP) series 1050 instrument (Hewlett-Packard, Avondale, PA, USA) using a silica gel column (Inertsil SIL, 5 μm, 4.6 mm i.d. $\times$ 250 mm, GL Science Inc., Tokyo, Japan), eluting with 3% ether in hexane at a flow rate of 1 ml/min. The eluate was monitored with a UV detector (HP series 1050) at 220 nm.

Gas Chromatography (GC). An HP 5890 Series II GC equipped with a FFAP column (0.56 mm i.d. $\times$ 15 m $\times$ 1 μm film, GL Science Inc., Tokyo, Japan) was used, with a temperature program of 50°C for 1 min, increased to 220°C at 5°C/min for preparative GC, with He carrier gas (5 ml/min) and thermal conductivity detection. Fractions were collected in dry ice cooled glass tubes (2 mm i.d. $\times$ 25 cm).

Gas Chromatography–Mass Spectrometry (GC–MS). A JEOL SX-102A GC–MS was used (Tokyo, Japan), with GC interface and ion source temperatures of 210 and 220°C, respectively. A capillary column (FFAP, 0.25 mm i.d. $\times$ 30 m $\times$ 0.25 μm film, GL Science Inc., Tokyo, Japan) was used with a temperature program of 80°C for 1 min, increased to 210°C at 7°C/min, with He carrier gas (1 m/min). A capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film, Betadex 120, Supelco, Bellefonte, USA) was used to separate enantiomers with a temperature program of 50°C for 1 min, increased to 180°C at 5°C/min. TC-1 and FFAP columns (0.25 mm i.d. $\times$ 30 m $\times$ 0.25 μm film, GL Science Inc., Tokyo, Japan) were used with a temperature program of 50°C for 1 min, increased to 220°C at 5°C /min. In the case of these three columns, samples were injected in splitless mode with He carrier gas (1 ml/min). Mass spectra in electron impact (EI) mode and high resolution mass spectra (HR-MS) were obtained at 70 eV. Isobutane was used as a reagent gas in the chemical ionization (CI) mode.

Nuclear Magnetic Resonance (NMR) Analyses. ^{1}H NMR spectra were obtained with a JEOL A600 NMR (^{1}H NMR, 600 MHz) or a Varian UNITY INOVA 500 NMR (^{1}H NMR, 500 MHz) in $CDCl_3$. Protons were assigned by COSY and HOHAHA spectra.

Hydrogenation. About 0.1 ml each of 3% hydrazine in EtOH and 0.1% hydrogen peroxide in EtOH were added to a sample. After incubation overnight at 60°C, the mixture was cooled with ice and quenched with 0.1 ml of 1 M HCl. The mixture was extracted three times with 0.1 ml ether, and the combined ether extracts were washed twice with 0.1 ml of water (Yamaoka et al., 1976).

Hydrolysis and Acetylation. Twenty thousand FDE of the isolated active component of *P. cryptus* were hydrolyzed and then acetylated to analyze the structure of the alcohol fragment of the active compound. The active component in hexane was concentrated under a stream of N_2, and then treated with 2 ml of 0.5 M potassium hydroxide in MeOH overnight at room temperature. The mixture was cooled with ice, then 2 ml of distilled water was added, and the solution was extracted

three times with 4 ml pentane. The pentane extract was dried over anhydrous sodium sulfate. The extract was concentrated with a rotary evaporator to about 2 ml followed by concentration under a stream of N_2. The residue was treated with 3 ml each of acetic anhydride and pyridine overnight at room temperature, then 50 ml of distilled ice water were added, and the solution was extracted three times with 50 ml cold pentane. The pentane extracts were washed with 50 ml of 5% HCl, then three times with 50 ml of distilled water, and the extract was dried over anhydrous sodium sulfate. One thousand FDE of the crude extract of *P. citri* were also hydrolyzed and acetylated by the same procedure.

Synthesis of (1R,3R)-3-Isopropenyl-2,2-Dimethylcyclobutylmethyl 3-Methyl-3-Butenoate. Reagents were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). To a stirred solution of (1*R*,3*R*)-3-isopropenyl-2,2-dimethylcyclobutane-methanol (1.20 g, 7.78 mmol) (Itagaki and Kuwahara, 2002) and 3-methyl-3-butenoic acid (1.17 g, 11.2 mmol) in dichloromethane (70 ml) were added 1,3-dicyclohexyl carbodiimide (2.89 g, 14.0 mmol) and 4-(dimethylamino)pyridine (0.095 g, 0.78 mmol) at room temperature. After 3 hr, the mixture was diluted with ether, and the ethereal solution was washed successively with 0.5 M aqueous HCl, saturated aqueous $NaHCO_3$, and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed over silica gel (50 g, eluting with pentane-ether, 30:1) to afford 1.67 g (91%) of the ester. $[\alpha]_D^{21} + 18.9^\circ$ ($c = 1.17$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): 0.81 (3H, s, 2-CH_3), 1.20 (3H, s, 2-CH_3), 1.61 (1H, q, $J = 10.7$ Hz, 4-H), 1.66 (3H, s, 3-CCH_3), 1.81 (3H, s, 3′-CH_3), 1.90 (1H, dt, $J = 10.7$, 7.6 Hz, 4-H), 2.19 (1H, dddd, $J = 10.7$, 9.0, 7.6, 6.2 Hz, 1-H), 2.39 (1H, dd, $J = 10.7$, 7.6 Hz, 3-H), 3.01 (2H, s, 2′-H_2), 3.96 (1H, dd, $J =$ 11.2, 9.0 Hz, 1-CH), 4.08 (1H, dd, $J = 11.2$, 6.2 Hz, 1-CH); 4.57 (1H, s, C═CH), 4.81 (1H, s, 3—C═CH), 4.85 (H, s, 4′H), 4.91 (H, s, 4′H). MS: m/z (relative intensity) 236 (47, M^+), 168 (34), 153 (20), 136 (37), 121 (39), 107 (22), 100 (41), 93 (32), 85 (55), 83 (31), 81 (22), 69 (100), 68 (44), 67 (20), 55 (45), 41 (38). HR-MS m/z (M^+): calcd for $C_{15}H_{24}O_2$, 236.1776; found, 236.1781.

Bioassays in Petri Dishes. The activity of each sample was determined with adult males (10–20) in a petri dish bioassay (Arai, 2000). Test samples deposited on filter paper (1 cm^2) (Toyo Roshi Co. Ltd., No. 1) consisted of either 2–200 FDE, or 10 ng of synthetic pheromone, or solvent (hexane). When the males ceased moving (10–60 min), numbers of males captured were counted. Experiments were replicated at least three times for each sample. The resulting percentage data were arcsine transformed to stabilize variance, and the transformed data were compared with Tukey–Kramer HSD or t tests (SAS Institute, 1995).

Bioassays in a Glasshouse. Attractiveness of test samples to adult males was tested using yellow sticky board traps (Arai, 2000) in a glasshouse (5-m long and 6.5-m wide) at the Department of Citrus Research, Kuchinotsu, National Institute of Fruit Tree Science. Ten ng of synthesized sex pheromone, solvent (hexane), and 10 FDE of volatiles or acetylated components were applied to a 1-cm^2 piece of

filter paper (Toyo Roshi Co. Ltd., No. 1), which was attached to the center of a yellow sticky board trap (20-cm width, 10-cm length). Traps were placed about 180 cm above ground in a row, and 140 cm apart. A control trap with a filter paper treated with solvent was set at the center. Sheets of tissue paper with emerging adult males were placed below the control trap at 1700 hr, and captured males were counted at noon the next day. Count data were log $(x + 0.5)$ transformed, and the transformed data were analyzed with Tukey–Kramer HSD tests (SAS Institute, 1995).

RESULTS AND DISCUSSION

Isolation. Following column chromatographic fractionation of the crude extract on Florisil, the 5% ether in hexane fraction showed the strongest activity (Table 1). Although the other fractions also showed some activity, and active components might be contained in those fractions, we decided to identify the active components in the 5% ether in hexane fraction first, because it was considered that some tailing from this fraction to other fractions might have caused the residual activity in other fractions. Thus, the 5% ether in hexane fraction was further fractionated by HPLC. The strongest activity eluted between 7 and 10 min (Table 1), and this active fraction was fractionated further by HPLC, with the fraction eluting between 7.5 and 8 min showing the strongest activity (Table 1). This fraction and the next fraction were further purified by preparative GC. The GC fraction at a retention time (t_R) of 20.5–21 min, which contained a single GC peak, showed the strongest activity (Table 1).

Gas Chromatography-Mass Spectrometry. The purified compound eluted at $t_R = 14.73$ min with GC–MS analysis. The Kovats retention indices (KI) were 1905 and 1526 on the FFAP and TC-1 columns, respectively. This component gave characteristic EI GC–MS fragment ions at m/z (relative intensity) 69 (100), 100 (32), 136 (22), 168 (26), and 236 (8). The molecular weight of the component was confirmed to be 236 by CI GC–MS. The precise molecular weight of 236.1808 from HR–MS indicated a molecular formula of $C_{15}H_{24}O_2$. When the active component was hydrogenated, a single product was observed at $t_R = 13.35$ min by GC–MS analysis (KI 1689 and 1495 on FFAP and TC-1 columns respectively). The EI mass spectrum of the hydrogenated product showed significant fragment ions at m/z 83 (100), 98 (69), 123 (10), 143 (12), and 170 (25), with no molecular ion. The molecular weight was determined to be m/z 240 from the CI mass spectrum, indicating that the active component had two sites of unsaturation.

Analysis of the Alcohol Part of the Active Component. In the basic and neutral fraction obtained by hydrolysis of the active component, a single peak was observed by GC–MS (KI 1601 and 1150 on the FFAP and TC-1 columns, respectively). The peak gave significant EI MS fragment ions at m/z 71 (100), 121 (27), 123 (9), and 139 (12), and the molecular weight was determined to be 154 by CI MS. The mass

TABLE 1. NUMBERS OF *P. cryptus* MALES ATTRACTED TO FILTER PAPERS IMPREGNATED WITH FRACTIONS FROM FLORISIL LC, HPLC, AND PREPARATIVE GC

Sample	Treatment[a,b]	Control	*P* value
Florisil column chromatography			
Hexane	2.1 ± 2.1 b	10.8 ± 5.8	ns
5% E/H	76.5 ± 20.0 a	0	**
15% E/H	54.4 ± 16.0 ab	2.1 ± 2.1	*
25% E/H	56.7 ± 23.3 ab	4.1 ± 2.1	ns
50% E/H	50.7 ± 9.1 ab	7.5 ± 4.9	*
HPLC			
0–7 min	20.1 ± 10.0 b	3.0 ± 3.0	ns
7–10 min	80.5 ± 5.2 a	0	**
10–16 min	66.6 ± 13.7 ab	3.7 ± 3.7	*
Blank[c]	60.0 ± 10.3 ab	0	**
HPLC between 7 and 10 min			
7–7.5 min	12.1 ± 12.1 b	4.2 ± 4.2	ns
7.5–8 min	74.8 ± 4.1 a	0	**
8–8.5 min	60.0 ± 10.0 ab	0	**
8.5–10 min	62.0 ± 16.1 a	0	**
Preparative GC			
0–15 min	3.0 ± 3.0 b	0	ns
15–18 min	2.6 ± 2.6 b	5.6 ± 2.8	ns
18–19.5 min	3.0 ± 3.0 b	0	ns
19.5–20 min	22.5 ± 8.0 b	0	**
20–20.5 min	5.6 ± 5.6 b	6.1 ± 3.1	ns
20.5–21 min[d]	79.6 ± 5.9 a	1.5 ± 1.5	**
Blank[c]	16.7 ± 8.3 b	0	ns

Note: ∗ and ∗∗ indicate that the number of adult males attracted to the baited filter paper was significantly greater than that to the control at 5 and 1% levels, respectively, by *t* test. Ns indicates that the difference was not significant.

[a] Average ± SE of three replicates.

[b] Treatments within a column followed by the same letter are not significantly different at the 5% level by Tukey–Kramer HSD test.

[c] Solvent.

[d] Data from five replicates.

spectrum was similar to that of the alcohol part (i.e., 3-isopropenyl-2,2-dimethyl -cyclobutanemethanol) of the sex pheromone of *Planococcus citri* (Bierl-Leonhardt et al., 1981), and the KIs were also close matches with those of the alcohol part of the *P. citri* pheromone (1601: FFAP, 1152: TC-1). Therefore, the isolated compound was deduced to be an ester of 3-isopropenyl-2,2-dimethylcyclobutanemethanol (molecular weight = 154) with an acid (molecular weight = 100) containing a double bond. To confirm the structure of the alcohol, the hydrolysis product was acetylated and compared directly with the pheromone of *P. citri* (3-isopropenyl-2,2-dimethylcyclobutanemethyl acetate). The acetylation product

TABLE 2. NUMBERS OF *P. citri* MALES ATTRACTED TO *P. citri* OR ACETYLATED *P. cryptus* PHEROMONE IN LABORATORY BIOASSAYS

Lures	Baited[a]	Control	Difference[b]
Crude pheromone of *P. citri*	52.8 ± 0.2	0	***
Acetylated *P. cryptus* component	58.9 ± 7.3	0	***
Acetylated *P. citri* pheromone component	56.9 ± 1.1	0	***

[a] Mean ± SE of three replicates.
[b] Asterisks indicate that the number of adult males attracted to the baited filter paper was significantly larger than that to the control at 0.1% level by *t* test.

was observed at $t_R = 10.63$ min as a single peak and gave the following EI MS fragment ions; m/z 68 (100), 86 (9), 121 (19), 128 (20), and 196 (1.6) (KIs of the product were 1702 and 1281 on FFAP and TC-1 columns, respectively). Its molecular weight was confirmed as 196 by CI GC-MS. The mass spectral data of the acetylation product were similar to that reported for the sex pheromone of *P. citri* (Bierl-Leonhardt et al., 1981), and the KIs of the acetate were exactly the same as those of the sex pheromone of *P. citri* (1702: FFAP, 1281: TC-1). Thus, the acetylation product derived from *P. cryptus* appeared to be identical to the pheromone of *P. citri*. To confirm this, the pheromonal activity of the acetylation product derived from *P. cryptus* was tested with males of *P. citri*. The acetylation product showed almost the same activity as the crude pheromone extracted from *P. citri* in petri dish bioassays (Table 2) and glasshouse bioassays (Table 3), confirming that its chemical structure was the same as that of the sex pheromone of *P. citri*, and that the alcohol part of the sex pheromone of *P. cryptus* had the same structure as that of the sex pheromone of *P. citri* (i.e., 3-isopropenyl-2,2-dimethylcyclobutanemethanol).

To confirm the absolute configuration of the alcohol, a synthetic sample of (1*R*,3*R*)-3-isopropenyl-2.2-dimethylcyclobutanemethanol having about 85% ee (Itagaki and Kuwahara, 2002) was resolved by GC on a chiral stationary

TABLE 3. NUMBERS OF *P. citri* MALES CAPTURED ON YELLOW STICKY BOARD TRAPS WITH DIFFERENT LURE SOURCES IN GLASSHOUSE BIOASSAYS

Lures	Mean ± SE (replicates)[a]
Acetylated *P. cryptus* component	133.3 ± 46.9 (4) a
Crude pheromone of *P. citri*	114.8 ± 44.5 (4) a
Control	0.3 ± 0.3 (4) b
Acetylated *P. citri* pheromone	59.4 ± 18.9 (5) a
Crude pheromone of *P. citri*	84.2 ± 26.2 (5) a
Control	0.6 ± 0.4 (5) b

[a] Values followed by the same letters are not significantly different at the 5% level by Tukey–Kramer HSD test.

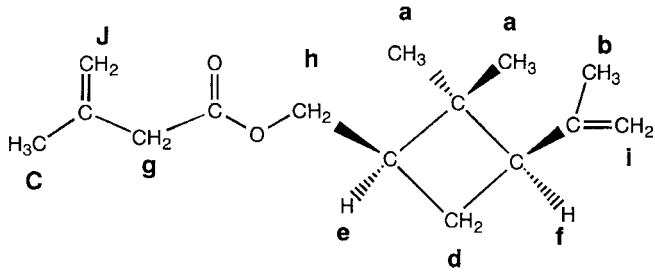

FIG. 1. Structure of a pheromone component of *Pseudococcus cryptus*.

phase (Betadex 120A), with the major (1*R*,3*R*)-enantiomer (94%) and the minor (1*S*,3*S*)-enantiomer (6%) giving KI values of 1375 and 1391, respectively. The alcohol derived from the pheromone of *P. cryptus* and the alcohol of known (1*R*,3*R*)-absolute configuration derived from *P. citri* pheromone both gave KI values of 1375, with no peak corresponding to the (1*S*,3*S*)-enantiomer. Thus, the structure of the alcohol portion of the *P. cryptus* pheromone was confirmed as (1*R*,3*R*)-3-isopropenyl-2,2-dimethylcyclobutanemethanol.

Nuclear Magnetic Resonance Analyses and Synthesis of the Pheromone. Each proton signal in the ^{1}H NMR spectrum [a: $\delta = 0.79$ (3H, s), 1.17 (3H, s), b: 1.63 (3H, s), c: 1.79 (3H, s), d: 1.56–1.66, 1.85–1.94 (2H, m), e: 2.17 (1H, m), f: 2.37 (1H, m), g: 2.99 (2H, s), h: 3.93, 4.08 (2H, m), i: 4.54, 4.79 (2H, s), j: 4.82, 4.89 (2H, s)] was assigned by COSY and HOHAHA spectra. Considering these data as well as the structure of the alcohol part elucidated above, the isolated component was identified as (1*R*,3*R*)-3-isopropenyl-2,2-dimethylcyclobutylmethyl 3-methyl-3-butenoate (Figure 1). As final confirmation, the (1*R*,3*R*)-stereoisomer was synthesized from (+)-α-pinene by the procedure of Itagaki and Kuwahara (2002) with the exception that the alcohol precursor, (1*R*,3*R*)-3-isopropenyl-2,2-dimethylcyclobutylmethanol, was esterified with 3-methyl-3-butenoic acid in the presence of 1,3-dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine (DMAP) instead of acetylation with acetyl chloride. The ^{1}H NMR spectrum of the synthetic material was virtually identical with that of the natural pheromone. The synthetic material was free from any isomers, as judged from its ^{1}H NMR spectrum (500 MHz). It is critically important to note that the use of only a catalytic amount of DMAP (about 0.1 equivalent) was essential for preventing the migration of the double bond of the acid moiety to the conjugated position; with one equivalent of DMAP, a 12:1 mixture of the desired ester and the conjugated isomer was obtained. The optical purity of the synthetic sample was first estimated to be about 85% from the comparison of the specific rotation value of α-pinene employed in this synthesis (+43.8°) with its maximum reported value (+51.6°) (Brown et al., 1982), and determined precisely to be 88% ee by GC analysis of the alcohol precursor as described above.

TABLE 4. NUMBERS OF *P. cryptus* MALES ATTRACTED TO DIFFERENT DOSES OF SYNTHESIZED PHEROMONE ON FILTER PAPER DISPENSERS

Amount of pheromone (ng)	Percentage of attracted males[a]		Difference[b]
	Baited	Control	
0.1	43.0 ± 13.3	3.2 ± 2.0	**
1	46.6 ± 2.5	0	***
10	64.1 ± 3.5	0	***
100	37.2 ± 1.9	0	***

[a] Mean $\pm$ SE of five replicates.
[b] ** and *** indicate that the numbers of adult males attracted to the baited filter paper were significantly larger than that to the control at 1 and 0.1% levels, respectively, by *t* test.

The pheromonal activity of the synthetic compound was confirmed in bioassays. It attracted more *P. cryptus* adult males than controls in petri dish bioassays (Table 4), and attracted as many adult males as the crude sex pheromone of *P. cryptus* in a glasshouse bioassay (Table 5). From these results, the female-produced sex pheromone of *P. cryptus* was confirmed to be (1*R*,3*R*)-3-isopropenyl -2,2-dimethylcyclobutylmethyl 3-methyl-3-butenoate. The 600,000 FDE of crude sex pheromone collected was estimated to contain about 2 mg of the pheromone, from which we obtained about 200 μg of the pure pheromone for ^{1}H NMR analysis.

To date, sex pheromones have been identified in four species of armored scales (Roelofs et al., 1977; Gieselmann et al., 1979a,b; Heath et al., 1979), three species of mealybugs (Bierl-Leonhardt et al., 1980, 1981; Negishi et al., 1980; Hinkens et al., 2001) and five species of the genus *Matsucoccus* (Lanier et al., 1989; Einhorn et al., 1990; Dunkelblum et al., 1993). The sex pheromones of *Aonidiella aurantii*, *A. citrina*, and *Pseudaulacaspis pentagona* that belong to the Diaspididae family share some structural similarities (Gieselmann et al., 1979b), as do the sex

TABLE 5. NUMBERS OF *P. cryptus* MALES CAPTURED BY YELLOW STICKY BOARD TRAPS WITH SYNTHESIZED OR CRUDE PHEROMONE OF *P. cryptus* IN GLASSHOUSE BIOASSAYS

Lures	Mean $\pm$ SE (replicates)[a]
Synthesized pheromone (10 ng)	9.7 ± 2.6 (7) a
Crude pheromone of *P. cryptus* (10 FDE)	10.9 ± 2.4 (7) a
Control	0 (7) b

[a] Values followed by the same letter indicate that the numbers of males caught was not significantly different at 5% level by Tukey–Kramer HSD test.

pheromones of *Matsucoccus* species (Dunkelblum et al., 1993). The structure of the sex pheromone of *P. cryptus* is similar to that of *P. citri*. Furthermore, the structure of the alcohol moiety of the sex pheromone of *Pseudococcus comstocki* is similar to that of *Planococcus ficus*, both of which are in the Pseudococcidae family. Although their pheromone structures do not have the cyclobutane structure found in other mealybug pheromones, there appears to be a relationship because the sex pheromones of these four mealybugs have an unusual connection of the isoprene units of the skeleton. The acid moiety of the sex pheromone of *P. cryptus* might be synthesized from isopentenyl pyrophosphate, the precursor of the isoprene unit.

Because two of the GC fractions showed activity, the sex pheromone of this mealybug might be composed of more than one component. However, the amount of crude pheromone of *P. cryptus* available was insufficient to identify any other components. Nevertheless, the identified component showed activity equal to that of the crude pheromone. Thus, it will be useful for developing a more efficient and exact monitoring system for this mealybug.

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RESPONSES OF THE APHIDS *Phorodon humuli* AND *Rhopalosiphum padi* TO SEX PHEROMONE STEREOCHEMISTRY IN THE FIELD

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Abstract—Gynoparous female and male damson-hop aphids, *Phorodon humuli* (Schrank), were caught in the field by water traps that were releasing the sex pheromone of this species, (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol. No behavioral activity was elicited by (4a*S*,7*S*,7a*R*)-nepetalactone, the major sex pheromone component of other aphid species such as *Megoura viciae* Buckton, even though olfactory cells were found in the secondary rhinaria on the third antennal segment of *P. humuli* that responded strongly to this compound. Gynoparous female *P. humuli* in the field responded less strongly to (1*R*,4*aS*,7*S*,7a*R*)-nepetalactol, the sex pheromone of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.), than they did to the (4a*R*,7*S*,7a*S*)-nepetalactols, but males responded only to the latter. The (4a*R*,7*S*,7a*S*)-nepetalactone showed no electrophysiological activity so was not used in field trials. Releasing either the (4a*S*,7*S*,7a*R*)-nepetalactone or the (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol with the (4a*R*,7*S*,7a*S*)-nepetalactols did not inhibit the response of *P. humuli* gynoparous females and males to the latter. Males of *R. padi* responded as strongly to the (4a*R*,7*S*,7a*S*)-nepetalactols as they did to (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol. Males of *P. humuli* and *R. padi* responded positively to an increased concentration of the (4a*R*,7*S*,7a*S*)-nepetalactols released from two vials compared with that from a single vial, as did *P. humuli* (in one of two experiments) and *R. padi* to the (1*RS*,4a*R*,7*S*,7a*S*)- and (1*R*,4aS,7S,7a*R*)-nepetalactols when released together.

Key Words—Aphid, sex pheromone, olfactory cell, stereochemistry, nepetalactone, nepetalactol, *Phorodon humuli*, *Rhopalosiphum padi*, semiochemical.

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INTRODUCTION

One of the advantages of using sex pheromones for the control of pests is their species selectivity. Many aphids utilize as sex pheromones either one or both of the cyclopentanoid monoterpenoids, (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and (4a*S*,7*S*,7a*R*)-nepetalactone (**I** and **II** respectively, Fig. 1), released in species-specific ratios (Dawson et al., 1990; Hardie et al., 1990, 1994; Pickett et al., 1992) (Fig. 1). At present, the only known exception is the damson-hop aphid, *Phorodon humuli* (Schrank), which produces a mixture of the diastereoisomers (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol (**III**, Fig. 1) (Campbell et al., 1990). In the field, males and gynoparous female *P. humuli* were highly responsive to a 7:3 synthetic mixture of these lactols (Campbell et al., 1990; Hardie et al., 1996; Lösel et al., 1996).

Although sexual females of *P. humuli* do not produce either **I** or its diastereoisomer (1*S*,4a*S*,7*S*,7a*R*)-nepetalactol, Hardie et al. (1997) caught

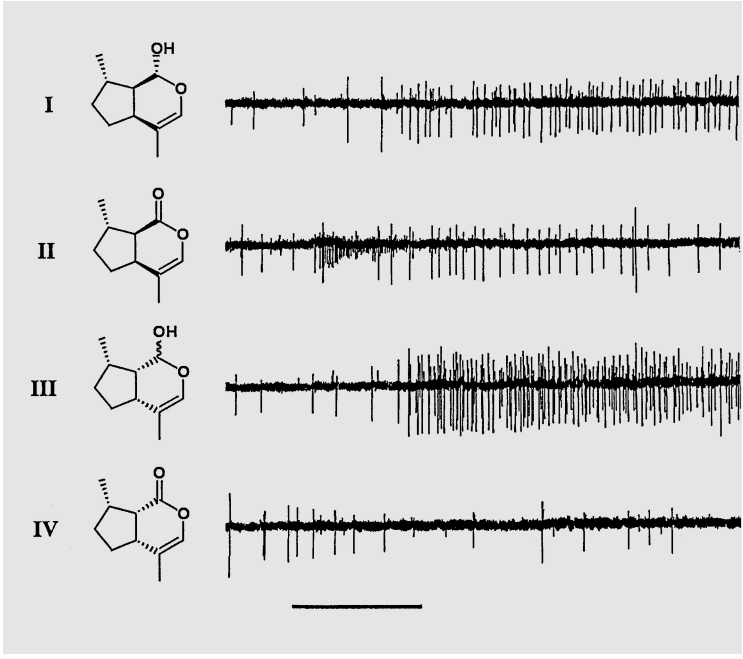

FIG. 1. Response of olfactory cells in a secondary rhinarium on the third antennal segment of a *Phorodon humuli* male to (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**I**), (4a*S*,7*S*,7a*R*)-nepetalactone (**II**), (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol (**III**), and (4a*R*,7*S*,7a*S*)-nepetalactone (**IV**). Compounds applied at 10^{-6} g in10 μl. Bar = 1 sec stimulus.

P. humuli males in traps releasing blends of these diastereoisomers derived synthetically from (*S*)-citronellol. The highest numbers of *P. humuli* were caught in the sample synthesized from the purest (*S*)-citronellol, and numbers dropped in line with declining purity of the (*S*)-citronellol used in the synthesis. Observing that *P. humuli* did not respond either to plant-derived **I** or to (4a*S*,7*R*,7a*S*)-nepetalactol derived from (*R*)-citronellol, these authors concluded that *P. humuli* was probably responding to traces of **III**, a by-product of synthesizing **I** from (*S*)-citronellol. If this explanation is confirmed, it suggests that males of *P. humuli* can discriminate **III** in an active space dominated by other stereoisomers of nepetalactol. Similarly, Hardie et al. (1997) showed that the numbers of male bird cherry-oat aphid, *Rhopalosiphum padi* (L.), and of the blackberry-cereal aphid, *Sitobion fragariae* (Walker), caught in water traps depends upon the concentration of their specific pheromones, and that these species were not inhibited by blends containing other stereoisomers of their pheromones. This contrasts with insects in some other orders, where the presence of stereoisomers of pheromone components inhibits their responses, e.g., diprionid sawflies (Anderbrandt et al., 1992) and moths (Kozlov et al., 1996).

The purpose of this study was to establish the electrophysiological activity in *P. humuli* of nepetalactol and nepetalactone stereoisomers in the laboratory and the influence of pheromone stereochemistry on the aphid's behavioral response in the field. Compounds **I**, **II**, and **III** were tested in various combinations and concentrations. During the course of the experiments, it was noted that *R. padi* was also caught in traps releasing **III**, and in subsequent experiments, the response of this species to its own pheromone **I** and **III** was recorded.

METHODS AND MATERIALS

Chemicals. (4a*S*,7*S*,7a*R*)-Nepetalactone (**II**) was prepared from an extract of the catmint *Nepeta cataria* (Lamiaceae) and reduced to the corresponding nepetalactol (**I**) using diisobutylaluminium hydride (Dawson et al., 1987). Similarly, (4a*R*,7*S*,7a*S*)-nepetalactone was prepared by steam distillation of *N. racemosa* (syn. *mussinii*) (Campbell et al., 1990). The crude extract was separated chromatographically on silica gel columns to give pure (4a*R*,7*S*,7a*S*)-nepetalactone, **(IV)** which was then reduced with diisobutylaluminium hydride to yield a 7:3 mixture of the 1*S*- and 1*R*- diastereoisomers of (4a*R*,7*S*,7a*S*)-nepetalactol (**III**).

Electrophysiology. For male and gynoparous female *P. humuli*, single cell recordings (SCR) from olfactory cells in the secondary rhinaria on the third antennal segment were made using tungsten microelectrodes (Boeckh, 1962). The indifferent electrode was placed in the first antennal segment, and the recording electrode was brought into contact with the surface of the secondary rhinarium

until impulses were recorded. Signals were amplified (UN-06, Syntech, The Netherlands) and displayed on an oscilloscope, and permanent copies of the action potentials generated by the olfactory cells were obtained by standard methods (Wadhams et al., 1982).

Stimulus Delivery. The delivery system, which employed a filter paper strip in a disposable Pasteur pipette cartridge, has been described previously (Wadhams et al., 1982). The stimulus (2-sec duration) was delivered into a purified air stream (1 l/min) flowing continuously over the preparation. Solutions of test compounds (0.1 mg/ml) were applied (10 μl) to filter paper strips, and the solvent was allowed to evaporate (30 sec) before the paper strip was placed in the cartridge. Fresh cartridges were prepared prior to each stimulation.

Field Experiments. Randomized complete block experiments were conducted in plantations of an unnamed genotype of dwarf hop growing on a 2.4-m high trellis at HRI-East Malling in 1993 and 1994. In 1993, the design comprised four blocks and five in 1994. The water traps were identical to those used by Hardie et al. (1997). They were constructed from clear plastic Petri dishes (14 cm diam. $\times$ 2 cm deep) and were filled with a dilute solution of Lipsol, a non-ionic detergent. Pheromones (10 mg) in 50 μl Et_2O were placed into glass vials (08-CPV Chromacol), with a 1-mm hole drilled in the cap. Control vials contained only Et_2O. The vials were suspended 4 cm above the center of the dish. Where single pheromone components were being tested against combinations, control vials were added as necessary so that all traps presented an identical visual image. Traps were spaced 2 m apart and 60 cm above the ground and sited centrally in the 3-m wide alleys between the rows of hops. Traps were emptied daily and their relative positions within each block were reallocated randomly. The numbers of gynoparous females and males of *P. humuli*, and male *R. padi* in 1994, were assessed in the laboratory.

Two experiments were conducted in 1993. The first, from September 18 to October 6, employed a factorial design with the three pheromones each at two levels (present and absent), except that the treatment combination **I** + **II** + **III** was omitted. Therefore, the treatments were (i) **I**, (ii) **II**, (iii) **III**, (iv) **I** + **II**, (v) **I** + **III**, (vi) **II** + **III**, and (vii) control. The results (see below) suggested that catches might have been affected by the number of vials of nepetalactol in traps irrespective of the stereochemistry. This possibility was explored in a second experiment from October 8 to November 6 in 1993 and in the 1994 experiment. The four treatments in 1993 were (i) **I** + **I**, (ii) **I** + **III**, (iii) **III** + **III**, and (iv) control. Five treatments were compared from October 20 to November 11 in 1994: (i) **I**, (ii) **III**, (iii) **I** + **III**, (iv) **III** + **III**, and (v) control.

Data Analysis. Data were examined by analysis of variance (ANOVA). In order to stabilize variances, the daily counts were transformed to square roots ($x^{0.5}$) and summed for the whole sampling period. A two-way classification of treatments within blocks was used for first-step analyses, with differences between

means compared against the least significant difference (LSD) only if the overall treatment terms were significant. Those data were reanalyzed subsequently with the variance partitioned into contrasts of interest and assessed by $F_{1,N}-$test where $N =$ error degree of freedom. Separate terms for block-to-block variance, main effects of each pheromone individually, two-pheromone interactions, and residual variance for the incomplete factorial design in 1993 were accumulated into analyses of variance from regressions using GenStat 5 (Payne, 2000).

RESULTS

For both males and gynoparous females of *P. humuli*, olfactory cells were found in the secondary rhinaria on the third antennal segment that responded to **I**, **II**, and **III**. Compound **I** was detected weakly by a medium amplitude cell, **II** by a strongly responding small amplitude cell, and **III** elicited a response either from a large amplitude cell or from a large and a medium amplitude cell (Fig. 1). Compound **IV**, as in other studies on the vetch aphid, *Megoura viciae* Buckton (Dawson et al., 1996), did not elicit a response from any of the preparations tested, and, therefore, was not included in the field experiments.

A total of 1323 gynoparous females and 1805 males of *P. humuli* were caught in the first field experiment which ran for 18 days ending on October 6, 1993 (Table 1). Sixteen gynoparous females and 623 males were caught in the second experiment (Table 2). This experiment ran for 34 days after the seasonal peak of migration for gynoparous females, which in the United Kingdom occurs around mid to late September. In the first-step (Blocks + Treatments) analysis, none of the individual treatment means for **I** in combinations without **III** differed significantly from the controls (Table 1). However, a total of 305 females were caught in the two treatments releasing **I** without **III**, 523 in the two treatments releasing **III** without **I,** and 186 in the two treatments in which neither **I** nor **III** was released. The factorial ANOVA of these data showed that gynoparous females responded significantly to **I** and **III** (**I**, $F = 6.59$, $P < 0.05$; **III**, $F = 44.54$, $P < 0.001$). The interaction between **I** and **III** was not significant.

Unlike the gynoparous females, males of *P. humuli* discriminated between the two nepetalactols, although they responded significantly to **I** and **III** (**I**, $F = 9.25$, $P < 0.01$; **III**, $F = 190.72$, $P < 0.001$). The interaction between these lactols was also significant ($F = 4.55$, $P < 0.05$). Partitioning the two-pheromone interactions revealed that males responded to **I** only when it was presented with **III** (**I**, $F = 2.03$, $P > 0.05$; **III**, $F = 41.95$, $P < 0.001$). Significantly more males were caught in traps releasing **I** and **III** together than in those releasing **III** alone ($F = 7.62$, $P < 0.05$) (Table 1). The nepetalactone **II** was no more active than the controls for either morph and, when released with nepetalactol **III**, did not affect the numbers caught compared to **III** alone.

TABLE 1. TOTAL NUMBERS OF *Phorodon humuli* MALES AND GYNOPAROUS FEMALES CAUGHT IN WATER TRAPS RELEASING (4a*S*,7*S*,7a*R*)-NEPETALACTOL (**I**), (4a*S*,7*S*,7a*R*)-NEPETALACTONE (**II**), AND (1*RS*,4a*R*,7*S*,7a*S*)-NEPETALACTOL (**III**)[a]

Treatment	Males	Females
Control	43 c	101 c
Nepetalactol (**I**)	81 c	175 bc
Nepetalactone (**II**)	56 c	85 c
Nepetalactol (**III**)	460 b	279 a
I + **II**	48 c	130 bc
I + **III**	698 a	309 a
II + **III**	419 b	244 a

[a] Numbers in a column followed by the same letter are not significantly different at $P < 0.05$ based on two-way ANOVA of $x^{0.5}$ transformed counts.

The significantly greater catch of males in traps releasing **I** and **III** together, than those releasing either **I** or **III** alone, suggested a possible synergism between the two pheromones, perhaps because releasing two stereoisomers together may have enhanced the size of the pheromone active space compared to that from a single vial. In the follow-up experiment, pairs of vials were used for all pheromone-release treatments (Table 2). As with the first experiment, *P. humuli* males responded to **III** ($F = 18.31$, $P < 0.01$) but not to **I** ($F = 0.00$, $P > 0.05$). The interaction between **I** and **III** was not significant ($F = 0.63$, $P > 0.05$). Although more gynoparous females were caught in the traps releasing **III** than in

TABLE 2. TOTAL NUMBERS OF *Phorodon humuli* MALES AND GYNOPAROUS FEMALES CAUGHT IN WATER TRAPS RELEASING (4a*S*,7*S*,7a*R*)-NEPETALACTOL (**I**), AND (1*RS*,4a*R*,7*S*,7a*S*)-NEPETALACTOL (**III**)[a]

Treatment	Males	Females
Control	9 b	2 a
I + **I**	30 b	1 a
I + **III**	277 a	3 a
III + **III**	307 a	10 a

[a] Numbers in a column followed by the same letter are not significantly different at $P < 0.05$ based on two-way ANOVA of $x^{0.5}$ transformed counts.

TABLE 3. TOTAL NUMBERS OF *Phorodon humuli* AND *Rhopalosiphum padi* MALES CAUGHT IN WATER TRAPS RELEASING (4a*S*,7*S*,7a*R*)-NEPETALACTOL (**I**), AND (1*RS*,4a*R*,7*S*,7a*S*)-NEPETALACTOL (**III**)[a]

Treatment	*P. humuli*	*R. padi*
Control	44 c	18 c
I	53 c	171 b
III	562 b	114 b
I + **III**	618 b	238 a
III + **III**	821 a	219 a

[a] Numbers in a column followed by the same letter are not significantly different at $P < 0.05$ based on two-way ANOVA of $x^{0.5}$ transformed counts.

other treatments, too few were caught for meaningful statistics, and even the response to **III** was not significant ($F = 4.27$, $P = 0.069$).

Significantly more *P. humuli* males were trapped when **III** was released from a pair of vials than from one vial only ($F = 11.70$, $P < 0.01$) (Table 3). A similar response to increased concentration of **III** was observed with *R. padi* ($F = 9.99$, $P < 0.01$). Indeed, *R. padi* males responded significantly to all of the pheromone-release treatments in this experiment, including the single vial of **III** ($F = 23.33$, $P<0.001$). Although 10% more *P. humuli* males were caught in the traps releasing **I** together with **III** than in those with a single vial of **III** (Table 3), this increase was not statistically significant. As in experiments 1 and 2, **I** alone was no more active than the control treatment for male *P. humuli* ($F = 0.52$, $P > 0.05$), whereas males of *R. padi* responded significantly to **I** ($F = 42.12$, $P < 0.001$). Addition of **III** did not increase catches ($F = 2.56$, $P>0.05$). However, the response of *R. padi* to a single vial of **I** was not greater than to a single vial of **III** ($F = 2.79$, $P > 0.05$).

DISCUSSION

Hardie et al. (1997) caught a total of 430 male *P. humuli* in water traps at Silwood Park, Berkshire, in 1995, a site where autumn migrants of this species are uncommon (Taylor et al., 1979), and at which only one individual male was caught in 1995 in the 12.2-m Rothamsted Insect Survey (RIS) suction trap operated at that site (R. Harrington, unpublished data). It was also observed that, although males of *P. humuli* responded significantly to **I** (the sex pheromone of *R. padi*), no response was found to **I** prepared semisynthetically from *N. cataria*. This finding

was confirmed in a further 2 years of trapping by Donato (2001). Hardie et al. (1997) concluded that *P. humuli* was responding to the presence of up to 8% of **III**, a by-product of the synthesis from (*S*)-citronellol. An 8% contamination of **I** by **III** in 10 mg is similar to the 1 mg loading that Lösel et al. (1996) found maximized the catch of *P. humuli* in response to **III** released from a single Chromacol vial, a type of dispenser common to all of these studies. All pheromone components in the present experiments were derived from plants. However, *P. humuli* males did not respond to **I** alone in any of the three field experiments, confirming the findings of Hardie et al. (1997).

Although gynoparous female *P. humuli* showed electrophysiological activity to **I**, **II**, and **III**, in the field they only responded to **I** and **III**, and most strongly to the latter, their conspecific sex pheromone (Table 1). Such discrimination may allow gynoparous females to locate potential host plants already colonized by sexual females of other aphid species. Although males of *P. humuli* also responded electrophysiologically to **I**, **II**, and **III** (Figure 1), in the field they responded behaviorally only to **III**, thus allowing discrimination between sexual females of different species. Surprisingly, males of *R. padi* responded behaviorally to both **I** and **III**. It is likely that interactions between specific plant volatiles and sex pheromones (Hardie et al., 1994) allow male aphids to discriminate between species, as it has been observed that a number of aphids utilize **I** alone as a sex pheromone (Hardie et al., 1999).

Corelease of even a small amount of **II** with **I** reduced catches of male *R. padi* to the level of the controls (Hardie et al., 1994). Similarly, when **I**, **II**, or blends of these were released, males of several other aphid species were caught in greatest numbers by the blend that most closely matched the pheromone released by conspecific sexual females (Hardie et al., 1999). The results from this study suggest that inhibition by **II** may be limited to those aphid species which utilize the (4a*S*,7*S*,7a*R*)- stereochemistry, as *P. humuli*, which utilizes the (4a*R*,7*S*,7a*S*) stereochemistry, was unaffected by the corelease of **II** with **III.**

The corelease of other nepetalactol stereoisomers with the respective species-specific compound did not inhibit responses by males of either *R. padi* or *P. humuli*, in our study or previously (Hardie et al., 1997; Donato, 2000). Similarly, Lösel et al. (1996) found that *P. humuli* responded almost as strongly to an unpurified mixture of nepetalactols, only half of which was **III**, as they did to pure plant-derived and pure synthetically derived **III**. The apparent behavioral indifference of these aphid species to geometric isomers of their sex pheromones contrasts with the strong inhibitory effects of traces of such compounds for other insect orders, e.g., diprionid sawflies (Anderbrandt et al., 1992) and moths (Kozlov et al., 1996). However, as both *P. humuli* and *R. padi* use nepetalactols alone as their sex pheromones, further work is needed to assess whether the majority of species utilizing species-specific blends of **I** and **II** show a similar insensitivity to inappropriate nepetalactol stereoisomers.

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MAMMALIAN EXOCRINE SECRETIONS. XVIII: CHEMICAL CHARACTERIZATION OF INTERDIGITAL SECRETION OF RED HARTEBEEST, *Alcelaphus buselaphus caama*

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Abstract—Gas chromatography, coupled gas chromatography-mass spectrometry (electron impact mode and chemical ionization with methane as reactant gas), gas chromatography-infrared spectroscopy, and derivatization techniques were used to identity 53 compounds in the interdigital secretion of the red hartebeest, *Alcelaphus buselaphus caama*. These compounds included alkanes, isoalkanes, alcohols, ketones, carboxylic acids, oxiranes, furanoid linalool oxides, and a large number of branched and unbranched saturated and unsaturated aldehydes. The secretion probably plays a role in demarcation of territories by dominant bulls.

Key Words—Red hartebeest, *Alcelaphus buselaphus*, semiochemicals, pheromones, mass spectra, branched-chain aldehydes, territorial marking.

INTRODUCTION

The red hartebeest, *Alcelaphus buselaphus*, is a member of the alcelaphine group of antelopes (Family Bovidae, Tribe Alcelaphini). Two main subspecies are distinguished: the North African Hartebeest, *Alcelaphus buselaphus buselaphus*, now extinct, and the Red Hartebeest, *Alcelaphus buselaphus caama*, a relatively rare antelope mostly encountered in the southern African subregion, from Namibia southeastwards to the Botswana border and in the north of the Northern Cape Province along the Botswana border. Their habitat is grassland, semidesert bush

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savanna, and open woodland. They avoid the more closed types of woodland (Kok, 1975; Skinner and Smithers, 1990).

Adult male red hartebeest have a mass of about 150 kg. Females are slightly smaller with a mean mass of 120 kg. High humped shoulders, sloping backs, and elongated heads, held high on their upright necks, characterize the shape of the body and head. Hairs are reddish-brown in general, with dark and black patches on the forehead, on the front of the shoulder, on forelegs, and hind legs.

Usually, males are territorial and herd females, actively defending their territory against other trespassing males. The social structure consists of harem herds (groups of females with their offspring, a dominant bull, and young bulls), bachelor herds (males of all ages), and, to a small extent, territorial bulls without followers (Kok, 1975). In territorial behavior, visual and olfactory techniques are used for demarcation. Olfactory demarcation is carried out by feces and urine, since harem bulls frequently lie down on dung patches, typical behavior by which their characteristic odor is spread. It is claimed that ground horning by bulls has a territorial function because it could facilitate transfer of preorbital secretion to the ground. Pawing and scratching with the foreleg before lying down is regularly performed by both sexes. This behavior also could play a role in marking by transferring secretion from the interdigital glands, situated between the front hooves, to the ground. In general, these glands occur in both sexes, but are more productive in territorial bulls (Kok, 1975).

The chemical compositions of the preorbital and interdigital secretions of the red hartebeest are unknown. This antelope is of interest from a nature preservation point of view, and the development of a semiochemical lure that can be used in translocation operations could play a future role in its conservation. Identification of the constituents of the interdigital secretion of the red hartebeest reported here is a first step toward development of such a lure.

METHODS AND MATERIALS

General. All Pyrex glassware was throughly cleaned with water and organic solvents, and heated at 500°C in an annealing oven to remove any trace of organic contaminants. Syringes and other apparatus that could not be heated to 500°C were cleaned with dichloromethane (Merck, Residue Analysis Grade). Interdigital secretions were extracted and extracts diluted, when necessary, with this solvent.

Collection and Sample Preparation. Interdigital secretions were collected individually from four male and four female red hartebeest captured in Kimberly, Northern Province, South Africa, during August 1999. Surgical gauze squares (ca. 25×25 mm) consisting of several layers of surgical gauze were extracted for five hr with dichloromethane (Residue Analysis Grade), dried in an atmosphere of activated charcoal purified N_2, and stored in glass-stoppered bottles. Interdigital secretion was collected by rolling a gauze square around the tip of dressing forceps,

inserting the forceps into the interdigital cavity, and collecting the secretion by rotating the forceps while removing it from the cavity. This procedure does not seem to be painful to the animals because they did not twitch during the procedure, nor attempted to kick themselves free from their handlers. The gauze pads with the secretion were stored at -30°C in glass bottles with Teflon-lined screw caps until used for analysis.

To extract the organic constituents of the secretion from the gauze, just enough CH_2Cl_2 to wet the gauze was added to one or more gauze pads in a suitable glass vial. The vial was closed with a screw cap with a Teflon-lined septum and was centrifuged for 1 min at 1500 rpm to enhance contact between the gauze and the small volume of solvent by compressing the gauze in the vial, after which the contents were sonicated for 2 min. The extract was separated from the gauze by centrifuging in a sintered glass filter insert suspended in a 5-ml Reacti-Vial. If a more concentrated solution was required, the solvent was evaporated by placing the vial containing the extract in a 2-1 glass beaker covered with aluminum foil and purging the solvent vapor from the beaker with a weak stream of purified (activated charcoal) nitrogen. Care was taken not to blow the purge gas directly into the vial containing the extract.

Analytical Methods. Gas chromatographic (GC) analyses were carried out on a Carlo Erba 5300 (Mega) gas chromatograph equipped with a flame ionization detector (FID) and a split/splitless injector. The FID was operated at 280°C. Glass capillary columns (40 m $\times$ 0.3 mm), drawn from Duran 50 glass (Schott, Mainz, Germany), were coated in our laboratory with the following phases: (1) PS-089-OH (DB-5 equivalent, film thickness 0.25 μm, (2) OV-1701-OH, film thickness 0.375 μm, and (3) Carbowax 20M, film thickness 0.25 μm. Hydrogen was used as carrier gas at a linear velocity of 50 cm/sec. Samples were injected in split mode (injector temperature 220°C) with an oven temp of $\leq$30°C. After injection, the oven was heated ballistically to 40°C, then at 2°C from 40–280°C. Temperature programming and data acquisition were started at 40°C. A programming rate of 4°C/min was used in GC analyses of synthetic compounds and intermediates.

Low-resolution electron impact mass spectra (EI-MS) were obtained at 70 eV on a Carlo Erba QMD 1000 GC-MS instrument using the above columns and conditions with He as carrier gas (linear velocity 28.6 cm/sec). The injector temperature was set at 220°C, the interface at 250°C, and the ion source at 180°C. A scan rate of 0.9 sec per scan with an interval of 0.1 sec between scans was employed. Chemical ionization mass spectra, with methane as reactant gas [CI(CH_4)-MS] were obtained on the same GC-MS instrument. The methane flow into the ion source was regulated to keep the source housing pressure at an optimum value of 8×10^{-5} torr.

Chiral GC and GC-MS analyses were done with a capillary column (30 m $\times$ 0.2 mm) coated with OV-1701-OH containing 13% (w/w) of the chiral selector

heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (synthesized in our laboratory) at a film thickness of 0.25 μm, using hydrogen and helium, respectively, as carrier gasses.

Solid-phase microextraction (SPME) was used to extract the volatile compounds from the headspace in a 50-ml vial containing the interdigital secretion on gauze pads as well as some hairs extracted from the interdigital cavity. The 100-μm polydimethylsiloxane SPME fiber was exposed to the secretion for 60 min at room temperature (22°C). Enriched volatiles were desorbed from the fiber for 5 min at an injector temperature of 220°C, cryotrapped on the column for 30 sec with dry ice, and analyzed by GC and GC-MS using the specified columns and conditions.

Mass spectral data were also obtained using a solventless sample introduction technique. About 10–15 hairs from the interdigital cavity were inserted into a glass injector liner. To circumvent the oxidation of sensitive compounds at 220°C in the presence of oxygen, the liner containing the hairs was introduced into the injector at 40°C, and the injector was heated to 220°C over a period of 4 min. The desorbed volatile organic compounds were cryotrapped on the column with dry ice. The liner and septum were quickly replaced by a clean liner and septum after which the cryotrapping was terminated and the oven temperature programs and data acquisition were started.

GC-IR data were obtained with an HP 5890 series II GC equipped with an HP IRD 5965B IR detector. Column 2, specified above, and a temperature program of 4°C/min from 40°C to 250°C were used. The temperature of the injection port was set at 250°C and the transfer line and flow cell at 220°C. The sample was injected splitless with a purge delay of 10 sec.

^{1}H and ^{13}C NMR spectra of synthetic compounds and intermediates were recorded at 299.905 and 75.42 MHz, respectively, at 25°C on a Varian VXR 300 NMR spectrometer.

Lithium Aluminium Hydride Reduction. An aliquot of the interdigital secretion extract was reduced with excess $LiAlH_4$ in diethyl ether. Thus, a sample (4 μl) of the concentrated extract was transferred to a Reacti-Vial. The solvent was gently evaporated. To remove residual CH_2Cl_2, two drops of diethyl ether were added to the vial and evaporated in a slow stream of nitrogen. This step was repeated, after which the residue was diluted with a few drops of ether and treated with two drops of a saturated solution of $LiAlH_4$ in ether. The reaction mixture was sealed and heated to 40°C for 1 hr, cooled, diluted with ether (1 ml), cooled in ice, and treated with a drop of distilled water. The mixture was centrifuged and the ether supernatant was removed with a 100-μl syringe and concentrated for GC-MS analysis.

Pyrrolidide Derivatives. A small sample (ca. 8 μl) of the extract was concentrated under nitrogen, The residue was dissolved in diethyl ether (1 ml, containing 10% methanol), and treated with diazomethane-diethyl ether solution until the mixture remained yellow. The mixture was concentrated under a stream of N_2.

The resulting mixture of methyl esters was dissolved in pyrrolidine (1 ml) and acetic acid (100 μl), and the solution was heated at 100°C for 30 min (Andersson and Holman, 1974). After cooling to room temperature, the amides were taken up in ether (2 ml), and the solution was washed three times with 2 M HCl and three times with distilled water. The organic phase was concentrated for GC-MS analysis under a stream of nitrogen.

Trimethylsilyl Derivatives. Approximately 5 μl of the extract were treated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Pierce, Rockford, IL), and the solution was heated at 40°C for 30 min. The resulting mixture of trimethylsilyl ethers was immediately subjected to GC-MS analysis (Donike, 1969).

Reference Compounds. Some of the compounds identified in the interdigital secretion of the red hartebeest were available from previous research projects, whereas others were obtained commercially. The following compounds were synthesized.

*2-Methylundecane **(29)***. Wurtz condensation of 1-bromoheptane and 1-bromo-3-methylbutane (Stander et al., 2002) gave a mixture of 2-methylundecane [MS *m/z* (%): 170 (1), 155 (1), 127 (4), 126 (5), 113 (2), 99 (5), 97 (3), 85 (23), 84 (3), 71 (48), 70 (7), 69 (6), 57 (86), 56 (18), 55 (16), 43 (100), 42 (19), 41 (44)], 2,7-dimethyloctane, and tetradecane.

*6-Methyl-1-heptanol **(27)***. A solution of phosphorous tribromide (13.5 g, 50 mmol) in dichloromethane (10 ml) was added over 30 min at −20°C to a solution of 4-methyl-1-pentanol (5.11 g, 50 mmol) and pyridine (0.5 g, 6 mmol) in dichloromethane (10 ml) (Tang et al., 1995) at −20°C. The reaction mixture was stirred for another 4 hr at 0°C, carefully treated with water (5 ml), and the organic product was washed with saturated sodium bicarbonate solution and brine. Isolation of the organic product in the usual manner and distillation (70°C/53 mm Hg) gave 1-bromo-4-methylpentane (4.6 g, 56%).

A solution of 1-bromo-4-methylpentane (4.5 g, 27 mmol) in dry THF (25 ml) was added drop-wise to magnesium turnings (648 mg, 27 mmol) in dry THF (15 ml), and the reaction mixture was stirred for 3 hr. Anhydrous CuI (500 mg, 2.6 mmol) was added to the resulting Grignard reagent at −10°C, and the reaction mixture was treated with gaseous ethylene oxide (6.2 g, 141 mmol), introduced by means of a tube with its tip about 15 mm above the surface of the mixture. The reaction mixture was stirred overnight at −20°C, then hydrolyzed with water (3 ml), the resulting solid material was filtered off and washed repeatedly with hexane, and the combined filtrate and washings were washed with HCl (3M) and brine. Work-up gave 6-methyl-1-heptanol **(27)** (2.7 g, 47%). MS: 98 (2), 97 (19), 84 (18), 83 (6), 70 (25), 69 (58), 68 (19), 57 (36), 56 (100), 55 (76), 43 (77), 41 (72).

*4-Methylpentanal **(11)***. Oxidation of 4-methyl-1-pentanol with pyridinium dichromate according to Tietze and Eicher (1981) gave 4-methylpentanal. MS: 100

(0.1), 99 (0.1), 85 (1), 82 (1), 81 (2), 72 (12), 71 (7), 67 (11), 58 (16), 57 (100), 56 (98), 55 (19), 44 (12), 43 (78), 41 (76).

3-Methyl-3-butenal ***(15)***. Oxidation of 3-methyl-3-buten-1-ol with pyridinium dichromate (Tietze and Eicher, 1981) gave 3-methyl-3-butenal. Because of its volatility, the product was not isolated but was used as a solution in ether. There was no detectable isomerization to the conjugated isomer. MS: 85 (8), 84 (99), 83 (54), 69 (7), 65 (2), 56 (13), 55 (100), 53 (25), 41 (71), 39 (79).

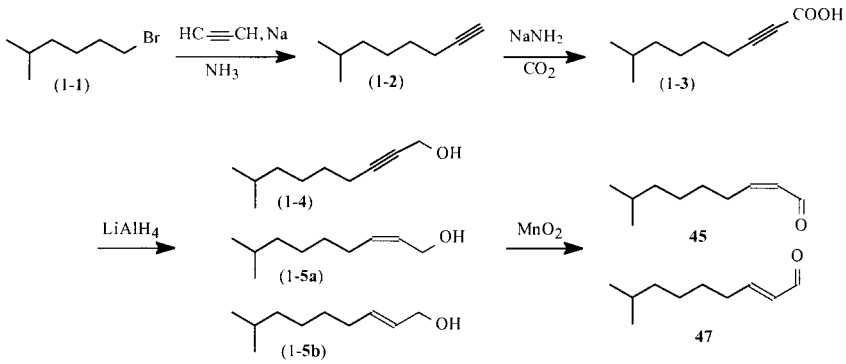
5-Methylhexanal ***(17)***. This compound was prepared as described by Andersson et al. (1979). Isobutyl magnesium bromide (16.1 g, 0.1 mol) in dry ether was added drop-wise over 20 min to a cold (10°C) stirred solution of acrolein diethyl acetal (13 g, 0.1 mol) and CuBr (0.5 g) in 100 ml THF. After 2 hr at room temperature, the reaction mixture was treated with saturated ammonium chloride, extracted with pentane, dried, and concentrated to give 5-methylhexanal diethyl acetal. The acetal was refluxed for 1 hr with aqueous acetone (35 ml, 75%) and a few drops of conc. HCl to hydrolyze the acetal. Standard isolation procedures gave 5-methylhexanal. MS: 96 (28), 86 (9), 81 (39), 71 (51), 70 (44), 57 (23), 56 (9), 55 (72), 44 (67), 43 (100), 41 (69).

6-Methylheptanal ***(22)***. Oxidation of 6-methyl-1-heptanol with pyridinium dichromate (Tietze and Eicher, 1981) gave 6-methylheptanal. MS: 110 (2), 100 (5), 96 (2), 95 (18), 85 (23), 84 (12), 82 (31), 71 (5), 69 (33), 67 (14), 57 (54), 56 (35), 55 (20), 44 (38), 43 (100), 42 (17), 41 (70).

8-Methylnonanal ***(39)***. Reduction of 8-methylnonanoic acid (Sigma Aldrich, Cape Town, South Africa) with $LiAlH_4$ in ether and subsequent oxidation of the alcohol with pyridinium dichromate (Tietze and Eiche 1981) gave 8-methylnonanal in almost quantitative yield. MS: 128 (3), 123 (5), 112 (4), 110 (5), 109 (2), 97 (6), 96 (6), 95 (23), 83 (10), 82 (41), 81 (23), 71 (28), 70 (20), 69 (38), 68 (19), 67 (23), 57 (100), 56 (52), 55 (53), 43 (93), 41 (76).

(E)- and (Z)-8-Methyl-2-Nonenal ***(45)*** and ***(47)***. These isomeric aldehydes were synthesized from 1-bromo-5-methylhexane (1-**1**) according to Scheme 1. The starting bromide for the synthesis was obtained by conventional methods from 1-bromo-3-methylbutane *via* alkylation of diethyl malonate, hydrolysis of the alkylation product, decarboxylation of the alkylated malonic acid, reduction of the resulting 5-methylhexanoic acid to 5-methyl-1-hexanol, and bromination with PBr_3.

1-Bromo-5-methylhexane (1-**1**) (37.8 g, 0.21 mol) was condensed with sodium acetylide in liquid NH_3 (500 ml) according to Ziegenbein (1963). The reaction mixture was stirred for 2 hr, *N,N*-dimethylformamide (200 ml) was added, the liquid NH_3 was allowed to evaporate, and the reaction mixture was stirred overnight at room temperature. The unreacted acetylide was hydrolyzed with ice-cold water (200 ml), extracted with pentane, and the extract was washed twice with water, then dried over anhyd. $MgSO_4$. Filtration, concentration, and distillation (bp. 142–143°C) gave 7-methyl-1-octyne (1-**2**) (21.0 g, 80%). MS:



SCHEME 1. Synthesis of (*Z*)- and (*E*)-8-methyl-2-nonenal.

109 (22), 95 (12), 82 (35), 81 (97), 79 (35), 69 (47), 68 (25), 67 (100), 57 (14), 56 (27), 55 (52), 54 (18), 53 (22), 43 (91), 41 (87), 39 (43).

7-Methyl-1-octyne (1-**2**) (9 g, 72 mmol) was added drop-wise to a suspension of $NaNH_2$ (9 g, 0.23 mol) in dry ether (80 ml), the reaction mixture was refluxed for 4 hr, poured onto dry ice (60 g), and allowed to stand overnight (Wotiz, 1954). The reaction mixture was treated with water and extracted with ether. The water phase was acidified with phosphoric acid, and extracted with ether. After drying and concentration, the residue (6 g) contained some unreacted 7-methyl-1-octyne and the target compound 8-methyl-2-nonynoic acid (1-**3**). MS: 135 (1), 126 (2), 125 (2), 109 (11), 97 (8), 95 (7), 81 (68), 69 (35), 67 (68), 57 (12), 56 (20), 55 (44), 53 (16), 44 (54), 43 (100), 41 (80). This product was used without further purification.

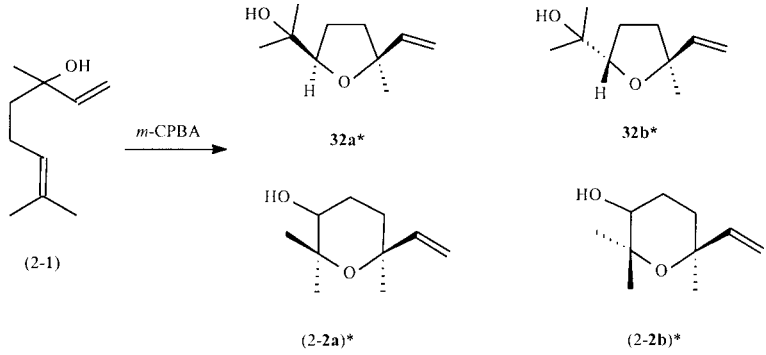
Reduction of 8-methyl-2-nonynoic acid (1-**3**) (0.14 g) with a clear solution of $LiAlH_4$ in ether for 22 hr at 22°C, and work-up in the conventional manner, gave a mixture of products (100 mg, 77%) containing 8-methyl-2-nonen-1-ol, 8-methyl-1-nonanol, and 8-methyl-2-nonyn-1-ol (91:4:5). 8-Methyl-2-nonen-1-ol (1-**5**): MS: 138 (3), 123 (9), 110 (7), 109 (9), 96 (14), 95 (42), 82 (56), 81 (32), 69 (43), 67 (42), 57 (100), 56, (41), 55 (55), 43 (86), 41 (88), 39 (37), 29 (30). ^{13}C NMR analysis showed that (*Z*)- and (*E*)-8-methyl-2-nonen-1-ol (1-**5a** and 1-**5b**, respectively) were present in a ratio of 1:10. (*E*)-8-methyl-2-nonen-1-ol (1-**5b**): ^{13}C NMR ($CDCl_3$): $\delta = 22.6$ (q, C-9, C-8 Me); 26.91 (t); 27.90 (d, C-8); 29.37 (t); 32.22 (t, C-4); 38.81 (t); 63.79 (t, C-1); 128.75 (d, C-2); 133.57 (d, C-3). (*Z*)-8-methyl-2-nonen-1-ol (1-**5a**): ^{13}C NMR ($CDCl_3$): $\delta = 58.57$ (t, C-1); 128.25 (d, C-2); 133.25 (d, C-3). (Other resonances not assigned.)

The mixture of alcohols (1-**5a**) and (1-**5b**) (100 mg) in hexane (250 μl) was added to a suspension of MnO_2 (250 mg) in hexane (250 μl) (Hickinbottom, 1957). The mixture was stirred for 20 hr at room temperature in darkness, then filtered and concentrated to give (*E*)-8-methyl-2-nonenal **(47)** (43 mg, 44%, purity 97.3%

(GC-MS). ^{13}C NMR did not show the presence of any (*Z*)-isomer in this product. Another 250 μl of hexane was added to the recovered MnO_2, and the suspension was stirred for a further 36 hr. Work-up as before gave a mixture (4 mg) of the (*E*)- and (*Z*)-aldehydes, that were separable on the apolar PS-089 column. This material, which also contained traces of the acetylenic alcohol (1–4), was not combined with the main product, but could be used for retention time comparison with the natural (*Z*)-aldehyde **(45)**. (*E*)-8-methyl-2-nonenal **(47)**. MS: 136 (5), 121 (18), 111 (10), 110 (7), 107 (8), 97 (20), 95 (23), 93 (15), 83 (44), 81 (20), 70 (58), 69 (50), 57 (43), 55 (57), 43 (98), 41 (100), 39 (52), 29 (32). ^{13}C NMR ($CDCl_3$): $\delta = 22.39$ (q, C-9, C-8 Me), 26.72 (t), 27.69 (d, C-8), 27.94 (t), 32.65 (t, C-4), 38.49 (t), 133.15 (d, C-2), 159.27 (d, C-3), 194.53 (d, C-1). (Z)-8-Methyl-2-nonenal (**45**). MS: 136 (4), 121 (13), 111 (7), 110 (5), 107 (7), 97 (13), 95 (15), 93 (10), 83 (39), 81 (14), 70 (55), 69 (47), 57 (40), 55 (66), 43 (100), 41 (97), 39 (49), 29 (34).

*2-Methyl-2,3-Epoxypentane (Ethyl-2,2-Dimethyloxirane) (**8**).* 2-Methyl-2-pentene (0.8 g, 9.5 mmol) was added in portions over 20 min to a stirred solution of *m*-chloroperbenzoic acid (MCPBA), (1.9 g, 11 mmol) in chloroform (20 ml) at 0°C (Vogel, 1989). The mixture was stirred overnight in the dark at room temperature, cooled to −10°C and the *m*-chlorobenzoic acid filtered off. The filtrate was washed with $NaHCO_3$ solution (10 ml, 10%), dried over anhydrous sodium sulfate, and concentrated to give the title compound. MS: 85 (34), 71 (10), 59 (100), 58 (85), 57 (53), 43 (86), 42 (80), 41 (89).

Linalool Oxides. cis- and *trans*-Furanoid linalool oxides (**32a**) and (**32b**), were prepared from linalool according to Scheme 2 (Tang et al., 1995). Linalool (0.15 g, 1 mmol) was added drop-wise over 15 min to a stirred solution of MCPBA



* Only one enantiomer of each isomer is shown.

SCHEME 2. Synthesis of furanoid and pyranoid linalool oxides.

(0.21 g, 1.2 mmol) in CH_2Cl_2 (8 ml) at 0°C. The mixture was stirred 5 hr at 0°C and overnight at room temperature. The solids were filtered off, and the filtrate was washed with aqueous NaOH (10%) and brine, and concentrated to give a mixture of *cis*- and *trans*-furanoid linalool oxides, (**32a**) and (**32b**), and *cis*- and *trans*-pyranoid linalool oxides, (2-**2a**) and (2-**2b**), in a ratio of 42:45:7:6, respectively. The furanoid linalool oxides, (**32a**) and (**32b**) have identical mass spectra. MS: 155 (7), 137 (6), 111 (26), 94 (40), 93 (31), 83 (14), 81 (16), 79 (12), 72 (6), 68 (27), 67 (29), 59 (100), 55 (41), 43 (57), 41 (34), as do the *cis*- and *trans*-pyranoid linalool oxides (2-**2a** and 2-**2b**). MS: 155 (7), 143 (2), 137 (5), 109 (6), 94 (71), 83 (15), 81 (16), 79 (23), 68 (100), 67 (67), 59 (69), 55 (28), 43 (65).

RESULTS AND DISCUSSION

To obtain reliable information on the composition of the interdigital secretion of the red hartebeest, three different gas chromatographic sample introduction techniques were used: (1) split injection of an extract of the secretion, (2) SPME, and (3) solventless sample introduction. Because the quality of the GC information and mass spectra obtained from the extract was by far the best, chromatographic and mass spectral data obtained with this method were used as reference data in the characterization of the secretion. Split injection was also the preferred injection technique because it was difficult to match retention times of the natural and synthetic reference compounds with the solventless sample introduction technique. However, because no solvent is used in the other two sample introduction techniques, they provided valuable information on early eluting compounds that were obscured by the solvent peak when extracts were analyzed.

A typical TIC of an interdigital secretion extract of a male hartebeest obtained with the OV-1701 column is shown in Figure 1. To obtain information on constituents that might coelute from this column, GC-MS analyses were also done with the apolar PS-089 column. The differences in peak shape and order of elution from the two columns provided additional diagnostic information. The secretion from females contained the same compounds, albeit in different relative amounts. A TIC of the headspace volatiles collected by SPME is shown in Figure 2A. The compounds identified in the interdigital secretion of the red hartebeest and the diagnostic methods employed in their identification are listed in Table 1.

Tentative identification of components was based on mass spectra comparisons with NBS, NIST, LECUS, and Wiley libraries. Reduction of samples of the crude secretion with $LiAlH_4$ gave proof of the presence of aldehydes, acids, and ketones, which were reduced to the corresponding alcohols. The molecular masses of a series of saturated and unsaturated aldehydes and carboxylic acids were confirmed by their CI(CH_4) spectra. In some cases, GC-IR provided invaluable information concerning the organic compound class to which some of the

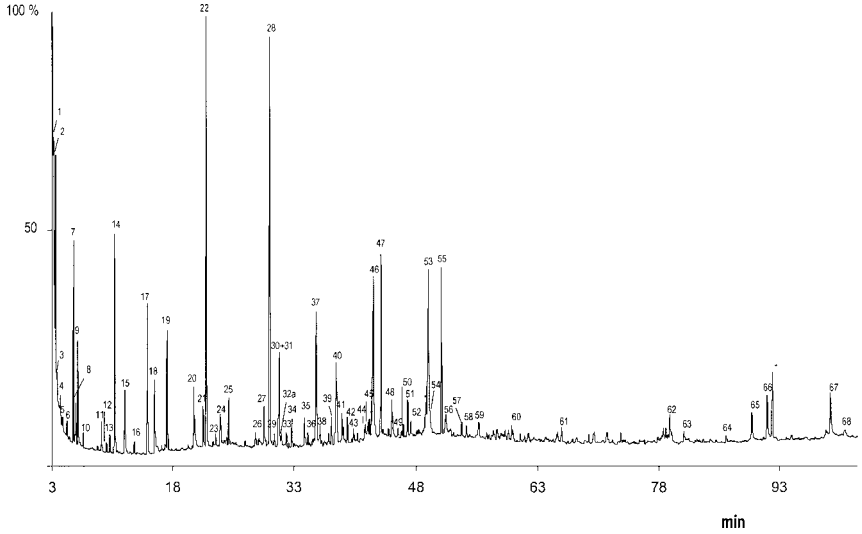


FIG. 1. Total ion chromatogram of an interdigital secretion extract of male red hartebeest, *Alcelaphus buselaphus caama* analyzed on the OV-1701-OH column, programmed at 2°C/min from 40°C to 280°C. Constituents are numbered as in Table 1. *Impurities.

unknowns belonged. Final proof of the structure of most compounds was provided by coinjection of commercially available or synthesized reference compounds.

The main compound classes represented in the secretion were alkanes and isoalkanes, primary alcohols, ketones, straight-chain, branched, and unsaturated aldehydes, carboxylic acids, and cyclic ethers. In addition to the straight- and branched-chain hydrocarbons listed in Table 1, the secretion contains traces of 2-methylalkanes from C_{10} to C_{17}. Methyl branching seems to be characteristic for this secretion as it also was present in other compound classes (e.g., alcohols, aldehydes, and carboxylic acids). Apart from 2-methylundecane, all the alkanes were commercially available.

Compound (**21**) coeluted with synthetic 5-methyl-1-hexanol but the natural compound had a mass spectrum that was also almost identical to that of 3-methyl-1-hexanol. The structure was confirmed to be 5-methyl-1-hexanol by GC-MS analysis of the trimethylsilyl ethers (TMS ethers) of 5-methyl-1-hexanol and the natural compound. The similarity of the mass spectra of constituents (**21**) and (**27**) and the retention time increment between these two compounds suggested that **27** could also be an isobranched primary alcohol. Constituent (**27**) was confirmed as 6-methyl-1-heptanol by coelution of the natural and synthetic compounds.

Aldehydes were the most abundant class of compounds present in the secretion. They are also plentiful in the interdigital secretions of the bontebok,

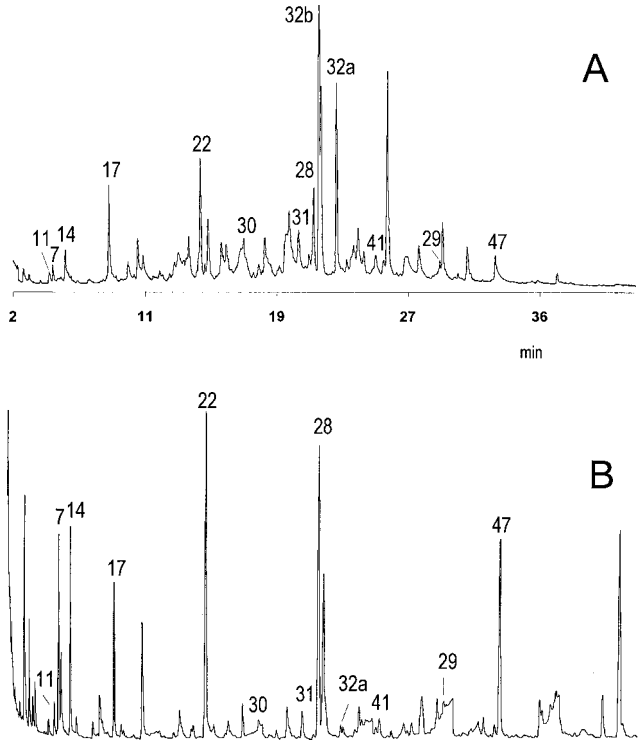


FIG. 2. Total ion chromatogram of (A) an SPME sample of the headspace of the interdigital secretion of male red hartebeest, *Alcelaphus buselaphus caama* and (B) an extract of the secretion. GC conditions for both analyses: PS-089-OH column programmed at 2°C/min from 40°C to 280°C. Constituents are numbered as in Table 1.

blesbok (Burger et al., 1999a,b), and black wildebeest (Burger et al., unpublished manuscript), albeit in lesser amounts. In contrast, aldehydes are rather rare in the preorbital secretions of the antelope that have so far been investigated (Stander et al., 2002).

In addition to straight-chain saturated and monounsaturated aldehydes, a large number of isobranched analogs also were present in the secretion. The unsaturated aldehydes were only tentatively identified because the mass spectra of their DMDS derivatives did not provide unequivocal diagnostic information. The isobranched aldehydes higher than 5-methylhexanal are not commercially available, and they are not represented in mass spectra libraries. Characteristically, the molecular ion is not present in the EI spectra of branched aldehydes. Diagnostic information can, however, be derived from ions at $[M-18]^+$ (loss of H_2O), $[M-28]^+$ (loss of C_2H_4), and $[M-33]^+$ (loss of H_2O plus CH_3). The CI (CH_4) spectra of the

TABLE 1. COMPOUNDS IDENTIFIED IN INTERDIGITAL SECRETION OF THE RED HARTEBEEST

No. in Fig. 1	Compound	EI mass spectral data, *m/z* (%)[a]	Identification	Relative quantities (%) Male	Female
	Alkanes				
2	2-Methylhexane		a,c,g	3.4	2.6
4	Heptane		a,c	0.5	0.3
7	2-Methylheptane		a,c,g,i	2.8	3.6
10	Octane		a,c	0.2	0.3
12	2-Methyloctane		a,c	0.1	0.2
29	2-Methylundecane		a,c	0.3	0.1
	Alcohols				
5	2-Methyl-3-buten-2-ol		a,c,i	0.3	0.2
16	2-Methyl-2-hexanol		a,c	0.2	0.3
21	5-Methyl-1-hexanol		a,c,f,j	1.0	1.0
27	6-Methyl-1-heptanol		a,c,g	0.9	1.0
	Aldehydes				
1	2-Methyl-2-propenal		a,c,g	4.3	0.9
3	Butanal		a,c	0.8	0.1
6	3-Methylbutanal		a,c	0.3	0.2
9	Pentanal		a,b,c,d,g,i	1.7	1.4
11	4-Methylpentanal		a,c,d,i	0.4	0.4
14	Hexanal		a,b,c,d,g,i,j	3.6	3.5
15	3-Methyl-3-butenal		a,b,c,g,i,j	1.4	1.7
17	5-Methylhexanal		a,b,c,d,g,i,j	2.7	1.7
19	Heptanal		a,b,c,d,g	2.1	1.9
22	6-Methylheptanal		a,b,c,d,g,i,j	8.0	5.5
23	(*E*)-2-Heptenal	112 (3), 111 (1), 97 (6), 94 (4), 84 (12), 83 (55), 70 (30), 69 (30), 68 (32), 57 (46), 56 (41), 55 (65), 41 (100)	a,b,c,d,i,j	0.3	0.3
25	Octanal		a,b,h	0.9	0.9
28	7-Methyloctanal		a,b,d,g,h,i	8.2	8.1
31	(E)-2-Octenal	111 (3), 108 (3), 98 (13), 97 (13), 95 (9), 93 (9), 83 (58), 82 (42), 70 (83), 69 (42), 58 (57), 57 (56), 55 (88), 43 (72), 41 (100), 39 (55)	a,c,h	*b*	*b*
34	Nonanal		a,b,c,d,i,j	0.6	0.4
38	(*E*)-7-Methyl-2-octenal	125 (5), 122 (3), 107 (26), 97 (13), 96 (24), 95 (9), 84 (12), 83 (34), 81 (26), 70 (63), 69 (43), 57 (32), 56 (32), 55 (59), 43 (94), 41 (100)	a,b,g,h	0.5	0.6
39	8-Methylnonal		a,b,c,d,g	0.7	0.5
41	(*E*)-2-Nonenal	122 (1), 112 (2), 111 (6), 97 (13), 96 (18), 83 (45), 70 (62), 69 (44), 57 (37), 55 (80)	a,b,c,d,g,i	0.6	0.7

TABLE 1. CONTINUED

No. in Fig. 1	Compound	EI mass spectral data, *m/z* (%)[a]	Identification	Relative quantities (%) Male	Female
45	(*Z*)-8-Methyl-2-nonenal	136 (2), 121 (11), 111 (8), 110 (7), 107 (6), 97 (11), 95 (16), 93 (11), 83 (54), 81 (15), 71 (68), 70 (69), 69 (41), 57 (39), 55 (69), 43 (83), 41 (100)	a,b,c,g,h	0.5	0.7
47	(*E*)-8-Methyl-2-nonenal	136 (2), 121 (14), 111 (8), 110 (6), 108 (5), 107 (7), 97 (16), 95 (19), 93 (12), 84 (10), 83 (33), 81 (15), 79 (10), 70 (49), 69 (44), 67 (18), 57 (44), 56 (33), 55 (57), 43 (100), 41 (96)	a,b,c,g,h,i	4.0	5.6
50	(*E*)-2-Decenal	136 (1), 121 (4), 111 (8), 110 (9), 107 (8), 98 (16), 97 (17), 83 (40), 70 (70), 69 (38), 57 (33), 56 (34), 55 (74), 43 (70), 41 (100)	a,b,c,h	0.3	0.4
52	Unidentified (nonadienal)	137 (1), 111 (12), 96 (52), 95 (59), 81 (19), 68 (36), 67 (100), 65 (13), 59 (27), 55 (33), 53 (31), 43 (63), 41 (67), 39 (53)	a,b,g	0.5	0.5
54	(*Z*)-9-Methyl-2-decenal	150 (2), 135 (11), 124 (8), 111 (8), 109 (7), 107 (7), 98 (10), 97 (13), 95 (17), 85 (62), 83 (87), 81 (41), 70 (100), 69 (49), 67 (23), 57 (57), 55 (66), 43 (65), 41 (79)	a,b,g,h	0.8	1.2
55	(*E*)-9-Methyl-2-decenal	150 (3), 135 (19), 124 (7), 121 (6), 111 (12), 109 (9), 107 (12), 97 (20), 95 (20), 83 (70), 82 (31), 70 (93), 69 (76), 67 (29), 57 (91), 55 (78), 43 (86), 41 (100)	a,b,g,h	3.8	5.7
57	(E)-2-Undecenal	150 (2), 124 (10), 121 (17), 111 (11), 98 (16), 97 (18), 83 (64), 82 (35), 70 (100), 69 (44), 57 (64), 56 (23), 55 (69), 43 (39), 41 (78)	a,g,h	0.3	0.6
	Ketones				
20	3-Methyl-2-hexanone	85 (4), 72 (25), 57 (11), 55 (3), 43 (100)	a,b,h	1.6	3.6
	Acids				
24	Pentanoic acid		a,b,c,d	0.7	1.0
30	Hexanoic acid		a,b,c,d	2.8[b]	3.0[b]
40	Heptanoic acid		a,b,c,d,j	2.9	3.6
46	6-Methylheptanoic acid		a,b,d,e,j	6.8	3.8
48	Octanoic acid		a,b,c,d,j	1.1	0.8

TABLE 1. CONTINUED

No. in Fig. 1	Compound	EI mass spectral data, *m/z* (%)[a]	Identification	Relative quantities (%) Male	Female
53	7-Methyloctanoic acid		a,b,d,e,j	6.3	3.9
56	Nonanoic acid		a,b,c,d,j	0.8	0.3
63	Tetradecanoic acid		a,c	0.4	—
64	Pentadecanoic acid		a,c	[c]	—
65	14-Methylpentadecanoic acid		a,b,c,d	1.0	1.6
66	Hexadecanoic acid		a,b,c,d	1.8	1.6
67	16-Methylheptadecanoic acid		a,b,c,d	2.1	4.1
68	Octadecanoic acid		a,b,c,d	0.3	1.9
44	Cyclohexanecarboxylic acid		a,c,g	0.5	0.6
	Cyclic ethers				
8	2-Methyl-2,3-epoxypentane	100 (3), 85 (17), 71 (19), 59 (100), 58 (100), 57 (35), 43 (92), 41 (89), 39 (51)	a,c,i	0.6	1.1
32a	*cis*-Furanoid linalool oxide	155 (11), 137 (12), 111 (28), 94 (38), 93 (32), 83 (19), 81 (19), 79 (17), 72 (20), 68 (36), 67 (39), 59 (100), 55 (49), 43 (92), 41 (46)	a,c,i	0.3	0.6
32b	*trans*-Furanoid linalool oxide	155 (5), 137 (8), 111 (30), 94 (44), 93 (42), 83 (18), 81 (30), 79 (19), 72 (9), 68 (39), 67 (45), 59 (100), 55 (42), 43 (63), 41 (33)	a,i	[d]	[d]
	Unidentified				
13	Unidentified	100 (2), 85 (78), 60 (24), 57 (20), 43 (77), 41 (100)		0.4	0.4
18	Unidentified	142 (3), 127 (3), 100 (5), 84 (20), 83 (13), 82 (13), 72 (33), 57 (18), 55 (15), 43 (100)		1.6	2.4
26	Unidentified	83 (5), 71 (100), 57 (17), 55 (28), 43 (38), 41 (44)		0.4	0.1
33	Unidentified	102 (11), 100 (6), 85 (5), 71 (12), 61 (22), 59 (92), 57 (13), 43 (100)		0.3	1.4
35	Unidentified	99 (23), 85 (10), 71 (100), 59 (20), 53 (16), 43 (94)		0.7	0.9
36	Unidentified	95 (4), 71 (100), 69 (11), 57 (17), 55 (21), 43 (23), 41 (44)		0.3	0.3
37	Unidentified	142 (3), 127 (1), 109 (6), 99 (18), 84 (21), 83 (13), 72 (17), 59 (11), 57 (11), 55 (16), 43 (100)		3.5	4.5

TABLE 1. CONTINUED

No. in Fig. 1	Compound	EI mass spectral data, *m/z* (%)[a]	Identification	Relative quantities (%) Male	Female
42	Unidentified	109 (5), 97 (6), 83 (5), 71 (100), 69 (20), 57 (26), 55 (50), 41 (58)		0.5	0.4
43	Unidentified	127 (9), 109 (22), 100 (5), 85 (22), 83 (18), 69 (5), 67 (7), 58 (25), 43 (100), 41 (39)		0.3	1.0
49	Unidentified	130 (4), 113 (2), 99 (17), 72 (18), 59 (62), 57 (13), 43 (100)		0.3	0.6
51	Unidentified	123 (2), 111 (2), 98 (2), 95 (6), 71 (100), 69 (26), 67 (14), 57 (27), 55 (40), 43 (57), 41 (60)		0.9	0.7
52	Unidentified (a nonadienal)	137 (1), 111 (12), 96 (52), 95 (59), 81 (19), 68 (36), 67 (100), 65 (13), 59 (27), 55 (33), 53 (31), 43 (63), 41 (67), 39 (53)	a,b,g	0.5	0.5
58	Unidentified	177 (8), 139 (7), 123 (24), 109 (8), 98 (20), 95 (20), 83 (34), 81 (19), 71 (98), 69 (43), 57 (39), 55 (85), 43 (100), 41 (90)		0.4	0.2
59	Unidentified	129 (16), 111 (6), 86 (12), 85 (8), 59 (100), 43 (47)		0.5	0.7
60	Unidentified (a decadienal)	125 (8), 109 (10), 97 (51), 95 (23), 85 (75), 81 (100), 79 (35), 71 (28), 67 (31), 59 (97), 57 (32), 43 (74), 41 (98), 39 (59)	a,b,g	0.5	1.8
61	Unidentified	168 (8), 142 (8), 125 (27), 111 (27), 109 (36), 107 (28), 96 (21), 95 (42), 93 (21), 83 (29), 77 (10), 69 (52), 55 (62), 43 (100)		0.5	0.4
62	Unidentified	210 (3), 195 (3), 177 (6), 153 (22), 137 (9), 135 (19), 121 (3), 111 (3), 97 (16), 83 (27), 69 (28), 55 (23), 43 (100)		0.8	1.3

Note: a, GC-MS analysis; b, CI (CH_4) spectra; c, retention time comparison with synthetic compounds; d, reduction with $LiA1H_4$; e, EI-spectra of pyrrolidine derivatives; f, EI-spectra of TMS derivatives; g, GC-IR analysis; h, retention time increment comparison; i, observed with SPME sample enrichment; j, observed with solventless sample introduction.

[a] MS data are included for compounds that were not identified and for a few compound types that are not well represented in spectra libraries.

[b] Coeluting compounds; the % for hexanoic acid includes (*E*)-2-octenal.

[c] Traces.

[d] Observed only with SPME sample enrichment.

branched aldehydes contain a prominent $[M\text{-}17]^+$ ion formed by the loss of water from the protonated molecular ion. All of the constituents under discussion were identified as aldehydes in GC-IR library searches. The available information is reconcilable with these compounds being isobranched aldehydes. This interpretation was confirmed by synthesis of the iso-C_6, C_7, C_8, and C_{10} aldehydes and their coelution with consituents (**11**), (**17**), (**22**), and (**39**). Constituent (**28**) was identified as 7-methyloctanal based on its mass spectrum and retention time increment comparison.

The identification of a series of compounds **(23)**, **(31)**, **(41)**, and **(50)** as straight chain (*E*)-2-alkenals was confirmed by their coelution with the commercially available compounds. In the case of (*E*)-2-undecenal **(57)**, retention time increment correlation was used. (*E*)-2-Octenal **(31)** coeluted with hexanoic acid on the OV-1701 column, but was well separated from other constituents on the PS-089 column.

A series of isobranched α,β-unsaturated aldehydes was present in the secretion. According to their IR spectra, **(47)** and **(55)** appeared to be unsaturated aldehydes. The IR spectra of constituents **(38)**, **(45)**, and **(54)** contained little diagnostic information due to their low concentration. Ions in their EI mass spectra at $[M-18]^+$ (loss of H_2O), $[M-33]^+$ (loss of H_2O plus CH_3), and ions at $[M+1]^+$ and $(M-17]^+$ in their CI(CH_4) mass spectra were used to determine their molecular masses. The *Z*-isomers of these compounds show more prominent ions at *m*/*z* 70 and *m*/*z* 83 than the later eluting *E*-isomers. The structures of **(45)** and **(47)** were confirmed by their GC coelution with synthesized (*Z*)- and (*E*)-8-methyl-2-nonenal, respectively. The identification of **(38)**, **(54)**, and **(55)** as (*E*)-7-methyl-2-octenal, (*Z*)-9-methyl-2-decenal, and (*E*)-9-methyl-2-decenal was based on their mass spectral and retention time data.

Two branched diunsaturated aldehydes could not be fully characterized. According to the (M+1) ions in their CI(CH_4) spectra, compounds **(52)** and **(60)** have molecular masses of 138 and 152, respectively, in agreement with the library search of GC-IR spectra, which gave branched, nonadienal, and decadienal as the most likely candidate structures. The mass spectra of their DMDS derivatives did not contain any useful diagnostic information, and these two constituents remain unidentified.

The mass spectra of constituents **(46)**, **(53)**, **(65)**, and **(67)** were typical of branched fatty acids, but did not contain ions that can be used to determine the position of branching. Comparison of mass spectra and retention times of available fatty acid standards allowed identification of constituents **(65)** and **(67)** as 14-methylpentadecanoic acid and 16-methylheptadecanoic acid, respectively. 6-Methylheptanoic acid **(46)** and 7-methyloctanoic acid **(53)** were converted to pyrrolidine derivatives, both of which have abundant $[M-15]^+$ and $[M-43]^+$ ions typical of pyrrolidine derivatives of isobranched fatty acids (Andersson and

Holman, 1975). These two ions are almost undetectable in the straight chain derivatives.

Figure 2A shows the TIC of the SPME-sampled headspace volatiles of the secretion, analyzed on the apolar PS-089 column. Many overlapping peaks and contaminants complicated the interpretation of the mass spectra obtained in this analysis. However, this chromatogram shows large peaks of compounds with the typical spectra of the *cis*- and *trans*-pyranoid linalool oxides of which only the *cis* isomer could be found in the extracted secretion. These compounds were recently identified in the castor sacs of the North American beaver, *Castor canadensis*, by Tang et al. (1995). The linalool oxides were synthesized as a mixture of isomers by treating linalool with *m*-chloroperbenzoic acid according to Scheme 2 to give the furanoid linalool oxides, (**32a**) and (**32b**), as well as the pyranoid linalool oxides (2-**2a**) and (2-**2b**), the formation of the furanoid oxides being considerably favored. The *cis*-furanoid linalool oxide and constituent **(32a)** coeluted on GC, proving its identity. It was concluded that constituent (**32b**), which was not found in extracts but was detected in the headspace of the secretion, is the *trans*-furanoid linalool oxide. The order in which the furanoid linalool oxide enantiomers elute from a chiral column coated with OV-1701-OH containing the chiral selector heptakis (2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin, has been determined by Kreis et al. (1996). By comparison of the chiral GC and GC-MS analyses of the natural linalool oxides and the synthetic compounds, *trans*-(2*R*,5*R*)-, *cis*-(2*R*,5*S*)-, and *cis*-(2*S*,5*R*)-linalool oxide were found to be present in the secretion in a ratio of 2.5:1:1.5, respectively, in the headspace of samples of the secretion collected on gauze.

The solventless sample introduction technique used in this study suffers from the problem that it is difficult to control the sample size. This resulted in peak broadening and overlapping of peaks so that it was impossible to obtain interpretable mass spectra from this analysis. Although the information obtained with this method was only used as supporting evidence, the solventless sample introduction of large secretion samples revealed the presence of compounds that were tentatively identified as 2-butylfuran, 2-pentylfuran, and 2-hexylfuran with base peaks at *m/z* 81, and the γ-lactones, hexan-4-olide, heptan-4-olide, and octan-4-olide, with base peaks at *m/z* 85. Because of insufficient diagnostic information in their mass spectra, these identifications remain tentative. Using large sample sizes, several other trace constituents with base peaks at *m/z* 85 were observed in the secretion, but full identification was not possible.

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SYNERGISM OF POLYGODIAL AND *trans*-CINNAMIC ACID ON INHIBITION OF ROOT ELONGATION IN LETTUCE SEEDLING GROWTH BIOASSAYS

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Abstract—A bicyclic sesquiterpene dialdehyde, polygodial did not inhibit root elongation up to a concentration of 12.5 μg/ml in a lettuce seedling assay: *trans*-Cinnamic acid inhibited the elongation by 50% at 1.2 μg/ml (8.1 μM). The inhibitory activity of *trans*-cinnamic acid was enhanced 17-fold when used in combination with 6.25 μg/ml (26.5 μM) of polygodial. A decrease in the pH of the medium was observed during normal seedling growth, indicating transport of protons from the cells by a plasma membrane H^+-ATPase. The inhibitory effect of *trans*-cinnamic acid on the elongation was reduced to some extent in 2 mM phosphate buffer (pH 7.0) during seedling growth. Although polygodial did not inhibit the activity of H^+-ATPase in the plasma membrane fraction of roots in normally growing seedlings, a decrease in activity was found in the fraction obtained from seedlings incubated with 20 μg/ml of polygodial. These results suggest that polygodial functions synergistically with *trans*-cinnamic acid in the inhibition of root elongation via restriction of proton transport from the cytoplasm of germinated cells.

Key Words—Allelopathy, synergism, polygodial, *trans*-cinnamic acid, seedling growth, *Polygonum hydropiper*, lettuce, *Lactuca sativa*, plasma membrane H^+-ATPase.

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INTRODUCTION

Two bicyclic sesquiterpene dialdehydes, polygodial (Barnes and Loder, 1962) and warburganal, have been isolated from the sprouts of *Polygonum hydropiper* (Polygonaceae) and the bark of *Warburgia ugandensis* and *W. stuhlmannii* (Canellaceae). More specifically, polygodial was first isolated as a pungent principle from the fresh sprouts of *P. hydropiper,* termed "tade" and used as a food spice for "sashimi" in Japan (Haraguchi et al., 1993). *P. hydropiper* plants are frequent in wet places like ditches and shore-lines (Staniforth and Bergeron, 1990). Subsequently, warburganal was also isolated from the same fresh sprouts of *P. hydropiper* in minute amounts. Warburganal was first isolated as the principal insect antifeedant, together with polygodial in minute amounts, from *Warburgia* plants (Kubo et al., 1976). The genus *Warburgia* consists of only two species, both of which are widely used as medicinal plants and food spices in Eastern Africa (Taniguchi et al., 1983). Although both warburganal and polygodial were isolated from *Polygonum* and *Warburgia* plants, these genera are taxonomically unrelated. However, we became aware of a natural phenomenon common among these plants. Specifically, they have few plants growing in their vicinity and they grow some distance from each other, indicating the possibility of competition, allelopathy, or both, or simply reflecting the characteristics of the soil. The, *P. hydropiper* community has strong growth potential because it is a rich source of biodiversity in its reservoir of germinable seeds of different species (Staniforth and Bergeron, 1990). In addition, the weed extracts of this species have been reported to inhibit the length of wheat plumule, suggesting a phytotoxic effect (Agarwal et al., 2002).

During our preliminary lettuce seedling assay using *Lactuca sativa*, var. "Grand Rapids," the drimane sesquiterpene dialdehyde polygodial was found to exhibit significant inhibition of germination and root elongation in seedling growth, and it was subjected to further study. Since polygodial appeared to be a more potent inhibitor when used in combination with other allelochemicals, the current study was performed. Here we report on the plant growth inhibitory activity of polygodial, describe its interaction with a common plant allelochemical *trans*-cinnamic acid, and speculate on the mechanism of this inhibition. Acquisition of such knowledge may ultimately provide a rational and scientific basis for the design of safe and effective herbicides.

METHODS AND MATERIALS

Chemicals. Polygodial was extracted and purified from the leaves of *P. hydropiper* as described previously (Kubo and Taniguchi, 1988). *trans*-Cinnamic acid was purchased from Aldrich (Milwaukee, WI). Other chemicals were reagent grade. All chemicals were dissolved in distilled water prior to use in experiments.

Growth Assay of Seedlings. Hypocotyl and root elongation experiments (Kamisaka, 1973; Quayyum et al., 1999) were performed with a slight modification as follows. Lettuce seeds (*Lactuca sativa*, var. "Grand Rapids" from Ferry Morse Co., USA) were germinated on two layers of filter paper moistened with distilled water for 24 hr under continuous fluorescent light at 25°C. Five milliliters of distilled water with a suitable concentration of the test compound was added to each petri dish (diam = 90 mm). Fifty intact seedlings, selected for uniformity, were placed onto two layers of filter paper in each dish. Seedlings was allowed to grow at 25°C under the same light condition as used for germination. After a 3-day incubation, the length of the hypocotyls and roots of the seedlings was measured. This seedling test was repeated at least three times. Values for the length are expressed as the means of lengths ± standard deviations ($N = 150$). IC_{50} was defined as the concentration of each inhibitor that halved the length of the hypocotyls or roots of the control after seedling growth. Combination data were obtained according to the checkerboard method (Norden et al., 1979). The results were used for calculating the fractional inhibitory concentration (FIC) index value of combined compounds. The FIC index is calculated as (IC_{50a}combination/IC_{50a}alone) + (IC_{50b}combination/IC_{50b}alone), where a and b are the two compounds used. The values of FIC indices were used to define the interaction of combined compounds; synergistic ($X \leq 0.5$), additive ($0.5 < X \leq 1$), indifferent ($1 < X \leq 4$), or antagonistic ($X > 4$).

Plasma Membrane ATPase Assay. Two hundred intact seedlings, germinated and grown as described above, were homogenized in a blender in 2 ml of grinding buffer (50 mM Tris–Mes, pH 8.0, 500 mM sucrose, 20 mM EDTA, 10 mM DTT, and 1 mM PMSF). The supernatants obtained from the samples by centrifugation at 20,000*g* for 20 min were used for the preparation of plasma membrane fractions as described by Simon-Plas et al. (1996). Polygodial-treated seedlings were obtained as follows. Two hundred intact seedlings were grown in distilled water containing 20 μg/ml of polygodial. Plasma membrane ATPase activity was measured at 37°C for 30 min in 100 μl of a reaction mixture containing 3 mM ATP, 30 mM Tris–Mes (pH 6.5), 3 mM $MgSO_4$, 50 mM KCl, and 1 mM NaN_3 with or without 500 μM V_2O_5, and the plasma membrane fractions prepared as described above. After incubation, inorganic phosphate liberated in the reaction mixture was measured with the BIOMOL GREEN™ reagent (BIOMOL Research Laboratories, Inc) based on malachite green.

RESULTS AND DISCUSSION

Polygodial completely inhibited the germination of lettuce seeds (*Lactuca sativa*, var. "Grand Rapids") at 50 μg/ml (data not shown). The IC_{50} of polygodial for hypocotyl elongation was 6.0 μg/ml (25 μM, data not shown). Although it

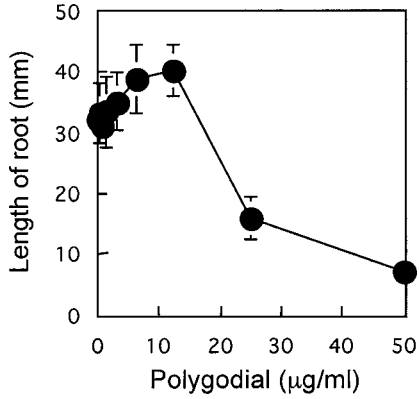

FIG. 1. Effect of polygodial on root elongation during the seedling growth of lettuce. After a 3-day incubation, the root lengths of 150 lettuce seedlings were measured. Values are the means of the length ± standard deviations ($N = 150$).

inhibited root elongation in a dose-dependent manner at concentrations higher than 12.5 μg/ml, a slight promotion of root elongation was observed at concentrations below 12.5 μg/ml as shown in Figure 1. The IC_{50} for root elongation was 25 μg/ml. The slight promotion of elongation at concentrations lower than that at which the inhibitory effect on root elongation was observed is well known for many allelochemicals (Rice, 1984).

Roots and seeds of *P. hydropiper* do not contain detectable amounts of polygodial (Hagendoorn et al., 1994). Polygodial-containing organs include the leaves and the petals (Hagendoorn et al., 1994). Generally, terpenoids are released and dispersed at some distance from these organs. Therefore, the concentration of polygodial in the soil surface around a community of *P. hydropiper* plants is thought to be lower than that showing inhibition of root elongation in the above *in vitro* assay. The weak inhibition of elongation caused by polygodial alone is difficult to explain an allelopathic effect of *P. hydropiper*. To enhance the inhibitory activity of polygodial, we investigated the synergism between polygodial and *trans*-cinnamic acid as a model. This acid exists in the roots of many plants, is thought to be released into soil, and is identified as one of the most common allelochemicals produced by higher plants (Bonner and Galston, 1944; Li et al., 1993; Chaves et al., 2001)

First, we confirmed the inhibitory effect of *trans*-cinnamic acid on root and hypocotyl elongation during seedling growth. The IC_{50} of *trans*-cinnamic acid for hypocotyl elongation was 15 μg/ml (101 μM, data not shown). As shown in Figure 2, *trans*-cinnamic acid slightly promoted root elongation between 0.0625 and 0.5 μg/ml suggesting common physiological properties of allelochemicals. The IC_{50} for this acid in root elongation was 1.2 μg/ml (8.1 μM).

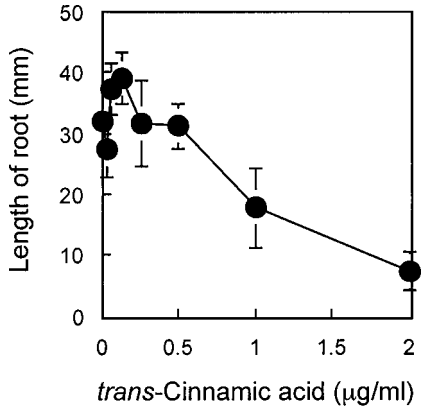

FIG. 2. Effect of *trans*-cinnamic acid on root elongation during the seedling growth of lettuce. After a 3-day incubation, the root lengths of 150 lettuce seedlings were measured. Values are the means of the length ± standard deviations ($N = 150$).

Next, we determined the synergism between polygodial and *trans*-cinnamic acid in root elongation during seedling growth using the checkerboard method (Norden et al., 1979). Synergistic effects, in terms of the IC_{50} for elongation, were observed at 0.78, 1.56, 3.13, and 6.25 μg/ml of polygodial combined with 0.23, 0.1, 0.09, and 0.07 μg/ml of *trans*-cinnamic acid. The FIC indices were calculated as 0.22, 0.14, 0.20, and 0.31, respectively, as shown in Figure 3. In

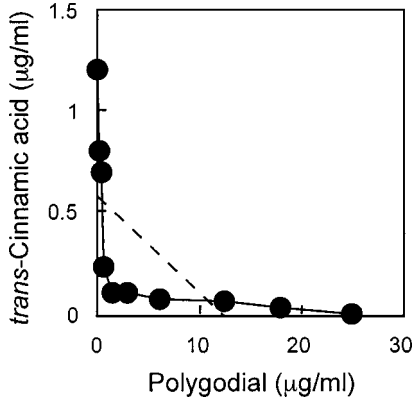

FIG. 3. Synergy between polygodial and *trans*-cinnamic acid in terms of the IC_{50} for root elongation during the seedling growth of lettuce. IC_{50} was defined as the concentration of the combined inhibitors that halved the length of the roots of control after a 3-day iucubation of seedlings. Data belonging to area left under of the dotted line show synergy on the IC_{50} of the root elongation.

other words, the IC_{50} of *trans*-cinnamic acid was enhanced 5-, 12-, 13-, and 17-fold when used in combination with 0.78, 1.56, 3.13, and 6.25 μg/ml of polygodial, respectively. When the IC_{50} values were calculated for other combinations, the effects were determined to be additive. Namely, when concentrations of polygodial or *trans*-cinnamic acid lower than 0.39 or 0.06 μg/ml, respectively, were used, the synergistic effect was not observed, indicating that there are threshold values for inhibition. No significant synergism in the inhibition of hypocotyl elongation of seedlings was observed (data not shown). These results strongly suggest synergism between polygodial and *trans*-cinnamic acid in the inhibition of root elongation during seedling growth in *Lactuca sativa*, var. "Grand Rapids."

We previously reported that polygodial exhibited fungicidal activity (Taniguchi et al., 1978) due to a potent inhibition of a plasma membrane H^+-ATPase, Pma1, in a yeast, *Saccharomyces cerevisiae* (Kubo et al., 2001). The fungicidal activity of sorbic acid, a fungistatic food preservative, against this yeast is enhanced 64-fold in combination with half of the minimum fungicidal concentration of polygodial (Kubo and Lee, 1998). Weak acids such as sorbic acid and benzoic acid in undissociated form enter yeast cells by passive diffusion and then lower the cytoplasmic pH to below the normal physiological range. Excess protons are pumped out by the plasma membrane H^+-ATPase to maintain neutral pH. Polygodial inhibits this pumping process. A decrease in the pH of the medium during germination and the subsequent seedling growth is also thought to indicate the efflux of excess protons produced in the cytoplasm (Young et al., 1998). The H^+-pumping ATPase of the plasma membrane is homologous in fungi and plants (Serrano et al., 1999; Serrano and Rodriguez-Navarro, 2001). Mutant plants of *Arabidopsis thaliana* containing a phloem-specific transgene encoding a plasma membrane H^+-ATPase with an altered carboxy terminus showed improved growth at low pH during seedling development (Young et al., 1998).

Phenolics, including *trans*-cinnamic acid, have also been reported to inhibit plasma membrane H^+-ATPases (Balke and Hodges, 1975). Polygodial in combination with *trans*-cinnamic acid might potentiate the elongation inhibitory effect on seedling growth by potent blockage of proton pumping mediated by plant plasma membrane H^+-ATPases, inducing a decrease in internal pH. However, polygodial did not inhibit ATPase activity in the plasma membrane fraction obtained from the 3-day-incubated seedlings not treated with polygodial (data not shown). A potent inhibitor of this enzyme, vanadate at 500 μM, inhibited the enzyme activity and both hypocotyl and root growth of seedlings completely (data not shown). On the other hand, the ATPase activity in the plasma membrane fraction from the seedlings incubated with 20 μg/ml of polygodial for 3 days was 23% of that incubated without the compound as described in Table 1. This enzyme is tightly regulated at transcription, translation, and enzymatic levels in plants (Morsomme and Boutry, 2000). Our result indicates that polygodial inhibits the expression of plasma membrane H^+-ATPase activity.

TABLE 1. ATPASE ACTIVITIES IN PLASMA MEMBRANE FRACTION OF ROOTS TREATED WITH OR WITHOUT POLYGODIAL

Treatment	ATPase activity	Relative activity (%)
None	213±31[a]	100
Polgodial (20 μg/ml)	49±8	23

[a] Values shown as μmol/mg protein/min are means ± SD of triplicate assays.

We monitored the decrease in pH of the medium during normal seedling growth as shown in Figure 4. The results indicate the possibility of the efflux of protons produced in the cytoplasm of cells into the medium during seedling growth induced by plasma membrane H^+-ATPase activity. The antimicrobial activity of sorbic acid has been reported to be weakened under neutral pH conditions because of the low permeability of the dissociated forms through the plasma membrane (Kubo and Lee, 1998). Next, we examined the effect of *trans*-cinnamic acid on root elongation in 2 mM phosphate buffer (pH 7.0) as shown in Figure 5. This buffer at a concentration higher than 2 mM itself strongly restricted germination of seeds and root elongation during seedling growth (data not shown) indicating the high sensitivity of lettuce seeds to salt stress. Root elongation in this buffer alone was 60% that of the control in deionized water. Phosphate buffer includes sodium and potassium ions. Such stress has been reported to restrict root elongation by disordering ion homeostasis (Serrano and Rodriguez-Navarro, 2001). The use of a medium with low pH has been reported to be necessary for the inhibition

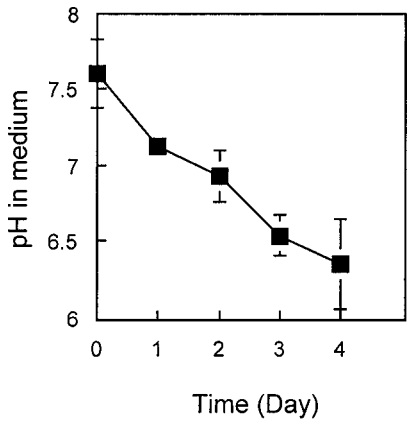

FIG. 4. Decrease in the pH of the medium during normal seedling growth of lettuce. One hundred intact seedlings were planted on two layers of filter paper in 90-mm petri dishes containing 5 ml of distilled water. The petri dishes were sealed tightly until measurement of the pH. Values are means of the pH ± standard deviations ($N = 3$).

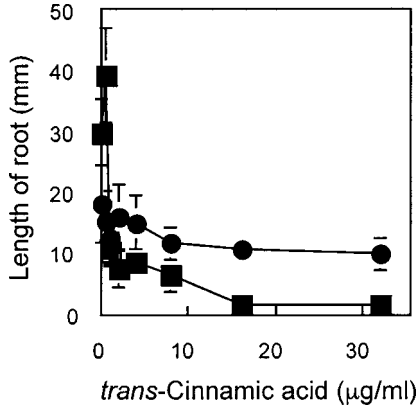

FIG. 5. Effect of 2 mM phosphate buffer (pH 7.0) on root elongation during the seedling growth of lettuce. The medium consisted of distilled water (■) or 2 mM phosphate buffer (pH 7.0, ●). Other experimental conditions were the same as for the growth assay of seedlings.

of K^+ influx in oat roots by phenolic acid (Harper and Balke, 1981). Although we could not directly compare the data obtained in the presence or absence of the buffer components, this buffer significantly weakened the inhibitory activity of *trans*-cinnamic acid against root elongation at concentrations higher than 2 μg/ml. Therefore, to some extent, a fixed neutral pH environment in the seedling growth medium negated the inhibition of root elongation by *trans*-cinnamic acid. This suggests that the undissociated form of *trans*-cinnamic acid is needed for incorporation into the seeds of *L. sativa*, var. "Grand Rapids" and expression of the inhibitory action.

Many phenolic acids have been identified in soils at low concentrations (Blum et al., 1989; Lehman-Mary and Blum, 1999). Many plant organs contain phenolics. Phenolics in seeds could control germination and seedling growth, but roots and seeds do not contain detectable amounts of polygodial in *P. hydropiper* (Hagendoorn et al., 1994). Polygodial-containing organs include leaves and petals (Hagendoorn et al., 1994), and polygodial secreted from leaves might express an allelophathic effect in coordination with phenolics on the soil surface around *P. hydropiper* plants or in seeds (Smolarz-Helena, 2000). Our results may explain why this species grows in communities apart from other species, but it is not an invasive colonizer (Sultan, 2001).

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CORRELATION BETWEEN PHYTOTOXICITY ON ANNUAL RYEGRASS (*Lolium rigidum*) AND PRODUCTION DYNAMICS OF ALLELOCHEMICALS WITHIN ROOT EXUDATES OF AN ALLELOPATHIC WHEAT

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Abstract—An improved allelopathic correlation between phytotoxicity measured in root growth bioassay upon annual ryegrass (*Lolium rigidum* Gaud.) and the concentrations of a selection of dynamically produced allelochemicals quantified in the root exudates of cv. Khapli wheat (*Triticum turgidum* ssp. *durum* (Desf.) Husn.) monitored during the first 15 days of wheat seedling growth in a sterile, agar–water medium, has been established. Changes over the 15-day growth period in the quantities of five exuded benzoxazinones and seven phenolic acids were measured simultaneously using GC/MS/MS. Substantiating pure compound dose–response measurements were conducted over a range of concentrations for the putative allelochemicals within the wheat exudates. One synergism-based proposal using the monitored compounds to explain the observed low-exudate-concentration phytotoxicity was explored, but was found to be experimentally inadequate.

Key Words—Allelopathic correlation, wheat root exudate, production dynamics, benzoxazinones, phenolic acids, allelochemicals, synergism, GC/MS/MS.

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INTRODUCTION

Crop plant root exudation has been chemically investigated from as early as 1921 when Lyon and Wilson (1921) studied the liberation of organic substances through the roots of growing plants. Rovira (1969) has written a review covering plant root exudation. Allelopathic secondary metabolites such as α-methoxyphenylacetic acid have been observed to move from one plant to another through their root systems (Preston et al., 1954), and as one of the important world crops, the root exudates from wheat have become a focus of allelopathic study.

The literature on wheat (*Triticum* spp.) contains a number of studies on its allelochemicals, singling out such compound types as the benzoxazinoids, the phenolic acids, and scopoletin (Baghestani et al., 1999) as the main active constituents. Some studies deal with the phenolics alone (e.g., Guenzi et al., 1966; Lodhi et al., 1987, Baghestani et al., 1999; Wu et al., 2001a,b), some with benzoxazinoids alone (e.g., Copaja et al., 1991; Perez and Ormeno-Nunez, 1991; Petho 1992; Wu et al., 2001c), while some deal with both phenolics and benzoxazinoids (e.g., Wu et al., 1999, 2000a). A small number of wheat studies include the production dynamics of allelochemicals (e.g., Argandona et al., 1981; Zuniga et al., 1990; Nicol et al., 1992; Nakagawa et al., 1995, Copaja et al., 1999), while a smaller number focus upon wheat root exudates (e.g., Perez and Ormeno-Nunez, 1991; Petho, 1992; Kobayashi, et al., 1996; Baghestani et al., 1999; Wu et al., 2000a,c). To our knowledge, no wheat study has been published that attempted to deal simultaneously with the dynamics of both phenolic acids and benzoxazinoids production within root exudate.

The significance of root exudates to plant–plant allelopathy lies in the fact that soil-borne exudates are the main source of contact between donor and receiver plants. Any serious examination of crop plants with allelopathic potential for weed control must necessarily focus upon exudate content. A simplistic quantification of allelochemical levels inside donor plant roots alone is not adequate (Perez and Ormeno-Nunez, 1991; Niemeyer and Perez, 1995; Wu et al., 2000a) because exudation appears to be an active metabolic process (Wu et al., 2001c) dependent upon genetic factors. Thus, of a global selection of 58 wheat cultivar exudates analyzed (Wu et al., 2001c), only 11 were found to exude detectable amounts of the hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), one of the main allelochemicals of wheat, despite the fact that 57 of those cultivars had measurable quantities of DIMBOA within their roots. Of the 58 cultivars, one in particular exuded relatively high concentrations of both phenolic acids and DIMBOA, viz., cv. Khapli (*Triticum turgidum ssp. durum* (Desf.) Husn.), an Indian wheat. Khapli, therefore, presented a favorable donor target for indepth allelochemical studies toward receiver annual ryegrass (*Lolium rigidum* Gaud.), and as a source of germplasm possibly suitable for breeding new wheats of high quality, high yield, and weed inhibiting ability.

Whether phenolic acids actually play a role in wheat allelopathy remains a vexing question. Presently, two schools of thought exist. One is based upon a large body of published material containing circumstantial evidence suggesting the assumption to be correct, while the second withholds belief until the definitive experiments are performed and proof of cause–effect convincingly demonstrated. That *Triticum aestivum* L. is allelopathic toward *L. rigidum* Gaud. (Wu et al., 2000b) and exudes phenolic acids from the roots is not contended (Kobayashi et al., 1996; Baghestani et al., 1999, Wu et al., 2000a, 2001b, 2002), and neither is the individual acids' phytotoxicities, as measured against a range of test plants (e.g., Rasmussen and Einhellig, 1979; Liebl and Worsham, 1983; Lodhi et al., 1987; Blum et al., 1992; Schulz et al., 1994). However, a point of contention surrounds the perception that previous dose–response studies performed on pure individual phenolic acids, such as ferulic or coumaric, require concentrations at 1.0 mM or above to cause significant toxicity, whereas the actual concentrations associated with real plant sources measured experimentally are less than this by a factor of 10–1000. A possible answer to this apparent concentration requirement is the idea of synergism (or multiple additivities), whereby in the usual plant-produced mixture, the constituents combine their biological activities to produce a concerted effect equivalent to a much higher concentration of the most active compound. A limited amount of support exists for this idea (e.g., Asplund, 1969, Rasmussen and Einhellig, 1979). Certainly, the concept of synergistic enhancement of biological activity has been long accepted in agriculture and applied to herbicidal mixtures (Sharma et al., 1982; Simpson and Stoller, 1996). However, the occurrence is rare, and determining the applicability of synergism to plant exudates containing a hundred or more components is a daunting task not yet known to have been undertaken; insufficient evidence presently exists to accept this explanation for any low-concentration phytotoxicity of phenolics.

Although the literature contains no reports (Eljarrat and Barcelo, 2001) on the simultaneous determination of the two distinct groups of allelochemicals in wheat (benzoxazinoids and phenolic acids) apart from our own limited results incorporating DIMBOA using a GC/MS/MS method (Wu et al., 1999, 2000a), we recognized the ability of this technique to handle simultaneously a range of compounds from both groups (Schulz et al., 1994) and to open new ground for exploring any causal relationship that might exist at the molecular level between observed phytotoxic effect in bioassay and the putative allelopathic agents.

The work of Petho (1992) and our own earlier unpublished findings on the benzoxazinones present in exudates from *Triticum aestivum* pointed to the presence of such constituents as DIMBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy,1,4-benzoxazin-3-one (HMBOA), and 2,7-dihydroxy-1,4-benzoxazin-3-one (DHBOA). Of these benzoxazinones, only DIBOA and DIMBOA are hydroxamic acids (hydroxyl group on the nitrogen atom at position 4). According to Hashimoto and Shudo

(1996), it is only such acids that possess the necessary level of allelochemical activity. However, the other three benzoxazinones have weak activity (Friebe, 2001) and for caution's sake deserved not to be automatically discounted in any causative exploration, particularly as Macias et al. (2002) found the lactam 2-deoxy-HMBOA to show hydroxamic acid-like activity against *L. rigidum*. All five benzoxazinones therefore, were, chosen, together with the seven phenolic acids of our earlier work (Wu et al., 2000a), for determination by GC/MS/MS on the exudate from Khapli wheat grown in a neutral, sterile, agar–water medium.

Our aims were to: (1) monitor changes in the quantities of identified phenolic acids and benzoxazinones in the exudates of seedlings of an allelopathic wheat during the course of the first 15 days growth in sterile agar–water; (2) record the accompanying changes in levels of phytotoxicity by bioassay against root growth of annual ryegrass; (3) investigate any correlation between wheat exudate allelochemical concentrations and the corresponding observed phytotoxicity on ryegrass; and (4) substantiate the phytotoxicities of the selected individual phytotoxins toward annual ryegrass using pure compound dose–response measurements.

METHODS AND MATERIALS

Plant Material. Seeds of wheat (*T. turgidum* ssp. *durum* cv. Khapli) [AUS #378] were obtained from the Australian Winter Cereals Collection, Tamworth. Seeds of annual ryegrass (*Lolium rigidum* Gaud.) were obtained commercially. Agar technical was purchased from Amyl Media Pty Ltd.

Sterilization and Pregermination. The pretreatment of wheat and ryegrass seeds has been previously described (Wu et al., 2000c). Wheat seeds were surface-sterilized by soaking them in 70% ethanol for 2.5 min, followed by four rinses with sterilized distilled water. Seeds were soaked in 2.5% sodium hypochlorite solution for 15 min, followed by five rinses with sterilized distilled water. The surface-sterilized seeds of wheat and ryegrass were each soaked in sterilized water for imbibition in light at 25°C for 24 hr and then rinsed with fresh sterilized water. Finally, wheat seeds were incubated in light at 25°C for another 24 hr, and ryegrass seeds incubated in light at 25°C for 48 hr.

Bioassay of Wheat Exudates. The method described previously (Wu et al., 2000a) was slightly modified to assess the allelopathic activity of wheat seedling exudates. Glass beakers (500 ml) containing 30 ml of 0.3% agar–water (nonutrients) were autoclaved. Twenty-four pregerminated wheat seeds were uniformly selected and aseptically sown on the agar surface in each beaker. Beakers were wrapped with parafilm to prevent contamination and evaporation from the agar surface and placed in a controlled environment growth cabinet with an L/D cycle of 13 hr/11 hr and a temperature cycle at 25/13°C. The fluorescent light intensity in the cabinet was 42.72 ± 1.92 μEm^{-2} s^{-1} (PAR) (Quantum/Radiometer/Photometer, model LI-185B, LI-COR, Inc.). Wheat seedlings were allowed to grow for 2, 4,

6, 8, 11, and 15 days. Seedlings were carefully removed from the agar, and 10 pregerminated seeds of ryegrass (*L. rigidum*) were aseptically sown onto each of the respective agar surfaces. Ryegrass sown on agar–water with no wheat beforehand was used as a control. Experiments were arranged in a randomized complete block design with three replicates. Ryegrass-containing beakers were wrapped with parafilm and placed back into the growth cabinet, and the seeds were allowed to grow for 7 days. At the end of this period, the lengths of ryegrass roots and shoots were determined as the means of the three replicates.

Collection of Root Exudates from the Agar Medium. Methods of collection and extraction of allelochemicals for wheat root exudates were identical to those reported previously (Wu et al., 1999). After the growth of 24 wheat seedlings in 0.3% agar–water medium (nutrient-free) for 2, 4, 6, 8, 11, and 15 days, the wheat seedlings were uprooted from the soft growth medium and their roots rinsed twice with 5-ml distilled water to remove any residual agar. Each of the respective washings was returned to its corresponding agar–water medium. The agar–water was collected, adjusted to pH 3.0 by dropwise addition of 0.01 M HCl, stirred thoroughly, and sonicated at 5°C for 15 min. The agar medium was extracted three times with 60-ml portions of diethyl ether. Ether layers were combined and evaporated on a rotary evaporator under reduced pressure at 35°C until the volume of residual solution was approximately 2 ml. The ether residue was transferred into a 2-ml minivial, and the solvent was removed by nitrogen gas blow-down.

Preparation of Silyl Derivatives and GC/MS/MS Analysis. Minivials containing agar extract samples were further dried in an air oven at 60°C for 30 min. After each vial had been cooled to ambient temperature, 1.0 ml of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added to each, and then the vial sealed with its septum cap. Vials were shaken well, heated at 60°C for 30 min to complete derivatization, and allowed to cool to ambient temperature in a desiccator over P_2O_5. Silylated samples were analyzed for selected phenolic acids and benzoxazinones under the same set of GC/MS/MS instrument parameters as earlier reported (Wu et al., 2000a). The MS/MS parameters chosen for determination of the five selected benzoxazinones (under identical GC conditions as for phenolics) are as stated in Table 1. Allelopathic compounds found in the wheat agar extracts were identified by comparing their retention times and daughter mass spectra with those in the user-library created earlier from authentic reference compounds, kindly supplied by Prof. D. Sicker, University of Leipzig. Quantifications were carried out in triplicate using multipoint external standard calibration curves based upon the same reference compounds.

Pure Compound Dose–Response Data. Defined concentrations of four benzoxazionones and five phenolic acids were tested for biological activity against annual ryegrass. Bioassays were performed in 9-cm petri dishes lined with one layer of filter paper, where 12 pregerminated ryegrass seeds were uniformly

TABLE 1. ION-TRAP MS/MS METHOD PARAMETERS FOR BENZOXAZINONES[a]

Benzoxazinone[b]	Retention time (min)	Precursor ion (m/z)	Excitation: Nonresonant amplitude (V)	Excitation: Storage level (m/z)	Quantifying product ion (m/z)
HBOA	13.04	309	50	75	266
DIBOA	15.73	310	50	75	164
HMBOA	17.28	339	46.5	75	222
DHBOA[c]	18.65	397	49	75	280
DIMBOA	20.18	340	46.5	75	194

[a] Mass isolation window (m/z) was set at 3 and excitation time at 20 ms.
[b] Identified as trimethylsilyl derivatives (TMS).
[c] Identified as *tris*-TMS derivative ($M = 397$) by mass spectral comparison with Woodward et al. (1979) and estimation based upon calibration for HMBOA.

distributed after complete evaporation of the solvents. Controls received only pure solvent. After 7 days of incubation in 4.0 ml water at 25°C; 15 hr light/9 hr dark cycle, measurements were taken of the ryegrass root lengths. Assays were repeated four times.

RESULTS

Allelopathic Effect. The effect of wheat root exudate upon the growth of annual ryegrass roots (means of three replicates) across a 15-day period of juvenile wheat growth is shown in Figures 1–3. While ryegrass root growth was inhibited

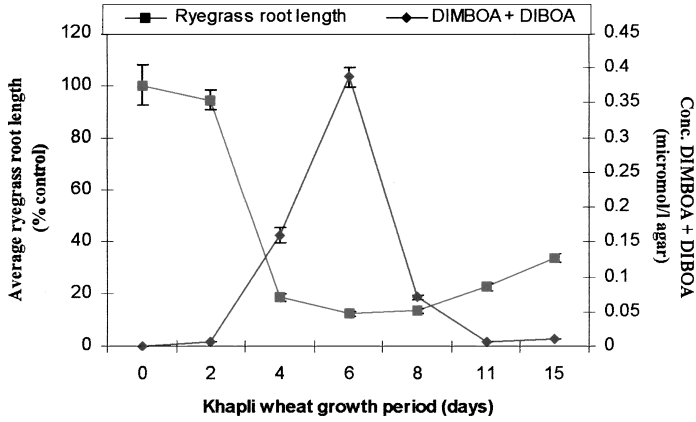

FIG. 1. Relation between phytotoxic effect on ryegrass root length and concentration of active hydroxamic acids in cv. Khapli wheat root exudate (means ± standard errors). Absence of error bars indicates that error bars are smaller than the symbol representing the mean.

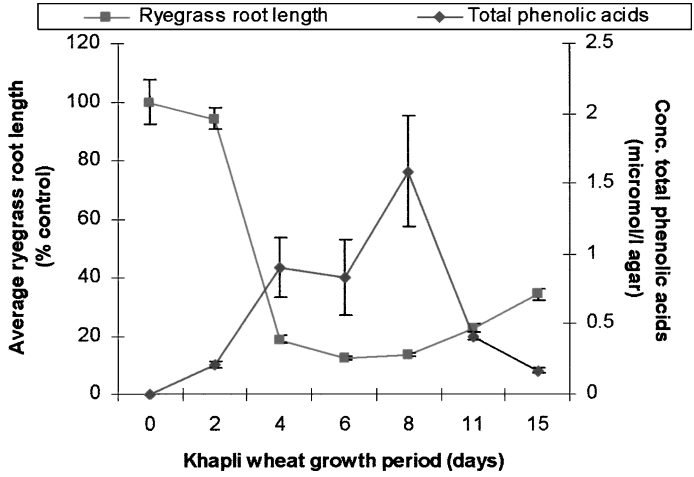

FIG. 2. Relation between phytotoxic effect on ryegrass root length and concentration of total phenolic acids in cv. Khapli wheat root exudate (means ± standard errors). Absence of error bars indicates that error bars are smaller than the symbol representing the mean.

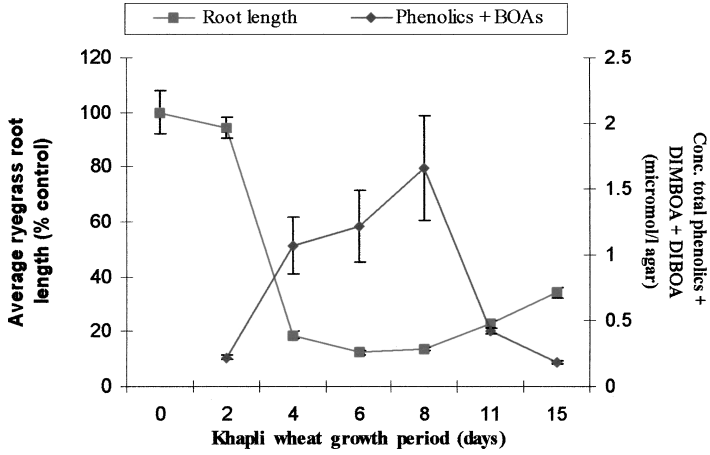

FIG. 3. Relation between phytotoxic effect on ryegrass root length and concentration of total measured phenolics + DIMBOA + DIBOA in cv. Khapli wheat root exudate (means ± standard errors). Absence of error bars indicates that error bars are smaller than the symbol representing the mean.

to a greater extent than shoot, both root and shoot inhibition was greatest in the day 6 to day 8 zone. This maximum coincides with the highest levels of measured allelochemicals (Table 2).

The parallels that exist between measured allelochemical concentrations in the wheat growth medium and the concurrent growth inhibition in the root length of test plant annual ryegrass are shown in Figures 1 and 2. Figure 1 shows an exact coincidence between the maxima of hydroxamic acids concentration and ryegrass root inhibition on day 6, while Figure 2 shows that the total measured phenolic acids maximize on day 8, a little later than maximum inhibition. The relation between ryegrass inhibition and total hydroxamic acids plus phenolic acids appears in Figure 3, where it is evident that as the combined allelochemical concentration drops in the latter days, so the allelopathic effect upon ryegrass roots also declines. These results suggested an association of the 12 measured allelochemicals with the phytotoxicity observed.

Quantitation of Allelochemicals. The concentrations of benzoxazinones and phenolic acids in Khapli wheat exudate at 6 different time periods across the first 15 days of seedling growth are given in Table 2. Both DIBOA and DIMBOA reached their highest concentrations on day 6 (0.146 ± 0.018 μmol/l and 0.241 ± 0.015 μmol/l agar–water, respectively. The highest level found for any of the benzoxazinones was 0.424 ± 0.023 μmol/l on day 8 for HBOA. All benzoxazinones reached maximal concentrations in the day 6 to day 8 zone before declining. Individual phenolic acids had more variability across the 15-day observation period, however the total concentration of phenolic acids showed a similar (though broader) maximum between day 4 and day 8. The highest combined concentration of measured allelochemicals from both classes occurred at day 8 with 2.312 ± 0.682 μmol/l agar.

Correlation between Allelochemical Concentration and Phytotoxicity. Prompted by the above parallels, a wide range of both single and multivariate regressions were attempted between the dependent variable (Y) "relative phytotoxicity" (defined as ryegrass root control length divided by ryegrass root test length under bioassay), and a range of independent variables (Xn) all involving some measure of allelochemical concentration within the agar wheat growth medium, and arranged in (nonchronological) order of ascending magnitude. The resulting plot represented a natural dose–response curve. After statistical testing, the best regression took the form of a "relative phytotoxicity" versus Ln (total phenolic plus hydroxamic acids) plot (one variate, nine compounds), which for a linear model gave $r^2 = 0.914$ ($P < .005$) (see Figure 4). Taken together, the concentrations of the seven phenolic acids plus DIBOA plus DIMBOA explain 91% of the variation in growth inhibition of annual ryegrass.

Response of Ryegrass to Pure Allelochemicals. Table 3 presents the results of the pure compound dose–response treatments for five phenolic acids (*cis* and *trans* isomers combined as one) ranging from 10^{-8} to 10^{-2} molar, based upon ryegrass root length measurements (4 replicates). Similar results appear for the

TABLE 2. QUANTIFICATION OF BENZOXAZINONES AND PHENOLIC ACIDS IN WHEAT SEEDLING EXUDATES[a]

Compound	2 Days[b]	4 Days	6 Days	8 Days	11 Days	15 Days
HBOA[c]	0.042 ± 0.002	0.189 ± 0.027	0.168 ± 0.022	0.424 ± 0.023	0.082 ± 0.016	0.129 ± 0.009
DIBOA	0.006 ± 0.001	0.083 ± 0.017	0.146 ± 0.018	0.070 ± 0.004	0.006 ± 0.001	0.010 ± 0.000
HMBOA	0.020 ± 0.001	0.092 ± 0.011	0.154 ± 0.020	0.209 ± 0.004	0.115 ± 0.013	0.103 ± 0.012
DHBOA[d]	ND	0.023 ± 0.002	0.026 ± 0.000	0.019 ± 0.000	0.016 ± 0.000	0.016 ± 0.000
DIMBOA	ND	0.076 ± 0.010	0.241 ± 0.015	ND	ND	ND
Total benzoxazinones	0.068 ± 0.002	0.463 ± 0.035	0.735 ± 0.38	0.722 ± 0.024	0.219 ± 0.021	0.258 ± 0.015
p-Hydroxybenzoic acid	0.031 ± 0.010	0.430 ± 0.353	0.418 ± 0.453	1.158 ± 0.680	0.051 ± 0.003	0.029 ± 0.011
Vanillic acid	0.012 ± 0.005	0.046 ± 0.032	0.030 ± 0.033	0.016 ± 0.004	0.048 ± 0.007	0.020 ± 0.006
cis-*p*-Coumaric acid	0.008 ± 0.008	0.037 ± 0.031	0.091 ± 0.083	0.081 ± 0.030	0.022 ± 0.012	0.004 ± 0.003
Syringic acid	0.025 ± 0.009	0.101 ± 0.049	0.077 ± 0.057	0.071 ± 0.020	0.047 ± 0.017	0.017 ± 0.006
cis-Ferulic acid	0.014 ± 0.002	0.027 ± 0.023	0.029 ± 0.026	0.014 ± 0.006	0.027 ± 0.017	0.014 ± 0.002
trans-*p*-Coumaric acid	0.075 ± 0.030	0.136 ± 0.056	0.098 ± 0.055	0.167 ± 0.028	0.098 ± 0.018	0.039 ± 0.011
trans-Ferulic acid	0.050 ± 0.016	0.130 ± 0.064	0.089 ± 0.059	0.083 ± 0.024	0.122 ± 0.033	0.046 ± 0.014
Total phenolic acids	0.215 ± 0.038	0.907 ± 0.370	0.832 ± 0.473	1.590 ± 0.682	0.415 ± 0.047	0.169 ± 0.023

Note: ND = not detected.
[a] Data expressed as mean concentration of compound ± SD in μmol/l agar-water ($N = 3$).
[b] Days growth of cv. Khapli wheat seedlings in sterile agar-water medium, 24 seeds per beaker.
[c] Determined as their trimethylsilyl ethers/esters (TMS).
[d] Estimated from the calibration curve for pure HMBOA.

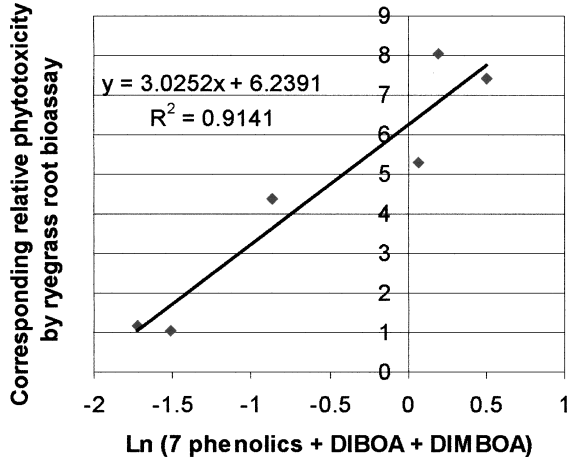

FIG. 4. Correlation between allelochemical concentrations (μmol/l) in Khapli wheat seedlings growth medium and phytotoxicity on annual ryegrass root growth ($P < 0.005$). Plot represents a natural dose–response to measured allelochemicals at points within an observed 15-day wheat growth period.

benzoxazinones whose concentrations ranged from 10^{-8} to 10^{-3} molar (DHBOA unavailable). The phenolic acids affected *Lolium rigidum* in a characteristic manner, exhibiting little phytotoxicity until their individual concentrations reached a value close to 10^{-3} molar (Schulz et al., 1994). In this respect, they are about as active as the natural lactam benzoxazinones HBOA and HMBOA that do not possess the seemingly important OH group on position 4 of the oxazinone ring. As expected, the two hydroxamic acids DIBOA and DIMBOA displayed higher levels of activity, quite noticeable at 10^{-4} molar. However, during the 15-day growth monitoring period, the sum of total phenolics plus total benzoxazinones never exceeded 3.02 μmol/l. This is a concentration 100 times less than what appears to be necessary for equivalent activity with pure allelochemicals applied to ryegrass in isolation. As synergism among these allelochemicals has been offered in the literature as one explanation for high bioactivity from a low-dose mixture, an artificial mixture with the day 6 exudate composition (minus DHBOA which was unavailable) was prepared at 1.55 μmol/l and tested against ryegrass root growth in our usual bioassay. Instead of root inhibition it resulted in a slight stimulation (Table 4). If bioactivity at low concentration on day 6 was due to synergism, we were not able to duplicate the correct mixture composition with the above design. Of course there may be a number of reasons for our not testing the "true synergistic mix," one of which may simply be the fact we chose only nine allelochemicals to combine. There may well be other unknown contributors present in the real exudate that have not been considered and play an essential role.

TABLE 3. DOSE–RESPONSE RESULTS FROM PURE PHYTOTOXIN TREATMENTS OF RYEGRASS SEEDLINGS

	Average root length of ryegrass (mm) (4 replicates)									
Concentration (μmol/l)	0.0 (control)[a]	0.01 (10^{-8} M)	0.1 (10^{-7} M)	1 (10^{-6} M)	10 (10^{-5} M)	100 (10^{-4} M)	1000 (10^{-3} M)	5000 (5×10^{-3} M)	10000 (10^{-2} M)	l.s.d. at 0.05 level
Phytotoxin										
p-Hydroxybenzoic acid	62.7	58.4	61.8	63.8	58.8	58.5	63.8	6.4	0.0	7.0
Vanillic acid	62.7	65.9	67.4	66.9	64.7	67.2	48.7	2.1	0.0	6.9
Coumaric acids	62.7	65.0	72.9	66.8	64.5	65.2	54.9	0.4	0.1	10.2
Syringic acid	62.7	74.4	71.6	68.5	72.6	65.8	63.0	0.2	0.0	4.5
Ferulic acids	62.7	61.5	57.8	62.4	64.4	61.1	59.7	24.6	0.1	4.4
Concentration (μmol/l)[b]	(control)	0.01 (10^{-8} M)	0.05 (5×10^{-8} M)	0.1 (10^{-7} M)	1 (10^{-6} M)	5 (5×10^{-6} M)	10 (10^{-5} M)	100 (10^{-4} M)	1000 (10^{-3} M)	
HBOA	62.7	62.8	65.8	75.4	73.1	69.7	66.2	66.2	49.3	6.2
DIBOA	62.7	69.7	65.2	70.2	68.8	67.3	69.1	53.2	1.7	5.7
HMBOA	62.7	68.8	70.3	69.8	64.3	65.2	70.6	70.2	47.5	5.8
DIMBOA	62.7	61.5	69.2	66.7	73.2	65.2	71.6	45.3	7.4	5.9

[a] Control was distilled water.
[b] DHBOA unavailable.

TABLE 4. ATTEMPTED COPY OF MAXIMUM PHYTOXICITY MIXTURE EFFECT

Phytotoxic compounds	Concentration phytotoxins on day 6[b] (μmol/l agar)	Day 6, % of control ryegrass root length
		15
p-Hydroxybenzoic acid	0.418	
Vanillic acid	0.03	
Coumaric acids	0.189	
Syringic acid	0.077	
Ferulic acids	0.118	
HBOA[a]	0.168	
DIBOA	0.146	
HMBOA	0.154	
DIMBOA	0.241	
Artificial Chemical Mix	Total conc. mix = 1.55	
Artificial Mix Result	Root length ryegrass = 68.5 mm, H_2O control = 62.7 mm (4 replicates)	

[a] DHBOA unavailable.
[b] Maximum phytotoxicity occurred at 6–8 days of Khapli wheat growth.

DISCUSSION

Under sterile agar conditions of the bioassay, the dynamics of exuded benzoxazinones displayed a rapid increase in concentration up to a maximum at 6–8 days of wheat seedling growth followed by a decline to low levels by 15 days (cf. Nicol et al., 1992; Copaja et al., 1999). The reason for the decline was not experimentally pursued, and is not well understood. One explanation could be associated with the limited half-life these compounds possess in near-neutral solution. For example, DIMBOA has a half-life of 5.3 hr at 28°C at pH 6.75 (Woodward et al., 1978) and decomposes to the more stable MBOA (a benzoxazolinone) that also has inhibitory activity, but to a lesser degree (Hashimoto et al., 1996; Burgos et al., 1999; Macias et al., 2002). While the phenolic acids as a group followed a similar overall change during the 15-day monitoring period, the concentration of individual compounds fluctuated. It is likely that their decline toward the end of the experimental period is due to reabsorption by the growing wheat plants—a process observed earlier in wheat Kobayashi et al., (1996). However, if absorption is a possibility, then even in sterile conditions a decarboxylase in wheat roots may be capable of converting *p*-hydroxycinnamic acid derivatives into their corresponding hydroxystyrenes (vinylphenols), which are more phytotoxic than their precursors (Liebl and Worsham, 1983). This could possibly help to sustain the observed effect even after the 15-day wheat growth period when phenolic acid (and benzoxazinone) concentration has become quite low. The present experiments were not designed to test this possibility. The observed decrease in ryegrass root inhibition after 8 days of wheat growth not only correlates with the

concentration of benzoxazinones and phenolic acids found herein, but is consistent with the findings of Schulz et al. (1994) and Macias et al. (2002).

Regarding the failure of the artificial phytotoxins mix to duplicate the response of ryegrass roots at wheat growth day 6 (Table 4), it is unlikely that the two bioassay systems used for comparison—agar/water/seedlings versus filter paper/water/pure phytotoxins—would behave differently, as each system is a simple polysaccharide/water matrix. The difference is more likely accounted for by the additional (mostly unknown) components within the agar assay that come from the wheat exudate. Other possible contributions to this difference may arise from the degree of spontaneous breakdown in the hydroxamic acids that can occur over a longer period of time in the agar system than in the filter paper chemoassay, thereby allowing accumulation of moderately toxic benzoxazolinones (BOA; MBOA) for the commencement of the 7-day ryegrass growth period.

While numerous statistical correlations have been reported in the plant allelopathy literature, only a few attempts have been made to study correlation between observed allelopathy effect and the actual concentrations of specific allelochemicals found in the donor exudate. This is understandable given the complexity of many allelopathic plant products. Such products often contain different primary and secondary metabolites (Vancura, 1964), many of which are unknown, and only a few of which have any recognized phytotoxicity against the test plant of interest. What allelopathic role is played by most of the individual constituents in these phytotoxic mixtures can only be guessed. Considering the large number of exuded compounds and the enormous number of possible combinations of these compounds that would need to be tested for phytotoxicity, the finding of proof for simple synergism (if that is the true picture) between root exudate components as a molecular explanation for plant allelopathy will prove to be a difficult task.

Nevertheless, causative explanations for allelopathic effects at a molecular level are considerably assisted by any significant correlations that can be found that link particular compounds to observed effects. Some limited progress in this area has been made (Ben-Hammouda et al., 1995, Burgos et al., 1999; Wu et al., 2002). The correlation of Burgos et al. (1999) showed a significant ($P = 0.04$), but weak ($r^2 = 0.50$) linear relationship between root growth of *Eleusine indica* (measured as dilutions of *Secale cereale* cultivar extracts causing 50% root growth inhibition), and Ln (DIBOA + BOA) in *Secale cereale* cultivar shoot tissue (μg/g tissue). The weak relationship prompted these workers to comment that "compounds other than hydroxamic acids may also be involved in growth suppression." However, in view of the usual transportation mechanism required in plant allelopathy, plant-parts studies are not as likely as exudate studies to yield strong correlations between observed phytotoxicity under bioassay, and the concentrations of active compounds. Better correlations are more likely found using root exudate concentrations because it is exudate components (or their soil metabolites) that are transported to the receiver plant.

The correlation between allelopathic wheat exudates (including cv. Khapli) and annual ryegrass toxicity that we achieved earlier (Wu et al., 2002) using DIMBOA plus seven phenolic acids in a linear model, 8-variate, multiple regression ($r^2 = 0.504$, $P < 0.01$) indicated a possible additive or synergistic effect among those (and other) compounds. The widening of the compound range in the present study to examine the effect of an additional four benzoxazinones has led to an improved univariate, linear correlation between toxicity on ryegrass and putative allelochemicals in wheat exudate where a significant ($P < 0.005$) and strong ($r^2 = 0.914$) association has been established, based upon the inclusion of one additional hydroxamic acid (DIBOA). Most correlations (uni- or bivariate) attempted between ryegrass toxicity and the two hydroxamic acids alone were unsatisfactory (not significant), with the best using as independent variable Ln (DIMBOA + DIBOA), which resulted in $r^2 = 0.678$ ($P < 0.05$), noticeably below the previous coefficient of determination (0.914) that also incorporates the phenolic acids. This suggests an interactive relationship between both classes of compound in accounting for the observed toxicity on ryegrass. A capacity to account for 91% of test-plant bioassay response in terms of defined molecular concentrations of natural allelopathic agents appears to be one of the best results of its type so far recorded in the allelopathy literature (Belz and Hurle, 2001). It is worth making the comparison between this natural dose–response result using a partially informed selection of exudate components and the artificial extreme obtained by observing the effects of a single pure allelochemical (DIBOA) dose–response treatment of the *Lolium* species *perenne* (Shulz et al., 1994) where a linear regression of "relative phytotoxicity" vs. DIBOA concentration was $r^2 = 0.965$ ($P < 0.001$) (our calculations). The comparison helps place a perspective upon the progress that is being made toward understanding the chemical basis of plant allelopathy.

Nevertheless, further improvement is likely to be gained in the case of Khapli wheat by the future inclusion of other possible bioactive exudate components such as the benzoxazolinones and bioactive coumarins like scopoletin. Scopoletin is a recognized phytotoxin toward Italian ryegrass (*Lolium multiflorum* Lam.) (Fay and Duke, 1977) and is an established constituent of *Triticum aestivum* exudate (Baghestani et al., 1999). Interestingly, Korableva et al. (1969) report that scopoletin is more effective as a growth retardant when used in combination with caffeic acid than when used alone. This fact raises speculation as to the joint roles of the potent phytotoxins (e.g., DIMBOA) and the less active phenolic acids within the overall allelopathic wheat mix. It appears that the primary effect of phenolic acids on plant roots is an alteration of water and nutrient uptake (Blum et al., 1999), perhaps by working through cell permeability, and that this effect has more to do with outer surface contact concentration than with the actual uptake of phenolics. If so, in exuded donor mixtures such as those we examined from wheat, the phenolic acids may be playing a cell permeability role on the root cells of the receiver plant,

and, thereby, opening the way for easier uptake of the more potent components like hydroxamic acids.

Another possiblity lies with the hydroxystyrene phytotoxins (Liebl and Worsham, 1983) that may be formed at the point of exudation from wheat by possible decarboxylase action on various cinnamic acids (Kobayashi et al., 1996) even under sterile conditions, and, therefore, deserves consideration in future study, as do any other newly discovered secondary metabolites in wheat exudate that are known to exhibit phytotoxicity. Further experimentation along these lines is presently being undertaken.

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EFFECTS OF MONOTERPENOIDS, ACTING ALONE OR IN PAIRS, ON SEED GERMINATION AND SUBSEQUENT SEEDLING GROWTH

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Abstract—We compared the potential allelopathic activity of 47 monoterpenoids of different chemical groups, by estimating their effect on seed germination and subsequent growth of *Lactuca sativa* seedlings. Apart from individual compounds, eleven pairs at different proportions were also tested. As a group, the hydrocarbons, except for (+)-3-carene, were the least inhibitory. Of the oxygenated compounds, the least inhibitory were the acetates; whenever the free hydroxyl group of an alcohol turned into a carboxyl group, the activity of the resulting ester was markedly lower (against both germination and seedling growth). Twenty-four compounds were extremely active against seedling growth (inhibiting it by more than 85%), but only five against seed germination. The compounds that were most active against both processes belonged to the groups of ketones and alcohols; they were terpinen-4-ol, dihydrocarvone, and two carvone stereoisomers. We used a model to investigate whether compounds acted independently when applied in pairs. The combined effect varied. In half of the cases, it followed the pattern expected under the assumption of independence; in the rest, either synergistic or antagonistic interactions were found in both germination and elongation. However, even in cases of synergistic interactions, the level of inhibition was not comparable to that of a single extremely active compound, unless such a compound already participated in the combination. The specific structural factors that operate and determine the activity of monoterpenoids still remain rather obscure. The same holds true for the combined effect; its character cannot in general be predicted on the basis of individual compounds acting alone.

Key Words—Allelochemicals, essential oils, allelopathy, phytotoxicity, *Lactuca sativa*, synergism, antagonism.

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INTRODUCTION

There is much literature on the phytotoxic properties of essential oils and pure monoterpenoids, their main constituents. Extensive reviews can be found in Evenari, 1949; Rice, 1974, 1979; Putnam and Tang, 1986; Waller, 1987; Reynolds, 1987; Harborne, 1988; Fischer, 1991. Evidence for considerable water solubility of monoterpenoids (Weidenhamer et al., 1993; Fischer et al., 1994; Li et al., 1998), despite previous beliefs, along with the persistence of their phytotoxicity in some types of soil (Dudai et al., 1999), provide insight into potential use as bioherbicides and a role in vegetation patterning. Regarding the latter, plants with mixtures of different classes of terpenoids, such as essential oils and resins, are associated with certain climatic and vegetation types (Langenheim, 1994). According to Ross and Sombrero (1991), 49% of the essential-oil producing genera, mostly of the families of Labiatae and Compositae, occur in regions with mediterranean-type climate.

The effect of an essential oil on seed germination and seedling growth is often explained in terms of the individual effects of some main constituents. However, an essential oil is a mixture of many compounds in different proportions, and it is often not known whether and how they might interact. In addition, there is considerable variability in the composition of the essential oil of a certain plant species; this variability may be seasonal (Vokou and Margaris, 1986a) or infraspecific, between different populations of the same species (Vokou et al., 1993), or even between individuals of the same population (Kokkini and Vokou, 1989; Tarayre et al., 1995). Given this variability, the overall effect of the essential oil of an aromatic plant cannot be predicted, unless we know both its exact composition and the type of interactions among its constituents. The same holds true, if we are to proceed to formulations of monoterpenoids or essential oils for application in agriculture.

According to An et al. (1993), an allelochemical is assumed to have two complementary attributes, stimulation and inhibition. These attributes need not necessarily have the same sites of action. As concentration changes, the relative dominance of these attributes alters, so determining the overall property. If this is true for one compound, we can assume that the biological activity of a mixture of compounds will be determined not only by their concentrations, but also by the interactions among them. Various researchers have suggested that synergistic actions probably result from combinations of identified allelochemicals, but little research has clearly demonstrated such synergistic action (Einhellig and Rasmussen, 1978). Even now, experiments involving combinations of isoprenoids are few (e.g., Kubo et al., 1992; Weidenhamer et al., 1994); combinations involving different proportions are even rarer.

We examined a large number of individual compounds (a few of them in various isomeric types) belonging to all major groups of monoterpenoids in an attempt to identify structural features, responsible for the activity expressed on germination and subsequent seeding growth, which would consequently allow

prediction of the level of inhibition. The compounds belonged to the chemical groups of hydrocarbons, alcohols, aldehydes, ketones, ethers, and acetates—acyclic or cyclic, with one of more rings—as well as phenols. After estimating the activity of each compound on seed germination and on growth of the seedlings that emerged, we examined 11 pairs of compounds in different proportions to explore further the nature of their combined effects. These combinations were chosen in such a way as to represent a number of different cases encountered in essential oils, and they consisted of compounds of similar or of different activities.

METHODS AND MATERIALS

Individual Monoterpenoids. A total of 47 commercial compounds (Aldrich Chemical Co., Sigma Chemical Co.) were examined. The test plant was *Lactuca sativa*. This species is one of the most commonly used in bioassays for allelochemicals and constitutes not only a good basis for comparisons but also, given its well known properties, an essential starting point for the type of research we are undertaking. The concentration of the compounds used in the experiments was chosen on the basis of our previous experience. In earlier studies, we had examined seed germination and seedling growth of various plant species in the presence of different amounts of essential oils within the range of 1–20 μl (Vokou and Margaris, 1982; Vokou and Margaris, 1986b). Amounts above 5 μl were usually very inhibitory. To reveal subtle differences among individual compounds in regard to phytotoxic properties, we chose a low quantity, that of 2.5 μl. An additional reason is that low concentrations are more likely to be found under field conditions. The number of seeds that germinated and the length of the seedlings that emerged in the presence of each compound were compared to those of the control. There were 6 acetates, 15 alcohols, 2 aldehydes, 2 ethers, 12 hydrocarbons, 8 ketones, and 2 phenols Table 1. All compounds were used as received without further purification.

Combinations of Compounds. The following 11 pairs, out of a set of 17 compounds in total, were examined : (*1R*)-(+)-α-pinene : (*1S*)-(−)-β-pinene; α-terpinene : γ-terpinene; (*R*)-(+)-limonene : (*S*)-(−)-limonene; *p*-cymene : (+)-3-carene; *p*-cymene : carvacrol; γ-terpinene : carvacrol; isopulegyl acetate : (−)-carvyl acetate; (*R*)-(+)-limonene : cineol; geraniol : (1*S*, 2*S*, 5*R*)-(+)-neomenthol; (−)-menthone : (*S*)-(+)-carvone; geraniol : terpinen-4-ol. These combinations consisted of compounds either of the same or different activity in inhibiting seed germination and subsequent seedling growth. In many cases, the effect on seed germination was different from that on seedling elongation. Pairs were tested at proportions 1:3, 1:2, 1:1, 2:1, and 3:1, but their sum was always maintained at 2.5 μl, which means that they were used at quantities 0.625, 0.833, 1.250, 1.667, and 1.875 μl, respectively. Taking into consideration that each compound was also tested alone, there were in fact seven proportions for each combination.

TABLE 1. ACTIVITY OF MONOTERPENOID COMPOUNDS AGAINST SEED GERMINATION AND SUBSEQUENT SEEDLING GROWTH OF *L. Sativa* (AVERAGE ± STANDARD ERROR)

Compounds tested	Germination	Percent of control	Maximum length (cm)	Percent of control
(−)-Bornyl acetate (97%)	$0.76 \pm 0.05^{*}$	79.1	$6.03 \pm 0.26^{**}$	57.1
(−)-Carvyl acetate (98%, mixture of isomers)	$0.57 \pm 0.05^{**}$	56.7	$4.70 \pm 0.44^{***}$	39.6
Geranyl acetate (98%)	$0.87 \pm 0.02^{**}$	86.7	$3.93 \pm 0.09^{***}$	36.2
Isopulegyl acetate (98%, mixture of isomers)	$0.79 \pm 0.05^{*}$	78.9	$7.63 \pm 0.09^{***}$	64.3
Linalyl acetate (97%)	0.87 ± 0.05 ns	86.7	$9.40 \pm 0.31^{**}$	79.2
(±)-Menthyl acetate (mixture of *cis* and *trans*)	$0.86 \pm 0.03^{**}$	85.6	$6.23 \pm 0.35^{***}$	52.5
Mean ± SE for acetates	$0.79 \pm 0.03^{***}$	78.9	$6.32 \pm 0.45^{***}$	54.8
[(1*S*)-Endo]-(−)-borneol (99%, solid)	$0.31 \pm 0.02^{***}$	35.0	$0.30 \pm 0.10^{***}$	3.2
DL-Isoborneol (85%, solid)	$0.21 \pm 0.03^{***}$	23.8	$0.18 \pm 0.11^{***}$	2.0
(−)-Carveol (97%, mixture of isomers)	$0.50 \pm 0.03^{***}$	50.0	$2.17 \pm 0.77^{***}$	18.3
Dihydrocarveol (95%, mixture of isomers)	$0.44 \pm 0.04^{***}$	44.4	$0.30 \pm 0.25^{***}$	2.5
β-Citronellol (95%)	$0.38 \pm 0.04^{***}$	42.5	$0.05 \pm 0.00^{***}$	0.5
(*R*)-(+)-β-Citronellol (98%)	$0.41 \pm 0.06^{**}$	46.3	$0.05 \pm 0.00^{***}$	0.5
(*S*)-(−)-β-Citronellol (95%)	$0.31 \pm 0.05^{***}$	35.0	$0.05 \pm 0.00^{***}$	0.5
Geraniol (98%)	$0.51 \pm 0.08^{*}$	57.5	$0.37 \pm 0.32^{***}$	3.3
Isopulegol (tech.)	$0.30 \pm 0.04^{***}$	31.4	$0.05 \pm 0.00^{***}$	0.5
(±)-Linalool (97%)	$0.38 \pm 0.03^{***}$	39.5	$0.05 \pm 0.02^{***}$	0.4
(±)-Menthol (99%, solid)	$0.29 \pm 0.02^{***}$	32.5	$0.07 \pm 0.01^{***}$	0.7
(1*R*, 2*S*, 5*R*)-(−)-Menthol (99%, solid)	$0.41 \pm 0.03^{***}$	46.3	$0.83 \pm 0.12^{***}$	8.9
(1*S*, 2*R*, 5*R*)-(+)-Isomenthol (99%, solid)	$0.47 \pm 0.03^{**}$	52.5	$2.40 \pm 0.78^{**}$	25.6
(1*S*, 2*S*, 5*R*)-(+)-Neomenthol (97%)	$0.54 \pm 0.03^{***}$	54.4	$1.70 \pm 0.15^{***}$	14.3
Terpinen-4-ol (97%)	0.00^{***}	0.0	—	—
Mean ± SE for alcohols	$0.36 \pm 0.02^{***}$	39.4	$0.61 \pm 0.14^{***}$	5.8
(*R*)-(+)-Citronellal (96%)	$0.36 \pm 0.05^{***}$	40.0	$0.05 \pm 0.00^{***}$	0.5
(1*R*)-(−)-Myrtenal (98%)	$0.34 \pm 0.04^{***}$	38.8	$0.05 \pm 0.00^{***}$	0.5
Mean ± SE for aldehydes	$0.35 \pm 0.03^{***}$	39.4	$0.05 \pm 0.00^{***}$	0.5
Cineol (99%)	$0.19 \pm 0.03^{***}$	18.9	$0.35 \pm 0.28^{***}$	3.2
(−)-Limonene oxide (97%, mixture of *cis* and *trans*)	0.53 ± 0.16 ns	55.8	$4.73 \pm 1.76^{*}$	45.8
Mean±SE for ethers	$0.36 \pm 0.11^{***}$	37.3	$2.54 \pm 1.26^{***}$	24.5
(+)-Camphene (tech., 80%, solid)	0.77 ± 0.04 ns	86.3	10.63 ± 0.84 ns	113.5
2-Carene (97%)	0.58 ± 0.09 ns	68.4	8.50 ± 0.50 ns	101.6
(+)-3-Carene (95%)	$0.39 \pm 0.09^{*}$	46.1	$1.10 \pm 0.76^{**}$	13.1
p-Cymene (99%)	0.99 ± 0.01 ns	103.5	11.90 ± 0.32 ns	96.7

TABLE 1. CONTINUED

Compounds tested	Germination	Percent of control	Maximum length (cm)	Percent of control
(*R*)-(+)-Limonene (97%)	$0.94 \pm 0.06^{*}$	94.4	9.20 ± 0.80 ns	77.5
(*S*)-(−)-Limonene (96%)	0.86 ± 0.08 ns	89.5	9.27 ± 0.50 ns	89.7
Myrcene (tech.)	0.91 ± 0.01 ns	95.4	7.63 ± 0.94 ns	73.9
α-Phellandrene	0.71 ± 0.08 ns	80.0	$8.37 \pm 0.41^{**}$	76.3
(*1R*)-(+)-α-Pinene (98%)	0.92 ± 0.03 ns	96.5	10.97 ± 0.19 ns	103.8
(*1S*)-(−)-β-Pinene (99%)	0.96 ± 0.01 ns	95.6	10.13 ± 0.38 ns	93.3
α-Terpinene (85%)	$0.78 \pm 0.04^{*}$	87.5	$9.47 \pm 0.23^{**}$	86.3
γ-Terpinene (95%)	0.97 ± 0.00 ns	101.2	11.20 ± 0.36 ns	91.1
Mean±SE for hydrocarbons	$0.82 \pm 0.03^{**}$	87.0	$9.03 \pm 0.47^{**}$	84.7
(+)-Camphor (96%, solid)	$0.23 \pm 0.02^{***}$	26.3	$0.10 \pm 0.00^{***}$	1.0
(*R*)-(−)-Carvone (98%)	$0.11 \pm 0.03^{***}$	11.2	$0.05 \pm 0.00^{**}$	0.4
(*S*)-(+)-Carvone (96%)	$0.01 \pm 0.01^{***}$	1.1	$0.02 \pm 0.02^{**}$	0.1
(+)-Dihydrocarvone (98%, mixture of isomers)	$0.01 \pm 0.01^{***}$	1.1	$0.02 \pm 0.02^{**}$	0.1
(*1R*)-(−)-Fenchone (98%)	$0.26 \pm 0.03^{***}$	26.7	$0.08 \pm 0.02^{***}$	0.7
(−)-Menthone (95%)	$0.48 \pm 0.06^{**}$	47.8	$4.10 \pm 1.27^{**}$	37.7
(*R*)-(+)-Pulegone (tech., 85%)	$0.11 \pm 0.03^{***}$	11.2	$2.63 \pm 0.80^{*}$	23.7
(−)-Thujone (tech.)	$0.26 \pm 0.06^{***}$	26.7	$3.30 \pm 0.83^{***}$	31.2
Mean±SE for ketones	$0.18 \pm 0.03^{***}$	19.0	$1.29 \pm 0.39^{***}$	11.9
Carvacrol (98%)	$0.41 \pm 0.01^{***}$	46.3	$0.53 \pm 0.19^{***}$	4.9
Thymol (98%)	$0.44 \pm 0.03^{***}$	50.0	$0.90 \pm 0.19^{***}$	9.6
Mean±SE for phenols	$0.43 \pm 0.02^{***}$	48.1	$0.72 \pm 0.14^{***}$	7.2

Germination and Growth Experiments. Thirty *Lactuca sativa* seeds were germinated in a Petri dish, on a filter paper, with 10 ml of water added. In a small aluminum container inside the Petri dish, 2.5 μl of each compound or combination were added such that seeds and monoterpenoids were in aerial contact only. Taking into account that the volume of the Petri dishes was approximately 100 cm^3, this quantity corresponds to 25 ppm. Solid compounds were tested in their solid state. The quantity used was based on the density of the liquid monoterpenoids, which is lower than 1 g cm^{-3} and higher than 0.8 g cm^{-3}. We assumed a mean density of 0.9 g cm^{-3}, and estimated the amount that would correspond to 2.5 μl, which is 2.25 mg. Petri dishes were closed with an adhesive tape so as to prevent escape of the volatile compounds, and were put in the dark at $23 \pm 2^{\circ}$. After 7 days, we counted the number of seeds that had germinated and measured the length of seedlings. For each treatment, including controls, 90 seeds were used (three replicate petri dishes $\times$ 30 seeds in each).

Germination is the percentage of seeds from which a radicle emerges. Relative germination is the germination of the treatment divided by that of the corresponding control. Elongation is defined in terms of maximum seedling length, estimated as the average of the maxima from each of the three replicates. Relative elongation is the elongation of the treatment divided by that of the corresponding control.

We used maximum rather than average seedling length as an estimator of elongation for the following reasons: (1) After analysis of our results, we found a bimodal pattern of elongation in all controls (Figure 3). From a statistical point of view, this bimodality will cause the variance around the average length to be high, consequently obscuring treatment effects. Maximum length does not suffer from this problem to the same extent as average length. (2) Bimodality means that there are two distinct groups, one of sizeable seedings and another of very small ones. Biologically, the latter group comes from seeds that do not have or have lost vigor, germinating late or giving poorly-growing seedlings. We were primarily interested in how the treatments would affect the first group of "fitter" seedlings. Maximum length is a natural way of characterizing the growth of this group without the need for introducing a (possibly arbitrary) means for assigning seedlings to one group or the other.

Using maximum seedling length to estimate growth in the presence of an allelochemical does not entirely separate the effect of reduced seedling elongation from that of delayed germination. But it gives insight on how the fittest of seedlings, i.e., the ones that readily emerge and grow vigorously, will behave in the presence of the allelochemical. It, thus, enables us to assess the allelopathic potential of a given compound (or set of compounds) against the growth of a target species subsequent to germination, as distinct from its effect on germination probability.

Statistical Analysis. The effects of individual compounds on germination and growth were compared with their respective controls by *t* test, whereas the effects of the chemical groups of compounds were compared with each other and with the control by one-way analysis of variance and *post hoc* tests (LSD multiple comparisons).

Modeling the Combined Effect. To explore the combined effect of two compounds, we used a model that indicates whether they act in a nonindependent way. Further statistical analysis was carried out in order to determine whether this could be ascribed to typical randomness and experimental error, or whether there was an interaction that could not be explained by chance alone. The model describes the amount of seed germination, γ, or seedling elongation, ε, relative to the control. For a pure compound, γ is assumed to be

$$\gamma(x) = \frac{1}{1 + \frac{x}{L}} \tag{1}$$

where x is the concentration of the compound, and L is the concentration that would be required to achieve 50% inhibition relative to the control. This model is based on the formulation of An et al. (1993), assuming negligible stimulation and a saturation shape factor of the simplest form ($S_m = 0$ and $q = 1$, respectively, in the notation of An et al.).

To examine the combined effect of two compounds acting independently in a mixture, Eq. (1) is extended in a simple fashion to

$$\gamma(x, y) = \frac{1}{1 + \dfrac{x}{L_1} + \dfrac{y}{L_2}} \tag{2}$$

where L_1 and L_2 are the values of L associated with compounds X and Y, respectively. The model assumes that the two compounds increase inhibition, as their concentration increases, in an independent manner. Effectively, we are assuming that the weaker compound behaves as if it were a dilute version of the stronger compound and, conversely, that the strong compound behaves like a concentrated version of the weaker one. Thus, if X is five times stronger than Y, the effect of adding 1 μl of X is the same as adding 5 μl of Y. Since this model describes the action of mixtures of compounds under the assumption of independence, it provides a benchmark, or "null" model, against which we can compare the degree of interaction of two compounds.

We parameterize Eq. (2) on the basis of the effect of pure compounds (endpoints of each graph in Figures 1 and 2). Since the total concentration of the two compounds has been set at 2.5 μl, starting with this equation, we can derive formulae for L_2 and L_1, for the special cases, where $x = 0$ and $y = 0$, which are respectively

$$L_2 = \frac{2.5\gamma_Y}{1 - \gamma_Y}, \qquad L_1 = \frac{2.5\gamma_X}{1 - \gamma_X} \tag{3}$$

where γ_X and γ_Y is the relative germination associated with each of the pure compounds.

The position of the data relative to the corresponding model curve gives an estimate of their departure from the null model of independent activity. When the departure from the null model cannot be explained by natural chance or experimental error, there is evidence of higher order effects, which can be understood as being caused by an extra term in the denominator of Eq. (2), $\gamma(x, y) = [1 + x/L_1 + y/L_2 + f(x, y)]^{-1}$. This will be a multiplicative term, in contrast to the other additive terms in the denominator of Eq. (2), and will reflect an interaction between the compounds themselves. If $f > 0$, the extra term is synergistic, since it increases the denominator and hence the inhibition; conversely, if $f < 0$, the extra term is antagonistic.

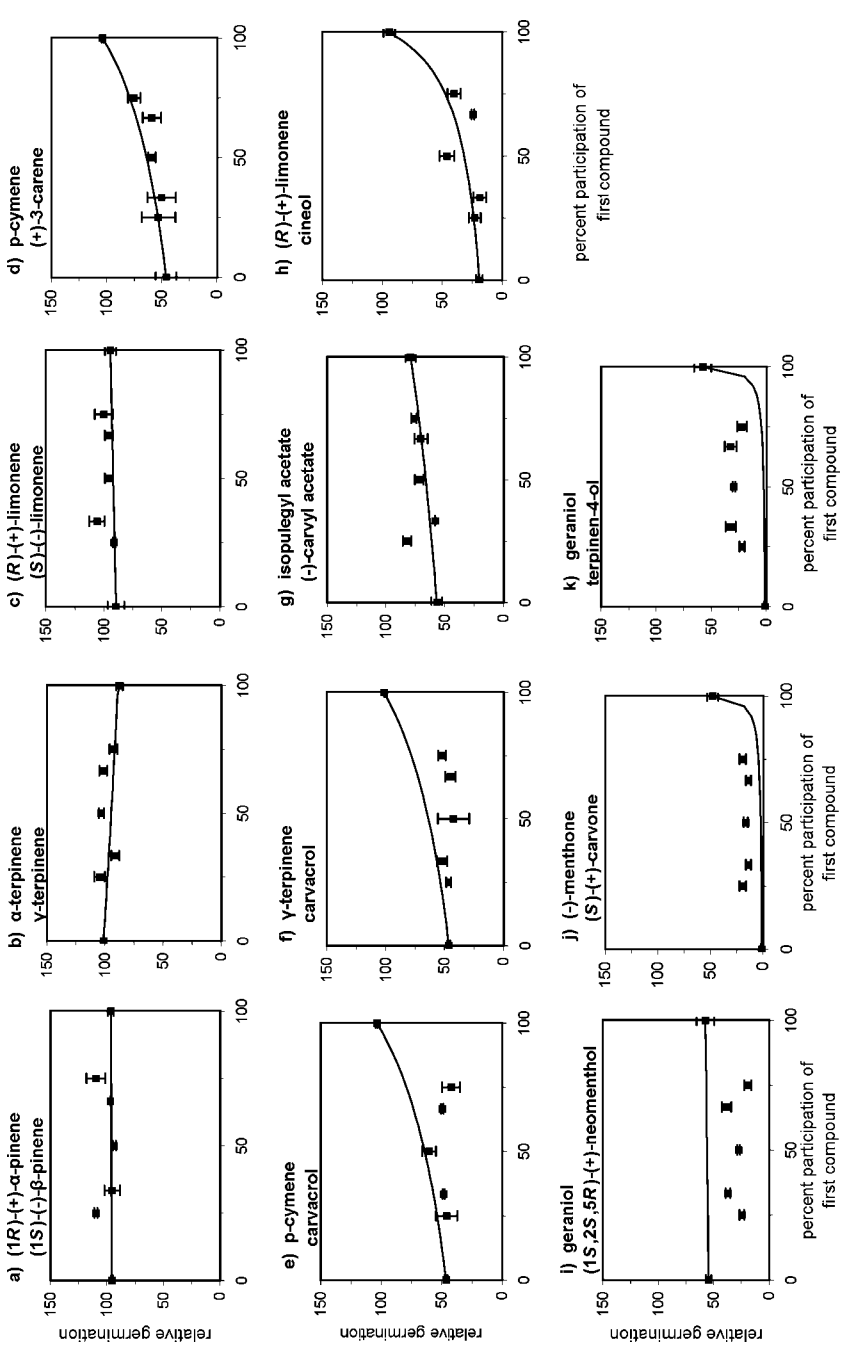

FIG. 1. (a–k). Relative germination of *L. Sativa* (after 7 days) for 11 pairs of monoterpenoids, tested at different relative proportions. Error bars denote ± standard deviation of three replicates. The curves represent the expected effects of the two substances acting independently, according to Eq. (2).

For relative elongation, we use the same basic form, Eq. (2), as for relative germination:

$$\varepsilon(x, y) = \frac{1}{1 + \dfrac{x}{L_3} + \dfrac{y}{L_4}} \tag{4}$$

Equation (4) can be parameterized in the same way as Eq. (2), using ε_X and ε_Y, the relative elongation associated with each of the pure compounds to find L_3 and L_4, respectively.

Significance of Departures. The effects of different treatments on germination and elongation were compared by simulation, carried out by programs written in the C^{++} programming language. The statistical aim was to determine the significance of departure from the null model: could it be due to chance alone? In each case, the null hypothesis is that compounds act independently. The analysis uses computer intensive methods to recreate a distribution of variability under the null hypothesis. We simulate replicates, for both germination and elongation, of the five mixture treatments. Note that we do not simulate replicates of the entire experiment, but only replicates of the mixtures, and we use the observed results for the pure compounds and the controls. Thus, the same values of L_1 and L_2 for germination (or L_3 and L_4 for elongation) apply to the replicates as for the original observations themselves.

(i) *Germination*. To test the combined effects on germination, we used Monte-Carlo simulation (Manly, 1997). Simulated replicates are created from a theoretical distribution known under the null hypothesis. In our case, the distribution is a binomial random variable with 90 trials, each trial having a probability of success given by $\gamma(x, y)g_c$, where $\gamma(x, y)$ is given by Eq. (2) and g_c is the germination of the control.

The details of the procedure are as follows:

1. For each pair of compounds, we compute L_1 and L_2 using Eq. (3) by calculating the relative germination in presence of the pure compounds.
2. For each of the five mixtures, we calculate the difference between the relative germination predicted by Eq. (2) and that observed, and calculate the RMS (Root Mean Square) deviation, D_0.
3. We generate a Monte-Carlo replicate set. This consists of the number of germination events that would be expected under the null hypothesis (with binomial distribution) for each of the five mixtures.
4. We calculate the RMS deviation of the relative germination of these replicate data from that predicted by Eq. (2); this is D_1.
5. We repeat steps 3–4, generating another 999 replicates, each time calculating an RMS deviation, so as to have D_1, D_2, D_3 $\ldots$, D_{1000}.

6. The significance of D_0, is the proportion of the entire set of D's [namely $\{D_0, D_1, D_2, \ldots, D_{1000}\}$] that are larger than D_0.

(ii) *Elongation*. In contrast to germination, we have no theoretical model for the variability of elongation under the null hypothesis. However, the method of bootstrapping (Manly, 1997) can be used in order to find a plausible distribution of natural variability of maximum length. In this method, replicates are constructed by sampling with replacement from the original observations.

The details of our procedure are as follows:

1. We calculate L_3 and L_4 on the basis of the relative elongation associated with each of the pure compounds using Eq. (3).
2. For each of the five mixtures, we calculate the difference between the relative elongation predicted by Eq. (4) and that observed, and calculate the RMS deviation, D_0.
3. We generate a bootstrap replicate set for elongation by resampling from the original data (Manly, 1997). All 90 observations from the three initial replicates are pooled. From this pool, 90 values are chosen at random; each value is not removed from the pool but is available for the next choice. The 90 new values are then divided randomly in three groups of 30 seedlings. This is done for each of the five mixtures.
4. We calculate the RMS deviation of the relative elongation of the five mixture replicates from their observed values; this is D_1.
5. We repeat steps 3–4, generating another 999 replicates, each time calculating an RMS deviation, so as to have $D_1, D_2, D_3, \ldots, D_{1000}$.
6. The significance of D_0, is the proportion of the entire set of D's [namely $\{D_0, D_1, D_2, \ldots, D_{1000}\}$] that are larger than D_0.

RESULTS

Individual Monoterpenoids. Results for seed germination and subsequent seedling growth are given in Table 1. Regarding germination, of all compounds tested, terpinen-4-ol, an alcohol, was the most inhibitory, totally suppressing germination. Pulegone, dihydrocarvone, and the two carvone isomers, all belonging to the group of ketones, followed, reducing germination to $<15\%$ of the control. All chemical groups examined inhibited germination, but to varying levels (Table 1). Ketones proved the most inhibitory (Tables 1 and 2). Aldehydes, ethers, alcohols, and phenols followed. Acetates and hydrocarbons were the least inhibitory.

Regarding growth of the seedlings that emerged, 24 compounds, representing all chemical groups examined except for the acetates, were extremely active;

TABLE 2. SIGNIFICANCE OF THE DIFFERENCES IN ACTIVITY OF THE GROUPS OF MONOTERPENOIDS AGAINST SEED GERMINATION OF *L. Sativa*

	Alcohols	Aldehydes	Ethers	Hydrocarbons	Ketones	Phenols
Acetates	***	**	**	ns	***	*
Alcohols		ns	ns	***	**	ns
Aldehydes			ns	***	ns	ns
Ethers				***	ns	ns
Hydrocarbons					***	**
Ketones						*

Note: ns = Nonsignificant.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

maximum seedling length in their presence was less than 15% of the control. The most phytotoxic among them were the three citronellol isomers, linalool, and isopulegol, from the group of alcohols, both aldeydes (citronellal and myrtenal), and the two carvone isomers and dihydrocarvone, from the ketones; in their presence, there was almost no growth at all of the seedlings that emerged (maximum length <0.5% of the control). All chemical groups inhibited seedling growth (Table 1). Ketones, alcohols, aldehydes, ethers, and phenols induced comparable effects (Tables 1 and 3). Their effect differed from those of acetates and hydrocarbons; the latter were the least active. Within this rather inactive group, there was one compound, (+)-3-carene, which was very inhibitory. We do not know which properties of this molecule are responsible for its potent activity. We should also note that, in some cases, hydrocarbons seemed to stimulate rather than inhibit germination and seedling growth (Table 1). Such a stimulation is often encountered in tests of phytotoxicity of various secondary metabolites, at low concentrations (Asplund, 1968; Lovett et al., 1989; An et al., 1993). However, in our experiments, this effect was not significant.

TABLE 3. SIGNIFICANCE OF THE DIFFERENCES IN ACTIVITY OF THE GROUPS OF MONOTERPENOIDS AGAINST GROWTH OF *L. Sativa* SEEDLINGS THAT EMERGED IN THEIR PRESENCE

	Alcohols	Aldehydes	Ethers	Hydrocarbons	Ketones	Phenols
Acetates	***	**	*	**	***	**
Alcohols		ns	ns	***	ns	ns
Aldehydes			ns	***	ns	ns
Ethers				***	ns	ns
Hydrocarbons					***	***
Ketones						ns

Note: ns = Nonsignificant.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4 illustrates the comparative overall effect of each compound on both seed germination and subsequent seedling growth. On the basis of the magnitude of their effect, compounds were grouped into five categories. They were considered as (i) inactive/slightly active, when the effect was not significant or when either seed germination or seedling growth remained at $>85\%$ of the control, (ii) moderately active, when activity fell to 85–50% of the control, (iii) strongly active, when it fell to 49–15%, and (iv) extremely active, when it fell to $<15\%$. It is obvious that a large number of monoterpenoids (about 50% of the compounds examined) were extremely active against growth of seedlings that emerged in their presence, whereas only a few (about 10%) were extremely active against seed germination. Hydrocarbons were represented by only one compound in the group of extremely active compounds. Acetates were not represented at all. In fact, this group was the least effective of the oxygenated compounds, second only to the rather inactive, non-oxygenated hydrocarbons.

Combinations of Monoterpenoids. Figure 1 shows the relative germination for the 11 pairs of compounds, while Figure 2 the relative elongation. The error bars are the standard deviation between the three replicates under each treatment. On the x-axis of each graph, the type of mixture is given in terms of increasing participation of the first compound of the combination (coinciding with the corresponding decrease of the second compound).

It is clear from these figures that the results for elongation contain far more instances of very inhibitory activity than do the results for germination. This was expected from the fact that in the case of compounds acting individually very few were extremely active against germination (in contrast to elongation). Also, there seems to be a greater variability in relative elongation, particularly in cases d, h, and i.

The reason for the large variability in some instances of elongation is evident from Figure 3, which shows the histograms of seedling elongation for all pairs of compounds at each different proportion, and of their respective controls. It is evident that the distribution is bimodal for all controls. We discern one group of sizeable seedlings and another of very small ones, which can be associated with vigorous and nonvigorous seeds, respectively. The same bimodality is also found in most treatments. When it is absent, it is because there are only small seedlings. If the treatment leads to only a few sizeable seedlings (as, for example, in Figure 3d, mixture 1a : 3b), the variability of maximum length may be quite large (Figure 2d, 25% participation of first compound), as it is possible that one (or two) of the replicates will contain no sizeable seedlings at all.

In combinations of inactive/slightly active compounds, this bimodal distribution, encountered in all controls, is retained at all proportions of the participating compounds (Figure 3a–c). This holds true in the case of combinations of moderately active with strongly active compounds (Figure 3g). In contrast, whenever one extremely active compound participates in the combination (Figure 3e,f, h–k),

TABLE 4. COMPARATIVE PERFORMANCE OF MONOTERPENOID COMPOUNDS AGAINST GERMINATION AND GROWTH OF *L. Sativa*

	Germination			
Elongation	Inactive/Slightly active (nonsignificant difference/ >85% of the control)	Moderately active (85–50% of the control)	Strongly active (49–15% of the control)	Extremely active (<15% of the control)
Inactive/slightly active (nonsignificant difference/ >85% of the control	(+)-Camphene 2-Carene *p*-Cymene (*R*)-(+)-Limonene (*S*)-(−)-Limonene Myrcene (*1R*)-(+)-α-Pinene (*1S*)-(−)-β-Pinene α-Terpinene γ-Terpinene			
Moderately active (85–50% of the control)	Linalyl acetate (±)-Menthyl acetate α-Phellandrene	(−)-Bornyl acetate Isopulegyl acetate		
Strongly active (49–15% of the control)	(−)-Limonene oxide Geranyl acetate	(−)-Carveol (−)-Carvyl acetate (*1S,2R,5R*)-(+)-Isomenthol	(−)-Menthone (−)-Thujone	(*R*)-(+)-Pulegone
Extremely active (<15% of the control)		Carvacrol Geraniol (*1S,2R,5R*)-(+)-Neomenthol Thymol	[(*1S*)-Endo]-(−)-borneol DL-Isoborneol (+)-Camphor Cineol (+)-3-Carene (*R*)-(+)-Citronellal β-Citronellol (*R*)-(+)-β-Citronellol (*S*)-(−)-β-Citronellol Dihydrocarveol (*1R*)-(−)-Fenchone Isopulegol (±) Linalool (±)-Menthol (*1R,2S,5R*)-(−)-Menthol (*1R*)-(−)-Myrtenal	(*R*)-(−)-Carvone (*S*)-(+)-Carvone (+)-Dihydrocarvone Terpinen- 4-ol[a]

[a] Germination was inhibited by 100%; therefore, there could not be seeding length measurements.

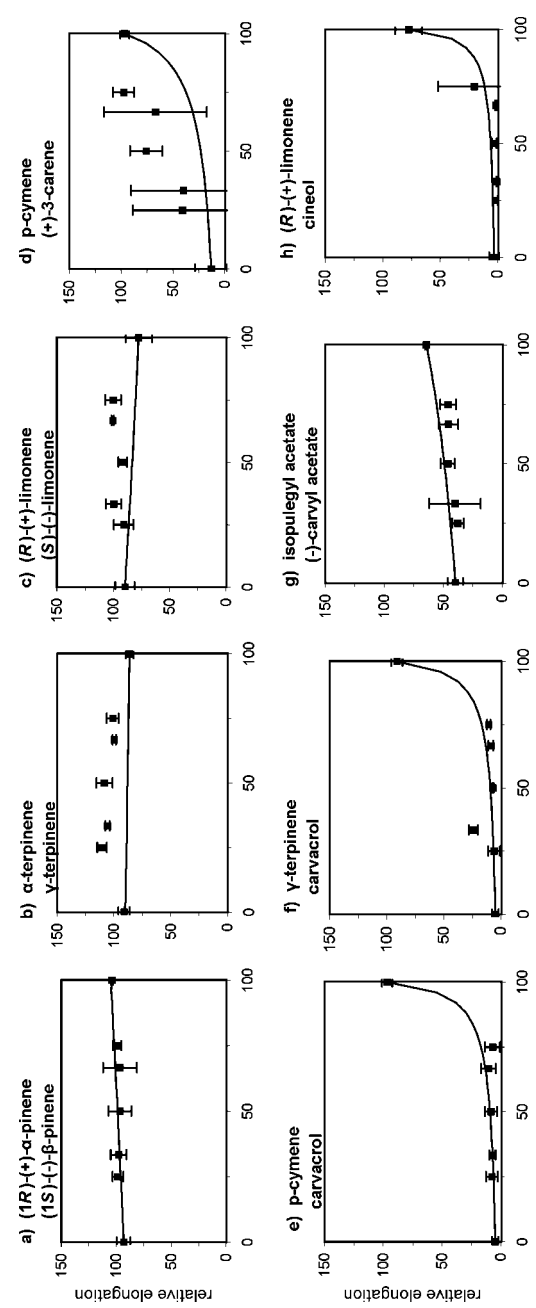

FIG. 2. (a–j). Relative elongation of seedlings of *L. sativa* (after 7 days) emerging in the presence of pairs of monoterpenoids. Ten pairs of monoterpenoids were tested at different relative proportions. Error bars denote ± standard deviation of three replicates. The curves represent the expected effects of the two substances acting independently, according to Eq. (4).

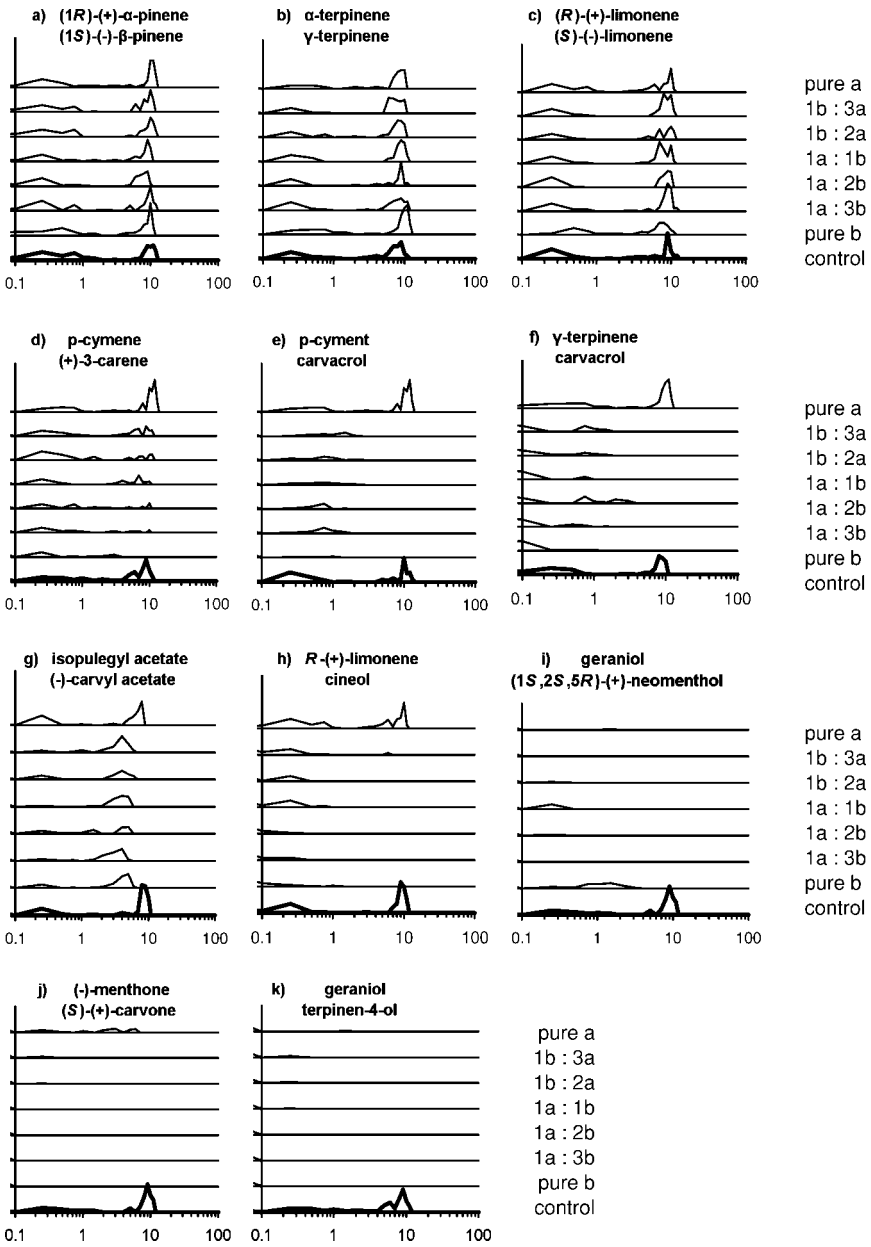

FIG. 3. (a–k). Histograms of seedling length of *L. sativa* (after 7 days) for 11 pairs of monoterpenoids, at each of the seven proportions tested, and corresponding control. The *x*-axis is seedling length in cm (log). The area under each histogram is equal to the proportion of germinated seeds.

even at the lowest concentration used, the group of sizeable seedlings virtually disappears. The exception to this rule is the combination of the extremely active (+)-3-carene with the inactive *p*-cymene (Figure 3d); here, the group of sizeable seedlings does not disappear but decreases with increasing concentrations of (+)-3-carene. This can be explained by the fact that the latter compound is on the borderline between strongly and extremely active compounds, reducing maximum length to 13.1% of the control, when all other extremely active compounds reduce it within the range of 4.9–0.1% of the control.

The models for germination and elongation, Eqs. (2) and (4), respectively, are also shown in Figures 1 and 2. In each panel, the heavy curve represents the amount of relative germination (or elongation) expected under the assumption that the compounds act independently of one another. All observations falling above this line have less inhibition than expected and, therefore, this suggests that the compounds are acting antagonistically. Conversely, observations below the curve reveal greater inhibition than expected and this suggests a synergistic interaction.

The significance of departures from independence is given in Table 5, along with the RMS deviation, for both germination and elongation. Two values of significance of the RMS deviation are offered for germination. The first (headed by P) assumes that all variability arises from the basic binomial model. As shown in the table, only in one out of the 11 cases is this deviation not significant. However, judging from the appearance of the graphs and error bars, there should be fewer

TABLE 5. THE RESULTS OF TESTS FOR STATISTICAL SIGNIFICANCE

	Germination				Elongation		
Compounds	RMS deviation	P	P^{**}	Effect	RMS deviation	P	Effect
α-Pinene : β-pinene	0.09	0.001	0.003	−	0.01	0.884	
α-Terpinene : γ-terpinene	0.07	0.001	0.033		0.15	0.006	−
(+)-Limonene : (−)-limonene	0.08	0.004	0.017		0.15	0.009	−
p-Cymene : 3-carene	0.07	0.214	0.554		0.94	0.008	−
p-Cymene : carvacrol	0.19	0.001	0.001	+	0.01	0.670	
γ-Terpinene : carvacrol	0.19	0.001	0.001	+	0.04	0.192	
Isopulegyl acetate : carvyl acetate	0.10	0.010	0.134		0.04	0.253	
(+)-Limonene : cineol	0.11	0.005	0.073		0.02	0.526	
Geraniol : neomenthol	0.28	0.001	0.001	+	0.03	0.001	+
Menthone : carvone	0.14	0.001	0.001	−	0.00	0.549	
Geraniol : terpinen-4-ol	0.25	0.001	0.001	−			

Note: The Units of Root Mean Square (RMS) deviation were Relative Germination and Relative Elongation of *L. Sativa*, respectively. The P^{**} column is the significance of germination RMS deviation when using the model with adjusted variability. Under effect, + indicates significant synergistic effect; − indicates significant antagonistic effect; blank indicates absence of significant interaction.

significant departures than this (for example, in the cases of terpinene and limonene combinations, Figure 1b and c, respectively). For this reason, we carried out an additional analysis of the variance in the numbers of successful germination events in the control groups. We found that variance was approximately twice as large as expected with the binomial model. It is, thus, likely that an extra source of variability is also present in the treatments. Therefore, we adjusted the model to take into account this extra source of variability. This was done by multiplying the proportion of germinating seeds in each Monte-Carlo replicate by a Normal random variable, having a mean equal to 1 and a variance equal to 2. The variability predicted by this adjusted model matched that of the controls, and provides a good basis for estimating the significance for the treatment results. These adjusted significance values (headed by P^{**}) are also given in Table 5.

Concerning elongation (Table 5), there are cases where the P-value is larger than expected from the appearance of the position of the curve relative to the observations and the error bars, as in the terpinene combination (Figure 2b). This is due to the fact that even though the variance of observations may be small, the variance of some of the bootstrap replicates used in the calculation of significance may be very large. This is related to the bimodal character of the distribution of data, as explained earlier.

Despite the rigorous standards of calculation of significance, we chose the conservative level of $P \leq 0.01$ as the threshold of significance. Thus, from Table 5 with Figures 1 and 2, it is evident that there are significant antagonistic and synergistic effects both in germination and in elongation. In particular, for germination, antagonistic effects were found in the following combinations: α-pinene : β-pinene; menthone : carvone; geraniol : terpinen-4-ol, whereas for elongation, in the terpinene and the limonene combinations, and in p-cymene : 3-carene. Synergistic effects for germination were found in both combinations involving carvacrol (with p-cymene or γ-terpinene), and in geraniol : neomenthol, whereas for elongation only for the geraniol : neomenthol combination.

DISCUSSION

In this study, we compare the potential allelopathic effect of monoterpenoids, acting alone or in pairs, as expressed against seed germination and subsequent seedling growth. In many cases, the degree of the compounds' activity on seed germination was not the same as that on growth.

Some of the most inhibitory compounds have been repeatedly reported as phytotoxic against a number of target species (Muller and Muller, 1964; Asplund, 1968; Vokou, 1986b; Reynolds, 1987; Fischer, 1991), though not always with the same level of activity. Reynolds (1987), also working with *L. sativa*, compared a large number of compounds belonging to different chemical groups as to their effect on seed germination. Therefore, that study constitutes a good basis with

which to compare our results. Trying to relate structure with activity, Reynolds stated that "the lipophilic nature of the molecule is one of the main contributing factors towards inhibitory activity . . . although the parent hydrocarbons with extreme lipophilicity and no functional group were not inhibitory" and that "it might be expected that more than one structural factor operates." In other words, lipophilicity is a necessary, but not a sufficient condition for activity. However, it is not yet clear what the specific structural factors are that operate in each case.

From this study of 47 monoterpenoids, we can draw one clear conclusion, which has been repeatedly reported, that as a group, non-oxygenated compounds are less active than oxygenated ones. Similarly, when we examine individual cases of similarly structured compounds, we observe that whenever an oxygen atom is added into the molecule of a hydrocarbon, the activity of the resulting oxygenated compound increases considerably. For instance, this is manifested in both processes, for the pairs *p*-cymene–carvacrol or *p*-cymene–thymol. What has not been reported, is that acetates, although they contain oxygen, are not as active as other oxygenated groups. In fact, their effect on seed germination was of a magnitude comparable to that of hydrocarbons. If we examine individual cases, such as the pairs, linalool–linalyl acetate, isopulegol–isopulegyl acetate, menthol–menthyl acetate, we observe that whenever the free hydroxyl group of the respective alcohol turns into a carboxyl group, there is a remarkable decrease in activity for both parameters examined.

There is no conclusive evidence that that the hydroxyl group contributes more to activity than the carbonyl group (Reynolds, 1987). In our study, the most active compound in inhibiting germination was an alcohol, terpinen-4-ol, but as a group, ketones, were more drastic than alcohols. When we examine individual cases of similarly structured compounds, we observe that in some, oxidation results in more severe effects against seed germination (e.g., dihydrocarveol–dihydrocarvone, carveol–carvone), whereas in others there is no considerable difference (e.g., menthol–menthone, borneol–camphor). Similarly, we did not find any considerable change of activity when the oxidation of the hydroxyl group resulted in not a ketone but an aldehyde (e.g., the pair of citronellol–citronellal).

As for other structural factors that might influence activity, there is no evidence that straight chain compounds are less active than cyclic ones, or that cyclic compounds with two rings are more active than those with one, as also reported by Reynolds (1987). As for the aromatic oxygenated compounds, thymol and carvacrol, although they strongly reduced seed elongation, they were not more effective against seed germination than the more saturated analogs. The differing activity values of stereoisomers (e.g., the two carvone and the two β-citronellol stereoisomers) might imply control of activity by stereoisomerism. Nevertheless, their effects were not significantly different (one-way analysis of variance and *post hoc* LSD tests).

Analysis of the combined effect of compounds acting as pairs at various concentrations showed that only in about half of the cases examined (11 out of 21) compounds acted independently. In the rest, there was evidence of interaction of both a synergistic and antagonistic character. This interaction effect was synergistic for germination in three pairs of compounds and for elongation in one pair; it was antagonistic for both germination and elongation in three pairs of compounds. However, only for one combination there was a significant interaction in both germination and elongation, for the geraniol : neomenthol combination. Nevertheless, in the absence of any pattern, these results cannot increase our predictive power as to the character of the combined effect of compounds other than the exact ones that we examined.

Notwithstanding interactions, the effect of any combination of inactive, slightly or moderately active compounds will be insufficient to achieve the same degree of suppression as one extremely active compound. Going a step further, we could argue that for an essential oil to be extremely active, a necessary prerequisite is the participation of one or more extremely active compounds among its constituents. However, its overall activity may be moderated depending on the character of the other constituents; we found antagonistic interactions, particularly in germination, when less active compounds were present.

The activity of monoterpenoids seems to be selective. For instance, cineol was found to be highly toxic against one grass (*Schizachyrium scoparium*) that occurs in the pineland communities of Florida but not on another (*Leptochloa dubia*) (Fischer et al., 1988). Whatever activity has been found for a certain compound against a certain target species will not necessarily be maintained against another target species, even of the same family or genus. This is an important feature, particularly for application as bioherbicides, where specificity of activity is required. Therefore, research is needed to identify the most drastic compounds for the different target species.

Except for a few cases (e.g., hydrocarbons, acetates), the specific structural factors that operate and determine the activity of monoterpenoids still remain rather obscure. The same holds true for the combined effect of monoterpenoid compounds. Our results show that in some cases compounds act independently, whereas in others they act synergistically or antagonistically. Nevertheless, the character of the interaction cannot in general be predicted on the basis of the individual compounds acting alone.

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BEHAVIORAL FORAGING RESPONSES BY THE BUTTERFLY *Heliconius melpomene* TO *Lantana camara* FLORAL SCENT

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Abstract—Floral color has been shown to influence flower selection by butterflies, but few studies have investigated the role of floral scent. In this study, adults of *Heliconius melpomene* L. (Lepidoptera: Nymphalidae: Heliconiinae) were tested in two-choice bioassays to investigate their ability to distinguish floral scent of the butterfly pollinated plant *Lantana camara* L. (Verbenaceae) from other plant scents. The relative importance of floral scent vs. color was also studied. Butterfly foraging behavior was measured as probing with proboscis. This probing, on floral models varying in scent and color, was timed. When given a choice of floral and vegetative scents of *L. camara*, newly emerged butterflies preferred floral scent, indicating an innate response to floral scents. When butterflies were conditioned to *L. camara* floral scent by offering the scent with yellow color and sugar water, yellow color elicited stronger feeding responses than did the floral scent. However, the floral scent of *L. camara* was preferred to that of the novel species *Philadelphus coronarius* L. (Hydrangiaceae). The floral scent of *L. camara* was dominated by tepenoid compounds, while that of *P. coronarius* by fatty acid derivatives, thus demonstrating totally different compositions. It is concluded that, while *H. melpomene* butterflies often use visual floral traits when selecting which flowers to visit, floral scents elicit behavioral responses that initiate and maintain foraging on flowers.

Key Words—Floral scent, behavior, food search, *Heliconius melpomene*, Nymphalidae, butterfly, Lepidoptera, *Lantana camara*, *Philadelphus coronarius*.

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INTRODUCTION

Olfactory and visual stimuli in flowers attract pollinating animals, which have been of pivotal importance in the reproductive success and radiation of angiosperms (Stebbins, 1970; Faegri and Pijl, 1979; Crepet, 1983; Pellmyr and Thien, 1986). The convergent evolution of suites of floral traits among unrelated plant species to fit specific ecological groups of pollinators has led to "pollination syndromes" (Faegri and Pijl, 1979). These syndromes range from fairly specialized to generalized, in terms of the pollinator utilized (Waser et al., 1996; Johnson and Steiner, 2000). In the butterfly pollination syndrome (psychophily), some typical floral traits are bright red or orange color, a landing platform, and a narrow deep corolla tube (Faegri and Pijl, 1979; Cocucci et al., 1992).

For a butterfly to be an effective pollinator, it has to remain constant in its visitation to a plant species. Goulson et al. (1997) found that adults of the butterfly *Thymelicus flavus* (Hesperiidae) prefer certain nectar plants and will remain constant to them if there is sufficient nectar. Such a foraging strategy seems advantageous to the butterfly as long as flower constancy reduces the flower handling time and, thereby, increases the available time for other important activities, such as reproduction. However, in a natural habitat with a diversity of available plant species, the ability of butterflies to be flexible in their foraging preferences is clearly of adaptive value and allows butterflies to effectively track the most rewarding flowers. Flexible foraging preferences have been observed in *Pieris napi* regarding its use of flower color (Goulson and Cory, 1993) and in *Heodes virgaureae* L. (Lycaenidae) regarding its use of complex visual cues (Douwes, 1978). When there is a shortage of the preferred plants, butterflies can shift to other rewarding ones, and forage from a wide range of different plant species, thus acting as generalists (Jennersten, 1984; Lewis and Lipani, 1990), but focus on only one plant species at a time, thus being specialist in the short term.

The visual appearance of flowers, especially color, seems to be important for nectar searching butterflies (Ilse, 1928; Lewis and Lipani, 1990; Goulson and Cory, 1993; Weiss, 1995a). Ilse (1928) demonstrated that butterflies have innate preferences to certain color, and that these preferences differ among butterfly families. Studies by Weiss (1997) suggest that in many butterfly species a combination of innate and learned behavior is used to locate and forage on flowers, as butterflies learn to associate reward more quickly with an innately preferred color. In addition, floral scent can be an important guide to butterflies (Ilse, 1928; Groth et al., 1987; Proctor et al., 1996; Honda and et al., 1998), but the relative reliance on color vs. scent varies among butterfly species (Ilse, 1928).

One plant that is typically butterfly pollinated is the woody shrub *Lantana camara* (Verbenaceae), which is native to tropical and subtropical America, and naturalized in other areas (Barrows, 1976; Denton et al., 1991). Flowers of *L. camara* change from yellow to red when pollinated (because of the appearance

of anthocyanin) and, thereby, direct pollinators, mainly butterflies, to unpollinated, rewarding yellow flowers (Weiss, 1995b). *Lantana camara* is a favorite nectar plant for several tropical butterfly species, including *Heliconius* spp. (Crane, 1955; Gilbert, 1972; Boggs et al., 1981). *Heliconius* butterflies have a well developed color vision (Swihart, 1972), which together with chemical signals, is of vital importance in the butterfly's defense, roosting, courtship, and oviposition (Crane, 1955; Benson et al., 1974; Brown, 1981; Spencer, 1988; Agosta, 1992).

The present study explores how adults of *Heliconius melpomene* use floral scent from *L. camara* to recognize food-rewarding flowers from the perspective of foraging behavior. Four questions were addressed in two-choice bioassays. (1) Do newly emerged butterflies prefer the scent of flowers over that of the vegetative parts of *L. camara*? (2) Do experienced butterflies previously rewarded (conditioned) with flowers of *L. camara* prefer the scent of flowers when offered a choice of scent from flowers and vegetative parts of *L. camara*? (3) Do butterflies associate reward more strongly with the floral scent than with the yellow flower color of *L. camara*? (4) Do they use floral scents to discriminate between *L. camara* and the non-butterfly visited plant *Philadelphus coronarius*? To address this last question, we analyzed the floral scent of *P. coronarius* and compared it with that of *L. camara*.

METHODS AND MATERIALS

Butterfly and Plant Species Heliconius melpomene. L. (Lepidoptera: Nymphalidae: Heliconiinae) pupae were obtained from the Stratford butterfly farm in Stratford-upon-Avon, UK. Adult butterflies were individually marked on their hind wings with permanent ink, and maintained in 60 × 60 × 60 cm net cages, with the cage floor covered with a grey polyester cloth and kept moist. In the cages, the butterflies were offered their host plant *Passiflora caerulea* L. (Passifloraceae) obtained from Grandiflora AB, Partihallarna, Göteborg, Sweden, flowering plants of *L. camara*, water (in black and white cups; 3 cm ID, 1.5 cm depth), and sugar water (sucrose: water, 1:4 by volume, in Eppendorf-vessels or sponges within floral models). *Lantana camara* L. (Verbenaceae) were cloned cultivars "Roma," native to the West-Indies, obtained from Grandiflora AB, Partihallarna, Göteborg. *Philadelphus coronarius* L. (Hydrangiaceae) was growing in a garden in Alingsås close to Göteborg.

Behavioral Response to Floral Scents—Bioassays. Bioassays were conducted inside a 60 × 60 × 60 cm net cage in the Butterfly House, Trädgårdsföreningen, Göteborg, on sunny days, between 8.00 and 11.00 A.M., since foraging activity was highest under such conditions and at that time period (H. Rönnmark, pers. comm.). RH was 30–70% and temperature 21–40°C.

TABLE 1. SUMMARY OF THE DIFFERENT PARAMETERS ASSOCIATED WITH EACH BIOASSAY

	Conditioning[a]			Two-choice models[b]					Individuals tested	
Bioassays	yel	gr	yel	Lc	fl	veg	Pc	fl	Group	Number[c]
I				yel		yel			a	35 (15; 17; 3)
II	6			gr		yel			a	14 (5; 6; 3)
III	2			yel		yel			b	18 (10; 7; 1)
IV	4	2		gr		gr			b	27 (11; 11; 5)
V	4	4		gr		yel			b	21 (9; 7; 5)
VI	4	4	8	yel			yel		b	22 (15; 7)

Recorded behavior: I: Duration of behavior; II: Duration of behavior; III: Visit frequency and Duration of behavior; IV: Duration of behavior; V: Duration of behavior; VI: Visit frequency and Duration of behavior

[a] Number of days with conditioning to rewarding (sugar water and/or real flowers) yellow: yel, or green: gr *L. camara* floral scented models (start from left).
[b] No reward in models; Lc: Lantana camara; Pc: *Philadelphus coronarius*; fl: floral scent; veg: vegetative scent; yel: yellow; gr: green.
[c] Total (female; male; undetermined gender).

Individual *H. melpomene* were tested for their behavioral responses to floral scents in two-choice bioassays (June and July 1998). During the bioassays, butterflies were offered choices between two types of flower models that contained no sugar water and differed in their combinations of scents (flowers and vegetative parts of *L. camara*) and colors (yellow and green cotton gauze) as summarized in Table 1. In bioassay VI, flower scent from the non-butterfly plant *P. coronarius* was used in one of the models. Flowers of *P. coronarius* are white, bowl-shaped, and rose-like (the plant being closely related to the family Rosaceae; Heywood, 1993) and contrast with the narrow tubed yellow-red flowers of *L. camara*.

For each bioassay, the two flower models were placed on the floor of the cage in the center. The distance from the flower models to the cage roof was 20–30 cm. One flower model was placed 20–50 cm from the other model; the positions of the two models were exchanged between every individual butterfly tested. Each flower model comprised a group of two to four units. Each unit was constructed from two black plastic film cans (5 cm length, 3 cm ID, with bottoms cut away) that were glued together, creating a hollow tube. One end of the tube (the top) was covered with four to six layers of cotton gauze, dyed yellow or green, to resemble the color of *L. camara* flowers and vegetative parts, respectively. The tube was placed over the scent source, i.e., flowers or vegetative parts, which was not visible through the layers of cotton gauze. Plant material was located at least 25 mm below the cotton gauze in order to be out of reach of the butterfly proboscis, which was approximately 15 mm long.

During bioassays, the behavioral responses of butterflies to the two models were evaluated as summarized in Table 1. In bioassays I, II, IV, and V, butterflies

showed a low motivation to visit the models; the individual butterflies were, therefore, manually placed once on each model, and the duration of the response behavior, demonstrated within 1 min, was timed. In bioassays III and VI, butterflies were highly motivated to visit the models and each individual was let free in the cage, allowing both the duration of visits and the number of visits to be recorded. For all visits to the models, two types of behaviors were recorded: 1) nonprobing, where the butterfly sat immobile, with proboscis uncoiled, on the model; (2) probing, where the proboscis was extended and repeatedly penetrated through the cotton gauze of the model. Deep probing, where the butterfly probed with the whole length of the proboscis, was also recorded and interpreted as an eager form of probing.

Bioassay I tested the responses of newly emerged individuals, whereas bioassays II–VI tested the responses of experienced (conditioned) individuals that had been previously rewarded with *L. camara* flowers. Females and males were tested in random order. The gender of an individual was determined after the bioassays were finished; however, some individuals escaped before their gender was determined. The same individual butterflies were used in consecutive bioassays, with one group (a) in bioassays I–II and a second group (b) in bioassays III–VI.

Bioassay I: Newly emerged individuals were tested to the floral vs. vegetative scent of *Lantana camara* (both combined with yellow). *Bioassays II–V*: Preconditioned butterflies were tested to floral scent vs. vegetative scent of *L. camara* (combined with different colors). *Bioassay VI*: Preconditioned butterflies were tested to floral scents of *L. camara* vs. *Philadelphus coronarius*.

Newly-Emerged Butterflies. Prior to testing, butterflies were given only water. In addition, to activate butterflies and determine if they were interested in foraging, they were held by their wings close to *L. camara* flowers for about 15 sec, during which they were allowed to touch the flowers with their legs, proboscis, and antennae, but not actually forage on the flowers. Individuals that showed foraging activity, by means of wing fluttering and/or starting to uncoil proboscis, to the real flowers were used in tests.

Preconditioned Butterflies. These butterflies were classified as being *L. camara* flower-rewarded. Prior to testing, they were allowed to forage on both fresh flowers and, for conditioning purposes, on rewarding *L. camara* flower-scented models that were either yellow or green, depending on the bioassay (see Table 1). In all conditioning, butterflies were rewarded on the models, each of which contained sugar water and flowers of *L. camara*; the floral scent could emanate from the models and the fresh flowers were within reach of the butterfly proboscis. The duration of conditioning varied between the bioassays, since the day of the bioassay was determined by weather conditions; only clear sunny days gave high butterfly activity and permitted testing. Two to 16 hr before a bioassay, i.e., the preceding afternoon or in the early morning of the day of bioassay, the rewarding flower models were removed from the breeding cage. Individuals demonstrating

foraging activity, i.e., wing fluttering and/or uncoiling of proboscis to real *L. camara* flowers held in front of them, were taken into the bioassay cage one by one.

Floral Scent Composition—GC–MS Analysis. The floral scent analysis of *L. camara* is reported elsewhere (Andersson et al., 2002). For the floral scent composition of *P. coronarius*, one twig with 25 flowers was enclosed in a polyacetate bag (Pingvin frying bags, Art.nr 352, Kalle Nalo GmbH D-65203 Wiesbaden, Germany), and two separate floral scent samples were collected. To identify background compounds, control samples were collected from within polyacetate bags without flowers, both from the vegetative parts of the plant and from the ambient air. Scent collection was conducted using the dynamic headspace adsorption method. Volatiles were drawn from the bag enclosure into cartridges containing the adsorbent Tenax-GR (85 mg, mesh 60/80, Chrompack) for 7 hr (11.30 A.M.–06.30 P.M.) by using a pump with an inlet flow rate of 100 ml/min. Prior to use, the adsorbent cartridges were cleaned with 2 ml of diethyl ether, dried with nitrogen gas, then wrapped in aluminum foil to protect from contamination. Trapped volatiles were eluted with 300–400 μl diethyl ether (Fluka, 99.8% pure) to a volume of 250 μl into a glass vial. Each sample was spiked with 20 ng methyl stearate for quantification, and stored at -18°C until analysis. Samples were analyzed by combined gas chromatography and mass spectrometry (GC–MS) using an Hewlett-Packard HP5890 II/5972. Two microliters of each sample were injected using an HP 7673 auto sampler, at 220°C. The splitter was off for 0.5 min, then split with a purge flow rate of 30 ml/min. The GC was equipped with a fused silica column (25 m $\times$ 0.25 mm ID), coated with 0.2 μm nitroterephtal acid derivative of polyethylene glycol (PEG), OV-351 as stationary phase. Helium was used as carrier gas at a constant flow rate of 30 cm/sec. The temperature of the GC oven was kept constant at 50°C for 5 min, increased by 8°C/min to 230°C, and then kept isothermal for 10 min. The GC–MS transfer line temperature was 225°C. Tentative identifications were made, by matching mass spectra and retention times to a library obtained with the same type of column, instrument, and temperature program as the samples. Other computer libraries (NIST) and reference literature (Adams, 1989) were also used in identification.

Statistical Analyses. To test for differences ($P < 0.05$) in the number of probing visits (used only for the first visit by each individual) between the two models in bioassay III and VI, the binomial sign test was used (Underwood, 1997). Differences ($P < 0.05$) in the duration of behavioral responses in all bioassays were tested with analysis of variance (ANOVA), followed by an all-pair-wise multiple comparison Tukey Test (SigmaStat 2.03. SigmaStat, SPSS Inc, Chicago, USA). Each data set was square root or ln transformed in order to get homogenous variances; significant differences were considered only when homogeneous variances were obtained (Underwood, 1997). To detect differences between sexes, a three-factor ANOVA was used, with model, behavior, and gender as the fixed factors, and

the data set was balanced by randomly excluding some individuals. If no difference ($P > 0.05$) was found between females and males, the difference in duration of behavior on the two models for all individuals, including those of undetermined gender, was tested with a two-factor ANOVA, with model and behavior as the fixed factors.

RESULTS

Behavioral Response to Floral Scents. Butterflies showed a high responsiveness to *Lantana camara* floral scent (fl-scent) in terms of probing activity (Figure 1). Newly emerged individuals preferred fl-scent to vegetative scent (veg-scent), of *L. camara* and the *L. camara* flower-conditioned individuals preferred the floral scent of *L. camara* to that of *P. coronarius*. However, for the *L. camara* flower-conditioned butterflies, color seemed to be ranked higher than floral scent. Females and males did not differ in visit duration, based on the results of gender-determined individuals.

Bioassay I: Newly emerged butterflies spent more time probing on yellow fl-scent than on yellow veg-scent. Furthermore, the duration of probing was greater than non-probing on the fl-scent, but similar on veg-scent. *Bioassay II*: Butterflies conditioned on *L. camara*+yellow probed longer on yellow veg-scent than on green fl-scent. *Bioassay III*: Butterflies conditioned on *L. camara*+yellow showed no difference in number of visits between the yellow fl-scent and yellow veg-scent (nine visits to each). Similarly, each visit was accompanied by probing, and the duration of probing did not differ between the models. *Bioassay IV*: Butterflies conditioned on *L. camara*+yellow (4 days) followed by +green (2 days) showed a preference for floral over vegetative scent on the green models; they spent more time probing on fl-scent than on veg-scent, and the duration of probing was greater than non-probing on fl-scent, but not on veg-scent. Furthermore, thirteen individuals (five females, five males, and three unsexed) made deep probes on fl-scent (mean = 2.7 probes/individual), but none on veg-scent. *Bioassay V*: Butterflies conditioned on *L. camara*+yellow followed by +green (4 days each) showed no difference in probing duration between green fl-scent and yellow veg-scent. However, eight individuals (three females, three males, and two unsexed) made deep probes on green fl-scent (mean = 5.2 probes/individual) compared to four (two females, one male, and one unsexed) on yellow veg-scent (mean = 2 probes/individual), indicating a higher importance of floral scent when combined with the most recent conditioned color. *Bioassay VI*: Butterflies conditioned on *L. camara*+yellow (4 days), +green (4 days), and then +yellow (8 days) directed more of their first visits accompanied by probing to yellow fl-scent of *L. camara* than to the yellow floral scent of *Philadelphus coronaries*. Of the 22 individuals tested, 16 (73%) made their first visits to *L. camara* compared to six (27%) individuals to *P. coronarius*. Each individual that visited a model immediately probed, and spent more time probing on

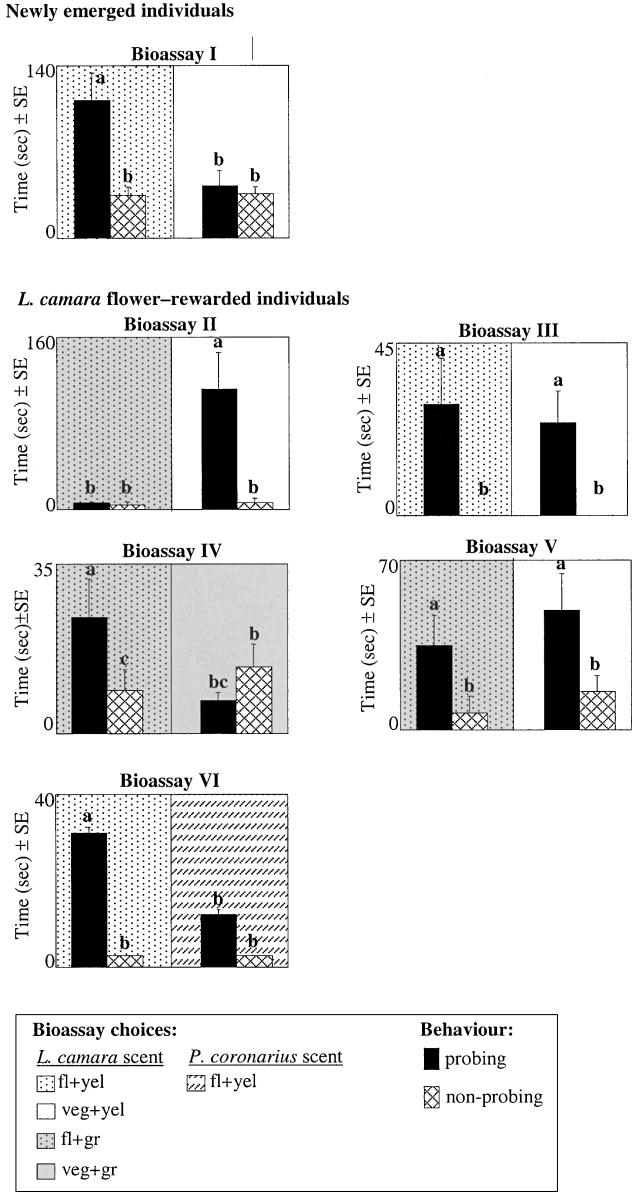

FIG. 1. Duration (mean ± SE) in seconds of probing and nonprobing responses in the two-choice bioassays. For conditioning of butterflies and replicates, see Table 1. Fl = floral scent, veg = vegetative scent, yel = yellow, and gr = green. Different letters on the bars indicate statistically significant differences (Tukey Test, $P < 0.05$).

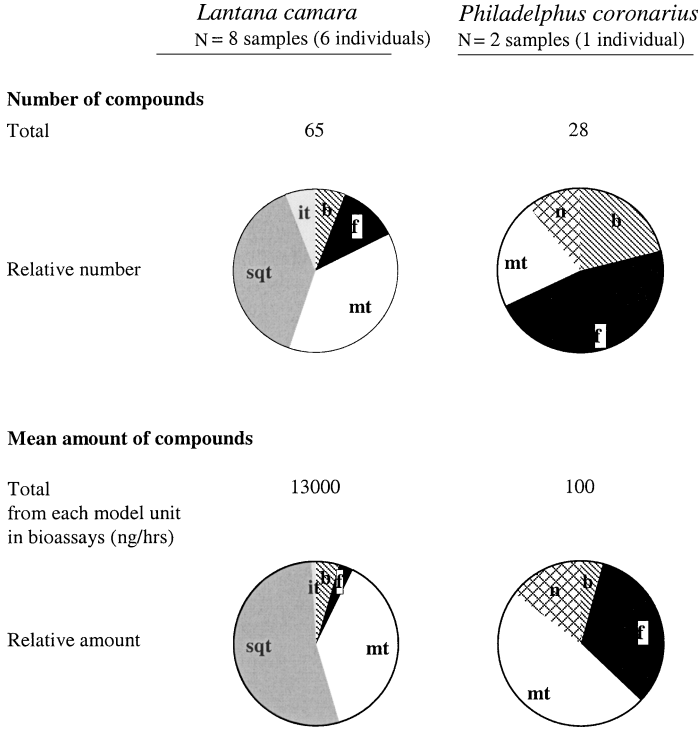

FIG. 2. The floral scent composition of *L. camara* and *P. coronarius* in terms of the number and amounts of floral scent compounds represented in chemical groups of different biosynthetic origin where, b = benzenoid compounds, f = fatty acid derivatives, mt = monoterpenes, sqt = sesquiterpenes, it = irregular terpenes, and n = nitrogen containing compounds.

the *L. camara* than *P. coronarius* floral scented model. Furthermore, four females made deep probes on the *L. camara* model (mean = 3 probes/individual), while none were observed on the *P. coronarius* model. However, the quantity of floral scent emitted from a single model unit in the bioassay was 100-fold greater for *L. camara* (13,000 ng/hr) than *P. coronarius* (100 ng/hr) (Figure 2).

Floral Scent Composition. Although the floral scent composition of *L. camara* has been reported in detail (Andersson et al., 2002), it is given for comparison in Table 2 together with that of *P. coronarius*. *L. camara* emits over twice as many different compounds (65) as *P. coronarius* (28). Sesquiterpenes and monoterpenes were most diverse in *L. camara*, and fatty acid derivatives in *P. coronarius* (Figure 2). In terms of the relative amounts of each compound, *L. camara* was dominated by sesquiterpenes, and *P. coronarius* by monoterpenes (Figure 2).

TABLE 2. FLORAL SCENT COMPOSITION OF *L. camara* (*Lc*) (ANDERSSON ET AL., 2002) AND P. CORONARIUS (*Pc*), AS MEAN RELATIVE AMOUNT (%) OF EACH COMPOUND

	Plant species	
	Lc	*Pc*
Number of samples (individuals)	8 (6)	2 (1)
Fatty acid derivatives[a]		
Hexanal	trace[b]	
2-Heptanone		1.3
trans-2-Hexenal	0.1	
cis-3-Hexenol	0.2[c]	
2-Nonanone		16.0
Nonanal	0.4	
2-Nonanol		0.9
Decanal		1.7
1-Octen-3-ol	0.1	
Pentadecane	0.5	0.1
Hexadecane	0.9	0.2
Heptadecane		0.3
2-Undecanone		0.2
Ethyl dodecanoate		2.4
Isopropyl myristate		0.2
Ethyl tetradecanoate		8.9
Ethyl hexadecanoate		0.2
Benzenoid compounds		
Benzaldehyde	3.9	0.9
Phenylacetaldehyde		trace
Methyl benzoate	0.4	
Ethyl benzoate (benzoic acid ethyl ester)		3.2
Methyl 2-hydroxybenzoate (methyl salicylate)	0.3	trace
Benzeneacetic acid ethyl ester		0.3
Benzylalcohol (phenylmethanol)	0.1	
4-Methoxy ethylbenzoate		trace
Monoterpenes		
α-Pinene	0.9	
α-Thujene	0.0	
β-Pinene	0.6	
Sabinene	3.7	
α-Phellandrene	0.5	
Myrcene	0.4	
Limonene	0.5	
β-Phellandrene	0.3	
cis-β-Ocimene	0.8	
trans-β-Ocimene	21.3	
Allo-ocimene	0.1	
Linalool oxide (furanoid) I	0.5	trace
trans-4-Thujanol	0.1	

TABLE 2. CONTINUED

	Plant species	
	Lc	*Pc*
Linalool oxide (furanoid) II	0.5	11.1
6-Ethenyltetrahydro-2,2,6-trimethyl-2H-pyran 3(4H)-one	0.2	22.2
trans-β-Ocimene epoxide	trace	
Linalool	0.6	trace
Neral	1.4	
Methyl geranate	0.2	
Linalool oxide (pyranoid) I	1.2	trace
Linalool oxide (pyranoid) II	1.6	14.6
trans-Ocimenol	trace	
Nerol	0.1	
Geraniol	0.5	
Geranylacetone	trace	
Sesquiterpenes		
α-Cubebene	0.2	
δ-Elemene	0.0	
α-Copaene	0.1	
β-Bourbonene	0.1	
β-Cubebene	1.0	
cis-α-Bergamotene	0.2	
α-Cedrene	0.2	
β-Elemene	0.9	
β-Caryophyllene	23.1	
Thujopsene	0.1	
α-Caryophyllene (α-humulene)	13.7	
trans-β-Farnesene	2.0	
Unidentified *m*/*z*: 93 119 121 161 204	2.8	
Germacrene D	1.7	
α-Zingiberene	2.9	
α-Muurolene	0.2	
Germacrene B	0.3	
trans, *trans*-α-Farnesene	1.0	
δ-Cadinene	0.6	
(+)-γ-Cadinene	0.2	
AR-curcumene	0.6	
γ-Elemene	trace	
Caryophyllene oxide	0.4	
trans-Nerolidole	1.2	
T-cadinol	trace	
Irregular terpenes		
4,8-Dimethyl-1,3-(*E*)-7-nonatriene	0.6	
6-Methyl-5-hepten-2-one	0.1	
4,8,12-Trimethyl-1,3-(*E*)-7-(*E*)-11-Tridecatetraene	0.1	

TABLE 2. CONTINUED

	Plant species	
	Lc	*Pc*
Dihydro-β-ionone	trace	
Nitrogen containing compounds		
N-phenyl-Formamide		11.5
Indole		1.0
Unidentified *m*/*z*: 66 93 121 149		2.3
Unidentified *m*/*z*: 43 59 81 95 107 135 168		0.3

[a] The compounds are arranged in chemical groups with similar biosynthetic origin, and listed in GC–retention time order within each group.
[b] Trace amounts are less than 0.05%.
[c] The relative mean amount of a compound is underlined for compounds that are exclusively emitted from flowers.

Monoterpenes were emitted in relatively large amounts from both species, and the six compounds in *P. coronarius* were also emitted from *L. camara*, which emitted 25 compounds (Table 2). The compounds emitted in largest relative amounts from *L. camara* were the monoterpene *trans*-β-ocimene (21.3%), and the sesquiterpenes β-caryophyllene (23.1%) and α-caryophyllene (13.7%), which were emitted from both the flowers and vegetative parts of the plant (Table 2). Compounds that were exclusively in the floral scent were in lower representation, and included, for example, the two linalool oxide pyranoid monoterpenes (1.2% and 1.6%, respectively) and the two benzenoid compounds methyl benzoate and benzyl alcohol (both less than 0.5%). The quantitatively dominant compounds in the floral scent of *P. coronarius* were more numerous and included the following: the monoterpenes 6-ethenyltetrahydro-2,2,6-trimethyl-2H-pyran-3(4H)-one (22.2%), linalool oxide (pyranoid) II (14.6%), and linalool oxide (furanoid) II (11.1%; the fatty acid derivative 2-nonanone (16%); and the nitrogen containing compound N-phenylformamide (11.5%). In contrast to*L. camara*, all of the dominant compounds were of floral origin exclusively.

DISCUSSION

Floral scent is vital in the recognition of food-rewarding flowers of *Lantana camara* by newly emerged *Heliconius melpomene* butterflies. Although many of the scent compounds in *L. camara* were emitted from both flowers and vegetative parts, butterflies showed an olfactory sensitivity that enabled them to differentiate between the floral and vegetative blends. Thus, scent compounds of exclusively floral origin, which included linalool and both linalool oxide pyranoids, are obviously important although they are present in the floral scent in low relative amounts (less

than 2%). In contrast, floral scent compounds emitted from both flowers and vegetative parts, some of which were emitted in relatively high amounts and dominated the floral scent (e.g., the monoterpene *trans*-β-ocimene, and the sesquiterpenes β-and α-caryophyllene) seem to be of less importance as food signals.

After butterflies were rewarded with sugar water presented together with *L. camara* floral scent in yellow models, the color yellow was more strongly associated with reward than floral scent, as judged by the duration of probing. Indeed, after being rewarded on yellow models with *L. camara* floral scent, butterflies preferred yellow models with vegetative scent over green ones with floral scent (bioassay II, Figure 1). Furthermore, they showed no preference when offered a choice of yellow models with floral scent as opposed to vegetative scent (bioassay III, Figure 1), which is opposite to newly emerged butterflies offered the same choice. Thus, floral scent is of primary and greater importance than color to newly emerged butterflies. In Flower-conditioned and, thus, foraging experienced butterflies, the color yellow seems to be the most important sign of food reward, with floral scent being of secondary importance.

When butterflies were conditioned to green scented models, they also learned to associate the green color with food, but they did so to a lesser extent than with yellow; floral scent was of greater importance than green. When both choice models were green in the tests, the scent of flowers was preferred over that of the vegetative parts (bioassay IV). When offered a choice between green floral-scented and yellow vegetative-scented models (bioassay V), which is the same choice as in bioassay II where the yellow-conditioned butterflies preferred the yellow model, the green-conditioned butterflies did not show any preference between the two models. This could be because of a diminished association of food with yellow to the advantage of green and/or to a stronger association of food with floral scent than with green. The greater number of individuals of *H. melpomene* that made deep probes on the floral-scented models than on the vegetative ones in bioassays IV and V emphasizes the crucial role and clearly stimulating effect of floral scent in the recognition of food-rewarding sources at a close distance.

The color yellow used in the bioassays was assumed to be a preferred color for *H. melpomene*, since yellow and orange were prefered to blue and green colors in feeding assays with the closely related species *Heliconius charitonius* and *H. erato* (Crane, 1955; Swihart and Swihart, 1970; Swihart, 1971, 1972). Several species of butterflies can be conditioned to select almost any portion of the human visible spectrum and to form search images for flowers consisting of several hues, except for shades of grey (Crane, 1955; Swihart and Swihart, 1970; Swihart, 1971, 1972), and color preferences can be easily switched in association with food reward (Goulson and Cory, 1993; Weiss, 1995a). However, red and yellow were most easily associated with food in the butterfly *Papilio xuthus* while other colors took a longer time to learn, most likely because of innate preferences to red and yellow (Kinoshita et al., 1999).

Two temperate butterfly species, *Inachis io* and *Aglais urticae* (Nymphalidae), show innate olfactory preference to floral scents of specific nectar plants over non-nectar plants (Andersson, 2003). A similar ability in newly emerged *H. melpomene* individuals remains to be tested. However, when *H. melpomene* butterflies were rewarded on *L. camara* in this study, they showed a clear preference for the floral scent of *L. camara* over that of *P. coronarius*, thus demonstrating an ability to discriminate species-specific floral scent blends. This preference was shown in the number of visits accompanied by probing, indicating discrimination at a distance, as well as in the duration of probing. Although the amounts of emitted floral scent were 100-fold greater in *L. camara* than *P. coronarius* flower-scented models, the scent of *P. coronarius* was easily perceived by the human nose and, thus, probably also by the butterflies. The underlying basis of the butterfly's selectivity towards the two scents is not entirely clear. Since butterflies were conditioned to *L. camara* floral scent, the preference could be the result of a discrimination between a butterfly and a non-butterfly pollinated plant, or of an associative learning to the previously rewarding *L. camara* floral scent, or to both. Scent compounds not normally found in food sources have been learned to be associated with food in a moth species (Hartliebet al., 1999), and it is possible that *H. melpomene* butterflies could likewise learn to associate the floral scent of *P. coronarius* with food even though the plant is not a butterfly nectar plant. However, this remains to be experimentally tested.

The honeybee, *Apis mellifera* (Hymenoptera), is capable of using one or more sensory traits to enhance its foraging efficiency in different environmental situations (Giurfa et al., 1994). Also this may be true for butterflies in their relative dependence on olfaction and vision in selecting floral food sources. Taken together, the results from this study demonstrate that *H. melpomene* integrates olfaction and vision in locating food sources. However, while newly emerged butterflies rely mainly on olfactory floral cues to select flowers, upon gaining foraging experience the butterflies learn to associate a food source more with visual stimuli, although the relative strength of this association and primary reliance on vision is subject to variation depending on the traits of the food source.

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ANTENNAL RESPONSES TO FLORAL SCENTS IN THE BUTTERFLY *Heliconius melpomene*

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Abstract—Floral scent, together with visual floral cues, are important signals to adult butterflies searching for food-rewarding plants. To identify which compounds in a floral scent are more attractive and, thus, of biological importance to foraging butterflies, we applied electrophysiological methods. Antennal responses of male and female adults of the tropical butterfly *Heliconius melpomene* L. (Lepidoptera: Nymphalidae: Heliconiinae) to individual compounds of natural floral scents and synthetic floral scent mixtures were investigated using gas chromatography–electroantennographic detection (GC-EAD). The natural floral scents included those of two tropical plant species, *Lantana camara* L. (Verbenaceae) and *Warszewiczia coccinea* (Vahl) Kl. (Rubiaceae), and two temperate species, *Buddleja davidii* Franchet (Loganiaceae) and *Cirsium arvense* (L.) Scop. (Asteraceae). The two synthetic floral scent mixtures contained many of the compounds found in the natural scents, but all in equal quantities. Compounds both present in relatively high abundance in the floral scents and detected exclusively in the floral parts of the plant, such as linalool, linalool oxide (furanoid) I and II, oxoisophoroneoxide, and phenylacetaldehyde, elicited the strongest antennal responses, suggesting that they may reflect adaptations by the plant to attract butterfly pollinators. However, other compounds also present in high abundance in the floral scent, but detected in the vegetative as well as floral plant parts, either elicited strong antennal responses, such as *trans*-β-ocimene and benzaldehyde, or failed to elicit antennal responses, such as the sesquiterpenes β-caryophyllene and α-humulene from *L. camara*. The most volatile monoterpene alkenes in the synthetic scent mixtures elicited only low or no responses. Furthermore, the overall antennal responses were stronger in females than in males. The findings suggest that several floral scent volatiles, especially those of exclusively floral origin, are of high biological

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significance to *H. melpomene* butterflies. These include compounds of different biosynthetic origins belonging to the benzenoids, monoterpenoids, and irregular terpenoids.

Key Words—Floral scent, *Heliconius melpomene*, Nymphalidae, Lepidoptera, *Lantana camara*, *Warszewiczia coccinea*, *Buddleja davidii*, *Cirsium arvense*, GC-MS, GC-EAD, benzenoid, terpenoids.

INTRODUCTION

Floral scents provide important guides in the nectar-seeking behavior of butterflies (Ilse, 1928; Groth et al., 1987; Honda et al., 1998). Scents emitted from flowers typically consist of complex mixtures of volatiles, with species–specific compositions (Knudsen et al., 1993). Some compounds may have evolved as adaptations to attract pollinating butterflies, and these would be expected to elicit a high attractiveness for foraging butterflies. To identify these behaviorally active compounds, the complex scent mixture first needs to be narrowed down to a list of potential candidate compounds, which can then be tested in behavioral experiments. This initial screening can be done using electrophysiological techniques, such as gas chromatography coupled with electroantennographic detection (GC-EAD) (Arn et al., 1975). By using the whole antennae of butterflies in GC-EAD, the summed neural activity of antennal olfactory receptors can be measured. Compounds eliciting a neural activity from the antennae are most likely of biological significance to the butterfly. Strong antennal responses to volatiles, measured by GC-EAD, have been shown to correspond closely with behavioral responses in various insects, such as in the attraction of parasitic wasps to bark beetle larvae (Pettersson, 2000), reactions of males of the solitary bee *Andrena nigroaenea* to copulation inhibiting volatiles in the cuticular extracts of unattractive females (Schiestl and Ayasse, 2000), and the attraction of the moth *Autographa gamma* to floral volatiles (Plepys, 2001). In general, behavior-mediating floral scent compounds show high electrophysiological activity (review, Schiestl and Marion-Poll, 2001), making electrophysiological analyses a useful tool for delimiting the compounds in a floral scent that should be behaviorally tested. However, dosage is a factor that may also influence the activity elicited in antennal receptors, as shown in the butterfly *Pieris rapae* L. (Pieridae), which demonstrated a stronger antennal response to phenylacetaldehyde compared to other compounds only when it was applied at a certain dosage level (Ômura et al., 1999).

The present study uses GC-EAD to explore the antennal responses of the tropical butterfly *Heliconius melpomene* to floral scents of butterfly nectar plants. *Heliconius* butterflies have a well-developed color vision (Swihart, 1972), which together with olfactory and gustatory senses is of vital importance in their lives, for example, in defense, roosting, courtship, and oviposition (Crane, 1955; Benson

et al., 1974; Brown, 1981; Spencer, 1988; Agosta, 1992). *Lantana camara* L. (Verbenaceae) is a favorite nectar plant for *Heliconius* spp., and for several other tropical butterfly species, and is also mainly pollinated by butterflies (Crane, 1955; Gilbert, 1972; Barrows, 1976; Schemske, 1976; Faegri and van deer Pijl, 1979; Boggs et al., 1981). *Warszewiczia coccinea* (Vahl) Kl. is another tropical plant frequently visited by butterflies (Crane, 1955), and butterflies and hummingbirds are the probable pollinators (Bawa and Beach, 1983). The floral scents from these two tropical plants, and from two temperate plants *Buddleja davidii* Franchet that are adapted to butterflies (Faegri and van deer Pijl, 1979; Proctor et al., 1996), and *Cirsium arvense* (L.) Scop, which is frequently visited by butterflies as well as bees (Douwes, 1978; Jennersten, 1980, 1984; Proctor et al., 1996), were tested for their impact on *H. melpomene* antennal activity. In addition, mixtures of synthetic compounds commonly encountered in butterfly-pollinated flowers were tested.

METHODS AND MATERIALS

Butterfly and Plant Species Heliconius melpomene. L. (Lepidoptera: Nymphalidae: Heliconiinae) pupae were obtained from the Stratford butterfly farm in Stratford-upon-Avon, UK. Adult butterflies were individually marked on their hind wings with permanent ink, and maintained in $60 \times 60 \times 60$ cm net cages until GC-EAD analysis. In the cages, the butterflies were offered their host plant *Passiflora caerulea* L. (Passifloraceae), which was obtained from Grandiflora AB, Partihallarna, Göteborg, Sweden; flowering plants of *Lantana camara* L. (Verbenaceae); water (in black and white cups; 3 cm i.d., 1.5 cm depth); and sugar water (sucrose: water, 1:4 by volume, in Eppendorf-vessels or sponges within floral models).

Floral scents tested on the butterflies were collected from four plant species: (1) *Lantana camara* L. (Verbenaceae), cloned cultivars "Roma," native to the West Indies, obtained from Grandiflora AB, Partihallarna, Göteborg; (2) *Warszewiczia coccinea* (Vahl) Kl. (Rubiaceae), growing in primary rainforest at the Tiputini Biodiversity Station (76°04′ W, 00°38′S, alt. ca. 200 m), Napo Province, Ecuador; (3) *Buddleja davidii* Franchet (Loganiaceae), growing in the Botanical Garden of Göteborg; and (4) *Cirsium arvense* (L.) Scop. (Asteraceae), growing in the vicinity of Göteborg.

Floral Scent Samples. Floral scent samples were collected from rooted plants of *L. camara* in the lab in Göteborg, September 1998, *W. coccinea* in the field in Ecuador, January 1997 (Voucher: Knudsen et al., 747, in GB: Göteborg, Sweden, and in QCA: Ecuador), *B. davidii* in the Botanical Garden of Göteborg, September 1998, and from cut plants of *C. arvense* in the lab in Göteborg, August 1998.

Floral scent collections were conducted using the dynamic headspace adsorption method. Flowers were enclosed in polyacetate bags (Pingvin frying bags, Art.nr 352, Kalle Nalo GmbH D-65203 Wiesbaden, Germany), and volatiles were

drawn from the enclosures into cartridges containing the adsorbent Tenax-GR (85 mg, mesh 60/80, Chrompack) for 10 hr during daytime by using a pump with an inlet flow rate of 100 ml/min. Prior to use, the adsorbent cartridges were cleaned with 2 ml of diethyl ether, dried with nitrogen gas, then wrapped in aluminum foil to protect from contamination. Trapped volatiles were eluted with 300–400 μl diethyl ether (Fluka, 99.8% pure) or pentane (Fluka, $>$99.5% pure) to a volume of 250 μl into a glass vial. Twenty nanograms methyl stearate were added to each sample for quantification, and the samples were stored at -18°C until analysis. To identify contaminations, control samples were collected both from the vegetative parts of the plant (polyacetate bags containing plants with no flowers) and from the ambient air (empty bags).

Scent Samples Used in GC-EAD. Floral scent eluates, representing the actual qualitative and quantitative composition of compounds emitted from the flowers, were used in the GC-EAD analyses. The amount of each compound presented to the antennae was estimated from GC-MS analysis of the eluates. In addition, two synthetic floral scent blends were prepared by mixing typical floral compounds, mainly monoterpenes, irregular terpenes, and benzenoids, in pentane; the amounts used were such that the antennae were exposed to about 35 ng of each compound in the GC-EAD analyses.

Floral Scent Composition–GC-MS Analyses. Floral scent samples were also analyzed by combined gas chromatography and mass spectrometry (GC-MS), using an Hewlett-Packard HP5890 II/5972, to identify the constituent volatiles. Two microliters of each sample were injected into the GC using an HP 7673 auto sampler, at 220°C. The splitter was off for 0.5 min, then split with a purge flow rate of 30ml/min. The GC was equipped with a fused silica column (25 m $\times$ 0.25 mm i.d.), coated with 0.2 μm nitroterephtal acid derivative of polyethylene glycol (PEG), OV-351 as the stationary phase. Helium was used as the carrier gas at a constant flow rate of 30 cm/sec. Temperature of the GC oven was kept constant at 50°C for 5 min, increased by 8°/min to 230°C, and then kept isothermal for 10 min. The GC-MS transfer line temperature was 225°C.

Tentative identification of compounds was made by matching mass spectra and retention times to those of a library obtained with the same type of column, instrument, and temperature program as the samples. Other computer libraries (NIST) and reference literature (Adams, 1989) were also used in identification. The GC-MS analyses used the similar stationary GC-phases and temperature program as in the GC-EAD (below) to allow identification of the compounds in GC-EAD.

Antennal Response–Electrophysiological Analyses. The combined GC-EAD was conducted in December 1998, using a Shimadzu 14A GC equipped with a fused silica column (25 m $\times$ 0.25 mm i.d.), coated with 0.2 μm PEG (CP-Wax 52 CB) as the stationary phase. Nitrogen was used as the carrier gas at a flow rate of 12 cm/sec (at 175°C). Samples were injected split-less for 0.5 min, thereafter split with a purge flow rate of 30 ml/min at an injector temperature of 200°C.

Temperature of the GC oven was kept constant at 35°C for 2 min, increased 8°/min to 200°C, then 30°/min to 230°C, and finally kept isothermal for 6 min. The GC was connected to both a flame ionization detector (FID) and an EAD, with the split ratio 1:1. The effluent from the GC-column was mixed with split-makeup gas (nitrogen with a flow rate of 20 ml/min) in a union T and passed into a glass Y splitter (quick seal splitter, Chrompack). Two deactivated fused silica columns of equal length connected the GC-column from the glass Y-splitter to the FID and the EAD. The FID temperature was set at 230°C. The temperature program of the GC-EAD transfer line was 50°C for 2 min, increased 15°/min to 230°C, and isothermal for 15 min. The EAD fused silica column discharged into a glass-tube, and a moistened air stream chilled and transported the GC effluent to the antenna with a constant flow rate (12 cm/sec, tube length 9 cm).

To prepare the antenna for the GC-EAD, it was removed from the butterfly, and the base was connected to the grounding electrode, while the tip, which was cut (ca. 2 mm removed) in order to get better contact, was connected to the recording electrode. Before and after each GC-EAD run, the responsiveness of the antenna to the whole scent blend was confirmed by electroantennogram technique (EAG): the whole floral scent was puffed directly on the antenna through a Pasteur pipette that was inserted into the glass interface between the GC exit and antenna. Identification of each floral scent compound was made by GC-MS (see above), and the approximate amount of each compound was calculated from the GC-MS analyses.

Statistical Analyses. Differences ($P < 0.05$) in the intensity of antennal response to compounds were tested with ANOVA, followed by a post hoc Tukey test (SigmaStat 2.03. SPSS Inc.). Each data set was square rooted or ln transformed in order to get homogeneous variances. Significant differences were considered only when the analyses gave homogeneous variances and a power above 0.80 at $\alpha = 0.05$ (Underwood, 1997). Differences in antennal responses by all individuals (males and females) to compounds in the natural floral scents were tested by a one-factor ANOVA; the individuals of each gender were too few to allow comparison of responses between males and females. Differences in responses between males and females to compounds in the synthetic blends were tested using a two-factor ANOVA.

RESULTS

The butterflies showed antennal responses to several of the compounds both in the natural floral scents of *Lantana camara*, *Warszewiczia coccinea*, *Buddleja davidii*, and *Cirsium arvense*, and in the synthetic floral scent mixtures (Figures 1 and 2). The overall antennal responses to compounds of the synthetic mixtures were significantly stronger in females than in males ($P < 0.05$) (Figure 2). With respect

Compounds from L. camara
♂ n=2
♀ n=6
α-pinene(mt)
sabinene(mt)
cis-β-ocimene(mt)
trans-β-ocimene(mt)
(E),7-nonatriene(it)
nonanal(f)
linox(fur)I(mt)
linox(fur)II(mt)+pyr-one(mt)
benzaldehyde(b)+β-cub(sqt)
linalool(mt)
sqt 1(sqt)
β-caryophyllene(sqt)
α-humulene(sqt)
neral(mt)+sqt 2(sqt)
germacreneD(sqt)
geranial (mt)
sqt 3 (sqt)
sqt 4 (sqt)
sqt 5(sqt)+pyran-ol(mt)
geraniol(mt)
caryophyllene oxide(sqt)
Compounds from W. coccinea
♂ n=3
♀ n=6
myrcene(mt)
limonene(mt)
cis-β-ocimene(mt)
trans-β-ocimene(mt)
(Z), 7-nonatriene(it)
(E), 7-nonatriene(it)
6-methyl-5-hepten-2-one(it)
nonanal(f)+allo-ocimene(mt)
linox(fur)I(mt)
benz(b)
linox(fur)II(mt)+pyr-one(mt)
β-bourbonene(sqt)
linalool(mt)
β-caryophyllene(sqt)
hexadecane(f)
(Z)-α-farnesene(sqt)
(E)-α-farnesene(sqt)
Compounds from B. davidii
♂ n=2
♀ n=4
cis-β-ocimene(mt)
trans-β-ocimene(mt)
6-methyl-5-hepten-2-one(it)
hexanol(f)
2-hydroxyheptan-3-one(f)
3-hydroxyheptan-2-one(f)
benzaldehyde(b)
β-cyclocitral(it)
phenylacetaldehyde(b)
oxoisophoroneoxide(it)
oxoisophorone(it)
(E)-α-farnesene(sqt)
dihydroxoisophorone(it)
geranyl acetone(it)
phenyl methanol(b)
2-phenyl ethanol(b)
Compounds from C. arvense
♂ n=2
♀ n=3
linox(fur)II(mt)
pyran-one(mt)
benzaldehyde(b)
linalool(mt)
phenylacetaldehyde(b)
linox(pyr)I(mt)
linox(pyr)II(mt)
methyl salicylate(b)
phenyl methanol(b)
2-phenyl ethanol(b)
methyl 2-methoxybenzoate(b)
Amount (ng)
Mean response (mV) ± SE
60 0 0.06 0.12

to the floral scents of *L. camara*, *B. davidii*, and *C. arvense*, female responses were not significantly higher (Figure 1), whereas for the floral scent of *W. coccinea*, male antennae responded more strongly than did those of the females.

Antennal Responses to Natural Floral Scents. For the GC-MS analyses of the floral scents, only compounds that were detected in the GC-EAD analyses are reported here (Figure 1); the detailed floral scent compositions have been reported previously (Andersson et al., 2002). Some of the volatiles detected in the GC-MS of floral scents did not separate in the GC-EAD analysis (Figure 1). These included three compound pairs in *L. camara*: linalool oxide (furanoid) II and 6-ethenyl tetrahydro-2,2,6-trimethyl-2H-pyran-3(4H)-one, benzaldehyde and β-cubebene, and neral and sesquiterpene 2; and two pairs in *W. coccinea*: nonanal and allo-ocimene, and linalool oxide (furanoid) II and 6-ethenyltetrahydro-2,2,6-trimethyl-2H-pyran-3(4H)-one. Consequently, antennal responses to each of these individual compounds could not be evaluated.

In general, volatiles that were both present in high amounts ($\geq$10 ng) in the natural scents and exclusively of floral origin elicited strong antennal responses. These included most notably linalool from *W. coccinea*, oxoisophoroneoxide, oxoisophorone, and phenyl methanol from *B. davidii*, and linalool oxide (furanoid) II and phenylacetaldehyde from *C. arvense*. Sharp exceptions to this pattern were β-caryophyllene and (*E*)-α-farnesene from *W. coccinea*, and linalool oxide (pyranoid) I from *C. arvense*, which elicited only low or no responses. With respect to compounds in large amounts but of both floral and vegetative origin, high responses were elicited by *trans*-β-ocimene from *L. camara* and *W. coccinea*, benzaldehyde in *B. davidii* and *C. arvense*, and 2-phenyl ethanol from *B. davidii*, while at the other

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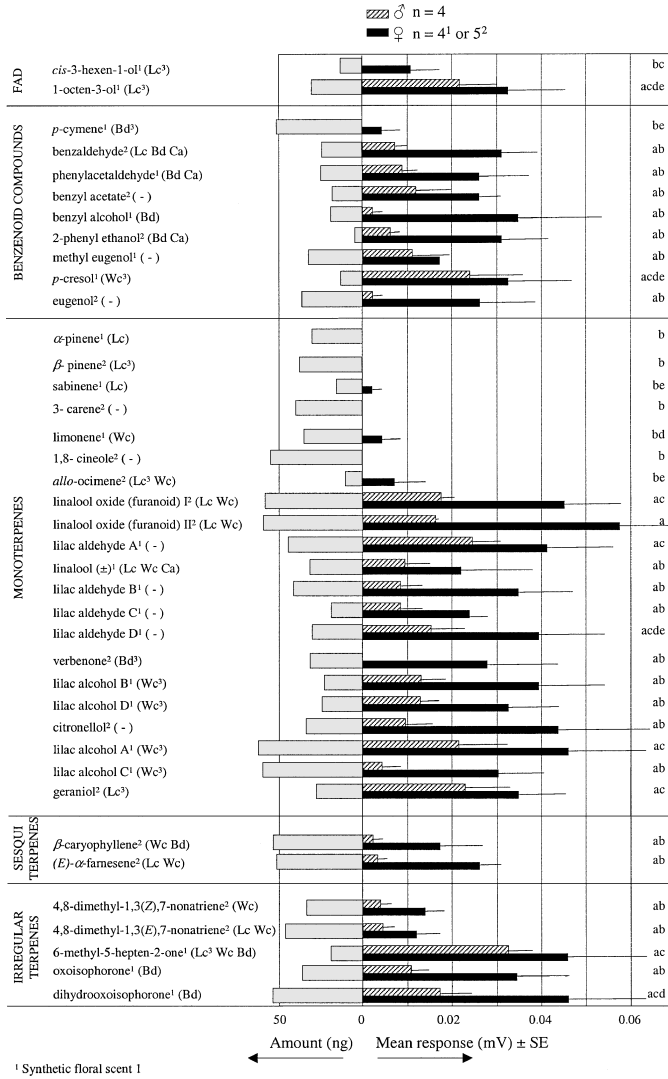
FIG. 1. EAD-responses (mean $\pm$ SE) in adult males and females of *Heliconius melpomene* (bars on right), when their antennae are exposed to the natural floral scent compounds (bars on left) of *Lantana camara*, *Warszewiczia coccinea*, *Buddleja davidii*, and *Cirsium arvense*. The compounds are listed in order of their GC retention times. Compounds that are of exclusive floral scent origin from a plant are underlined. Abbreviations of compound names; (*E*),7-nonatriene: 4,8-dimethyl-1,3-(*E*), 7-nonatriene; linox(fur)I: linalool oxide (furanoid) I; linox(fur)II: linalool oxide (furanoid) II; sqt: unidentified sesquiterpene; pyrone: 6-ethenyltetrahydro-2,2,6-trimethyl-2H-pyran-3(4H)-one; β-cub: β-cubebene; pyranol: 6-ethenyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol; benz: unidentified benzenoid m/z 91 119 134 77 105; linox(pyr)I: linalool oxide (pyranoid) I; and linox(pyr)II: linalool oxide (pyranoid) II. α-humulene = α-caryophyllene, and β-cyclocitral = 2,6,6-trimethyl-1-cyclohexene-1-carboxal. The biosynthetic origin of each compound is noted in brackets; mt: monoterpene; it: irregular terpene; b: benzenoid compound; sqt: sesquiterpene; and f: fatty acid derivative. For each plant species, responses marked with different letters indicate significant differences between the responses of all individuals to the compound in question (Tukey test, $P < 0.05$).



[1] Synthetic floral scent 1
[2] Synthetic floral scent 2
[3] The compound is emitted from the plant species (Andersson et al., submitted) but was not detected in the GC-EAD analysis

FIG. 2. EAD-responses (mean ± SE) in adult males and females of *Heliconius melpomene* (bars on right), when their antennae were exposed to similar amounts of compounds (bars on left) in the synthetic floral scents. The overall antennal responses to compounds of the synthetic mixtures were significantly stronger in females than in males. Responses marked with different letters indicate significant differences between the average responses of both males and females to the compounds (Tukey test, $P < 0.05$). The compounds are listed in order of their GC retention times within each biosynthetic group. The natural floral scents in which the compounds were found are noted in brackets. Lc: *Lantana camara*; Wc: *Warszewiczia coccinea*; Bd: *Buddleja davidii*; and Ca: *Cirsium arvense*.

extreme low or no responses were elicited by the sesquiterpenes β-caryophyllene and α-humulene from *L. camara*.

Interestingly, some floral scent compounds emitted in relatively small amounts ($<$10 ng) elicited strong responses in the antennae. Among the exclusively floral volatiles were linalool from *L. camara* and *C. arvense*; and linalool oxide (furanoid) I, linalool oxide (furanoid) II paired with 6-ethenyltetrahydro-2,2,6-trimethyl-2H-pyran-3(4H)-one, and nonanal paired with allo-ocimene from *W. coccinea*; and dihydroxoisophorone from *B. davidii*. Only a few compounds emitted in relatively small amounts and of both floral and vegetative origin, such as nonanal from *L. camara* and 6-methyl-5-hepten-2-one from *B. davidii*, elicited strong antennal responses.

Antennal Responses to Synthetic Floral Scent Mixtures. Although the synthetic mixtures were prepared so that equal amounts of each compound would reach the antennae, the actual quantities varied somewhat, from 5 to 67 ng (Figure 2), probably because of physical factors in the GC system. Compounds that elicited the strongest responses ($\geq$0.04 mV for females and/or $\geq$0.02 mV for males) included the fatty acid derivative 1-octen-3-ol; the benzenoid *p*-cresol; the monoterpenes linalool oxide (furanoid) I and II, lilac aldehyde A, lilac alcohol A, citronellol, geraniol, and marginally lilac aldelhyde D; and the irregular terpenes 6-methyl-5-hepten-2-one, and dihydrooxoisophorone. One notable pattern was the remarkably low levels of antennal response recorded for the most highly volatile monoterpene alkenes and the benzenoid *p*-cymene.

DISCUSSION

This study shows that several compounds of exclusively floral origin and present in relatively high amounts in the floral scents of four plant species—such as linalool, linalool oxide furanoids, oxoisophorone, dihydrooxoisophorone, and phenylacetaldehyde—elicited clear and, in most cases, strong antennal responses in the butterfly *Heliconius melpomene*. Thus, this tropical butterfly demonstrated strong antennal responses to volatiles in the floral scent of the tropical plants *Lantana camara* and *Warszewiczia coccinea*, as well as of the temperate plants *Buddleja davidii* and *Cirsium arvense*. This suggests that these compounds may be important attractants to butterflies and that they may reflect plant adaptations to pollinators. Moreover, the presence of floral scent compounds that are attractive to *H. melpomene* in both tropical and temperate plants could represent convergent evolution of floral scent composition to the general olfactory sensitivities of butterflies.

However, in contrast to the findings here for *H. melpomene*, the temperate butterflies *Inachis io* L. (Nymphalidae), *Aglais urticae* L. (Nymphalidae), and *Gonepteryx rhamni* L. (Pieridae) show higher antennal responses to phenylacetaldehyde and 2-phenyl ethanol than to linalool oxide (furanoid) I and II

(Andersson, 2003). This suggests that there are differences in antennal receptor sensitivity between the tropical *H. melpomene* and the temperate butterflies. Interestingly, this differential antennal receptivity is also reflected in the floral scent compositions of the tropical and temperate plants, where phenylacetaldehyde and 2-phenyl ethanol are released in relatively high amounts from the temperate *B. davidii* and *C. arvense*, while not at all from the tropical *L. camara* and *W. coccinea*. Thus, we see possible adaptations of the floral scent composition towards local pollinators. However, a strikingly low—and even absence of—antennal response to the most volatile monoterpene alkenes is found in both *H. melpomene* and the temperate butterflies. In fact, the most volatile monoterpene alkenes are usually not emitted in very large relative amounts from butterfly pollinated plants, which may demonstrate a common adaptation to butterfly pollination (Andersson et al., 2002).

Although analysis of butterfly olfactory receptor activity in response to certain compounds, as revealed by GC-EAD, narrows the list of potentially attractive food related compounds, behavioral studies of butterfly foraging responses are essential to establish which compounds are part of the food signal to the butterfly. Such behavioral studies are few, but some have been performed with the butterfly *Pieris rapae* L. (Pieridae) by Honda et al. (1998) and Ômura et al. (1999). Interestingly, this butterfly made many visits accompanied by proboscis extension to flower models scented with either 2-phenyl ethanol or phenylacetaldehyde, but the antennal receptivity (EAG) to these two compounds was not significantly stronger than to other compounds unless the antennae were tested with higher doses, which yielded a proportionately higher antennal response to phenylacetaldehyde. These findings stress the importance of conducting behavioral tests together with electroantennogram studies to identify the ecological impact of a compound.

With respect to variation in EAD responses related to gender, antennal responses in *H. melpomene* were constantly stronger in females, which may reflect the greater need for females to be highly sensitive to chemicals of their surroundings in order to successfully reproduce. In butterflies, sex pheromones are mainly produced by the males, and the females probably judge a male by his odor (Vane-Wright and Ackery, 1989); during oviposition, females use volatile plant compounds to choose the right host plant (van Loon et al., 1992; Baur et al., 1993). In the context of food searching, *Heliconius* butterflies feed on both nectar and pollen from flowers. However, females feed more often on pollen than do males, which may increase a females nutritional investment in eggs (Gilbert, 1972; Boggs et al., 1981), and females might, thus, have a more refined ability to chemically locate pollen sources. In addition, the higher responses by females than males of *I. io* to nectar chemistry (Erhardt and Rusterholz, 1998) suggests that female butterflies in general may also be more sensitive to floral chemicals at the gustatory as well as the olfactory level.

In conclusion, several benzenoids, monoterpenoids, and irregular terpenoids in the floral scents of *Lantana camara*, *Warszewiczia coccinea*, *Buddleja davidii*, and *Cirsium arvense* seem to be of biological significance for the butterfly *Heliconius melpomene*, as suggested by their elicitation of strong antennal responses. However, behavioral studies are now needed to determine which of these, and possibly other, floral scent compounds are most attractive to foraging *H. melpomene* in the ecological context.

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RESPONSES OF *Glossina morsitans morsitans* TO BLENDS OF ELECTROANTENNOGRAPHICALLY ACTIVE COMPOUNDS IN THE ODORS OF ITS PREFERRED (BUFFALO AND OX) AND NONPREFERRED (WATERBUCK) HOSTS

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Abstract—In a previous study, comparison of the behavior of teneral *Glossina morsitans morsitans* on waterbuck, *Kobus defassa* (a refractory host), and on two preferred hosts, buffalo, *Syncerus caffer*, and ox, *Bos indicus*, suggested the presence of allomones in the waterbuck odor. Examination of the volatile odors by coupled gas chromatography–electroantennographic detection showed that the antennal receptors of the flies detected constituents common to the three bovids (phenols and aldehydes), as well as a series of compounds specific to waterbuck, including C_8–C_{13} methyl ketones, δ-octalactone, and phenols. In this study, behavioral respones of teneral *G. m. morsitans* to different blends of these compounds were evaluated in a choice wind tunnel. The flies' responses to known or putative attractant blends (the latter comprising EAG-active constituents common to all three animals and those common to buffalo and ox, excluding the known tseste attractants, 4-methylphenol and 3-*n*-propylphenol), and to putative repellent (the blend of EAG-active compounds specific to the waterbuck volatiles), were different. A major difference related to their initial and final behaviors. When a choice of attractant blends (known or putative) and clean air was presented, flies initially responded by flying upwind toward the odor source, but later moved downwind and rested on either side of the tunnel, with some preference for the side with the odor treatments. However, when presented with a choice of waterbuck-specific blend (putative repellent) and clean air, the flies' initial reaction appeared random; flies flew upwind on either side, but eventually settled down on the odorless side of the tunnel. Flies that flew up the odor plume showed an aversion behavior to the blend. The results lend further support to previous indications for the existence of a tsetse repellent blend

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in waterbuck body odor and additional attractive constituents in buffalo and ox body odors.

Key Words—*Glossina morsitans morsitans*, tsetse fly, buffalo, ox, waterbuck, behavior, wind tunnel, host preferences, repellents, attractants, allomones.

INTRODUCTION

Feeding preferences of different species of tsetse (*Glossina* spp.) bear no relationship to the relative abundance of the vertebrate hosts in a given habitat (Weitz, 1963; Turner, 1987; Snow et al., 1988; Clasen et al., 1998). The gradation in host preference or nonpreference has been confirmed by direct observations and by bloodmeal analyses (Turner, 1987; Moloo, 1993; Clausen et al., 1998). For example, in Zimbabwe *Glossina morsitans morsitans* showed nonpreference for the impala (*Aepyceros melampus*) with respect to landing and engorgement compared to ox (*Bos indicus*), bushpig (*Potamochoerus porcus*), and warthog (*Pharcochoerus aethiopicus*) (Vale, 1974b). Similarly, in Kenya, *G. pallidipes* preferred to alight and engorge on buffalo (*Syncerus caffer*) or ox but not on waterbuck (*Kobus defassa*), even when these animals were kept in adjacent enclosures in the field (Grootenhuis, 1986; Grootenhuis and Olubayo, 1993).

Preferred hosts are located and recognized through olfactory and visual cues. In general, the fly is activated by the host's odor beyond its visual range, and after orientation, flies upwind until it comes to the vicinity of the host, where visual cues such as shape, size, color contrast, movement, and close-range olfactory stimuli may mediate alightment on the host (Vale, 1974a; Vale and Hall, 1985a; Sutcliffe, 1987; Lehane, 1991; Willemse and Takken, 1994; Gouteux et al., 1995; Leak, 1998). Odors attractive to tsetse emanate from excretory products such as urine, dung, breath, or glandular secretions and exudates of hosts (Chorley, 1948; Owaga, 1984, 1985; Vale et al., 1986; Warnes, 1990; Mihok et al., 1996). Attractants identified from host's breath include carbon dioxide, acetone, and 1-octen-3-ol (Vale, 1980; Bursell, 1984; Hall et al., 1984; Vale and Hall, 1985b). Those identified from buffalo or ox urine and skin secretions from cattle include 4-methylphenol and 3-*n*-propylphenol (Hassanali et al., 1986; Bursell et al., 1988; Owaga et al., 1988; Vale et al., 1988; Warnes, 1990; Saini et al., 1993). Although combinations of these kairomones have been used successfully as baits with traps and targets for the control of *G. pallidipes* and *G. m. morsitans* in the field (Vale et al., 1988; Brightwell et al., 1991; Willemse, 1991), they are still less than 50% as attractive as the natural cattle odor. This implies that there are other unidentified attractive components of the host odor (Vale, 1981; Willemṡe and Takken, 1994; Hargrove et al., 1995; Torr et al., 1995; Holmes, 1997).

Until recently, no studies designed to elucidate the basis of nonpreference of certain vertebrates by tsetse flies were reported. Factors such as body size and

grooming behavior of different vertebrates, or their relative abundance in different habitats, do not account for the frequency with which they are fed on by tsetse (Vale, 1974a; Hargrove, 1976; Turner, 1987; Snow et al., 1988; Baylis, 1996). In a previous study, we compared the behavior of teneral *G. m. morsitans* confined in small experimental cages on waterbuck and ox, and on feeding membranes with and without smears of waterbuck sebum (Gikonyo et al., 2000). Our results suggested the presence of volatile and short-range or contact allomones from the waterbuck. Recently, we compared the composition of the body volatiles of waterbuck, including their EAG-active constituents (on *G. m. morsitans* and *G. pallidipes*), with those of buffalo and ox (Gikonyo et al., 2002). Of the known tsetse attractants (Hassanali et al., 1986; Bursell et al., 1988; Owaga et al., 1988), 4-cresol (4-methylphenol) and 3-*n*-propylphenol were detected, although in varying relative amounts in the three bovids. The gas chromatography–electroantennographic (GC-EAD) profiles (with *G. m. morsitans* antennae) of the odors from the two preferred hosts were comparable, comprising phenols and medium-chain, saturated or unsaturated aldehydes. Waterbuck odor gave a richer profile, consisting of fewer aldehydes but more phenolic components, δ-octalactone, and a series of methylketones (C_8—C_{13}), which were either not detected or present in trace amounts in the two preferred hosts (Gikonyo et al., 2002). The occurrence of different combinations of EAG-active compounds in these animals suggested that they play a role in host detection and discrimination. We proposed that the blend of waterbuck-specific EAG-active constiuents may function as a long- or medium-range allomone against tsetse flies, and that the blend of aldehydes may be a hitherto unidentified part of the attractant system for these insects. This study was designed to compare the behavioral responses of *G. m. morsitans* to different blends of EAG-active constituents of the three bovids in a choice wind tunnel.

METHODS AND MATERIALS

Insects. Pupae of *G. m. morsitans* were obtained from the insectaries of the Intenational Atomic Energy Agency (IAEA), Vienna, Austria, and the International Livestock Research Institute (ILRI), Nairobi, Kenya. After emergence at the International Center of Insect Physiology and Ecology (ICIPE) insectary, teneral females *G. m. morsitans* were placed in $20 \times 15 \times 15$ cm plexiglass cages and kept at $25 \pm 2^{\circ}$C and $70 \pm 5\%$ RH until they were 3-d-old. On the day of experiments, flies were individually transferred into cylindrical PVC release cages (4-cm long and 3-cm diam) closed on one side with dark PVC gauze. The open end was plugged loosely with a small piece of cotton wool.

The Wind-Tunnel. Tests were conducted in a cylindrical plexiglass tunnel (180-cm long, 24-cm diam) in a bioassay room maintained at $25 \pm 2^{\circ}$C and $70 \pm 5\%$ RH. A duct (20-cm diam) in the middle of the tunnel was connected to a PVC

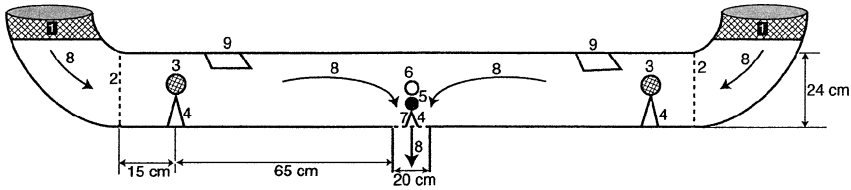

FIG. 1. The wind tunnel: 1–activated charcoal; 2–PVC gauze; 3–odor dispenser; 4–metallic rack; 5–insect release cage; 6–window for introducing insect release cage; 7–metallic wire mesh; 8–air flow direction, and 9–window for introducing odor dispenser.

pipe in the terminal end of which an air-extracting fan was mounted. The duct in the middle divided the tunnel into two equal arms with a 20-cm wide middle zone where air from arms mixed (Figure 1). When the fan was switched on, air flowed into the tunnel from both arms, thereby making the middle of the tunnel downwind. The upwind ends of the tunnel were closed with white PVC gauze, and the downwind end with a metallic wire mesh cover. The two upwind ends were connected to air filters made of PVC containing activated charcoal (4–14 mesh, Sigma Chemical Co.). The wind tunnel had three windows, one on each arm (15×10 cm) for introducing sample dispensers and one in the middle (4.7-cm diam) for introducing a release cage.

Light from fluorescent tubes and bulbs controlled with a dimmer switch was diffused through frosted glass sheeting placed 35 cm above the tunnel, giving about 1000 lux incident light. A white sheet of paper with black stripes marked about 2 cm apart was placed beneath the tunnel floor to provide contrast to a fly during anemotaxis. The wind speed in the tunnel was adjusted to 10 cm/sec.

Test Odor. Test odors consisted of selected blends of compounds identified in body volatile odors of buffalo, ox, and waterbuck that were previously shown to elicit electroantennographic (EAG) responses from the antenna of *G. m. morsitans* (Gikonyo et al., 2002). The following odor blends were tested (Table 1):

A: all EAG-active compounds in waterbuck body volatiles;
B: compounds present in waterbuck but absent in both ox and buffalo volatiles;
C: compounds common in waterbuck and ox or buffalo volatiles;
D: compounds present in buffalo and ox volatiles minus known tsetse attractants, 4-methylphenol and 3-*n*-propylphenol and;
E: known tsetse fly attractants previously identified from ox breath, body, and urine (acetone, 1-octen-3-ol, 4-methylphenol, and 3-*n*-prophylphenol) in the ratio 1000:10:4:1 (Owaga et al., 1988; Saini and Hassanali, 1992; Paynter and Brady, 1993;).

The flies' responses to the above odor blends were compared in the following ways: (a) three or four doses (0.01, 0.1, 1.0, and 10.0 mg) in 200-μl paraffin oil

TABLE 1. COMPOUNDS AND CORRESPONDING RATIOS CONSTITUTING TEST ODORS IN WIND TUNNEL EXPERIMENTS

Compound	Blend[a]				
	A	B	C	D	E
Acetone	—	—	—	—	1000.0
1-Octen-3-ol	—	—	—	—	10.0
4-Methylphenol	4.5	—	1.4	—	4.0
3-*n*-Propylphenol	3.3	—	1.0	—	1.0
(*E*)-2-Heptenal	13.4	—	4.0	1.0	
Octanal	—	—	—	9.2	
Nonanal	18.9	—	5.7	19.9	
Decanal	14.8	—	4.5	11.1	
Undecanal	10.0	—	3.0	6.9	
Dodecanal	—	—	—	8.4	
2-Octanone	4.4	4.4	—	—	
2-Nonanone	6.9	6.9	—	—	
2-Decanone	1.0	1.0	—	—	
2-Undecanone	1.0	1.0	—	—	
2-Dodecanone	2.9	2.9	—	—	
δ-Octalactone	9.3	9.3	—	—	
(*E*)-6, 10-Dimethyl-5,9-undecadien-2-one	2.6	2.6	—	—	

[a] Blend: A = EAG-active compounds in waterbuck body odor. B = EAG-active compounds present in waterbuck but absent in both ox and buffalo body odors. C = EAG-active compounds present in waterbuck, and ox or buffalo body odors. D = EAG-active compounds present in buffalo and ox odors less the known attractants, 4-methylphenol and 3-*n*-propylphenol. E = Known tsetse fly attractants previously identified in ox breath and urine (or body odar).

of blends A, B, C, and D set against a control (200-μl paraffin oil); (b) 1.0 mg of blend E in 200-μl paraffin oil tested against the control; and (c) a blank test involving equal volumes of paraffin oil (200-μl) at both upwind ends.

Odor Dispensers. The odor dispenser consisted of a black cloth that was secured with Soxhlet-cleaned (dichloromethane) rubber bands placed on one end of an open-ended plexiglass tube (5-cm long and 4.5-cm diam). The dispensers were each placed via the window on the upper side of either arm of the tunnel onto a metallic rack positioned 15 cm from the upwind end such that they were at mid-height with the cloth facing downwind. The appropriate dose of the odor in paraffin oil was pipetted onto the center of the cloth on one of the dispensers, while air was allowed to flow at 10 cm/sec. For the control, the same volume of paraffin oil was pipetted onto a similar dispenser placed in the other arm of the tunnel. All windows of the tunnel were closed and air allowed to flow for 5 min before the first fly was introduced.

Behavioral Experiments. A release cage containing a quiescent 3-d-old teneral female tsetse fly was introduced cautiously through the window in the middle of the tunnel onto a metallic rack in the middle of the mesh wire cover at the downwind end (middle zone). The cotton-plugged end of the release cage was positioned facing the observer. The plug was removed gently with forceps, and the window was closed tightly so that only air from the charcoal filters carrying odor from the dispensers passed over the release cage. The behavior of each fly was observed and recorded for 3-min, after which the fly was aspirated out with a vacuum pump. The following observations were made by the same person throughout the study:

i) activation (walking or hopping within or out of the release cage)
ii) initial direction of flight
iii) distance covered in flight
iv) turning 180° and downwind flight
v) flying out of the plume (gliding against wall of tunnel while in flight or attempting to fly through the wall of the tunnel) herein referred to as "escape behavior," and
vi) landing and final resting position (odor/odorless arm of the tunnel) at the end of the response cycle.

In each replicate cycle, involving one dose of a given test blend in 1 d, the responses of about 10 individual flies were recorded after which the tunnel, the metallic racks, and the release cages were cleaned with water and then with 70% ethanol. With the humidifier switched off, the bioassay room temperature was raised to above 30°C and air was exhausted from the room for about 6 hrs by a powerful extraction fan fixed on the room wall. At the same time, the tunnel was exhausted at maximum wind speed overnight. Blank tests with clean paraffin oil were conducted to confirm no residual effects of previous test materials. Once a week, the tunnel walls also were flushed with hot air from a heat gun. Between five and eight replicates (corresponding to 50–85 flies observed) were carried out for each treatment. To avoid possible bias, the arms of the tunnel were used alternately for control or test blend in successive replicates.

Statistical Analyses. The differences in proportions of flies that were engaged in specific behaviors (activation, departure from midsection, flight preferences, upwind progression, and avoidance) in the choice wind tunnel were analyzed by chi-square (SAS, 1996).

RESULTS

Activation Behavior. Table 2 shows the responses of *G. m. morsitans* to the five different odor blends and to the blank (in which two odorless dispensers were

TABLE 2. BEHAVIORAL RESPONSES OF 3-D TENERAL FEMALE *G. m. morsitans* TO CHOICES OF CLEAN AIR AND GC-EAG-ACTIVE ODOR BLENDS ASSOCIATED WITH WATERBUCK, BUFFALO AND OX IN A WIND TUNNEL

Test odor	Dose (mg)[b]	N	Percent flies activated	Percent files departing from midsection	Percent flies showing flight preference in the wind tunnel[a]				Average upwind distance (cm ± SE) covered during flight in the tunnel[a]	
					Upwind flight		Final resting position			
					C	*T*	*C*	*T*	*C*	*T*
Blank	0.00	50	94.0	76.0	50.0	50.0	48.6	51.4	26.74 ± 4.28	28.24 ± 4.56
Blend A[c]	10.00	71	88.7	43.6	51.6	48.4	50.0	50.0	20.42 ± 5.11	30.39 ± 4.76
	1.00	50	82.0	56.0	50.0	50.0	70.8	29.2*	25.43 ± 5.08	20.32 ± 4.78
	0.10	55	83.6	50.9	46.4	53.6	53.8	46.2	18.82 ± 4.62	30.36 ± 6.13
Blend B[c]	10.00	57	93.0	66.6	44.7	55.3	70.0	30.0*	24.15 ± 4.13	23.08 ± 4.02
	1.00	59	88.1	62.7	54.1	45.9	70.4	29.6*	31.80 ± 4.12	19.97 ± 4.14*
	0.10	59	96.6	55.9	54.5	45.5	69.2	30.8*	33.48 ± 4.87	22.61 ± 4.43*
	0.01	57	94.7	68.4	51.3	48.7	52.9	47.1	23.26 ± 4.42	20.82 ± 3.69
Blend C[c]	10.00	55	85.5	43.6	29.2	70.8*	45.5	54.5	20.13 ± 5.70	32.22 ± 5.89
	1.00	40	90.0	55.0	22.7	77.3**	28.6	71.4*	13.53 ± 3.08	24.33 ± 4.32*
	0.10	47	91.5	57.5	48.1	51.9	41.7	58.3	24.22 ± 6.11	23.56 ± 5.00
Blend D[c]	10.00	53	81.1	47.1	24.0	76.0**	53.3	46.7	14.16 ± 4.94	18.60 ± 4.93
	1.00	59	81.4	54.2	31.2	68.8*	42.3	57.7	18.31 ± 4.45	23.47 ± 4.31
	0.10	49	81.6	40.8	40.0	60.0	29.4	70.6†	10.90 ± 3.41	31.95 ± 6.66**
Blend E[c]	1.00	85	80.0	44.7	26.3	73.7**	35.3	64.7†	17.95 ± 4.12	35.76 ± 4.75**

[a] *,** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, while † at $P < 0.09$ (χ^2 test), between the control and treated sections.
[b] In 200 μl of paraffin oil; *C* = Control; *T* = Treated.
[c] Blend A, B, C, D, and E = Blends of EAG-active compounds present in waterbuck odor, waterbuck only, waterbuck and ox or buffalo, buffalo and ox less known attractants, and standard attractants from ox breath and urine, respectively.

placed on both sides of the wind tunnel) during the 3-min observation periods. The number of flies activated by each blend did not vary with the dose. However, this behavior was dependent on test odor ($P < 0.01$). The known tsetse attractant (Blend E), and the putative tsetse attractant (Blend D) induced lower ($P < 0.05$) activation behavior than the blank. The proportion of flies activated by the EAG-active blends specific to waterbuck (Blend B) was similar to that of the blank ($P = 0.819$). Those activated by the EAG-active blends of all EAG-active constituents of waterbuck odor and EAG-active consituents common to waterbuck and ox or buffalo (Blend C) also were not significantly different from the blank ($P = 0.102$ and 0.283, respectively).

Departure from Midsection. Between 40 and 75% of the flies flew upwind toward the treatment or the control, while others remained in the midsection. The proportions of *G. m. morsitans* that flew upwind followed a pattern comparable to that of activation behavior, with significant dependence on the test odor ($P < 0.001$) but not the dose. The proportions of flies that flew in the presence of the different blends were lower than the blank ($P < 0.001$, 0.01, 0.001, 0.001 for A, C, D, and E, respectively), except in the case of waterbuck-specific blend ($P = 0.088$).

Upwind Flight. The initial choice of direction of flight (to either treated or control side) was dependent on the test odor ($P < 0.01$) but not on the dose. Clear differences were observed in flight direction selection among blends containing waterbuck-specific EAG constituents (A and B) and those that comprised constituents common to the three bovids or were specific to buffalo and ox (C and D). No significant direction selection was observed in the former. However, in the latter, more flies flew towards the odor sources than the control ($P < 0.05$–0.01), similar to the choice made in the presence of the known tsetse attractant blend (E).

Resting Position. At the end of the observation time (3 min), the resting position of the flies was dependent on the treatment ($P < 0.001$). Again, this was independent of the dose. Comparison of the proportions of flies resting in either control or treated zones showed that fewer flies rested in the treated zone when waterbuck-specific blend (B) was dispensed compared to the control zone ($P < 0.05$). A similar result was obtained at one of the doses of the blend of all EAG-active constituents of this bovid odor. Responses to the putative and known tsetse attractants (C, D, and E), on the other hand, were less discriminate, although some preference for the odor zone was evident (Table 2).

Upwind-Flight Progression. Flies released in the tunnel when no odor was dispensed moved similar distances along the two zones, as expected (Table 2). In the presence of odors, however, differences were observed, which were significant at some doses. In the presence of waterbuck-specific blend (B), at 1.0- and 0.1-mg doses, the flies flew shorter distances in the odor zone than in the control arm ($P < 0.05$). On the other hand, flies exposed to the attractant blend (E) and putative

TABLE 3. TURNING AT 180° AND DRAGGING AGAINST WIND TUNNEL WALL BY 3-D TENERAL *G. m. morsitans* FEMALES EXPOSED TO WATERBUCK-SPECIFIC ODOR

Dose (mg)[b]	*N*		Percentage responding[a]		
			X[c]	*E*[c]	*EX*[c]
10.00	57	Treated	10.5	26.3*	31.6*
		Control	3.5	12.3	15.8
1.00	59	Treated	13.6*	20.3	28.8
		Control	3.4	11.9	15.3
0.10	59	Treated	20.3***	16.9**	32.2***
		Control	1.7	3.4	5.1
0.01	57	Treated	17.5**	22.8***	33.3***
		Control	3.5	1.8	5.3
0.00	50	Treated	2.0	16.0	18.0
		Control	2.0	16.0	18.0

[a] *,**,*** indicate significant differences at $P < 0.05$, $P < 0.01$ or $P < 0.001$(χ^2 test), respectively, between the control and treated zones.
[b] In 200 μl of paraffin oil.
[c] *X*-Turning at 180° in flight; *E*-Gliding or fanning wings while stationary against the tunnel wall; EX "Avoidance behavior" (turned or dragged)

attractant blends (C and D) showed higher upwind progression at specific doses than the control arm ($P < 0.05$–0.01).

Avoidance Behavior. Flight behavior of flies in the wind tunnel was characterized by straight upwind flight, zigzag flight in and out of plume, short hops, turning at 180° in flight and gliding, or fanning wings against wall while stationary (ostensibly attempting to fly out of the tunnel). The latter two behaviors, hereafter referred to as "avoidance," were found to differ significantly between the treated and control sides of the tunnel when waterbuck-specific blend (B) was dispensed (Table 3). This behavior was particularly pronounced at lower doses.

DISCUSSION

The present results show clear differences in some of the behavioral responses of teneral *G. m. morsitans* in the presence of the putative allomone and kairomone, respectively. When a choice of the putative attractant blend (C) and clean air was presented in the wind tunnel, significantly more flies flew up the odor plume (at two of the three doses) (Table 2). Similar results were obtained when choices of clean air were presented with the known tsetse attractant blend (E) or the blend of EAG-active constituents common to all the three bovids (C). The distances covered in the tunnel arm containing the odor plumes were generally longer than in the control arms (Table 2). Thus, the aldehyde blend (D) elicits more or less

the same behavior as the known tsetse attractant blend (E). On the other hand, when presented with a choice of waterbuck-specific blend (B) and clean air, the flies' initial responses appeared random, with no significant differences between the numbers that flew in the control arm and the arm containing the odor plume (Table 2). The behavior of flies in the odor arm of the tunnel was characterized by sharp 180° turns while in flight and/or gliding or fanning wings against the tunnel wall, ostensibly attempting to fly out of the tunnel (Table 3). No such behavior was elicited by the putative or known attractant blends. Moreover, the distance covered in the tunnel arm in the presence of the putative repellent was generally shorter than in the control arm. In combination, these results indicate an aversion toward the waterbuck-specific blend.

The subsequent behavior of the flies that flew upwind was also consistent with the attractive or aversive nature of the two respective odor blends. In the presence of waterbuck-specific blend, the flies moved downwind and settled mostly in the odorless, control arm of the tunnel ($P < 0.05$ at doses 0.10, 1.00, and 10.0 mg). In the presence of attractant blends, they were less discriminating, although there was some indication of preference for the tunnel arm with the odors.

The flies' responses to the blend of EAG-active constituents of waterbuck odor, comprising both attractive and aversive compounds (blend A), warrant special comment. Upwind flight choices of the flies showed no discrimination between this odor blend and clean air (Table 2). On the other hand, more flies eventually settled on the odorless control arm of the tunnel than those on the odor arm at one of the doses (1.00 mg, $P < 0.05$). The first response suggests masking of the effects of the attractive constituents by the waterbuck-specific compounds (Davis, 1985; Ouden et al., 1996). The second involves orientation away from the total blend implying repellency in the sense defined by Dethier et al. (1960). The actual mechanism that operates in nature remains to be established through detailed observations in the field. However, preliminary results from our field studies suggest an active repellent effect. Moreover, an additional feature in the volatile odor chemistry of waterbuck is the presence of moderate amounts of C_5—C_9 straight-chain fatty acids, which were not present in ox or buffalo odors (Gikonyo et al., 2002). Although none of these fatty acids elicited EAG responses, some are known to reduce tsetse fly trap catches in the field (Vale, 1980; Torr et al., 1996). We have proposed that C_5—C_9 acids may act additively or in concert at the antennal receptors that result in a repellent action, and may augment the effects of the blend of ketones, octalactone, and phenols (Gikonyo et al., 2002). Our preliminary field results confirm that the fatty acid mixture contributes to the overall repellency of waterbuck-specific constituents.

This study was undertaken primarily to compare the responses of *G. m. morsitans* to putative repellent and attractant blends of EAG-active constituents associated with nonpreferred and preferred hosts, respectively. However, some of the observations of fly responses in the wind tunnel may provide additional hints as to

the flies' host-location behavior. First, although it has been recognized that tsetse flies are sensitive to wind direction, upwind anemotactic flight has been previously associated only with the presence of host or synthetic odor plumes (Bursell, 1984, 1987; Gibson and Brady, 1988; Torr 1988b, 1989; Colvin et al., 1989; Gibson et al., 1991). In the absence of odor, previous observations have suggested that tsetse flies take off largely downwind, and that this may constitute the primary mechanism of the fly's "ranging" behavior in search of an odor plume (Vale, 1980; Gibson et al., 1991) similar to onion flies (Judd and Borden, 1989). On the other hand, we found a large proportion of the flies released in the two-choice wind tunnel taking off upwind. The reason underlying this apparent paradox is unclear. It may be related to a number of factors, including the hunger and physiological status of the insects observed in different studies, and the precise conditions under which observations were made. The relative merit of upwind or downwind "ranging" by an insect in search of an odor plume has been analyzed theoretically (Sabelis and Schippers, 1984; Dusenbery, 1989). Although it has been proposed that downwind ranging may represent a more cost-effective tactic for encountering an odor plume under variable wind conditions, for a fast flier like tsetse (5 m/sec; Brady et al., 1990), this may not be so under all conditions (Gibson et al., 1991). Interestingly, Torr and Mangwiro (1996) provided evidence that in the absence of a host at the odor source, tsetse flew up as well as down the odor plume, possibly searching for the host. Our results suggest that the insect may also deploy both these tactics in an initial search for host odor.

Another surprising result of this study relates to the relative proportions of flies activated in the absence and presence of odors, and, particularly, the proportions that flew upwind (Table 2). Lower numbers of flies (80–81%) exposed to either the known tsetse attractant blend (E) or the blend of EAG-active aldehydes present in the odors of preferred hosts (Blend D) were activated than those that had not encountered any blends (94%). The flies were not similarly affected by the waterbuck-specific blend of EAG-active constituents (Blend B). The effect on the proportions of flies that took off up wind in the presence of some of the blends was even more marked (Table 2). There is some parallel here with observations reported by Torr (1988a) that resting flies in the field are not readily activated by odor. In that study, less than 20% of the flies that were exposed to ox odor were activated and took off. Torr (1988a) suggested that the initial activation of resting flies is primarily mediated by endogeneous stimuli, rather than host cues, consistent with the widely accepted belief that the onset of the fly's host-seeking behavior is determined by its hunger state (Brady, 1975; Willemse and Takken, 1994). Our results show that even within a group of flies at more or less the same nutritional state, variations occur in their behavioral thresholds depending upon the presence or absence of odor stimuli. In the absence of host odor, flies may be more inclined toward opportunistic flights to detect host odor even if they are not looking for an immediate bloodmeal. Whether such flights confer significant advantage to

the flies to locate potential hosts remains to be demonstrated. However, there is some evidence that tsetse flies undertake exploratory flights even when not actively looking for a bloodmeal. For example, Langley and Wall (1990) found that not all flies approaching a trap enter it. Interestingly, flies caught around a trap with electric nets, but not disposed toward entering it, had significantly lower mean fat and haematin levels (and, therefore, were less hungry) than did those caught in the trap. The latter group of hungrier flies may have been actively looking for a bloodmeal, while the former may have been simply exploring.

In conclusion, this study corroborates our previous inferences that (i) the blend of EAG-active constituents specific to tsetse-refractory waterbuck is allomonal to the fly; and (ii) the aldehyde blend associated with tsetse hosts is attractive to the fly and may be a significant component of the kairomone system of the insect. Additionally, our study suggests a greater tactical diversity in the odor- and host-location behavior of tsetse that had been previously suspected.

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IDENTIFICATION OF HOST FRUIT VOLATILES FROM FLOWERING DOGWOOD (*Cornus florida*) ATTRACTIVE TO DOGWOOD-ORIGIN *Rhagoletis pomonella* FLIES

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Abstract—Solid-phase microextraction and gas chromatography coupled with electroantennographic detection were used to identify volatiles from fruit of flowering dogwood, *Cornus florida*, as key attractants for *Rhagoletis pomonella* flies originating from dogwood fruit. A six-component blend containing ethyl acetate (54.9%), 3-methylbutan-1-ol (27.5%), isoamyl acetate (0.9%), dimethyl trisulfide (1.9%), 1-octen-3-ol (9.1%), and β-caryophyllene (5.8%) was identified from flowering dogwood fruit that gave consistent EAD activity. In a flight tunnel assay there was no significant difference in the response of individual dogwood flies exhibiting upwind anemotactic flight to volatile extracts from dogwood fruit and the six-component synthetic mixture. Dogwood flies also displayed significantly greater levels of upwind flight to sources with the dogwood volatile blend than with previously identified volatile blends from domestic apple or hawthorn fruit. Selected subtraction assays showed that the three-component mixture of 3-methylbutan-1-ol, 1-octen-3-ol, and β-caryophyllene elicited levels of upwind flight to the source equivalent to the six-component mixture. Our study adds to previous ones showing that populations of *Rhagoletis pomonella* flies infesting apple, hawthorn, and flowering dogwood fruit are attracted to unique mixtures of fruit volatiles, supporting the hypothesis that host fruit odors could be key traits in sympatric host shifts and establishing host fidelity within members of the *Rhagoletis pomonella* species complex.

Key Words—Solid-phase microextraction gas chromatographic electroantennographic detection, flight tunnel, *Rhagoletis*, flowering dogwood, *Cornus florida*, fruit volatiles.

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INTRODUCTION

The *Rhagoletis pomonella* species group (Bush, 1966; Berlocher, 2000) is a complex of closely related races, sibling species, and one morphologically distinct species (*R. cornivora*) that infest different fruits of host plants in North America. The apple maggot fly, *R. pomonella*, exists as two host races, one on native hawthorn (*Crataegus*) and one on introduced apple (McPheron et al., 1988; Feder, 1998). A closely related fly to the *R. pomonella* host races occurs on flowering dogwood, *Cornus florida* (Berlocher et al., 1993; Berlocher, 1999, 2000). Genetic analysis and life history data suggest that the fly infesting flowering dogwood may be of species status, even though some interpopulation gene flow is occurring (Berlocher, 1999, 2000).

A key feature of the biology of *Rhagoletis* flies is that mating occurs on or near host fruit that females will oviposit in, a concept called "host fidelity" (Feder, 1998). We have been investigating the role of host fruit odors in establishing and maintaining host fidelity within the group (Zhang et al., 1999; Berlocher and Feder, 2002; Nojima et al., 2003). Previous studies have shown that apple-origin flies are attracted to the odor of apples (Prokopy et al., 1973), and to synthetic compounds isolated from apple fruit (Fein et al., 1982; Reissig et al., 1982; Averill et al., 1988; Zhang et al., 1999). More recently we showed that hawthorn-origin flies respond to a blend of hawthorn fruit volatiles that is different from the mixture characterized from domestic apple fruit (Nojima et al., 2003). Here, we present evidence that flowering dogwood flies respond behaviorally to yet a different blend of volatiles, identified from dogwood fruit, using solid-phase microextraction (SPME) (Supelco™) combined with gas chromatography and electroantennographic detection (GC-EAD) for the analysis of headspace volatiles from fruit.

METHODS AND MATERIALS

Insects. Dogwood-origin larvae were collected from fruit at Granger, Indiana. Post-diapause pupae (Feder et al., 1994) were shipped to the lab in Geneva, New York, and kept in an environmental chamber at 23–24°C, 16L:8D photoperiod, and 65–70% relative humidity. Adults were maintained in similar conditions on an artificial diet (Fein et al., 1982; water, sugar, vitamins, casein hydrolysate, and salt mixture). Adult flies at 0–7 and 10–21 days old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively.

Fruit. Dogwood fruit were collected at Granger and Racoon Lake Recreational Area, Indiana, during September and October 2000 and 2001 and shipped to the Geneva lab. Fruit were held in cold storage (4°C, never more than 2 weeks) until sampled. Fruit were taken from cold storage and placed in the sampling containers (see below) at room temperature at least 1 hr before SPME sampling. A minimum of nine SPME samples were taken from each fruit collection.

Adsorbent Sampling. A glass chamber (3 l; Analytical Research Systems, Gainesville, Florida) with a charcoal filter tube in the inlet was used for collection of volatiles. Fruit were placed in the apparatus, and the volatiles from the head-space were drawn (about 0.8 l/min) by a vacuum pump onto ORBO solvent de-sorption tubes (150-mg active coconut charcoal, 20/40 mesh, 4 mm i.d., 70-mm length; Supelco Inc., Bellefonte, Pennsylvania) in the outlet. Two tubes were set in series to provide a backup in case one tube broke. Adsorbent samplings were made from 1 kg of fruit for 9 d at room temperature. The ORBO tubes were changed every 3 d. Volatiles were eluted with 1-ml methylene chloride (nanograde, Mallinckrodt), dried over anhydrous sodium sulfate, and then combined. Extracts were kept in a freezer (-20°C) and subjected to GC-MS analysis and flight tunnel bioassay.

SPME Sampling. A glass jar (500 ml) with Teflon liner screwcap (Wheaton, Milliville, New Jersey) was used for a sampling container. Fruit (about 150 g) were placed in the container at least 1 hr before the SPME sampling. A carboxen-polydimethylsiloxane coated SPME fiber (film thickness 85 μm; Supelco Inc., Bellefonte, Pennsylvania) was used for head-space volatile collection. The fiber was conditioned in the GC injector (280°C) for 20 min, and then passed through the small hole on the cap into the head-space of the jar. After 10–30-min exposure, collected volatiles on the fiber were immediately subjected to GC-EAD analysis. For the chemical analysis of the volatiles with GC-MS, SPME samplings were made for a longer time (a few hours to overnight) if necessary.

Coupled Gas Chromatographic–Electroantennographic Detection (GC-EAD) Analysis. An Hewlett Packerd 5890 series II gas chromatograph equipped with a nonpolar SPB-1 capillary column (30 m $\times$ 0.25 mm i.d., 0.25-mm film thickness; Supelco Inc., Bellefonte, Pennsylvania) or a polar EC-WAX Econo-Cap capillary column (30 m $\times$ 0.25 mm i.d., 0.25-mm film thickness; Alltech, Deerfield, Illinois) in the splitless mode was used for GC-EAD analysis. For SPME sample injection, a 0.75-mm i.d. glass inlet liner (Supelco Inc., Bellefonte, Pennsylvania) was used, while for liquid sample injection a 4-mm i.d. liner was used. Nitrogen was the carrier gas at a head pressure of 138 kpa (flow rate, 2.0 ml/min). The time for splitless injection was 0.25 min for SPME samples and 1.0 min for liquid samples. Oven temperature was programmed for 40°C for 2 min, and then increased at 15°C/min to 250°C, where it was held for 10 min. Injector and detector temperature were set at 280°C. Septum purge flow rate was set at 3 ml/min and total flow rate at 60 ml/min.

The column effluent was combined with nitrogen makeup gas (30 ml/min) and then split 1:1 to the flame ionization detector (FID) and EAD with a TCD capillary column adapter to facilitate ease in changing columns (Nojima et al., 2003). The EAD outlet was secured in a charcoal-filtrated and humidified air stream, which was refrigerated by a modified condenser flushed with 0°C water, flowing at about 500 ml/min over a *Rhagoletis* fly antennal preparation. A fly head, separated at the prothorax, was mounted on a custom acrylic holder, equipped with

gold wire electrodes (Nojima et al., 2003). Drosophila ringer solution (46 mmol NaCl, 182 mmol KCl, 3 mmol $CaCl_2$, and 10 mmol Tris-HCl at pH 7.2) was used for making electrical conductivity between electrodes and antennae. The holder was placed inside of a cooling condenser and maintained about 5°C.

The output signal from the antennae was amplified by a customized single step high-input impedance DC amplifier at ×100 (Nojima et al., 2003). The signal was recorded on an HP 3390 A and synchronized with a GC integrator. As we found previously with hawthorn-origin flies (Nojima et al., 2003), preliminary experiments showed no significant qualitative differences in EAD responses between apple-origin *R. pomonella* flies from our lab colony and dogwood flies from nature, or between sexes within each race. Because of limited availability of dogwood flies needed for behavioral testing, key host volatiles from dogwood fruit were first estimated using the lab colony flies reared on apple, and then the results were confirmed with dogwood flies from nature.

Chemical Analysis. GC-MS analyses were performed as in Nojima et al., (2003) on a Shimadzu GC-17A equipped with a nonpolar DB-1ms capillary column (30 m × 0.25 mm i.d., 0.25-mm film thickness; J&W Scientific, Folsom, California) or a polar EC-WAX Econo-Cap capillary column (30 m × 0.25 mm i.d., 0.25-mm film thickness; Alltech, Deerfield, Illinois) and coupled to a Shimadzu QP 5050A quadrupole mass spectrometer running in the EI (electron ionization at 70 eV) scan mode. Helium was the carrier gas at an initial head pressure of 58 kpa at constant flow rate (1.0 ml/min). Oven temperature was programmed for 40°C for 2 min, increased at 15°C/min to 250°C, and held for 10 min. The injector and interface temperature were set at 280 and 260°C, respectively. Volatiles were identified by mass spectral matches to library spectra as well as by retention time matches to available known standards. The EAD activity of compounds was verified by the GC-EAD analysis with authentic standards. Quantification of the relative ratios of the EAD active compounds was made from the absorbent collections. The compounds were quantified from their ion abundances or FID responses of GC-MS or GC analyses according to the standard curves made from each authentic standard.

Chemicals. 3-Methylbutan-1-ol, isoamyl acetate, 1-octen-3-ol (a racemic mixture), and ethyl acetate were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin), and purities were >98%. Dimethyl trisulfide was purchased from Acros Organics (Pittsburgh, Pennsylvania), and purity was >98%. β-Caryophyllene, a mixture of β and α isomers at a ratio of 91:9 was purchased from ICN Biomedicals, Inc. (Irvine, California). Net proportion of isomers was >95% based on GC analysis.

Flight Tunnel. The response of flies to host fruit volatiles was measured in a sustained-flight tunnel (Nojima et al., 2003). Flight tunnel conditions were 23–24°C, 50–70% relative humidity, 35 cm/sec wind speed, 1000-lux light intensity. Adult Flies were tested between the 3rd and 11th hours of the 16 hr photophase period (6 A.M. to 10 P.M. EST). Adult flies (mixed sex, 10–21 days old) were

selected from holding cages located in a separate, environmentally controlled room, placed singly in glass vials, taken to the room housing the flight tunnel, and allowed to acclimate for 15 min. Individual flies were transferred to a screen release cage, which was placed on a screen stand inside the flight tunnel. The stand was positioned 1 m downwind of a red sphere used as a release point for volatile fruit blends (see below). The release cage was open at one end, with the open end facing in the upwind direction. Flies were given 1 min to respond, and scored for the following behaviors: 1) taking flight = flight from the release cage (100 cm from the source, may or may not be odor-stimulated); 2) upwind flight = odor-stimulated upwind flight oriented toward the sphere for at least 20 cm (80 cm from the source); and 3) reaching sphere = odor-stimulated upwind flight to and landing on the red sphere holding the source (0 cm). Flies were recovered after testing and used again, but never on the same day or for more than four trials.

Solutions of volatile extracts in methylene chloride, or synthetic compounds prepared in hexane, were applied to red rubber septa (Thomas Scientific, Swedesboro, New Jersey, Cat. No. 1780J07) that had been acetone-washed. The rubber septum was attached to a 7.5-cm red plastic sphere (Gempler's Inc., Mt. Horeb, Wisconsin) and hung from the ceiling at the upwind end of the tunnel. The proportions of chemicals in the dogwood blend are shown in Table 1. For the hawthorn blend (Nojima et al., 2003), the chemicals and proportions were ethyl acetate (2000), 3-methylbutan-1-ol (100), isoamyl acetate (40), 4,8-dimethyl-1,3(*E*),7-nonatriene (2), butyl hexanoate (1), and dihydro-β-ionone (2). For the apple blend (Zhang et al., 1999), the chemicals and proportions were butyl butanoate (10), propyl hexanoate (4), butyl hexanoate (37), hexyl butanoate (44), and pentyl hexanoate (5). For comparative purposes, the reported dosage of the dogwood synthetic blend and volatile extract, and the hawthorn blend, reflects the amount of 3-methylbutan-1-ol, with the other components added in the proportions

TABLE 1. RATIO OF ACTIVE CHEMICALS FROM FLOWERING DOGWOOD FRUIT AS DETERMINED BY INTEGRATION OF GC-FID, GC/MS TOTAL ION CURRENT USING ADSORBENT SAMPLE[a]

Chemicals	GC/FID; GC/MS (%)	FT (%)
Ethyl acetate	54.9	200
3-Methylbutan-1-ol	27.5	100
Isoamyl acetate	0.9	3
Dimethyl trisulfide	1.9	7
1-Octen-3-ol	9.1	30
β-Caryophyllene	5.8	20

[a] FT indicates the percentage of each compound in the synthetic blend used in the flight tunnel.

shown above. The reported dose of the apple mixture reflects the total amount of the compounds. Sources were prepared 60 min prior to a test, and were clipped onto the bottom of the red sphere. Blank spheres used for control experiments contained rubber septa treated with hexane. Fresh sources and red spheres were used for each test period.

Experiment 1. The response of dogwood flies to a 50-μg dose of the natural volatile extract from dogwood fruit was compared with a 50- and 200-μg dose of the complete six-component dogwood fruit blend identified from SPME-EAD analysis. The 50-μg dose was selected on the basis of analysis of the extract showing that it contained approximately 0.25 μg/μl 3-methylbutan-1-ol, resulting in 50 μg of the compound when 200 μl of the solution was applied to the rubber septum.

Experiment 2. Dogwood flies were tested to 200-μg dose of the six-component dogwood blend, the 6-component hawthorn blend (Nojima et al., 2003), and the five-component blend identified from apple (Zhang et al., 1999).

Experiment 3. The response of dogwood flies to a 200-μg dose of the six-component dogwood blend was compared with selected treatments in which single or multiple compounds were removed from the complete blend.

In all experiments, 10–15 flies were tested each day to each treatment, until a minimum of 40 different flies had been tested/treatment (with the exception of treatments 6 and 7, experiment 3, where $N = 15$). Over the course of the studies, a total of 35 flies also were tested to a control red sphere containing a solvent treated rubber septum.

Statistical Analysis. The total number of flies in each treatment that exhibited each behavior in the flight sequence was converted to a percent value for graphical display. Selected 2×2 comparisons were made *a posteriori* between values for different treatments within each experiment, using Fisher's exact test, according to the JMP statistical analysis program ($P < 0.05$).

RESULTS

Identification of Key Host Volatiles from Dogwood Fruit. A total of seven different fruit samples from Granger and Racoon Lake, Indiana, were analyzed using SPME with GC-EAD. Three fruit samples were collected from September to October 2000, and four during the same months in 2001. A total of 229 different antennal pairs were tested for the study (1–8 runs/pair; 129 female and 68 male colony apple-origin flies; 19 female and 13 male dogwood flies). Comparison of EAD samples from dogwood fruit with the Indiana dogwood flies and New York apple-origin flies allowed us to determine the key group of volatiles that consistently gave EAD responses. The corresponding six active compounds were identified as ethyl acetate, 3-methylbutan-1-ol, isoamyl acetate, dimethyl trisulfide, 1-octen-3-ol, and β-caryophyllene.

A single adsorbent extract of volatiles from whole ripe fruit collected at Racoon Lake in September 2000 was made for quantitative analysis of key volatiles and flight tunnel analysis. The relative ratio of the EAD active compounds in this sample, estimated with GC-FID and GC-MS, are listed in Table 1. Flight tunnel tests (see below) confirmed that the proportion of flies responding to this adsorbent collection was equivalent to a sample of the six-component synthetic blend at a comparable dose.

Behavioral Responses of Dogwood Flies. Only 2 of the 35 flies tested individually to a blank control treatment (red sphere with solvent treated septum) took flight from the release cage, and neither of these flies initiated upwind flight. Thus, as in previous tests with hawthorn flies (Nojima et al., 2003), there was no directed flight response of dogwood flies to the visual stimulus of the red sphere from the release distance of 1 m.

Experiment 1. Behavioral Responses of Dogwood Flies to an Adsorbent Collection Extract and the Synthetic Blend. A total of 44% of the dogwood flies tested reached the sphere when it contained the 50-μg source containing volatiles from whole fruit. This value was not significantly different from the 53% of flies reaching spheres containing the 50-μg source with the six-component synthetic blend (Figure 1; Fisher's exact $P = 0.655$, df $= 1$). However, more flies (85%) flew

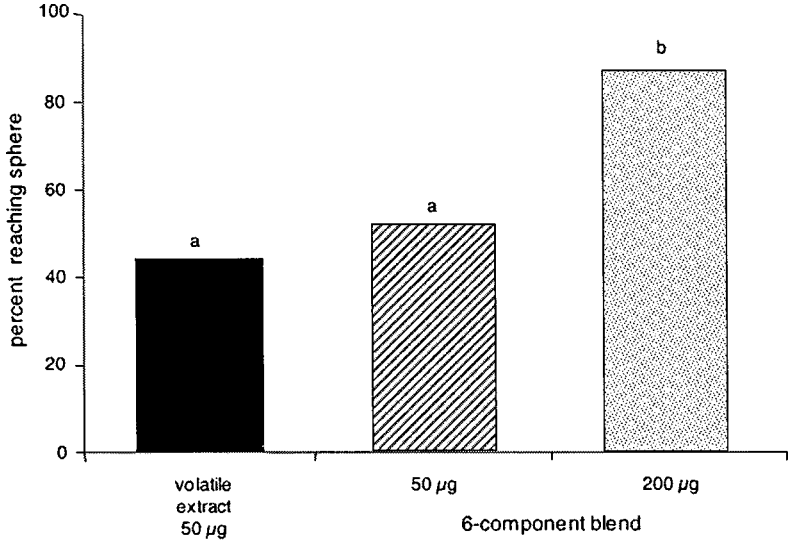


FIG. 1. The percentage of dogwood flies flying upwind and reaching spheres containing a 50-μg dose of the dogwood fruit volatile extract, and 50- and 200-μg doses of the 6-component synthetic blend of dogwood volatiles. $N = 40$ flies tested/dose. Values with different letters represent significant differences; Fisher's exact test, $P < 0.05$.

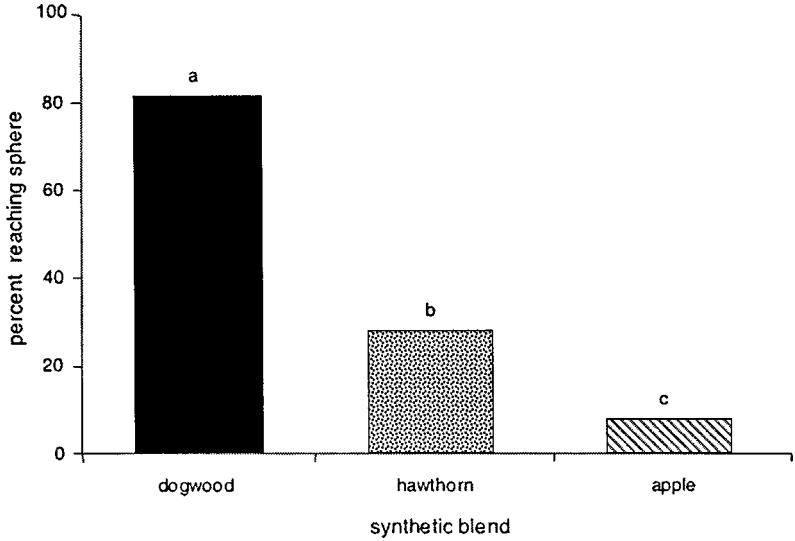
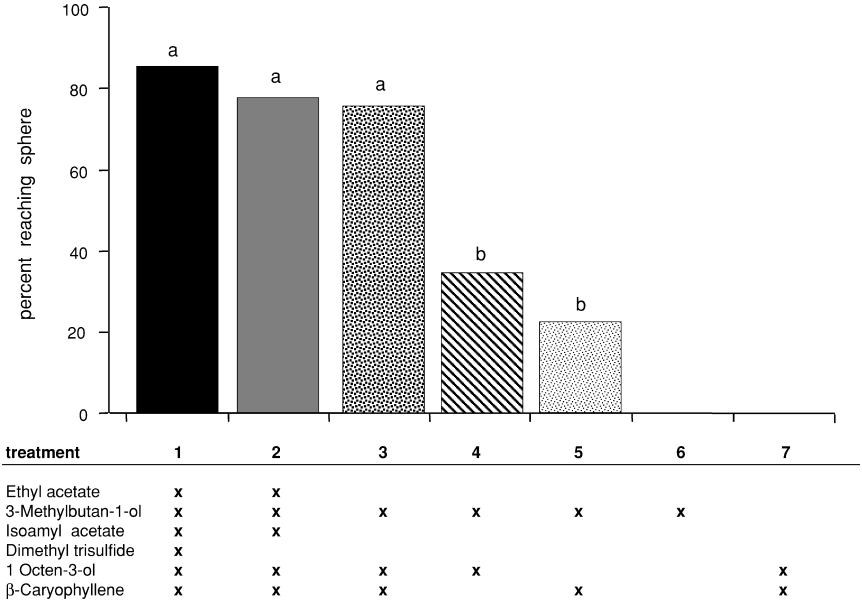


FIG. 2. Percentages of dogwood flies flying upwind and reaching spheres to a 200-μg dose of the 6-component dogwood blend, the 6-component hawthorn blend, or the 5-component apple blend (see Table 1 and methods for chemicals and proportions). $N = 42$ flies tested to dogwood blend, 49 flies hawthorn blend, and 43 flies to apple blend. Values with different letters represent significant differences; Fisher's exact test, $P < 0.05$.

upwind to a 200-μg dose of the 6-component blend, compared with the 50-μg dose of the synthetic blend (Fisher's exact $P = 0.005$, df $= 1$), or the 50-μg dose of the volatile collection (Fisher's exact $P = 0.003$, df $= 1$).

Experiment 2. Behavioral Response of Dogwood Flies to Dogwood, Hawthorn, and Apple Volatile Blends. With the 200-μg source of the six-component dogwood blend, 84% of dogwood flies reached the sphere (Figure 2). This value was greater than the 25% response value observed with the 200-μg six-component hawthorn blend (Fisher's exact $P = 0.001$, df $= 1$), and the 4% response value for the five-component apple blend (Fisher's exact $P = 0.001$, df $= 1$). Further, the 25% response value observed with the hawthorn blend was greater than the 4% for five-component apple blend (Fisher's exact $P = 0.008$, df $= 1$). The decreased response of dogwood flies to the hawthorn and apple blends was manifested at different behavioral stages in the orientation response. The lower response to the hawthorn blend primarily resulted from a decrease in the proportion of flies initiating upwind flight compared with those taking flight (74% vs. 34%; Fisher's exact $P = 0.001$, df $= 1$). In contrast, the lower proportion of flies reaching the sphere with the apple blend was mostly due to fewer flies taking flight,



treatment	1	2	3	4	5	6	7
Ethyl acetate	x	x					
3-Methylbutan-1-ol	x	x	x	x	x	x	
Isoamyl acetate	x	x					
Dimethyl trisulfide	x						
1 Octen-3-ol	x	x	x	x			x
β-Caryophyllene	x	x	x		x		x

FIG. 3. Percentage of dogwood flies flying upwind to a red sphere containing a 200-μg dose of the 6-component dogwood blend, or 6 different partial blends/single compounds. $N = 40$ flies tested/treatment for treatments 1–5, and $N = 15$ each for treatments 6 and 7. Values with different letters represent significant differences in selected 2×2 comparisons (Fisher's exact test, $P < 0.05$; see text).

compared with the hawthorn blend (73% for hawthorn vs. 23% for apple; Fisher's exact $P \leq 0.001$, df $= 1$).

Experiment 3. Behavioral Responses of Dogwood Flies to Partial Mixtures of the Synthetic Blend. There was no difference in the proportion of upwind flights by dogwood flies to a three-component mixture of 3-methylbutan-1-ol, 1-octen-3-ol, and β-caryophyllene, compared to the response observed to the full six-component blend (85% vs. 76%; treatment 3 vs. 1) (Fisher's exact $P = 0.237$, df $= 1$), or the six-component blend minus dimethyl trisulfide (78%, treatment 2; Fisher's exact $P = 0.315$, df $= 1$) (Figure 3). Responses to the two-component blends of 3-methylbutan-1-ol + 1-octen-3-ol (treatment 4) and 3-methylbutan-1-ol + β-caryophyllene (5) were not different from each other (36% and 22%; Fisher's exact $P = 0.306$ df $= 1$), but the proportion of flies reaching the sphere with the two-component blend of 3-methylbutan-1-ol + 1-octen-3-ol (treatment 4) was lower compared with the response to the six-component blend (treatment 1; Fisher's exact $P = 0.001$, df $= 1$). Finally, 3-methylbutan-1-ol (treatment 6) or 1-octen-3-ol + β-caryophyllene (7) did not elicit any upwind flight when presented as single compounds.

DISCUSSION

Using a protocol involving SPME and GC-EAD analysis previously employed for apple- and hawthorn-origin flies (Zhang et al., 1999; Nojima et al., 2003), we have identified a blend of volatile compounds emitted from flowering dogwood fruit that gave consistent EAD activity from *Rhagoletis pomonella* flies infesting dogwood fruit: ethyl acetate, isoamyl acetate, 3-methylbutan-1-ol, dimethyl trisulfide, 1-octen-3-ol, and β-caryophyllene. Adult dogwood flies responded in a flight tunnel assay at equivalent levels to 50-μg doses of an extract of whole fruit and a synthetic blend of the six chemicals. This supports the conclusion that our synthetic blend contains all of the key compounds resulting in attraction of flies to the sphere. However, based on results of subtraction assays, a three-component mix of 3-methylbutan-1-ol, 1-octen-3-ol, and β-caryophyllene also induced similar behavioral activity in flies to that of the six-component blend.

Our results add to previous findings (Zhang et al., 1999; Nojima et al., 2003) with different members of the *R. pomonella* species complex showing that adult flies detect and orient to unique mixtures of volatile odors from their natal host fruits. This behavior is likely to play an important role in the establishment and maintenance of host fidelity, the tendency of adult flies to mate on and oviposit into the same species of host fruit that they infested as larvae. Dogwood flies flew upwind and reached the red sphere in significantly greater numbers with the dogwood blend than with equivalent amounts of previously identified volatile blends from apple or hawthorn fruit (Zhang et al., 1999; Nojima et al., 2003). The dogwood blend is more similar to the hawthorn than the apple blend, with both blends sharing the key volatile 3-methylbutan-1-ol. The presence of this key volatile in the dogwood and hawthorn blends may partly explain the higher proportion of dogwood flies taking flight to the hawthorn than the apple blend. The compounds 1-octen-3-ol and β-caryophyllene were not found in hawthorn fruit, and this could account for the significant decrease in the number of upwind flights by dogwood flies to the hawthorn blend compared with the dogwood mixture. Butyl hexanoate, a key behavioral component in the apple blend for apple-origin *R. pomonella*, was identified in much smaller quantities in the hawthorn blend compared with apple (1% vs. 37% of the mixture, respectively), but was not present in volatiles from flowering dogwood. Tests of the effects of combined volatile blends, as well as effects of individual nonnatal fruit chemicals on the flight behavior of apple, hawthorn, and dogwood flies to their respective blends are currently being conducted.

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HOSTPLANT SUITABILITY AND DEFENSIVE CHEMISTRY OF THE CATALPA SPHINX, *Ceratomia catalpae*

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Abstract—The growth and survival of the Catalpa sphinx, *Ceratomia catalpae* (Sphingidae), were measured on five different species of *Catalpa: C. bignonioides, C. bungeii, C. fargeseii, C. ovata*, and *C. speciosa*. Larval growth varied significantly among these host plant species; however, survival did not differ. Quantification of the iridoid glycoside content of larvae, pupae, adults, larval frass, and leaves of the larval host plant, *C. bignonioides*, by gas chromatography showed that leaves contained both catalpol and catalposide; larvae, pupae, and frass contained only catalpol; and the adults contained no detectable iridoid glycosides. Amounts were highest in the larvae and declined in the pupal stage. Very small amounts of catalpol were detected in adults of the parasitoid, *Cotesia congregata*, and in the silken cocoons. The hemolymph in which the parasitoid larvae grew contained over 50% dry weight catalpol. Larvae of *C. catalpae* often regurgitate when disturbed. This may serve as a defense against predators. A comparison of the growth of larvae pinched with forceps to induce regurgitation with those that were not so treated showed that larvae that were pinched, and usually regurgitated, grew significantly more slowly than those that were not.

Key Words—Bignoniaceae, *Catalpa*, catalpol, catalposide, *Cotesia congregata*, iridoid glycosides, parasitoid, regurgitation, sequestration.

INTRODUCTION

Insects exhibit an impressive diversity of defenses against predators, ranging from crypsis and camouflage, to potent stings, to distastefulness and toxicity (e.g., Cott, 1940; Edmunds, 1974; Duffey, 1980; Blum, 1981; Bowers, 1993; Nishida, 2002). Some distasteful or toxic insect species owe their defense to sequestration of

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chemical compounds from the plants on which they feed (reviewed in Bowers, 1993; Nishida, 2002). These aposematic insects typically advertise their unpalatability by attributes such as conspicuous coloration, gregariousness, and sedentary behavior.

For holometabolous insects that sequester defensive compounds from their hostplants, there may be different ecological and evolutionary forces acting on different life stages, resulting in differences in the types of predator avoidance strategy used by these different stages. For example, in some lepidopteran species, the larvae are aposematic, while the pupae and adults are not; whereas in other species, both larvae and adults are aposematic (Bowers, 1993). Unpalatable caterpillars may enhance the effectiveness of their defense capability by reflex-bleeding, regurgitation, or other defensive behaviors, while adults may not have this ability. As a result, the kinds of natural enemies that are most important in shaping these defenses may also vary among life stages.

The Catalpa Sphinx, *Ceratomia catalpae* L. (Sphingidae), is a specialist on *Catalpa* G. Don (Bignoniaceae) species (as its name suggests). In some areas, it may be an important pest of these ornamental trees (Baerg, 1935). Members of the genus *Catalpa* contain a diversity of iridoid glycosides (Boros and Stermitz, 1990; Von Poser et al., 2000), and these compounds may serve as antifeedants for generalist insect species or attractants for specialist species (Bowers, 1991, 1993). Nayar and Fraenkel (1963) suggested that certain iridoid glycosides (that they called catalposides) were responsible for the monophagous feeding habit exhibited by *C. catalpae*.

Adults of the Catalpa Sphinx are a drab gray and appear relatively cryptic, while the larvae are brightly colored black, white, and yellow. The adults are palatable to one model predator, the Grey Jay (*Perisoreus canadensis*, Corvidae) (Bowers and Farley, 1990), while larvae have not been tested for palatability. Thin-layer chromatography (TLC) indicated that the larvae of the Catalpa Sphinx contained iridoid glycosides (Bowers and Puttick, 1986). Iridoid glycosides are sequestered by a variety of insect species and render them unpalatable to both vertebrate and invertebrate predators (Bowers and Farley, 1990; Camara, 1997; Theodoratus and Bowers, 1999).

In this paper, I examine several aspects of the hostplant relationships and chemical ecology of the Catalpa Sphinx: 1) larval growth of *C. catalpae* on five species of *Catalpa*; 2) iridoid glycoside content of larvae, pupae, adults, and frass of the Catalpa sphinx reared on *C. bignonioides*; 3) iridoid glycoside content of pupal cocoons and adults of the parasitoid, *Cotesia congregata* (Braconidae); and 4) growth consequences of regurgitation for Catalpa sphinx larvae.

METHODS AND MATERIALS

Study Organisms. A laboratory culture of *C. catalpae* was started with larvae that were collected in Cape May, New Jersey. By exposing larvae to a long

daylength (16 hr L : 8 hr D), I was able to obtain several generations. The colony was maintained on leaves of *Catalpa bignonioides* collected from Cambridge, Massachusetts. Adults were mated in large mesh flight cages ($1 \times 1 \times 1$ m). Eggs were laid in large masses on leaves or sides of the cages.

Growth Rate on Different Catalpa Species. Newly hatched larvae of *C. catalpae* were weighed in groups of 10 and placed in large petri dishes (15-cm diam). Larvae were weighed at 5, 10, 15, and 20 days; on day 5 as a group, and thereafter as individual larvae. At 5 days, larvae were separated into individual petri dishes, and fed on one of the following five species of *Catalpa*: *C. bignonioides*, *C. bungeii*, *C. fargeseii*, *C. ovata*, and *C. speciosa*. The experiment began with 30 larvae on each hostplant species. Leaves of all species were collected from the Arnold Arboretum, located in Jamaica Plain, Massachusetts.

Iridoid Glycoside Content in C. catalpae. To assess sequestration of iridoid glycosides in different life stages of *C. catalpae*, I used gas chromatography (Gardner and Stermitz, 1988; Bowers and Stamp, 1997) to analyze larvae, pupae, adults, and larval frass of the Catalpa Sphinx, and leaves of *C. bignonioides*, the plant on which the larvae were fed. *Catalpa bignonioides* contains several different iridoid glycosides (Iwaga et al., 1991; Von Poser et al., 2000); however, I quantified only two of these, catalpol and catalposide. To prepare samples for analysis, each was extracted overnight in methanol. The solid material was filtered out, and the extract evaporated to dryness. An internal standard, phenyl-β-D-glucose (=PBG), was added, and the sample was partitioned between water and ether. The ether fraction, containing lipophilic substances, was discarded, and the water fraction, containing the iridoid glycosides, was evaporated to dryness. An aliquot of this was derivatized using Tri-Sil Z™ (Pierce Chemical Co., Rockford, IL), prior to injection onto the gas chromatograph.

Iridoid Glycoside Contents in Cotesia congregata. Cotesia congregata parasitizes larvae of several species in the Sphingidae. Adults oviposit into the gut cavity, and larvae develop there, bathed in hemolymph of the host. In a sample of Catalpa sphinx larvae collected in the field, the number of parasitoids ranged from 3 to 184 per individual, with a mean of 56.9 per individual ($N = 17$). I analyzed iridoid glycoside content of hemolymph of Catalpa sphinx larvae, *C. congregata* adults, and silken pupal cases. The hemolymph samples were collected by cutting the larval abdominal "horn" and collecting the hemolymph with capillary tubes. These hemolymph samples were immediately put into 95% methanol and this was evaporated to dryness. Dry weights of hemolymph were the weight of this sample. Iridoid glycosides were analyzed as described above.

Growth Consequences of Regurgitation. One common defense of Catalpa sphinx larvae is to regurgitate. This is such a common response to disturbance that it is often difficult to handle them without their regurgitating (pers. obs.). To examine the consequence of regurgitation for these larvae, I began with newly molted third instars. Larvae were reared individually. Catalpa sphinx larvae are

gregarious in the early instars, but beginning in the third instar they begin to disperse.

There were two treatments: 1) control and, 2) larvae pinched with forceps to mimic attack by predators (see Cornell et al., 1987), once in the morning and once in the afternoon. This treatment often elicited regurgitation, but not always. Newly molted third instars were weighed at the start of the experiment and fed *ad libitum*. Larvae were weighed again after 5 and 10 days. The experiment began with 15 larvae in each treatment.

RESULTS

Growth Rate on Different Catalpa Species. Repeated measures ANOVA showed that larval weight increased over time (they grew) ($F = 242.970$, $P < 0.001$) and that there was an effect of hostplant species on larval growth ($F = 7.454$, $P = 0.005$) (Figure 1). The drop in weight on day 20 was due to weight loss prior to pupation; most larvae pupated at 21 or 22 days. Survival to day 20 was as follows: *C. bungei* = 50%, *C. ovata* = 47%, *C. bignonioides* = 70%, *C. fargesii* = 53%, *C. speciosa* = 53%. Survival did not vary among larvae reared on the different hostplant species ($\chi^2 = 1.78$; $df = 4$; $P > 0.50$).

Iridoid Glycoside Content of C. catalpae Reared on C. bignonioides. Leaf samples contained a mean of just over 6% total iridoid glycosides, with there being approximately eight times more catalposide than catalpol (Figure 2). Larvae,

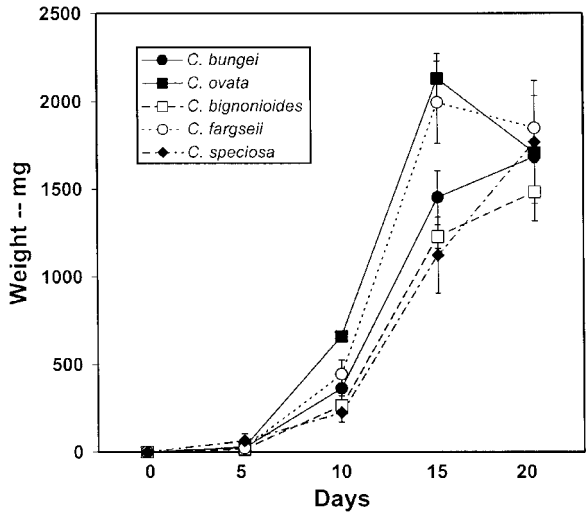

FIG. 1. Growth of *Ceratomia catalpae* larvae on five different *Catalpa* species. Means ± SE are shown.

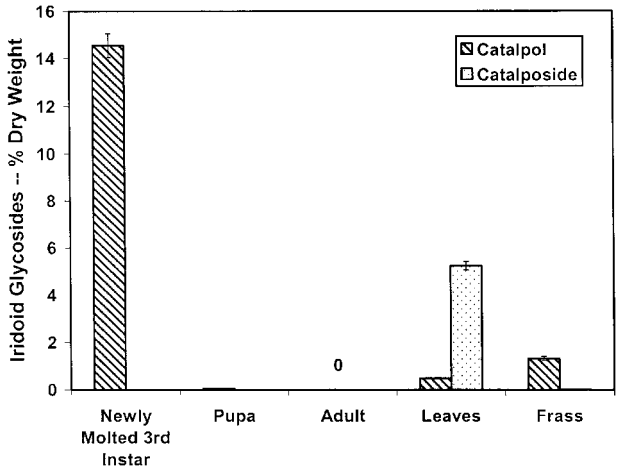

FIG. 2. Iridoid glycoside concentration of larvae, pupae, adults, and frass of *Ceratomia catalpae*, and leaves of the hostplant, *Catalpa bignonioides*. Means ± SE are shown.

pupae, and frass, however, contained only catalpol. Newly molted third instars contained over 14% dry weight catalpol, pupae were low in catalpol, and adults contained no detectable iridoid glycosides at all. The presence of catalpol in the frass indicates that larvae do not sequester all the iridoid glycosides present in the leaves.

Iridoid Glycosides in Cotesia congregata. Hemolymph of *C. catalpae* contained a mean of almost 50% catalpol (mean = 47.68% ± 7.79 SE) and no catalposide. There were traces of catalpol in parasitoid silken pupal cases (mean = 0.67% ± 0.065 SE) and even smaller traces in adults (mean = 0.11% ± 0.065 SE).

Growth Consequences of Regurgitation for Larvae of C. catalpae. T tests on the weights of larvae at days 0, 5, and 10 showed that the two groups (regurgitating and nonregurgitating) did not differ in weight at the start of the experiment (day 0, $P = 0.820$) (Figure 3). However, larvae that regurgitated weighed significantly less than those that did not at both days 5 ($P = 0.010$) and 10 ($P = 0.019$) (Figure 3). Thirteen of the larvae in the nonregurgitation treatment survived to day 10, and 10 of the larvae in the regurgitation treatment survived this long.

DISCUSSION

Larvae of *Ceratomia catalpae* will accept and feed on any of the five *Catalpa* species that they are offered and in the field have been destructive on all of these species (Baerg, 1935). While there was a significant effect of hostplant on larval

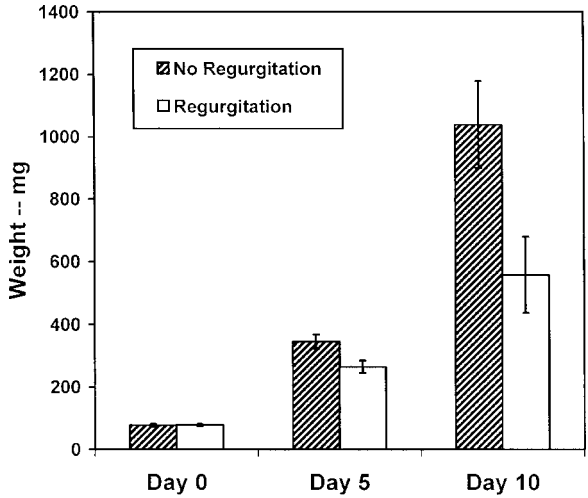

FIG. 3. Weight of *Ceratomia catalpae* larvae that were either pinched with forceps to induce regurgitation (regurgitation treatment) or not so pinched (no regurgitation treatment). Means ± SE are shown.

weight, survival did not differ among the five plant species, indicating that all would serve as adequate food resources.

Larvae of the Catalpa sphinx sequester iridoid glycosides from *Catalpa*. Although both catalpol and catalposide were found in the host, *C. bignonioides*, only catalpol was found in larvae, pupae, and frass. No iridoid glycosides were found in the adult. Thus, catalposide is probably converted to catalpol in the gut and the catalpol sequestered. Similar conversions have been found in the checkerspot butterfly, *Euphydryas anicia* (Nymphalidae), in which an ester of catalpol, 6-isovanillylcatalpol, is metabolized to catalpol and then sequestered (Gardner and Stermitz, 1988). Other catalpol esters, such as veronicoside and verproside, are also likely metabolized to catalpol, and then the catalpol is sequestered (Gardner and Stermitz, 1988). Similarly to these compounds, catalposide is an ester of catalpol; it is likely to be metabolized to catalpol by *C. catalpae* and then sequestered.

Ceratomia catalpae exhibits a sequestration pattern similar to that of the Buckeye butterfly, *Junonia coenia* (Nymphalidae), in which larvae sequester relatively large amounts of iridoid glycosides, pupae are significantly lower, and the adult contains no iridoid glycosides at all (Bowers and Collinge, 1992). Such a decline in iridoid glycoside content in the pupal stage also has been observed in other iridoid sequesterers, such as the Baltimore checkerspot, *Euphydryas phaeton* (Nymphalidae) and the arctiid, *Spilosoma congrua* (Arctiidae) (Bowers,

unpublished data). These data suggest that there may be a cost to retaining iridoid glycosides through to the pupal stage. During the pupal stage, larval structures are resorbed, and there is growth and differentiation of the imaginal disks. During such time, there may be problems with autotoxicity of sequestered compounds, especially those such as iridoid glycosides that are stored primarily in the hemolymph, that will bathe all developing structures.

Ceratomia catalpae stores iridoid glycosides in the hemolymph, as has been found for other iridoid glycoside sequestering species (Strohmeyer et al., 1998). In this study, iridoid glycoside concentration was approximately 50% of the dry weight of the hemolymph. A previous study showed that larvae of the Buckeye butterfly, *Junonia coenia*, also stored substantial amounts of iridoid glycosides in the hemolymph: 63.6 μg/μl ($\pm$1.3 SE). This means that the larvae of the parasitoid, *Cotesia congregata*, that were growing in the caterpillars were bathed in hemolymph that was high in iridoid glycosides. Although I did find low levels of catalpol in both adult *Cotesia congregata* and in the silken cocoons that remained after adult emergence, these amounts were small, especially when compared to that of the hemolymph. Other studies that have documented the occurrence of secondary compounds in parasitoids have likewise found relatively small amounts of these compounds (Duffey et al., 1986). For example, cardiac glycosides were found in larvae of the tachinid, *Zenilla adamsonii*, that parasitized larvae of the Monarch, *Danaus plexippus* (Nymphalidae) (Reichstein et al., 1968), and several pyrrolizidine alkaloids were found in the braconid wasp *Microplitis* sp. reared from an arctiid that had fed upon *Senecio spathulatus* (Asteraceae) (Benn et al., 1979). The alkaloid tomatine was found in the ichneumonid parasitoid, *Hyposoter exiguae*, that parasitized *Heliothis zea* (Noctuidae) (Campbell and Duffey, 1979, 1981). Similar to my results with *C. congregata*, another study with this same parasitoid species showed that nicotine in the diet of *Manduca sexta* (Sphingidae) was passed on to the parasitoid and was found in the silk of the cocoon (Barbosa et al., 1982).

Regurgitation has been observed to be a common response to predators in many lepidopteran and sawfly larvae (e.g., Stamp, 1982; Larsson et al., 1986; Cornell et al., 1987; Peterson et al., 1987). The regurgitant may contain plant secondary compounds that may serve as repellents (e.g., Larsson et al., 1986; Peterson et al., 1987), and the regurgitant itself may be viscous and sticky, thus gumming up mouthparts of attacking predators. Although we did not quantify iridoid glycoside content of the regurgitant of *C. catalpae*, TLC comparison of standards of catalpol and catalposide with the regurgitant indicated that the regurgitant contained both catalpol and catalposide. The data on growth of *C. catalpae* larvae that were pinched with tweezers and regurgitated indicated that there was a cost to this defensive behavior. Larvae thus disturbed and that typically regurgitated grew more slowly than larvae that were not. Similar costs have been documented in other lepidopteran and sawfly larvae that use regurgitation as a defense (e.g.,

Larsson et al., 1986; Peterson et al., 1987). Thus, larvae that regurgitate may repulse potential predators, but may also grow more slowly and, therefore, increase their exposure to other natural enemies.

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COMPARATIVE BENZENE-INDUCED FATTY ACID CHANGES IN A *Rhodococcus* SPECIES AND ITS BENZENE-SENSITIVE MUTANT: POSSIBLE ROLE OF MYRISTIC AND OLEIC ACIDS IN TOLERANCE

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Abstract—A Gram positive bacterium of the genus *Rhodococcus* was isolated from a contaminated site in Sydney, Australia, for its ability to tolerate and degrade high concentrations of benzene. To identify fatty acids that may impart this *Rhodococcus* sp. with tolerance to toxic solvents, a benzene-sensitive strain, labeled M2b, was isolated using EMS mutagenesis. A comparative analysis of fatty acid profiles showed that strain M2b was unable to increase its saturated:unsaturated ratio of fatty acids to the level achieved by the *w-t* strain when both strains were challenged with benzene. This was due to M2b's increased abundance of myristic acid, and decreased abundance of oleic acid. In addition, by measuring the generalized polarization of the fluorescent membrane probe laurdan using fluorescence spectroscopy, we have shown for the first time the effects of an aromatic hydrocarbon on the membrane fluidity of a *Rhodococcus* sp. The fluidity of the membranes increased after only 0.5 hr of exposure to benzene, thus suggesting the partitioning of benzene within the lipid bilayer. The response of this *Rhodococcus* sp. to benzene may suggest a mechanism for how other microorganisms survive when toxic solvents are released within the vicinity of their environment.

Key Words—Benzene, membrane fluidity, bioremediation, fatty acids, myristic acid, oleic acid, *Rhodococcus*, cell membrane, solvent tolerance.

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INTRODUCTION

Aromatic compounds are present in natural ecosystems as common components of many secondary plant metabolites and as products released from the partial decomposition of lignin and protein (Claus and Walker, 1964; Middlehoven et al., 1992). Because of human intervention, however, many aromatic solvents have been introduced into the environment in high concentrations, often via crude oil spills and leaky underground petroleum tanks. Benzene, a derivative of crude oil, is one of the most toxic environmental pollutants recognized. It has been ranked as the fifth most hazardous pollutant based on a total of 466 different volatile organic compounds found at 888 contaminated sites in the United States and other countries (Siegrist, 1992). Previously, our group isolated a Gram positive bacterium from a contaminated site near a chemical plant in Sydney, Australia, based on its ability to tolerate and efficiently degrade concentrations of benzene, reaching its maximal solubility (1800 mg/l) in aqueous media (Paje et al., 1997). The organism was partially characterized and found to belong to the *Rhodococcus* genus. Like the pseudomonads, many species within this genus have been recognized to tolerate and degrade various pollutants. They have been described as robust and have been found inhabiting a wide range of environments (Warhurst and Fewson, 1994).

The isolation of solvent-tolerant microorganisms has opened a new era of investigation directed toward elucidating the mechanisms that confer solvent-resistance. This should help to further our understanding of how they are able to exist in environments that are ridden with toxic chemicals where most other microorganisms would not survive. Numerous reports in the literature, mainly from the past two decades, have shown that various hydrocarbon-tolerant microorganisms produce qualitative and quantitative changes in their membrane fatty acids in response to toxic organic compounds (Heipieper et al., 1994; Sikkema et al., 1995; Weber and de Bont, 1996). Such changes have been shown to decrease the fluidity of cell membranes. This has been proposed to give them greater structural rigidity and be a mechanism for tolerating organic solvents (Sikkema et al., 1995). To date, only three reports exist that have studied the effects of aromatic hydrocarbons on the fatty acid composition in any one *Rhodococcus* spp. (Hamilton et al., 1995; Gutierrez et al., 1999; Tsitko et al., 1999). We have shown that our *Rhodococcus* strain increases its ratio of saturated:unsaturated cellular fatty acids during growth with benzene (Gutierrez et al., 1999). Such changes are often associated with decreasing the fluidity of cell membranes, and are suggested to reduce the toxicity of solvents like benzene. Although changes in the abundance of various fatty acids have been shown to lead to the fluctuations observed in the sat:unsat ratio, the specific fatty acids that play a contributing role in the tolerance of this organism to toxic solvents in the environment remain to be elucidated.

The present study was conducted to identify fatty acids in a *Rhodococcus* sp. that may be involved in the adaptive response associated with tolerating exposure

to benzene. For this, a benzene-sensitive mutant, M2b, was isolated and its fatty acid profiles determined and compared to those of the parent strain (*w-t*) when both strains were exposed to benzene. We also report for the first time the effects of an aromatic hydrocarbon on the cell membrane fluidity of a *Rhodococcus* sp.

METHODS AND MATERIALS

Organism and Growth Conditions. A highly benzene-tolerant *Rhodococcus* sp., previously isolated from a contaminated site near Port Botany in Sydney, Australia (Paje et al., 1997), was used. A benzene-sensitive mutant, labeled strain M2b, was isolated from this *Rhodococcus* (*w-t*) as described below. Stock cultures were maintained at 4°C on Luria-Bertani (LB) agar medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar per liter of water). Inocula were prepared in 50 ml of minimal medium (MM), with colonies from a fresh plate, and supplemented with mannitol (1%) and/or benzene (supplied via the vapor phase) as required (Paje et al., 1997). Cells were grown to the mid-exponential phase (28°C, 190 rpm) prior to use as inocula at a final level of 4% (v/v).

Isolation of Benzene-Sensitive Mutants. The method for generating benzene-sensitive mutants from the *w-t* was performed using chemical mutagenesis with ethyl methanosulfonate (EMS), as adapted from the method by Eisenstadt et al. (1994). This involved subjecting the cells to a 4% solution of freshly prepared EMS (Sigma), prewarmed to 37°C, for 2 hr. The cells were then washed twice with 0.1 M phosphate buffer. To select for nongrowing benzene-sensitive mutants, the cells were resuspended in a 50-ml volume of LB broth supplemented with 200 mg/l of presolubilized benzene and 2 mg/l of ampicillin. After 4 hr of incubation (28°C, 190 rpm), the cells were harvested and appropriate dilutions performed for replica spread-plating on LB agar medium. Plates were incubated at room temperature in a cotton wool-plugged desiccator for 72 hr with benzene supplied via the vapor phase. For a comparative selection of sensitivity to the benzene, duplicates of these plates were maintained under identical conditions without benzene. Colonies of less than 0.5 mm in diameter were selected as benzene-sensitive mutants from plates incubated in the desiccator with the benzene.

Effects of Benzene on the Fatty Acid Profiles of Strain M2b. To identify fatty acids involved in conferring the *w-t* with a tolerance to benzene, the total lipid profiles of the benzene-sensitive mutant, strain M2b, were compared to those previously published for cells of the *w-t* grown under identical conditions (Gutierrez et al., 1999). For this, three cotton wool-plugged 250-ml Erlenmeyer flasks containing 50 ml of MM were used. Culture 1 contained mannitol, Culture 2 contained benzene, and Culture 3 contained both mannitol and benzene, as carbon sources. Each was inoculated with the 4% inoculum and incubated (28°C, 190 rpm) until a cell turbidity (OD_{600}) of approximately 0.4 (0.1 dilution) was

reached. The cells were then harvested prior to use for lipid extraction and fatty acid analysis.

For lipid extraction, whole cell lysates were prepared of wet pellet (triplicate samples, 400 mg) by saponification in a solution of 13% NaOH in 50% (v/v) aqueous MeOH at 100°C for 30 min. The fatty acids were then methylated in a solution of 3.25 M HCl in aqueous methanol at 80°C for 10 min. The fatty acid methyl esters (FAME) were extracted with hexane/methyl tertiary butyl ether (MTBE) and base washed in a dilute solution of 1.2% NaOH. Triplicate 0.1 ml samples of the hydrophobic phase, constituting the fatty acid fraction in hexane and MTBE, were then dispensed into 1 ml glass vials and hermetically capped with Teflon-lined rubber septa for gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) analysis.

Benzene's Effect on the Membrane Fluidity of the w-t. Two types of culture treatments were prepared to determine benzene's short-term effect on the cell membrane fluidity of the *w-t*. These were performed in hermetically closed screw-capped 250-ml Teflon tubes containing 100 ml of MM and inoculated with a washed 50-ml suspension of benzene-unexposed cells in the exponential phase. One culture contained 1% (v/v) mannitol (no benzene), and the other contained presolubilized benzene (200 mg/l) plus the 1% mannitol. Samples for fluorescence analysis were taken at the point of inoculation and periodically thereafter.

To determine benzene's long-term effect on the membrane fluidity of the *w-t*, three types of culture treatments were prepared — one with mannitol, the second with benzene, and the third with both mannitol and benzene as carbon sources. Each was inoculated with the 4% inoculum (benzene-unexposed cells) and grown to the exponential phase prior to sampling for spectrofluorometric analysis.

Membrane fluidity was assessed by measurement of generalized polarization (GP) using the fluorescent membrane probe laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) as developed for application with microorganisms (Parasassi et al., 1990; Learmonth and Gratton, 2002). When necessary, cell cultures were diluted to $OD_{600} = 0.3$ (0.1 dilution) for fluorescent probe labeling. Five microliters of laurdan (Molecular Probes, Eugene, OR), from a 1-mM stock solution in ethanol, was added to 5 ml of cell culture. This was incubated for 30 min at room temperature in the dark prior to spectrofluorometric analysis.

Analytical Procedures. GC analysis was performed with an Hewlett Packard 5890A GC as described elsewhere (Gutierrez et al., 1999). Peaks were quantified with DAPA and/or Waters Millennium Scientific software. Individual components were identified using GC–MS (see below) by comparing retention time data with those obtained for authentic and laboratory standards. The standard deviations (SD) were $\leq$5% of the mean value determined for each individual fatty acid.

GC–MS analyses were performed on a Fisons MD 800 GC-MS (Manchester, UK) fitted with an on-column injector. The GC–MS was operated in scan mode

with an ionizing voltage of 70 eV. The GC was fitted with a capillary column similar to that described above. Helium was used as the carrier gas.

To locate the double-bond positions, dimethyl disulfide (DMDS) adducts of monounsaturated fatty acids (as FAME) were formed for selected samples by treating the total FAME with DMDS (Dunkelblum et al., 1985; Nichols et al., 1986). Adducts were extracted using hexane/chloroform (4:1 v/v) and analyzed by GC–MS as described elsewhere (Gutierrez et al., 1999).

For spectrofluorometry, fluorescent intensities were measured in a SLM-Aminco Bowman Series 2 luminescence spectrometer (SLM Instruments, IL, USA) using 10 × 10 mm PMMA 4-sided cuvettes with the following instrument conditions: excitation wavelength (Ex) = 340 nm; fluorescence emission wavelength (Em) = 440 and 490 nm. Cells were kept in suspension during measurements using a cuvette stirrer. Cuvette temperature was maintained at 28°C with a circulating water bath (Thermoline, Sydney) to reduce the possibility of fluidity changes as a result of a change in temperature. The spectrofluorometer photomultiplier gain was optimized using labeled cells with Ex 340 nm and Em 440 nm. Intensity readings were taken every 30 sec over 180 sec, thus, each intensity recorded was the mean of six readings. To determine background fluorescence, a labeled cell suspension was filtered (0.22 μm) and fluorescence emission was measured as described above. Generalized Polarization (GP) was calculated as previously described (Parasassi et al., 1990). The theoretical maximum and minimum values of GP are 1.0 and −1.0, respectively. GP can be used as a numerical index of membrane fluidity — the higher the value the lower the fluidity (Parasassi et al., 1990).

RESULTS AND DISCUSSION

Isolation of Benzene-Sensitive Mutants. The mutation frequency for generating benzene-sensitive mutants from this *Rhodococcus* sp. was estimated to be 2.7×10^{-3} using the EMS mutagenesis and mutant selection procedure described. Five colonies (<0.5 mm in diameter) were selected as candidates for benzene-sensitivity. These were tested for sensitivity to different concentrations of benzene. One mutant, labeled strain M2b, was found to have a 60–70% sensitivity as compared to the *w-t* strain (results not shown). This mutant was selected for further investigation.

Effect of Benzene on the Fatty Acid Profiles of Strain M2b. Table 1 shows the six fatty acids that dominated the fatty acid profiles of strain M2b when the cells were grown on MM containing mannitol, benzene, and both carbon sources, as obtained from two separate duplicate experiments, Experiment 1 and Experiment 2. The results of Experiment 2 are used simply to show the consistency in the fatty acid trends, henceforth, the discussion will focus on Experiment 1.

TABLE 1. COMPOSITIONAL (%) CHANGES OF SIX DOMINANT FATTY ACIDS IN THE BENZENE-SENSITIVE MUTANT, M2b, WHEN GROWN ON MANNITOL, BENZENE, AND MANNITOL+BENZENE IN MM

	Experiment 1			Experiment 2		
Fatty acid	Man[a] (Avg. ± SD)	Benz[b] (Avg. ± SD)	Man + Benz[c] (Avg. ± SD)	Man (Avg. ± SD)	Benz (Avg. ± SD)	Man + Benz (Avg. ± SD)
14:0	15.5 ± 0.4	10.5 ± 0.2	9.4 ± 1.9	13.8 ± 2.5	10.9 ± 0.3	10.5 ± 0.4
16:0	27.1 ± 0.6	39.1 ± 0.9	37.1 ± 1.2	28.7 ± 1.8	42.0 ± 0.4	36.7 ± 0.3
10Me18:0	3.4 ± 0.2	6.2 ± 0.4	7.0 ± 1.4	4.2 ± 1.3	6.2 ± 0.3	6.1 ± 0.1
Sum Sat	53.3 ± 1.6	60.3 ± 2.1	58.1 ± 6.5	54.6 ± 7.4	61.6 ± 1.6	57.6 ± 1.6
16:1ω7c	12.0 ± 0.3	4.0 ± 0.1	4.5 ± 0.8	10.9 ± 1.7	4.5 ± 0.0	4.8 ± 0.1
16:1ω6c	13.6 ± 0.3	11.1 ± 0.3	12.6 ± 2.2	12.0 ± 1.9	9.0 ± 0.1	13.9 ± 0.1
18:1ω9c	16.4 ± 0.3	18.8 ± 0.3	20.0 ± 1.4	17.8 ± 1.9	19.1 ± 0.1	19.4 ± 0.1
Sum unsat	46.7 ± 1.3	39.7 ± 2.7	41.9 ± 5.4	45.4 ± 6.2	38.4 ± 1.1	42.4 ± 0.7
Other	0.0 ± 0.0	1.9 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sat/unsat ratio	1.1	1.5	1.4	1.2	1.6	1.4
Total	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

Note: Data shown are for two separate experiments. Avg. ± SD = average percentage from three replicates, ± standard deviation of replicates.

[a] Man = mannitol-grown cells.

[b] Benz = benzene-grown cells.

[c] Man + Benz = mannitol+benzene-grown cells.

The total level of saturated fatty acids changed from $53.3 \pm 1.6\%$ for mannitol-grown cells (no benzene) to $60.3 \pm 2.1\%$ for benzene-grown cells (no mannitol). The total level of unsaturated fatty acids changed, respectively, from $46.7 \pm 1.3\%$ to $39.7 \pm 2.7\%$. All of the fatty acids (16:0, 10Me18:0, and 16:1ω7c) that were found to increase the sat:unsat ratio of the *w-t* from 1.1 (for mannitol-grown cells) to 1.5 (for benzene-grown cells) (Gutierrez et al., 1999), were also shown to have the same effect in strain M2b under identical growth conditions.

Interestingly, strain M2b did not increase its sat:unsat ratio to a higher level during growth in MM containing both carbon sources (Table 1) — a growth condition that has been shown to effectively expose the cells to higher concentrations of benzene (143 to 154 mg/l) (Gutierrez et al., 1999). This ratio was 1.4 with M2b. Under identical growth conditions, it was 1.8 for the *w-t* (Gutierrez et al., 1999). Thus, the mutant did not show the dose-related increase in fatty acid saturation that had previously been observed with the *w-t* (Gutierrez et al., 1999), and that had also been observed in *Rhodococcus opacus* when exposed to toluene and phenol (Tsitko et al., 1999). The lower sat:unsat ratio achieved by strain M2b appears to be due to a decrease in the abundance of myristic acid (by 39.4%) and increase in oleic acid (by 22.0%) compared to that in the *w-t* when grown under identical conditions (Gutierrez et al., 1999). Hence, a mutation in the expression of one or both of these fatty acids may account partly or totally for M2b's sensitivity to benzene, and either or both of these fatty acids may play a role in providing the *w-t* with some degree of tolerance to benzene. Mannitol, along with benzene, also may have contributed to the changes in the sat:unsat ratios observed when both compounds were present in the medium. However, at the concentrations used (1%), this readily metabolizable and relatively innocuous sugar should have no significant effect on the fatty acid saturation compared to that of benzene.

Sampling each culture for fatty acid analysis when the optical density reached approximately 0.4 minimized the artifact that fatty acid compositional variation might be due to cells being analyzed at a different stage of growth. Hence, the fatty acid changes observed are concluded to be effectively because of the difference in the treatment (i.e., presence or absence of benzene). Since no changes in the ratio of *cis* to *trans* monounsaturated fatty acids were detected, and all unsaturated fatty acids were *cis* monoenoic, the results show that saturated fatty acids were the main components involved in changes observed with the sat:unsat ratio in response to benzene.

Effect of Benzene on the Membrane Fluidity of the w-t. Table 2 shows the effect of benzene on the GP value of the membranes in cells of the *w-t* when grown for 18–20 hr on MM containing mannitol, benzene, and both carbon sources, as obtained from duplicate cultures — Experiments 1 and 2. The results obtained from the duplicate, Culture 2, are shown to demonstrate the consistency in the GP trends. Hence, the discussion focuses on the results from Experiment 1.

TABLE 2. GENERALIZED POLARIZATION (GP) OF LAURDAN-LABELED CELLS OF THE *Rhodococcus w-t* DURING GROWTH IN MM SUPPLEMENTED WITH MANNITOL, BENZENE, AND MANNITOL+BENZENE

Treatment	Experiment 1	Experiment 2
Man[a]	0.51 ± 0.002	0.49 ± 0.001
Benz[b]	0.60 ± 0.005	0.58 ± 0.003
Man + Benz[c]	0.64 ± 0.005	0.60 ± 0.002

Note: Experiment 2 is a duplicate of Experiment 1.
[a] Man = mannitol-grown cells.
[b] Benz = benzene-grown cells.
[c] Man + Benz = mannitol+benzene-grown cells.

The increase in the GP value of the membranes, as induced by the presence of benzene, is correlated with a decrease in membrane fluidity (Parasassi et al., 1990; Learmonth and Gratton, 2002). Theoretically, an increase in the sat:unsat ratio of fatty acids has been proposed to lower the fluidity of membranes and reduce the accumulation of solvents at this cellular site (Sikkema et al., 1995; Weber and de Bont, 1996). Therefore, this benzene-induced decrease in membrane fluidity complements previous results where we showed that benzene induced an increase in the ratio of sat:unsat fatty acids (Gutierrez et al., 1999). These types of fatty acid changes have been associated with the decreasing fluidity of cell membranes in various microorganisms when exposed to toxic organic solvents (Sikkema et al., 1995; Weber and de Bont, 1996), as well as in model phospholipid membrane systems (Cullis and Hope, 1985). No fluorescence was detected in the laurdan-uninoculated controls (data not shown), which eliminates the existence of background fluorescence by other components of cellular or culture medium origin.

Benzene (200 mg/l) was found to have an almost immediate effect on the fluidity of the membranes in the *w-t* strain (Figure 1). After only a 0.5 hr incubation, the GP decreased. This correlated with an increase in the fluidity of the membranes. Although there are few reports available describing the position of benzene in a membrane (Simon et al., 1982; Ward et al., 1986; Boden et al., 1991), its influence at this site is widely accepted to produce an increase in fluidity (Sikkema et al., 1994). A decrease in the fluidity of the cell membrane, to counter the fluidizing effect of the benzene, was not evident over the duration (6 hr) of this experiment. Since fluidity was observed to decrease after the cells had been exposed to the benzene for approximately 18–20 hr (Table 2), this suggests that an adaptive response, set to mitigate benzene toxicity, is induced only after long exposure. With respect to the organism's tolerance during the first 6 hr, this *Rhodococcus* may have a well-adapted inherent mechanism to survive an initial challenge with benzene. Such a mechanism would be beneficial to the organism at times when

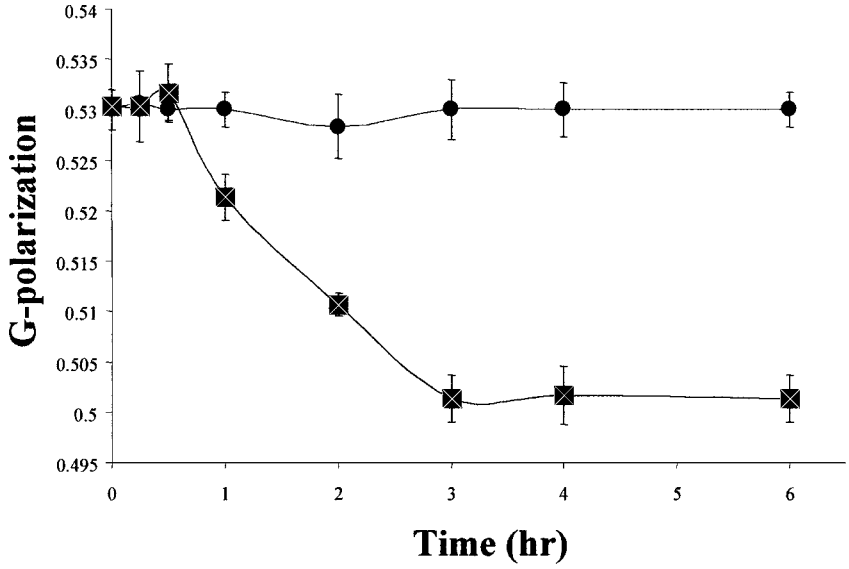

FIG. 1. Change in the generalized polarization (GP) value of laurdan-labeled cells of the *Rhodococcus w-t* during exposure to 200 mg/l benzene (■) in MM containing 1% (v/v) mannitol, compared to the benzene-unexposed control (●).

"shock" concentrations of benzene, and other toxic solvents, enter the vicinity of this organism in the environment.

In this study we have shown that a *Rhodococcus* species produces benzene-induced changes in the sat:unsat ratio of fatty acids, influenced particularly by myristic and oleic acids. These changes may be associated with mitigating solvent toxicity in the cell membrane interface, thus enabling this organism to exist at contaminated sites. The presence of solvent-tolerant microorganisms at contaminated sites is of ecological benefit to the environment because they can contribute to the natural bioremediation or detoxification of these compounds — a process that involves the microbial conversion of the contaminants into innocuous products.

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ATTRACTION OF THE FRUIT-EATING BAT *Carollia perspicillata* TO *Piper gaudichaudianum* ESSENTIAL OIL

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Abstract—We performed field tests using mimetic *Piper* fruits with and without essential oil extracted through hydrodistillation from *Piper gaudichaudianum* ripe fruits in order to evaluate the role of odor in *Carollia perspicillata* attraction and capture in mist-nets. During the field tests, 26 *C. perspicillata* were captured, 21 (80.7%) in nets with the essential oil of *P. gaudichaudianum* and five (19.3%) in nets without oil. Other bat species, *Artibeus* spp. (67), which is specialized on fruits of Moraceae, and *Sturnira lilium* (10), specialized on those of Solanaceae, were also captured, but they exhibited no significant preference for nets with or without oil. We conclude that odor is pre-eminent over visual cues in food location by *C. perspicillata* in a field situation. Based on the result, we propose the extraction and use of essential oils of chiropterochoric fruits as a useful approach to improve autoecological studies on fruit-eating bats and to promote

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tropical forest restoration through the attraction of frugivorous bats to degraded areas.

Key Words—Essential oil, *Piper gaudichaudianum*, short-tailed fruit bat, *Carollia perspicillata*, odor detection, bat attraction, mist-netting, frugivory, seed dispersal, forest restoration.

INTRODUCTION

Fruit consumption and seed dispersal by the Neotropical family of leaf-nosed bats (Phyllostomidae) are key-processes for plant reproductive success, forest diversity maintenance, and forest recovery after mild or severe disturbances (Fleming, 1988; Medellin and Gaona, 1999). In addition to echolocation, fruit-eating bats use vision and olfaction for spatial orientation, obstacle avoidance, and food searching (Fleming, 1988; Thies et al., 1998). The importance of odor in foraging activities by phyllostomids has been recognized and investigated for more than 50 years (e.g., van der Pijl, 1957), revealing that olfactory accuracy is high in this family. The evidence is based on histological and anatomical studies (e.g., Bhatnagar and Kallen, 1975), laboratory experiments (e.g., Laska, 1990), and field observations (e.g., Fleming, 1988; Rieger and Jakob, 1988; Thies et al., 1998).

Some authors also consider olfaction to be the main sense used by the short-tailed fruit bat, *Carollia perspicillata*, a specialist on the genus *Piper*, to locate ripe fruits (Fleming, 1988; Thies et al., 1998).

Here, we present results of a field test designed to evaluate the efficiency of the essential oil of *Piper gaudichaudianum* ripe fruits in attracting and capturing *C. perspicillata* in a small forest remnant of southern Brazil. The experiment studied the role of olfaction in fruit location by bats, and presents a new technique to attract fruit-eating bats to degraded or disturbed areas in order to improve forest regeneration through seeds contained in bat feces.

METHODS AND MATERIALS

Study Area. The study was conducted in the Parque Estadual Vila Rica do Espírito Santo (PEVR) (23°55′S–51°57′W), Fênix, Paraná state, Brazil. This area, completely surrounded by agricultural land, represents one of the last forest fragments (354 ha) of Semideciduous Seasonal Forest in the mid-west Paraná state. The mean altitude of PEVR is 330 m; temperature is variable throughout the year (mean temperature—summer = 25°C; winter = 16°C); frosts are rare; annual rainfall is about 1500 mm, with a peak between December and February. A detailed description of the study area and its plant species is in Mikich and Silva (2001).

Piper gaudichaudianum Kunth. Pepper plants of the pantropical genus *Piper* (Piperaceae) are shrubs or small trees relatively common and conspicuous as

pioneer species in regeneration areas, but also occurring in forest understory, edges, and gaps (Fleming, 1988; Thies et al., 1998). *Piper* infructecenses (drupes grouped in spikes; herein called fruits) have several characteristics associated with bat dispersal (chiropterochory), such as strong odor, exposure outside the foliage, dull color (van der Pijl, 1957), and ripening in the late afternoon (Thies et al., 1998; Mikich, personal observation). In the PEVR, the genus *Piper* is represented by nine species distributed along roads, trails, the forest interior and its edges. *P. gaudichaudianum*, one of the most abundant species in this area, has strongly curved fruits (100 mm long, 6 mm wide), containing up to 2000 seeds (1.0×0.8 mm). It has two annual periods of fruit availability, the most intense being between November and February (wet season) and the other between May and July (dry season) (Mikich and Silva, 2001).

Carollia perspicillata (Linnaeus, 1758). The short-tailed fruit bat, *Carollia perspicillata*, ranges from Mexico to southern Brazil (Fleming, 1988) and is common in the PEVR (Bianconi, personal observation). It is basically frugivorous, and *Piper* fruits are its preferred food (Fleming et al., 1977; Fleming, 1988; Mikich, 2002).

Essential Oil Extraction. The essential oil of ripe fruits of *P. gaudichaudianum* was obtained through hydrodistillation by using an adapted Clevenger for 4 hr. The oil was stored in vials with water until field tests were run. Fruits were collected in the PEVR in January/2000, July/2000, and December/2001 and were kept frozen until extraction, which was conducted, respectively, in November/2000, December/2000, and April/2002. Since each individual *Piper* plant holds few (up to three) ripe fruits each night, and since fruit removal is fast and efficient (approximately 100%) (Fleming et al., 1977; Mikich, personal observation), 30 *P. gaudichaudianum* plants were marked, and their near-ripe fruits were wrapped with plastic bags until the ripening process was completed.

Field Tests. To test the efficiency of the essential oil of *P. gaudichaudianum* ripe fruits for attracting *C. perspicillata*, a field test was conducted in the PEVR. Three replications were done in December/2000, June/2001, June/2002, months in which this plant holds ripe fruits in the study area. Tests used one set of 16 mist-nets opened (zig-zag design) in a forest interior road for six hr after dark, on four consecutive days, totaling 28,476 $m^2 \cdot hr$. A mimetic fruit made with green foam rubber was fixed in the middle of each net numbered from 1 to 16; half (even net numbers) were soaked with essential oil diluted in water, and the other half (odd numbers) were soaked with water only. Considering that all nets had mimetic fruits as visual cues, we evaluated whether nets containing the essential oil of *P. gaudichaudianum* as olfactory cues captured more *C. perspicillata* bats than nets with no oil.

Statistical Analysis. The difference between the number of bats of each species captured in nets with oil and without oil was calculated using a chi-square test ($df = 1$) with Yates' Correction, whenever necessary.

TABLE 1. FRUIT-EATING BATS CAPTURED IN THE PARQUE ESTADUAL DE VILA RICA DO ESPÍRITO SANTO, FÊNIX, BRAZIL, IN MIST-NETS WITH AND WITHOUT ESSENTIAL OIL EXTRACTED FROM *Piper gaudichaudianum* RIPE FRUITS

Bat species	Number of bats captured in mist-nets			Differences (χ^2)
	Total	With essential oil	Without essential oil	
Carollia perspicillata	26	21	5	χ^2 Yates CofT = 8.65; $P = 0.003$
Artibeus spp.	67	33	34	$\chi^2 = 0.015$; $P = 0.903$
Sturnira lilium	10	5	5	—

RESULTS AND DISCUSSION

During the field tests, 26 *C. perspicillata* were captured, 21 (80.7%) in nets with the essential oil of *P. gaudichaudianum* and five (19.3%) in nets without oil, indicating that the oil provided significant attraction (Table 1). This result is in agreement with previous studies that found olfaction to be one of the main senses used by *C. perspicillata* to locate food. Rieger and Jakob (1988) manipulated the odor and shape of banana during mist-net tests and concluded that odor was the main factor responsible for fruit-eating bat attraction and capture. Thies et al. (1998) evaluated the attraction of *Piper* fruits on *Carollia* spp. in semi-natural conditions. They tested the response of *C. perspicillata* and *C. castanea* to variations in texture, form, position, and odor of *Piper marginatum* fruits. Both species exhibited positive responses to mimetic fruits of *P. marginatum* containing odor of smashed real ripe fruits, but both ignored real unripe and odorless mimetic fruits. They concluded that odor is more important than form and texture for food location and choice by *Carollia* spp. Additionally, preference tests conducted in the laboratory by Laska (1990) and in the field by Fleming et al. (1977) showed that bats are able to distinguish different stages of ripeness of a fruit.

As seen in Table 1, other fruit-eating bats were also captured during the trials, but only *C. perspicillata*, a specialist in *Piper* fruits, responded positively to the odor of *P. gaudichaudianum* ripe fruits. This result might be related to the preferences of *Artibeus* spp. and *Sturnira* spp. for other fruit species, since *Artibeus* spp. are specialists on fruits of *Ficus* spp. (Moraceae) (Fleming et al., 1977; Mikich, 2002), and *Sturnira lilium* consumes mainly fruits of *Solanum* spp. (Solanaceae) (see references in Mikich, 2002). These findings open the possibility of using essential oils of chiropterochoric fruits to improve the capture of focal bat species for biological or population studies, by using the oils of their preferred fruit species as bait.

We can now conclude that the essential oil of *P. gaudichaudianum* ripe fruits is efficient for attraction and capture of *C. perspicillata* under natural conditions, even in the presence of natural ripe fruits of the same species. Our study corroborates the hypothesis that *C. perspicillata* uses mainly olfactory cues during foraging activities and suggests an important tool for conservation programs or ecological studies by attracting fruit-eating bats to disturbed areas (Bianconi et al., unpublished data) or for improving the capture of interest species. Following up on these perspectives, our research group is presently conducting tests with different chiropterochoric species of *Piper*, *Ficus*, and *Solanum*, both in the forest and in wide opened areas, such as agricultural fields.

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ANALYSIS OF ANAL DROPLETS OF THE WESTERN FLOWER THRIPS *Frankliniella occidentalis*

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Abstract—Larvae of the western flower thrips *Frankliniella occidentalis* are known to produce an anal droplet containing decyl acetate (10Ac) and dodecyl acetate (12Ac), which act as an alarm pheromone. Analysis by GC showed that the combined mass of 10Ac and 12Ac per droplet increased with age of second instars from 2 ng to 18 ng, while the mass ratio of 10Ac:12Ac increased from 0.4:1 to 1.1:1 (molar ratio 0.4:1 to 1.2:1). Droplet volume increased from 0.4 nl to 2.1 nl with age, but only about 1% of this was accounted for by 10Ac and 12Ac. The remainder contained water. Analysis by GC-MS using SPME revealed no other volatile components. Whole bodies contained about two to four times as much 10Ac and 12Ac as a droplet, but in a similar ratio.

Key Words—*Frankliniella occidentalis*; alarm pheromone; kairomone; integrated pest management; decyl acetate; dodecyl acetate; water; GC-MS.

INTRODUCTION

The western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), is a major worldwide horticultural pest that is difficult to control (Kirk, 2002). Teerling et al. (1993b) found that second instars, when attacked by a predator, produce an anal droplet containing decyl acetate (10Ac) and dodecyl acetate (12Ac). These compounds repel walking larvae and adults, reduce oviposition

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rate, increase take-off rate, and decrease landing rate (Teerling et al., 1993a,b; MacDonald et al., 2002). They are also used as a prey-finding kairomone by two predators (Teerling et al., 1993a) and may be involved in causing larvae to seek refuge from predators Pallini et al., 1998; Venzon et al., 2000). These compounds have potential for enhancing various control measures, for example sprays of 12Ac, alone or in combination with an insecticide, significantly reduced larval numbers in field infestations (Cook et al., 2002). Teerling et al. (1993b) reported that the molar ratio of 10Ac:12Ac in two whole-body extracts from second instars were 2:1 and 1.5:1 (mass ratios 1.8:1 and 1.3:1), but did not report the quantities present or the ratios in droplets. This paper provides details of the quantities and ratios in anal droplets and whole bodies of precisely aged larvae.

METHODS AND MATERIALS

Rearing. A culture of *F. occidentalis* from commercial glasshouses in the UK was maintained on pot chrysanthemums (*Dendranthema grandiflora* Tzvelev) at 25 $\pm 2^{\circ}$C and L18:D6. Larvae of known age were obtained by moving about 100–150 adults for 24 hr to 500 ml pots containing French bean pods (*Phaseolus vulgaris* L.) and pine pollen (*Pinus brutia* Tenore). Larvae hatching from the pods 3–4 d after oviposition were moved to new pots at 25 $\pm 0.3^{\circ}$C and L18:D6. At 4–5 d after hatching, some larvae remained feeding on the pods (feeders), while others that were nearer to molting wandered away to find a pupation site (wanderers) (MacDonald, 2002). Wanderers were sampled separately because they are behaviorally different from the earlier feeding stages.

Sample Collection. Larvae in the pots were anaesthetized first with carbon dioxide to ensure they did not produce an anal droplet when moved. Whole-body extracts were obtained by transferring individual larvae with a fine hexane-rinsed brush to clean glass vials on ice. The vials were immediately sealed after adding 5 μl of hexane (>95%, residue analysis grade, Fisher Scientific Ltd., UK). Anal droplets were collected from larvae by transferring anaesthetized individuals to a clean glass petri dish and taking a 1.5 mm square of glass micro-fibre disk (GMFS) (first cleaned by hexane rinse and baked overnight at 200°C) with hexane-rinsed forceps and touching it against the dorsal surface of a larva after it became active. If the larva appeared to have placed an anal droplet on the GMFS, the GMFS was transferred to a glass vial, which was immediately sealed after adding 10 μl of hexane. The vials were stored at -20°C until GC analysis. Control samples without larval contact confirmed the absence of any contamination of 10Ac or 12Ac. The larvae were examined later to verify instar and check the sex (Heming, 1991). Droplet volumes were determined by measuring droplet diameters from video recordings of known-age larvae and calculating a volume based on the assumption that the droplets were spherical. To test for components other than 10Ac and 12Ac,

droplets were collected directly on a 20 mm long 50/30 μm DVB/Carboxen SPME fiber assembly (57348-U, Supelco, USA) conditioned at 270°C and immediately analyzed by GC-MS. This method is solventless, so a solvent peak cannot hide any compounds. Because the SPME fiber was smaller than the GMFS, it needed to be touched against the larva more precisely, so larvae were stuck on the edge of a sticky trap, with the abdomen projecting, to immobilize them during droplet collection.

GC and GC-MS Analysis. The anal droplets collected on GMFS and the whole-body extracts were analyzed by GC as described previously (MacDonald, 2002). Peak areas were converted to masses from a calibration curve using standards of 10Ac (>98%, Sigma, UK) and 12Ac (>97%, Aldrich, UK). It was not always easy to see whether an anal droplet had been produced and had made good contact with the GMFS. If neither 10Ac or 12Ac were detected on the GMFS, the sample was omitted. The droplets collected by SPME were analyzed by GC-MS as described previously (MacDonald, 2002). The GC-MS was set up in EI mode and scanned a mass range of 40–500 amu. Mass ratios were calculated as 10Ac/12Ac and were converted to molar ratios by multiplying by 1.14. The data for mass of pheromone were $\log_{10}(x)$ transformed to normalize the residuals for statistical analysis.

RESULTS AND DISCUSSION

Anal droplets and whole-body extracts from larvae of the same cohort were analysed by GC (Table 1). First instars produced visible droplets, but they were too small to be collected. All the analyses were of females, apart from one of a

TABLE 1. ANALYSIS OF INDIVIDUAL ANAL DROPLETS AND WHOLE-BODY EXTRACTS OF KNOWN-AGE FIRST INSTAR LARVAE (LI) AND SECOND INSTAR LARVAE (LII) OF *F. occidentalis*[a]

		Mass of 10Ac + 12Ac (ng)[c]		Mass ratio of 10Ac/12Ac[c]		
Instar	Age (d)[b]	droplet ($N = 4$)	body ($N = 5$)	droplet ($N = 4$)	body ($N = 5$)	Droplet volume (nl) ($N = 4$–10)
LI	0–1	—	0.54 ± 0.08	—	0.33 ± 0.03	0.4 ± 0.2
LI	1–2	—	0.66 ± 0.14	—	0.22 ± 0.08	0.3 ± 0.0
LII	2–3	1.9 ± 0.4	8.5 ± 1.8	0.37 ± 0.06	0.45 ± 0.07	0.8 ± 0.2
LII	3–4	8.9 ± 3.1	17.8 ± 3.4	0.65 ± 0.22	0.86 ± 0.10	0.9 ± 0.2
LII	4–5 f	10.4 ± 4.9	19.5 ± 1.6	0.65 ± 0.38	1.0 ± 0.1	1.5 ± 0.3
LII	4–5 w	17.9 ± 4.7	28.8 ± 5.6	1.1 ± 0.1	1.1 ± 0.0	2.1 ± 0.7

[a] Untransformed mean ± SE.
[b] Days post hatching. f: feeders; w: wanderers.
[c] 10Ac: decyl acetate; 12Ac: dodecyl acetate. All data are for females.

male (4–5 d post hatching, wanderer) sampled for whole-body extract. It produced similar amounts of 10Ac and 12Ac (mass: 34.1 ng; mass ratio: 1.4:1) as females of the same age. Analysis of whole-body extracts from another cohort on bean pods and from second instars on chrysanthemums also gave similar results for masses and mass ratios.

The mass of both compounds increased with larval age for droplets (regression $F_{(1,14)} = 10.5$, $P = 0.006$), and whole bodies ($F_{(1,28)} = 124.3$, $P < 0.001$). The mass in droplets was highest in the wandering larvae and in these ranged from 8.2 ng to 28.5 ng. The mass in whole bodies was about two to four times that in droplets, which supports the observation that larvae can often produce two or three droplets in succession if provoked. The mass ratio of 10Ac/12Ac was variable, ranging from 0.0 to 1.4 (molar ratio 0.0 to 1.6) in droplets and 0.0 to 1.2 (molar ratio 0.0 to 1.4) in whole bodies. The ratio increased with age for droplets ($F_{(1,14)} = 4.7$, $P = 0.048$), and whole bodies ($F_{(1,28)} = 88.2$, $P < 0.001$). Three droplets contained only 12Ac, and one whole body contained only 12Ac. However, behavioral experiments have shown that 12Ac alone is at least as effective as a synthetic blend (Teerling et al., 1993b; MacDonald et al., 2002). No droplets or whole bodies contained only 10Ac.

Droplet volume increased with larval age ($F_{(1,40)} = 35.3$, $P < 0.001$), but the mass of 10Ac and 12Ac accounted for only about 1% of the volume of the droplets (given a density of 860 ng nl^{-1}. To test for other compounds, five droplets from 4–5 d old wandering second instars were collected on an SPME fiber and analyzed by GC-MS. This was compared with one droplet collected in the same way. Peaks for 10Ac and 12Ac were about five times as large in the first sample as in the second, as expected. A few other peaks were present, but all were likely contaminants from the air, and none were near to five times larger in the first sample than in the second, which would have indicated they came from the droplets. There was no evidence for any volatile compounds other than 10Ac and 12Ac in the droplets. Droplets evaporated quickly when larvae placed them on a glass surface, and no sticky residue was observed, which would have indicated high molecular mass compounds. The most likely other constituent is water and this was confirmed with a crystal of white anhydrous copper sulfate, which turned blue (Cotton et al., 1995) when a larva placed an anal droplet on it.

The change in the ratio of alarm pheromone with larval age could have ecological consequences. For example, the mite *Neoseiulus cucumeris* (Oudemans) uses the alarm pheromone as a prey-finding kairomone (Teerling et al., 1993a) and could use the ratio to distinguish the first instars, which are its main prey, from older larvae. The possibility that the change in ratio with age is exploited by predators or parasitoids needs experimental study.

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SEX PHEROMONE OF THE LEAF-MINER *PHYLLONORYCTER PLATANI*: (*Z*10)-TETRADECENYL ACETATE

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Abstract—A female produced sex pheromone of the leaf-miner pest *Phyllonorycter platani* (Staudinger) (Lepidoptera: Gracillariidae) was identified as (*Z*10)-tetradecenyl acetate. Field trapping trials confirmed activity of this compound. The presence of the geometrical isomer (*E*10)-tetradecenyl acetate, a pheromone component of other *Phyllonorycter* species, reduced trap efficacy.

Key Words—*Phyllonorycter platani*, leaf-miner, sex pheromone, monitoring, (*Z*10)-tetradecenyl acetate.

INTRODUCTION

At the beginning of the 20th century, *Phyllonorycter platani* (Staudinger) (Lepidoptera: Gracillariidae), a leaf-miner pest on *Platanus* spp., was only known to occur in North Africa, Asia, and South Europe. Subsequently, it colonized

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central Europe, and in the last 50 years has been recorded in many parts of northern Europe (Šefrová, 2001). We, therefore, undertook studies to identify the sex pheromone of *P. platani*, as pheromone traps would provide a useful tool to monitor the movement of this species and possibly in IPM control programes.

METHODS AND MATERIALS

Insects. Mined leaves of *Platanus orientalis* were collected in late autumn of 2001 in Sofia, Bulgaria and held under natural conditions until February 2002. At this time, *P. platani* pupae were removed from the leaves, sexed by the place and shape of the genital opening, and female pupae were stored at about 10°C. Each day, 80 pupae were transferred to 22°C under a L:D 14:10 photoperiod and checked daily. Newly-emerged insects were placed in individual test tubes closed with moistened cotton. As calling starts at the onset of the photophase (Mircheva, unpublished), calling females were collected about 1 hr after lights-on and freeze-killed. The ovipositors (with the pheromone gland) were immediately excised, and 10–12 were then extracted in 40 μl hexane for 5–15 min. Subsequently, all extracts were combined to provide a 1000 female equivalent sample for chemical analyses.

Electrophysiology. Electroantennogram (EAG) recordings were made using Ag-AgCl glass electrodes filled with saline solution (composition as in Maddrell, 1969, but without glucose). An antenna was excised and suspended between the two electrodes. The signals were passed through a high impedance amplifier (UN-06, Syntech, the Netherlands) and analyzed with a customized software package (Syntech). The stimulus was delivered into a purified air stream (1 l/min) flowing continuously over the preparation.

Coupled Gas Chromatography-Electroantennography (GC-EAG). The coupled GC-electrophysiology system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the volatiles was achieved on an AI 93 GC (AI Scientific, Cambridge, UK) equipped with a cold on-column injector and an FID, using a 30 m $\times$ 0.3 mm i.d. HP-WAX column. The oven temperature was maintained at 40°C for 1 min and then programmed at 10°C/min to 220°C. The carrier gas was hydrogen. The outputs from the EAG amplifier and the FID were monitored simultaneously and analyzed with the Syntech software package.

Analysis. Extracts were analyzed by combined gas chromatography-mass spectrometry (GC-MS), following published procedures (Schulz et al., 2002). An HP 5908 (Hewlett Packard) was linked to a double focussing sector field mass spectrometer, VG 70-250 SE (Vacuum Generators, Manchester, UK), operated in the EI-mode at 70 eV. Determination of double bond positions in unsaturated compounds by methylthiolation followed the protocol of Buser et al. (1983).

Synthesis. To synthesize (*Z*10)- and (*E*10)-tetradecenylacetate (*Z*10-14:Ac, *E*10-14:Ac), the tetrahydropyranyl derivative of commercially available 10-undecynol (Lancaster) was deprotonated using butyllithium and chain elongated with 1-iodopropane (Brandsma, 1988). Lindlar-hydrogenation of the protected 10-tetradecynol, deprotection, and acetylation with acetic anhydride gave a 90% overall yield of *Z*10-14:Ac with a chemical purity of 97% (ca. 3% of the saturated compound) containing less than 1% of the *E*-isomer. Deprotection of the protected 10-tetradecynol, reduction of the triple bond with lithium aluminate-trahydride (Rossi and Carpita, 1977), followed by acetylation, gave an 80% yield of *E*10-14:Ac, with 98% chemical purity, containing less than 2% of the *Z*-isomer.

To confirm the synthesis, 1H NMR-spectra were recorded with a Bruker AMX 400 (Karlsruhe, Germany); chemical shifts δ are given in [ppm] relative to tetramethylsilane (internal standard, 0 ppm)

1H-NR (400 MHz, $CDCl_3$): $\delta = 0.87$ (t, 3H, H-14); 1.23–1.37 (bm, 14H, $-CH_2-$); 1.55–1.63 (m, 2H, $-CH_2-$); 1.98 (m, 4H, H-9, HJ-12); 2.01 (s, 3H, O=C $-CH_3$); 4.02 (t, 2H, H-1); 5.33 (m, 2H, H-10, H-11).

1H-NMR (400 MHz, $CDCl_3$): $\delta = 0.86$ (t, 3H, H-14); 1.25–1.40 (m, 14H, $-CH_2-$); 1.59 (m, 2H, $-CH_2-$); 1.90–1.96 (m, 4H, H-10, H-11); 2.02 (s, 3H, O=C $-CH_3$); 4.02 (t, 2H, H-1); 5.31–5.41 (m, 2H, H-10, H-11).

Field Tests. Grey rubber septa were loaded with hexane solutions (1 μg in 10 μl) of either *Z*10-14:Ac or *E*10-14:Ac, as well as different ratios of the two compounds. The caps were fixed to a cardboard strip (8 $\times$ 3 cm) and placed in Delta traps made of transparent PVC foil and a PVC insert covered with Tanglefoot (USA) insect glue. Traps were set out at three different sites in Sofia and installed at about 2 m above the ground on branches of *P. orientalis* trees. At each site, there were at least three replicates of each treatment, together with empty traps as controls. Traps were checked at least twice a week. The number of males captured per sampling interval in the different treatments was compared after $\log(x + 1)$ data transformation, using ANOVA followed by Duncan's New Multiple Range Test.

RESULTS AND DISCUSSION

Coupled GC-EAG investigations on *P. platani* male antennae found one electrophysiologically active component in the female gland extract (Figure 1). On the HP-WAX column, when co-injected with a solution of alkanes, the compound eluted (Kovats Index 2166.6) between C21 and C22, and on GC-MS showed a mass spectrum identical to that of (*Z*10)-tetradecenyl acetate (Mozuraitis et al., 1997). Treatment of the crude extract with dimethyl disulfide (Buser et al., 1983) yielded a bis(thiomethyl) derivative of 10-tetradecenyl acetate ($M^+ = 348$, 6%). The mass spectrum of this compound showed an abundant signal at m/z 103 (21%, CH_3-CH_2-CH_2-$CHSMe^+$) and the complementary diagnostic fragment at m/z 245

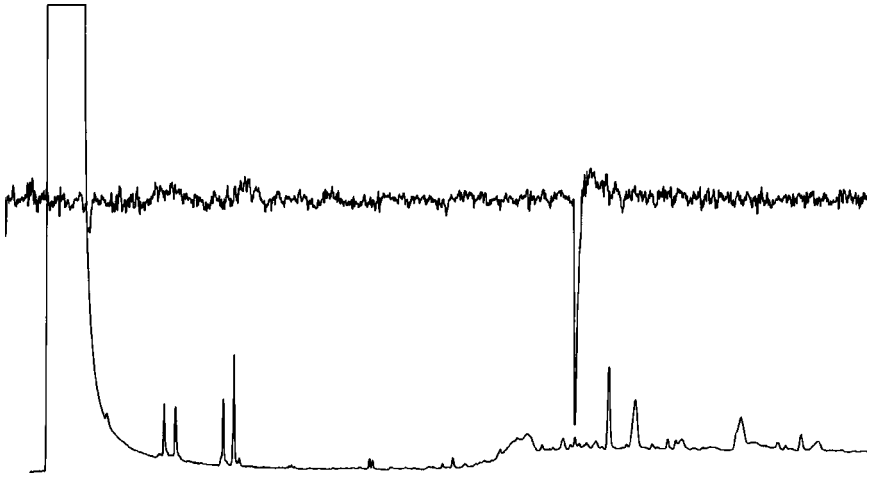

FIG. 1. Coupled GC-EAG on *Phyllonorycter platani* male antenna. Lower trace: GC of female gland extract; upper trace: EAG response.

(24%, CH_3-CO_2-$(CH_2)_9$-$CHSMe^+$), which proved that the double bond was retained at position 10. With FFAP as the stationary phase, the non-derivatized *Z*10-14:Ac coeluted with the EAG-active natural product (Ret. Index 2138,5), while *E*10-14:Ac eluted earlier (Ret. Index 2120,5).

Field tests, carried out during the end of the third generation at places of relatively low population densities, confirmed *Z*10-14:Ac to be the sex pheromone, as traps baited with *Z*10-14:Ac caught significantly more males than the controls or *E*10-14:Ac (Table 1). Furthermore, the presence of *E*10-14:Ac significantly

TABLE 1. *Phyllonorycter platani* MALES (MEAN ± SE OF UNTRANSFORMED DATA) CAPTURED PER SAMPLING INTERVAL IN TRAPS BAITED WITH DIFFERENT RATIOS OF *Z*10-14:AC AND *E*10-14:AC IN SOFIA.

Compound, μg *Z*:*E*	I 28/8–30/9, 2002 3 Replicates	II 27/8–4/9, 2002 3 Replicates	III 2/9–13/9, 2002 4 Replicates	IV 6/9–23/9, 2002 3 Replicates
100:0	3.1 ± 0.7a	5.8 ± 1.2a	1.9 ± 0.7a	2.8 ± 0.7a
100:10	—	—	—	0.7 ± 0.1b
100:50	—	—	—	0.3 ± 0.1c
100:100	1.4b ± 0.4b	1.9 ± 0.4b	0.4 ± 0.2b	—
0:100	0.1 ± 0.06c	0.2 ± 0.1c	0.1 ± 0.1b	—
control	0.04 ± 0.04c	0.2 ± 0.1c	0b	0.1 ± 0.1c

Note: Values within a column followed by the same letter are not significantly different at $P = 0.05$ Duncan's NMRT. **I**: Higher Forest Institute; **II**: Sitnjakovo; **III**: Borisova Gradina Park; **IV**: Sitnjakovo.

reduced the attractiveness of *Z*10-14:Ac, with an antagonistic effect even when present at only 10% (Table 1). Our field data suggest that traps baited with 100 μg of *Z*10-14:Ac would be effective for monitoring *P. platani* populations, but that contamination by *E*10-14:Ac must be avoided.

The pheromones of only a few *Phyllonorycter* species have been identified (Mozuraitis et al., 1997, 2000; Ferrao et al., 1998). *Z*10-14:Ac has been know as an attractant or pheromone (component) in other *Phyllonorycter* species but also as a pheromone in some tortricid species (Arn et al., 1992). (*Z*10,*Z*12)-Tetradecenyl acetate a pheromone of *P. crataegella* strongly reduces response of *P. mespilella* males to its female produced sex pheromone, (Ferrao et al., 1998). Mutual antagonism caused by pheromone components, including (*Z*10)- and (*E*10)-tetradecenyl acetate has been described for *P. acerifoliella*, *P. heegerella*, and *P. ulmifoliella* (Mozuraitis et al., 2000), and may play a role in reproductive isolation of related species. Interestingly, our findings on *P. platani*, including the inhibition by the geometric isomer, are the same as those reported for *P. ulmifoliella* (Mozuraitis et al., 1997), although these species exploit different host plants.

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INTRASPECIFIC AND INTERSPECIFIC INTERACTIONS MEDIATED BY A PHYTOTOXIN, (−)-CATECHIN, SECRETED BY THE ROOTS OF *Centaurea maculosa* (SPOTTED KNAPWEED)

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Abstract—*Centarea maculosa* Lam. (spotted knapweed) is one of the most destructive invasive weeds in the western United States, particularly in pastures and rangelands. One of the components that may contribute to its invasiveness is the naturally produced, root-secreted allelochemical (−)-catechin. This compound has been shown to have broad-spectrum phytotoxic activity, possibly assisting *C. maculosa* in displacing native plant communities. As a recently characterized phytochemical, little is known about the specific effect of (−)-catechin on either *C. maculosa* or other plant species. We have found that, *in vitro*, *C. maculosa* begins to secrete phytotoxic levels of (−)-catechin within 2–3 weeks of seedling emergence. Furthermore, (−)-catechin concentrations consistent with those naturally secreted by *C. maculosa* were sufficient to inhibit germination in all species tested, including *C. maculosa*. These concentrations were also often either phytotoxic or growth inhibitory to seedlings in a range of plant species, while having no negative effects on the growth of *C. maculosa* seedlings. However, our results also indicate that different levels of resistance and susceptibility to (−)-catechin exist in plant populations, suggesting that the capability of *C. maculosa* to invade an area through allelochemistry may be dependent on the age and species composition of plants in that area.

Key Words—Centaurea maculosa, allelopathy, (−)-catechin.

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INTRODUCTION

Exotic plants are a major economic problem and threat to ecological diversity throughout the world. Many invasive species are uncommon in their native range, but become abundant in their new habitats and competitively exclude natives (Louda et al., 1990). There are several factors involved in determining the invasive potential of nonnative plant species. The lack of regulation by natural predators, pathogens, and competitors leading to an increase in distribution and abundance of the exotic species is one commonly accepted mechanism for invasion (Williamson, 1996). Another hypothesis argues that the fast relative growth rates of invasive plants allow for more effective resource competition (Pattison et al., 1998; Grotkopp et al., 2002). These hypotheses have been credited as major determining factors in the ability of an exotic plant species to displace native plant communities. Another mechanism contributing to invasiveness, although not as widely accepted, is the chemical suppression of competing plant species through allelopathy. Some plant species release allelopathic chemicals into their surroundings that may have deleterious or even deadly effects on neighboring plants (reviewed in Whittaker and Feeney, 1971), thus allowing the allelopathic plant less impeded access to natural resources.

One highly invasive weed in much of the western United States is spotted knapweed (*Centaurea maculosa* Lam.), a Eurasian native. Areas that have been invaded by *C. maculosa* are characterized by dense stands of the invading plant and a nearly complete competitive exclusion of native plant species (Callaway et al., 1999). When the first *C. maculosa* plants were found in Montana in the 1920s, no one recognized their invasive potential. Now Montana has about 5 million acres infested with this species and another 29 million acres that are vulnerable to its continued spread (Western Governor's Association, 2000). Many of the studies pertaining to *C. maculosa*'s invasive behavior have focused on resource competition as the major cause for displacement of native plants. Jacobs and Sheley (1999) suggest that spatial and temporal similarities among forbs places *C. maculosa* in direct competition with many indigenous forbs. Seedling studies indicate that *C. maculosa* is able to outcompete and displace indigenous perennial grasses (Sheley and Jacobs, 1997; Velagala et al., 1997), and Kedzie-Webb et al. (2001) showed that indigenous perennial grass cover and species diversity in a *Pseudoroegneria spicatum*/*Festuca idahoensis* habitat were inversely related to *C. maculosa* cover. The invasive success of *C. maculosa* has also been attributed to its rapid growth rate, prolific seed production, and high biomass (Watson and Renney, 1974; Sheley et al., 1998). Recently, new findings provide strong evidence that allelopathy may also play a role in the ability of this plant to successfully disrupt native communities (Ridenour and Callaway, 2001; Bais et al., 2002).

Although a study as early as 1832 suggested allelopathy as the displacing mechanism for *C. maculosa* (De Candolle, 1832), and at least one ecological

study has been conducted to determine the importance of allelopathy in the invasiveness of *C. maculosa* (Ridenour and Callaway, 2001), it was not until recently that a phytotoxic, root-secreted compound was isolated and identified. Bais et al. (2002, 2003) determined that root exudates of *C. maculosa* inhibit germination and cause wilting, lack of root and shoot differentiation, and eventually mortality in seedlings of a broad range of plant species, including *Arabidopsis thaliana*, several noxious weeds, and common crops. Through testing of fractions obtained by HPLC analysis, they discovered that the active fraction of the exudate was composed of racemic catechin, a 50:50 mixture of (+)-catechin and (−)-catechin. Further separation of the enantiomers proved that (−)-catechin was solely responsible for phytotoxic activity, while (+)-catechin provided antimicrobial properties. Bais et al. (2002) have also shown racemic catechin to be present in soil samples collected from spotted knapweed-invaded fields, linking their *in vitro* studies to invasiveness in the field.

Although the ecological significance of allelopathy is still in question, several plants have been shown to produce chemicals displaying allelopathic activity under controlled conditions. However, rather than examining the link between allelopathy and plant invasions, much of our research has focused on allelochemicals produced by agricultural crops that can be used as a potential means to suppress weeds. Phytotoxic substances from the root exudates and extracts of *Cucumis sativus* have been shown to inhibit the growth of other *C. sativus* plants (Yu and Matsui, 1994; Yu et al., 2000), as well as exhibiting more detrimental effects by influencing membrane peroxidation, ion uptake, stomatal opening, and photosynthesis (Yu et al., 2002). Root exudates of rice (*Oryza sativa*) are inhibitory to the growth of cress and lettuce (Kato-Noguchi et al., 2002), and several compounds from root exudates of buckwheat (*Fagopyrum esculentum*) reduce weed biomass (Iqbal et al., 2002). Sundiversifolide, a compound identified from seed exudates of sunflower (*Helianthus annum*), showed phytogrowth inhibitory activity against the weed cats-eye (*Euphoria malaiense*) (Ohno et al., 2001).

In this paper, we explore the intra- and interspecific effects that (−)-catechin has on germination and seedling growth. We plotted a 5-week growth curve to determine how the concentration of (−)-catechin present in media extracts varies with the age of the secreting *C. maculosa* plant. Additionally, *in vitro* tests were used to determine the effects of varying concentrations of (−)-catechin on germination and growth of several different plant species and on *C. maculosa* itself.

METHODS AND MATERIALS

Plant Culture Conditions. Seeds of *Centaurea maculosa* and *Gaillardia aristata* were obtained from natural populations in Missoula, Montana. Seeds of *Gaillardia grandiflora*, *Lobelia erinus*, *Papaver orientale*, and *Stipa tenuissima*

were obtained from Botanical Interests, Inc. (Broomfield, CO), and *Digitalis purpurea*, *Matricaria recutita*, and *Physalis alkekengi* were purchased from W. Atlee Burpee & Co. (Warminster, PA). The native grasses *Festuca idahoensis* and *Koelaria cristata* were purchased from Wind River Seed (Manderson, WY). Seeds were washed in running tap water and surface-sterilized using sodium hypochlorite (0.3% v v^{-1}) for 30 min, followed by 3–4 washes in sterile distilled water. Surface-sterilized seeds were inoculated on static MS (Murashige and Skoog, 1962) basal media in petri dishes for germination. Seeds were allowed to germinate for 10 days after roots and shoots emerged. The temperature within the growth chamber was $25 \pm 2^{\circ}$C and light intensity was 4.41 J m^{-2} s^{-1}, with a 16:8 L:D photoperiod. Ten-day-old seedlings were transferred to sterilize 50-ml culture tubes with 5 ml of liquid MS basal media or 12-well plates with 2 ml of liquid MS basal media depending on the size and physiological characteristics of the species. Plants were then placed at $25 \pm 2^{\circ}$C on an orbital platform shaker set at 90 rpm and exposed to 16 hr daily of 4.41 J m^{-2} s^{-1} light intensity and allowed to stabilize in the liquid media for 5 days prior to treatment with (−)-catechin. (−)-Catechin was obtained from Sigma Co. (St. Louis, USA), and all other chemicals and solvents were of analytical and HPLC grade.

Plant Growth Inhibitory Bioassays with (−)-Catechin. A stock solution of 10 mg ml^{-1} of (−)-catechin was prepared by dissolving (−)-catechin in 50 μl of methanol per 10 mg of (−)-catechin and adjusted to a final volume with sterile H_2O. To ensure that the (−)-catechin retained activity, fresh stock was prepared prior to each experiment. The solution was filter-sterilized and added to the liquid media containing the various plant species at concentrations of 10, 50, 150, 250, and 400 μg ml^{-1}. *C. maculosa* plants were treated with 50, 100, 200, 400, 600 μg ml^{-1}, 1 μg ml^{-1}, and 2 mg $ml^{-1}$of the (−)-catechin solution. Control plants were treated with a filter-sterilized solution of methanol and sterile H_2O in proportions equivalent to the (−)-catechin solution, and added at the same volume as the highest concentration of treated plants. Plant cultures were maintained at $25 \pm 2^{\circ}$C on an orbital platform shaker set at 90 rpm and exposed to 4.41 J m^{-2} s^{-1} light intensity for 7 days. Seven days posttreatment, root, shoot length, and fresh weight were recorded, and all plants were scored for mortality. Plants were grouped on the basis of minimum concentration of (−)- catechin required for mortality. Plants were categorized as susceptible ($\leq$50 μg ml^{-1}), moderately resistant (150 μg ml^{-1}), and highly resistant (400 μg ml^{-1}).

Growth Curve Association of (−)-Catechin Exudation. C. maculosa seeds were surface sterilized and germinated as described above. Ten-day-old seedlings were transferred to sterile 50-ml culture tubes containing 10 ml of MS basal media. Twenty *C. maculosa* cultures were incubated and maintained as described. Each week four plants were removed, and the root and shoot length and the fresh weight were recorded. Root exudates were extracted from the media and (−)-catechin was identified and quantified using HPLC as described below.

Extraction. Root exudates from *C. maculosa* plants used for the 5-week growth curve were extracted using 5 ml of hexane (Fisher Co.). Extracts were vortexed and stored for 24 hr at 30°C. The supernatant was transferred with a Pasteur pipette to a separate test tube, and concentrated under N_2. The concentrated powder was resuspended in 200 μl of absolute methanol (Fisher Co.) for HPLC analyses.

HPLC Analysis. Compounds in the root exudates were chromatographed by gradient elution on a reverse phase 5 μm, C_{18} column (25 cm × 4.6 mm) (Supelco Co). The chromatographic system (Summit Dionex) consisted of P_{580} pumps (Dionex) connected to an ASI-100 Automated Sample Injector (Dionex Co). The visible absorbance at 210 nm was measured by a PDA-100 Photodiode array variable UV/VIS detector (Dionex). Mobile phase Solution A consisted of double distilled water and Solution B (acetonitrile) (Fisher Co). A multistep gradient was used for all separations with an initial injection volume of 15 μl and a flow rate of 1 ml min^{-1}. The multistep gradient was as follows: 0–5 min 5.0% B, 5–10 min 20.0% B, 15–20 min 20.0% B, 20–40 min 80.0% B, 40–60 min 100% B, 60–70 min 100% B, 70–80 min 5.0% B.

Germination Inhibitory Assays. Assays were conducted to determine the inhibitory effects of (−)-catechin on the germination of four plant species, chosen because of their varying degree of susceptibility to (−)-catechin as noted in *in vitro* assays of 15-day-old seedlings. The plant species chosen were *C. maculosa* (self), *M. recutita* (highly resistant), *G. aristata* (moderately resistant), and *K. cristata* (susceptible). Static MS basal media was added to one compartment of partitioned petri dishes (VWR) to serve as a control. A filter-sterilized solution of (−)-catechin was added, in concentrations matching those used for the corresponding seedling studies, to unsolidified MS basal media and used to fill the remaining three compartments of the plate. Three surface-sterilized seeds were placed in each compartment, and there were four replicates/treatment. Plates were placed in a growth chamber under previously described conditions. Total numbers of germinated seeds were recorded every 5 days for 20 days. Germination in *C. maculosa* was monitored for 30 days because of a slower germination rate.

RESULTS

Growth Curve Association of (−)-Catechin Exudation. *C. maculosa* plants were grown in liquid media for 1–5 weeks. At the end of each time period, plants were measured for growth (Figure 1A) and the (−)-catechin present in the media (Figure 1B), root, and shoot, extracts was quantified. *In vitro*, plants grown in 5 ml of MS basal media for 4 and 5 weeks began to show signs of declining health. These plants displayed necrotic shoots and swelling or tip death in the roots. The amount of (−)-catechin secreted from the roots of *C. maculosa* plants

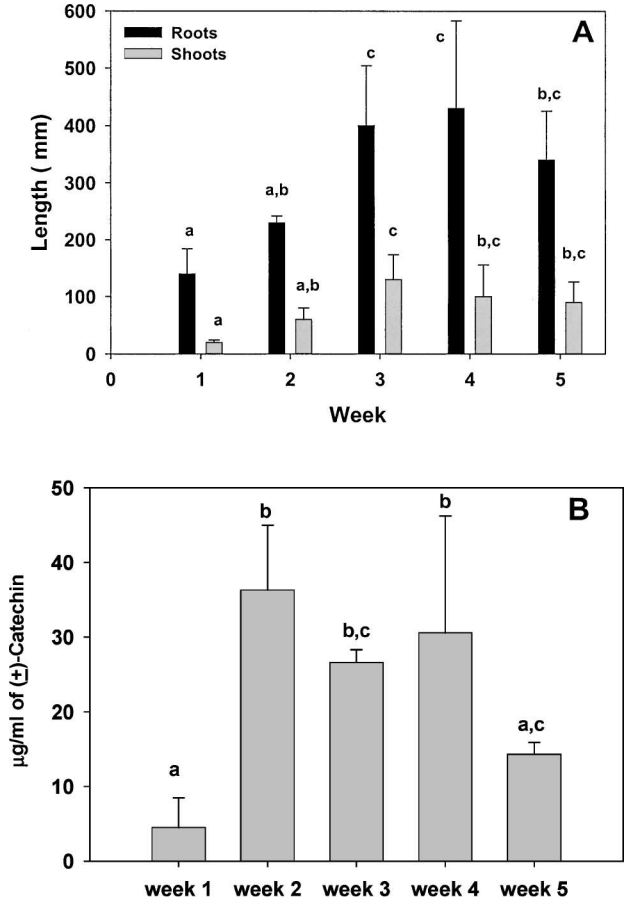

FIG. 1. (A) Mean root and shoot growth (mm) of replicate *Centaurea maculosa* plants grown *in vitro* plotted over a 5-week time course. (B) The amount of catechin (μg/ml) present in hexane-partitioned media extracts containing root exudates of *C. maculosa* were quantified by HPLC analysis. Error bars show +1 SD. Significantly different means are denoted by different letters; ANOVA, $P < 0.05$.

varied with the age and health of the plants, with actively growing plants producing more (−)-catechin than declining plants. We also determined that (−)-catechin is found in *C. maculosa* root exudates but is not found in significant quantities in the roots or shoots of the plant (Bais et al., 2002; data not shown). Overall root length consistently increased in the first 4 weeks of the experiment; however, plants grown in liquid media for 5 weeks showed a shorter average root length (ANOVA,

$F = 6.54$; df = 4, 19; $P = 0.003$). Shoot growth followed a similar pattern with slight declines in overall shoot length beginning to appear in plants grown in liquid media for 4 weeks (ANOVA, $F = 4.82$; df = 4, 19; $P = 0.011$). The (−)-catechin present in the plant root exudates, as determined by HPLC analysis, increased significantly from I to II week after plants were transferred to liquid media. There was less (−)-catechin present in media extracts of plants grown in liquid media for 5 weeks, which corresponded with the overall decline in growth and fitness of these plants (ANOVA, $F = 5.00$; df = 4, 19; $P = 0.009$). There was also considerable variability in the amount of (−)-catechin present in media extracts of replicate plants, with the greatest difference (190 μg ml^{-1}) occurring within the IV week replicates. This indicates that factors other than the age of the plant are involved in determining (−)-catechin secretion. Under natural conditions we have also seen that in the rhizosphere of *C. maculosa,* a range of (−)-catechin concentrations can occur (195 ± 14 to 146 ± 9 μg/g soil) (Bais et al., 2002, 2003, in press). There are several potential reasons that may explain the described variation in (−)-catechin. A gradual breakdown of (−)-catechin over time (catechin is photosensitive) and phenotypic variation in catechin production are both possible explanations.

Inhibition of Growth and Germination of C. maculosa Treated with (−)-Catechin. Bais et al. (2002) reported that (−)-catechin was phytotoxic to a number of plant species at 50 μg ml^{-1} but that it had no effect on *C. maculosa* at this concentration. To determine if *C. maculosa* had a threshold level of resistance to (−)-catechin, we tested seven different concentrations of (−)-catechin (50, 100, 200, 400, 600 μg ml^{-1}, 1 mg ml^{-1}, and 2 mg ml^{-1}) on 10-day-old seedlings (Figure 2) and germinating seeds of *C. maculosa* (Figure 3). There were no significant effects on the root (ANOVA, $F = 2.07$; df = 7, 23; $P = 0.109$), or shoot growth (ANOVA, $F = 0.823$; df = 7, 23; $P = 0.582$) of *C. maculosa* in response to any of the concentrations of (−)-catechin 7 days posttreatment. Physiological symptoms such as necrosis of the leaves and discoloration of the roots were seen at the 600 μg ml^{-1} concentration; however, plants were still viable after 7 days. Treatments with 1 mg ml^{-1} of (−)-catechin and higher caused mortality in *C. maculosa* seedlings after 7 days. Concentrations of (−)-catechin that caused mortality after 7 days were much higher than the concentrations that were found in the media extracts of *C. maculosa* plants, indicating that the natural secretion of (−)-catechin by the plant generally occurs at levels that are not autotoxic.

Plates of solid MS media infused with the same seven concentrations of (−)-catechin were used to determine the effect on germinating seeds of *C. maculosa*. Only 40% of control seeds germinated over a period of 30 days. Significant inhibition of germination (ANOVA, $F = 3.78$; df = 7, 34; $P = 0.006$), in comparison to seeds grown on media free of (−)-catechin, was noted in (−)-catechin concentrations of 400 μg ml^{-1} and higher (Figure 3). Inhibitory concentrations of (−)-catechin were higher than those found in media extracts (Figure 1B), and soil from *C. maculosa* invaded fields (Bais et al., 2002, 2003, in press) suggesting that

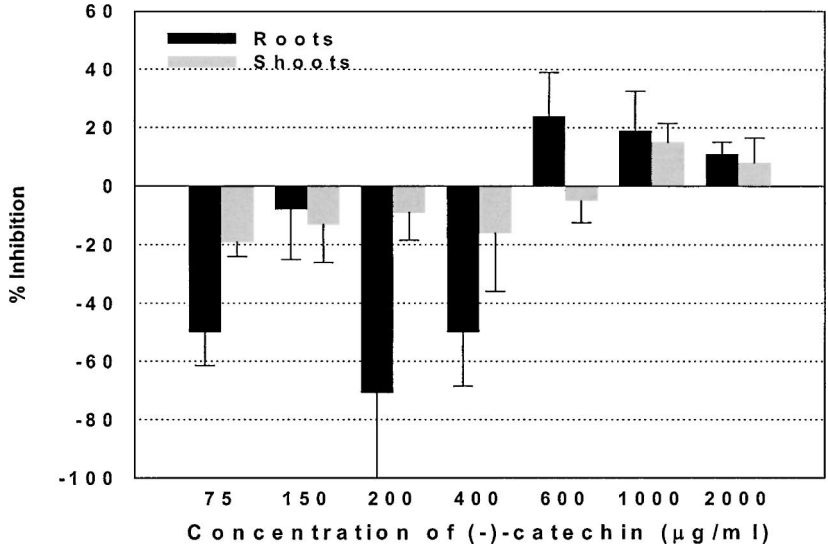

FIG. 2. *Centaurea maculosa* seedlings treated with seven different concentrations of (−)-catechin and measured for root and shoot growth after 7 days. This figure represents the mean percent inhibition relative to untreated control plants. Error bars show +1 SE. Root and shoot growth was not significantly enhanced or inhibited by the addition of (−)-catechin; although, mortality was noted at concentrations of 1 mg ml^{-1} and higher.

germination of *C. maculosa* seeds in the field is probably only inhibited by the presence of a number of (−)-catechin secreting *C. maculosa* plants.

Interspecific Plant Growth Inhibitory Bioassays with (−)-Catechin. Although all of the plants tested were affected by the presence of (−)-catechin to some degree, the 10 plant species that were treated with (−)-catechin were separated into three groups based on mortality: highly resistant, moderately resistant, and susceptible (Table 1). Half of the plants tested were susceptible, with mortality occurring at 50 μg ml^{-1} of (−)-catechin. *Digitalis purpurea* and *Papaver orientale* showed both root burning and necrosis of the shoots (10–50 μg ml^{-1}); however, the three grasses, *Festuca idahoensis, Stipa tenuissima*, and *Koeleria cristata* showed necrosis primarily in the root system. *D. purpurea*, *F. idahoensis*, and *S. tenuissima* also showed significant decreases in root growth (Figure 4A and B) over the 7-day time course (ANOVA, $F = 3.63$; df = 4, 23; $P = 0.019$; $F = 4.60$; df = 4, 23; $P = 0.007$; $F = 3.88$; df = 4, 23; $P = 0.015$), although none of the susceptible plants showed significant decreases in shoot growth. Three plants were classified as moderately resistant: *Physalis alkekengi, Gaillardia aristata*, and *Lobelia erinus*. Only *L. erinus* showed significant inhibition of root growth (ANOVA, $F = 9.45$; df = 4, 23; $P < 0.001$), however, mortality was observed at 150 μg ml^{-1} for all

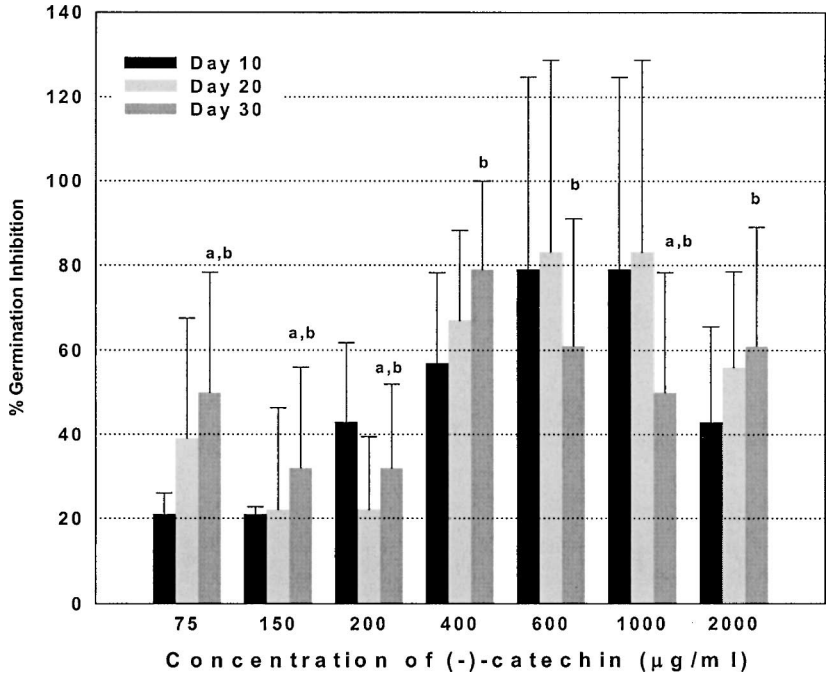

FIG. 3. *Centaurea maculosa* seeds germinated on plates containing solidified MS basal media with seven different concentrations of (−)-catechin incorporated. Germinated seedlings were counted every 10 days for 1 month. Mean germination rates of *C. maculosa* seeds for each concentration and time period are displayed as percent inhibition relative to control plants germinated on plates containing no (−)-catechin. Error bars show +1 SD. Significantly different means are denoted by different letters; ANOVA, $P < 0.05$.

three species. Two of the moderately resistant plants, *G. aristata* and *L. erinus*, actually showed significant increases in shoot growth at lower concentrations of (−)-catechin (ANOVA, $F = 3.34$; df = 4, 23: $P = 0.026$; $F = 4.3$; df = 4, 23; $P = 0.009$) (Figure 4C and D). Visual observations revealed that nonfatal concentrations of (−)-catechin (10–50 μg ml^{-1}) made the leaves and stems of *G. aristata* plants thinner and more elongated in appearance. Also, all three species of plants designated as moderately resistant produced aerial roots to some extent. This may have allowed the plants to survive longer in culture with toxic amounts of (−)-catechin present. Finally, two of the plants tested were considered to be highly resistant to (−)-catechin treatments after 7 days. No significant inhibition of root growth in chamomile, *Matricaria recutita* (ANOVA; $F = 0.99$; df = 4, 23; $P = 0.451$) was noted. No *M. recutita* plants showed mortality after 7 days, regardless of the (−)-catechin concentration. In fact, there were no symptoms such

TABLE 1. TEN DIFFERENT PLANT SPECIES WERE TREATED WITH FIVE CONCENTRATIONS OF (−)-CATECHIN

Plant Species	Minimun mortality concentration (μg/ml)	% Mortality
Susceptible		
Digitalis purpurea	50	25
Festuca idahoensis	50	100
Koelaria cristata	50	100
Papavar orientale	50	75
Stipa tenuissima	50	75
Moderately resistant		
Gaillardia aristata	150	75
Lobelia erinus	150	100
Physalis alkekengi	150	25
Lightly resistant		
Gaillardia grandiflora	400	50
Matricaria recutita	No mortality	0

Note: On the basis of the minimum concentration required for mortality, the plants were separated into categories of susceptible, moderately resistant and highly resistant.

as root tip burning or shoot necrosis. The second highly resistant plant, *Gaillardia grandiflora*, showed no significant inhibition of root growth (ANOVA, $F = 1.03$; df $= 4, 23$; $P = 0.431$), however, mortality was observed in 50% of the plants at a (−)-catechin concentration of 400 μg ml^{-1}. At 50 μ gml^{-1} of (−)-catechin and higher, root damage was visible as a darkening of the roots, but in most cases newly formed undamaged lateral roots were obvious.

Germination Inhibition of a Susceptible, Moderately Resistant, and Highly Resistant Plant Species. The five concentrations used to treat 10-day-old seedlings were also used to determine inhibition of germination rates in seeds of three plant species, *K. cristata* (susceptible), *G. aristata* (moderately resistant), and *M. recutita* (highly resistant). After 20 days, the levels of inhibition of germination reflected levels of seedling resistance determined in the previous experiment. *K. cristata* showed significant inhibition of germination beginning at 50 μg ml^{-1}, the mortal concentration in seedlings (Figure 5A) (ANOVA, $F = 42.14$; df $= 5, 24$; $P < 0.001$). At (−)-catechin concentrations of 250 μg ml^{-1} or more, there was a nearly 100% inhibition of germination for this plant. Microscopic inspection of the ungerminated seeds showed that the embryo was killed prior to seedling emergence at these concentrations (data not shown). At all concentrations, seedlings died within a few days of emergence. *G. aristata*, which showed some seedling resistance, also showed significant germination inhibition (Figure 5B) (ANOVA, $F = 17.08$; df $= 5, 24$; $P < 0.001$). An interesting effect

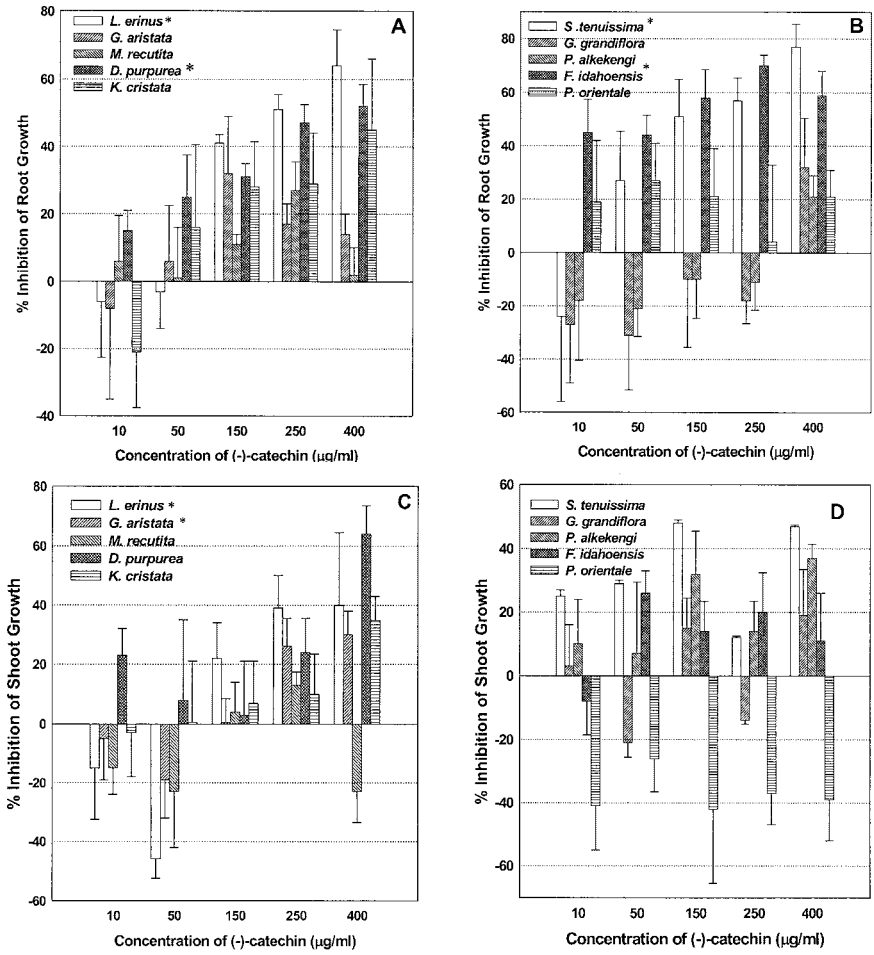

FIG. 4. The mean percent of root inhibition (A, B) and shoot inhibition (C, D) of plants treated with (−)-catechin relative to methanol treated control plants. Error bars show +1 SE. Treated plants showing significant differences in mean root and/or shoot growth are denoted with an asterisk; ANOVA, $P < 0.05$.

of the lower concentrations (10–50 μg ml^{-1}) of (−)-catechin on emerging seedlings of *G. aristata* was callus formation in the roots (Figure 5C). At (−)-catechin concentrations of 150 μg ml^{-1} and higher, seedlings died shortly after emergence, but plants with callused root systems were alive and continued to grow through the duration of the experiment. Finally, (−)- catechin had the least effect on germination of *M. recutita* (Figure 5D). There was no significant inhibition noted

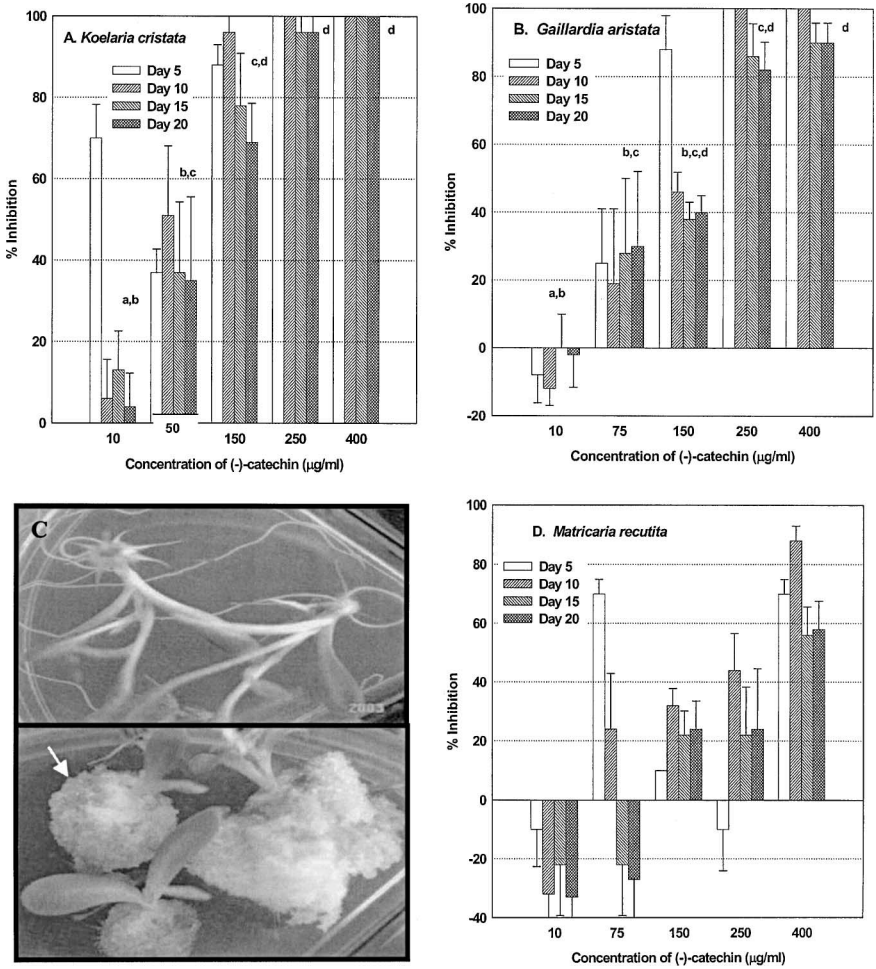

FIG. 5. Seeds of one plant from each category (susceptible, moderately resistant, and highly resistant) were germinated on solidified MS basal media supplemented with (−)-catechin. Mean germination rates of the seeds, for each concentration and time period, are displayed as percent inhibition relative to control plants grown on MS basal media with no (−)-catechin incorporated. (A) *Koelaria cristata*, (B) *Gaillardia aristata*, (C) roots of *G. aristata* control plants compared to callused roots germinated on media containing 10 μg ml^{-1} of (−)-catechin, (D) *Matricaria recutita*. Error bars show +1 SD. Treated plants showing significant differences in mean germination inhibition are denoted with different letters; ANOVA, $P < 0.05$.

at any of the concentrations of (−)-catechin (ANOVA, $F = 2.01$; df $= 5, 24$; $P = 0.124$). However, similar to the susceptible plant species, none of the seedlings that emerged were able to survive, suggesting that the ability to resist (−)-catechin is dependent on the age of the plant.

DISCUSSION

Many plants produce compounds that provide a useful function in their interaction with their environments (Paiva, 2000). *C. maculosa* provides an example of how a root-exuded allelochemical may provide a competitive advantage to the plant by inhibiting the growth of a broad range of plant species, as well as modulating its own growth, facilitating its ability to invade an area. Like most invasive weed species, *C. maculosa* tends to invade and become established in disturbed ecosystems. One hypothesis explaining this tendency is that areas of poor species diversity result in the incomplete use of limiting resources, increasing the invasive ability of aggressive species (McNaughton, 1993; Robinson et al., 1995; Tilman, 1997). Although this may certainly be true in the case of *C. maculosa,* the effectiveness of the root-secreted phytotoxin, (−)-catechin, at inhibiting the germination and growth of a variety of plant seedlings most likely contributes to its invasiveness. Our studies show that seedlings of *C. maculosa* begin secreting growth-inhibiting levels of (−)-catechin within 2–3 weeks of emergence. This effectively produces a "ring of death" around *C. maculosa* seedlings, allowing them uninhibited access to available water and nutrients in the soil.

In addition to the interspecific effects, we observed inhibition of *C. maculosa* seed germination in the presence of (−)-catechin, suggesting a mechanism by which *C. maculosa* plants avoid intraspecific competition in areas already saturated by *C. maculosa*. Artificially buried seeds of *C. maculosa* showed an average *in situ* germination rate of 51% after 8 years, and 90% of the ungerminated seeds that were recovered remained viable (Davis et al., 1993). Davis et al. (1993) hypothesized that this slow germination and long-term seed viability may be influenced by the availability of water for imbibition, partially explaining why *C. maculosa* is so adapted to well-drained soils where seeds do not imbibe rapidly and respond prematurely to short-term germination opportunities. Our results indicate that the presence of (−)-catechin in the soil may also play a role in modulating growth, germination, and flowering in *C. maculosa*. Observed spatial segregation of mature *C. maculosa* plants suggest that successful germination requires soil where less (−)-catechin is present. Furthermore, in garden experiments, a large percentage (up to 93%) of *C. maculosa* plants bolt after 1 year (Schirman, 1981); however, experiments done in natural *C. maculosa* populations showed none of the 1-year plants bolting, and only 10% of the 2-year-old plants bolted (Story et al., 2001). This delayed maturation observed under natural field conditions may also be an

effect by of (−)-catechin present in the soil of a well-established *C. maculosa* site that does not occur under controlled conditions where there would be no residual (−)-catechin in the soil.

It is known that some compounds act as plant growth regulators, exhibiting hormesis, or concentration-dependent stimulatory or inhibitory effects on seedling growth (Einhellig et al., 1983). Demuner et al. (1996) discovered that two diterpenes isolated from seed oils of *P. polygalaeflorus* stimulated radicular growth in *C. sativus* at 100 ppm, but had a significant inhibitory effect at 1000 ppm. Root-secreted (−)-catechin has a similar effect on some of the plants that we tested, showing increased growth of shoots at lower concentrations of (−)-catechin. In addition, root callus formation visible when *G. aristata* seeds were germinated on media treated with 10–50 μg ml^{-1} of (−)-catechin indicate that this phytochemical can act as a plant growth regulator.

Finally, we discovered that there is a species and age-dependent gradient of plant susceptibility to (−)-catechin. While many of the seedlings that we tested succumbed to (−)-catechin *in vitro*, some plants appeared to resist the chemical to varying degrees. However, even very low concentrations of (−)-catechin either inhibited germination or caused mortality in newly emerged seedlings. The exact mechanism by which (−)-catechin kills the susceptible plants and by which resistant plants, including *C. maculosa*, evade (−)-catechin, will be the focus of follow-up work. It is tempting to speculate that some resistant plants might have adapted a counter root exudation process, possibly to detoxify (−)-catechin for better survival. We have seen that reactive oxygen species (ROS) play an important role in the initial signaling events leading (−)-catechin induced cell death (Bais et al., in press). However, application of ascorbic acid in combination with (−)-catechin blocked the ROS signal and the subsequent phytotoxicity in the roots of *Arabidopsis thaliana* and *Centaurea diffusa*. This result raises the possibility that plants that secrete high levels of organic acids, or other antioxidants, may be more resistant to (−)-catechin.

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PHYSIOLOGICAL ASSESSMENT AND PATH COEFFICIENT ANALYSIS TO IMPROVE EVALUATION OF ALFALFA AUTOTOXICITY

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Abstract—Reseeding of alfalfa is affected until autotoxic chemicals break down or are dispersed, often requiring a year or more. Bioassays of seed germination and early seedling growth, on agar medium in petri dishes, were conducted to evaluate autotoxic responses of 20 alfalfa germplasms to water-soluble extracts of alfalfa leaf tissue. Root length, 120 hr after placing imbibed seed on agar, was more sensitive to the autotoxin(s) than was hypocotyl length, germination speed, and final germination percentage. Path coefficient analyses showed variation in root length had 7–17 times more effect than variation in hypocotyl length in determining autotoxic effects on total seedling length. Although variations in seed size and germination rate were negatively associated ($P < 0.05$) with final root length, the autotoxin had little effect on these factors relative to that on root length. Germplasms in the control differed ($P < 0.05$) in root length, requiring tolerance to be evaluated as percent of control. Germplasms, as percent of control, differed significantly ($P < 0.05$) at extract concentrations of 1.0 and 4.0 g l^{-1}, but the range and LSD were more favorable for selection at 1.0 g l^{-1}. Root length is appropriate for genetic assessments of tolerance to the autotoxin when expressed as percent of control.

Key Words—Alfalfa, autotoxicity, aqueous extracts, bioassay, seed germination, early seedling growth, path coefficient analysis.

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INTRODUCTION

Autotoxicity occurs when a plant species releases chemical substances that inhibit or delay germination and growth of the same plant species (Putnam, 1985). Alfalfa (*Medicago sativa* L.) is known to contain water-soluble substance(s) that are autotoxic (Klein and Miller, 1980; Jensen et al., 1981; Li, 1981; Miller, 1983; Hall and Henderlong, 1989; Hedge and Miller, 1992a,b; Chung and Miller, 1995a,b).

When reseeding, or interseeding alfalfa to thicken an older stand, the soil will have accumulated autotoxic substances (Jennings and Nelson, 2002). Incorporation of residues of old plants into the soil also releases water-soluble inhibitory substances that can affect new seedlings (Miller, 1996). Miller (1983) reported that the best crop preceding alfalfa establishment is corn (*Zea mays* L.), followed by various grains and soybean (*Glycine max* L.); the most detrimental crop preceding alfalfa is alfalfa. Klein and Miller (1980) also indicated that alfalfa was difficult to establish without rotating to another crop first.

Several reports (Henderlong and Li, 1981; Jensen et al., 1981; Miller, 1983) on the basis of greenhouse, laboratory, and field evaluations have emphasized the need for an intervening crop between alfalfa stands. These reports contend that stand densities may be lower and yields may be reduced for several years when alfalfa is reestablished after itself without waiting a year (Jennings and Nelson, 2002). Interest in interplanting, double cropping, no-till planting, and nonrotational cropping systems increase the need to understand allelopathy (Duke et al., 2002), and in this case also autotoxicity.

Some researchers have evaluated management practices to reduce autotoxicity or to exploit allelopathy as an additional weed management strategy. One approach has been to utilize rotational crops or companion plants in annual or perennial cropping systems. Another is to develop cultivars of alfalfa that are tolerant or resistant to the autoallelochemicals (Klein and Miller, 1980; Miller, 1983). Genetic variation among alfalfa germplasms has been established (Chung and Miller, 1995b; Chon et al., 2000). A goal has been to screen for tolerant or resistant types in germplasm collections such that this character can be transferred into cultivars by either conventional breeding or genetic transfer techniques (Chung and Miller, 1995b; Miller, 1996).

Alfalfa exhibits differential autotoxic effects as influenced by experimental procedures and conditions (Chon et al., 2000; Chon and Nelson, 2001). The autotoxic chemical(s) is produced mainly in the leaves and flowers with lesser amounts in the roots, stems, and seeds (Miller, 1996), but the contributing chemical may differ among plant parts. Chon and Kim (2002) identified nine causative phenolic compounds as water-soluble allelochemicals that were extracted from leaves, stems, roots, and seed of alfalfa. An appropriate bioassay to distinguish the autotoxic factors and to separate their effects from those of competition or inherent

growth properties of alfalfa is needed to screen germplasms and to assess results from management practices in the field.

The usual response to the autotoxin is reduced root growth, but at higher concentrations it also reduces the rate and final percentage of germination (Chon et al., 2000). Thus, the responses are partly confounded, as a delay in germination results in a shorter time interval for root growth in conventional bioassays.

Cultivars with genetic tolerance to the autotoxic chemical(s) would have potential for interseeding into alfalfa or for reseeding shortly after killing an old stand. Miller (1992) suggested that the autotoxicity problem in alfalfa might be solved by selecting new cultivars that do not produce various allelochemicals or those that are resistant to these compounds. There is limited information available on breeding efforts in alfalfa for reducing autotoxicity or allelopathy (Goplen and Webster, 1969; Miller, 1983; Wyman-Simpson et al., 1991). Chung and Miller (1995b) have recently classified some alfalfa cultivars according to the tolerance of seedlings to allelochemicals extracted from alfalfa herbage, but to date there has been little success in field tests.

The objectives of this study were to (a) evaluate the effects of alfalfa autotoxin(s) on the sequential steps in germination and early seedling growth of alfalfa, and (b) test the relative effects of the autotoxin during each step. We compared characters of seed germination and seedling growth of 17 germplasms and 3 cultivars. This information should improve the bioassay sensitivity for alfalfa autotoxicity, provide a better understanding of the mechanisms of alfalfa allelopathy or autotoxicity, and give insight for evaluating the fate of the chemical(s) in the field.

METHODS AND MATERIALS

Preparation of Extracts. Three-year-old alfalfa (cv. Cody) plants were harvested at a vegetative stage from a field near West Plains, Missouri in November 1995 and oven dried at 40°C for 120 hr Dried tissue was separated into leaves (blades and petioles) and stems. Leaf samples were ground to pass a 1-mm screen and then stored at 2°C until used. Ground tissue (20 g) was extracted by soaking in 1 l deionized water for 24 hr at 24°C. Extracts were filtered through four layers of cheesecloth to remove fibrous debris, and centrifuged at 3000 rpm ($\times$2063 g) for 4 hr The supernatant was vacuum-filtered through a sheet of Whatman 42 paper (Whatman Co., Clifton, NJ). Extract concentrations are reported as gram per liter of dry leaf tissue (hereafter as g l^{-1}).

Medium. Bacto agar (Difco Laboratories, Detroit, MI) (16 g l^{-1}) was autoclaved for 30 min at 125°C and then equilibrated in a 50°C water bath along with a glass flask containing stock leaf extract and another containing sterile deionized water. An aliquot of stock extract (20 g l^{-1}) was diluted with the sterile water to

twice the test concentration desired and then mixed in a 1:1 ratio with the agar solution to give the final concentration. About 10 ml of extract–agar or water–agar (control) were poured into 9-cm-diam plastic petri dishes and allowed to solidify for 4 hr at room temperature.

General Bioassay. Seeds were surface-sterilized for 15 min in sodium hypochlorite (0.525 g l^{-1}), rinsed, imbibed for 12 hr in deionized water at 22°C, and blotted by placing them between layers of a folded paper towel for 30 min. Twenty swelled seeds were evenly placed onto the agar surface in each petri dish. The plates were covered, the edges sealed with parafilm, and placed into a growth chamber programmed at 24°C during the 14-hr light period and 22°C during the dark period. Plates were illuminated at 350 μmol $m^{-2}sec^{-1}$ photosynthetically active radiation (PAR) provided by incandescent and fluorescent lamps (Chon et al., 2000).

Cumulative germination was determined at 12-hr intervals by counting the number of seeds with a radicle 1-mm long over a defined period. Hypocotyl length, root length, and total length (hypocotyl plus root lengths) were measured on all seedlings in each petri dish at 120 hr after placing seed on the medium. The experiment had four replicates.

Effects of Leaf Extracts on Seed Germination. Twenty-five seeds from 20 alfalfa germplasms were imbibed in distilled water for 12 hr and placed onto the agar surface with extract concentrations of 1.0 and 4.0 g l^{-1}. Agar containing distilled water was used for the control. Four replicates were used in a randomized complete block design. Cumulative germination was determined by counting the number of germinated seeds at 12-hr intervals over a 72-hr period and transformed into percent germination. Data were graphed, and then GT_{50}, the time to reach 50% of the final germination, was calculated for each germplasm by interpolation. To determine if seed size of germplasms is associated with autotoxicity, weights of four replicates of 100 seeds each were determined before imbibition.

Effects of Leaf Extracts on Seedling Growth. Twenty-five seeds from the 20 germplasms were imbibed in distilled water for 12 hr and placed onto the agar surface with leaf extract concentrations of 0.0 (distilled water), 1.0, and 4.0 g l^{-1}. Root and hypocotyl lengths of all seedlings in each petri dish were measured 120 hr after the transfer of seed to agar. Root length data were transformed into relative growth rates, i.e., (final length minus 1 mm)/(120 hr minus GT_{50}), and to percent of control for the same germplasm because of genetic differences in growth rates among seedlings in the control treatments. When the F-test for extract concentration or germplasms was significant ($P < 0.05$), means were separated on the basis of least significant difference (LSD) at the 0.05 probability level.

Path Coefficient Analysis of Alfalfa Autotoxicity. To better characterize the mechanism of autotoxicity involved in alfalfa seedling growth, path coefficient analysis was used following the methods of Li (1956) and Dewey and Lu (1959). Relationships among growth parameters for each extract concentration were computed to separate the direct and indirect effects of hypocotyl length and root length

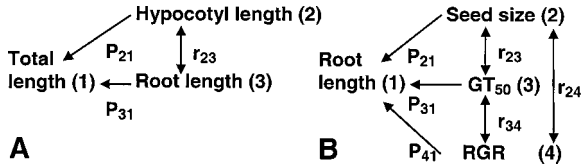

FIG. 1. Path diagrams showing direct and indirect effects of components on total seedling length (A) and final root length (B). A. Casual relationships are described between the predictor variables, hypocotyl length and root length, and the response variable, total seedling length. B. Casual relationships are shown between the predictor variables, seed size, GT_{50}, and RGR (Root growth rate), and the response variable, final root length.

on total seedling length (Figure 1A), and of GT_{50}, seed size, and root growth rate on root length (Figure 1B) at 120 hr Data for all 20 germplasms were included for path coefficient analysis.

RESULTS

Effect of Leaf Extracts on Seed Germination. Seed size differed significantly ($P < 0.05$) among germplasms, ranging from 205 to 340 mg per 100 seeds (Figure 2A). No significant difference was observed among germplasms in final germination percentage for the control or either extract concentration (data not shown). At 4.0 g l^{-1}, the GT_{50} of entry numbers 12, 13, and 17 was 39.6, 37.7, and 31.7 hr, respectively, showing the extract caused a delay in seed imbibition or in germination rate compared to the control (Figure 2B).

Effect of Leaf Extracts on Seedling Growth and Growth Rate. In the 1.0 and 4.0 g l^{-1} extracts, respectively, hypocotyl length of germplasms averaged 94 (NS) and 84% ($P < 0.05$) of the control (Figure 3A). In contrast, root length averaged only 57 and 15% (both $P < 0.05$) of the control (Figure 3B), and root growth rate averaged 56 and 12% (both $P < 0.05$) of the control (Figure 3C). On a relative basis, root length was inhibited much more by the extract than was hypocotyl length or percentage final germination.

Both root length (35 mm) and root growth rate (0.31 mm/hr) within the control treatment were highest for entry 3. But at 1.0 g l^{-1}, entry 7 had the longest root length (24.4 mm) and the highest root growth rate (0.21 mm/hr). At 4.0 g l^{-1}, entry 7 also had the longest root length (11.2 mm) and the highest root growth rate (0.09 mm/hr). The data indicate that it is necessary to use a relative value to rank germplasms because relative hypocotyl lengths, root lengths, and root growth rates of the extract treatments were not consistent for the same germplasms in the control.

Effect of Leaf Extracts on Relative Root Length. Root length for each germplasm was expressed as percent of control because of inherent genetic differences among control seedlings. At an extract concentration of 1.0 g l^{-1}, the

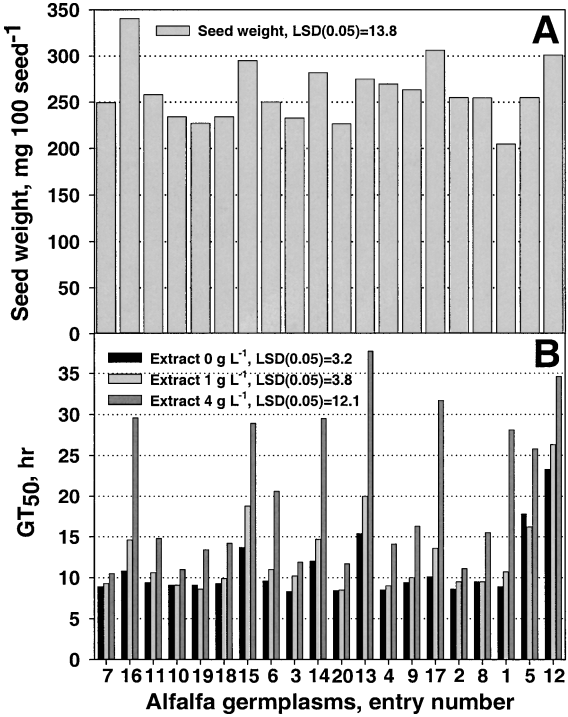

FIG. 2. Seed weight (A) and time to reach 50% of the final germination (GT_{50}) (B) of 20 germplasms. Germplasms are arrayed according to the root length response at 1.0 g l^{-1} extract concentration (Figure 3B).

root lengths of germplasms ranged from 37 to 92% of their control, indicating true genetic variation in response to the extract. However, root lengths at 4.0 g l^{-1} ranged only from 9 to 36% of the control. The correlation between responses at the two concentrations was $r = 0.74$, but the respective LSD was lower relative to the mean of 60.6% at 1.0 g l^{-1}, than to the mean of 15.5% at 4.0 g l^{-1} (Table 1). Germplasms were ranked in the following order of tolerance on the basis of relative root length at 1.0 g l^{-1}: a tolerant group (95–70% of the control) with entries 15, 16, 7, 11, and 14; an intermediate group (69–56% of the control) with entries 10, 19, 12, 6, 18, 5, and 20; and a susceptible group (below 55% of the control) with entries 13, 17, 3, 4, 8, 9, 2, and 1.

Cultivars, entry numbers 18 (Innovator), 19 (Magnum IV), and 20 (WL 252) belonged to the intermediate group (Table 1). The tolerance of germplasms to the alfalfa autotoxin showed more than a two-fold range among 17 germplasms and 3 cultivars at 1.0 g l^{-1} extract concentration. Some were more tolerant than the representative cultivars suggesting potential for genetic improvement.

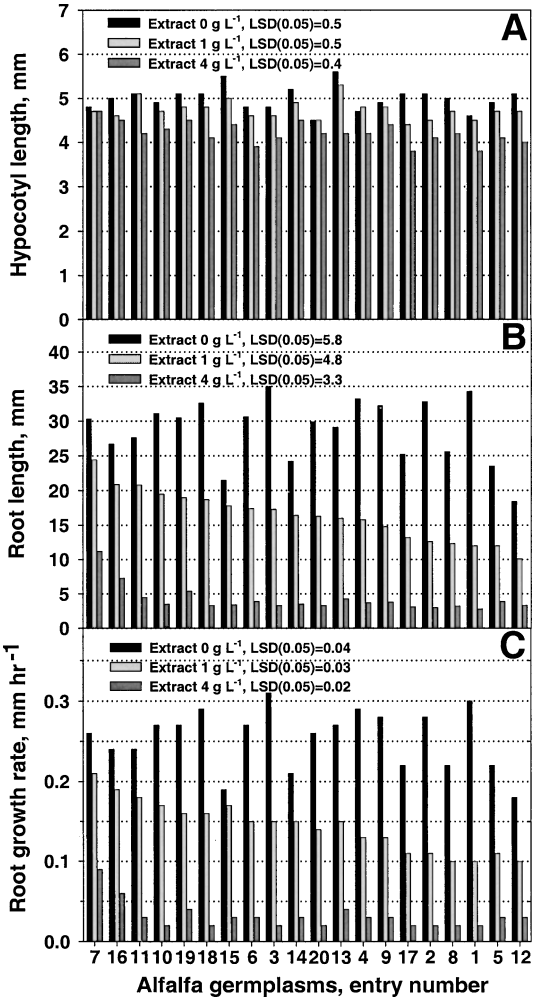

FIG. 3. Hypocotyl length (A), root length (B) and root growth rate (C) of 20 germplasms 120 hr after placing imbibed seed on agar containing leaf extract. Germplasms are arrayed according to the root length response at 1.0 g l^{-1} extract concentration (Figure 3B).

Path Coefficient Analyses of Growth Factors. Because there was little variation in hypocotyl length, the direct correlation between hypocotyl length and total length was very low at both the control (−0.02) and 1 g l^{-1} extract treatment (−0.09) (Table 2). Therefore, the direct effect of hypocotyl length (P_{21}) on total length was small (0.06, NS) at both the control and 1.0 g l^{-1} extract concentrations. The correlation coefficient (r_{21}) at 4.0 g l^{-1} was higher (0.69) than for other

TABLE 1. ROOT LENGTH RELATIVE TO THE CONTROL FOR 20 GERMPLASMS 120 HR AFTER PLACING SEED ON AGAR CONTAINING LEAF EXTRACTS OF ALFALFA[a]

Germplasm entry number	Extract Concentration(%)	
	1.0 g l^{-1}	4.0 g l^{-1}
15	91.4 a	17.3 bc
16	81.0 ab	28.6 ab
7	80.0 ab	36.4 a
11	78.4 abc	17.6 bc
14	69.9 abcd	14.9 c
10	64.4 bcde	11.2 c
19[b]	64.2 bcde	18.5 bc
12	64.0 bcde	20.7 bc
6	61.1 bcdef	13.7 c
18[b]	58.3 bcdef	10.3 c
5	58.2 bcdef	18.4 bc
20[b]	57.2 bcdef	11.4 c
13	55.2 cdef	14.7 c
17	54.2 cdef	12.4 c
3	49.9 def	9.5 c
4	49.5 def	11.2 c
8	48.9 def	12.9 c
9	46.3 def	12.1 c
2	42.1 ef	9.6 c
1	37.2 f	8.5 c
Mean	60.6	15.5
LSD, 0.05	24.3	12.4

[a] Means with the same letter are not significantly different ($P < 0.05$).
[b] Cultivars are entries 18 = "Innovator," 19 = "Magnum IV," and 20 = "WL252."

treatments, but the direct effect from hypocotyl length (0.13) was low. The indirect effect of hypocotyl length on total seedling length at 4.0 g l^{-1} was high (0.56) because the correlation (−0.61) between hypocotyl length and root length was inverse and high at this concentration (Table 2). Hypocotyl length had greater effect on total length at 4.0 g l^{-1} because root length was relatively more inhibited than was hypocotyl length.

When total length was partitioned into direct and indirect effects contributed from the root length component, the correlation (r_{31}) was very high (over 0.995) (Table 2), such that the direct effect (P_{31}) of root length for the control and each extract concentration was 0.92–1.00. Therefore, variation in root length due to extract treatment had 7–17 times more influence than did variation in hypocotyl length for determining total seedling length.

TABLE 2. PATH COEFFICIENT ANALYSIS FOR TOTAL SEEDLING LENGTH OF 20 GERMPLASMS BECAUSE OF AUTOTOXIC EFFECTS ON HYPOCOTYL LENGTH AND ROOT LENGTH (SEE FIGURE 1A)

Pathway	Extract Concentration		
	0.0 g l^{-1}	1.0 g l^{-1}	4.0 g l^{-1}
Hypocotyl length vs. total length, r_{21}	−0.017	−0.087	0.688**
Direct effect, P_{21}	0.061	0.059	0.125
Indirect effect via root length, $r_{23}P_{21}$	−0.078	−0.146	0.563[a]
Root length vs. total length, r_{31}	0.998**	0.998**	0.995**
Direct effect, P_{31}	1.003	1.007	0.919
Indirect effect hypocotyl length, $r_{23}P_{31}$	−0.005	−0.009	0.076
Hypocotyl length vs. root length, r_{23}	−0.078	−0.145	−0.613**
P_{31}/P_{21}^{b}	16.4	17.1	7.4

[a] The indirect effect of hypocotyl length on total length is 0.56 because the correlation (−0.61) between hypocotyl length and root length is inversely high at 4.0 g l^{-1} extract concentration ($P < 0.01$).

[b] Variation in root length is 16.4–17.1 times more important at 0.0 and 1.0 extract concentrations than variation in hypocotyl length, but only 7.4 times more important in defining seedling length at 4.0 g l^{-1}.

*** $P < 0.01$ (18 *df*).

Final root length in the control treatment was positively correlated with root growth rate (r_{41} = around 0.99), negatively with GT_{50} ($r_{21} = -0.63$), and negatively with seed size ($r_{31} = -0.39$) (Table 3). However, because of the relatively small effects of GT_{50} and seed size, root length at all treatments remained positively correlated with root growth rate (RGR, r_{41} =around 0.99) (Table 3). This suggests that large seed size is not a factor that confers tolerance to the autotoxin. Direct and indirect effects on final root length, when partitioned into its component parts of seed size, GT_{50}, and root growth rate, were similar for all the extract concentrations (Table 3). The direct effect of root growth rate (P_{41}) on final root length for the control, 1.0, and 4.0 g l^{-1} extract concentrations was 0.88, 1.00, and 0.98, respectively.

The direct effects of seed size (P_{21}) and GT_{50} (P_{31}) were very low for all the extract concentrations (Table 3). Larger seed size was correlated ($P < 0.05$) with the longer time needed to reach 50% of final germination in the extract treatments (r_{23} = about 0.48), but not in the control. Root length at 120 hr and root growth rate among germplasms were not affected by seed size in either the control or extract solutions. Large seed size may sustain root growth for a longer time, which would be beneficial. The larger seed size; however, delayed emergence of the radicle for a few hours (Figure 2B), perhaps because the seed required more water to initiate germination or the young, developing root had further to grow within the seed before it was exposed by 1 mm. Overall, final root length for autotoxic effects of extracts was influenced directly to a much greater degree by variation in root growth rate than by variation in either seed size or GT_{50}.

TABLE 3. PATH COEFFICIENT ANALYSES FOR FINAL ROOT LENGTH AMONG 20 GERMPLASMS AS AFFECTED BY SEED SIZE AND THE EFFECTS OF AUTOTOXIC EXTRACTS ON TIME TO REACH 50% OF THE FINAL GERMINATION (GT_{50}) AND ON ROOT GROWTH RATE (RGR) (FIGURE 1B)

Pathway	Extract Concentration		
	0.0 g l^{-1}	1.0 g l^{-1}	4.0 g l^{-1}
Seed size vs. root length, r_{21}	−0.394	−0.029	0.101
Direct effect, P_{21}	−0.005	−0.008	−0.012
Indirect effect via GT_{50}, $r_{23}P_{31}$	−0.053	−0.058	−0.050
Indirect effect via RGR $r_{24}P_{41}$	−0.332	−0.037	−0.163
GT_{50} vs. root length, r_{31}	−0.632**	−0.039	−0.244
Direct effect, P_{31}	−0.178	−0.119	−0.106
Indirect effect via seed size, $r_{23}P_{21}$	−0.002	−0.004	0.006
Indirect effect via RGR, $r_{34}P_{41}$	−0.447	−0.084	−0.132
RGR vs. root length, r_{41}	0.987**	0.991**	0.993**
Direct effect, P_{41}	0.884	1.000	0.977
Indirect effect via GT_{50}, $r_{34}P_{31}$	0.002	−0.000	−0.002
Indirect effect via seed size, $r_{24}P_{21}$	0.009	−0.010	0.014
Seed size vs. GT_{50}, r_{23}	0.296	0.485*	0.475*
GT_{50} vs. RGR, r_{34}	−0.375	0.037	0.167
Seed size vs. RGR, r_{24}	−0.506*	0.084	−0.135

* $P < 0.05$ (18 *df*). ** $P < 0.01$ (18 *df*).

DISCUSSION

The bioassay to select tolerant or susceptible germplasms to the autotoxin showed that root length and root growth rate were more responsive to the extracts than were hypocotyl length, GT_{50}, or final germination percentage, and were the best parameters for comparing sensitivities among germplasms or cultivars. These results are supported by Cope (1982) and Peterson (1986) who reported that germination inhibition in other legumes was a less sensitive measure of phytotoxicity or allelopathic effect than was root growth. GT_{50} showed more variation among germplasms as an indicator of germination rate than did final germination percentage. Larger seed size was significantly associated with longer GT_{50}, the time needed to reach 50% of final germination, but had no effect on root growth rate, and did not discriminate the extract effect on germplasms at concentrations that reduced root growth by nearly 50%. Root growth rate and root length were highly correlated, making root length the choice for screening germplasms based on its simpler measurement.

Genetic differences in root length occurred among untreated seedlings. Thus, expressing responses to the extract as percent of control was needed and showed genetic variation in autotoxic sensitivities among germplasms. This differs from

an earlier study that used cultivars and concluded that there was little genetic variation in tolerance (Goplen and Webster, 1969; Wyman-Simpson et al., 1991). Chung and Miller (1995b) evaluated effects of extracts from seven cultivars of alfalfa on seed germination and early seedling growth of the same cultivars. It is not clear, however, whether the noted difference in tolerance was due to genetic variation in the production of the chemical by the donor plants, tolerance of the seedlings to the chemical, or a combination of both.

In a routine screening program using root length relative to the control, it is critical that seed in the control and extract treatments need to germinate near the same time to be equal in age at final measurement. This did not appear to be a major problem with imbibed seed in a petri dish. Path coefficient analyses suggested that final root length for autotoxic effects of extracts was not influenced greatly by genetic variation in seed size or GT_{50}. Therefore, because of minimal effects from other factors, final root length at 1.0 $g\,l^{-1}$ of extract, and its value relative to the control, would be an accurate and convenient parameter for evaluating tolerance of alfalfa germplasms to autotoxic effects of alfalfa.

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ISOLATION AND STRUCTURAL CHARACTERIZATION OF A WATER-SOLUBLE GERMINATION INHIBITOR FROM SCOTCH THISTLE (*Onopordum acanthium*) CYPSELAS

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Abstract—Cypsela dormancy in Scotch thistle (*Onopordum acanthium*) may be affected by the presence of chemical inhibitors. To investigate this phenomenon, a leachate from *O. acanthium* cypselas was tested for its ability to inhibit germination of the cypselas from which it was derived (i.e., autoinhibition). Leachates varied in their degree of autoinhibition, depending on the cypsela population from which they were prepared. Overall, removal of leachate from a group of *O. acanthium* cypselas increased their germinability. Using lettuce (*Lactuca sativa*) cypselas as an indicator species, bioactivity-guided fractionation was used to isolate a water-soluble, *para*-substituted benzamide from *O. acanthium* cypselas, which caused germination inhibition. Various chromatographic, spectroscopic, and spectrometric techniques were applied to the characterization of the bioactive compound.

Key Words—Bioactivity-guided fractionation, chemical analysis, cypsela dormancy, germination inhibitor, *Lactuca sativa*, leachate, lettuce, *Onopordum acanthium*, population, Scotch thistle.

INTRODUCTION

Many weeds cause problems in agricultural fields by the production of dormant seeds. These seeds become incorporated into a long lasting, persistent soil seed bank and germinate over a long period of time (Parsons, 1973; Radosevich et al., 1997) and often intermittently (Cavers et al., 2000). Dormancy in such seeds can be caused by the presence of inhibitors, either in the embryo or the embryo coverings

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or by mechanical restraint by the embryo coverings (Bewley and Black, 1994). This type of seed dormancy can be classed as morpho-physiological, a category often associated with the presence of germination inhibitors (Nikolaeva, 1977). Embryo coverings can either provide inhibitors to the embryo or prevent the escape of inhibitors from the embryo (Bradbeer, 1988).

A wide range of chemical groups, including alkaloids, amino acids, coumarins, flavonoids, proteins, terpenes, and phenolic acids, can act as germination inhibitors (Evenari, 1949; Friedman, 1995) in different plant families (Evenari, 1949; Edwards et al., 1988; Harborne, 1988). Small and Gutterman (1991) found that a leachate from lettuce (*Lactuca sativa*) cypselas (dry indehiscent fruits) induced into dormancy by high temperatures, reduced the germination percentages of nondormant cypselas of this species. Similarly, inhibitors isolated from dormant embryos of European ash (*Fraxinus excelsior*) (Nikolaeva and Vorob'eva, 1979) and sycamore maple (*Acer pseudoplatanus*) (Webb and Wareing, 1972) inhibited the germination of nondormant seeds of their own species.

In the Asteraceae, many weedy thistles produce cypselas with different germination patterns (Parsons and Cuthbertson, 2000). Among thistles, the inhibitory potential of nodding thistle (*Carduus nutans*) cypselas on the germination of pasture species, both on filter paper and in soil, has been studied (Wardale et al., 1991). Scotch thistle (*Onopordum acanthium*) is another species of the Asteraceae that produces cypselas with different degrees of intraspecific and intercypselar dormancy (Qaderi and Cavers, 2000). It causes agricultural loss by interfering with crops or preventing animals from grazing on pastures (Hooper et al., 1970; Auld et al., 1979). This species can produce cypselas with both embryo- and coat-imposed dormancy (Qaderi, 1998), which may be related to chemical constituents. Previous studies have proposed the presence of a water-soluble germination inhibitor(s) in the cypselas of this species (Scifres and McCarty, 1969; Young and Evans, 1972), however, no attempt has been made to characterize it (them).

In this report, we demonstrate the presence of a water-soluble germination inhibitor in cypselas of *O. acanthium*. Using the inhibition of lettuce cypsela germination as a guide, we isolated a chemical responsible for germination inhibition in *O. acanthium*, and provide partial identification based on spectroscopic analyses.

METHODS AND MATERIALS

Cypsela Collection. Cypselas were collected in bulk from three geographically distinct *O. acanthium* populations originally located in or near London, Ontario (Table 1) in 1996 and subsequently planted at the Environmental Sciences Western (ESW) Research Station in 1998. They were excavated and replanted approximately 200 m apart in 1999 to prevent cross-pollination. Cypselas were subsequently collected from the ESW-grown plants, cleaned manually, and stored dry at -20°C for up to 847 days until used.

TABLE 1. TIME-COURSE OF EVENTS FOR SCOTCH THISTLE (*O. acanthium*) CYPSELAS FROM THREE POPULATIONS MATURED UNDER FIELD CONDITIONS IN LONDON, CANADA IN 1999

Population[a]	Cypselas set to germinate	Seedlings transferred to flats	Hardening of seedlings started	Seedlings/rosettes planted outside at ESW	Rosettes transplanted at ESW	Cypselas collected
ESW	May 08, 98	May 22, 98	Jun 23, 98	Jul 28, 98	Apr 08, 99	Aug 05, 99
Ola	May 08, 98	May 22, 98	Jun 23, 98	Jul 28, 98	Apr 08, 99	Aug 05, 99
GP	May 08, 98	May 22, 98	Jun 23, 98	Jul 28, 98	Apr 08, 99	Aug 05, 99

[a] ESW = Environmental Sciences Western, Ola = Olalondo Road, GP = Gibbons Park. In 1996, plants from the Ola population had grown *in situ*, while those of the ESW and GP populations were transplanted to the ESW Research Station (on May 17 and June 4, respectively), because they would have been destroyed in their original sites. On September 4, 1996, cypselas were collected and stored until set to germinate in May 1998.

Autoinhibition of Cypsela Germination. For the field-matured cypselas of the Olalondo Road (Ola), Environmental Sciences Western (ESW), and Gibbons Park (GP) populations, 10 lots of 100 cypselas were tested for germination as described by Qaderi et al. (2002) with the following modifications: On day seven, filter papers containing leachate were removed from five Petri dishes and replaced with fresh moistened filter papers, while the remaining five Petri dishes were left alone (except for the addition of water as required). The experiment was terminated once a period of 5 days or more with no germination (typically after 45 days) had elapsed.

Isolation of a Water-Soluble Germination Inhibitor from Cypselas. A 500 g sample of cypselas from the Ola population (which showed the highest degree of autoinhibition of germination *in vitro*) was extracted twice with Milli-Q™ water (2 and 1 l, respectively) on a shaker (250 rpm) over 48 hr at room temperature. Extracts were mixed and concentrated *in vacuo* at $< 40^{\circ}$C, filtered through Whatman No. 1 filter paper to remove debris, and adjusted to 22.5 ml/g residue. The filtered crude extract (approximately 175 ml) was loaded (5 ml per each run) onto an LH-20 column (2×100 cm) pre-equilibrated with filtered Milli-Q™ water, using a Bio-Rad Biologic HR Chromatography System fitted with a model 2128 fraction collector (Bio-Rad Laboratories, Inc., California, USA), and eluted isocratically for 180 min with Milli-Q™ water at a flow rate of 1 ml min^{-1}. The eluent was monitored at 280 nm, and fractions were collected every 5 min. Fractions that showed bioactivity (see below) from the first round of LH-20 chromatography were pooled and rechromatographed on the same LH-20 column as above. After each step of purification, analytical HPLC was used to estimate fraction purity (see below).

Final purification was achieved using preparative HPLC on a Beckman HPLC consisting of a model 126 binary pump, a model 168 diode-array detector, a model 508 autosampler (Beckman Instruments, Inc., Fullerton, CA), and a preparative Nucleosil C-18 reversed phase column (10×250 mm, 7 μm; Macherey-Nagel, Düren, Germany). Solvent A was 1.5% (v/v) aqueous phosphoric acid; Solvent B was MeOH:CH_3CN:H_2O (1:1:1). Compounds were eluted with a gradient of 0–35% B in A over 50 min. The flow rate was 1 ml min^{-1} and the injection volume 250 μl. The eluent was monitored at 270 nm, and peaks were collected manually into 50-ml screw cap tubes. The bioactive compound was rechromatographed over LH-20 (as above) to remove phosphate, to yield 3.3 mg.

Analytical HPLC was performed on a Beckman HPLC (as above) using a Nucleosil C-18 reversed phase analytical column (4.6×150 mm, 5 μm; Varian, California, USA). Solvent A was 1.5% (v/v) aqueous phosphoric acid; Solvent B was MeOH:CH_3CN:H_2O (1:1:1). Compounds were eluted with a gradient of 0–50% B in A over 15 min. The flow rate was 1 ml min^{-1} and the injection volume 20 μl. The eluent was monitored over the range of 200–350 nm, with 240 nm used for calibration and quantification.

Bioassay of Fractions. For bioassay, selected fractions (100 μl) were applied directly (without concentration) on five replicates, each consisting of five *L. sativa* cypselas and five *O. acanthium* cypselas in a 96-well plate. Plates were covered with lids and sealed with Parafilm. Cypselas were incubated at 25/10°C, 14-hr L and 10-hr D, respectively. After 1 week, germinated cypselas were counted, and the experiment was terminated.

Characterization of the Bioactive Compound. The bioactive compound was characterized using proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy, fourier transform infrared spectroscopy (FTIR), UV spectroscopy, and mass spectrometry (MS). ^{1}H and ^{13}C NMR spectra were generated in deuterated methanol (MeOH-d_4) at 300 and 75 MHz, respectively, using a Varian Inova 400 instrument. Spectra for total correlation, H-C connectivity (HSQC) and long-range connectivity (HMBC) were obtained in deuterated dimethyl sulphoxide (DMSO-d_6) at 600 MHz using a Varian Inova 600 instrument.

Infrared data were obtained in the wave-number range of 600–4000 cm^{-1} as a NaCl pellet. A Finnigan MAT 8400 instrument was used for mass spectrometry. The compound was dissolved in dichloromethane (CH_2Cl_2) and subjected to either electron impact ionization (EI) or chemical ionization (CI) (using isobutane as an ionizing reagent) via a direct exposure probe. EI and CI were recorded at 70 electron volts (eV). For fast atom bombardment mass spectrometry (FAB-MS), glycerol was used as a matrix, and the sample was bombarded with neutral xenon (Xe) atoms at 8 keV using an Ion Tech FAB instrument.

Tissue Distribution. To determine whether the bioactive compound was present in the embryo and/or cypsela coat, embryos from the Ola population were separated from their cypsela coats. From each category, a 500 mg sample was taken and extracted with 5 ml Milli-Q™ water on a shaker (250 rpm) for 48 hr at room temperature. Extracts were centrifuged at 17,300 g, and 20 μl from the supernatant were analyzed by analytical HPLC (as above).

Statistical Analyses. All data, except for chromatographic fractions, were analyzed by means of a one-way ANOVA and a Tukey's test to determine differences among treatments (Sokal and Rohlf, 1995; SAS Institute Inc., 1999). Germination percentages were transformed to the arcsine square root of the percentage (Zar, 1999) before analysis.

RESULTS

Autoinhibition of Cypsela Germination. Overall, the transfer of cypselas from Petri dishes containing their own leachate to fresh moistened filter papers resulted in their increased germinability. In the Ola population, the final germination percentage was significantly higher ($F = 69.46$, $P < 0.001$) for cypselas removed from their leachate (87.2% ± 1.6) than for those remaining in it (24.2% ± 6.6) (Figure 1). Within 2 days of transfer to fresh dishes (i.e., 9 days after plating), the

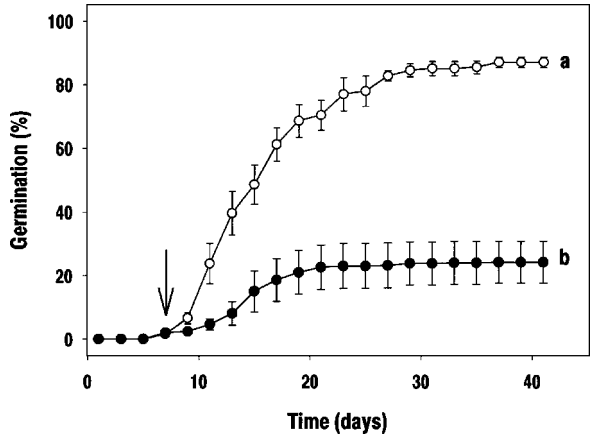

FIG. 1. Autoinhibition of the germination of Scotch thistle (*O. acanthium*) cypselas (Ola population) collected from the ESW field plots. Cypselas were allowed to germinate on filter papers moistened with distilled water in Petri dishes. On day seven (arrow), any leachate from the cypselas was removed from half of the Petri dishes (−○−) by replacing the filter paper and remoistening with distilled water. The remaining cypselas (−●−) were left to germinate in the presence of their own leachate. Lines followed by different letters are significantly different ($P < 0.05$) according to Tukey's test.

difference in germination percentage was significant (Figure 1). Similarly, with the ESW population, removal of cypselas from dishes with leachate resulted in significantly increased germinability ($F = 9.92$, $P = 0.014$; Figure 2A). However, with cypselas from the GP population (a highly germinable population), there was no significant difference in germination percentage for cypselas removed from their leachate (90.6% ± 1.5) compared to those remaining in dishes containing leachate (87.2% ± 1.4) (Figure 2B).

Bioactivity-Guided Fractionation of a Germination Inhibitor. Water extracts prepared from cypselas of the Ola population were chromatographed on LH-20. Bioassay of fractions using *L. sativa* cypselas revealed the greatest inhibition of germination associated with two later-eluting peaks (Figure 3A). These fractions were pooled and rechromatographed on LH-20. Bioassay with *L. sativa* cypselas was used again to collect the most active peak (Figure 3B), which consisted of two compounds as determined by analytical HPLC (Figure 4A). After preparative HPLC to isolate these two compounds, the later-eluting compound (designated Compound 1) caused 100% of the germination inhibition in bioassay with *L. sativa*, and completely inhibited germination of *O. acanthium* (Ola population) under the same conditions.

Analytical HPLC analysis of the pure bioactive compound (i.e., Compound 1) revealed a single peak (Figure 4B) at 8.38 min with strong absorbance peaks at 310, 258, and 209 nm. Analytical HPLC analysis of crude water extracts suggests

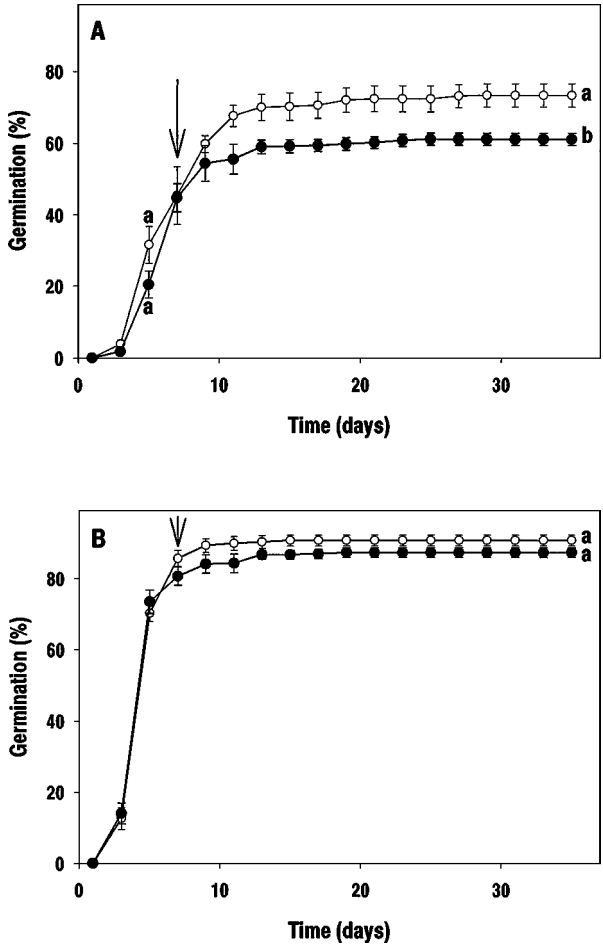

FIG. 2. Autoinhibition of cypsela germination of two populations of Scotch thistle (*O. acanthium*): (A) ESW and (B) GP collected from the ESW field plots. Cypselas were allowed to germinate on moist filter paper in Petri dishes. On day seven (arrow), any leachate was removed from half of the Petri dishes (−○−) by replacing the filter paper and remoistening with distilled water. The remaining cypselas (−●−) were left to germinate in the presence of their own leachate. Lines in each graph followed by different letters are significantly different ($P < 0.05$) according to Tukey's test.

that Compound 1 is present almost entirely in the cypsela coat (data not shown), albeit at very low levels.

Spectroscopic Characterization of the Bioactive Compound. The proton NMR spectrum of Compound 1 (recorded in CD_3OD) revealed two doublets at δ 7.015 ($J = 6$ Hz) and 6.820 ($J = 6$ Hz) ppm with each integrating for

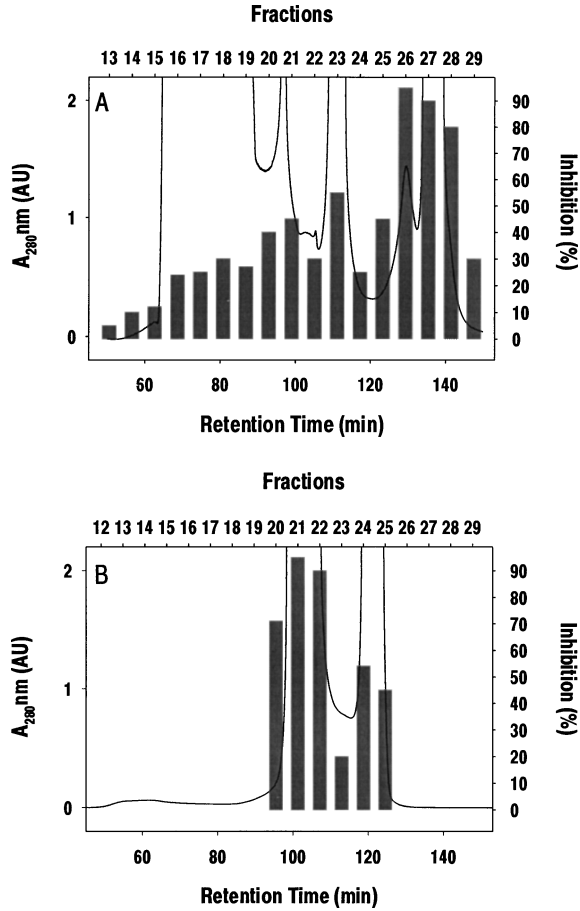

FIG. 3. Isolation of a water-soluble bioactive compound from Scotch thistle (*O. acanthium*) cypselas. (A) LH-20 chromatograph of a crude water extract prepared from the cypselas from the Ola population collected from the ESW field plots on August 5, 1999. (B) Rechromatography of the most bioactive fractions from (A). The solid lines indicate A_{280} while the vertical bars indicate % inhibition of the germination of lettuce (*L. sativa*) cypselas by individual fractions.

2H, suggesting a *para*-substituted aromatic ring system (Table 2). The carbon-13 NMR spectrum revealed signals at δ 113.4, 116.0, 126.8, 153.2, 159.7, and 164.0 ppm, with the signals at 113.4 and 116.0 likely representing 2-C each, again suggesting a *para*-substituted aromatic ring system and an 8-carbon parent molecule (Table 2). However, in dimethyl sulphoxide $(CD_3)_2SO$, ^1H NMR revealed two additional signals at δ 8.9 and 10.1 ppm, each integrating for 2H, indicating the presence of two distinct amine groups ($-NH_2$). The position of the hydrogen

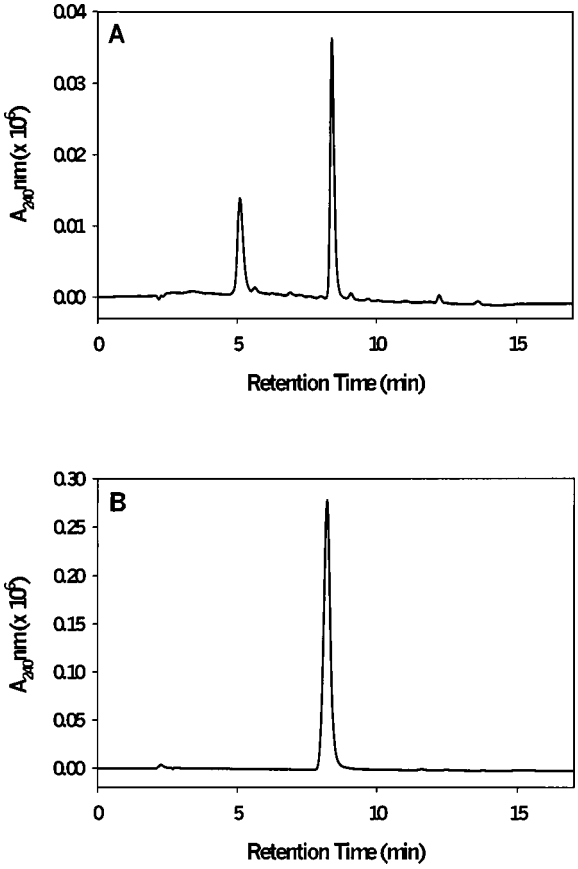

FIG. 4. Analytical HPLC of bioactive fractions from LH-20 (A) and preparative HPLC (B) column chromatography. Compounds were separated on a Nucleosil C-18 column as described in Methods and Materials.

atoms and the potential substitution on the benzene ring were established by a combination of heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC) experiments. Thus, H2/6 ($\delta = 6.47$ in DMSO-d_6) showed cross peaks with C-2/6 (HSQC) and C-1, C-4, C-3/5, and C-7 (HMBC), while H3/5 ($\delta = 6.67$) showed connectivity with C-3/5 (HSQC) and C-1 and C-4 (HMBC). In addition, H3/5 showed a strong cross peak with a C signal at δ157.5 (C-8), although no cross peak between H3/5 and C2/6 was observed (Figure 5).

FTIR analysis (IR ν_{max} (NaCl) cm^{-1}) supported the presence of carbonyl and amine groups (1555 and 1646 cm^{-1}) in the structure of Compound 1. Additional peaks were observed at 3403, 3093, 2943, 2644, 2542 1481, 1427, 1387,

TABLE 2. ^{1}H AND ^{13}C NMR SPECTRAL DATA FOR A WATER-SOLUBLE GERMINATION INHIBITOR ISOLATED FROM SCOTCH THISTLE (*O. acanthium*) CYPSELAS OF THE OLA POPULATION COLLECTED FROM FIELD- GROWN PLANTS ON AUGUST 5, 1999 IN LONDON, CANADA

Position	^{1}H δ	^{13}C δ
1	—	148.5
2/6	7.025 (2H, *d*, $J_{H2/6-H3/5} = 6.67$ Hz)	105.5
3/5	6.820 (2H, *d*, $J_{H3/5-H2/6} = 6.47$ Hz)	115.5
4	—	134.5
7	—	163.0
8	—	157.5

1277, 1141, 1106, 980, 906, 864, 788, and 759. Both EI- and CI-MS support a parent mass of 155 amu (data not shown), corresponding to molecular formulas of $C_8H_{11}O_3$, $C_8H_{13}NO_2$, or $C_8H_{15}N_2O$. Additional EI-MS peaks were observed at 137 (88.33%), 109 (70.28%), 81 (52.22%), and 53 (34.17%) amu. FAB-MS data were inconclusive.

DISCUSSION

Autoinhibition of Cypsela Germination. Onopordum acanthium cypselas placed on moist filter papers under conditions favorable for germination, often fail to do so (Qaderi and Cavers, 2000). However, when poorly germinating cypselas (e.g., from the Ola population used in this study) are removed from the filter papers on which they were sown, and placed on fresh, moist filter papers, they

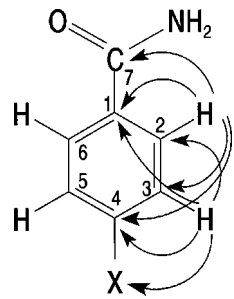

FIG. 5. Tentative structure for Compound 1. The partial structure of Compound 1 (a *para*-substituted benzamide) is consistent with the NMR and FTIR data obtained. The identity of substituent "X" remains unknown, but based on NMR and FTIR data it should include second $-C=O$ and $-NH_2$ groups. Arrows indicate long-range $H-C$ connectivities.

germinate readily. In this example, cypselas from the Ola population germinated to >80% after transfer, whereas only 20% of those left to sit in their own leachate germinated (Figure 1). Conversely, cypselas from the GP population used in this study readily germinated to >80%, whether transferred to new filter papers or not (Figure 2B). These observations suggest that *O. acanthium* cypselas from some populations produce a water-soluble germination inhibitor during cypsela development and that this inhibitor has an autoinhibitory effect on germination. To our knowledge, this is the first attempt to characterize a germination inhibitor in thistles, in general, and in *O. acanthium*, in particular. In their discussion of chemical inhibitors of germination, Baskin and Baskin (1998) do not mention any members of the Asteraceae.

Using a bioactivity-guided fractionation strategy in which the inhibition of lettuce cypsela germination was used to identify column fractions with germination inhibition potential, we isolated a potent germination inhibitor from *O. acanthium*. This strategy worked because the lettuce cypselas readily germinate to 100% within a reasonable time frame (e.g., 7 days) under the conditions used, unless they are incubated with an inhibitory compound(s). Thus, after several repetitive chromatographic steps on LH-20 and preparative reversed-phase HPLC, we isolated 3.3 mg of a white powder that chromatographed as a single peak using analytical reversed-phase HPLC (Figure 4B), herein refered to as Compound 1. Importantly, this compound was an effective inhibitor of *O. acanthium* cypsela germination. While we have not been able to accurately quantify Compound 1 in individual cypselas, our *in vitro* germination assays suggest that there is sufficient amount of it in some populations to inhibit germination (Figures 1 and 2). Once we have established a reliable analytical protocol for the quantitative analysis of Compound 1, we will be able to track its accumulation in cypselas and investigate the environmental conditions that result in its biosynthesis. It is important to keep in mind that more fractions showed inhibition of lettuce cypselas than just those containing Compound 1 (Figure 3), and that other compounds may be involved in the overall germination patterns observed in *O. acanthium* cypselas. However, Compound 1 appeared to be the most effective (and abundant) inhibitor, at least in the Ola population used in this study.

Characterization of the Bioactive Compound. The proton and carbon NMR spectra of Compound 1 revealed a 1,4-di-substituted benzene ring system (Table 2) with two distinct exocyclic functional groups. Two sharp, strong peaks in the IR spectrum indicated the presence of an amine ($-NH_2$) group. A sharp, strong peak at 1646 $cm^{-1}$with a shoulder at 1619 cm^{-1} in the fingerprint region suggested a carbonyl group ($-C{=}O$). Based on the total correlation and H—C connectivity data, the attachment of an amide carbonyl group to C—1 can be inferred. Based on these data, a tentative core structure for Compound 1 is proposed (Figure 5).

However, when the MS data, which predict a parent mass of 155 amu, are taken into account, the molecular formula for Compound 1 can only be $C_8H_{11}O_3$, $C_8H_{13}NO_2$, or $C_8H_{15}N_2O$. It is possible that Compound 1 is heat labile, and its

introduction into the mass spectrometer via heating a direct exposure probe (to make the sample volatile) may have caused its degradation prior to analysis. To address this possibility, Compound 1 was subjected to FAB-MS, which yielded potential parent ions with m/z of 225 or 277, neither of which is consistent with the spectroscopic data. At this stage, therefore, the spectroscopic data for Compound 1 are not consistent, and in order to propose a complete structure for Compound 1, further investigations are required.

Implications. In many plant species, water-soluble germination inhibitors, including phenolics, occur in embryos or embryo coverings of dormant seeds (Bradbeer, 1988). Even if other environmental conditions (e.g., moisture, light, temperature) are suitable for germination, the seed coat can impose dormancy by supplying inhibitors to the embryo, preventing the exit of inhibitors from the embryo, or limiting oxygen supply to the embryo (Villiers, 1975; Bewley and Black, 1994). Several studies have shown that dark-colored seeds have higher amounts of phenolics and lower percent germination than light-colored seeds (Bewley and Black, 1982; Cavers and Bough, 1985; Khan et al., 1996; Doucet and Cavers, 1997), a phenomenon also noted for *O. acanthium* cypselas (Qaderi and Cavers, 2000). Furthermore, our preliminary results suggest that Compound 1, which causes inhibition of germination, is present in the cypsela coat.

Taken together, the data strongly implicate a water-soluble inhibitor in the complex interactions that regulate *O. acanthium* germination patterns. While it remains to be seen whether Compound 1 is present in sufficient quantities under field conditions to have an effect on cypsela germination, or indeed whether there is any correlation between the amount of Compound 1 present in a cypsela and its ability to germinate under favorable conditions, our *in vitro* data strongly implicate Compound 1 in the process.

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L-LEUCINE METHYL ESTER: THE FEMALE-PRODUCED SEX PHEROMONE OF THE SCARAB BEETLE, *Phyllophaga lanceolata*

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Abstract—The female-produced sex pheromone of the scarab beetle *Phyllophaga lanceolata* was identified as the methyl ester of an essential amino acid, L-leucine. During field testing, 239 male *P. lanceolata* were caught in traps baited with L-leucine methyl ester. L-Isoleucine and L-valine methyl esters, similar in structure to L-leucine methyl ester and previously identified as female-produced sex pheromone compounds employed by other *Phyllophaga* species, were also tested. Addition of L-valine or L-isoleucine methyl esters to the L-leucine methyl ester in 1:1 ratios completely inhibited attraction of *P. lanceolata* males. Males of *P. squamipilosa* were also captured using L-leucine methyl ester. This is the first record of *P. squamipilosa* from Kansas.

Key Words—L-Valine, L-isoleucine, L-leucine methyl ester, sex pheromone, electroantennogram, *Tostegoptera, squamipilosa*, Scarabaeidae, Melolonthinae.

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INTRODUCTION

The genus *Phyllophaga* (*sensu lato*) (Coleoptera: Scarabaeidae: Melolonthinae), encompasses about 200 species in the United States north of Mexico (Woodruff and Beck, 1989). Only two species are found in the subgenus *Tostegoptera*, *P*. (*Tostegoptera*) *lanceolata* and *P*. (*Tostegoptera*) *squamipilosa* Saylor. Whereas *P. lanceolata* was described in 1824, *P. squamipilosa* was not described until 1936 by L.W. Saylor, who discovered the species in a series of *P. lanceolata* from Texas (Saylor, 1936). Nothing is known of the life history or biology of *P. squamipilosa*.

P. lanceolata is unusual among the *Phyllophaga* in that it is diurnal, and the females are flightless (Travis, 1939; Reinhard, 1940). Travis (1939) reported that on days that are "hot and sunny," the greatest number of beetles "are visible between 8 and 10 o'clock in the morning." He further reports that "On many occasions, males were noted feeding, apparently unconcerned, on the same leaf or plant with a female until a female extruded her genitalia. Immediately, males within a radius of 15 to 20 yards flew toward her."

P. lanceolata is distributed throughout the Great Plains including Colorado, Iowa, Kansas, Missouri, Nebraska, Nevada, New Mexico, Oklahoma, South Dakota, Texas, and Wyoming. It has also been reported from the province of Ontario, Canada (Luginbill and Painter, 1953). It has been an important pest species in both the larval and adult stages on wheat, cotton, corn (Hayes, 1919), and sunflowers (Rogers, 1978). Reinhard (1940) listed a series of truck crops on which *P. lanceolata* fed including spinach, lettuce, carrot, cowpea, rape, turnip, mustard, onion, beans, and Irish potato. We present here the identification of the female-produced sex pheromone of *P. lanceolata* and the results of field trapping tests.

METHODS AND MATERIALS

Pheromone Collections. Third-instar larvae of *P. lanceolata* were dug from infested cattle range on a ranch in Kiowa County, Kansas, in late April 2000. Larvae were individually housed in ~30-ml plastic cups in a 3:1 mix of greenhouse sand and screened peat moss raised to about 12% moisture. The cups were housed in a controlled environment room maintained at 25°C during the 16-hr photophase and 20°C during the 8-hr scotophase. After pupation and adult emergence, females were placed in observation cages. When females were observed calling (abdominal pheromone gland everted, see Figure 1) (Leal et al., 1993) during the photophase, they were removed from the cages, and the glands were excised and soaked in 200 μl of dichloromethane. After 20 min, the glands were removed and the extract concentrated under a nitrogen stream to a volume of about 20 μl.

GC-EAD Analysis. GC-EAD instrumentation used in this study was the same as described previously (Nojima et al., 2003), except for using an acrylic holder

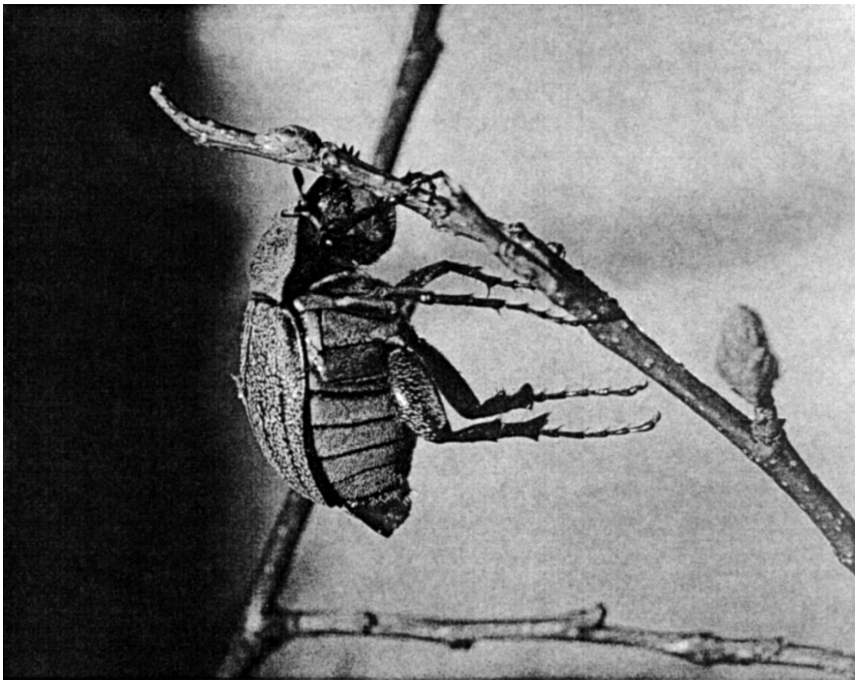


FIG. 1. Female *P. lanceolata* in calling posture with sex pheromone gland everted.

designed for a beetle's antenna. A nonpolar SPB-1 capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; Supelco, Bellefonte, PA) or a polar EC-WAX Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; Alltech, Deerfield, IL) was used for GC-EAD analyses. Nitrogen was used as carrier gas (flow 2.0 ml/min). The GC was programmed for 40°C for 5 min, then 15°C/min to 250°C and held for 10 min. Injector and detector temperatures were 260°C. A β-DEX 120 chiral capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; Supelco, Bellefonte, PA) was used for resolution of enantiomers, with N_2 carrier gas (flow 2.0 ml/min). The GC program was 40°C for 5 min, 4°C/min to 130°C, then 10°C/min to 220°C and held for 20 min. Injector and detector temperatures were 260°C. Six individual virgin female gland extracts were subjected to GC-EAD analysis, and antennae from four different males were used for EAD recordings.

Chemical Analysis. GC-MS analysis was performed on a Shimadzu QP 5050A equipped with a nonpolar DB-1ms capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; J&W Scientific, Folsom, CA) or a polar EC-WAX Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness;

Alltech, Deerfield, IL) in EI mode (70 eV) and the same conditions as those of GC-EAD analyses, but with helium as the carrier gas at constant flow (1.0 ml/min). The EAD active compound was tentatively identified by mass spectral matches to library spectra. The identity was confirmed by comparing GC retention times, mass spectra, and EAD activity with those of an authentic sample.

Chemicals. L-Leucine, L-valine, and L-isoleucine methyl esters were obtained as hydrochloric acid salts (Aldrich, Milwaukee, WI). Free amino acid methyl esters were regenerated by dissolving the salts in an aqueous alkali solution and extracting the mixture with three portions of ether. Ether layers were combined, washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The resulting methyl esters were used without further purification. D-Leucine methyl ester was prepared by methyl esterification of D-Leucine (Aldrich, Milwaukee, WI) according to Anand and Vimal (1998).

Preparation and Field Evaluation of Synthetic Lures. Lures were formulated by dissolving the neat compounds in hexane (20 μg/μl), dispensing appropriate amounts into 5-mm rubber septa (Thomas Scientific, Swedesboro, NJ), and allowing the hexane to evaporate in a fume hood. Lures were deployed in the field in lab-constructed cross-vane traps.

The six treatments tested included the methyl esters of L-leucine, L-isoleucine, and L-valine, each tested alone at 4 mg/septum, L-leucine + L-isoleucine (1:1) at 4 mg each/septum, L-leucine + L-valine (1:1) at 4 mg each/septum, and a solvent only control.

L-Isoleucine and L-valine methyl esters have been reported as sex pheromone components for other *Phyllophaga* species (Zhang et al., 1997; Leal et al., 2003). They were included in the field test because when antennae of *P. lanceolata* males were challenged with these two compounds in GC-EAD assays, they elicited antennal responses similar to those produced by the L-leucine methyl ester (Figure 2B).

Three replicates of the six treatments were deployed June 1, 2001, on cattle range at the ranch from which the larvae had been obtained and where adult activity had been observed in previous years. Each replicate of the six treatments was deployed in a line with traps placed about 15 m apart. Traps were hung on metal stakes such that the bottom of the trap touched the ground. Trap positions within a line were randomized at initial placement and rerandomized each time they were checked. Traps were checked nine times between June 2 and June 16, when the traps were removed from the field. The pooled data were log transformed $(x + 1)$ to insure homogeneity of variance before calculating one sample t confidence intervals (0.95) for those treatments that had catches greater than 0.

RESULTS AND DISCUSSION

Pheromone Identification. GC-EAD analyses of pheromone gland extracts of female *P. lanceolata* using both nonpolar and polar columns consistently revealed

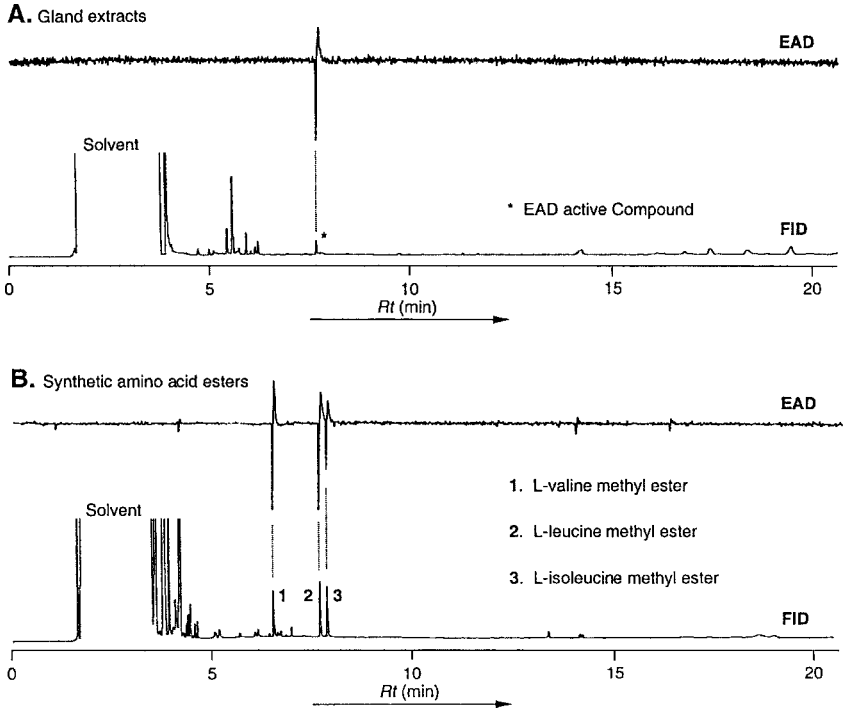


FIG. 2. GC-EAD traces using the antennae of male *P. lanceolata* stimulated with the female pheromone gland extracts (A) and synthetic amino acid methyl esters (B) on a nonpolar capillary column.

one EAD active compound (Figure 2A). The mass spectrum of the compound matched that of leucine methyl ester. The identity was confirmed by comparing GC retention times and MS spectra with those of authentic samples. The chirality of the leucine methyl ester was confirmed by means of a chiral GC-EAD analysis. When run on a chiral column, authentic L-leucine methyl ester had a retention time (32.43 min) and EAD activity similar to that of the natural compound (32.48 min), whereas the D-leucine methyl ester did not (31.98 min).

Field Evaluation of Synthetic Lures. Of the 240 male *P. lanceolata* captured in field testing of the compounds, 239 were captured in the L-leucine methyl ester baited traps (Table 1). No female beetles were taken in the traps. We conclude that L-leucine methyl ester is the sex pheromone of *P. lanceolata*. The four treatments that compared the L-isoleucine and L-valine methyl esters, each alone and each in combination with the L-leucine methyl ester, yielded only a single male *P. lanceolata* that was captured in a trap baited with L-valine methyl ester. The confidence intervals calculated for the two treatments that caught beetles indicated the catch

TABLE 1. AVERAGE NUMBER OF MALES CAPTURES/TREATMENT (±SE)

L-Leucine	L-Isoleucine	L-Valine	L-Leucine + L-Isoleucine	L-Leucine + L-Valine	Solvent control
79.7 ± 18.6	0[a]	0.33 ± 0.33[a]	0[a]	0[a]	0[a]

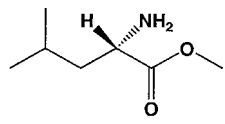
[a] Not significantly different, one sample *t* confidence interval, 95%.

from the traps baited with the L-valine methyl ester (a single beetle) were not significantly different from those traps that captured no beetles (Table 1), whereas the catches from the L-leucine methyl ester baited traps were different. L-Valine and L-isoleucine methyl esters are clearly nonattractive to the males when presented alone and demonstrated antagonistic effects when presented in combination with L-leucine methyl ester, the sex pheromone of *P. lanceolata.*

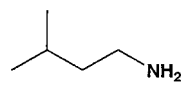
In light of the identification of L-leucine methyl ester as the sex pheromone of *P. lanceolata*, the work of Travis (1939) is of particular note. In that publication, Travis reported results of field tests of a series of 24 volatile compounds assessing whether they would attract *P. lanceolata* adults. One of the compounds tested was isoamylamine. Travis states.

> The last named (isoamylamine) was the only one of these compounds that proved attractive to the males, and none of them seemed to stimulate the females. Isoamylamine produced a male response similar to that made to crushed or to sexually active females. The crushed and receptive females appeared to produce an emanation or odor that was rapidly disseminated by air movement, as male activity was always greater on the leeward side of the female. The same type of response was secured when isoamylamine was released.

A comparison of the structures of L-leucine methyl ester, the authentic pheromone, and isoamylamine (3-methyl-butylamine), (Figure 3) demonstrates that the work of Travis presaged the identification of L-leucine methyl ester as the sex pheromone of *P. lanceolata*. Future field tests will compare the relative attractancy of the methyl ester of L-leucine and isoamylamine to *P. lanceolata* males.



L -leucine methyl ester



isoamylamine

FIG. 3. Structures of L-leucine methyl ester and isoamylamine.

Of additional interest is the capture of *P. squamipilosa* using L-leucine methyl ester. In an unreplicated test in Stevens County in southwestern Kansas in mid-June of 2001 (G.A.S., personal observation), a septum loaded with 4 mg of L-leucine methyl ester was hung (using a piece of wire) in low brush in grassy rangeland habitat. No trap was used. Beetles were captured both crawling on the septum as well as while flying upwind in the pheromone plume. The 16 male beetles captured were subsequently identified as *P. squamipilosa.* This is the first record of *P. squamipilosa* from the state of Kansas. Very little is known regarding the degree of overlap in the ranges of *P. lanceolata* and *P. squamipilosa*, and nothing is known about their interspecific relationships where they occur sympatrically.

Methyl esters of essential amino acids are employed as sex pheromones in the genus *Phyllophaga* (Zhang et al., 1997; Leal et al., 2003) as well as the closely related genus *Holotrichia* (Leal et al., 1992). They are termed essential amino acids because they are generally required for growth and development by insects and other animals and cannot be synthesized *de novo* (McFarlane, 1985). Therefore, prior to utilization of these compounds as sex pheromones (subsequent to methyl esterification) they must be sequestered from plants or produced by endosymbionts. Virgin female *P. anxia* (LeConte) individuals are capable of emitting L-valine and L-isoleucine methyl esters before any adult feeding has taken place (P.S.R., unpublished data). Furthermore, the sex pheromone of *Costelytra zealandica* (White), another melolonthine scarab beetle, identified as phenol (Henzell and Lowe, 1970), was later found to be produced by bacterial endosymbionts (Hoyt and Osborne, 1971). Further work is planned to identify the source of the essential amino acids used by these *Phyllophaga* species to make sex pheromone compounds.

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SEX PHEROMONE OF THE PERSIMMON FRUIT MOTH, *Stathmopoda masinissa*: IDENTIFICATION AND LABORATORY BIOASSAY OF (4*E*, 6*Z*)-4,6-HEXADECADIEN-1-OL DERIVATIVES

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Abstract—Three electroantennogram (EAG)-active components were detected by gas chromatography coupled to an electroantennographic detector (GC–EAD) analysis of a hexane extract of the pheromone glands of the persimmon fruit moth, *Stathmopoda masinissa*. These compounds were identified as (4*E*,6*Z*)-4,6-hexadecadienal (E4,Z6-16:Ald) and the corresponding acetate (E4,Z6-16:
OAc) and alcohol (E4,Z6-16:OH) by mass spectral, GC retention time (RT), and microchemical test data. The characteristic base peak of the aldehyde at m/z 84 provided a crucial piece of information suggesting the possibility of a 4,6-diene structure. The (4*E*,6*Z*)-isomer elicited the strongest EAG responses among the four geometrical isomers of each synthetic 4,6-hexadecadienyl compound. In a laboratory bioassay, only E4,Z6-16:OAc elicited male moth behavioral activity significantly different from the control; the activity of the acetate was not affected by addition of the aldehyde and alcohol. A preliminary field trial confirmed that E4,Z6-16:OAc as a single component attracted male moths. The possible roles

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of E4,Z6-16:Ald and E4,Z6-16:OH as components of lures for field use remain to be determined.

Key Words—*Stathmopoda masinissa*, sex pheromone, (4*E*,6*Z*)-4,6-hexadecadienyl acetate, (4*E*,6*Z*)-4,6-hexadecadienal, (4*E*,6*Z*)-4,6-hexadecandien-1-ol, GC–EAD.

INTRODUCTION

The persimmon fruit moth, *Stathmopoda masinissa* Meyrick (Lepidoptera: Oecophoridae) is a pest of persimmon (*Diospyros kaki* Thumb.). This moth is distributed throughout Honshu, Shikoku, and Kyushu Islands in Japan and has two generations a year in the central Honshu Island. The adults appear in late spring and summer and the fifth instars overwinter. Eggs are laid on succulent sprout shoots and hatch about 1 week after being laid. The early instars feed inside the shoots, and the third to last (fifth) instars bore into the core of the persimmon fruit around the calyx, and subsequently the damaged fruits rot and drop to the ground (Ishihara, 1948; Okudai, 1961; Oda, 1983, 1986).

In Japan, insecticides are used to control this species, with the most effective application being carried out in the limited period when the larvae move from the shoots to the fruits. Monitoring population densities of this species is laborious because it is necessary to directly count the moths on persimmon leaves during the day because they are minimally attracted to light traps. Sex pheromone baited traps might provide a much easier method of monitoring this species.

Oda and Sugiura (1982) found that female *S. masinissa* showed a distinct calling behavior at dawn, and that males were strongly attracted to traps baited with virgin females. Recently, Naka et al. (2001) reported the mating behavior of this species in detail and confirmed that males are attracted to a crude extract of the pheromone glands. Although no female sex pheromones of species in the genus *Stathmopoda* have been identified, Nagano et al. (1979) reported attraction of one species (*S. auriferella*; formally, *S. theoris*) to hexadecenyl and hexadecadienyl acetates in a random screening test utilizing several synthetic lures known as lepidopteran pheromones. In general, the structures of the sex pheromones of taxonomically closely related species are similar (Arn et al., 1992, 1997). On the basis of this information, we analyzed the chemical structure of the sex pheromone secreted by *S. masinissa*, and successfully identified three compounds, including a unique conjugated diene system. In addition to structure determination of the pheromone components, we report the results of electrophysiological and laboratory bioassays with the synthesized pheromone.

METHODS AND MATERIALS

Insects. More than 2000 fifth instar larvae of *S. masinissa* were collected from several locations in Gifu and Nara Prefectures from June to August 1996–2002

and individually reared on sliced unripe persimmon fruit in 50 ml plastic cups under laboratory conditions (25 ± 1°C, 15L:9D) until emergence of the adults. Insects were sexed at the adult stage on the basis of their copulatory organs (Naka and Tsuchida, 2001). The male and female adults were separately placed in 200 ml plastic cups and kept under conditions identical to those for the larval and pupal stages. Adults were supplied *ad libitum* a nonalcoholic beverage, Pocari Sweat® (Ohtsuka Pharmaceuticals Co., Ltd.). Overwintering larvae were also collected in orchards in Gifu Prefecture from December to March. Larvae were held under laboratory conditions without food until the adults emerged.

Pheromone Extraction. One- to 5-d-old virgin females were anesthetized with CO_2 9 hr after lights-off (Naka et al., 2002), and extruded abdominal segments 8–10 were excised and extracted in *n*-hexane (ca. 1 μl/female equivalent (FE)) for 30 min. A portion of the crude extract was used for bioassays and chemical analysis without purification. Column chromatography of an aliquot of the extract (500 FE) was carried out on 200 mg of Florisil® (100–200 mesh, Floridin Co.) in a Pasteur pipette using successively 1 ml of *n*-hexane, 5%, 20%, and 50% ether in *n*-hexane as eluting solvents.

Chemicals. C_{16} and C_{18} alcohols (cetyl and stearyl alcohols) (Aldrich Chemical Co.) were acetylated with acetic anhydride in pyridine, or oxidized to the corresponding aldehydes by treatment with pyridinium chlorochromate (PCC) in CH_2Cl_2. (*Z*)-11-Hexadecenyl acetate (Aldrich Chemical) and (7*Z*,11*Z*)-7,11-hexadecadienyl acetate (Shin-etsu Chemical Co., Tokyo, Japan) were saponified with KOH in ethanol to yield the corresponding alcohols, which were oxidized to aldehydes by PCC oxidation. Bombykol ((10*E*,12*Z*)-10,12-hexadecadien-1-ol) and its derivatives had been synthesized previously (Ando et al., 1988). All geometric isomers of 4,6-hexadecadienyl compounds were synthesized using modifications to a previously described method for conjugated diene synthesis (Ando et al., 1985, 1988). Details of these syntheses were reported elsewhere (Nishida et al., 2003).

Analytical Instruments. The electroantennogram (EAG) activity of natural and synthetic pheromone components was determined with an HP-5890 Series II gas chromatograph (Hewlett-Packard, Wilmington, DE) coupled to an electroantennographic detector (GC–EAD, Struble and Arn, 1984). The GC was equipped with a capillary column (0.25 mm × 30 m, DB-23, J&W Scientific, Folsom, CA, or HP-5, Hewlett-Packard). The carrier gas was He. The oven temperature was maintained at 100°C for 2 min and programmed at 8°C/min to 240°C and held for 15 min. The column effluent was split between a flame ionization detector (FID) and the EAD at a ratio of 1:1. An antenna was excised at the base from a *S. masinissa* male and a few distal segments were cut off. Each end of the antenna was placed in a droplet of saline solution on the electrodes of the EAD device so that the sensilla faced the effluent flow from the GC. Each EAD analysis was replicated at least five times.

An HP-5973 mass spectrometer in electron impact (EI) mode (70 eV) interfaced to an HP-6890 gas chromatograph that was used to obtain mass spectra, using the same columns (DB-23 and HP-5), temperature program, and carrier gas as in GC–EAD analyses. The ion source temperature was 230°C. For the analysis of adducts formed by reaction of the conjugated diene pheromone components and 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) (Young et al., 1990), an HP-5 column was used, programmed from 120°C for 2 min, 10°C/min to 300°C, and held for 10 min.

Chemical Reactions with Pheromone Extracts. Catalytic hydrogenation of the 5% ether column chromatography fraction of the gland extract (100 FE) was carried out with Pd-$BaSO_4$ in *n*-hexane (0.5 ml) for 1 hr at room temperature. After filtration and concentration, the hydrogenated products were analyzed by GC–MS. Another aliquot of this fraction (100 FE) was treated with 1 N methanolic KOH (0.5 ml) for 1 hr at room temperature. After neutralization with 1 N HCl, the hydrolyzed products were extracted with *n*-hexane and analyzed by GC–MS. In addition, an aliquot of crude extract (100 FE) was dissolved in CH_2Cl_2 (50 μl) and treated with a CH_2Cl_2 solution of MTAD (1.0 mg/ml, 2.5 μl) for 1 hr at room temperature. After changing the solvent to *n*-hexane, the MTAD adducts were analyzed by GC–MS as described above. To determine the retention times (Rts) of the adducts, a mixture of synthetic 4,6-hexadecadienyl compounds (0.1 μg each) was treated with the MTAD solution in the same manner and the products analyzed.

Laboratory Bioassays. Each bioassay was replicated 3–5 times with groups of 9–13 male *S. masinissa* (2- to 6-d-old) released in a 200 ml plastic cup. Because the males were most responsive to crude extracts (Naka et al., 2002) from 8.5 to 10.5 hr after lights-off, the bioassay was started 9 hr after lights-off. Responses of the males to pheromone gland extracts and synthetic compounds were observed in darkness under a red light (10 lux). A piece of filter paper (3 × 30 mm) was loaded with each test material dissolved in hexane, and the biological activity was examined after evaporation of the solvent. Mating behavior was divided into four categories: vigorous antennal swing (AS), orientation flight (OF), mating dance (MD), and abdominal contact with the pheromone source (AC) (Naka et al., 2002).

RESULTS

GC–EAD Analysis of the Pheromone Extract. The results of the GC–EAD analysis of the crude extract (10 FE) on a DB-23 column are shown in Figure 1. The antennae of male *S. masinissa* were stimulated only by three components (A, B, and C). The amounts of these components were estimated to be about 20–50 pg/female from the FID peak areas. However, the exact ratios could not be defined because of large variations among extracts. In column chromatography of the extract on Florisil®, components A and B were eluted with 5% ether in hexane, and component C with 10% ether in hexane, indicating component C was more

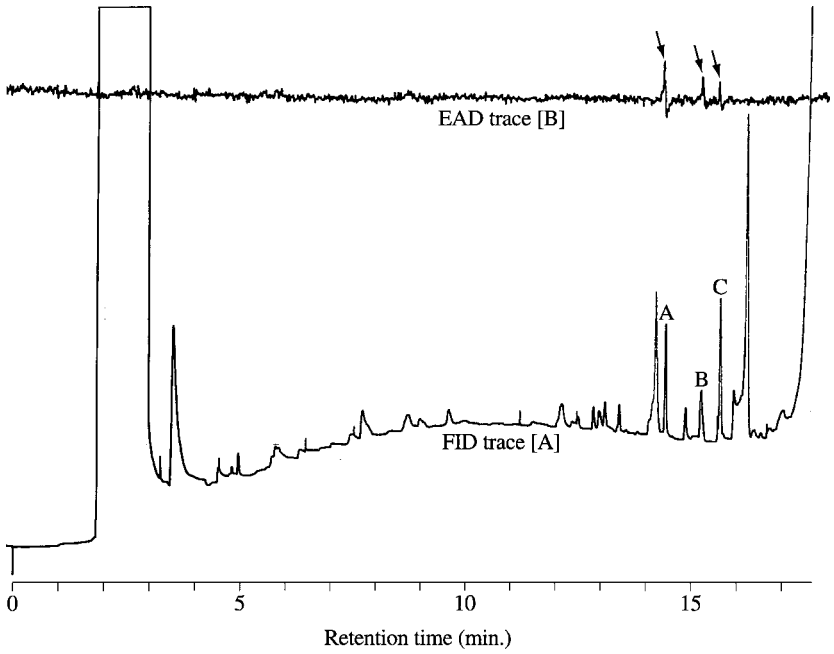

FIG. 1. GC analysis (DB-23) of a crude extract of the pheromone glands of *Stathmopoda masinissa* (100 FE) with a flame ionization detector (FID) (A) and an electroantennographic detector (EAD) (B). EAD responses of three components A–C are indicated by arrows.

polar than the others. Components A and B were tentatively designated as acetate and/or aldehyde, and component C as an alcohol, on the basis of the chromatographic behaviors of synthetic hexadecenyl and hexadecadienyl standards.

GC–MS Analysis of the Pheromone Extract and Synthetic 4,6-Dienes. The mass spectra of components A, B, and C are shown in Figure 2A, B, and C, respectively. In addition to the molecular ions (M^+), their characteristic fragment ions suggested that the three components were analogs with straight chain C_{16} dienes. Component A, with M^+ at m/z 236 (relative intensity: 13.8%) was tentatively assigned as a hexadecadienal, component B with M^+ at m/z 280 (6.4%) and $[M\text{-}60]^+$ at m/z 220 (19.9%) as a hexadecadienyl acetate, and component C with M^+ at m/z 238 (23.3%) and $[M\text{-}18]^+$ at m/z 220 (6.6%) as a hexadecadien-1-ol. Table 1 shows the Rts of the three insect-produced components and synthetic standards on DB-23 and HP-5 GC columns. Conjugated dienes elute later than isolated dienes from a GC column, particularly from the polar DB-23 column. The fact that the Rt of each component was considerably longer than that of a nonconjugated 7,11-hexadecadienyl standard with the comparable functional group indicated that

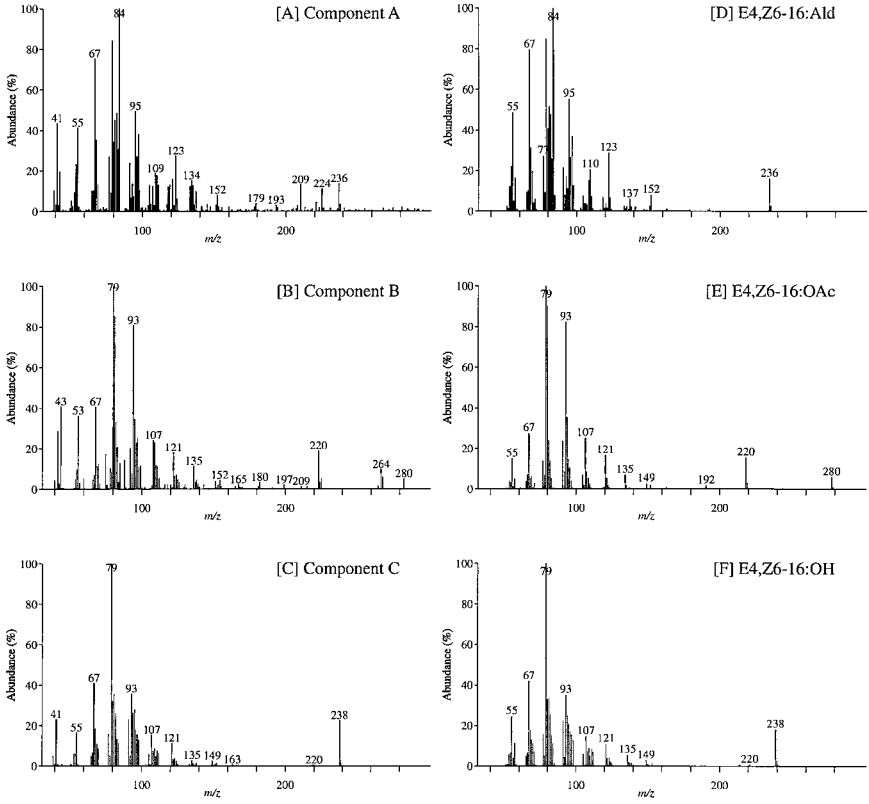

FIG. 2. Mass spectra of components A–C in a crude extract of the pheromone glands of *Stathmopoda masinissa* females (A–C) and those of synthetic (4*E*,6*Z*)-4,6-hexadecadienyl compounds (D–F).

the two double bonds in components A–C were conjugated. The Rts of the natural components were slightly different from those of the 10,12-dienes, suggesting that the dienes were in some other position.

EI mass spectra have been reported for a series of conjugated hexadecadienyl aldehydes, acetates, and alcohol including their diene systems at the 9,11- or higher positions (Ando et al., 1988), but none of them matched those of the components from *S. masinissa*. Whereas the base peaks of the synthetic dienes appeared at m/z 67, 81, or 95, those of components A–C appeared at m/z 84 or 79. The characteristic base peak at m/z 84 of component A might arise from formation of a 2,3-dihydropyranyl structure ($C_5H_8O^+$) formed by cyclization after hydrogen rearrangement and a bond cleavage between carbons 5 and 6 of a 4,6-diene aldehyde (Nishida et al., 2003). The four geometric isomers of the

TABLE 1. KOVÁTS RETENTION INDICES (RETENTION TIME, MIN) OF NATURAL PHEROMONE COMPONENTS OF *S. masinissa* AND SYNTHETIC STANDARDS ON DB-23 AND HP-5 GC COLUMNS[a]

	Natural component	C_{16} compound					C_{18} saturated compound
		Saturated	(*Z*)-11-Ene	(7*Z*,11*Z*)-Diene	(10*E*,12*Z*)-Diene	(4*E*,6*Z*)-Diene	
DB-23 column							
Aldehyde	2407 (comp. A)	2229 (11.59)	2278	2311	2445	2407 (13.23)	2452 (13.60)
Acetate	2506 (comp. B)	2344 (12.69)	2390	2422	2551	2505 (14.07)	2556 (14.50)
Alcohol	2552 (comp. C)	2366 (12.86)	2415	2451	2579	2551 (14.47)	2583 (14.74)
HP-5 column							
Aldehyde	1839 (comp. A)	1818 (14.43)	1806	1791	1857	1840 (14.71)	2021 (16.95)
Acetate	2022 (comp. B)	2010 (16.84)	1998	1982	2047	2022 (16.98)	2211 (19.11)
Alcohol	1903 (comp. C)	1881 (15.23)	1871	1857	1919	1904 (15.52)	2084 (17.70)

[a] Capillary column (0.25 mm ID × 30 m); temperature program: 100°C for 2 min, 8°C/min to 240°C, and 240°C for 15 min.

4,6-hexadecadienyl compounds were synthesized on the basis of this speculation. Figure 2D–F shows mass spectra of (4*E*,6*Z*)-4,6-hexadecadienal (E4,Z6-16:Ald), (4*E*,6*Z*)-4,6-hexadecadienyl acetate (E4,Z6-16:OAc), and (4*E*,6*Z*)-4,6-hexadecadien-1-ol (E4,Z6-16:OH), which matched those of the insect-produced compounds. The mass spectra of the four geometric isomers of each compound were similar, so the retention times were crucial in distinguishing the isomers. Only the (4*E*,6*Z*)-isomers exhibited the same Rts as the natural components.

Chemical Derivatization of Pheromone Components. After catalytic hydrogenation, GC–MS analysis of the 5% ether fraction indicated the production of hexadecyl acetate showing a characteristic $[M\text{-}60]^+$ at m/z 224 (Rt on a DB-23 column: 12.69 min), probably produced from component B. The partially purified extract used for this experiment also included component A, but the corresponding saturated aldehyde (hexadecanal) was not detected in the hydrogenated products. After saponification with 1 N methanolic KOH, only component B disappeared from the fraction that included components A and B, but the expected hexadecadien-1-ol derived from component B was not confirmed in GC–MS analyses. GC–MS analysis of the synthetic standards treated with an MTAD solution gave mass spectra of the MTAD adducts of E4,Z6-16:Ald (Rt on HP-5 column: 18.55 min), m/z (relative intensity) 349 (M^+, 5), 305 (100), 292 (35), 222 (49), of E4,Z6-16:OH (Rt: 19.19 min), m/z 351 (M^+, 12), 292 (100), 224 (85), and of E4,Z6-16:OAc (Rt: 19.84 min), m/z 393 (M^+, 25), m/z 292 (100), m/z 266 (26). These characteristic fragment ions unequivocally confirmed that the dienes of the original ccompounds were in the 4,6-position (Young et al., 1990). However, no MTAD adducts were detected in the analysis of the pheromone extract treated with solution.

GC–EAD Analysis of Synthetic 4,6-Dienes. The geometric isomers of each synthetic 4,6-hexadecadienyl compound were analyzed by GC–EAD. The (4*Z*,6*E*)-, (4*E*,6*Z*)-, (4*Z*,6*Z*)-, and (4*E*,6*E*)-isomers eluted in that order from the DB-23 and HP-5 columns, with the isomers being particularly well separated on the polar DB-23 column. Table 2 shows the Rts and EAG activity of each compound. Among the four isomers, the greatest activity was always observed for the (4*E*,6*Z*)-isomer. The EAG responses to the (4*E*,6*E*)-isomers were higher than those to the (4*Z*,6*E*)- and (4*Z*,6*Z*)-isomers, but weak in comparison to the activity of the (4*E*,6*Z*)-isomers.

Mating Behaviors Induced by Synthetic (4E,6Z)-4,6-Dienes. The activity of synthetic E4,Z6-16:Ald, E4,Z6-16:OAc, and E4,Z6-16:OH was examined using a laboratory bioassay. Table 3 shows the male responses to each of the individual components, their 1:1 and 1:1:1 mixtures, and a crude extract (5 FE). Among the synthetic compounds tested, E4,Z6-16:OAc as a single component had the greatest effect on males and was not significantly different from the the crude extract.

TABLE 2. CHROMATOGRAPHIC BEHAVIORS AND EAG ACTIVITY OF SYNTHETIC 4,6-DIENYL COMPOUNDS (10 ng) AND NATURAL PHEROMONE COMPONENTS (A–C, 10 FE)

Compound	Kováts RI (Rt, min)		Relative EAG (%)[b]
	DB-23[a]	HP-5[a]	
Aldehyde			
(4*Z*,6*E*)-Isomer	2402	1843	12 ± 5 c
(4*E*,6*Z*)-Isomer	2407	1839	74 ± 9 a
(4*Z*,6*Z*)-Isomer	2418	1861	10 ± 5 c
(4*E*,6*E*)-Isomer	2438	1872	28 ± 9 b
Natural (comp. A)	2406 (14.36)	1839 (16.23)	11 ± 4
Acetate			
(4*Z*,6*E*)-Isomer	2499	2025	21 ± 6 bc
(4*E*,6*Z*)-Isomer	2505	2023	100 a
(4*Z*,6*Z*)-Isomer	2420	2047	13 ± 7 c
(4*E*,6*E*)-Isomer	2549	2066	35 ± 11 b
Natural (comp. B)	2505 (15.23)	2023 (18.41)	38 ± 9
Alcohol			
(4*Z*,6*E*)-Isomer	2539	1905	13 ± 10 cd
(4*E*,6*Z*)-Isomer	2552	1904	64 ± 8 a
(4*Z*,6*Z*)-Isomer	2569	1925	9 ± 3 bc
(4*E*,6*E*)-Isomer	2586	1936	23 ± 9 b
Natural (comp. C)	2551 (15.57)	1904 (17.01)	26 ± 5

[a] Capillary column (0.25 mm ID × 30 m); temperature program: 100°C for 2 min, 8 °/min to 240°, and 240° for 15 min.

[b] Mean EAG activities (%) recorded from at least five antennae comparing relative to that of E4,Z6-16:OAc (0.16 ± 0.6 mV). Within the geometrical isomers possessing the same functional group, values followed by a different letter are significantly different at $P < 0.05$ (Tukey–Kramer test).

Dose Responses of Males to Synthetic E4,Z6-16:OAc. Table 4 shows the male responses to synthetic E4,Z6-16:OAc at seven different doses from 0.01 to 100 ng/paper. At doses of 0.01 and 0.1 ng/paper, the males did not respond. In all four behavioral categories, the percentages of the responses rose as the dose was increased to 10 ng/paper, and an abrupt increment was observed from 0.1 to 1 ng/paper. The activities of the synthetic pheromone components seemed to reach a peak around 5 or 10 ng/paper, although the overall activity was slightly lower than that of the crude extract.

DISCUSSION

Using GC–EAD, three distinct EAG-active components A–C were found in a crude extract of pheromone glands of *S. masinissa* females (Figure 1). Although the extract included a lot of impurities and the amounts of the components were

TABLE 3. MATING BEHAVIORS OF *S. masinissa* MALES INDUCED BY SYNTHETIC STANDARDS OF PHEROMONE COMPOUNDS

Composition of lure (ng)			Behavioral response (%, mean ± SE)[a]			
E4,Z6-16:Ald	E4,Z6-16:OAc	E4,Z6-16:OH	AS	OF	MD	AC
0	0	0	0e	0c	0c	0b
10	0	0	37.8 ± 3.9 cd	7.0 ± 6.1 bc	13.7 ± 15.2 abc	3.3 ± 5.8 ab
0	10	0	86.3 ± 5.5 ab	68.5 ± 12.3 a	37.8 ± 10.7 ab	16.7 ± 15.3 ab
0	0	10	38.2 ± 16.0 cd	3.7 ± 6.4 c	0c	0b
5	5	0	68.2 ± 20.5 abc	68.5 ± 12.3 a	30.4 ± 19.5 abc	13.3 ± 11.6 ab
5	0	5	23.7 ± 10.9 d	17.0 ± 5.1 bc	3.7 ± 6.4 bc	0b
0	5	5	44.8 ± 15.0 cd	68.5 ± 12.3 a	30.4 ± 19.5 abc	6.7 ± 11.6 ab
3.3	3.3	3.3	55.2 ± 5.0 bcd	34.8 ± 13.0 ab	3.7 ± 6.4 bc	3.7 ± 6.4 ab
Crude extract (5 FE)			98.3 ± 3.7 a	64.0 ± 11.7 a	45.2 ± 12.4 a	37.7 ± 10.2 a

[a] AS: vigorous antennal swing, OF: orientation flight, MD: mating dance, AC: abdominal contact on pheromone source. All tests were replicated at least three times. Values reported are means of the % response obtained from 9–13 males used in each replicate. Numbers in the same column with the same letters are not significantly different at the 5% level by Scheffé's test.

TABLE 4. MATING BEHAVIORS OF *S. masinissa* MALES INDUCED BY SYNTHETIC (4*E*,6*Z*)-4,6-HEXADECADIENYL ACETATE (E4,Z6-16:OAC, COMPONENT B)

Lure dose (ng)	Behavioral response (%, mean ± SE)[a]			
	AS	OF	MD	AC
0.01	3.3 ± 5.8 e	3.3 ± 5.8 c	0c	0b
0.1	14.1 ± 7.1 cd	7.0 ± 6.1 c	0c	0b
0.5	38.5 ± 17.8 cd	24.1 ± 5.3 abc	20.7 ± 10.1 ab	6.7 ± 11.6 ab
1	72.2 ± 6.9 bcd	47.4 ± 21.8 ab	30.7 ± 8.9 ab	13.7 ± 5.5 ab
5	75.6 ± 7.7 bc	54.4 ± 19.0 a	37.8 ± 3.9 ab	21.1 ± 18.4 ab
10	86.3 ± 5.5 ab	68.5 ± 12.3 a	37.8 ± 10.7 ab	16.7 ± 15.3 ab
100	41.1 ± 8.4 cd	27.8 ± 6.9 abc	17.4 ± 6.5 b	3.7 ± 6.4 ab
Crude extract (5FE)	98.3 ± 3.7 a	64.0 ± 11.7 a	45.2 ± 12.3 a	37.7 ± 10.2 a

[a] AS: vigorous antennal swing, OF: orientation flight, MD: mating dance, AC: abdominal contact on pheromone source. All tests were replicated five times. Values reported are mean % behavioral response produced by 10–13 males per replicate. Numbers in the same column with the same letters are not significantly different at the 5% level by Scheffé's test.

low, they were identified by a combination of mass spectral and retention time matches on two columns, and microchemical tests. The exact positions of the double bonds could not be experimentally confirmed from the MTAD adducts because the amounts were too low to yield a sufficient amount of the MTAD adducts for GC–MS analysis. Fortunately, the diagnostic base peak at m/z 84 of the aldehyde component suggested the possibility of a 4,6-diene. Among the four geometrical isomers of each 4,6-hexadecadienyl compound, only the (4*E*,6*Z*) isomer matched the GC–MS data of the insect-produced components. Further experiments confirmed that the base peak at m/z 84 was diagnostic only for 4,6-hexadecadienal, and that any other positional isomers with a conjugated diene system did not exhibit the base peak at m/z 84 (Nishida et al., 2003).

The strongest EAG response was also recorded for the (4*E*,6*Z*)-isomer among the geometric isomers of each compound, with the acetate yielding stronger responses than the corresponding aldehyde (E4,Z6-16:Ald) and alcohol (E4,Z6-16:OH) (Table 2). In a laboratory bioassay, only lures containing the acetate elicited responses significantly different from the control (Table 3). When presented with a filter paper treated only with E4,Z6-16:OAc, more than 20% of the tested males rubbed their abdomens against the paper (Table 4). The attraction of males to E4,Z6-16:OAc was also verified in a preliminary field bioassay: a sticky trap baited with a rubber septum impregnated with 0.6 mg E4,Z6-16:OAc caught 80 males from May 16 to 30, 2002 at a persimmon garden in Gifu Prefecture. The possible role of the aldehyde and alcohol as synergists is currently being investigated in field trials.

Lepidopteran sex pheromones have been chemically defined from more than 500 species, and compounds with a conjugated diene system in a C_{10}—C_{18} straight

chain are among the most important groups. The double bonds are usually located towards the hydrocarbon end such as with bombykol, a 10,12-diene with a C_{16}chain (Arn et al., 1992, 1997). 3,5-Dienes with a C_{12} or C_{14} chain have been identified as exceptions from a Cochylidae species, *Bonagota cranaodes* Meyrick (Unelius et al., 1996; Eiras et al., 1999; Coracini et al., 2001). However, the 4,6-diene structure is not entirely unique, because 4,6,10- or 4,6,11-trienols, acetates, and aldehydes have been identified as pheromone components of a Gracillariidae species, the cocoa pod borer moth (*Conopomorpha cramerella* Snellen) (Beevor et al., 1986), and from a Saturniidae species, the eri-silkworm (*Samia cynthia ricini* Donovan) (Bestmann et al., 1989). The Oecophoridae family, to which *S. masinissa* belongs, is taxonomically closer to the Gracillariidae than to Saturniidae. The structural similarities of the 4,6-dienes and 4,6,10-trienes suggests the possibility that they are produced by similar biosynthetic pathways, but confirmation awaits further research.

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FIELD TRIALS WITH THE SYNTHETIC SEX PHEROMONE OF THE OAK PROCESSIONARY MOTH *Thaumetopoea processionea*

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Abstract—The biological activity of synthetic (*Z,Z*)-11,13-hexadecadienyl acetate, the major pheromone component found in female gland extracts of the oak processionary moth *Thaumetopoea processionea*, was evaluated in field trials. Traps baited with 10 mg of the chemical efficiently attracted a large number of males provided they were placed in the upper crown region of the oaks. Devices positioned 10–15 m high in the trees attracted significantly more males than those traps installed at 2 or 6–8 m above the ground. Pherocon traps were slightly more efficient than Delta traps, and lower or higher amounts of the attractant in the baits did not significantly influence the number of moths caught. The importance of the stereomeric purity of the lure and the easy isomerization of the (*Z,Z*)-acetate to other isomers, particularly to the *E,E* isomer, should be considered for the development of efficient formulations in the field.

Key Words—Sex pheromone, oak processionary moth, *Thaumetopoea processionea*, Lepidoptera, Thaumetopoeidae, field trials, pheromone traps.

INTRODUCTION

The oak processionary moth *Thaumetopoea processionea* (L.) (Lepidoptera, Thaumetopoeidae) is a serious forest pest in many areas of western, southern,

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and central Europe. The larvae hatch during spring synchronously with sprouting oak leaves, develop within 2–3 months, and pupate in nests in early summer. The insect is univoltine, and the adults emerge in July–August, although the flight period is often restricted to a few weeks. In addition to the damage caused to oak trees, larvae disperse highly urticating microscopic hairs into the environment causing strong allergic reactions in animals and humans. Quite frequently, the skin and eyes are affected, and irritation of the respiratory mucous membranes can cause asthma symptoms and anaphylactic shocks (Lamy and Novak, 1987; Lamy, 1990). In public areas, therefore, control of the pest is mandatory, but thus far an efficient method to detect infested areas is lacking. In this regard, a promising way for monitoring the appearance and population of the pest might be through utilization of the sex pheromone.

In a previous paper, we reported the characterization of the sex pheromone of the pest as a mixture of (*Z,Z*)-11,13-hexadecadienyl acetate (**I**), (*E,Z*)-11,13-hexadecadienyl acetate (**III**), and (*Z,Z*)-11,13-hexadecadienol (**V**) (Figure 1) in a 88:7:5 ratio from female pheromone gland extracts (Quero et al., 2003). Acetate **I** and alcohol **V** had been previously reported as possible pheromone components (Frérot and Démolin, 1993), while Germinara et al. (2001) found compound I in gland extracts of females. However, no biological activity was disclosed. We found that in a wind tunnel the major compound **I** induced males to display all behavioral responses, and that (*E,E*)-11,13-hexadecadienyl acetate (**IV**) reduced the number of contacts with the source when mixed with the major compound in several ratios (Quero et al., 2003). In the field, although Tibery and Niccoli (1984) mentioned that traps baited with (*Z*)-13-hexadecen-11-ynyl acetate, the sex pheromone of the pine processionary moth *Thaumetopoea pityocampa*, also captured males of *T. processionea*, we (Breuer and De Loof, 1997) and others (Montoya and Hernández, personal communication, 1986) found that this compound was inactive in field trials carried out in Belgium and Spain, respectively. In this paper, we report a series of field tests conducted with the major compound I, as well as the effect of different parameters on the number of catches.

METHODS AND MATERIALS

Chemicals and Dispensers. The pheromone compounds were synthesized and encapsulated in polyethylene vials (3 × 1.1 cm i.d.) by Sociedad Española de Desarrollos Químicos (SEDQ, Barberá del Vallés, Barcelona, Spain). Most of the trials were done with baits containing the following composition: (*Z*,*Z*)-11,13-hexadecadien-1-yl acetate (**I**, 93.7%), (*Z*,*E*)-11,13-hexadecadien-1-yl acetate (**II**, 3.9%), (*E*,*Z*)-11,13-hexadecadien-1-yl acetate (**III**, 2.2%), and (*E*,*E*)-11,13-hexadecadien-1-yl acetate (**IV**, 0.1%) (Figure 1). To study the activity of the *E*,*E* isomer, a second formulation consisting of a mixture of (*E*,*E*)-acetate **IV**

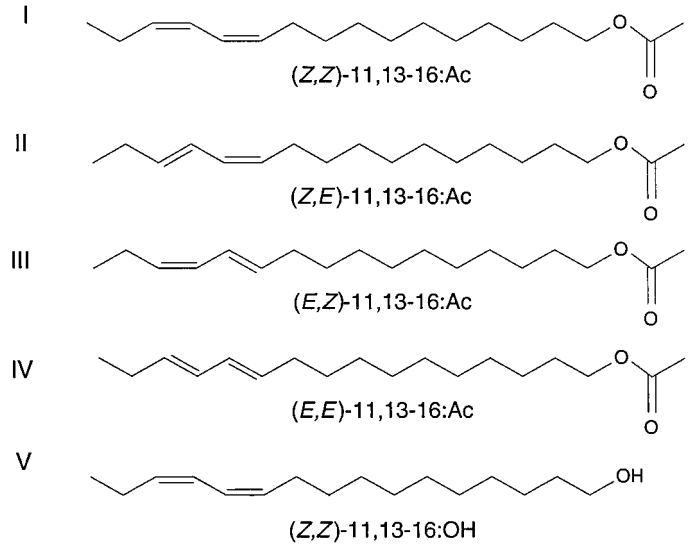

FIG. 1. Structures of pheromone compounds and analogues considered.

(72.8%), (*E*,*Z*)-acetate **III** (11.1%), (*Z*,*E*)-acetate **II** (3.1%), and (*Z*,*Z*)-acetate **I** (2.9%) was also tested. In both formulations, 10 mg of the major component, either the *Z*,*Z* or *E*,*E* isomer, and 1% of BHT (*t*-butylhydroxytoluene) as stabilizer, were encapsulated in polyethylene vials, packed in aluminum foil, and stored at -20°C until use. Stereochemical analyses were performed by GC either on a cross-linked HP-FFAP capillary column (25 m $\times$ 0.2 mm i.d., 0.33 μm) (Agilent Technologies, Barcelona, Spain) or on an HP-1 column (12 m $\times$ 0.2 mm i.d., 0.33 μm) (Agilent Technologies, Barcelona, Spain).

Pheromone Traps. All trials were conducted using Delta traps (12 cm high) (Servei de Protecció dels Vegetals, Generalitat de Catalunya, Spain) containing a nondrying adhesive base (10 $\times$ 10 cm) (Tangle-Trap Paste, The Tanglefoot Company, USA). In addition and to study the effect of other types of traps, Pherocon traps (10 cm high) (Trécé Inc., California, USA) with a sticky bottom (10 $\times$ 10 cm) were also used. All traps were provided by SEDQ (Barcelona, Spain).

Field Trials. The tests were carried out in three different oak forests near Genthin, Germany (NE of Magdeburg) from July 23 to September 12, 2001, in a region that had been highly infested in previous years by the oak processionary moth. The main purpose of the trials was the evaluation of the effects on catches of the following parameters: (a) trap height, (b) charge of dispenser, (c) trap design, (d) infestation level, and (e) pheromone components.

Most of the traps were suspended on oak trees along arbitrary lines through the forest to facilitate compilation of data. The distance between two adjacent traps

was 30–50 m, according to the forest structure, a distance considered sufficient to prevent one trap from "poaching" moths from the plume of the closest trap. Poaching is suppression of the trap catches occurring from effects of overlapping active spaces of other traps relative to the catches by a single isolated trap. For the first series of experiments, traps were installed at 2, 6–8, and 10–15 m above the ground. For the two lower heights, the traps were provided with hooks, which were hung on the tree branches either directly by hand or with the help of a telescopic bar. To reach the upper crown region (10–15 m high), a thread was shot over a branch. The trap was fixed at one end of the thread and lifted into the crown of the tree by pulling the other end. In this way, further control of these traps could be easily done. For the other experiments, traps were all installed in the upper crown region, since more individuals were caught at this maximum height (see below).

During the flight period, traps were inspected at intervals of 3–7 days, the moths were counted, and the adhesive bottom was renewed if necessary. Except for the experiment on the trap height, traps were rotated on each inspection to minimize the possible effects of their location in the forest.

To estimate the degree of infestation in the different study areas, the percentage of leaf mass consumed by *T. processionea* in at least 10 trees randomly selected in each area was determined and averaged relative to healthy unattacked trees.

RESULTS AND DISCUSSION

In the three study areas, the flight periods of the moths were similar. Most males were caught in the period July 24 – August 29, with a peak of catches during the 1st week of August. It is worth noting that in the more open stands, the maximum number of catches occurred earlier than in the more closed forest areas (August 2–5 vs. August 8–9, respectively). The temperature in all areas ranged from 10 to 36°C through the season.

The position of the traps on the trees had a noteworthy effect on the number of males caught. Devices positioned at the upper crown of the tree (10–15 m) attracted more males than did those installed at 6–8 or 2 m. The difference between the lowest and highest elevation was significant at $P < 0.05$ (Mann–Whitney U test) (Figure 2). These are important findings for practical application. At least two possibilities may explain these results. First, males may preferentially fly at the highest altitude of the trees. Leafy twigs may play a role in orientation. It has been observed that males fly to and around such structures, and traps attach to foliated branches attract more moths than those on brittle branches (H.-G. Kontzog and M. Breuer, unpublished). Second, in a forest, wind intensity usually increases with height and, therefore, more pheromone molecules may be perceived by the male antennal receptors orienting males to the traps. In a few other pest insects, an improvement of catches by placing traps in or even over the top of trees has

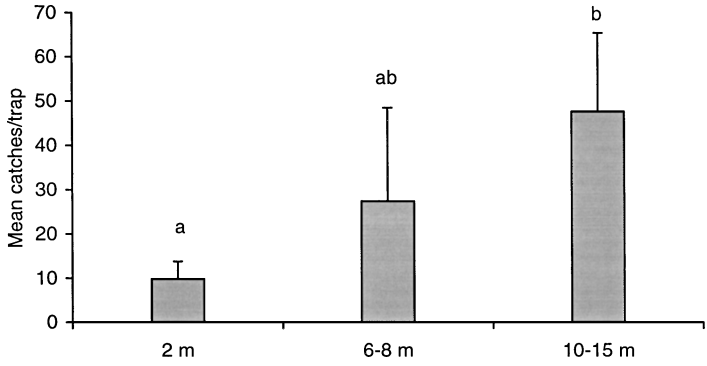

FIG. 2. Mean number of catches of *Thaumetopoea processionea*♂♂ (±SD) in Delta traps placed at different heights on the tree. The dispensers were loaded with 10 mg of (*Z*,*Z*)-11,13-hexadecadienyl acetate (**I**) (stereochemical purity: *Z*,*Z* 93.7%; *E*,*E* 3.9%; *E*,*Z* 2.2%; *E*,*E* 0.1%). Similar letters on top of the bars indicate no significant differences at $P < 0.05$ (Mann–Whitney *U* test).

been reported. Pasqualini et al. (1992, 1999) found that the level of capture of the leopard moth *Zeuzera pyrina* was improved by setting the traps at least 1 m above the apple or pear orchard. The authors speculated that in this position males may find it easier to locate traps, and at the same time competition with females may also be lower than when traps are placed inside the orchard. Similarly, Riedl et al. (1979) demonstrated that male codling moth catches are highest when traps were positioned near the top of the apple tree canopy.

Increasing or decreasing the load (5, 10, and 15 mg) of the major component **I** had no effect on the number of male catches (data are not shown). On the other hand, Pherocon traps worked more efficiently (twofold) than Delta traps, catching 59 males/trap vs. 28 males/trap, respectively. The difference was significant at $P < 0.05$ (Mann–Whitney *U* test) (Figure 3). It is well known that trap size and shape is one of the critical parameters for a given active formulation to achieve an efficient level of male captures and, consequently, a more successful competition with virgin females (Cardé and Elkinton, 1984; Ridgway et al., 1990).

There was a clear relationship between the level of infestation (measured as defoliation caused by the larvae) and the number of moths caught (Figure 4). Traps in the center of an infested forest area attracted more males than those hanging in areas slightly damaged by the pest. The level of catches diminished when traps were placed 50–100 m away from the infested area, suggesting that the attractive range of the pheromone preparation is apparently limited. This is in contrast with the close species, *T. pityocampa*, the pine processionary moth, which was attracted to pheromone even at 1400 m away from the pine grove (A. Shani, unpublished). It is possible that, under summer conditions, the easy isomerization

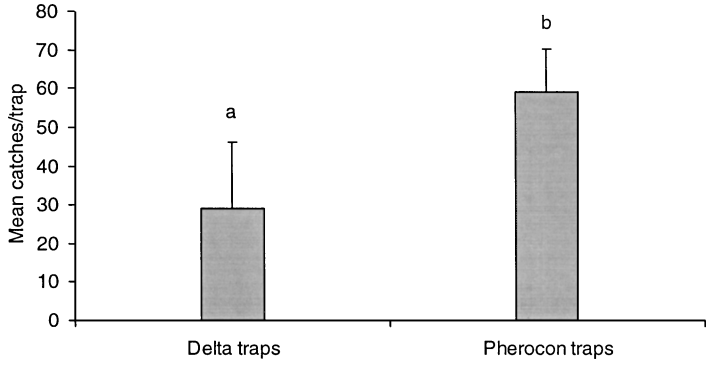

FIG. 3. Mean number of catches of *Thaumetopoea processionea* ♂♂ (±SD) in Delta and Pherocon traps baited with 10 mg of (*Z*,*Z*)-11,13-hexadecadienyl acetate (**I**) (stereochemical purity: *Z*,*Z* 93.7%; *Z*,*E* 3.9%; *E*,*Z* 2.2%; *E*,*E* 0.1%). Different letters on top of the bars indicate significant differences at $P < 0.05$ (Mann–Whitney *U* test).

and relatively low thermal stability of the *Z*,*Z*-conjugated system in isomer **I** of the oak processionary moth pheromone, in contrast to the much more stable enyne group present in the pine processionary pheromone, may account for the difference in attractiveness and potency of both attractants. In this regard, in wind tunnel experiments, the *E*,*E* isomer (**IV**), the most stable isomer into which the active *Z*,*Z* isomer can degrade, has been found to be an inhibitor of contacts with the source when males are subjected to mixtures of compounds **I** and **IV** in 1:1 and 1:10 ratios in comparison to the activity of compound **I** alone (Quero et al., 2003).

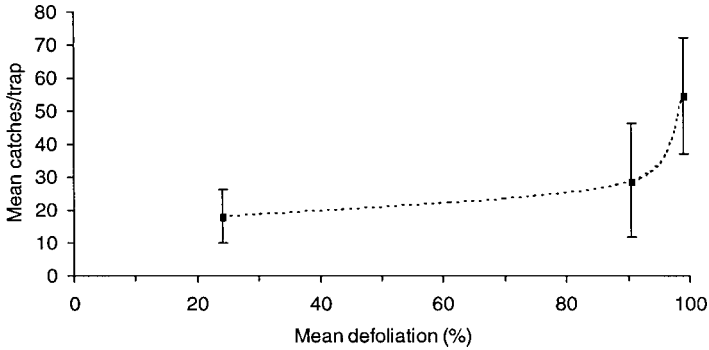

FIG. 4. Relationship between the percent of defoliation in the study areas caused by *Thaumetopoea processionea* larvae and the number of catches per trap (±SD). Delta traps baited with (*Z*,*Z*)-11,13-hexadecadienyl acetate (**I**) (stereochemical purity: *Z*,*Z* 93.7%; *Z*,*E* 3.9%; *E*,*Z* 2.2%; *E*,*E* 0.1%) were used.

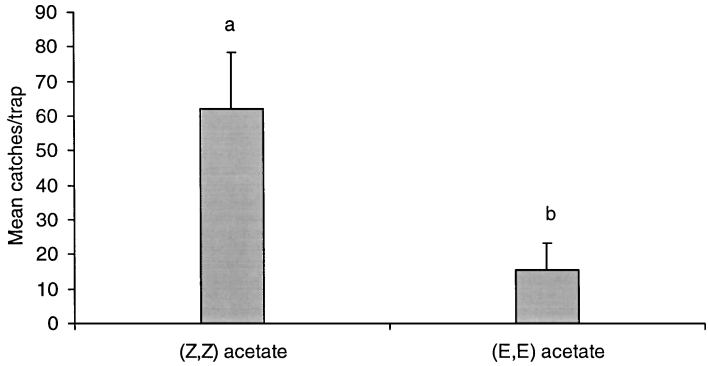

FIG. 5. Mean number of catches of *Thaumetopoea processionea*♂♂ (±SD) in Delta traps loaded with (*Z*,*Z*)-11,13-hexadecadienyl acetate (**I**) (stereochemical purity: *Z*,*Z* 93.7%; *Z*,*E* 3.9%; *E*,*Z* 2.2%; *E*,*E* 0.1%) and (*E*,*E*)-11,13-hexadecadienyl acetate (**IV**) (stereochemical purity: *E*,*E* 72.8%, *E*,*Z* 11.1%, *Z*,*E* 3.1%, *Z*,*Z* 2.9%). Different letters on top of the bars indicate significant differences at $P < 0.05$ (Mann Whitney *U* test).

To confirm the importance of the stereomeric purity of the lure, the activity of traps baited with a formulation enriched with the *E*,*E* isomer (stereochemical purity: *E*,*E* 72.8%; *E*,*Z* 11.1%; *Z*,*E* 3.1%; *Z*,*Z* 2.9%) was compared with that of the standard formulation. While the former attracted on average only 16.0 males/trap, the standard lure caught 62.2 males/trap (Figure 5). These data confirm the importance of the stereomeric purity of the pheromone, and the very low activity, if any, of the *E*,*E* isomer (the low content (2.9%) of the *Z*,*Z* isomer present in the second formulation may be responsible for the number of males caught). Because of the chemical and thermally easy interconversion of the isomers, the possible inhibitory effect of the *E*,*E* isomer in the field has not been tested, but, nevertheless, the results from the wind tunnel suggest that stereomerically pure *Z*,*Z* isomer is advisable for an efficient formulation, and also that measures (e.g., stabilizers, UV-protecting agents) should be taken to prevent isomerization to the *E*,*E* isomer under field conditions.

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(*S*)-2,3-DIHYDROFARNESOIC ACID, A NEW COMPONENT IN CEPHALIC GLANDS OF MALE EUROPEAN BEEWOLVES *Philanthus triangulum*

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Abstract—The chemical composition and functional significance of pheromones of solitary Hymenoptera is much less well known compared to social species. Males of the genus *Philanthus* (Sphecidae) are territorial and scent mark their territories to attract females. Because of inconsistent results of earlier studies, we reanalyzed the content of the cephalic glands of male European beewolves, *Philanthus triangulum* F. Besides a variety of alkanes and alkenes, four major compounds were found. Two of these, (Z)-11-eicosen-1-ol and (Z)-10-nonadecen-2-one, had been previously described as constituents of the cephalic glands. We identified 1-octadecanol as a new component of the cephalic gland, and a fourth compound, enantiopure (S)-2,3-dihydrofarnesoic acid, was identified for the first time in nature. Structural elucidation and enantiomeric analysis were performed by HRGC-MS and HRGC-FTIR as well as enantioselective gas chromatography and by means of authentic reference compounds. Occurrence and function of the four compounds in insect chemistry are discussed.

Key Words—*Philanthus triangulum*, Sphecidae, Hymenoptera, scent marking, pheromone, cephalic gland, (*S*)-2,3-dihydrofarnesoic acid, (*Z*)-11-eicosen-1-ol, (*Z*)-10-nonadecen-2-one, 1-octadecanol, HRGC-MS, HRGC-FTIR, enantioselective gas chromatography.

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INTRODUCTION

Although pheromone communication has been extensively studied in social Hymenoptera with regard to chemical composition, functional significance, and evolutionary aspects (reviewed by Vander Meer et al., 1998; Ayasse et al., 2001), much less is known about chemical communication in solitary Hymenoptera. Solitary wasps and bees exhibit a great complexity in the chemical composition of their glandular secretions, but only a few sex pheromones have been identified completely and little is known about their evolution (Ayasse et al., 2001). Knowledge of chemical communication in solitary Hymenoptera might provide information about the situation in the predecessors of the highly evolved chemical communication of social species.

Many species of the Philanthinae, a subfamily of the Sphecidae (digger wasps), exhibit an unusual mating system among Hymenoptera (Thornhill and Alcock, 1983; Evans and O'Neill, 1988; Strohm and Lechner, 2000). Male philanthine wasps establish small territories, where they scent mark plants or other structures with cephalic secretions. Receptive females approach territories and most matings take place in the territory or on nearby vegetation (Evans and O'Neill, 1988). The volatiles probably attract receptive females and provide species recognition cues (Simon Thomas and Poorter, 1972; Alcock, 1975; Gwynne, 1978; O'Neill, 1979, 1983; Schmidt et al., 1985; Evans and O'Neill, 1988; Strohm, unpublished observations).

Previous studies on the male cephalic secretions of species of the philanthine genera *Eucerceris* and *Philanthus* have shown a broad variety of components (Schmidt et al., 1985; McDaniel et al., 1987, 1992; Clarke et al., 2001). The content of the marking glands of male European beewolves, *Philanthus triangulum* F. (Hymenoptera, Sphecidae), has been studied twice (Borg-Karlson and Tengö, 1980; Schmidt et al., 1990). These two studies reported completely different compositions. Therefore, we reanalyzed the cephalic glands of male European beewolves. Our analysis of a population in central Germany revealed considerable differences to both of these earlier studies. Furthermore, we identified a component that was hitherto unknown from secretions of any animal species.

METHODS AND MATERIALS

Insects and Sampling. Beewolf males were obtained from a field population nesting in the vicinity of the Biocenter of the University of Würzburg or from a laboratory population reared under controlled conditions (see Strohm and Linsenmair, 1997a,b; Strohm et al., 2001, for more details on the study site and rearing conditions). The males were killed and stored in a freezer at -20°C. Since anatomical analyses (E. Strohm, unpublished data) suggest that the responsible

gland in European beewolf males is not a mandibular gland (as suggested, e.g., in Evans and O'Neill, 1988), we use the term *cephalic gland*. Three methods of extraction were used. First, the large cephalic glands were dissected and extracted in distilled hexane or dichloromethane for 4 hr. Second, entire heads of beewolf males were extracted in the same way. Known amounts of octadecane were added to all samples to provide an internal standard. Third, SPME fibres (SUPELCO, Deisenhofen, Germany; coated with a 100-μm polydimethylsiloxane film) (Arthur and Pawliszyn, 1990) were loaded by drawing the fibers through dissected cephalic glands.

Capillary Gas Chromatography – Mass Spectrometry (HRGC-MS). HRGC-MS was performed with a Fisons Instruments GC 8000 Series gas chromatograph (Fisons, Egelsbach, Germany) coupled to a Fisons Instruments MD 800 quadrupol mass detector. The GC was equipped either with a J & W DB-5 fused silica capillary column (30 m $\times$ 0.25 mm ID; df = 0.25 μm; J & W, Folsom, CA, USA; temperature program: from 60 to 310°C at 5°C/min and held for 10 min at 310°C), or with a J & W DB-1 fused silica capillary column (30 m $\times$ 0.25 mm ID; df = 0.25 μm; J & W; temperature program: from 60 to 150°C at 10°C/min, from 150 to 350°C at 5°C/min and held for 10 min at 350°C). Helium was used as carrier gas at a constant pressure of 90 kPa. Injection was carried out at 250°C in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV and a source temperature of 220°C.

Chemical ionization mass spectra (CI-MS) were obtained on the same GC-MS system using the J & W DB-5 column (temperature program as described above). Iso-butane with a pressure of 1 bar was used as ionization gas, and the source temperature was 150°C.

The software Xcalibur (ThermoFinnigan, Egelsbach, Germany) for windows was used for data acquisition.

Capillary Gas Chromatography – Fourier Transform Infrared Analysis (HRGC-FTIR). HRGC-FTIR spectra were obtained using an HP 5890 GC (Agilent Technologies, Böblingen, Germany) coupled to an FTS 575C Tracersystem (BioRad, Hercules, CA, USA). GC separation was performed using a DB-1 capillary column (30 m $\times$ 0.25 mm ID; df = 0.25 μm; J & W Scientific, Folsom, CA, USA). Temperature was programmed from 80 to 270°C with 4°C/min heating rate. Helium was used as carrier gas with a constant flow of 1–2 ml/min. Injection was carried out using a split/splitless injector at 250°C in the splitless mode for 60 sec. Injection volume was 0.1 μl.

IR spectra were recorded by scanning 256 times in a frequency range from 4000 to 700 cm^{-1} with a resolution of 1 cm^{-1}. Data system was a Dell Optiplex GX110-PC with BioRad WinIR Pro (Version 2.7) Tracer Software and Sadtler IRSearchMaster.

Enantioselective Capillary Gas Chromatography (Enantio-HRGC). Enantio-HRGC was carried out with a Carlo Erba 5160 GC using a fused silica capillary

column coated with 30% 2-methyl-3-ethyl-6-di-O-*t*-butyldimethylsilyl-β-cyclodextrin in (85–88%) dimethyl-(12–15%)-diphenylsiloxane copolymer silanol terminated (PS086) (25 m $\times$ 0.25 mm ID; df = 0.15 μm; temperature program from 50 to 160°C at 2°C/min and from 160 to 240°C at 5°C/min). Split injection (1:20) and an injector temperature of 230°C were employed. Hydrogen was used as carrier gas with an average linear velocity of 50 cm/sec. The temperature of the FID detector was 250°C. Samples of authentic racemic and (*R*)-methyl 2,3-dihydrofarnesoate (Ho and Millar, 2001a,b) as well as a methylated sample of beewolf head extract in hexane were analyzed.

Chemicals. Solvents (Fluka, Deisenhofen, Germany) were distilled and checked for purity by GC-MS prior to use. 11-Eicosen-1-ol was purchased from ICN Biomedicals (Irvine, CA, USA), and 1-octadecanol as well as the alkanes (C18 to C30) were purchased from Aldrich (Deisenhofen, Germany). Racemic methyl 2,3-dihydrofarnesoate and pure (*R*)-methyl 2,3-dihydrofarnesoate were kindly provided by Jocelyn G. Millar (Ho and Millar, 2001a,b). 10-Nonadecen-2-one was synthesized (see below).

Synthesis of (Z)-10-Nonadecen-2-one. This compound was synthesized from oleic acid and methyl lithium (Fluka, Deisenhofen, Germany) as described by Bestmann et al. (1975). To a solution of 5 g oleic acid in diethyl ether, 2 g methyl lithium in diethyl ether was added slowly under nitrogen at 0°C and stirred for 4 hr. The reaction mixture was diluted with 5% sulfuric acid. After removing the organic layer, drying over sodium sulfate, filtering, and evaporating the solvent, the residue was distilled. EI-MS (70 eV): m/z (%) 41 (67), 43 (100), 55 (87), 71 (87), 82 (63), 96 (61), 111 (26), 125 (42), 135 (13), 149 (7), 184 (4), 198 (3), 222 (3), 262 (2), 280 (2).

Dimethyl Disulfide (DMDS) Derivatizations. DMDS derivatization was carried out to determine the position of double bonds according to the method of Dunkelblum et al. (1985).

Methylation. A hexane solution of a beewolf male head extract was carefully evaporated under a stream of nitrogen to dryness and redissolved in 50 μl methanol. Then, 50 μl trimethylsulfonium hydroxide (TMSH) (Aldrich) was added, and 1 μl of the mixture was injected into the GC without further treatment.

RESULTS

Qualitative differences among the extracts made with hexane, dichloromethane, or SPME fibers were not found. We also did not find differences between males from the field population and those reared in the laboratory. However, the contents of the cephalic glands differed somewhat among individual males (Herzner et al., unpublished data). In the following, minor components that were found only in some males will not be reported. The heads of 25 males from the laboratory population were individually analyzed. A typical total ion

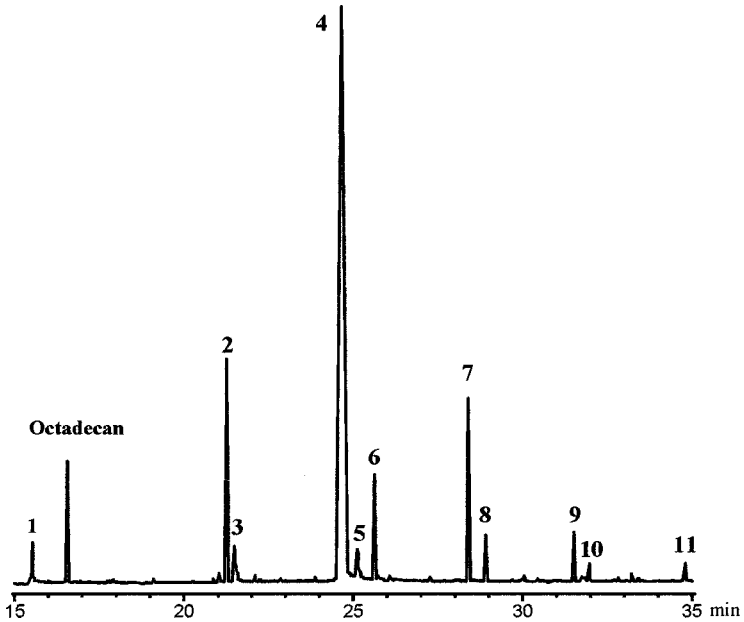

FIG. 1. Gas chromatogram of a hexane extraction of the cephalic gland of an individual beewolf male (**1** = (*S*)-2,3-Dihydrofarnesoic acid, **2** = (*Z*)-10-nonadecen-2-one, **3** = 1-octadecanol, **4** = (*Z*)-11-eicosen-1-ol, **5** = (*Z*)-9-tricosene, **6** = tricosane, **7** = (*Z*)-9-pentacosene, **8** = pentacosane, **9** = (*Z*)-9-heptacosene, **10** = heptacosane, **11** = nonacosane).

chromatogram of an hexane extract of the glands of an individual male is shown in Figure 1. The mean (± SD) total amount of the entire pheromone was 353 ± 167 μg. The mean relative amount of each constituent and its standard deviation is given in Figure 2.

(S)-2,3-Dihydrofarnesoic Acid. Compound **1** was identified as methyl 2,3-dihydrofarnesoate after methylation with TMSH by comparing the chromatographic and GC-MS as well as the GC-FTIR data with that of an authentic reference: *m*/*z* (%): 41 (71), 55 (16), 59 (15), 69 (100), 81 (18), 95 (18), 109 (56), 123 (18), 151 (2), 177 (6), 209 (18). In the untreated extract, this compound showed the following EI-MS data: *m*/*z* (%): 41 (41), 55 (12), 69 (100), 81 (12), 95 (11), 109 (32), 123 (20), 135 (2), 151 (1), 177 (2), 195 (19), 223 (1). CI-MS confirmed the molecular mass of 238. HRGC-FTIR analysis revealed 1704 (−COOH) and 987 (*trans* band) (Attygalle et al., 1995). The stereochemistry was determined by HRGC enantioseparation of the methylated racemate (Bicchi et al., 2002). The first eluted peak was the (*R*)-enantiomer according to an authentic (*R*)-reference (Ho and Millar, 2001a,b). The methylated gland extract contained exclusively

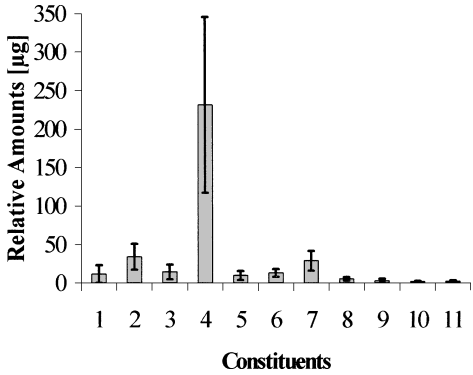

FIG. 2. Relative amounts of the constituents of the male cephalic secretion of *Philanthus triangulum*, means (±SD) of 25 beewolf head extracts. Numbers of components are the same as in Figure 1.

the methyl (*S*)-2,3-dihydrofarnesoate. Thus, compound **1** of the beewolf male pheromone was identified as enantiomerically pure (*S*)-2,3-dihydrofarnesoic acid (Figure 3).

(Z)-10-Nonadecen-2-one. Peak **2** of the cephalic gland extract was identified as (*Z*)-10-nonadecen-2-one. After derivatization with DMDS, it showed characteristic EI-MS peaks at 173 and 201 and M^+ at 374. The configuration of the double bond was determined by HRGC-FTIR (Attygalle et al., 1994). GC-FTIR: 3001 (*cis* compound of RCH═HCR′), 1703 (—C═O), 721 (*cis* compound of RCH═HCR′).

1-Octadecanol. (**3**) *m*/*z* (%): 41 (62), 43 (82), 55 (100), 69 (88), 71 (41), 83 (97), 97 (84), 111 (42), 125 (19), 139 (6), 154 (3), 168 (2), 182 (1), 196 (1), 224 (2), 252 (1).

(Z)-11-Eicosen-1-ol. The major compound **4** in the extract was identified as (*Z*)-11-eicosen-1-ol. The position of the double bond was determined after DMDS derivatization. M^+ of the derivatized compound was 390, and the major mass peaks were found at 173 and 217. The GC-FTIR data of the extract revealed the geometry of the double bond (Attygalle et al., 1994). *m*/*z* (%): 41 (58), 43 (45), 55 (99), 67 (61), 69 (65), 82 (100), 96 (75), 109 (30), 123 (17), 138 (9), 152 (4), 166 (2), 180 (1), 222 (1), 250 (1), 278 (3). GC-FTIR: 3326 (O—H), 3001 (*cis* compound of RCH═HCR′), 1054 (—CH_2—OH), 721 (*cis* compound of RCH═HCR′).

Alkanes. All alkanes were identified by comparing retention times and mass spectra of the beewolf head extracts with a mixture of purchased alkanes.

(Z)-Alkenes. The corresponding alkenes were identified by their typical mass spectra. The position and the geometry of the double bond was determined by the

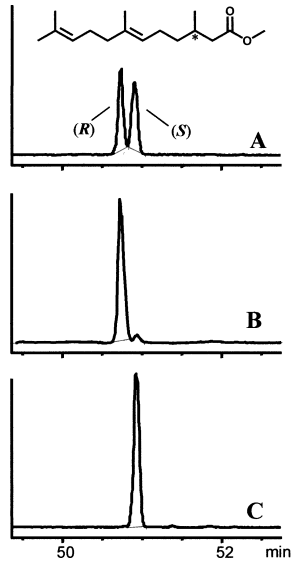

FIG. 3. Assignment of the absolute configuration of methyl 2,3-dihydrofarnesoate by enantioselective gas chromatography. (A) racemic, (B) synthetic methyl (*R*)-, and (C) methyl (*S*)-2,3-dihydrofarnesoate from male beewolf cephalic secretion.

same methods as described above. All alkenes have a double bond at position 9 with *cis* configuration.

DISCUSSION

The cephalic glands of *Philanthus triangulum* males from a population at the Biocenter in Würzburg contain a complex mixture of at least 11 components. The major compound is (*Z*)-11-eicosen-1-ol, which is consistent with a previous study on beewolf males of a population from France, South of Bordeaux (Schmidt et al., 1990). Also in agreement with this study, we found (*Z*)-10-nonadecen-2-one. However, contrary to Schmidt et al. (1990), we did not find any nonadecenal, eicosenal, octadecenoic, or octadecanoic acid in the extracts. Another study, which was done on head extracts of a population from Öland, Sweden, found 2,5-dimethyl-3-propylpyrazine and 2,5-dimethyl-3-isopentylpyrazine in both males and females (Borg-Karlson and Tengö, 1980). We did not find these compounds either. The different results of these three studies might be partly due to different methods or might reflect differences among populations. Quantitative or qualitative differences in pheromone compositions among populations are known from several species of Lepidoptera (Löfstedt et al., 1986; Hansson et al., 1990; Toth et al., 1996; Kawazu et al., 2000), click beetles (Coleoptera, Elateridae) (Yatsynin et al., 1996), and the European pine sawfly (Hymenoptera, Diprionidae) (Anderbrant et al., 2000).

Schmidt et al. (1990) found another six compounds including three not further specified hydrocarbons. Detailed examination was not carried out because these compounds were ubiquitously found in extracts of head, thorax, and abdomen of male and female wasps. The dissected cephalic glands analyzed in our studies contained a variety of alkanes and alkenes from C23 to C29 (Figure 1).

The major compound (*Z*)-11-eicosen-1-ol (**4**) is well known from the honey bee, *Apis mellifera*. Its function as a component of the alarm pheromone and its attractiveness to conspecifics has already been shown (Free et al., 1982, 1983; Pickett et al., 1982). Its occurrence in honeybees, which represent the exclusive prey of females of the European beewolf, as well as in the gland of male beewolves, might have implications for the evolution of the male sex pheromone of *P. triangulum* (Schmitt et al., unpublished data). Furthermore, (*Z*)-11-eicosen-1-ol is a major component of the venom of *Apis cerana* (Schmidt et al., 1997) and has been detected in the thoracic glands of male carpenter bees (*Xylocopa micheneri*) (Andersen et al., 1988). In the two latter cases, the function of (*Z*)-11-eicosen-1-ol is not known.

(*Z*)-10-Nonadecen-2-one (**2**) has only been found twice in arthropodes. It has been described as a trace constituent isolated from lipid fractions of the total extract of the ant *Iridomyrmex humilis* (Cavill et al., 1980) and as a component of the defensive secretion of the New Zealand tenebrionid beetle *Uloma tenebrionides* (Gnanasunderam et al., 1985).

The newly identified compound (*S*)-2,3-dihydrofarnesoic acid (**4**) has not been definitively identified in nature before. The occurrence of 2,3-dihydrofarnesoic acid was described from trichomes of *Lycopersicon hirsutum*, a wild relative of the tomato, but its stereochemistry was not established (Snyder et al., 1993). Interestingly, the methyl ester of (*R*)-2,3-dihydrofarnesoic acid is a component of the male sex pheromones of the stink bug species *Chlorochroa ligata*, *C. uhleri*, and *C. sayi* (Ho and Millar, 2001 a,b). Closely related substances such as 2,3-dihydrofarnesal and 2,3-dihydrofarnesol have been found in secretions from labial glands of males of several bumblebees (*Bombus*) and are used for scent marking, possibly to attract mates (Bergström et al., 1967; Bergström and Svensson, 1973; Svensson and Bergström, 1977, 1979; Bergman and Bergström, 1997).

1-Octadecanol (**3**), another new compound in the cephalic glands of the European beewolf, is known as a minor constituent of the glands of the congener *P. barbatus*, a species from North America. This alcohol was also found as a component of the alarm pheromone of *A. mellifera* (Free, 1987; Free et al., 1989) and in the venom of *A. cerana* (Schmidt et al., 1997).

The classes of compounds found in the cephalic glands of male European beewolfs (alcohols, a terpenoid, ketones, alkanes, alkenes) differ somewhat from those in males of several North American *Philanthus* species. The evolutionary significance of this difference is not yet understood. Assuming the pheromone provides species recognition cues, one would even expect stronger differences

among the North American species that often occur sympatrically than between these and the European beewolf that is the only representative of the genus in most of its geographical range (e.g., Ayasse et al., 2001; Borg-Karlson et al., 2003).

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OLFACTORY SENSITIVITY OF THE GILTHEAD SEABREAM (*Sparus auratus* L) TO CONSPECIFIC BODY FLUIDS

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Abstract—The potential for intraspecific chemical communication in the gilthead seabream (a marine perciform) was investigated by assessing the olfactory sensitivity to conspecific body-fluids (water occupied by conspecifics, intestinal fluid, urine, semen, egg fluid) by multiunit electrophysiological recording from the olfactory nerve. The olfactory system was responsive to water previously occupied by conspecifics, and the active compound(s) could be extracted by solid-phase extraction. The olfactory system was extremely sensitive to body fluids of sexually mature conspecifics: thresholds of detection were $1{:}10^{7.4}$ (intestinal fluid), $1{:}10^{6.1}$ (gametes), and $1{:}10^{4.2}$ (urine). The olfactory system was also sensitive to amino acids with thresholds of detection from $10^{-8.1}$ M (L-leucine) to $10^{-6.1}$ M (L-phenylalanine). However, a range of other known fish odorants (steroids, bile acids, and prostaglandins) failed to evoke significant responses. Given the high olfactory sensitivity to intestinal fluid and the low urine release rates of marine compared with freshwater fish, we suggest that chemical communication is likely to be mediated *via* compounds present in the intestinal fluid rather than urine. Furthermore, the types of chemicals involved are likely to be different from those of freshwater fish. Their exact chemical identity and biological roles remain to be established.

Key Words—Chemical communication, marine, teleost, urine, intestinal fluid, gametes, olfaction, seabream, electrophysiology.

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INTRODUCTION

Chemical communication is generally accepted to play many important roles in various aspects of fish physiology and behavior such as spawning synchrony (Sorensen et al., 1998), gonadal priming (Stacey et al., 1989), and the alarm response (Smith, 1992). However, the vast majority of evidence to date has been obtained from freshwater species. To assess the potential for chemical communication in a marine teleost, this study investigated the olfactory sensitivity of gilthead seabream to water previously containing conspecifics and conspecific body fluids.

Despite their biological diversity and economic importance, very little is known about olfaction in marine teleosts. Those studies that have investigated sensitivity of the olfactory system of seawater fish have focussed almost exclusively on the detection of amino acids (Goh et al., 1979; Goh and Tamura, 1980; Kobayashi and Goh, 1985; Ishida and Kobayashi, 1992). In common with freshwater species (Hara, 1994; Sorensen and Caprio, 1998), these fish are able to detect amino acids at concentrations down to about 10^{-9} M. Several studies have implicated olfaction as being important in several types of behavior, chiefly food-searching (e.g., Knutsen, 1992; Fishelson, 1995), but very few have identified the chemicals involved (Mackie et al., 1980).

The gilthead seabream (*Sparus auratus* L; a coastal and estuarine sparid of the Mediterranean Sea and western Atlantic Ocean) is a fish of high economic importance, especially as a species of aquaculture, in southern Europe. It is a protandrous, hermaphroditic fish (Zohar et al., 1978, 1984), wherein the sex ratio and oocyte development appear to be under social control (Happe and Zohar, 1988; Meiri et al., 2002). Given the lack of obvious external sexual dimorphism, this is probably mediated by chemical communication and/or behavior. This study provides evidence that gilthead seabream have high olfactory sensitivity to substances released by conspecifics and suggests that chemical communication may, indeed, be important in this species.

METHODS AND MATERIALS

Experimental Animals. Gilthead seabream (*Sparus auratus* L; 330–430 g, 25.3–28.0 cm nose-fork length) were bought from a local fish farm (TiMar – Culturas em Água, Lda., Fuzeta, Portugal) and maintained in recirculating sea water in 400 l tanks under a winter photoperiod (9L:15D) at temperatures 15–20°C. All fish used in electrophysiological experiments were male (gonadosomatic index GSI = 0.35–1.08 %), some spermiating spontaneously. The fish were fed once a day with commercial fish-food.

Collection of Water Samples. Water samples were taken weekly from a 13 m^3 tank containing 212 seabream of both sexes at TiMar; these fish were kept in an open-circuit of water, obtained directly from the sea, which had been coarse-filtered (sand) and sterilized (uv light). Control water samples were also taken

from the inlet. All samples were taken at the same time of day (between 9 and 10 a.m.) before the fish were fed. A portion of each sample was frozen immediately (-20°C) of which 1 l was passed through a C-18 reverse phase chromatography column (Waters Sep-Pak® Vac 20 cc C18 cartridges) previously conditioned with ethanol (10 ml) and distilled water (10 ml). The column was then washed (10 ml distilled water) and eluted (10 ml ethanol). The eluate was evaporated (under nitrogen) and the residue stored at -20°C, as was a sample of the filtrate. The eluate was dissolved in 10 ml of sea water (i.e., concentrated $\times 100$ from original source water) prior to use. The thawed filtrate and untreated water samples were applied undiluted. Water samples were taken from isolation tanks (50 l) before and after sexually mature individuals were placed inside for 1 hr. These samples were immediately frozen (-20°C) until use, when they were thawed and used untreated.

Collection of Body Fluids. Body fluids (urine and intestinal fluid) and gametes (sperm and eggs) were obtained from lightly anaesthetized (50 μ1/l 2-phenoxy-ethanol) brood-stock fish (1800–3500 g) kept under natural photoperiod and temperature in open-circuit seawater tanks (3000 l) at the aquaculture station of the Universidade do Algarve. Collections took place in November 1999, during the natural spawning season. Urine, intestinal fluid, and sperm or eggs were collected by gently squeezing the abdomen anteriorly to and just above the anus and urogenital openings. Samples were collected in plastic tubes and kept on ice until return to the laboratory where they were kept frozen (-20°C) until use. Fish were judged to be sexually mature by spontaneous release of semen or eggs during this procedure, and only samples from sexually mature fish were used. Unfortunately, because of the proximity of the urinary and genital openings, it was impossible to obtain urine samples from sexually mature males that were not mixed with semen. Thus, a sample with only minimal semen contamination was chosen. In addition, one mature male and one mature female fish were killed by overdose of anaesthetic to collect bile fluid from the gall bladder, and to assess the gonado-somatic index (male GSI = 2.4%, female GSI = 4.71%). Immediately prior to experiments, the samples were thawed, vortexed (30 secs), and centrifuged briefly (5 mins at 20,000 rpm). The samples were diluted in charcoal-filtered seawater; the same seawater used to superfuse the olfactory epithelium during recording (see following sections).

Extracellular Recording from the Olfactory Nerve. The surgery and method used for recording from the olfactory nerve were as described previously (Hubbard et al., 2000), except that, after initial anaesthesia by immersion in sea water containing 100 mg/l 3-aminobenzoic acid ethyl ester (MS222), some of the fish were anaesthetized by an intraperitoneal injection of 250 μl/100 g body weight Saffan™ (Schering-Plough Animal Health, Welwyn Garden City, U.K.) during the recording. These fish did not receive the intramuscular injection of gallamine (0.3 mg/100 g body weight) that the other fish did.

Briefly, olfactory nerve activity was recorded using two parylene-coated tungsten electrodes (resistance 0.08–0.12 MΩ, World Precision Instruments, Stevenage,

U.K.) inserted into the olfactory nerve, and connected to an A.C. preamplifier (Neurolog NL104, Digitimer Ltd., Welwyn Garden City, U.K.). The signal was filtered (low-pass; 3000 Hz, high pass; 200 Hz, Neurolog NL125) and integrated (time constant 1 sec, Neurolog NL703). Direct and integrated signals were digitized (Digidata 1200, Axon Instruments, Inc., Foster City, CA, U.S.A.) and displayed on a P.C. running Axoscope 1.1™ software. Stimuli were delivered to the olfactory epithelium *via* a gravity-fed glass tube, held just within the inhalant nare. Sparids in general have clearly differentiated inhalant (smaller, circular, and anterior) and exhalent (larger, slit-like, and posterior) nares (Mana and Kawamura, 2002). The stimulus-delivery tube was connected to a computer-controlled three-way valve (flow rate 6 ml/min), which allowed switching between control and stimulus-containing solutions without interruption of the flow over the olfactory epithelium. The optimal positions for both stimulus-delivery tube and electrodes were ascertained using 10^{-3} M L-serine as a stimulus. This was generally found to be laterally, mid depth, just prior to the junction with the olfactory bulb. Stable and reproducible recordings could be obtained up to 8 hr after the initial surgery. All stimuli were applied for 10 sec. At least 1 min was allowed between successive stimuli to ensure complete washout. The stimuli were applied in a randomized order, but dilutions of each stimulus were given in order of increasing concentration.

Treatment of Data. To correct for "blank" responses, a blank stimulus (treated in the same way as the stimulus solutions but without addition of body fluids) was applied to each fish prior to each group of samples. The amplitude of the integrated response was then subtracted from the amplitude of each of the subsequent integrated responses. To correct for variations in olfactory sensitivity among fish, and possible differences in the recording position, the recorded amplitudes of the integrated responses were normalized to that of a "standard" stimulus of 10^{-3} M L-serine similarly blank-subtracted. Blanks and standards were run repeatedly (every six stimuli or so) throughout the recording session. Only complete concentration-response curves for a given stimulus on a given fish were used in subsequent analysis.

Statistical Analysis. All statistical analysis was carried out on normalized data. A semilogarithmic plot (or log–log plot in the case of the amino acids depending on goodness of fit) was made of the normalized concentration/response data from each body fluid and subjected to linear regression analysis (Zar, 1984) using only those concentrations of stimulus that evoked responses significantly above zero. Olfactory detection thresholds were calculated from the intercept with the x axis. Slopes and line elevations were compared using Tukey's test for multiple comparisons (Zar, 1984). The amplitude of responses to fish-conditioned water, its eluate and filtrate were compared to their respective controls using Student's t test for paired data; responses to bile fluid and intestinal fluid were compared using Student's t test. In all analyses, $P < 0.05$ was taken to represent statistical significance.

RESULTS

Olfactory Sensitivity to Amino Acids. The olfactory system of seabream was sensitive to all eight amino acids tested (Figure 1). The amplitude of the integrated activity of the olfactory nerve in response to stimulation of the olfactory epithelium was strongly concentration-dependent, giving near-linear concentration

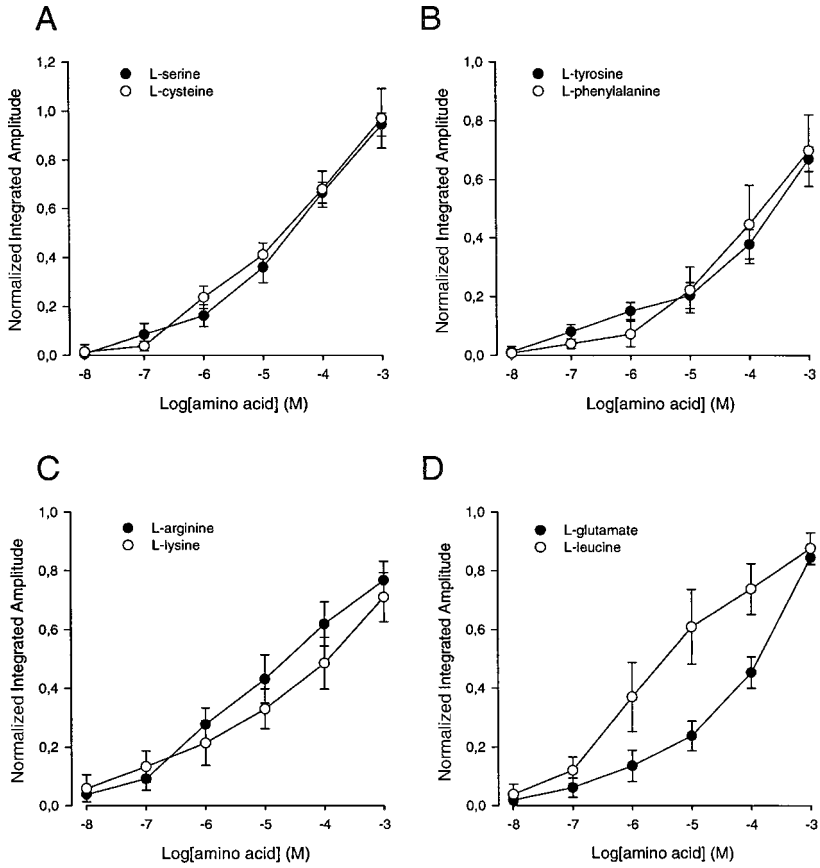

FIG. 1. Olfactory sensitivity to amino acids in *Sparus auratus*. Semilogarithmic plots of the normalized integrated activity recorded from the olfactory nerve in response to stimulation of the olfactory epithelium with (A) neutral short-chain amino acids L-serine ($N = 7$) and L-cysteine ($N = 7$), (B) aromatic neutral amino acids L-tyrosine ($N = 7$) and L-phenylalnine ($N = 6$), (C) basic amino acids L-arginine ($N = 7$) and L-lysine ($N = 6$), and (D) the acidic amino acid L-glutamate ($N = 7$), and neutral amino acid L-leucine ($N = 7$). Thresholds of detection are given in the text. All data are shown as mean ± SEM.

response curves when plotted semilogarithmically. The two exceptions to this were L-glutamate and L-leucine, producing exponential and quasisigmoidal curves, respectively. The estimated thresholds of detection were: L-serine $10^{-7.2}$ M, L-cysteine $10^{-7.2}$ M, L-tyrosine $10^{-7.3}$ M, L-phenylalanine $10^{-6.1}$ M, L-arginine $10^{-7.8}$ M, L-Lysine $10^{-7.6}$ M, L-leucine $10^{-8.1}$ M, and L-glutamate $10^{-6.3}$ M. Thus, in terms of both amplitude and threshold, the most potent amino acids were short-chain neutral (L-serine, L-cysteine, and L-leucine) followed by basic (L-arginine and L-lysine). Little or no olfactory responses were evoked by the steroids (17,20β-P [17α,20β-dihydroxy-4-pregnen-3-one] and cortisol), bile acids (lithocholic acid [5β-cholanic acid-3α-ol], taurolithocholic acid [5β-cholanic acid-3-α-ol *N*-(2 sulphoethyl)-amide], deoxycholic acid [5β-cholanic acid-3α,12α-diol], and chenodeoxycholic acid [5β-cholanic acid-3α,7α-diol]) or prostaglandins ($PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$) tested, even at concentrations as high as 10^{-5} M (data not shown).

Olfactory Responses to Water Samples. The olfactory system of seabream was found to be sensitive to substances present in normal sea water, giving consistently strong responses to the control water samples (Figure 2A). This is not surprising, but unfortunately gives no information on what these substances could be. However, the amplitude of the integrated responses to water samples from the tank containing conspecifics was significantly greater than that of the control samples (Figure 2A), suggesting that the olfactory system of seabream is sensitive to chemicals released by conspecifics. These substances are extractable using solid phase extraction (Figure 2B). The eluates of tank water evoked strong olfactory responses that lessened predictably with dilution (data not shown) and that were consistently greater in amplitude than those of the control (inlet) samples. The responses to the filtrates, however, showed no difference in amplitude (Figure 2C). The large olfactory responses to fish-conditioned water (Figure 2D) suggest that the source of the odorants is, at least in part, the fish themselves.

Olfactory Responses to Intestinal Fluid. The olfactory system of seabream was found to be extremely sensitive to the intestinal fluid of both male and female conspecifics (Figure 3), evoking consistent and large-amplitude responses to dilutions as low as 1:1,000,000. The predicted threshold of detection by linear regression of the data was $1{:}10^{6.4}$ (female intestinal fluid) and $1{:}10^{7.4}$ (male intestinal fluid). The form of the response was biphasic (Figure 3A and B); an initial phasic (rapidly adapting) component at stimulus onset, followed by a tonic phase of lower amplitude that lasted for the duration of the stimulus (10 sec). This form is typical of olfactory responses in fish. The amplitude of the integrated response was large; at dilutions of 1:100 and 1:1,000, it was consistently larger than that of 10^{-3} M L-serine. However, the two regression lines (male and female) were not significantly different (Table 1).

Olfactory Responses to Conspecific Gametes. The olfactory system of seabream was highly sensitive to dilutions of conspecific seminal and egg fluids (Figure 4). The responses were similar in form to those of intestinal fluids; a rapid

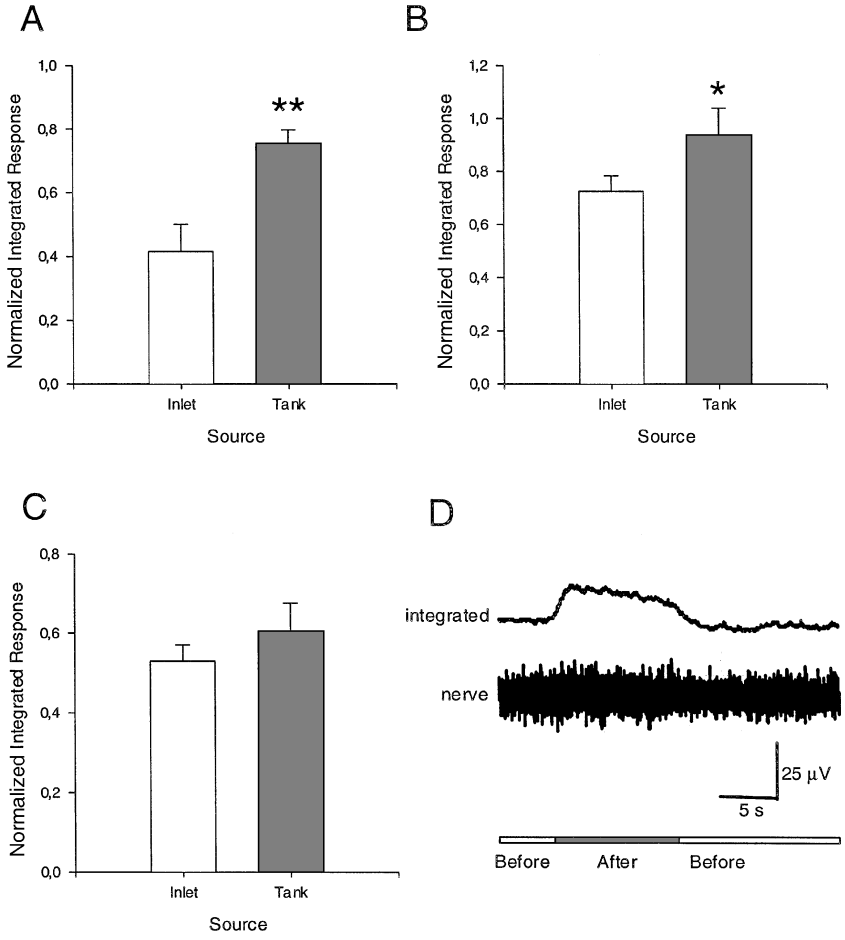

FIG. 2. Olfactory nerve responses of *Sparus auratus* to water from tanks containing conspecifics. (A) Histogram showing normalized amplitude of integrated activity of olfactory nerve after stimulation with untreated water (inlet) and the same water after being conditioned by the presence of conspecifics (tank). (B) Histogram showing normalized amplitude of integrated activity of olfactory nerve after stimulation with the eluate of solid-phase extracted control water (inlet) and the fraction of water after being conditioned by the presence of conspecifics (tank). (C) Histogram showing normalized amplitude of integrated activity of olfactory nerve after stimulation with the filtrate of solid-phase extracted control water (inlet) and the filtrate of water after being conditioned by the presence of conspecifics (tank). Data are shown as mean ± SEM. ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control (inlet) group. (D) Typical olfactory nerve activity (lower trace) and integrated activity (upper trace) in response to control water (before) and water conditioned by the presence of a sexually mature female conspecific (after). Representative of three independent experiments.

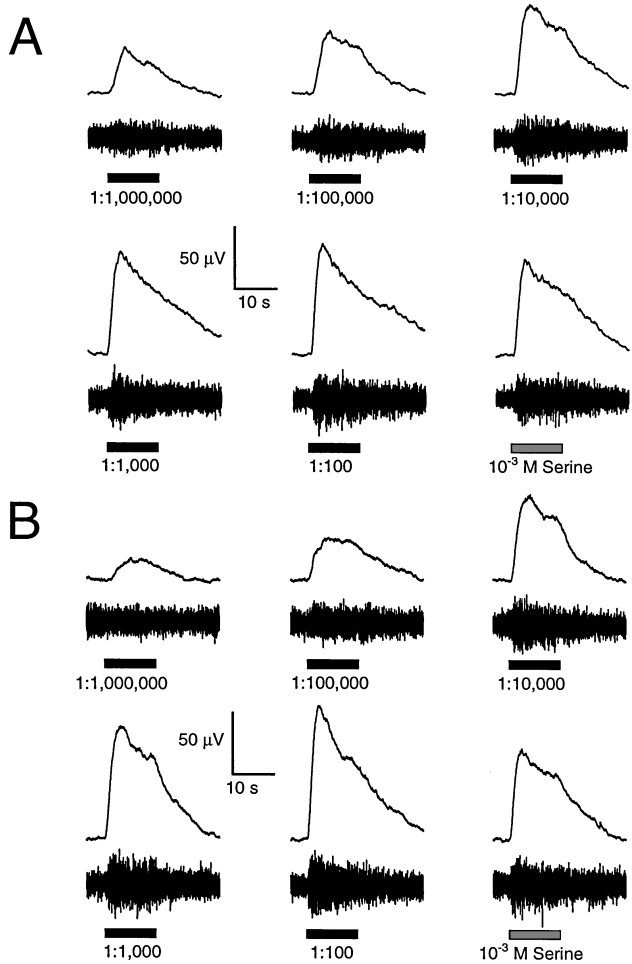

FIG. 3. Olfactory nerve responses of *Sparus auratus* to intestinal fluid of sexually mature conspecifics. (A) Typical extracellular recordings from the olfactory nerve (lower traces) and integrated activity (upper traces) to exposure of the olfactory epithelium to increasing concentrations of intestinal fluid from a sexually mature male conspecific (black horizontal bars). (B) Similar series of recordings in response to intestinal fluid from a sexually mature female conspecific (black horizontal bars). Responses to 10^{-3} M serine in the same fish are shown for comparison (grey horizontal bars). The dilution factor is given for each trace. The amplitude of the integrated response is measured in arbitary units (vertical scale = 4 units), but all traces in A and B are to the same scale. (C) Normalized, pooled data (mean ± SEM) from five (male intestinal fluid) and six (female intestinal fluid) plotted as a function of the dilution factor. Linear regression analysis of the data predicted the threshold of detection to be $1{:}10^{7.4}$ for male intestinal fluid, and $1{:}10^{6.4}$ for female intestinal fluid.

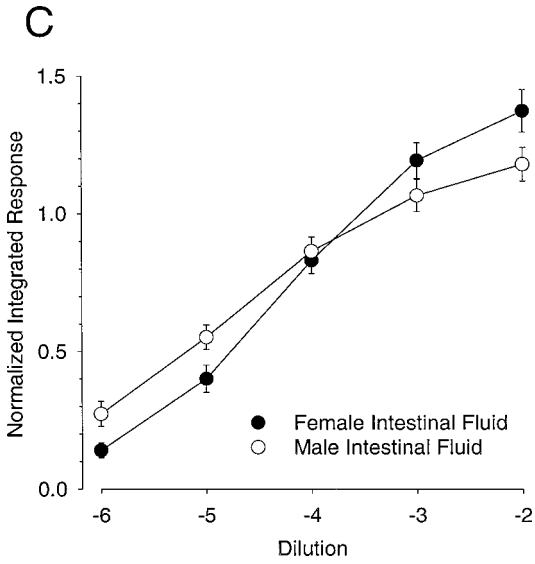

FIG. 3. Continued.

onset, large amplitude phasic component that accommodated rapidly, followed by a lower amplitude, more sustained, tonic component that continued as long as the stimulus was applied. However, only at dilutions of 1:100 was the amplitude of the integrated responses larger than that of 10^{-3} M serine, suggesting that the gamete fluids are slightly less potent olfactory stimuli than the intestinal fluids. This is reflected in the predicted thresholds (1:$10^{5.6}$; seminal fluid, 1:$10^{6.1}$; egg fluid) being significantly higher than the respective values for intestinal fluids (Table 1). The

TABLE 1. LINEAR REGRESSION STATISITICS FOR EACH OLFACTORY STIMULUS AND MULTIPLE COMPARISONS AMONG SLOPES AND LINE ELEVATIONS

Stimulus	R^2_{adj}	F value	Y Intercept (± SE)	t value	Slope (±SE)[a]	t value	Line elevation[b]
Male IF	0.90	$F_{1,23} = 205.83$*	1,72 ± 0.07	24.95*	0.23 ± 0.02 a	14.35*	a
Female IF	0.93	$F_{1,28} = 364.83$*	2.09 ± 0.07	28.89*	0.33 ± 0.02 ab	19.10*	a
Seminal fluid	0.90	$F_{1,18} = 162.34$*	1.90 ± 0.1	19.40*	0.34 ± 0.03 b	12.74*	b
Egg fluid	0.94	$F_{1,23} = 409.33$*	1,61 ± 0.06	28.94*	0.27 ± 0.01 ab	20.23*	b
Male urine	0.90	$F_{1,18} = 172.18$*	1.63 ± 0.09	18.52*	0.31 ± 0.02 ab	13.12*	c
Female urine	0.65	$F_{1,13} = 26.71$*	1.03 ± 0.15	6.90*	0.25 ± 0.05 ab	5.17*	d

[a] Different letters indicate significant statistical differences among slopes, $P < 0.05$.
[b] Different letters indicate significant statistical differences among regression line elevations, $P < 0.001$.
* $P < 0.001$.

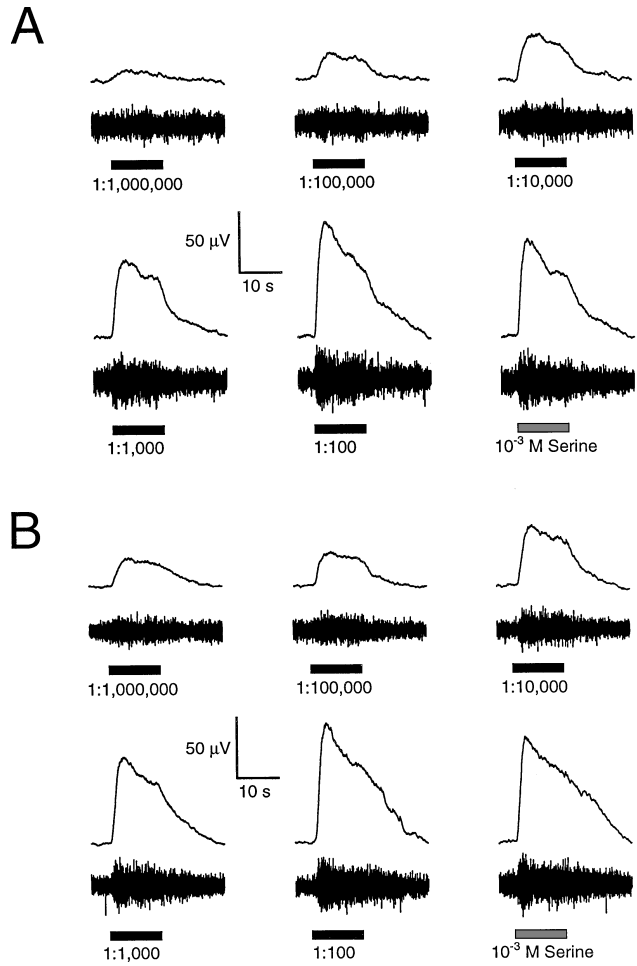

FIG. 4. Olfactory nerve responses of *Sparus auratus* to gamete fluid of sexually mature conspecifics. (A) A series of typical extracellular recordings from the olfactory nerve (lower traces) and integrated activity (upper traces) to exposure of the olfactory epithelium to increasing concentrations of seminal fluid from a sexually mature male conspecific (black horizontal bars). (B) A similar series of recordings in response to egg fluid from a sexually mature female conspecific (black horizontal bars). Responses to 10^{-3} M serine in the same fish are shown for comparison (grey horizontal bars). The dilution factor is given for each trace. The amplitude of the integrated response is measured in arbitary units (vertical scale = 4 units), but all traces in A and B are to the same scale. (C) Normalized, pooled data (mean $\pm$ SEM) from five independent experiments plotted as a function of the dilution factor. Linear regression analysis of the data predicted the threshold of detection to be $1{:}10^{5.6}$ for seminal fluid, and $1{:}10^{6.1}$ for egg fluid.

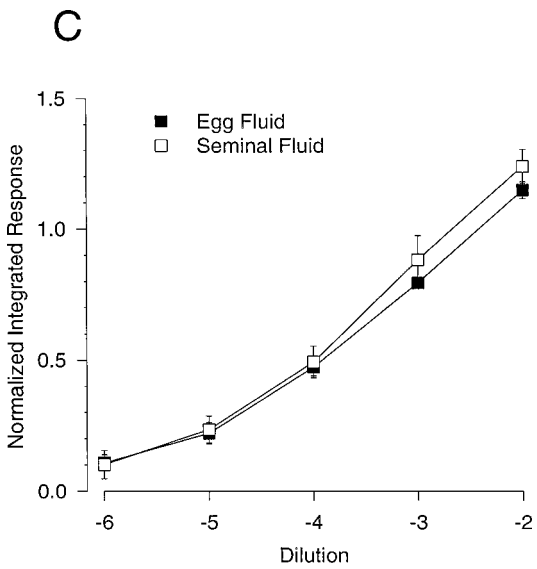

FIG. 4. Continued.

amplitude of the integrated responses was similar for egg and seminal fluid. This, of course, does not necessarily mean that the odorants contained in these fluids are the same.

Olfactory Nerve Responses to Conspecific Urine. The olfactory system of seabream proved to be less sensitive to conspecific urine (Figure 5; Table 1) than to either intestinal fluid or gametes: the amplitude of the integrated responses to dilutions of female urine was less than that of 10^{-3} M serine even at 1:100 (Figure 5B), and dilutions higher than 1:10,000 failed to give responses consistently larger than that of blank stimuli. The threshold of detection, as predicted by linear regression of the data, was $1{:}10^{4.2}$. The olfactory responses to male urine were consistently larger than that of female urine (Figure 5A), and this is reflected in the predicted threshold of detection, $10^{-5.2}$ (Table 1). However, as it was impossible to collect urine from sexually mature males without it being contaminated by spontaneously released semen (see Methods and Materials), this difference was attributed to the presence of seminal fluid in the male urine. Nevertheless, we cannot exclude a true difference in the olfactory potency of male and female urine.

Olfactory Nerve Responses to Bile Fluid. The olfactory system of several species of fish is known to be sensitive to conspecific bile acids (Døving et al., 1980; Li et al., 1995; Li and Sorensen, 1997; Zhang et al., 2001). To establish whether the large amplitude and high sensitivity of responses to intestinal fluids could be

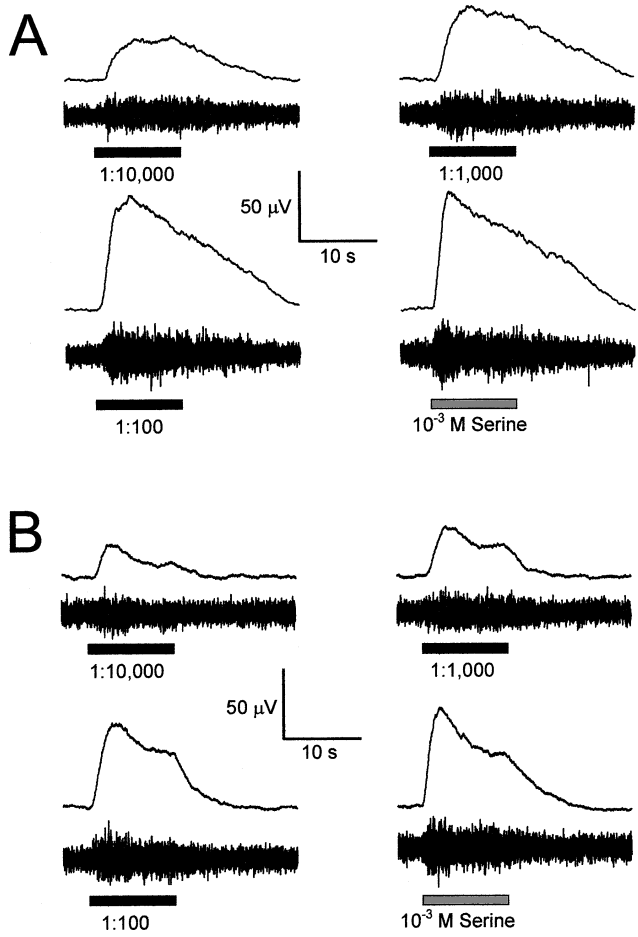

FIG. 5. Olfactory nerve responses of *Sparus auratus* to urine of sexually mature conspecifics. (A) A series of extracellular recordings from the olfactory nerve (lower traces) and integrated activity (upper traces) to exposure of the olfactory epithelium to increasing concentrations of urine from a sexually mature male conspecific (black horizontal bars). (B) A similar series of recordings in response to urine from a sexually mature female conspecific (black horizontal bars). Responses to 10^{-3} M serine in the same fish are shown for comparison (grey horizontal bars). The dilution factor is given for each trace. The amplitude of the integrated response is measured in arbitary units (vertical scale = 4 units), but all traces in A and B are to the same scale. (C) Normalized, pooled data (mean ± SEM) from five independent experiments plotted as a function of the dilution factor. Linear regression analysis of the data predicted the threshold of detection to be $1{:}10^{5.2}$ for male urine, and $1{:}10^{4.2}$ for female urine. The male urine sample was contaminated with seminal fluid (see text).

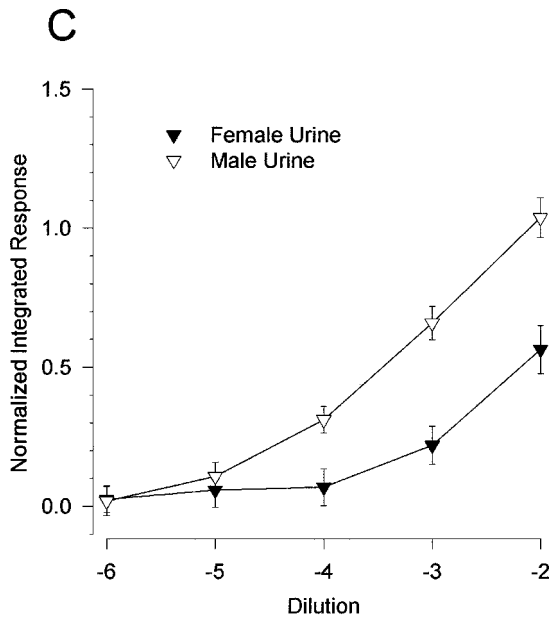

FIG. 5. Continued.

explained by the presence of bile acids in the intestinal fluid, we investigated the olfactory sensitivity of seabream to dilutions of conspecific bile fluid (Figure 6). Contrary to expectations, although the olfactory system was highly sensitive to dilutions of conspecific bile fluid, the amplitudes of these responses were slightly lower than those of the intestinal fluids (the bile fluid and intestinal fluid were collected from the same female). Assuming that the bile acids are significantly diluted on release from the gall bladder into the intestinal lumen, this would suggest that the large olfactory responses observed after stimulation with intestinal fluid are only in part due to olfaction of conspecific bile acids, or that the bile acids are chemically modified into more potent olfactory stimuli during their transit down the gut.

DISCUSSION

The olfactory system of the seabream was sensitive to all eight amino acids tested in this study (Figure 1) with thresholds of detection in the range of 10^{-8}–10^{-6} M, similar to many other species of fish. Amino acids are believed to be detected in freshwater fish by at least four olfactory receptor mechanisms (Caprio and Byrd, 1984; Friedrich and Korsching, 1997) relatively specific for amino acids with short and neutral, long and neutral, basic, and acidic side chains, respectively.

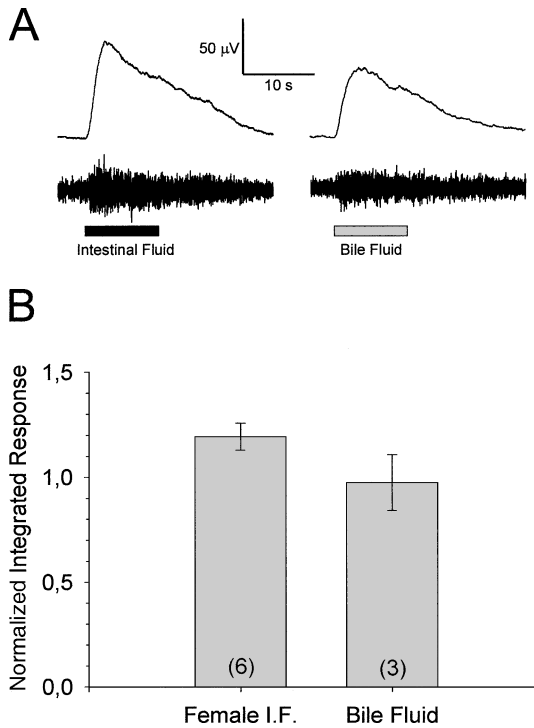

FIG. 6. Olfactory nerve responses of *Sparus auratus* to bile fluid of sexually mature conspecifics. (A) Typical consecutive olfactory nerve (lower traces) and integrated activity (upper traces) recordings in response to dilutions (1:1,000) of intestinal fluid (left panel; black horizontal bar) and bile fluid (right panel; grey horizontal bar). Note that the amplitude of the integrated response to bile fluid is lower than that of the intestinal fluid (both samples were collected from the same fish). (B) Histogram of the pooled data (mean ± SEM) from several similar experiments normalized to the response to 10^{-3} M serine. Numbers in parentheses refer to number of observations. The two sets of data are not statistically different (Student's *t* test; $P = 0.092$).

Although not tested directly, the similarity of responses to chemically similar amino acids within these groups suggests that this may be occurring in the seabream too. The more potent amino acids were the basic (L-arginine and L-lysine) and short-chain, neutral (L-serine, L-cysteine, and L-leucine) ones. This is broadly similar to one other study that investigated this phenomenon in detail in another marine species (the rabbitfish, *Siganus fuscescens* Houttuyn; Ishida and Kobayashi, 1992), despite the diets of the two species being quite different (molluscs and crustaceans vs. algae, respectively). Although likely, it is not yet certain that seabream use olfaction of amino acids to locate or identify potential food items.

The olfactory system of seabream was sensitive to water containing conspecifics, and the active substances could be extracted by solid-phase extraction (Figure 2). Furthermore, the source of these odorants was likely to be the fish themselves (Figure 2D). The olfactory system of seabream was extremely sensitive to dilutions of various conspecific body fluids down to 1:1,000,000. This compares favorably with the studies of Kitamura and Ogata (1989) who recorded electroencephalogram (EEG) responses to dilutions of "urogenital fluid" (chiefly urine) down to 1:100,000 from the olfactory bulb of the salmon (*Oncorhynchus rhodurus*), and Frade et al. (2002) who assessed the olfactory potency of feces ($1{:}10^{4.6}$) and urine ($1{:}10^{5.2}$) in the Mozambique tilapia (*Oreochromis mossambicus*) by electro-olfactogram (EOG). It is possible that any or all of the body fluids used in this study contain putative pheromones, but the ability of the olfactory system to detect them gives no information on their possible identity or biological function(s). Previous work has shown that the urine is a potent source of candidate pheromones (Scott et al., 1994; Waring et al., 1996; Appelt and Sorensen, 1999; Frade et al., 2002). However, most of this work has been carried out on freshwater species. For reasons of fluid and ion balance, freshwater fish tend to produce a large volume of dilute urine, whereas marine teleosts produce much smaller volumes of concentrated urine (about 0.3 ml/kg/hr; Brown et al., 1980; Claiborne et al., 1994; McDonald et al., 2000), which is released highly sporadically (less than once in 3 days in the plaice *Pleuronectes platessa*; Fletcher, 1990). Perhaps, for this reason, pheromonal systems have evolved in marine fish that utilize other routes of release into the water, and this may be why female urine evoked relatively weak olfactory responses compared to the intestinal and gamete fluids. Given the extreme sensitivity of seabream to dilutions of conspecific intestinal fluid, and considering the relative rates of release, our data suggest that this is a possible source of pheromones in this species, or perhaps even marine teleosts in general (Table 2; calculated "active space" of intestinal fluid is at least two orders of magnitude greater than that of urine).

In several species of fish, bile acids have been suggested as potential pheromones or markers of natal rivers in the case of migratory spawners (Li et al., 1995, 2002; Zhang et al., 2001)—certainly the olfactory system of fish is generally highly sensitive to bile acids (e.g., Døving et al., 1980; Michel and Lubomudrov, 1995; Li and Sorensen, 1997; Frade et al., 2002). Contrary to expectations, the olfactory response of seabream to dilutions of conspecific bile fluid was actually slightly weaker than that of intestinal fluid (Figure 6). Assuming that bile acids are highly concentrated in the gall bladder and are diluted on release into the intestinal lumen, this suggests that the olfactory system is responding to components of intestinal fluid other than bile acids or that bile acids are metabolized into more potent odorants during their passage down the intestine. Gut fauna have been implicated as responsible for the production of an aggregation pheromone in the locust (Dillon et al., 2000). It is possible that gut fauna specific to *Sparus auratus* produce a putative pheromone in a similar manner, thus immediately identifying

TABLE 2. CALCULATION OF "ACTIVE SPACE" (VOLUME OF WATER OCCUPIED AT A CONCENTRATION AT OR ABOVE THE OLFACTORY THRESHOLD OF DETECTION) FOR BODY FLUIDS OF THE GILTHEAD SEABREAM

Fluid	Release rate	Predicted detection threshold	Active space
Female urine	0.3[a,b,c] ml/kg/hr	$1{:}10^{4.2}$	5 l/hr
Male urine	0.3[a,b,c] ml/kg/hr	$1{:}10^{5.2}$	50 l/hr
Egg fluid	2.0[d] ml/spawning	$1{:}10^{6.1}$	2,500 l/spawning
Semen	2.0[d] ml/spawning	$1{:}10^{5.6}$	800 l/spawning
Female IF	0.5[e] ml/kg/hr	$1{:}10^{6.4}$	1,250 l/hr
Male IF	0.5[e] ml/kg/hr	$1{:}10^{7.4}$	12,500 l/hr

Note: Numbers are based on a sexually mature individual of 2 kg assuming equal and uniform diffusion of olfactory stimulant(s). IF: intestinal fluid.
[a] Claiborne et al., 1994.
[b] McDonald et al., 2000.
[c] Brown et al., 1980.
[d] approximate figures for *Sparus auratus* based on samples collected for this study.
[e] Wilson et al., 1996.

conspecifics in conditions of reduced visibility. Certainly, while none of the bile acids used in this study evoked significant olfactory responses in the seabream, all of them have been shown to be potent odorants in a number of freshwater fish (e.g., Michel and Lubomudrov, 1995; Zhang et al., 2001; Frade et al., 2002). If bile acids are important odorants to the seabream, then their structures are likely to be different from their freshwater counterparts. The same is probably true of both steroids and prostaglandins.

Sparus auratus is a protandrous, hermaphroditic species (Zohar et al., 1978, 1984), and the ratio of functional males to female appears to be under social control (Happe and Zohar, 1988). During the spawning season, the development of the oocytes is dependent on the presence of males (Meiri et al., 2002). It is possible that both these processes are mediated by chemical communication. The high sensitivity to the intestinal fluid implicates this as a likely source of the compounds involved. The olfactory sensitivity of seabream to both egg fluid and semen suggests that these fluids also may contain chemical cues important in spawning. The ability of males to detect seminal fluid with a similar sensitivity to egg fluid (Figure 4 and Table 2) is perhaps more difficult to explain—if the chemical cues in egg fluid and semen are different in nature, this may enable spermiating males to avoid other males and focus their attention on the ovulating females. Before these questions are addressed, the chemical identity of the olfactory stimulant(s) in each fluid needs to be determined. This work is currently underway in our laboratory.

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SYNERGISTIC EFFECTS OF THREE *Piper* AMIDES ON GENERALIST AND SPECIALIST HERBIVORES

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Abstract—The tropical rainforest shrub *Piper cenocladum*, which is normally defended against herbivores by a mutualistic ant, contains three amides that have various defensive functions. While the ants are effective primarily against specialist herbivores, we hypothesized that these secondary compounds would be effective against a wider range of insects, thus providing a broad array of defenses against herbivores. We also tested whether a mixture of amides would be more effective against herbivores than individual amides. Diets spiked with amides were offered to five herbivores: a naïve generalist caterpillar (*Spodoptera frugiperda*), two caterpillar species that are monophagous on *P. cenocladum* (*Eois* spp.), leaf-cutting ants (*Atta cephalotes*), and an omnivorous ant (*Paraponera clavata*). Amides had negative effects on all insects, whether they were naïve, experienced, generalized, or specialized feeders. For *Spodoptera*, amide mixtures caused decreased pupal weights and survivorship and increased development times. *Eois* pupal weights, larval mass gain, and development times were affected by additions of individual amides, but increased parasitism and lower survivorship were caused only by the amide mixture. Amide mixtures also deterred feeding by the two ant species, and crude plant extracts were strongly deterrent to *P. clavata*. The mixture of all three amides had the most dramatic deterrent and toxic effects across experiments, with the effects usually

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surpassing expected additive responses, indicating that these compounds can act synergistically against a wide array of herbivores.

Key Words—Synergy, amides, *Piper*, herbivory, chemical defense, specialists, generalists, caterpillars.

INTRODUCTION

Manipulative experiments examining the effects of plant defenses on herbivores often examine individual defenses in isolation, despite evidence that many defenses are additive or synergistic (Hay et al., 1994; Nelson and Kursar, 1999). Synergistic plant defenses are broadly defined as effects of multiple compounds that are greater than expected based on projected additive values of each individual compound (Berenbaum et al., 1991; Jones, 1998; Nelson and Kursar, 1999). Some authors use the term "potentiation" to distinguish those synergistic effects where compounds are each separately toxic to the insect but the toxicity of the combination is greater than expected additive effects (Stewart, 1998). Synergisms between different chemical defenses have been documented where deterrence, toxicity, or other fitness effects of one compound are increased dramatically (i.e., significantly greater than additive effects) in the presence of other compounds (Kumar and Parmar, 1996; Jones, 1998; Scott et al., 2002). One documented mechanism of synergy is that inactive metabolites impede an herbivore's or pathogen's ability to metabolize or eliminate toxins (Berenbaum and Neal, 1985; Kubo and Muroi, 1993; Stermitz et al., 2000). Secondary metabolites are always present in plants as simple or complex mixtures, and there are many hypotheses for this so called phytochemical redundancy (Jones and Firn, 1991; Romeo et al., 1996). Since synergy may be a common phenomenon in plant defenses and one important reason for phytochemical redundancy, it warrants investigation in any system where plant–herbivore interactions are well studied. We chose to study synergy in *Piper cenocladum* C. DC. (Piperaceae), which is emerging as a model system for studies of plant defense and herbivory.

A wide array of secondary compounds has been isolated from *Piper* spp., including amides of a characteristic type referred to as "*Piper* amides" (reviewed by Parmar et al., 1997). These amides contain a phenyl moiety with a variable length carbon side chain (typically with at least one unsaturation) ending in a carbonyl carbon. The nitrogen containing portion of the amide is derived from piperidine, pyrrole, or an isobutyl group and may contain an unsaturation, an epoxide, or a carbonyl group. *Piper cenocladum* contains three amides at high concentrations (total amide content can be as high as 3.8% dry weight): piplartine, 4′-desmethylpiplartine, and cenocladamide (Figure 1; Dodson et al., 2000). Many *Piper* amides that have been investigated for biological activity have been demonstrated to be insecticidal (Su and Horvat, 1981; Miyakado et al., 1989; Gbewonyo

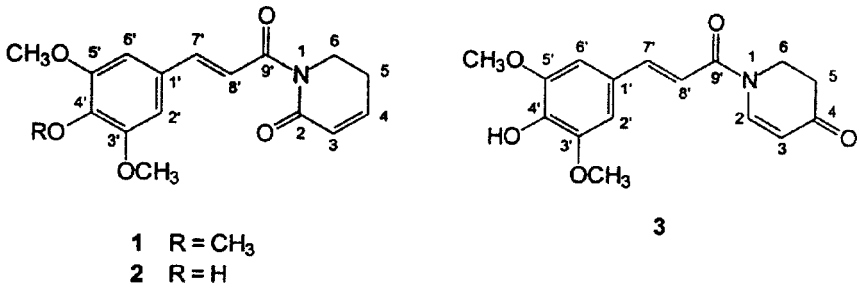

FIG. 1. *Piper cenocladum* amides: piplartine (1), 4′-desmethylpiplartine (2), and cenocladamide (3).

et al., 1993; Scott et al., 2002) or deterrent to leaf-cutting ants (Capron and Wiemer, 1996; Dyer and Dodson, unpublished data). One of the amides in *P. cenocladum*, piplartine, is cytotoxic *in vitro* (Duh et al., 1990). Amides are found in higher concentrations when mutualistic ants are absent from *P. cenocladum* (Dodson et al., 2000; Dyer et al., 2001), and measures of herbivory (Dyer and Letourneau, 1999a,b) have suggested that amides in *P. cenocladum* are deterrent to arthropods.

As with most apparently redundant chemical defenses in plants, it is unknown whether amides are enemy-specific in their deterrence or toxicity (as in Lindroth and Hwang, 1996) or whether they act in a synergistic fashion, as has been indicated for furanocoumarin defenses in apiaceous plants by Berenbaum and colleagues (Berenbaum and Neal, 1985; Berenbaum et al., 1991; Berenbaum and Zangerl, 1993). To test the hypothesis that *Piper* amides act synergistically, we performed a series of experiments examining the preference and performance of generalist and specialist insects on amide-manipulated diets.

METHODS AND MATERIALS

Natural History. Piper cenocladum are understory shrubs common in lowland wet forests throughout Costa Rica (Burger, 1971). Opalescent food bodies, rich in lipids and proteins, are produced on the adaxial side of sheathing leaf bases (hollow petioles) when occupied by *Pheidole bicornis* Forel ants (the plants are not always occupied by these ants). There are dozens of herbivores associated with *P. cenocladum* (Dyer and Letourneau, 2003). The most common external folivores include specialist and generalist lepidopterans (Geometridae, Hesperiidae, Apatelodidae, and Limacodidae), specialist coleopterans (Chrysomelidae and Curculionidae), generalist orthopterans (Tettigoniidae), and leaf-cutting ants (*Atta cephalotes* L.). More extensive natural history of this system, relevant to this paper, is described in Dyer and Letourneau (1999b) and Letourneau (1998).

Experiments—Overview. Four primary experiments were performed to examine the synergistic effects of *P. cenocladum* amides on four different arthropod herbivores: *Spodoptera frugiperda* (Lepidoptera: Noctuidae), the leaf-cutting ant *Atta cephalotes* (Hymenoptera: Formicidae), two species in the genus *Eois* (Lepidoptera: Geometridae), and the omnivorous ant *Paraponera clavata* Fabricius (Hymenoptera: Formicidae). The experiments with lepidopteran herbivores tested the performance of generalists and specialists on diets containing amides from *P. cenocladum*, and the experiments with ants assessed whether these amides were deterrent to a generalist herbivore and an omnivore. To assess the potential role of synergistic interactions between amide compounds, the effects of single amides on herbivore choice or performance were compared to the effects of mixtures in each of these experiments. Controls for all experiments were administered along with experimental diets and the methods for control and experimental diet applications were identical. For experiments that required synthetic material, amides were synthesized at Mesa State College, Colorado (Richards et al., 2001).

Experiment 1—Naïve Generalist. Spodoptera frugiperda JE Smith is a naïve (to *P. cenocladum*) generalist herbivore native to tropical regions of the western hemisphere and is a major crop pest in several regions of North America. Eggs of *S. frugiperda* were acquired from Agripest, North Carolina. Upon hatching, 50 larvae were placed on one of eight artificial diet treatments. The control diet consisted of 35 g powdered Fall Armyworm Diet (Southland Products, Lake Village, AR) mixed with 201 ml distilled water and 1.5 ml linseed oil. For experimental diets, a single amide or a combination of amides was added to the powdered control diet. Amide quantities added were 0.203 g of commercially available isolated piplartine (Sigma-Aldrich, St. Louis, MO), 0.1575 g of synthetically produced 4′-desmethylpiplartine, and 0.1155 g of synthetically produced cenocladamide. The three-way and two-way amide combinations contained additive amounts of the amides (e.g., the three-way combination contained the addition of 0.476 g of dry amide material). The quantity of amides added represents the upper concentration limits found in non-ant containing plants of *P. cenocladum* (piplartine: 0.58% leaf dry mass, 4′-desmethylpiplartine: 0.45% leaf dry mass, cenocladamide: 0.33% leaf dry mass; Dodson et al., 2000). First instars were placed in 1.25 oz opaque plastic cups and reared in a growth chamber (28°C, 14 hr L). Larval mass was measured every fourth day, and larvae were given fresh food every second day. At time of pupation, a final mass was obtained and gender determined. Date of adulthood was recorded upon eclosion.

Pupal mass and development times of *S. frugiperda* were analyzed with ANOVAs, using amide treatment as the independent variable. We were unable to include caterpillars fed the mixture of all three amides and the piplartine-4′-desmethylpiplartine combination in these analyses because of the almost complete mortality within these treatments. Treatment responses were compared with *post hoc* pairwise comparisons of each treatment employing modified Bonferroni

correction (McDonald et al., 2002). Survival data were analyzed with a series of log-rank tests, also employing a modified Bonferroni α to correct for multiple comparisons.

Experiment 2—Leaf-Cutting Ants. To assess deterrent effects of *P. cenocladum* amides on a generalist herbivore that commonly encounters this plant, we performed a series of field choice experiments with the leaf-cutting ant, *Atta cephalotes* at the La Selva Biological Station, Heredia Province, Costa Rica, (10°25'N 84°05'W). In these experiments (modeled after Folgarait et al., 1996), we presented foraging *A. cephalotes* colonies with a selection of leaf disks coated with amides in 2 mg/ml methanol solutions. The treatments included piplartine, 4′-desmethylpiplartine, cenocladamide, a mix of all three compounds, and a control containing only methanol. *Hyeronima alchorneoides* Allemao (Euphorbiaceae), a species shown to be palatable to *A. cephalotes* (Folgarait et al., 1996), was used for all leaf disks. Young leaves were cut into 1×0.5 cm disks and coated with 5 μl of one of the treatment solutions, creating a disk with total amide content of about 1% dry weight. The disks were allowed to air-dry and were presented to the ants soon after preparation to ensure freshness.

Twenty-five distinct (spatially separated by at least 200 m) *A. cephalotes* colonies were presented with five disks of each of the five treatments (for a total of 25 leaf disks per trial). Disks were offered simultaneously on a piece of paper (to control for the background on which the disks were offered) to foraging ants in a cafeteria-style display. Trials continued for 15 min or until all five disks of one of the treatments had been removed by the ants. We performed each trial at least 30 m from the entrance to the nest to ensure that any single ant did not participate in the choice test multiple times. Thus, each leaf disk or each leaf-cutting ant could be considered a replicate.

Because of the difficulties in analyzing results from multiple choice preference tests (Lockwood, 1998 and references therein), we analyzed the feeding preference of leaf-cutting ants (*A. cephalotes*) using the method advocated by Folgarait et al. (1996) that takes into account lack of independence in leaves simultaneously offered to ants. Each of the five treatments was included as a dichotomous variable (disk taken or not taken) in a loglinear model, along with appropriate interactions (i.e., all interactions with the control and all interactions that were necessary for the model to fit the data). We utilized nonhierarchical models for testing specific hypotheses, a maximum likelihood method to estimate parameters, and chi-square statistics for hypothesis testing (Dyer, 1995).

Experiment 3—Specialist Herbivores. To test the effects of elevated *P. cenocladum* amide concentrations on a specialized herbivore we conducted a performance experiment with two undescribed species of geometrid larvae in the genus *Eois* that appear to be oligophagous (Dyer, personal observations) at La Selva Biological Station in Costa Rica. In pilot feeding studies, the two species did not exhibit significant differences in development times, pupal weights, parasitism

rates, or mortality (Dyer and Gentry, unpublished data), so they were lumped together for this experiment. We collected 508 early instars (primarily second) from the field, measured their length and mass, and randomly assigned them to petri dishes with 9-cm disks of fresh leaf material harvested from ant-containing *P. cenocladum* plants growing in the field. Before adding the caterpillars, we made a series of 100 ml methanol–amide solutions and pipetted 1 ml onto the underside of each leaf disk, covering the entire surface, and let the methanol evaporate. Treatments consisted of 0.17 mg (per leaf) piplartine ($N = 109$), 0.14 mg 4′-desmethylpiplartine ($N = 70$), 0.1 mg cenocladamide ($N = 79$), a mixture of the three amides (with each amide added at the same mass as for individual treatments; $N = 82$), and a control with only methanol ($N = 168$). Shrubs with ants have a mean concentration of 0.41% dry weight total amides in the leaves, thus the concentrations that we used supplemented existing amide concentrations so that they were within the range of concentrations found in leaves of plants in the field without ants (the mean value for total amides in fragments without ants is 1.74% dry weight; Dyer et al., 2001) and were based on mean dry weights of leaf disks this size (30.4 mg, $N = 20$). Caterpillars were reared at ambient temperature and light. All larvae were reared until they eclosed as adults. The status of individuals was checked each day, and mortality, the time until pupation, and pupal mass were recorded.

Chi-square tests were used to determine whether mortality and parasitism differed among each of the amide treatments and controls, and ANCOVAs were used to assess treatment effects on total weight gain, development time, and pupal mass with initial length (mm) as a covariate. Tukey's HSD studentized range tests were used to conduct *post hoc* comparisons of means for each treatment group incorporating corrections for the elevated probability of Type I error.

Experiment 4—Bioassay with Omnivorous Ant. From January 2000 to July 2001 in rainy season months (June–August, October–December), we conducted a bioassay in which we offered amides in artificial nectaries (sugar water in a 2.5 ml microcentrifuge tube) along with control nectaries to *P. clavata* colonies at La Selva. Detailed methods for this bioassay are in Dyer et al. (2003). We assessed responses of ants to piplartine (0.44 and 1.06% dry weight of the nectary), 4′-desmethylpiplartine (0.36 and 1.06%), cenocladamide (0.26 and 1.06%), and a mixture of all three amides at the lower concentration (1.06% total amide concentration). The lower amide concentrations used in these nectaries were adjusted to mimic those that are normal for *P. cenocladum* shrubs with ants at La Selva (Dodson et al., 2000; Dyer et al., 2001). The higher concentrations (i.e., 1.06%) were used in assays to assess whether the amides act additively, where 1.06% concentrations of the mixture should exhibit a similar response to 1.06% of any amide alone, or synergistically, in which case the mixture may be far more deterrent than the high concentration of any single compound. In addition to the tests

of synthetic amides, methanol extracts of *P. cenocladum* plants without ants were also tested. The response variable in this bioassay is the adjusted consumption difference (ACD), which ranges from -1 to 1, with values from 0 to 1 indicating an unpalatable extract (i.e., ants consume more control than experimental nectar) and values from 0 to -1 indicating a palatable extract (Dyer et al., 2003). Two tailed t-tests were used to determine if ACD values were significantly different from zero. The sample size for these tests was always 15 (based on the number of ant colonies, not on the number of extracts made) for each extract type.

RESULTS

Experiment 1. *Spodoptera frugiperda* exhibited a variety of responses to *P. cenocladum* amides, but it was clear that amides caused dramatic increase in caterpillar development time ($F_{5,171} = 96.9$, $P < 0.001$), reduction in adult mass ($F_{5,171} = 38.0$, $P < 0.001$), and decreased survival of *S. frugiperda*. In general, diets containing piplartine by itself or in any combination with other amides were the most detrimental in terms of survival, pupal mass, and development times (Figure 2). However, 4′-desmethylpiplartine also reduced pupal mass, especially in mixtures (Figure 2), and appeared to increase development time (though this was not significant at a modified Bonferroni corrected α of 0.0035). Analysis of survival was complicated by the fact that early instar mortality was high on the control diet because of drowning of early instars in condensate. However, log-rank comparisons of mortality curves beginning at day 10 showed that caterpillars reared on the mixture of all three amides, piplartine alone, and the piplartine—-4′-desmethylpiplartine mixture all experienced significantly greater mortality than those on control food (Figure 3). In contrast, those feeding on diets containing only cenocladamide experienced significantly lower probabilities of mortality (Figure 3). Regressions of days to development, pupal mass, and relative growth rates against total amide concentration were all highly significant ($F_{1,180} = 39.5$, $F_{1,157} = 63.9$, $F_{1,221} = 109.2$; all $P < 0.001$) but the variance explained was relatively low ($R^2 = 0.180$, 0.289, 0.335, respectively).

Experiment 2. Analysis of the *A. cephalotes* choice tests revealed that control and piplartine disks were the most attractive to the ants, and the piplartine by control interaction was the strongest of all effects that included the control (Figure 4, Table 1), reflecting the fact that if high numbers of control disks were taken, then high numbers of piplartine disks were also taken. The 4′-desmethylpiplartine, cenocladamide, and mixture disks were taken less frequently than controls, which was reflected in the significant interaction terms (i.e., there were higher than expected cell frequencies for control disks taken and no amide disks taken). Leaves coated with the mixture of all three *P. cenocladum* amides were removed least often by

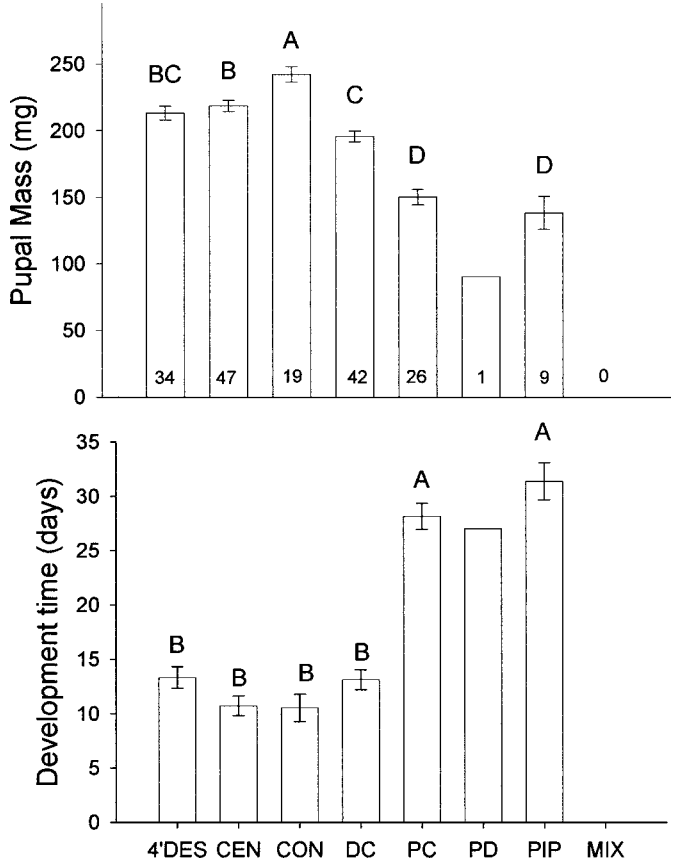

FIG. 2. *Spodoptera frugiperda* pupal mass and development times when reared on artificial diet spiked with a control solvent, a single amide compound, or combinations of the compounds. MIX = mixture; CEN = cenocladamide; CON = control; PIP = piplartine; 4′DES = 4′-desmethylpiplartine; DC = 4′DES + CEN; PC = PIP + CEN; PD = PIP + 4′DES. Numbers on the bars indicate sample sizes (not all larvae survived to pupation). Different letters indicate significant differences.

foraging ants (Figure 4), which was evident from the fact that the three mixture interaction terms contributed the most to the fit of the loglinear model (Table 1).

Experiment 3. All measures of fitness for the specialist geometrids, *Eois* spp., were significantly affected by leaf diets spiked with amides, but the mixture of amides was the only leaf diet associated with increased parasitism (16% vs. 7% or lower for controls and individual amides; $\chi^2 = 5.5$, DF = 1, $P = 0.02$), and increased mortality (65% vs. 37% or lower for controls and individual amides; $\chi^2 = 17.1$, DF = 1, $P < 0.001$). Mortality and parasitism were not different for

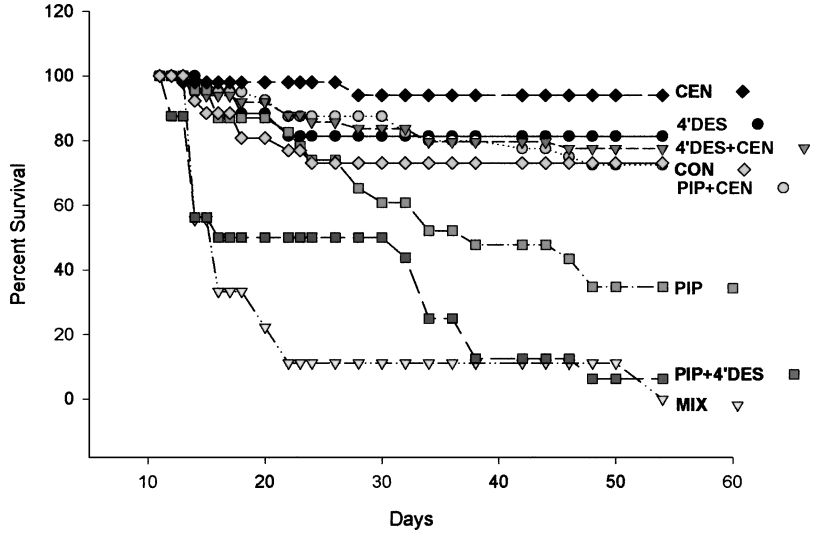

FIG. 3. Percent survival of *Spodoptera frugiperda* larvae feeding on artificial diet spiked with either a control solvent (CON), a single amide compound (piplartine (PIP), 4′-desmethylpiplartine (4′DES), cenocladamide (CEN)), or combinations of the compounds (MIX).

individual amides vs. the controls ($\chi^2 < 1$, DF $= 1$, $P > 0.05$ for all comparisons). Total weight gain, development time (days to pupation), and final pupal mass all differed among treatments (Table 2). Caterpillars feeding on leaves to which

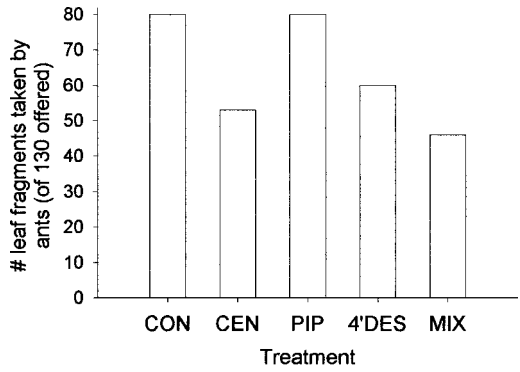

FIG. 4. Frequency of individual leaf disks taken by *Atta cephalotes* foraging ants. Disks were spiked with a control solvent, a single amide compound (piplartine, 4′-desmethylpiplartine, cenocladamide), or a mixture of the compounds.

TABLE 1. LEAF-CUTTING ANT (*Atta cephalotes*) RESPONSES TO COMBINATIONS OF LEAF DISKS SPIKED WITH DIFFERENT AMIDES

Source[a,b]	Parameter estimate[c]
Mixture × 4′-desmethylpiplartine	5.7064**
Cenocladamide × Mixture	5.0720**
Control × Piplartine	4.2127**
Control × 4′-desmethylpiplartine	3.8747**
Control × Cenocladamide	3.4456**
Control × Mixture	2.4048*

[a] Results are from a parsimonious log-linear model, which was a significant fit to the data ($\chi^2 = 7.5$, DF $= 7$, $P = 0.38$).
[b] The mixture included all three amides, piplartine, cenocladamide, and 4′-desmethylpiplartine.
[c] Parameter estimates are standardized, and P values are $^{**}P < 0.001$, $^{*}P < 0.02$, based on chi-square statistic and 1 degree of freedom for each.

cenocladamide was added gained half the weight of those on leaves with no amide addition. Development time exhibited a different pattern, with caterpillars feeding on piplartine laced leaves developing more slowly than those on 4′-desmethylpiplartine laced leaves (Figure 5). Finally, pupal mass was lowered on leaf diets with all amide combinations except piplartine alone (Figure 5).

Experiment 4. None of the single amide sugar solutions were deterrent to *P. clavata* ants, regardless of their concentration. In fact, concentrated solutions of cenocladamide (1.06%), appeared to be more attractive to ants than the straight sugar solution controls (Table 3). However, the sugar solution containing all three amides was avoided relative to controls, and *P. clavata* strongly avoided sugar solutions containing whole plant extracts.

TABLE 2. EFFECTS OF HEIGHTENED *P. cenocladum* AMIDE CONCENTRATIONS ON GROWTH AND DEVELOPMENT OF SPECIALIST *Eois* spp. CATERPILLARS[a]

Response variable	Source	*F*	*DF*	*P*
Weight gain	Amide treatment	3.36	4, 260	0.0106
	Initial length	19.97	1, 260	<0.001
Development time	Amide treatment	4.03	4, 260	0.004
	Initial length	27.80	1, 260	<0.001
Pupal mass	Amide treatment	17.45	4, 313	<0.001
	Initial length	1.20	1, 313	0.274

[a] Based on survivors of 508 individual rearings.

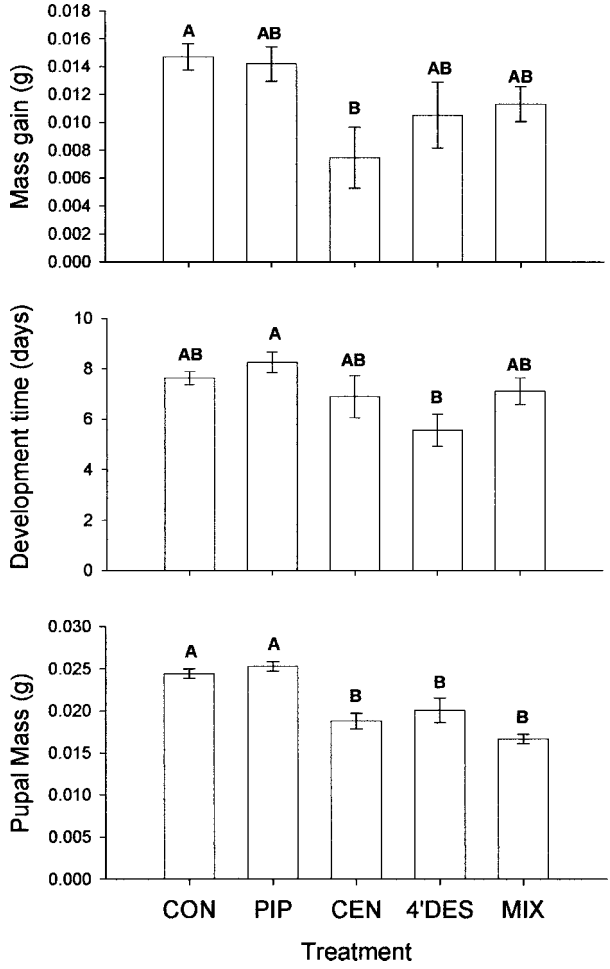

FIG. 5. Mass gain, pupal mass, and development times of *Eois* spp. larvae feeding on leaf diet spiked with a control solvent, a single amide compound (piplartine, 4′-desmethylpiplartine, cenocladamide), or a mixture of the compounds. Different letters indicate significant differences.

DISCUSSION

Amides in *P. cenocladum* are deterrent and toxic to both specialist and generalist herbivores. We detected several responses to amides including: (1) toxicity, (2) repellency, (3) attractance, (4) developmental effects, and (5) fitness effects. The first two were synergistic, while the latter were either independent (attractance)

TABLE 3. RESULTS OF BIOASSAY USING *Paraponera clavata* TO ASSESS THE RELATIVE PALATABILITY OF THREE SYNTHETIC AMIDES AND THE PLANT FROM WHICH THEY ARE DERIVED

Compound (% dry mass) or plant	ACD[a]	SE[b]
Cenocladamide (1.06)	*−0.10*	0.002
Piplartine (0.44)	*−0.049*	0.004
Cenocladamide (0.26)	*−0.047*	0.01
4′-Desmethylpiplartine (1.06)	*−0.04*	0.002
Piplartine (1.06)	*−0.03*	0.002
4′-Desmethylpiplartine (0.36)	*−0.019*	0.008
Mixture of the three amides (1.06)	**0.065**	0.01
Piper cenocladum without ants	**0.3**	0.01

[a] ACD = adjusted consumption difference (described in the text). Italic ACD values are attractant to ants while those in bold are deterrent.
[b] SE = 1 standard error of the mean.

or additive (developmental and fitness effects). The most compelling support for the synergy hypothesis came from the *P. clavata* bioassay, in which the mixture was deterrent, while individual amides at both the natural concentrations and at the increased mixture concentration were neutral or attractive to the ants. The deterrence value for the mixture was greater than three times the values (i.e., expected additive effects) for individual amides (Table 3). The strong preference of *P. clavata* for controls relative to whole plant extracts of *P. cenocladum* suggests that synergistic interactions may also occur among the three amides studied and other unidentified compounds in *P. cenocladum*.

For the caterpillar and *Atta* experiments, mixtures of amides offered to herbivores were triple the concentration of single amide treatments, suggesting that the observed effects were additive rather than synergistic. This could be true for some important response variables such as caterpillar pupal weights and development times; it is clear that individual amides do affect these attributes. However, there are several strong results that justify rejecting this alternative hypothesis for the most important variable measured—caterpillar mortality. For *Eois*, only the mixture affected parasitism and mortality, with χ^2 values being more than triple the values for individual amides. For the *Spodoptera* experiments, linear regressions of performance measures against total amide concentrations leave the majority of variance unexplained, which suggests that effects were more than additive. Furthermore, the mixture caused 100% mortality, which was more than triple the mortality of all treatments except piplartine alone and the piplartine—4′-desmethyl piplartine mixture; all the double amide treatments with cenocladamide had no effects. These results suggest that the synergy involved in *Spodoptera* mortality requires the presence of piplartine. These potent effects of amide mixtures are comparable to effects of commercial pesticides as well as other natural products. For example,

other studies using *S. frugiperda* report the same (100%) or lower (0–85%) mortality for diets spiked with compounds present at concentrations varying from 0.0025–1% dry weight (Belofsky et al., 1995; Cespedes et al., 2001; Wheeler et al., 2001).

One important example of synergy that requires comment is the work of Scott et al. (2002). They documented synergistic insecticidal effects of *Piper tuberculatum* amides against the mosquito *Aedes atropalpus* L. Our work corroborates theirs but differs in two important ways. We tested the amides of *P. cenocladum* against four different ecologically relevant herbivores instead of a human parasite. These synergistic amide mixtures probably evolved to defend *Piper* spp. from herbivores, not mosquitoes; thus, our system is relevant to the chemical ecology of *Piper* spp. Second, Scott et al. (2002) hypothesize that synergy in *P. tuberculatum* amides may be due to the presence of methylene dioxyphenyl groups (MDP). Inactive molecules containing MDP groups are commonly added to pesticides to synergistically enhance their lethality. The MDP group is known to inhibit P450 mixed function oxidases and allow the insecticide to avoid metabolic inactivation. The amides used in this study do not contain this moiety (Figure 1), thus, the synergy observed is not due to the effect of MDP groups on mixed function oxidases.

Plant chemical defense theory has generated many hypotheses on differences in responses of specialist versus generalist herbivores to specific defenses. It is interesting to consider the same hypotheses for synergy. Did synergistic compounds evolve in response to specialist herbivores that have circumvented the toxicity of individual defenses? Do the mechanisms of synergy usually involve general physiological reactions that are the same for all herbivorous and omnivorous insects? In our experiments, specialists were better equipped to circumvent the synergistic toxicity and developmental effects of the amides than generalists, nonetheless, amides functioned as a synergistic defense against specialists. While mixed diets caused the greatest mortality for both specialists and generalists, the mechanism of mortality was different; for specialists, the amides enhanced parasitism, while for the generalists, there was a more direct toxicity.

Individual, additive, and synergistic effects are possible for all categories of animals tested in this study: generalists, specialists, herbivores, and omnivores, but the mechanisms involved are likely to be different. Differences in the effects of individual *P. cenocladum* amides on the different organisms were interesting. For *S. frugiperda*, any mixture containing piplartine was detrimental, while for *A. cephalotes* and *Eois* spp., piplartine was ineffective. Cenocladamide was effective on its own against *Eois* spp. and *A. cephalotes*, but it had no effect on *S. frugiperda*. No individual compounds were effective against *P. clavata*. These differences support the idea that there is no "magic bullet" of defensive chemistry. Broad synergistic defense in combination with toxicity of individual compounds to targeted species may be the best adaptation to surviving attack by multiple species of herbivores and pathogens (but see Jones and Firn 1991 for an alternative hypothesis).

The importance of synergistic interactions among plant secondary compounds is only beginning to be explored (Berenbaum and Neal, 1985; Berenbaum et al., 1991; Kubo and Muroi 1993; Stermitz et al., 2000; Calcagno et al., 2002; Scott et al., 2002). The scarcity of examples of synergistically acting secondary compounds is likely due to the lack of research on this topic or weak statistical methods (Nelson and Kursar, 1999), and we suggest synergistic effects may be the rule rather than the exception. This may explain the apparent lack of defensive properties that have been indicated for a variety of plant secondary compounds, which have no known function (Harborne, 1988; Ayres et al., 1997) and the explanation for the redundancy observed in chemical defenses. Tests of antiherbivore (or other) activity of specific plant secondary metabolites must be supplemented with appropriate tests (e.g., Jones 1998; Nelson and Kursar 1999) of pertinent mixtures and whole plant extracts.

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ASSOCIATIVE OLFACTORY LEARNING OF HONEYBEES TO DIFFERENTIAL REWARDS IN MULTIPLE CONTEXTS—EFFECT OF ODOR COMPONENT AND MIXTURE SIMILARITY

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Abstract—Discrimination among differentially rewarding flowers allows honeybees to maximize their foraging efficiency. We studied how honeybees are able to relate to differential positive rewards when the odor representations are either structurally dissimilar, structurally similar (or form a substrate–product duo *in planta*), or form a binary mixture sharing a common constituent. Bees were tested both in conditioning of the proboscis-extension response (PER) and in a free-flying context. Our results point to honeybees using olfactory associative learning to differentiate between two positively rewarded odors. In PER, subjects discriminated best between dissimilar odors; they initially generalized between similar odors, but eventually learned to discriminate between them. The discrimination between mixtures sharing a common constituent remained poor. Likewise, the difference in visits to low- and high-rewarding flowers of free-flying bees was greater for dissimilar odors than for binary mixtures sharing a common constituent. Consequences of the operant conditioning nature of the free-flying context are discussed.

Key Words—Honey bee, *Apis mellifera*, Hymenoptera, Apidae, conditioned proboscis extension, free-flying foragers, olfactory discrimination, floral volatiles.

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INTRODUCTION

The ability of honeybees, *Apis mellifera* L., to discriminate among differential rewards in natural settings is mostly based on assessment of the flower bouquet in relation to reward (Masson et al., 1993). Odors may be either attractive or repellent to honeybees, sometimes as a function of their relative concentration and abundance (Henning et al., 1992), but mostly through their association to a more profitable nectar or pollen reward (Menzel, 1993; Dobson et al., 1996). In this manner, honeybees can discriminate among different genotypes of the same species (Wolf et al., 1999) or among different flowering stages of a particular genotype (Pham-Delegue et al., 1989). Based on circadian, diurnal, temperature dependent, or asynchronous emissions of flower odors (Loughrin et al., 1991, 1993; Hansted et al., 1994; Nielsen et al., 1995), honeybees learn to associate certain constituents of a bouquet with current reward (Blight et al., 1997).

The associative learning capability of honeybees has been extensively studied through the proboscis-extension response (PER) paradigm (Bitterman et al., 1983; Menzel and Muller, 1996). In PER conditioning, bees are harnessed so that they can freely move their mouthparts and antennae. Sucrose stimulation to the antennae serves as an unconditioned stimulus (US) and elicits proboscis extension as the unconditioned response (UR). If an odor as a conditioned stimulus (CS) is properly paired with the US, the odor itself becomes capable of eliciting proboscis extension as a conditioned response (CR). Many phenomena relating to the behavior of honeybees have been elucidated with this experimental paradigm (Hammer and Menzel, 1995; Shafir et al., 1999; Hosler and Smith 2000; Wright et al., 2002).

In free-flying bees, Jakobsen et al. (1995) found that honeybees, in contrast to bumblebees, disregarded unreliable positional cues for reward, and used odoriferous stimuli to locate a food source on a rotating arena. Laska et al. (1999) demonstrated that free flying honeybees distinguished among a large number of compound pairs bearing structural similarity to each other. In both these studies, conditioning was performed by associating a particular odor with sucrose reward against a background of a nonrewarding odor.

Only a few attempts have been made to test associative conditioning in multiple contexts. Gerber et al. (1996) found that experienced foragers could transfer their experience to the PER context, and Sandoz et al. (2000) showed transfer from PER conditioning to orientation in a free-walking olfactometer (Sandoz et al., 2000).

When restrained bees are conditioned to a specific odor, they sometimes generalize and extend their proboscis when confronted with a novel odorant; the level of generalization depends upon the structural similarity of the novel odorant to the conditioned one (Getz and Smith, 1990). In the field, honeybees are able to discriminate among closely related flowers and recognize which of these is most rewarding (Pham-Delègue et al., 1989). The bees often pick salient major

components of the bouquet and disregard the other components in their associative acquisition of an odor-reward pairing (Blight et al., 1997; Le Metayer et al., 1997). This strategy saves the need to relate to each of the odors in the myriad of olfactory stimulations in the field. Separate analysis of components of a mixture, in addition to relating to its configural properties (Chandra and Smith, 1998; Smith, 1998; Hosler and Smith, 2000), facilitates discrimination among volatiles. However, the recognition of some compounds may be affected by their association to other compounds in a mixture (Laloi et al., 2000).

This raises the question as to how honeybees assess natural situations such as bouquets of closely related genotypes where components may be identical or are linked as substrate-product duos. For example, evolutionary development of floral "scent genes" has facilitated the production of novel floral odors. In *Clarkia breweri*, (*S*)-linalool is produced in a one-step reaction catalyzed by (*S*)-linalool synthase from its ubiquitous precursor, geranyl pyrophosphate (GPP). Its appearance in the bouquet clearly defines it from a closely related species, *Clarkia concinna* (Pichersky et al., 1995). The production of benzyl acetate from benzyl alcohol by the action of acetyl CoA:benzyl alcohol acetyltransferase (Dudareva et al., 1998), makes benzyl acetate a major constituent of the *Clarkia breweri* bouquet. Linalool and benzyl acetate are some of the most common odor components in flowers, yet in many instances benzyl acetate co-occurs in the volatile emission of the flowers together with its substrate, benzyl alcohol (Knudsen et al., 1993).

In the common application of PER, discrimination between two odorants is tested by associating one odorant with an appetitive (positive) reward and the other with no reward or with a negative stimulus. When both odorants are appetitively but differentially rewarded, associative learning theory predicts different learning curves and asymptotic values for the two odorants (Rescorla and Wagner, 1972; Pearce and Bouton, 2001). The ability to discriminate between differentially rewarding odorants has clear relevance to decision making in foraging honeybees that are rarely confronted with an all or nothing reward ensemble.

We tested the ability of honeybees to discriminate between odors associated with differential appetitive rewards in the PER paradigm and in a free-flying context. We tested discrimination between odor combinations that were either structurally dissimilar, structurally similar (or formed a substrate–product duo), or required discrimination between two-compound mixtures having one common constituent.

METHODS AND MATERIALS

Insects. We tested the ability of honeybees (*Apis mellifera L.*) to discriminate among various differentially rewarded odor combinations using PER conditioning (Bitterman et al., 1983; Smith, 1998). Departing bees were collected into individual

glass vials from the entrance of the hive. The bees were chilled for 2–3 min until they ceased moving and then harnessed into holders in a manner that inhibited movement of the thorax yet enabled full movement of the head (Shafir et al., 1999). Five minutes after being restrained, the bees' antennae were touched with a 1 μl drop of 45% w/v sucrose solution. Bees usually extended their proboscides following this stimulation, were allowed to consume the drop, and were included in the experiment. The few bees that failed to extend their proboscides were excluded.

Chemicals. We tested compounds from several chemical groups (Table 1) in combinations that varied in the degree of chemical structure similarity and in whether they represent a substrate–product relationship in the flower (Table 2). Geraniol and 1-hexanol were from Sigma (St. Louis, USA) whereas racemic linalool, benzyl alcohol, benzyl acetate, methyl salicylate, isoeugenol, and methyl isoeugenol were from Merck (Mannheim, Germany).

PER Conditioning Procedure. Experiments were performed between July and October 2000 (late summer) in an air-conditioned room maintained at 21–25°C. The various experimental conditions tested are shown in Table 2. We performed replicates of each experimental condition on several days, dispersed throughout the experimental period. Training commenced after a 1–1.5 hr habituation period. Odor delivery was performed by applying 3 μl of pure odorant onto a strip of filter paper placed inside a 1 ml glass syringe, connected to a computer-controlled air pump. In experiments that tested discrimination between two mixtures that share a common component, we added 3 μl of odor C to filter papers with odors A and B, thus forming the combinations AC and BC. In all experiments, odor A was associated with a high reward "H" (1 μl, 45% w/v sucrose), and odor B was associated with a low reward "L" (0.5 μl, 15% w/v sucrose).

Eight bees were tested concurrently, and each odor was presented in six trials to each bee with an intertrial interval (ITI) of 7 min. The trials were conducted in the following pseudorandom order: ABABBABAABAB. The CS (odor) duration was 3.5 sec and the US (sucrose) was delivered 2 sec after onset of the CS, so that it overlapped with the CS for 1.5 sec. We recorded whether the subject extended its proboscis during the interval between onset of the CS and onset of the US.

Choice Experiments with Free-Flying Bees. We used four honey bee colonies kept in 10-comb standard Langstroth hives. They were concurrently introduced into a 12 $\times$ 7-m screened enclosure in Rehovot, Israel, and remained there throughout the duration of the experiments between September-November 2000. In May 2001, four colonies were again introduced into the enclosure. In order to sustain the colonies, and to maintain a constant motivation for foraging, we fed them twice a week with 0.5 l of 100% w/v sugar syrup and a 100 g pollen supplement patty.

The Artificial Flowers Patch. During an experiment, bees foraged from a patch of 40 artificial flowers, distributed along four rows (like crops in agricultural fields), with 1 m separation between rows and between flowers. The ten flowers

TABLE 1. CHEMICAL GROUP AND STRUCTURE OF VOLATILE ODORS USED IN THIS STUDY

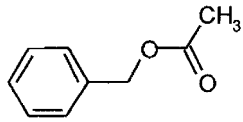
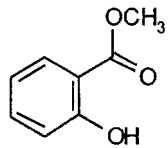
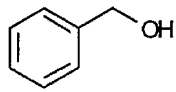
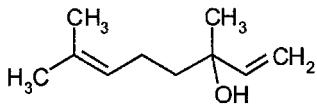
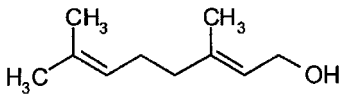
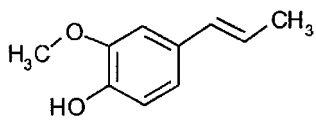
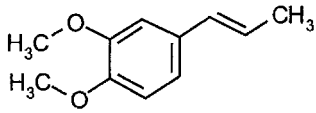
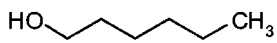
Name of compound	Chemical group	Chemical structure
Benzyl acetate	Aromatic ester	
Methyl salicylate	Aromatic ester	
Benzyl alcohol	Aromatic alcohol	
Linalool	Monoterpene	
Geraniol	Monoterpene	
Isoeugenol	Phenylpropanoid	
Methylisoeugenol	Phenylpropanoid	
1-Hexanol	Aliphatic alcohol	

TABLE 2. COMBINATIONS OF COMPOUNDS TESTED IN PER EXPERIMENTS AND THEIR RELATEDNESS[a]

Compound A	Compound B	Compound C	Chemical structure similarity	Substrate-product pair (*in-planta*)
Geraniol	1-Hexanol	—	No	No
Benzyl acetate	1-Hexanol	—	No	No
Linalool	1-Hexanol	—	No	No
Benzyl acetate	Benzyl alcohol	—	Yes	Yes[b]
Methylisoeugenol	Isoeugenol	—	Yes	Yes[c]
Methyl salicylate	Benzyl acetate	—	Yes	No
Geraniol	1-Hexanol	Benzyl acetate	No	No
Methylisoeugenol	1-Hexanol	Benzyl acetate	No	No

[a] Compound A was associated with the high reward (1 μ1, 45% sucrose solution), and compound B with the low reward (0.5 μl, 15% sucrose solution). In the odor-mixture experiments, compound C was added to compounds A and B to form mixtures AC and BC. Compound pairs are classified as similar if they share functional groups (e.g., benzyl acetate and benzyl alcohol have a phenyl group in common), and instances of compounds that form a substrate–product pair *in planta* are noted.
[b] Dudareva et al., 1998.
[c] Wang and Pichersky, 1998.

on each of two alternate lines offered a high reward, and the ten flowers on each of the other two lines offered a low reward (see experiment descriptions for details). Flowers were constructed using Plexiglas. A 10-mm thick, 6-cm diam, round flower was mounted on a 3-mm thick, 14.5 $\times$ 14.5-cm, green base. At the center of each flower, an 8.5-mm deep well, 5-mm diam, could potentially hold about 100 μl of sugar solution. Tubing reached the well by a tunnel drilled through the flower. Flowers were covered by yellow circular labels with four blue strips acting as nectar guides, pointing towards the center. The well at the center was marked by a blue circle 2 cm in diameter. Two strips of filter paper, 20 $\times$ 5-mm each, were glued on either side of the feeding well, with the odorants (3 μl per strip) administered onto the strips with a dispensing micropipette. Thus, all the flowers looked the same, but the flowers in the high and the low rewarding lines were distinguished by the odorants applied to them, according to the specifications of each experiment.

Sucrose solution was administered to the artificial flowers by four syringes (2 $\times$ 50 ml and 2 $\times$ 20 ml) mounted on a syringe pump (SP 200, World Precision Instruments Inc., Sarasota, USA). The 50-ml syringes were filled with a 45% w/v sucrose solution (H lines), and the 20-ml syringes were filled with a 15% w/v sucrose solution (L lines). The flow rates were 0.2 ml/min and 0.1 ml/min for the H and L lines, respectively. This amounted to a total flow of 20 μl/flower/min for the H line and 10 μl/flower/min for the L line. Thus, the H line received six times the total sugar reward of line L. Each syringe was connected to two pieces of a

6-m long, 1.6-mm internal diam (ID) Tygon tubing (Fisher Scientific, Pittsburgh, USA). The tubing was spread in four parallel rows, alternating between H and L lines. Artificial flowers were connected to the main lines with 20-cm long, 0.8-mm ID tubing and an infusion tap that controlled flow into each flower.

Procedure. The entrances of the hives were blocked approximately 30 min before first light, except for the hive that participated in the experiment on that day. Experiments began 30–90 min after first light, as soon as the ambient temperature reached 22°C or 19°C in September–November 2000 and May 2001, respectively. Each session lasted for approximately 70 min, with the ambient temperature at the end of each session reaching 22–25°C. This ensured that evaporation of both sugar solution and odors was moderate and almost constant throughout the experimental session.

At the beginning of every session, one researcher applied the odorants (3 μl per strip, two strips per flower), while another operated the automatic syringe pump to start the flow of sucrose solution into the flowers. To assess the bees' ability to discriminate between high and low rewarding flowers, a researcher moved from flower to flower along each row and counted for 10 sec the number of bees that touched the inner blue circle of each flower. Each round of counting the bees on all 40 flowers ($\sim$10 min) constituted a count episode. Each day we performed one replicate of an experiment, during which six count episodes were conducted consecutively, with a short break between the third and fourth count episodes during which a second round of odorant application was performed to compensate for evaporation. We performed three replicates of experimental condition 2000-A versus A and four replicates of each of the other experimental conditions (Table 3). We conducted the experiments on consecutive days in each year, alternating between experimental conditions and hives in a balanced design. Thus, each replicate of each experimental condition was with bees from a different hive, and several days

TABLE 3. COMBINATIONS OF ODORANTS TESTED IN THE SCREENED ENCLOSURE EXPERIMENTS WITH FREE-FLYING BEES[a]

	Odor			
Experimental condition	A	B	C	Replicates (days)
2000-A versus B	Linalool	1-Hexanol	—	4
2000-A versus A	Benzyl acetate	Benzyl acetate	—	3
2001-A versus B	Linalool	1-Hexanol	—	4
	1-Hexanol	Linalool	—	4
2001-AC versus BC	Linalool	1-Hexanol	Benzyl acetate	4
	1-Hexanol	Linalool	Benzyl acetate	4

[a] Odor A was always associated with the high reward and odor B with the low reward. In 2001, the odors associated with A and B were counterbalanced between replicates of each experimental condition. In the two odor-mixture conditions, a common odor C was added to each of the other two odors.

elapsed after a hive was used until it was tested again. In addition, we alternated the position of the high and low rewarding rows between days to control for potential position effects, such as positional learning. After the sixth count episode of each morning, the entrances of all the hives in the enclosure were opened and the bees were allowed to scout the nonrewarding artificial flowers.

In experiment 2000-A versus B, the syringe pump was on for the entire duration of the experiment. In contrast, in experimental condition 2000-A versus A and all the experiments in 2001, the syringe pump was turned off after the fourth count episode, and count episodes 5 and 6 became extinction episodes.

Differential Reward with Different Odors without Extinction (2000-A vs. B). Under this experimental condition, the high- and low-rewarding flowers were marked by different odors, and the syringe pump was on throughout the duration of the experiment.

Differential Reward with Identical Odor with Extinction (2000-A vs. A). Sucrose solution was dispensed to the high- and low-rewarding rows via the syringe pump system, which was turned off after count episode 4, so that count episodes 5 and 6 became unrewarding, extinction episodes. The odor we used in both high- and low-rewarding flowers was benzyl acetate.

Differential Reward with Different Odors with Extinction (2001-A vs. B). These experiments tested how well bees discriminated between low- and high-rewarding lines with different odors. In half of the replicates, 1-hexanol was associated with the L lines and linalool with the H lines; the odors were reversed in the other half of the replicates. The balanced design allowed differentiating the influence of possible spontaneous preference to a particular odor from that of odor-reward associative learning. Bees may have an innate preference for linalool over 1-hexanol, because the appearance of these compounds in natural bouquets is disproportionately in favor of linalool (Knudsen et al., 1993). Dispensing of reward was as in experiment 2000-A versus A. These experiments reflected the ability of bees in their natural settings to detect predictive differences in reward salience via associative learning.

Differential Reward with Odor Mixtures Having a Common Constituent (2001-AC vs. BC). In these experiments, we tested the ability of bees to discriminate between the H and L lines when the lines were marked by odor mixtures that had a common constituent. One line was associated with linalool and benzyl acetate, and the other line was associated with 1-hexanol and benzyl acetate, in a balanced design across replicates with regards to reward value. Dispensing of reward was as in experiments 2000-A versus A and 2001-A versus B. These experiments reflected the ability of bees to overcome odor mixture similarity, exemplified by the presence of a common odorant, in discriminating differentially rewarding flowers.

Statistical Analyses. In the PER experiments, the measure of performance was the proportion of subjects that extended their proboscides to the CS in each

trial, prior to delivery of the US. In each experimental condition, we were interested in testing the discrimination of subjects between the two differentially rewarding odors (or mixtures). Since learning curves typically show a logistic growth function, we used logistic growth curve analysis in SAS (Hartz et al., 2001) to compare the learning slopes between the low- and high-rewarding odors.

We assigned 1 to a response and 0 to a lack of response, and calculated the level of preference for the high-rewarding odor (or mixture) for each subject in each trial number as response to high minus response to low. Hence, a difference of 1 reflects a preference for high, −1 a preference for low, and 0 indifference. We compared the preferences for the high-rewarding odors between two odors, A and B, that were similar, dissimilar, or that appeared in a binary mixture (AC vs. BC) with a common component, C. We combined data from each experimental condition group (similar, dissimilar, mixture), and calculated mean preferences for trials 2–6 (subjects are naive about the rewards in the first trial). We used Tukey's test in a one-way ANOVA to compare mean preferences between experimental condition groups in trial 6 (JMP 4, SAS Institute).

In the experiments with free-flying bees, for each count episode of each day, we calculated the mean number of bees counted on the 20 A flowers and on the 20 B flowers. For each count episode in each experimental condition, we calculated the mean total number of bees visiting the flowers (A + B), the difference in the number of visits to flowers of type A and B (A − B), and the proportion of visits to flowers of type A (A/A + B). We compared separately the performance of the bees in the rewarding count episodes 1–4, and in the nonrewarding count episodes 5–6. Data were transformed as needed to achieve greater homoscedasticity (Sokal and Rohlf, 1981), and means were compared by t tests. Unequal-variance t tests were used when necessary (JMP 4, SAS Institute).

RESULTS

Harnessed Bees

Bees quickly learned to discriminate structurally dissimilar odors, and showed a clear preference for the odor associated with the higher reward (Figure 1a, slope difference = 0.36, $P < 0.005$; Figure 1b, slope difference = 0.57, $P < 0.001$; Figure 1c, slope difference = 0.78, $P < 0.001$).

It was more difficult for bees to discriminate between structurally similar compounds (Figure 2). The association to the higher-rewarding odor A was acquired in a similar manner to that attained between dissimilar odors (Figure 1). However, there was strong generalization during early trials, with response to odor B matching the response to odor A; discrimination occurred only by the third or fourth trials. The learning curves slope differences for Figures 2a, 2b, and 2c, were 0.41 ($P < 0.001$), 0.47 ($P < 0.01$), and 0.50 ($P < 0.005$), respectively.

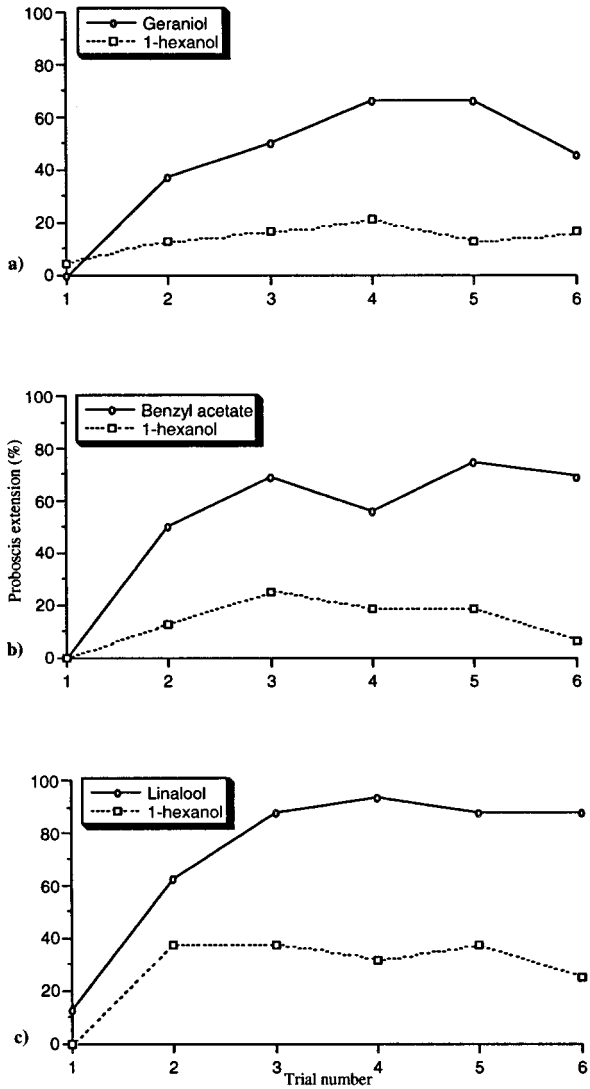


FIG. 1. Discrimination in PER between structurally dissimilar odors, one associated with a high reward (1 μl of a 45% sucrose solution; circles), and the other with a low reward (0.5 μl, 15% sucrose; squares). The odors associated with the high and low rewards, respectively, were a) geraniol and 1-hexanol ($N = 24$), b) benzyl acetate and 1-hexanol ($N = 16$), and c) linalool and 1-hexanol ($N = 16$).

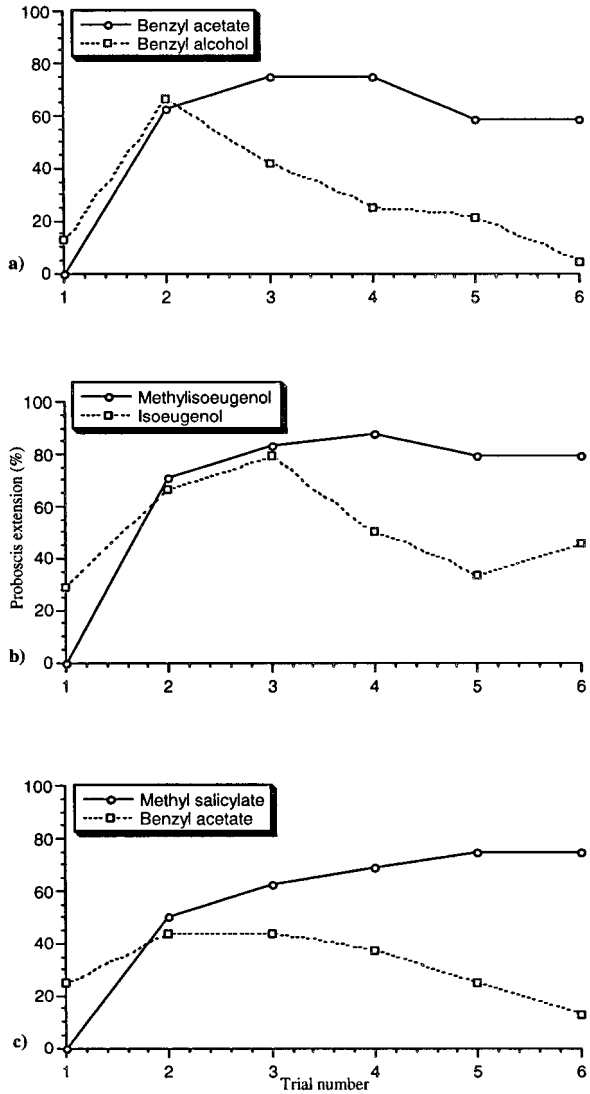


FIG. 2. Discrimination in PER between structurally similar odors, one associated with a high reward (1 μl, 45% sucrose; circles), and the other with a low reward (0.5 μl, 15% sucrose; squares). The odors associated with the high and low rewards, respectively, were a) benzyl acetate and benzyl alcohol ($N = 24$), b) methylisoeugenol and isoeugenol ($N = 24$), and c) methyl salicylate and benzyl acetate ($N = 16$).

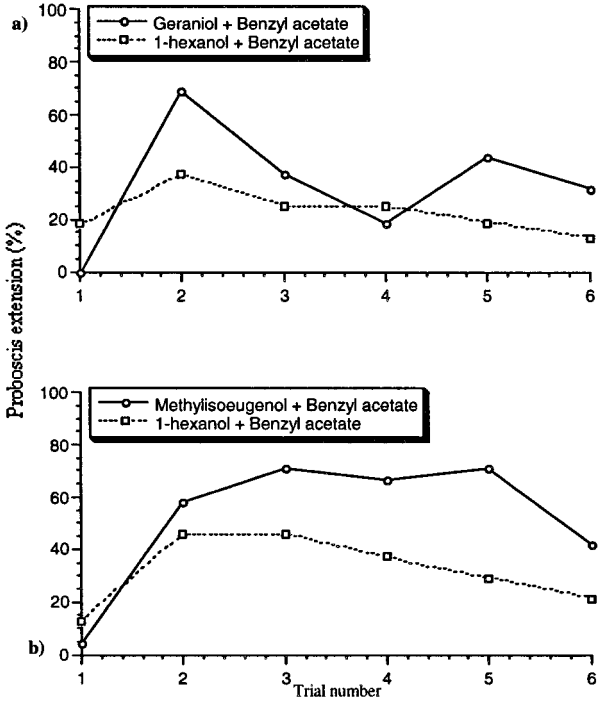


FIG. 3. Discrimination in PER between binary odor mixtures containing a common constituent, one mixture associated with a high reward (1 μl 45% sucrose; circles), and the other with a low reward (0.5 μl, 15% sucrose; squares). The mixtures associated with the high and low rewards, respectively, were a) geraniol plus benzyl acetate and 1-hexanol plus benzyl acetate ($N = 16$), and b) methylisoeugenol plus benzyl acetate and 1- hexanol plus benzyl acetate ($N = 24$).

Discrimination was especially poor between binary odor mixtures containing a common constituent. The addition of benzyl acetate to both geraniol and 1-hexanol greatly reduced the ability of the bees to discriminate between them (compare Figures 1a and 3a). Indeed, the difference between the learning curves to the two mixtures was only 0.15 (NS). The difference between the learning curves to two other mixtures sharing a common constituent was also relatively low (Figure 3b, slope difference $= 0.25$, $P < 0.005$). Overall, slope differences of the learning curves declined from a mean of 0.57 for two dissimilar odors, to 0.46 for similar odors, to 0.20 for binary mixtures containing a common constituent.

Figure 4 shows the difference in each trial of percent proboscis extension to the high- and low-rewarding odor (or mixture) for the three experimental condition groups. Bees showed good discrimination for the dissimilar odors already from the second trial. The discrimination between similar odors was poor at the beginning,

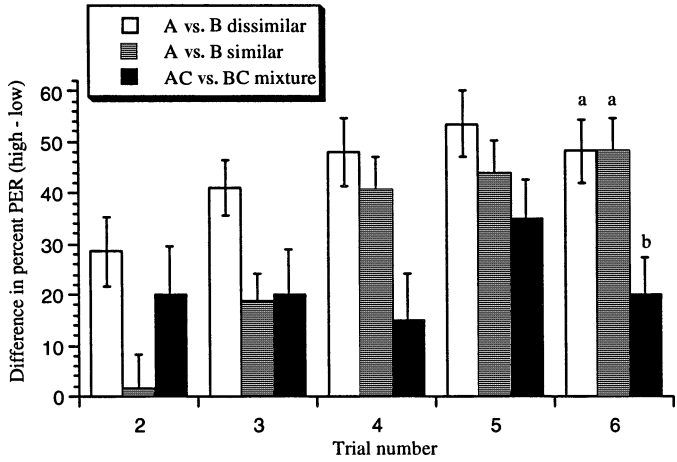


FIG. 4. Summary of PER discrimination experiments, showing mean (±SE) difference in percent response between the high- and low-rewarding odors when they were structurally dissimilar (white), similar (gray), or mixtures with a common constituent (black). Different letters in trial number 6 represent means that are statistically different (Tukey's test, $P < 0.05$).

but steadily improved; by trial 6 bees discriminated equally well between similar and dissimilar odors. The discrimination between binary mixtures that shared a common constituent remained generally weak, and at trial 6 was significantly lower (Turkey's test, $P < 0.05$) than between the dissimilar and the similar odors. A nonparametric test comparing the former with each of the latter, yielded similar results (Wilcoxon tests, $Z > 2.6$, $P < 0.01$).

Free-Flying Bees

Experiments of 2000. The total number of bees visiting flowers (both low and high) during the rewarding count episodes 1–4 was greater in experimental condition 2000-A versus B than in experimental condition 2000-A versus A (Figure 5a; $t = 3.76, df = 26, P < 0.001$). In experimental condition 2000-A versus A, the syringe pump was turned off at the end of count episode 4, but the number of foragers remained relatively constant during count episodes 5 and 6 ($t = 1.5, df = 16$, NS).

The mean number of visits to the high-rewarding flowers was greater than to the low-rewarding flowers in both conditions (Figure 5b). During the rewarding count episodes 1–4, the difference was greater in condition 2000-A versus B than in condition 2000-A versus A (unequal-variance t test, $t = 2.1, df = 24, P < 0.05$). In condition 2000-A versus B the flowers continued to provide sucrose solution in episodes 5 and 6, and the preference for the high-rewarding flowers was similar in count episodes 1–4 and 5–6. In condition 2000-A versus A, the syringe pump

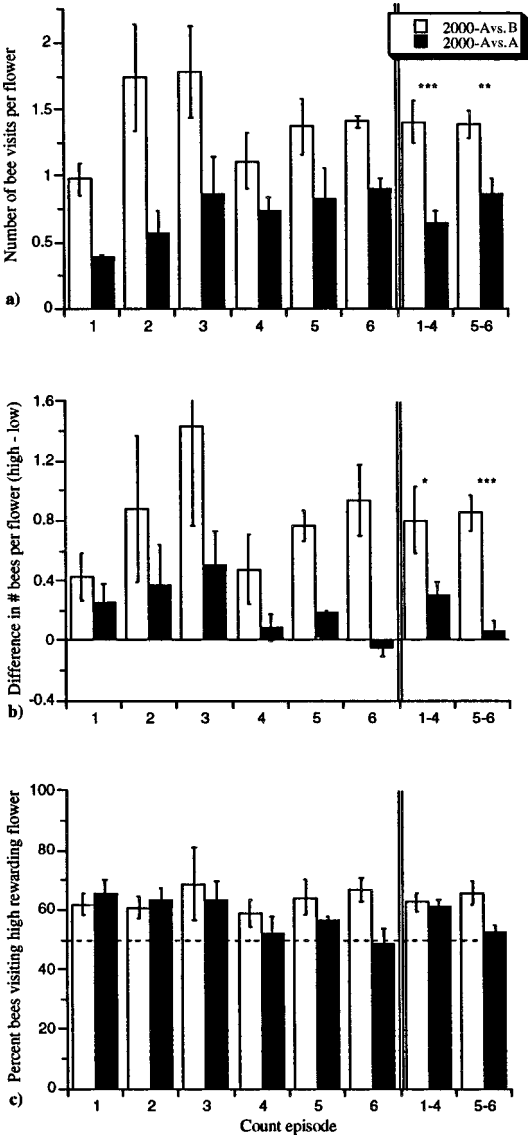


FIG. 5. Visits to the artificial flowers by free-flying bees in experiments of 2000 during each count episode, and summarized for count episodes 1–4 and 5–6. Data are means (±SE); means in the summarized count episodes that are significantly different are marked ($^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$). a) The total number of visits to the high- and low-rewarding flowers. b) The difference in the number of bee visits between the high- and low-rewarding flowers. c) The percent of bee visits to the high-rewarding flowers.

was turned off after count episode 4, and the difference in the mean number of bees visiting the high- and low-rewarding flowers decreased in count episodes 5–6 (unequal-variance t test, $t = 2.03$, $df = 15$, $P = 0.06$).

A greater proportion of bees visited the more rewarding flowers during count episodes 1–4 in both experimental conditions (Figure 5c; 2000-A versus B: $t = 4.9$, $df = 15$, $P < 0.001$; 2000-A versus A: $t = 3.7$, $df = 11$, $P < 0.005$). In condition 2000-A versus B the proportion was similar in count episodes 5–6. In condition 2000-A versus A, however, the syringe pump delivering sucrose solution was turned off in count episodes 5–6, and the proportion of visits to the previously high-rewarding flowers was not different from 50% ($t = 1.3$, $df = 5$, NS).

Experiments of 2001. Choice behavior was not affected by which odorant, linalool or 1-hexanol, marked the low- and high-rewarding flowers in each experimental condition. The total number of bees visiting the low- and high-rewarding flowers over all count episodes was similar when either odorant marked the two types of flowers (2001-A versus B: $t = 1.04$, $df = 46$, NS; 2001-AC versus BC: $t = 1.71$, $df = 46$, NS). The difference between the number of visits to the low- and high-rewarding flowers was also similar (2001-A versus B: $t = 0.80$, $df = 46$, NS; 2001-AC versus BC: $t = 0.99$, $df = 46$, NS), and so was the percent visits to the high-rewarding flower (2001-A versus B: $t = 1.08$, $df = 46$, NS; 2001-AC versus BC: $t = 0.07$, $df = 46$, NS). We, therefore, pooled the data within each experimental condition for further analyses.

The total number of bees visiting the low- and high-rewarding flowers was similar in conditions 2001-A versus B and 2001-AC versus BC during the rewarding count episodes 1–4 ($t = 0.41$, $df = 62$, NS) and the unrewarding count episodes 5–6 ($t = 1.34$, $df = 30$, NS; Figure 6a). The number of foragers decreased after the syringe pump was turned off at the end of count episode 4 in experimental condition 2001-A versus B, ($t = 2.31$, $df = 46$, $P < 0.03$), but remained constant in experimental condition 2001-AC versus BC ($t = 0.15$, $df = 46$, NS).

The mean number of visits to the high-rewarding flowers was greater than to the low-rewarding flowers in both conditions (Figure 6b). During the rewarding count episodes 1–4, the difference was greater in condition 2001-A versus B than in condition 2001-AC versus BC ($t = 2.4$, $df = 62$, $P < 0.02$), showing that the bees learned to relate to the difference in reward better when there was no confounding odor. This was especially pronounced during the latter rewarding count episodes 3 and 4. During the unrewarding count episodes 5–6, the difference was similar in the two conditions ($t = 0.89$, $df = 30$, NS).

In each count episode of both experimental conditions, a greater proportion of bees visited the more rewarding flower (Figure 6c). The proportions tended to be greater in condition 2001-A versus B than in condition 2001-AC versus BC, especially in the latter rewarding count episodes 2–4. Overall, the proportions were not significantly different between the two conditions during rewarding ($t = 1.27$, $df = 62$, NS) and unrewarding ($t = 0.40$, $df = 30$, NS) count episodes.

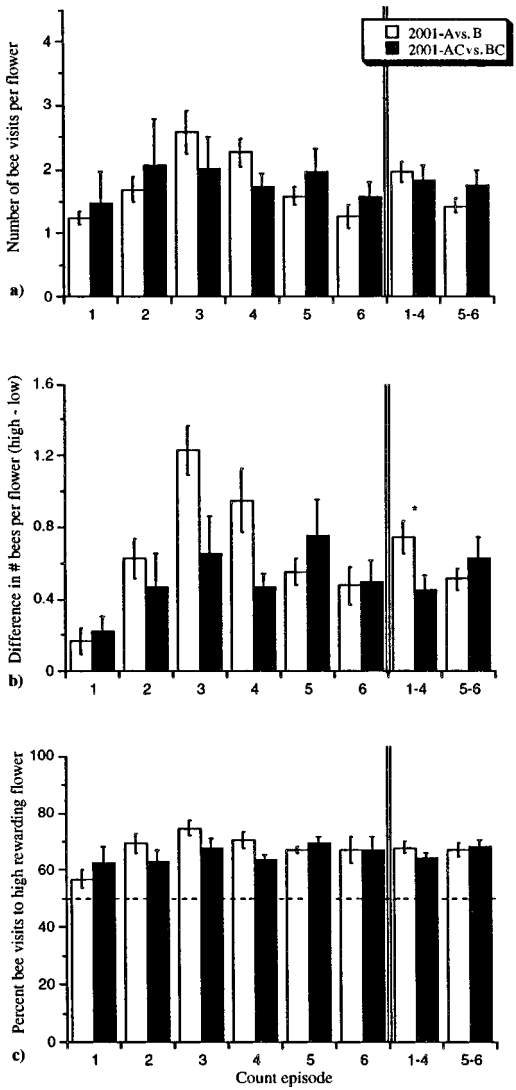


FIG. 6. Visits to the artificial flowers by free-flying bees in experiments of 2001 during each count episode, and summarized for count episodes 1–4 and 5–6. Data are means (±SE); means in the summarized count episodes that are significantly different are marked ($^{*}P < 0.02$). a) The total number of visits to the high- and low- rewarding flowers. b) The difference in the number of bee visits between the high- and low-rewarding flowers. c) The percent of bee visits to the high-rewarding flowers.

DISCUSSION

In this study, we addressed aspects relating to the ability of honeybees to forage efficiently in a patch containing differentially rewarding flower types. Despite the general predictive association between floral volatile emissions and nectar rewards, naturally occurring variation in volatile emission is probably quite common among genotypes both spatially and temporally (Borg-Karlson et al., 1994; N. Paldi unpublished observations). This may confuse pollinators if every combination is regarded as unique. Indeed, the ability of bees to recognize both individual components of blends and their ratios in blends (Joerges et al., 1997; Smith, 1998) probably lies at the basis of bees' ability to recognize those constituents of a bouquet that are most often associated with positive reward (Blight et al., 1997).

Honeybees readily discriminate odors associated with an appetitive stimulus from those associated with an aversive stimulus (Hammer and Menzel, 1995). Foragers, however, rarely encounter such diametrically opposed differences in floral rewards. Nectar rewards in different plant species or morphs differ in the mean and in the spread of their volume and concentration distributions (e.g., Petanidou and Smets, 1995; Wolf et al., 1999). We tested the ability of bees to discriminate between odors associated with different mean rewards, and compared their discrimination ability between structurally similar and dissimilar compounds, and compound mixtures sharing a common constituent.

In PER experiments in which structurally unrelated compounds were associated with differential rewards, bees quickly learned to respond to the compound associated with the high reward (Figure 1). They also learned to respond to odors associated with a low reward, but the asymptotic response levels reached were lower than those for the high reward. Hence, the salience of the unconditioned stimulus affected the asymptote level of the response curve in accordance with theories of associative learning, such as the Rescorla–Wagner model (Rescorla and Wagner, 1972; Pearce and Bouton, 2001). The different levels of response to the low- and high-rewarding odors were apparent already from the second trial and were maintained until the sixth trial.

A different pattern of responses was observed for structurally related compounds. Bees quickly learned to respond to the high-rewarding odor (Figure 2), as in the experiments with structurally unrelated compounds. However, generalization initially occurred to the similar odor associated with the low reward. In the first trial with this odor, which followed one trial with the odor associated with the high reward, between 13% and 29% of the bees responded. With structurally unrelated odors, in contrast, there was practically no generalization (Figure 1). Hosler and Smith (2000) also found weak generalization between structurally dissimilar compounds, such as between geraniol and 1-hexanol, which we also tested (Figure 1a), and greater generalization between more similar compounds.

Benzyl acetate, for example, was initially poorly differentiated from its floral precursor benzyl alcohol (Dudareva et al., 1998), and the response levels to the two odors were still similar in the second trial (Figure 2a). In later trials, bees learned to discriminate between the two odors and reduced their response to the low-rewarding benzyl alcohol. Appearance of benzyl acetate in floral bouquets is rhythmically (Loughrin et al., 1991) or developmentally (Dudareva et al., 1999) regulated, allowing bees to perceive it as a novel odorant. The two most closely structurally related compounds tested, methylisoeugenol and isoeugenol, which also form a substrate–product duo in-planta (Wang and Pichersky, 1998), were the most poorly differentiated by the bees (Figure 2b), although these compounds smell substantially different to a human nose. Initial generalization was high, and bees only learned to discriminate between the two odors after three training trials. Bees initially generalized between the two aromatic esters, benzyl acetate and methyl salicylate (Figure 2c). Response levels to the two odors only started to diverge in the third trial, with response to the low-rewarding benzyl acetate continually declining in later trials.

Overall, the relative difficulty in discriminating structurally similar compounds is consistent with the emerging synthesis of honeybee glomerular processing of odors (Joerges et al., 1997; Linster and Smith, 1997; Stopfer et al., 1997; Faber et al., 1999; Galizia et al., 2000; Hosler and Smith, 2000; Galizia and Menzel, 2001). While the mean difference in response to the low- and high-rewarding odors was initially small for structurally similar compounds relative to structurally dissimilar compounds, bees eventually learned to discriminate between the similar and dissimilar odors equally well (Figure 4). Our results regarding substrate–product duo discrimination have interesting implications *in-planta*, and for the evolutionary role of floral scents (Dudareva and Pichersky, 2000; Pichersky and Gang, 2000). For example, the evolution of enzymes that create a derivative that is slightly modified will be slower than that of enzymes that create a derivative that is more highly modified, because in the former case the new derivative will be less well differentiated from the original derivative than in the later case. Moreover, the evolution of structurally dissimilar derivatives may be a selective force in sympatric speciation.

Adding a common odor, benzyl acetate, to each of two structurally dissimilar compounds, greatly affected the learning curves to the low- and high-rewarding mixtures (Figure 3). Generalization in the first trial increased relative to when dissimilar compounds were tested alone. For example, the 4% generalization between 1-hexanol and geraniol (Figure 1a) increased to 19% when benzyl acetate was added to each compound. Overall, generalization levels were similar to those observed between structurally similar compounds (Figure 2). Unlike with similar compounds, however, discrimination did not show a consistent improvement with additional trials. The difference in response to the low- and high-rewarding options remained relatively constant between trials 2 and 6 (Figure 4). By the end

of training, the difference in response was smaller than for similar and dissimilar single compounds. Therefore, *in-planta*, the presence of identical compounds in the bouquets of two differentially rewarding flowers may continuously hinder the ability of bees to discriminate between them.

The experiments with free-flying bees complemented and reinforced the conclusions drawn from the PER experiments in a more complex context, more similar to natural foraging. Free-flying bees could use spatial cues in addition to olfactory ones to discriminate between differentially rewarding flowers. Furthermore, these experiments incorporated an instrumental learning component, in which subjects control their rate and sequence of foraging. Indeed, the method we used is a good simulation of the natural situation whereby nectar is often continuously secreted into the flowers regardless of whether or not bees visit and empty them.

In condition 2000-A versus A, during count episodes 1–4 the floral reward magnitudes were identical to those in condition 2000-A versus B, yet only about half as many foragers visited the patch (Figure 5a). The number of recruits arriving at a patch is a function of the intensity of dances performed in the colony, and low recruitment is a consequence of low perceived profitability of the patch by the foragers (Waddington, 1982; Seeley, 1995). Hence, at the colony level, a patch of one type of flower (same color, shape, and odor) that offers unreliable rewards (low and high) is perceived as less valuable than a patch of two types of flowers (different odors) that also provide low and high rewards, but reliably with regards to floral odor. Interestingly, the lower recruitment to the patch in condition 2000-A versus A, relative to condition 2000-A versus B, was not due to an inability of the bees to discriminate between the low- and high-rewarding flowers. Even though they could not use olfactory cues to discriminate between the flowers, the bees still visited the high-rewarding flowers preferentially (Figures 5b and 5c). They could have used spatial cues, or followed other bees that were feeding at the flowers. The smaller absolute difference in bees visiting the high- versus low-rewarding flowers, compared to condition 2000-A versus B, may be attributed to the overall fewer foragers working the patch. The percent visits to the high-rewarding flowers tended to decrease by count episode four, but was overall similar to that in condition 2000-A versus B. Hence, it appears that even when bees are able to preferentially visit the more profitable flowers, their subjective evaluation of a flower offering variable rewards is reduced relative to that of a less variable one. This is consistent with the general risk-aversion of bees toward variability in nectar rewards (Real, 1981; Shafir et al., 1999; Shafir, 2000; Shapiro, 2000; Shapiro et al., 2001).

When the syringe pump was turned off after count episode 4 of experimental condition 2000-A versus A, and the flowers were no longer rewarding, the number of foragers did not immediately decline (Figure 5a). However, the bees no longer discriminated between the flowers that were previously low- or high-rewarding (Figures 5b and 5c). They could have remembered the location of the differentially rewarding flower lines, but apparently, in the absence of local cues present when

the flowers were rewarding, the bees relied on (uninformative) olfactory cues. Olfaction is highest in the hierarchy of potential cues that bees employ (Gould and Gould, 1988; Jakobsen et al., 1995).

The balanced design of the experiments in 2001 showed that the differential foraging on the two flower types in each condition was due to their differential rewards, and not due to preexisting odor preferences. Linalool is by far more prevalent in floral bouquets than 1-hexanol (Knudsen et al., 1993). Nevertheless, the bees preferred the more highly rewarding flowers, regardless of whether they were associated with linalool or with 1-hexanol. Thus, associative learning dominates potential preexisting odor preferences in bee choice for differentially rewarding options.

The number of bees foraging at the patch and the preference for the more highly rewarding flowers reached a peak at count episode 3, and decreased at count episode 4. This pattern occurred in all experimental conditions (Figures 5 and 6). The initial gradual increase in the number of foragers is anticipated due to the bees' effective recruitment system. The large number of foragers utilizing the patch subsequently reduced the experienced nectar amounts. Thus, the distribution of foragers on the low- and high-rewarding flowers by count episode 4 may reflect an equilibrium between a low nectar flow rate, with few foragers, and a higher nectar flow rate, with more foragers.

Introduction of a common odorant to two structurally different compounds tended to subdue the increase in the total number of foragers in the later rewarding count episodes 3 and 4 (Figure 6a). The effect was less pronounced, but qualitatively similar to that of the two flower types being marked by the same odor (Figure 5a). The difference in visitation to the low- and high-rewarding flowers was greatly reduced with the addition of a common odorant (Figure 6b), similar to when there was only one odor (Figure 5b). Apparently, as in condition 2000-A versus A, the reduced ability to discriminate between the flowers in condition 2001-AC versus BC increased the flowers' perceived variability and diminished their attractiveness. This is consistent with the diminished ability of the individual forager to distinguish among flowers expressing a common odorant.

Floral visits during the nonrewarding count episodes 5 and 6 tended to decrease in condition 2001-A versus B, but not in conditions 2001-AC versus BC and 2000-A versus A (Figures 5a and 6a). Under the former experimental condition, the conditioned stimuli were more clearly distinguished from each other, and, thus, bees had clear expectations; when these expectations were not met in the nonrewarding count episodes, relatively quick extinction followed. In the latter conditions, the greater similarity between the conditioned stimuli resulted in greater ambiguity in expected rewards. Thus, the nonrewarding episodes were less surprising to the bees, and extinction was slower. Slower extinction to partial reinforcement is a well-documented phenomenon in animal learning (Bitterman, 1975), including in honeybees (Ishida et al., 1992). Unlike the lack of discrimination between

identically smelling flowers in experiment 2000-A versus A, however, bees continued preferentially to visit the odor mixture that had been more rewarding, as in Lee and Bitterman (1990).

Our results show that as in Laloi et al. (2000), PER conditioning reflects similar phenomena that occur in a free-flying context. However, the operant nature of free-flying conditioning introduces additional factors that affect flower visitation and subsequent pollination effectiveness. Honeybees are important pollinators of agricultural crops (Robinson et al., 1989), but their ability to discriminate between similar cultivars may hamper successful cross-pollination (Pham-Delègue et al., 1989). Our results imply that the introduction of common constituents to cultivars requiring cross-pollination may reduce the ability of bees to discriminate between them, and hence perform more cross-visits. The increased perceived variability of differentially rewarding cultivars that appear more similar to the bees may decrease their overall perceived profitability, but may reduce the effect of extinction when floral rewards decrease, thus achieving more efficient pollination over the long-term.

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VOLATILE EMISSIONS TRIGGERED BY MULTIPLE HERBIVORE DAMAGE: BEET ARMYWORM AND WHITEFLY FEEDING ON COTTON PLANTS

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Abstract—Plants are commonly attacked by more than one species of herbivore, potentially causing the induction of multiple, and possibly competing, plant defense systems. In the present paper, we determined the interaction between feeding by the phloem feeder silverleaf whitefly (SWF), *Bemisia tabaci* Gennadius (B-biotype = *B. argentifolii* Bellows and Perring), and the leaf-chewing beet armyworm (BAW), *Spodoptera exigua* Hübner, with regard to the induction of volatile compounds from cotton plants. Compared to undamaged control plants, infestation with SWF did not induce volatile emissions or affect the number and density of pigment glands that store volatile and non-volatile terpenoid compounds, whereas infestation by BAW strongly induced plant volatile emission. When challenged by the two insect herbivores simultaneously, volatile emission was significantly less than for plants infested with only BAW. Our results suggest that tritrophic level interactions between cotton, BAW, and natural enemies of BAW, that are known to be mediated by plant volatile emissions, may be perturbed by simultaneous infestation by SWF. Possible mechanisms by which the presence of whiteflies may attenuate volatile emissions from caterpillar-damaged cotton plants are discussed.

Key Words—*Bemisia argentifolii*, silverleaf whitefly, *Spodoptera exigua*, beet armyworm, *Gossypium hirsutum*, cotton, herbivore-induced volatiles.

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INTRODUCTION

Studies on induced plant defenses have mostly addressed the effects of damage by a single species of herbivore (Karban and Baldwin, 1997). These studies have shown that herbivore infestation of plants typically induces metabolic pathways that lead to altered levels of certain allelochemicals (Karban and Baldwin, 1997) or emission of volatile compounds that attract natural enemies of the herbivores (Dicke and Vet, 1999). These plant responses differ based on the type of feeding damage inflicted by the species of herbivores (Walling, 2000). Leaf-chewing insects typically induce the jasmonic acid (JA) pathway in plants, whereas phloem-feeding insects induce the salicylic acid (SA) pathway (Walling, 2000; Bostock et al., 2001). This specificity in a plant's response to herbivore damage is particularly important because plants throughout their life cycle are often exposed to simultaneous damage by multiple species of herbivores (e.g., Thompson, 1998).

Induction of a specific plant metabolic system by a particular herbivore may be altered if the plant is simultaneously challenged with one or more additional species of herbivores. For instance, induction of the SA defense pathway may inhibit the JA pathway, and vice versa (e.g., Fidantsef et al., 1999; Thaler et al., 2002). Consequently, we might expect that plant defense response to infestation by a specific herbivore would be perturbed upon simultaneous infestation by additional herbivores, particularly if they belong to different feeding guilds. Stout et al. (1998), for example, found that aphids (phloem feeders) caused tomato plants to become more susceptible to caterpillar damage.

Perturbations in plant defense responses when challenged by more than one herbivore species may impact tritrophic level interactions, i.e., interactions between plants and the natural enemies of herbivores. In particular, damage by more than one species of herbivore may alter emission of plant volatiles that attract natural enemies of the herbivores (Shiojiri et al., 2001; Vos et al., 2001). Shiojiri et al. (2001) found that cabbage plants damaged by two species of Lepidoptera emit a different blend of volatiles compared to those infested by a single species, e.g., the amount of (*E*)-4,8-dimethyl-1,3,7-nonatriene from plants infested by both herbivores was not as high as that from plants infested by a single species. Yet, we are not aware of any studies that have investigated the effects of combined damage by two species of insects with different feeding habits on the emission of volatiles in plants.

In cotton, feeding by leaf-chewing caterpillars and piercing-sucking hemipterans induces the production of volatiles (McCall et al., 1994; Rodriguez-Saona et al., 2002). Herbivore damage may induce volatile emissions in cotton by activation of the JA-pathway (Rodriguez-Saona et al., 2001). Also, herbivore feeding may increase volatile emissions in plants by inducing changes in the number, density, or chemistry of pigment glands (McAuslane et al., 1997; Agrawal and Karban, 2000), where large amounts of constitutive terpenoid volatiles are stored

(Elzen et al., 1985). For instance, damage to pigment glands by caterpillar feeding causes the release of high quantities of these stored compounds (Paré and Tumlinson, 1997; McAuslane and Alborn, 2000). However, it remains unknown whether these responses are also induced when cotton plants are damaged by phloem-feeding (Homoptera) insects.

This study investigates volatile emissions in cotton following feeding by the phloem feeder silverleaf whitefly (SWF), *Bemisia tabaci* Gennadius (B-biotype = *B. argentifolii* Bellows and Perring) (Homoptera: Aleyrodidae), when feeding alone or in combination with the leaf-chewing caterpillar beet armyworm (BAW), *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). Both insects are common pests on cotton and share similar habitats across the United States. We addressed the following specific objectives: First, we determined if SWF infestation induces volatile emissions or affects the number and density of pigment glands in cotton plants. Second, we investigated whether SWF infestation affects induction of volatiles by BAW damage and if so, if BAW leaf consumption was altered when feeding on SWF-damaged plants.

METHODS AND MATERIALS

Plants and Insects. Glanded cotton, *Gossypium hirsutum* L. cv. Delta Pine 5415, was grown as described in Rodriguez-Saona et al. (2001) and used for all experiments. Plants were grown in greenhouses under natural light supplemented with 2000 μmol/m^2/sec max daily photosynthetically active radiation and fertilized three times a week with 750 ml of a 2-g/l solution of 20-20-20 N-P-K fertilizer (Grow More, Gardena, California). A whitefly, *B. tabaci* B-biotype, colony was maintained on cotton plants in greenhouse cages (90 $\times$ 90 $\times$ 90 cm) at 30°C and 30% RH. *Spodoptera exigua* larvae were reared on an alfalfa-based diet as described by Rodriguez-Saona et al. (2001). Both colonies were maintained at the Western Cotton Research Laboratory, Phoenix, AZ.

Volatile Collections. Volatiles were collected in greenhouses as described by Rodriguez-Saona et al. (2001). The collection apparatus consisted of a push/pull system (Heath and Manukian, 1994) where charcoal purified air enters the top of a 42.5 cm high and 18 cm diam glass chamber over a plant at a rate of 5 l/min. Volatiles were collected on Super-Q adsorbent (Alltech, Deerfield, IL) filter traps by pulling air from the chambers at 1 l/min. The remaining air was vented from the chambers through an opening around the stem of the plant loosely sealed with cotton. Four independent chambers allowed for simultaneous collections of volatiles from four different cotton plants.

Volatile samples were analyzed as described in Rodriguez-Saona et al. (2001). Cotton volatiles were eluted from traps with 180 μl of methylene chloride. An internal standard (600 ng of *n*-octane in 5 μl of methylene chloride) was added to the extract. One-microliter aliquots were injected into an Hewlett-Packard gas

chromatograph (GC model 6890) equipped with a capillary injector system, a flame ionization detector, an auto injector model 7683, and an HP1 methyl siloxane column (30 m $\times$ 0.32 mm ID, 0.25-μm film). The GC was programmed in a split mode (25:1), and helium was used as a carrier gas at a linear flow velocity of 40 cm/sec. Following injection, oven temperature was maintained at 50°C for 3 min, programmed to rise to 190°C at 5°C/min, and then maintained at this temperature for 5 min. Data were analyzed with Hewlett-Packard ChemStation software.

For each sample, amounts of the detected volatiles were based on comparison of their peak areas with that of the internal standard. Individual compounds were identified by comparing their retention times with those of commercially available standards and by comparing their mass spectra standards to those available in a database from the National Institute of Standards and Technology.

Volatiles from Whitefly-Infested Plants. Cotton seedlings (2-wk old) with two cotyledons and two expanded leaves were exposed for 48 hr to *B. tabaci* adults (approx. 500–600) for oviposition. This density ensured high levels of infestation without killing the plant. Insects were caged with plants in 26 cm diam and 60 cm high Plexiglass cylinder chambers with the top of each chamber covered by a fine nylon mesh to allow air circulation. The chamber also had a 14-cm diam opening in the middle covered with nylon that allowed for manipulation of plants and insects inside the chambers. After oviposition, all SWF adults were removed from plants with the use of an aspirator, and plants were allowed to grow for 3–4 wk before volatiles were collected, such that the majority of nymphs (>80%) had reached the 3rd or 4th stadium. We collected volatiles from cotton infested with SWF nymphs or nymphs and adults because plants can respond differently to feeding by these two stages (van de Ven et al., 2000). In cases where volatiles were collected from plants infested by adults and nymphs, *B. tabaci* adults were reintroduced into the chambers 4 d prior to volatile collections and removed as described above on the day volatiles were collected. Control plants were subjected to the same conditions (caged), but had no whiteflies. Volatiles from SWF-infested cotton plants, which included the plant, whitefly nymphs, and nymphal secretions, and from SWF-free plants were collected in a greenhouse concurrently for two consecutive days during the times of maximal emissions (1000–1800 hr; Rodriguez-Saona et al., 2001). Each plant was considered a replicate, and treatments were replicated 3–4 times.

It is possible that whitefly infestation might affect volatile emissions in plants because of changes in number or density of cotton glands, where large amounts of terpene volatiles are stored (Elzen et al., 1985). Thus, we compared the number and density of glands on whitefly-infested and noninfested plants. After volatiles were collected, all leaves from each SWF-infested and SWF-free plant were excised, bagged, and labeled. Leaf area from each leaf was measured using a leaf area meter (LI-3100, LI-COR Inc., Lincoln, NE). In addition, total number of nymphs and pigment glands from a 3.88− and 1.0−cm^2 leaf disk, respectively, were recorded

from each leaf. Leaf disks were taken from an area located at the center of the leaf, left of the central vein, and on the abaxial surface (Naranjo and Flint, 1994; Chu et al., 2000). Both SWF nymphs and pigment glands were counted with the aid of a microscope. To aid in pigment gland counting, leaf disk surfaces were gently scrapped.

Interactions between Insects. Amounts of *S. exigua*-induced cotton volatiles when feeding on SWF-free and SWF (nymphs)-infested plants were investigated. Cotton plants were exposed to *B. tabaci* as described above, such that plants were infested with nymphs for 3–4 wk before they were presented to BAW for feeding. After treatment, plants were placed in volatile collection chambers at 1000 hr with 10 3rd instar BAW. Larvae were allowed to feed on plants for 24 hr, while volatile emissions were collected continuously during the same time period. Total leaf area removed by caterpillar feeding was obtained by scanning leaves and measuring area consumed using RE-650N computer software (Canon, Lake Success, NY). Each treatment was replicated 4–5 times.

Statistical Analysis. Differences in total volatile production and consumption among treatments were analyzed using a completely randomized analysis of variance (ANOVA; Systat ver. 9, 1998; SPSS Science, Chicago, IL). Differences in volatile emissions among undamaged control plants, plants infested with SWF nymphs and adults, and those infested only by nymphs were compared using ANOVA. Similarly, we used ANOVA to compare volatile emissions between plants damaged only by caterpillars to those from plants damaged by caterpillars and nymphs. Each plant was treated as a replicate in the analyses. In cases where significant differences were detected among treatments, a multiple comparison Tukey test was used. A nested ANOVA design was used to analyze the number and density of glands from SWF exposed or unexposed plants (PROC MIXED; SAS, 1999; SAS Institute, Cary, NC). Differences among treatments were assessed using multiple t-tests and adjusted using the Tukey method. Two factors were tested: treatment (uninfested controls, nymph and adult SWF-infested plants, and nymph SWF-infested plants) and leaf position within a plant (from top to bottom: pos1 = leaves on nodes 1 and 2; pos2 = leaves on nodes 3 and 4; pos3 = leaves on nodes 5 and 6; pos4 = leaves on nodes 7 and 8; pos5 = leaves on nodes 9 and 10). Trials were treated as a random variable in the model. The effects of insect density on plant volatiles and gland production were tested using linear regressions. Data were log transformed (natural logarithm) when necessary to correct for heterogeneity of variances.

RESULTS AND DISCUSSION

Volatiles from Whitefly-Infested Plants. There is little evidence pertaining to the relationship between insect feeding habit and the induction of plant volatile

emissions. To our knowledge this is the first report that addresses the impact of the phloem-feeding SWF on induction of volatiles in cotton. Under the experimental system we employed, SWF infestation did not lead to induction of plant volatile compounds relative to nondamaged control plants (Figure 1). It is well-documented that leaf-chewing caterpillars induce the synthesis and subsequent emission of terpenoid compounds in cotton (Figure 1; McCall et al., 1994; Paré

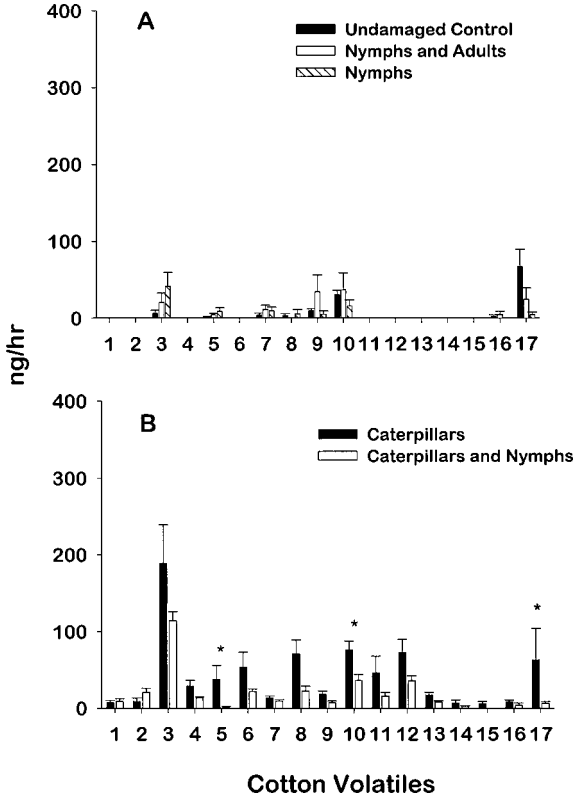

FIG. 1. Volatiles collected from noninfested cotton plants (control) and plants infested with *Bemisia tabaci* B-Biotype nymphs or nymphs and adults (A) and from plants damaged by *Spodoptera exigua* larvae with and without *B. tabaci* nymphs (B): **1** = (*E*)-2-Hexanal; **2** = (*Z*)-3-Hexen-1-ol; **3** = α-Pinene; **4** = β-Pinene; **5** = Myrcene; **6** = (*Z*)-3-Hexenyl acetate; **7** = Limonene; **8** = (*E*)-β-Ocimene; **9** = Linalool; **10** = (*E*)-4, 8-Dimethyl-1, 3, 7-Nonatriene; **11** = Indole; **12** = (*E*)-β-Caryophyllene; **13** = α-Humulene;**14** = (*E*)-β-Farnesene; **15** = (*E*, *E*)-α-Farnesene; **16** = Nerolidol; **17** = (*E*, *E*)-4, 8, 12-Trimethyl-1, 3, 7, 11-Tridecatetraene. Bars represent the mean + 1 SE ($N \geq 3$). An asterisk denotes significant differences among treatments (ANOVA, $P \leq 0.05$).

and Tumlinson, 1997). In contrast, cotton plants infested by SWF nymphs, or adults and nymphs, emitted only slight amounts of the inducible volatiles (*E*)-β-ocimene, linalool, (*E*)-4, 8-dimethyl-1, 3, 7-nonatriene, and (*E*, *E*)-4, 8, 12-trimethyl-1, 3, 7, 11-tridecatetraene at levels that were not different from undamaged control plants (ANOVA; $P > 0.05$; Figure 1). Likewise, the total volatile amounts emitted from SWF-infested plants did not differ from controls ($F = 0.42; df = 2, 8; P = 0.67$; Total volatile emissions: control = 116.8 ± 25.6 [ng/hr ± SE]; nymphs = 135.6 ± 53.8; nymphs and adults = 90.0 ± 36.1; Figure 1A) and was not affected by varying insect density, i.e., higher levels of SWF infestation did not increase volatile production in cotton (Linear Regression: $F = 2.06; df = 1, 4; P = 0.23; r^2 = 0.34$). Our results are comparable to Turlings et al. (1998) who reported high amounts of volatile emissions from maize attacked by caterpillars, but no measurable emissions of volatiles from plants heavily infested by aphids.

Volatiles emitted from whitefly-infested plants can serve as olfactory cues for parasitoids in host location. For example, *Encarsia formosa* Gahan, a parasitoid of the greenhouse whitefly, *Trialeurodes vaporariorum* Westwood, was more attracted to whitefly-infested bean plants, *Phaseolus vulgaris* L., compared to noninfested plants (Guerrieri, 1997). Birkett et al. (2003) showed that feeding by adults of *T. vaporariorum* elevated the amounts of (*Z*)-3-hexen-1-ol, (*E*)-4, 8-dimethyl-1, 3, 7-nonatriene, and 3-octanone emitted from bean plants compared to noninfested plants. These compounds increased the attractiveness of *E. formosa* towards the odor source (Birkett et al., 2003). Noldus and van Lenteren (1990), however, found a lack of discrimination by *E. formosa* between odors from whitefly-infested and noninfested tomato and cucumber plants. In cotton, cues other than odors emitted from SWF-infested plants, such as visual and contact chemical cues, might play a more important role in host-searching behavior of their parasitoids (e.g., van Lenteren et al., 1976; Shimron et al., 1992; Guerrieri, 1997; Sütterlin and van Lenteren, 2000).

Because insect herbivory may induce production and/or filling of pigment glands that store constitutive volatiles in plants (e.g., McAuslane et al., 1997; Agrawal and Karban, 2000), we tested whether SWF infestation induces changes in the number and density of pigment glands in cotton. In general, pigment glands decreased in density ($F = 29.9; P < 0.01$; Figure 2A) and number ($F = 35.3; P < 0.01$; Figure 2B) with leaf age. The number of SWF nymphs per cm^2 ranged from 0 on top leaves to 150 on leaves from position 4 of infested plants (Figure 2D).

Significant differences among treatments were marginal and only found for gland density at leaf positions 2 and 3 (nested ANOVA; Position within treatment: $F = 29.93; df = 12, 73; P < 0.01$; *t*-test for position 2: $t = 2.3; df = 73; P = 0.02$; *t*-test for position 3: $t = 2.85; df = 73; P < 0.01$; Figure 2A). The nymph density at leaf positions 2 and 3 was small (Figure 2D), and it is likely that the increased gland density at these leaf positions was due to smaller leaf area

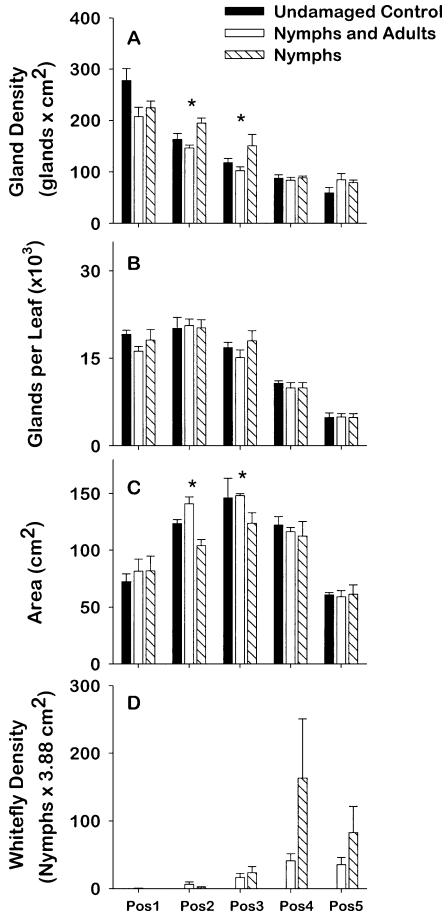

FIG. 2. Gland density (A), leaf area (B), glands per leaf (C), and whitefly density (D) on healthy cotton plants and plants infested with *Bemisia tabaci* B-biotype nymphs or nymphs and adults. Bars represent the mean + 1 SE ($N \geq 3$). The x-axis indicates the position of leaves in the plant (from top to bottom): pos1 = leaves on nodes 1 and 2; pos2 = leaves on nodes 3 and 4; pos3 = leaves on nodes 5 and 6; pos4 = leaves on nodes 7 and 8; pos5 = leaves on nodes 9 and 10. An asterisk denotes significant differences among treatments within leaf position (nested ANOVA, $P \leq 0.05$).

(nested ANOVA; Position within treatment: $F = 17.63; df = 12, 73; P < 0.01$; t-test for position 2: $t = 3.34; df = 73; P < 0.01$; t-test for position 3: $t = 2.2$; $df = 73$; $P = 0.03$; Figure 2C). There was no effect of SWF infestation on gland number at any leaf position (Figure 2B). Overall, our results indicate that there is little to no effect of SWF infestation on leaf gland number or density.

Interactions between Insects. Given that feeding by multiple species of herbivores can influence emissions of volatiles in plants (Shiojiri et al., 2001; Vos et al., 2001), we investigated how SWF infestation affected the production of volatiles induced by BAW in cotton. Although the blend of compounds emitted from plants infested with SWF nymphs plus BAW was qualitatively similar to those damaged by BAW alone, we found a 60% reduction in the magnitude of volatile emissions from plants attacked by SWF plus BAW as compared to those damaged only by BAW ($F = 9.17; df = 1, 7; P = 0.02$; Total volatile emissions: BAW $= 713.4 \pm 131.6$ [ng/hr $\pm$ SE]; BAW plus SWF $= 311.3 \pm 27.1$; Figure 1B). Such a reduction in volatile emissions may have important consequences for the attraction of natural enemies of herbivores. For example, Fritzsche et al. (2002) tested the attractiveness of the parasitoid *Cotesia marginiventris* (Cresson) to varieties of maize that differ in the quality and quantity of emitted volatiles. They reported that cultivars that emitted lower amounts of volatiles upon herbivory were less attractive to parasitic wasps. In our experiments, the majority of individual compounds from the cotton blend were emitted in lower amounts from BAW- plus SWF-infested plants as compared to plants damaged by BAW alone (Figure 1B). In particular, three compounds, myrcene, (*E*)-4, 8-dimethyl-1, 3, 7-nonatriene, and (*E*, *E*)-4, 8, 12-trimethyl-1, 3, 7, 11-tridecatetraene, were emitted in significantly lower amounts ($P < 0.05$; Figure 1B).

At least three nonmutually exclusive mechanisms may operate to reduce caterpillar-induced volatiles from SWF-infested plants. First, SWF feeding may affect the number or density of pigment glands that store constitutive volatiles, or the amounts of volatile chemicals stored in glands. We found little support for an effect of SWF feeding on the number or density of glands in cotton leaves (Figure 2), however, changes in gland chemistry were not investigated.

Second, because amounts of leaf area consumption by caterpillar damage is positively correlated with plant volatile emissions (e.g., Gouinguené et al., 2003), we hypothesized that decreased leaf consumption by BAW in the presence of SWF may be a factor associated with the observed decrease in volatile emissions. For instance, Inbar et al. (1999) reported reduced growth and development of young, but not older, instars of *Trichoplusia ni* (Hübner) on SWF-infested collard plants. Although larval consumption was not measured, they indicated that whitefly-infested plants could reduce the caterpillar's growth through direct physical interference and/or by the elevated foliar levels of chitinases, ß-1, 3-glucanases, and peroxidases on SWF-infested collard leaves as compared to uninfested controls. We found a trend towards reduced amounts of foliage consumed by caterpillars from SWF-infested plants as compared to uninfested plants, however, the difference between the two treatment means was not statistically significant ($F = 0.702; df = 1, 6; P = 0.434$; Leaf area removed: control $= 219.1 \pm 50.9$ [$cm^2 \pm$ SE]; SWF $= 119.6 \pm 14.1$).

Finally, SWF feeding may induce a response in plants that perturbs the induction of volatile emissions by BAW. Phloem-feeding herbivores, such as the SWF, induce the SA-signaling pathway in plants (Mayer et al., 1996; Walling, 2000). There is evidence that the induction of the SA pathway can negatively impact the induction of the JA pathway (Fidantsef et al., 1999; Thaler et al., 2002). Considering that the JA-signaling pathway is involved in the production of caterpillar-induced volatiles in cotton (Rodriguez-Saona et al., 2001), the decrease in volatile emissions in the presence of SWF could be associated with the induction of the SA pathway by SWF. Further research will be needed to accept or reject fully any of these mechanisms.

In summary, we found that volatiles emitted from plants in response to insect damage can vary with insect feeding habits (also see Turlings et al., 1998). Specificity in plant volatiles induced by different herbivores may have important implications for the types of cues natural enemies use in host location (e.g., De Moraes et al., 1998; Röse et al., 1998). Our data show that SWF feeding did not induce volatile production in cotton plants. However, we found evidence that SWF infestation did reduce amounts of volatile emissions triggered by the leaf-chewing BAW. Recently, Shiojiri et al. (2000) and Vos et al. (2001) showed that the presence of nonhosts on a host-infested plant can interfere with the attraction of parasitoids, weakening interaction strengths between parasitoids and their hosts. In the present study, we have shown that damage by a phloem-feeder such as SWF can potentially interfere in the production of volatiles induced by chewing caterpillars. However, it remains to be determined whether these interguild interactions result in changes in the host searching behavior of natural enemies.

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PHYTOCHEMICAL DETERMINATION FOR LEAF FOOD CHOICE BY WILD CHIMPANZEES IN GUINEA, BOSSOU

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Abstract—The feeding selection of leaves by chimpanzees was investigated from the perspective of phytochemistry and leaf availability. Field data were collected for 6 months from 1995 to 1996 and 8 months from 1997 to 1998 in Bossou, Guinea. Time budgets of leaf consumption by chimpanzees were analysed and the abundance of young leaves of each tree species for each month was estimated. Analyses of dried materials, stratified by leaf maturity and edibility, for the relative amounts of crude protein, crude fat, neutral detergent fiber, ash, and condensed tannin were determined. It was found that 1) leaves consumed by chimpanzees had higher levels of ash and lower levels of condensed tannin than leaves that were not eaten; 2) feeding selectivity was correlated with crude protein content; and 3) the availability of young leaves was not correlated with the percentage of feeding time, neither by seasonal changes nor by tree species. Chimpanzees preferred leaves containing higher protein content, but did not consume leaves containing high condensed tannin regardless of protein content. It seems reasonable to consider that a different rule exists for "whether chimpanzees eat it or not" and "whether they prefer it or not" in phytochemical determinants of leaves. Some Moraceae species, which had low levels of condensed tannins, even in mature leaves, were an important food source for the Bossou chimpanzees.

Key Words—Chimpanzee, feeding selectivity, leaf consumption, leaf maturity, Moraceae, phytochemistry.

INTRODUCTION

Chimpanzees are one of the largest mammalian frugivores. According to the law of Kleiber (1961), Jarman (1974), and Bell (1971), a larger animal can eat lower quality foods such as higher fiber herbs and leaves, because energy requirements

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are a function of metabolic body weight ($W^{0.75}$). Thus, a larger animal on a lower nutrient diet can extract the same amount of nutrients per unit of body weight as a smaller animal on a higher nutrient diet (Caughley and Sinclair, 1994). Primates also fit this relationship between body size and diet. Species that eat insects tend to be relatively small, whereas those that eat leaves tend to be relatively large (Fleagle, 1999). Larger bodied primates, with their relatively slow metabolic turnover rates, can tolerate the long lag between ingestion and digestion that leaves require, and might also have difficulty finding sufficient quantities of insects on a regular basis (Strier, 2000). In this respect, chimpanzees, which have a relatively large body without having adopted a leafy diet, are a paradoxical mammal.

The diet of chimpanzees consists largely of fruits, and consumption of foliage accounts for only 20–30% of the time spent feeding (Hladik, 1977a; Wrangham, 1977; Yamakoshi, 1998; Newton-Fisher, 1999). In general, leaves are relatively rich in protein, whereas fruits are rich in carbohydrates, but poor in protein (Hladik, 1977a; Milton, 1981; Marks et al., 1988; Rogers et al., 1990; Kool, 1992; Dasilva, 1994; Mowry et al., 1996; Matsumoto-Oda and Hayashi, 1998; Takemoto, 2002). Hladik (1978) pointed out that because fruits yield an average of only 5% protein, then larger primates more than 2 kg should eat leaves because a fruit–insect diet is insufficient for protein. Chimpanzees are fairly large-bodied compared to other primates; therefore, they require a large amount of protein, and leaves are thought to be an important source. Chimpanzees must choose high-quality leaves within a relatively lower time allocation in the diet to meet protein requirements. However, the selection of leaf foods has not been investigated from the perspective of phytochemistry.

Several studies have discussed the reasons for selective feeding on fruits or terrestrial herbaceous vegetation (THV) from the perspective of phytochemistry in chimpanzees. These results showed that chimpanzees preferred food items with high sugar content or caloric intake rate, regardless of protein content (Wrangham et al., 1991, 1993; Reynolds et al., 1998; Matsumoto-Oda and Hayashi, 1999). Tannins significantly reduced the palatability of many unripe fruits for chimpanzees (Wrangham and Waterman, 1983). In contrast, Reynolds et al. (1998) reported that chimpanzees ingested fruit or bark that contained considerable quantities of tannins. Tannins combine with protein, often irreversibly, and inhibit the digestibility of protein. As a result, they are effective as antifeedants (Robbins et al., 1991), although this is not always the case. Tannin-protein precipitation once formed, however, can dissolve, but most animals tend to reject astringent taste foods, such as those containing tannins (Mole and Waterman, 1987). If leaves are an essential food for protein, chimpanzees are likely to avoid those leaves with high tannin content.

Since the primary biochemical processes within a leaf are practically identical in all green plants, the relative amounts of sugars, lipids, polysaccharides, amino acids, and proteins are inevitably similar. Physiological processes (e.g.,

senescence) are more likely than anything else to affect the nutritional status of a plant (Harborne, 1993). Compared to mature leaves, young leaves have a higher protein content and a lower content of fiber or secondary compounds (Coley, 1983). Although chimpanzees prefer young leaves, there are several species whose young leaves they do not consume. These species have been noted in habitat inventories for different groups of chimpanzees (Sugiyama and Koman, 1992; Moor, 1994; Moutsanboté et al., 1994; Tutin et al., 1994; Yumoto et al., 1994). Two sets of analyses, i.e., "within young leaves" and "within mature leaves," will be needed to clarify the chemical differences between food source leaves and nonfood source leaves.

When fruit is in short supply, Bossou chimpanzees allocate less time to feeding (Takemoto, 2002), and their diet does not become more diverse (Yamakoshi, 1998; Takemoto, 2002). These results deviate from the general expectation of primate behavior, proposed by Dunbar (1988), that time spent feeding should increase and feeding items should become more diverse when there is a food shortage. At least, they can digest cellulose more efficiently than predicted by body weight (Milton and Demment, 1988). It is unlikely that a physiological process to digest fiber content, such as shorter retention time or insufficient fermentation in the hind gut, affects chimpanzee feeding behavior. Why do chimpanzees never eat large quantities of leaves when fruit is scarce, even though leaves appear freely available? Answering this question requires not only a phytochemical investigation, but also an estimation of seasonal changes in the availability of food source leaves, especially of young leaves.

According to optimal foraging theory, the time spent on each food item depends upon its availability (Pyke et al., 1977; Post, 1984). Optimal foraging theory has been applied with some success to carnivores, but not to herbivores, whose potential food species display more complex chemical variations, both in nutritional content and in the diverse array of substances they contain that inhibit digestion, some of which are toxic (Rhoades and Cates, 1976; Bryant et al., 1992). Choice, based on a combination of food quality and availability, can make the determination of optimal foraging strategy more difficult (Post, 1984). Accordingly, feeding selectivity indices were used (i.e., the relative use of specific food items compared to their availability) to compare with phytochemistry, which represents food quality.

The purpose of this study was to interpret leaf selection by chimpanzees in terms of leaf chemistry. Specifically, I sought to 1) clarify the phytochemical differences between young leaves from food and nonfood species, and between mature leaves from food and nonfood species, respectively; 2) examine the relationships between phytochemical content and feeding selectivity of leaves from food species; and 3) investigate the influence of young-leaf availability on the amount of time allocated to leaf consumption. On the basis of the results, I discuss phytochemical cues for leaf selection, important foliage species for chimpanzees, and why the time spent feeding on leaves does not increase when fruits are scarce in Bossou.

METHODS AND MATERIALS

The field survey was conducted in Bossou, Guinea, West Africa. Data were collected from June to November 1996 and November 1997 to June 1998. Annual rainfall in Bossou averaged 2190 mm between 1995 and 1998. The dry season, during which rainfall averaged <100 mm per month, lasted from November to March, while the wet season extended from April to October. Fruit availability in the forest varies throughout the year and peaks between January and April (Yamakoshi, 1998; Takemoto, 2002). Basic observation methods are described in Takemoto (2002). The activity budgets of three individuals (one subadult male [*VI*] and two adult females [*Jr*, *Vl*]) were recorded by focal animal sampling (Altman, 1974), following one chimpanzee for an entire day or as long as possible to avoid introducing observation bias associated with grouping patterns or visibility. The total observation time was 379 hr over 121 days. Only observations that lasted for >1 hr in a day were used for the analyses, and no bias of observation time across months nor individuals was found.

Estimating Availability of Young Leaves. Transects that measured 1600 m in total length were established during the first half of the study period and 2200 m during the second half. The trees surveyed in these transects were used to estimate the availability of young leaves. Young-leaf availability was represented as a percentage of young leaves to total leaves in the maximum volume of a tree. All trees in the transects were assigned a relative leaf abundance score between 0 and 3 twice monthly, and the relative abundance of young leaves among all the leaves on a tree was also scored between 0 and 3. Leaves were considered young from bud emergence until they had fully expanded and acquired adult coloring and toughness, following the definition of Coley (1983). The abundance of young leaves in the transects was described by

$$\mathrm{YLA} = \left[\sum (B_\mathrm{k} \times L_\mathrm{k} \times YL_\mathrm{k}) \Big/ \sum (B_\mathrm{k} \times 3 \times 3) \right] \times 100,$$

where YLA represents young-leaf availability (%), B_k is the basal area of tree k (cm^2), L_k is the leaf score of tree k (0–3), and Y_Lk is the score for the relative abundance of young leaves on tree k (0–3). When feeding selectivity was calculated, young-leaf availability of species i in a given month (YLAfi) compared with all food species (YLAf) was described by

$$\mathrm{YLAfi} = \left[\sum (B_\mathrm{ij} \times L_\mathrm{ij} \times Y_\mathrm{Lij}) \Big/ \sum \sum (B_\mathrm{ij} \times L_\mathrm{ij} \times YL_\mathrm{ij}) \right] \times 100$$

where YLAfi is the availability of tree species i (%), B_ij is the basal area of tree j of species i (cm^2), L_ij is the leaf score of tree j of species i (0–3), and Y_Lij is the relative abundance of young leaves on tree j of species i (0–3). The monthly averages of YLAfi represent the availability of species i throughout the study period.

Plant species' designations follow Adam (1971–83), and descriptions of plant life forms follow Letouzey (1982–83) and Keay (1989).

Relative Use of Each Tree Species. All observations of leaf consumption were recorded, including consumption of mature leaves, although the chimpanzees fed almost exclusively on young leaves. Feeding on petioles was excluded from the analyses (e.g., *Elaeis guineensis*, piths of THV, or Pteridophyta), because petioles and leaf blades may have different nutritive values (Struhsaker, 1975). If the chimpanzees consumed both the leaves and piths of a species (e.g., *Gonglonema latifolia*, *Musanga cecropioides*, and *Myrianthus spp*.), cases in which the chimpanzees ate only the petiole were excluded. June and November were excluded when monthly correlations were calculated (i.e., $N = 10$).

Estimation of Feeding Selectivity. All tree species recorded along the transects were divided into two categories (i.e., food and nonfood species) based on the observed diets of chimpanzees during the study periods. The percentage of time spent feeding on each species was calculated, and this percentage was considered to be the relative use of the species, i (ri); however, feeding of less than 1 min on any item was excluded because it was too short a time to estimate the selectivity index. The availability of young leaves of species i (pi) was calculated as the average of the monthly availability of species i (YLAfi).

Selectivity for each tree species was estimated using Ivlev's selectivity index (Ivlev, 1955) and Johnson's rank (Johnson, 1980). Ivlev's selectivity index (Ei), given by $(\mathrm{ri} - \mathrm{pi})/(\mathrm{ri} + \mathrm{pi})$, ranges from -1 to $+1$. An Ei of 0 indicates random feeding, an Ei of 0–1 indicates preferred items, and an Ei of -1 to 0 indicates food items that are avoided. Johnson's rank uses the differences between availability rank (pi) and usage rank (ri) in a given diet (e.g., $\mathrm{ri} - \mathrm{pi}$). Increasing use relative to availability (i.e., higher selectivity) results in increasing the negative value, because a higher use rank results in a smaller value for ri and a lower availability rank results in a larger value for pi.

Chemical Analysis. Leaves of trees on which chimpanzees foraged were collected, regardless of whether they were found along the transects. If the leaves were young, the mature leaf of that species was also collected after growing. In addition, young and mature leaves from transect species with a higher relative dominance ratio (SDR, Table 1) were collected, regardless of their edibility to chimpanzees. Nonfood young leaves were gathered when they were about half-expanded.

Collected materials were immediately placed in sealed plastic bags to prevent desiccation and taken to the field station. At the station, samples were weighed, wrapped in paper bags, and dried, using either a metal oven heated by kerosene stoves (Hladik, 1977b) or by solar drying during the dry season. Water content was estimated after weighing the dried samples.

Nutrient content was analyzed in the Primate Research Institute of Kyoto University. Samples were redried to a constant weight in a vacuum oven at 50˚C. Phytochemical contents of dried and milled samples were determined for crude

TABLE 1. STRUCTURES OF PLANT FAMILY IN TRANSECT

Family name	No. of species	BA (%)	density (/ha)	SDR[a]
Sterculiaceae	6	22.82	137.5	17.34
Moraceae	12	9.58	165.0	11.91
Mimosaceae	9	13.46	82.5	10.29
Sapotaceae	4	13.63	33.0	8.24
Anacardiaceae	4	10.84	62.7	8.13
Caesalpinaceae	5	2.61	111.1	6.10
Meliaceae	6	3.87	67.1	4.83
Euphorbiaceae	10	3.48	70.4	4.77
Apocynaceae	8	3.55	44.0	3.67
Papilionaceae	5	1.09	59.4	3.11
Rubiaceae	7	1.35	56.1	3.09
Bignoniaceae	2	0.99	39.6	2.20
Sapindaceae	3	0.76	38.5	2.04
Combretaceae	2	2.61	7.7	1.64
Agavaceae	1	1.14	23.1	1.57
Arecaceae	1	2.09	5.5	1.28
Annonaceae	2	0.32	19.8	1.01
Hypericaceae	1	0.28	18.7	0.95
Bombacaceae	1	1.02	9.9	0.94
Simaroubaceae	3	0.52	14.3	0.88
Myristicaceae	1	0.53	9.9	0.69
Lauraceae	2	0.76	4.4	0.57
Unknown	—	0.13	11.0	0.54
Burseraceae	1	0.47	6.6	0.52
Ulmaceae	1	0.37	5.5	0.42
Rhizophoraceae	1	0.17	7.7	0.42
Verbenaceae	2	0.55	3.3	0.42
Solanaceae	1	0.09	6.6	0.33
Icacinaceae	1	0.09	6.6	0.33
Rutaceae	2	0.15	5.5	0.31
Samidaceae	1	0.13	5.5	0.30
Lecythicaceae	2	0.11	5.5	0.29
Octoknemaceae	1	0.30	2.2	0.25
Flacourtiaceae	1	0.04	3.3	0.16
Loganiaceae	2	0.02	2.2	0.11
Hippocrateaceae	1	0.02	2.2	0.10
Violaceae	1	0.02	2.2	0.10
Rhamnaceae	1	0.02	1.1	0.06
Myrtaceae	1	0.01	1.1	0.05
Tiliaceae	1	0.01	1.1	0.05
Total	116	100.00	1159.4	100.00

[a] SDR: summed dominance ratio (average on BA and density).

protein (CP), crude fat (CFA), neutral detergent fiber (NDF), ash (ASH), and condensed tannin (CT), on the basis of dry matter weight. Crude protein was determined using the Kjeldahl procedure for total nitrogen and multiplying by 6.25. Crude fat was measured as diethyl-ether extract using the Soxhlet method. Ash was determined by ashing at 550˚C. Neutral detergent fiber was estimated following the methods of Van Soest (1994). Condensed tannin was extracted with 70% aqueous acetone (Hagerman, 1988), and determined by the buthanol-HCL method (Porter et al., 1986a,b; Porter, 1989).

Phytochemical contents of food and nonfood species were compared among young leaves and among mature leaves by using two-tailed Mann–Whitney *U* test. Spearman's rank correlation was used to evaluate the relationship between phytochemical composition of the leaves and feeding selectivity.

RESULTS

Forest Structure and Seasonal Changes in Availability of Young Leaves. In total, 116 tree species were found in the transects (not including unknown species). Members of the Sterculiaceae family had the highest summed dominance ratio (SDR; Table 1) and the largest total basal area, whereas species belonging to the Moraceae had the greatest density and numbers.

Figure 1 shows seasonal changes in young-leaf availability (YLA) in the transects. YLA increased during the dry season, beginning in November, peaked in

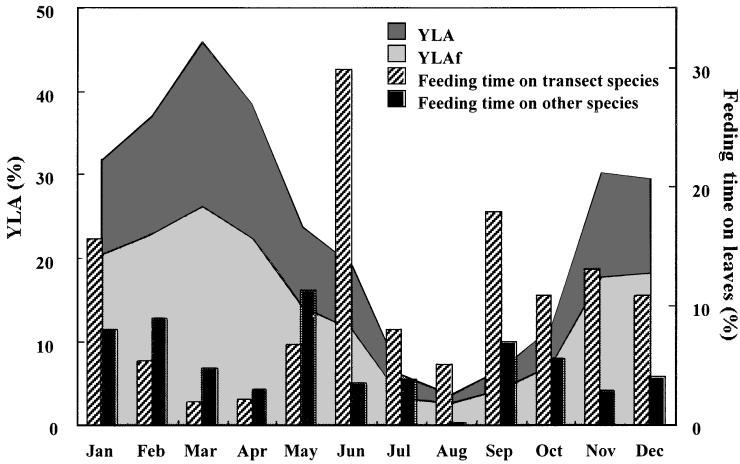

FIG. 1. Seasonal change in young leaf availability (YLA), feeding species (YLAf), and feeding time on leaves divided by transect species (species found in the transect) and other species. The average values of two different years are shown for June and November.

March, and then declined. The lowest index values were from June to October, during the wet season. Leaves from food species (YLAf) showed the same seasonal changes as YLA. Young leaf availability had strong correlation with fruit availability in the forest (FAI), which was shown in Takemoto (2002) ($rs = 0.90$, $N = 10$, $P < 0.007$).

Food Species and Young-Leaf Availability. Chimpanzees were observed to consume the leaves of 52 plant species during the study period (Table 2), and spent the most time feeding on *Baphia sp.*, *Ficus exasperata*, *Antiaris africana*, *Chlorophora excelsa*, and *Bosquia angorensis.* Many species of Moraceae and Papilionaceae were consumed, and the two families constituted 80% of total leaf consumption time (Moraceae: 60%, Papilionaceae: 20%; Table 3).

When food species were separated by plant life form, the leaves of high trees were where the greatest amount of time was spent feeding, whereas epiphytes were fed on the least (Table 4). This result is thought to be concordant with the biomass of the leaves. The chimpanzees ate only one species of epiphyte leaves, *Nephropsis biserrata* (Pteridophyta), which grows on oil palms. They consumed this plant intensively in rice fields between February and March, during the late dry season.

Chimpanzees consumed the mature leaves of five species: *Baphia sp.*, *Bosquia angolensis*, *Ficus exasperata*, *Polycephalium capitatum*, and *Ficus thoningii*. They ate mature leaves of *Polycephalium capitatum* as frequently as young leaves, while the mature leaves of the other four species were consumed much less often than the young leaves.

Figure 1 also shows the monthly percentages of time spent feeding on leaves. The mean proportion of time spent feeding on leaves was 15.9% of the total feeding time. Seasonal changes in time spent feeding on leaves were not correlated with YLA or YLAf (YLA: $rs = -0.15$, $N = 10$, $P > 0.05$; YLAf: $rs = -0.27$, $N = 10$, $P > 0.05$). Time spent feeding on the leaves of transect species (species recorded in the transect) also was not correlated with YLA or YLAf (YLA: $rs = -0.38$, $N = 10$, $P > 0.05$; YLAf: $rs = -0.46$, $N = 10$, $P > 0.05$). The time spent feeding on the leaves of species that were not recorded on transects did not increase when young leaves were scarce in the transects.

Feeding Selectivity. Of the 52 tree species consumed by chimpanzees, 18 were found on the transects. These 18 species were considered food species. Figure 2 shows seasonal changes in the young-leaf availability of these. The area occupied by a species in this graph indicates the relative young-leaf availability of species i (annual average for YLAfi = pi; see Methods and Materials).

Availability (pi), usage (ri), and selectivity (Ei and Johnson's rank) for each food species are shown in Table 5. Availability and usage were not correlated ($rs = -0.26$, $N = 18$, $P > 0.05$). The highest selectivity was found in *Baphia sp.* Eight species (six species of Moraceae, *Baphia sp.*, and *Ceiba pentandra*) were considered "preferred" using Johnson's rank estimator.

TABLE 2. RELATIVE USE OF LEAF FOOD SPECIES BY CHIMPANZEES

Species	Family	Relative use (%)	Life form
Baphia sp.[a]	Papilionaceae	11.29	Small tree
Ficus exasperata[a]	Moraceae	10.73	Small tree
Antiaris africana[a]	Moraceae	9.51	High tree
Chlorophora excersa[a]	Moraceae	8.62	High tree
Bosquia angolensis[a]	Moraceae	6.54	Medium-sized tree
Ficus variifolia[a]	Moraceae	6.41	High tree
Ficus mucso[a]	Moraceae	6.04	Medium-sized tree
Leptoderris fasciculata	Papilionaceae	5.96	Woody liana
Ficus umberrata[a]	Moraceae	3.56	Medium-sized tree
Musanga cecropioides[a]	Moraceae	3.34	Medium-sized tree
Celtis mildbraudii[a]	Ulmaceae	2.88	High tree
Ficus thoningii	Moraceae	2.45	Medium-sized tree
Ceiba pentandra	Bombacaceae	2.18	High tree
Smilax klaussiana	Smilacaceae	2.15	Woody liana
Myrianthus arboreus[a]	Moraceae	1.69	Medium-sized tree
Mesoneuron bentamianum	Caesalpinaceae	1.59	Woody liana
Polycephalium capitatum[a]	Icacinaceae	1.20	Woody liana
Triplochiton scleroxron[a]	Sterculiaceae	1.15	High tree
Aningueria altissima[a]	Sapotaceae	1.09	High tree
Unknown (liana)	—	0.97	Woody liana
Dalbergia afzeliana	Papilionaceae	0.93	Woody liana
Gonglonema latifolium	Asclepiadaceae	0.87	Woody liana
Erythrina mildbraedii[a]	Papilionaceae	0.79	Medium-sized tree
Albizia zygia[a]	Mimosaceae	0.68	Medium-sized tree
LUKOSU (liana)	—	0.63	Herbaceous liana
Myrianthus libericus	Moraceae	0.58	Medium-sized tree
Piper guineensis	Piperaceae	0.57	Woody liana
Ipomaea sp.	Convolvulceae	0.56	Herbaceous liana
Carica papaya	Caricaceae	0.54	Small tree
Craterispermum laurinum[a]	Rubiaceae	0.49	Small tree
Raphiostylis beninensis	Icacinaceae	0.47	Woody liana
Pseudospondias microcarpa[a]	Anacardiaceae	0.43	Medium-sized tree
unknown	—	0.43	Small tree
Glyphaea brevis	Tiliaceae	0.36	Small tree
Ceropegia sp. (*jhonsoni?*)	Asclepiadaceae	0.31	Terrestrial herb
liane (gnegneinsoon)	—	0.27	Herbaceous liana
Nephrolepsis biserrata	Polypodiaceae	0.26	Epiphyte
Illigera pentaphylla	Hernandiaceae	0.23	Woody liana
Tryunpheta heudelotii	Tiliaceae	0.23	Small tree
Voacanga africana	Apocynaceae	0.16	Medium-sized tree
Vigna sp. (*bakolo*)	Papilionaceae	0.15	Woody liana
Triumpheta rhonboidea	Tiliaceae	0.14	Small tree
unknown (herb)	—	0.13	Terrestrial herb
Homalium molle	Samidaceae	0.10	Small tree
Monodora tenuifolia[a]	Annonaceae	0.08	Medium-sized tree
Alchornea cordifolia	Euphorbiaceae	0.07	Woody liana
Milletia sp.[a]	Papilionaceae	0.06	Small tree

TABLE 2. CONTINUED

Species	Family	Relative use (%)	Life form
Unknown (tree)	—	0.03	Small tree
Celts adorfi-frederici[a]	Ulmaceae	0.02	Medium-sized tree
Justicia tennera	Acanthaceae	0.02	Terrestrial herb
Dialium denklagei	Caesalpinaceae	0.01	Small tree
Hibiscus esculentus	Malvaceae	0.00	Terrestrial herb

[a] The species that was used for nutritional analysis.

Phytochemical Differences Between Food Species and Nonfood Species. Figure 3 shows the phytochemical differences within leaf categories, stratified by maturity and edibility. Among mature leaves, leaves from food species had a higher

TABLE 3. RELATIVE USE OF LEAVES FOR EACH PLANT FAMILY BY CHIMPANZEES

Family	Relative use	No. of species
Moraceae	59.48	11
Papilionaceae	19.19	6
Ulmaceae	2.90	2
(Unknown)	2.46	(6)[a]
Bombacaceae	2.18	1
Smilacaceae	2.15	1
Icacinaceae	1.67	2
Ceasalpinaceae	1.60	2
Asclepiadaceae	1.19	2
Sterculiaceae	1.15	1
Sapotaceae	1.09	1
Tiliaceae	0.73	3
Mimosaceae	0.68	1
Piperaceae	0.57	1
Convolvulceae	0.56	1
Caricaceae	0.54	1
Rubiaceae	0.49	1
Anacardiaceae	0.43	1
Polypodiaceae	0.26	1
Hernandiaceae	0.23	1
Apocynaceae	0.16	1
Samidaceae	0.10	1
Annonaceae	0.08	1
Euphorbiaceae	0.07	1
Acanthceae	0.02	1
Malvaceae	0.005	1
Total	100.00	52

[a] Parentheses denote unidentitied plant species.

TABLE 4. RELATIVE USE OF LEAF FOOD SPECIES BY CHIMAPNZEES DIVIDED BY PLANT LIFE FORM

Life form	Relative use	No. of species
High-tree	31.85	7
Medium-sized Tree	26.37	13
Small Tree	24.42	12
Woody Liana	15.18	12
Herbaceous Liana	1.46	3
Terrestrial Herb	0.48	4
Epiphyte	0.26	1
Total	100.00	52

ash content (U test, $N_1 = 5$, $N_2 = 32$, $z = -2.36$, $P < 0.019$) and a lower condensed tannin content than leaves of nonfood species (U test, $N_1 = 4$, $N_2 = 18$, $U = 9$, $P < 0.021$). Among young leaves, leaves from food species had a higher ash content than nonfood species (U test, $N_1 = 12$, $N_2 = 9$, $U = 11$, $P < 0.002$). There was no significant difference between tannin content of young leaves from food species and nonfood species ($N_1 = 9$, $N_2 = 9$, $U = 24.5$, $P > 0.05$). No differences in protein content among mature leaves and young leaves were found (mature leaves: $N_1 = 4$, $N_2 = 32$, $z = -0.71$, $P > 0.05$; young leaves: $N_1 = 12$, $N_2 = 9$, $U = 47$, $P > 0.05$). Tannin was present in inverse proportion to ash across

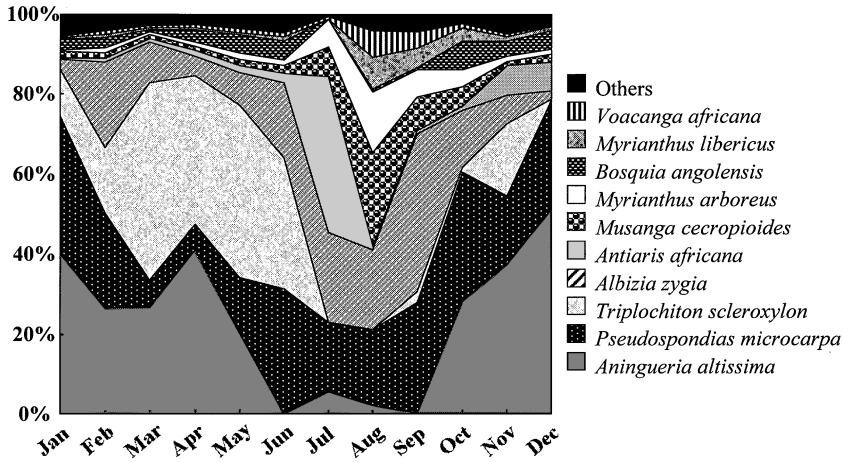

FIG. 2. Relative availabilities for each tree species regarded as chimpanzees' food in the transect. The area for each species shown in the graph represents young leaf availability of the species (pi). Eight species with availability less than 1% are combined and presented as "Others."

TABLE 5. IVEV'S SELECTIVITY INDEX AND JOHNSON'S RANK FOR EACE LEAF FEEDING SPECIES IN TRANSECT

Species	Ivlev's Selectivity Index			Johnson's Rank		
	pi	ri	Ei[a]	pi	ri	Deference[a]
Aningueria altissima	23.35	1.93	−0.85	1	12	11
Pseudospondias microcarpa	21.39	0.75	−0.93	2	16	14
Triplochiton scleroxylon	17.76	2.03	−0.79	3	11	8
Albizia zygia	14.23	1.20	−0.84	4	13	9
Antiaris africana	5.33	10.63	*0.33*	5	4	*−1*
Musanga cecropioides	4.64	5.88	*0.12*	6	7	1
Myrianthus arboreus	3.52	2.42	−0.19	7	10	3
Bosquia angolensis	2.95	10.52	*0.56*	8	5	*−3*
Myrianthus libericus	1.82	1.03	−0.28	9	14	5
Voacanga africana	1.54	0.28	−0.70	10	17	7
Craterispermum laurinum	0.89	0.86	−0.02	11	15	4
Ceiba pentandra	0.86	2.91	*0.54*	12	9	*−3*
Chlorophora excelsa	0.70	11.35	*0.88*	13	3	*−10*
Ficus exasperata	0.59	15.32	*0.93*	14	2	*−12*
Baphia sp.	0.15	19.88	*0.99*	15	1	*−14*
Ficus mucso	0.13	8.53	*0.97*	16	6	*−10*
Homalium sp.	0.10	0.18	*0.31*	17	18	1
Ficus thonningii	0.06	4.31	*0.97*	18	8	*−10*

[a] Italic numbers show preferred species estimated by each indicator.

leaves of which chemical contents were determined, and leaves eaten had less tannin and more ash content (Figure 4). The other phytochemical components showed no differences between leaves from food species and leaves from nonfood species.

Relation Between Feeding Selectivity and Phytochemistry. Correlations between Johnson's rank and phytochemical content were investigated (Figure 5). Correlation coefficients with negative values indicate positive correlations between feeding selectivity and each chemical, because Johnson's rank decreases as selectivity increases (see Methods and Materials). Selectivity was positively correlated with crude protein content. Selectivity also had a positive correlation coefficient with condensed tannin content, although sample size was small. All correlations between selectivity and water content, crude fat, ash, and neutral detergent fiber were weak.

DISCUSSION

No single phytochemical criterion seems to characterize "food sources and nonfood sources" nor "avoided and preferred leaves" within food sources. Chimpanzees preferred leaves with a higher protein content, but no other chemical contents were correlated to selectivity. There were differences in ash and condensed

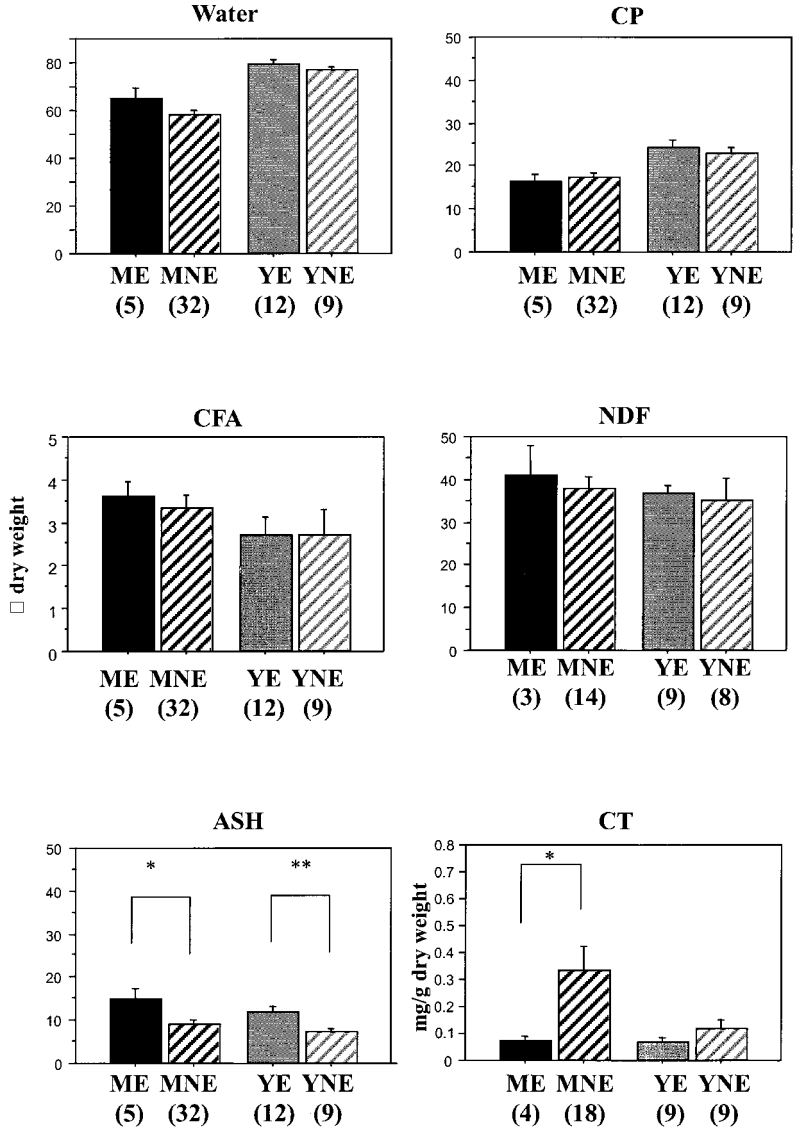

FIG. 3. Mean (+SEM) phytochemical content for food sources versus nonfood sources for chimpanzees stratified by leaf maturity. Condensed tannins (CT) are expressed as milligrams per gram dry weight in sample. Neutral detergent fiber (NDF), ash (ASH), crude fat (CFA), and crude protein (CP) are expressed as % dry weight. ME = mature leaves eaten, MNE = mature leaves not eaten, YE = young leaves eaten, YNE = young leaves not eaten. Numbers inside parentheses represent number of species. $^{*}P < 0.05$, $^{**}P < 0.01$.

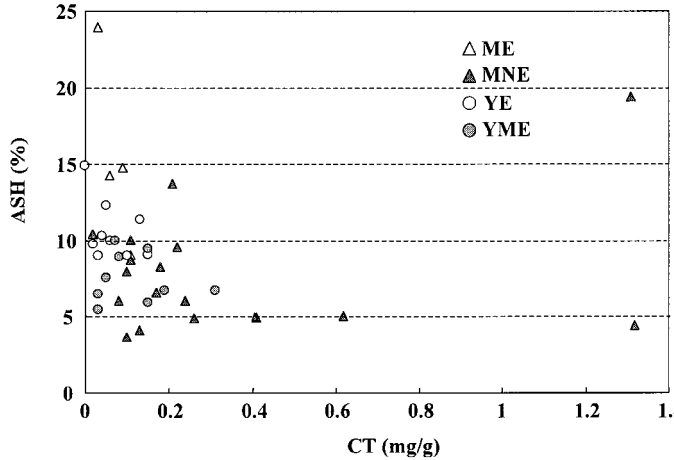

FIG. 4. Relation between ash and condensed tannin (CT) content within each leaf. ME = mature leaves eaten, MNE = mature leaves not eaten, YE = young leaves eaten, YNE = young leaves not eaten.

tannin between food and nonfood sources, but no other differences in chemical content were apparent. Chimpanzees preferred leaves containing higher protein content, but did not consume the leaves containing high condensed tannin regardless of protein content.

The data presented here are derived from observations targeting three individuals. Since each individual may not have the same experience of how to mix diets of different plant species (Provenza et al., 2002), it is difficult to generalize about the Bossou population. According to the long-term study of Sugiyama and Koman (1992) and other previous studies on Bossou chimpanzees (Yamakoshi, 1998), however, the species found to be consumed in large quantity in this study are known to be common food sources for the entire population at Bossou. Additionally, the Bossou population size has been small and stable since the 1970s (around 20 individuals) and female bonds are strong (Sugiyama, 1988; Muroyama and Sugiyama, 1994). No interindividual or interfamily biases in diet choice is expected from the protocol and sample size, as they often feed in close association with each other. Short durations of observation time for leaf eating behavior were eliminated from the analysis to alleviate possible bias from rare behaviors dependent upon individual or observational bias. It can be assumed that the results of this study are reasonably representative of the entire population.

Phytochemical Determination of Leaf Consumption by Chimpanzees. Tannins, which deter herbivory, can reduce the digestibility of food, reduce growth, and damage the intestine and kidneys of mammals, sometimes acting as toxins (Mole and Waterman, 1987; Hagerman and Butler, 1991; Robbins et al., 1991; Robbins,

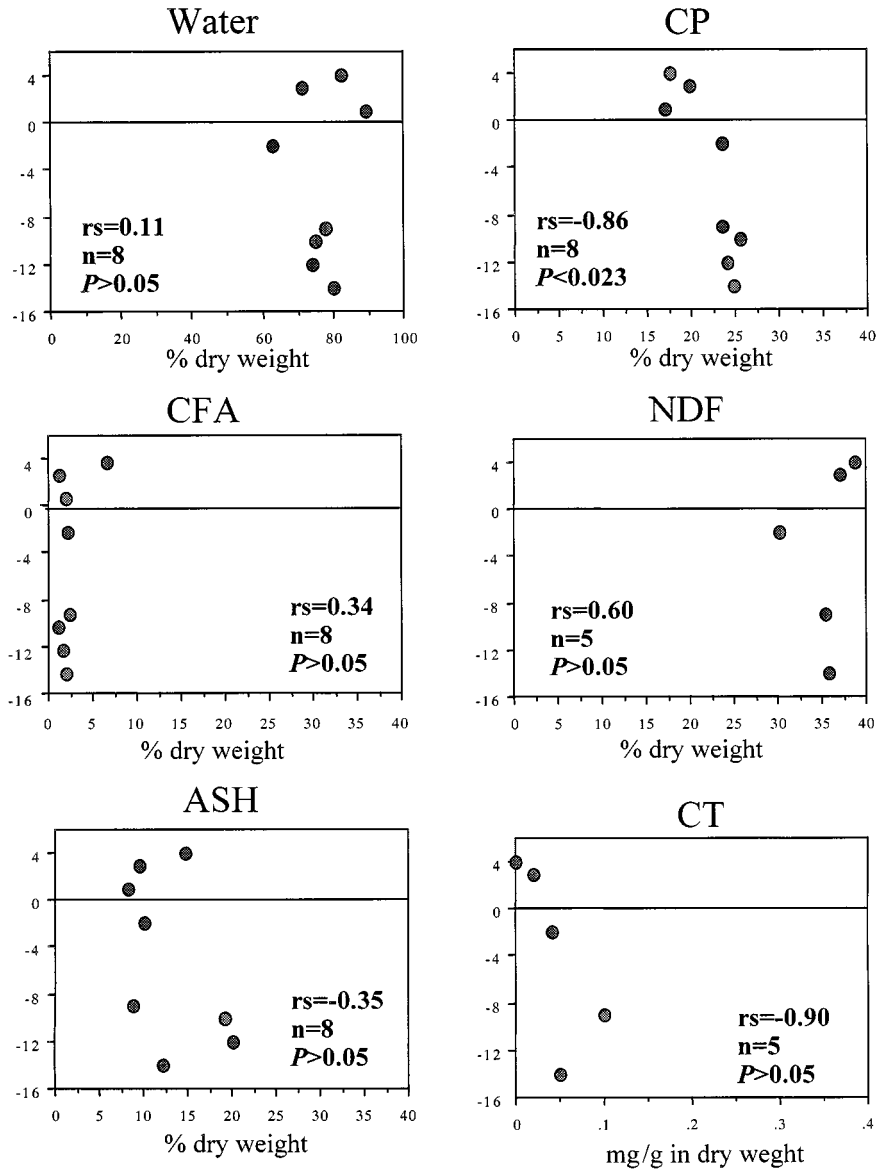

FIG. 5. Relation between each phytochemical content in young leaves eaten and selectivity by Johnson's rank (vertical axis). Refer to Figure 3 for definitions of abbreviations for phytochemical contents. Note the horizontal axis of CT, which represents mg/g.

1993). Many studies have indicated that animals, including primates, tend to reject astringent foods (for primates: Oates et al., 1977, 1980; Mckey and Gartlan, 1981; Wrangham and Waterman, 1981; Kool, 1992). Wrangham and Waterman (1983) suggested that tannins significantly reduced the palatability of many unripe fruits for chimpanzees. In contrast, Reynolds et al. (1998) reported that chimpanzees ingested fruit or bark that contained considerable quantities of tannins. The influence of tannins as feeding deterrents, however, depends on which food types they are contained in (Behmer et al., 2002). Other plant secondary metabolites also affect the food choice of herbivores. In their diet as a whole, chimpanzees have a lower intake of antifeedants, such as tannin or total tannins, than do *Cercopithecines*. Moreover, chimpanzees do not increase their intake of condensed tannin when ripe fruit is scarce (Wrangham et al., 1998). Thus, chimpanzees appear to avoid antifeedants, including tannins.

Tannic acid probably has a greater deterent effect on feeding of herbivorous insects when the amount of tannic acid increases compared to protein content (Behmer et al., 2002). When tannin infusions were greater, the preference of lambs shifted to higher protein food (Villalba et al., 2002). In field observations, browsing ruminants in the South African savanna rejected leaves with thresholds exceeding 5% condensed tannins, but protein-precipitating polyphenols did not influence palatability (Cooper and Owen-Smith, 1985). Experiments of response to tannin taste for primates also show that there are clear avoidance thresholds (for lemurs: Iaconelli and Simmen, 2002; for Gorillas: Remis and Kerr, 2002).

In food source leaves, chimpanzees showed higher selectivity for leaves with a higher protein content. The influences of other antifeedants (cellulose, silica, other secondary compounds, etc.) must be considered: by avoiding the leaves with high concentrations of tannins, the efficiency of protein absorption is likely to be increased. Although taste, smell, and the visceral system of chimpanzees as a frugivorous mammal are not the same as herbivorous insects and mammals, leaf eating behavior of chimpanzees seems to display phytochemical recognition responses regarding food choices observed in other herbivores. Feeding frequency and the protein or nitrogen content of leaves are positively correlated in leaf-eating monkeys (*Colobus satanas*: Mckey and Gartlan, 1981; *Procolobus badius*: Mowry et al., 1996). Leaves of food species had higher ash contents than did nonfood leaves. Because meat-eating behavior is rare at Bossou (Sugiyama and Koman, 1987), the chimpanzees may be deficient in some minerals. Ash contains many kinds of minerals; however, the mineral requirements of nonhuman primates have not been well studied (Yeager et al., 1997). Since condensed tannin content tended to be inversely proportional to ash content in this study (Figure 4), it is possible that avoiding high levels of condensed tannin would result in the consumption of a high concentration of ash in the leaves of food species.

Young leaves contained more protein and water, and less fiber and tannins, concordant with previous studies (Milton, 1979; Glander, 1981; Kool, 1992;

Mowry, 1996; Reynolds et al., 1998). However, this alone does not explain the selection for leaves, since there were young leaves that were never eaten and mature leaves that were commonly eaten. Young leaves that were never eaten contained as much protein as young leaves that were eaten, whereas mature leaves that were eaten contained lower protein content, similar to mature leaves that were never eaten. Unless such an analysis is stratified by leaf maturity, it will lead to an increase in the number of young leaves in samples of food resources, and will inevitably result in a higher protein content compared with nonfood resources, simply because chimpanzees consume mainly young leaves. Two hypotheses have been proposed to explain dietary generalism among mammalian herbivores: nutrient constraints (Westby, 1974) and detoxification limitations (Freeland and Janzen, 1974). According to the "satiety hypothesis" (Provenza, 1995, 1996), there is a fine line between satiety and aversion, which depends on an animal's morphology, physiology, and nutritional requirements. Odor, taste, and food texture combined with the postingestive effects of nutrients and toxins will determine the palatability of a particular item. Consequently, a complex of phytochemicals, both of nutritional and toxic value, narrows or widens feeding diversity. Besides the highly preferred leaf species (Table 5), chimpanzees consume leaves of as many as 52 other plant species. This may result from the interactions between nutrients and various feeding deterrents, as well as other features such as edible leaf availability.

Seasonal Changes in Availability of Young Leaves and Time Spent Feeding on Leaves. There was no relationship between the availability of young leaves and the time spent feeding on leaves. This must be because chimpanzees chose fruits when they were available, although they did consume young leaves of *Antiaris africana* and *Darbergia afzeliana* in large quantities for a short period. In Bossou, fruit in the forest is most abundant from January to April, and least abundant from May to August (Yamakoshi, 1998). Leaves that were consumed in large amounts by chimpanzees were the young leaves of a few species, and availability of these leaves was lower when fruits were scarce. Nearly mature leaves that contain high levels of tannin are difficult to ingest, and chimpanzees appear unable to consume large amounts of mature leaves, even when fruits and young leaves were scarce.

Analyses of the energy budgets of Bossou chimpanzees have shown several points incongruent with optimal foraging theory (Takemoto, 2002). The three focal chimpanzees, when fruits were abundant, increased energy expenditure to maximize energy intake (searched more and fed more). When fruits were scarce, they reduced energy expenditure to compensate for low energy intake (rested more and moved less). According to optimal foraging theory, animals should make an effort to feed more when food is scarce. Because mature leaves and bark contain more antifeedants than do fruits (Wrangham and Waterman, 1981; Calvert, 1985; Reynolds et al., 1998), edible foods for chimpanzees are likely to be scarcer in the fruit scarce season. Antifeedants may prevent chimpanzees from

adopting the strategy of eating more low-quality food in the fruit scarce season. In addition, temperature or climate can affect behavior of chimpanzees (Takemoto, in press). The fruit scarce season at Bossou occurs simultaneously with the rainy season and low temperatures. Colder temperatures increase energy requirements for thermoregulation (Oates, 1987), and the effective temperature may also decline following exposure to rain and wind. The climatic effects of the rainy season probably encourage low activity (such as sleeping in day beds) of chimpanzees to conserve body reserves. Few studies on primates have considered the relationship between seasonal changes in the availability of young leaves and feeding on young leaves. Some young leaves were correlated with feeding frequency, but in general, young leaves showed no relation to feeding time in *Colobus polykomos* (Dasilva, 1994) or Howler monkeys (Glander, 1981). Both of these species preferred fruits or seeds, so the time spent feeding on leaves was influenced by the availability of fruits or seeds. Leaf consumption in chimpanzees may be affected even more by fruit availability, as chimpanzees tend to be more dependent on fruits than the other two species.

Differences in Selectivity for Each Tree Species and the Importance of Moraceae Species. The leaves selected by chimpanzees were concentrated in several taxa. Of the 10 species that chimpanzees spent the most time feeding on, 8 belonged to the Moraceae and 2 belonged to the Papilionaceae. Similarly, of the 8 species that had a positive selectivity index among the 18 food species, 6 were from the Moraceae. In some cases, mature Moraceae leaves were consumed, so the selectivity index of Moraceae species may have been overestimated. Mature Moraceae leaves are consumable because they contain a higher ash content, but a lower tannin content, even as they mature. The chimpanzees studied fed on 11 Moraceae species, representing the highest number of feeding species among plant families in Bossou. Consequently, the Moraceae represent the most important family for Bossou chimpanzees.

Moraceae ranked second in summed dominance ratio (SDR), and had a high density and species diversity in the Bossou forest (Table 1). *Ficus exasperata* and *Bosquia angolensis* flushed young leaves constantly throughout the year, although they fluctuated in abundance. The dominance of Moraceae species and their suitability as a protein source, even when fruits are scarce, may result in a favorable nutritional environment for Bossou chimpanzees throughout the year. In addition, most Moraceae fruits are also eaten by the Bossou chimpanzees (Sugiyama and Koman, 1992). *Ficus* fruits are essential foods for chimpanzees in some study sites (Nishida and Uehara, 1983; Wrangham et al., 1996), and *Musanga* fruit is an important food in seasons of fruit scarcity in Bossou (Yamakoshi, 1998). The younger age of first birth, shorter interbirth intervals, and the high infant survival rate of chimpanzees in Bossou (Sugiyama, 1994) may be partly explained by the high density and large biomass of Moraceae food species.

In general, food species or species with a high selectivity index are considered to be pioneer species. According to Swain and Hall (1983), secondary forest species that had a high selectivity index in this study, include *Chlorofora excelsa*, *Ficus mucso*, *Ceiba pentandra*, and *Musanga cecropioides*. *Albizia zygia*, which had a low selectivity index in this study, was categorized as a primary species. In addition, *Ficus exasperata* was found mostly in forest gaps and was pioneer-like. Coley (1983) indicated that pioneer species lack chemical defenses, such as tannins or total phenols, but develop physical defenses such as hairs on the surface of leaves. Therefore, the leaves of pioneer species may be suitable food sources for chimpanzees, which tended to avoid high tannin concentrations in this study.

Such a preference for certain groups also has been observed in captive chimpanzees. Captive chimpanzees were found to select young leaves or leaf buds of deciduous trees, such as the Ulmaceae or Fagaceae, in a compound that contained planted trees and where chimpanzees could eat any leaves *ad libitum* (Takemoto, 1996). Feeding on particular types of leaves is probably a general feature of chimpanzees, to some extent, based on their digestive system, nutrient requirements, or palate.

The main phytochemical differences between the leaves of pioneer and persistent species, or among taxa, are found not in primary metabolic products, such as protein or fiber content, but rather in secondary metabolites, such as alkaloids or tannins (Howe and Westley, 1988; Harborne, 1993). The evident preference of chimpanzees for the leaves of certain tree species highlights the importance of secondary compounds in their selection of leaves.

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EFFECTS OF PLANT FLAVONOIDS ON FECUNDITY, SURVIVAL, AND FEEDING OF THE FORMOSAN SUBTERRANEAN TERMITE

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Abstract—Fecundity, mortality, and food consumption of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, were evaluated in response to five plant flavonoids (genistein, biochanin A, apigenin, quercetin, and glyceollin). Apigenin fed at 50 μg/primary reproductive pair proved to be the most toxic flavonoid. Biochanin A was most effective in reducing fecundity. Subsequently, these two flavonoids were tested through oral feeding and topical application at 100-μg dose. Significant reduction in the numbers of progeny was evident for biochanin A in both treatment methods. Choice feeding tests with termite workers showed that initially termites were attracted to filter paper treated with biochanin A, but over a period of 72 hr, consumed significantly less material when compared to controls. Biochanin A is a promising phytochemical with ability to reduce fecundity in primary reproductives of the Formosan subterranean termite, but it does not elicit phagostimulant activity.

Key Words—*Coptotermes formosanus*, feeding, fecundity, termites, flavonoid, flavone, isoflavone, genistein, biochanin A, apigenin, quercetin, glyceollin, survival.

INTRODUCTION

Flavonoids are phenolic compounds commonly found in many plants, vegetables, and flowers. Although flavonoids are ubiquitous, isoflavones are found only in a few legumes, particularly soybean. Flavonoids are able to modulate growth and reproduction of herbivores by directly interacting with steroid hormone systems

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(Oberdorster et al., 2001). In insects, several classes of phytochemicals, including the flavonoids, interfere with molting, reproduction, feeding, and behavior (Reyes-Chilpa et al., 1995; Musayimana et al., 2001; Simmonds, 2001). Insecticidal activity of flavonoids has been shown against the western corn rootworm (Mullin et al., 1992), the corn earworm (Widstrom and Snook, 2001), and the common cutworm (Morimoto et al., 2000). Reyes-Chilpa et al. (1995) determined that two flavonoids, castillen D and castillen E, showed concentration-dependent feeding deterrence, but were not toxic to *Cryptotermes brevis*. In a review of chemicals found to impart natural resistance in wood, Rao (1982) reported that flavonoids, including isoflavonoids and neoflavonoids, were important.

Coptotermes formosanus Shiraki is a serious urban pest causing extensive damage to wooden structures and live trees in several of the southern states and Hawaii in the United States. To date, no studies have examined sustainable management of *C. formosanus*, using inexpensive phytochemicals, including flavonoids. Flavonoids have been shown to interact with the ecdysone receptor using a reporter-gene assay and ecdysone-responsive cell lines (Oberdorster et al., 2001). Considering the ability of flavonoids to bind with the ecdysone receptor, flavonoids may affect other biological systems in termites. In this study, effects of oral administration of five selected flavonoids on mortality and fecundity of primary reproductives of the Formosan subterranean termite were determined. The five flavonoids (Figure 1) tested include the isoflavones glyceollin, genistein, and biochanin A, and the flavones apigenin and quercetin. In a subsequent test, only biochanin A and apigenin were evaluated after oral and topical application. The two flavonoids were also tested for feeding preference by termite workers in a no-choice and choice tests.

METHODS AND MATERIALS

Chemicals. Apigenin, genistein, quercetin, and biochanin A were obtained from Indofine Chemical (Somerville, NJ). Glyceollin (mixture of glyceollins I, II, III in a 6:2:1 ratio) was isolated using a procedure developed in this center (Burow et al., 2001). Soybean seeds (*G. max* cv. Pioneer 95B41, 50 g) were sliced and inoculated with *Aspergillus sojae*. After 3 days, glyceollin was extracted from the inoculated seeds with 80% ethanol. Glyceollin was isolated using preparative scale HPLC and confirmed by UV–vis spectrophotometry and electrospray mass spectrometry. Solvents [acetone and dimethyl sulfoxide (DMSO)] were purchased from Sigma-Aldrich (Milwaukee, WI). Water treated with a Millipore system was used during sample preparation procedures and HPLC analyses.

Insects. In Louisiana, *C. formosanus* swarm during April–June each year. Alates or primary reproductives were collected in 2001 and 2002 around light traps set up in New Orleans, LA. The dealates after losing their wings were sexed and used in the tests within a day. Termite workers were collected from ground

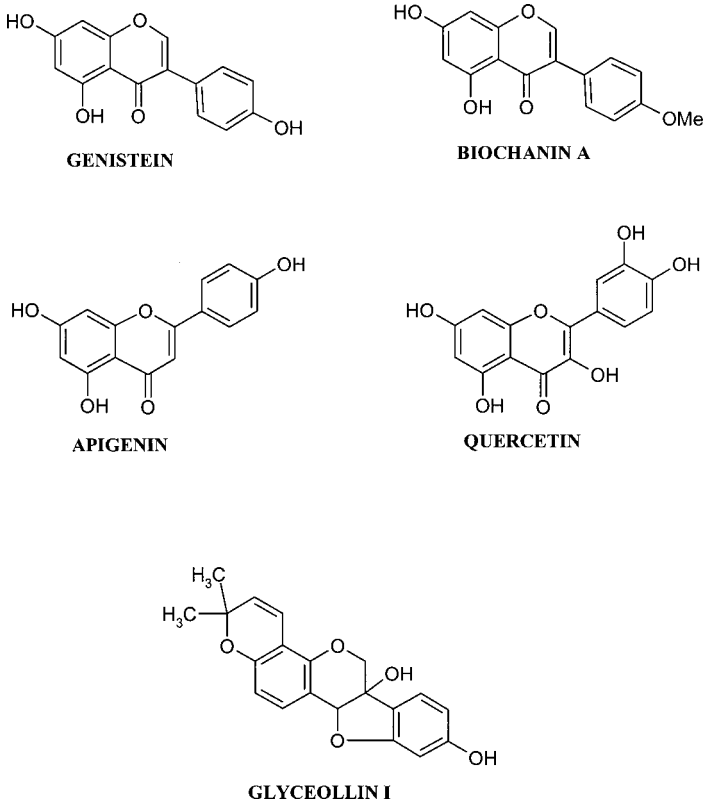

FIG. 1. Flavonoid/isoflavonoid structures. Only the structure of the predominant glyceollin isomer, glyceollin I, is shown.

traps setup in the City Park area, brought to the laboratory, and fed on pieces of pine wood in an incubator maintained at 28°C and 70% RH, until they were used in the tests.

Fecundity Bioassays. An initial test with five flavonoids was carried out in 2001. Except for apigenin, the chemicals (50 μg), were dissolved in acetone (5 μl) and applied to 12.2-mg bait matrix (0.41% by weight; bait matrix composition described in Rojas and Morales-Ramos, 2001). Apigenin was predissolved in DMSO before making the acetone solution (10% DMSO). Twenty pairs of dealates together with the treated bait matrix were placed singly in tight fitting, 50 × 9 mm Falcon Petri dishes (Becton Dickinson, NJ) containing agar–sawdust medium (Raina et al., 2003). Control insects were provided with bait matrix treated with 5-μl acetone. Observations on mortality and fecundity were recorded 30 days after treatment.

On the basis of the results from the first test, a second test was conducted in 2002. Only biochanin A and apigenin were used in both oral and topical applications. For oral application, Biochanin A (100 μg) was dissolved in acetone (5-μl) and applied to a 10-mg piece of Kimwipes™ (Kimberly-Clark, GA). Apigenin was predissolved in DMSO before making the acetone solution (10% DMSO). After allowing the solvent to evaporate, the Kimwipe™was treated with distilled water (10-μl) before being placed into a 50 $\times$ 9 mm Petri dish together with a pair of dealates. Thirty dealate pairs were allowed to feed for 72 hr, then transferred to Petri dishes containing diet as described above. Observations on mortality and total progeny were recorded after 30 days. Control insects were fed on Kimwipes™ treated with acetone followed by water. For topical applications of biochanin A and apigenin, each chemical was dissolved in DMSO at a concentration of 10 μg/0.1 μl. The solution (0.1 μl) was applied to both males and females after cooling them on ice. Thirty dealate pairs were tested and observations were recorded after 30 days.

Attractant and Feeding Bioassays. Only biochanin A was tested with termite workers in no-choice and choice tests. The tests were conducted in plastic Petri dishes (90 $\times$ 18 mm), each filled with sand (15-g) moistened with distilled water (1.5-ml). Filter papers (Whatman Grade 5, 2.5 cm diam) were weighed and treated with 50 μl of a 1% (w/w) acetone solution of biochanin A and then air-dried. Control filter papers were treated with 50-μl acetone. For the no-choice test, the treated and control filter papers were placed in separate Petri dishes with 100 workers per dish. Test dishes were placed in an incubator for 72 hr. In the choice test, a treated and a control filter paper disk were both placed in the same Petri dish with 200 workers per dish. Distribution of the termites was visually monitored for 1 hr, photographed after 1 hr, and the Petri dishes were placed into the incubator for 72 h. After this period, the filter papers were removed, washed, oven-dried (60°C for 1 hr), and weighed to determine consumption. The test was replicated three times.

Data Analysis. Significance tests, comparing treatments with controls for each experiment, were evaluated by a one-way Analysis of Variance (ANOVA) using a Dunnett's Multiple comparison posttest at the 5% level. Statistical tests were run on Prism software (GraphPad Prism version 2.1, San Diego, CA.)

RESULTS

Fecundity and Mortality. Addition of flavonoids did not appear to deter the primary reproductives from feeding on the bait matrix, with low to moderate feeding in all the treatments including control. The fecundity results from an initial screening experiment using the five flavonoids at 50 μg added to the bait matrix are presented in Figure 2. Only biochanin A and genistein significantly decreased the number of progeny (29.5 and 18.6%, respectively) produced from each alate pair after 30 days when compared to untreated controls. There was no effect of any of the flavonoids on egg hatch (data not shown). Apigenin was

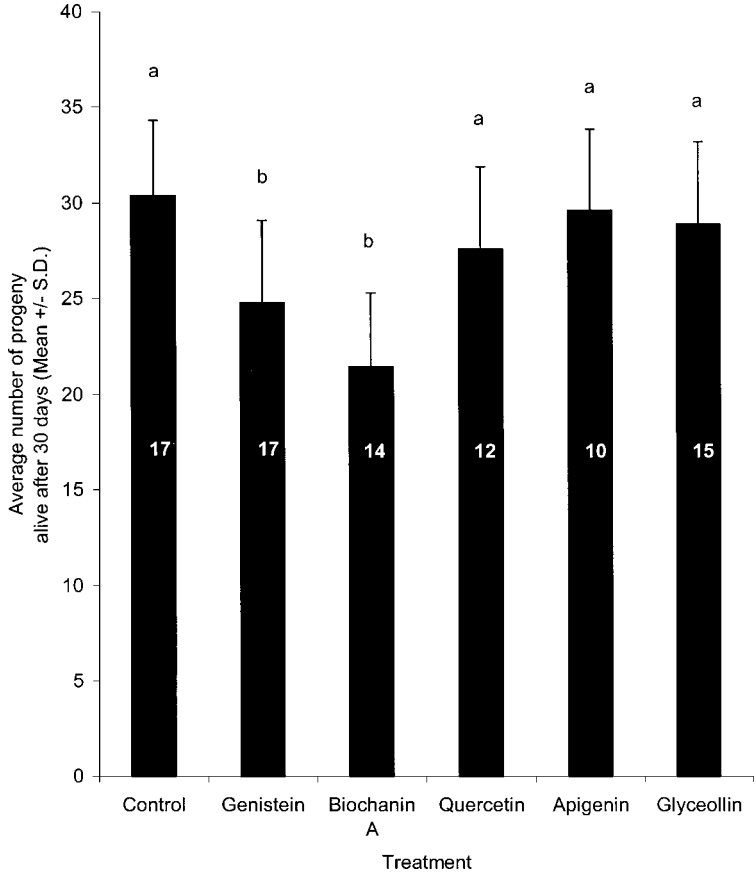

FIG. 2. Fecundity of primary reproductives of the Formosan subterranean termite 30 days following feeding on five flavonoids (50 μg). Bars with different letters are significantly different ($P < 0.01$, Dunnett's multiple comparison test). Numbers within bars indicate the sample size.

the most toxic flavonoid tested, resulting in 50% mortality among the primary reproductives (Table 1). Quercetin caused 40% mortality compared to 20% in the control group.

Biochanin A and apigenin were selected for further testing using 100-μg and 10-μg doses for oral and topical applications, respectively. In the oral treatment, total progeny after 30 days was significantly lower in both biochanin-A- and apigenin-treated groups compared to control (Figure 3). Of the two treatments, biochanin A was superior, causing about 32% decrease in the progeny. With topical application, only biochanin A caused a significant decrease in the progeny, and

TABLE 1. EFFECT OF ORAL FEEDING AND TOPICAL APPLICATION OF SELECTED PLANT FLAVONOIDS ON MORTALITY AMONG PRIMARY REPRODUCTIVES OF THE FORMOSAN SUBTERRANEAN TERMITE

Treatment	Percent mortality (N)		
	Oral 50 μg[a]	Oral 100 μg[b]	Topical 10 μg
Control	20 (17)	20 (30)	26.7 (30)
Genistein	15 (17)	—	—
Biochanin A	30 (14)	26.7 (30)	36.7 (30)
Quercetin	40 (12)	—	—
Apigenin	50 (10)	33.3 (30)	30 (30)
Glyceollin	25 (15)	—	—

[a] Flavonoids except apigenin were dissolved in acetone and applied to 12.2 mg bait matrix. Apigenin was predisolved in DMSO. Observations were recorded after 30 days.
[b] Flavonoids dissolves as above and applied to 10-mg Kimwipes™ tissue.

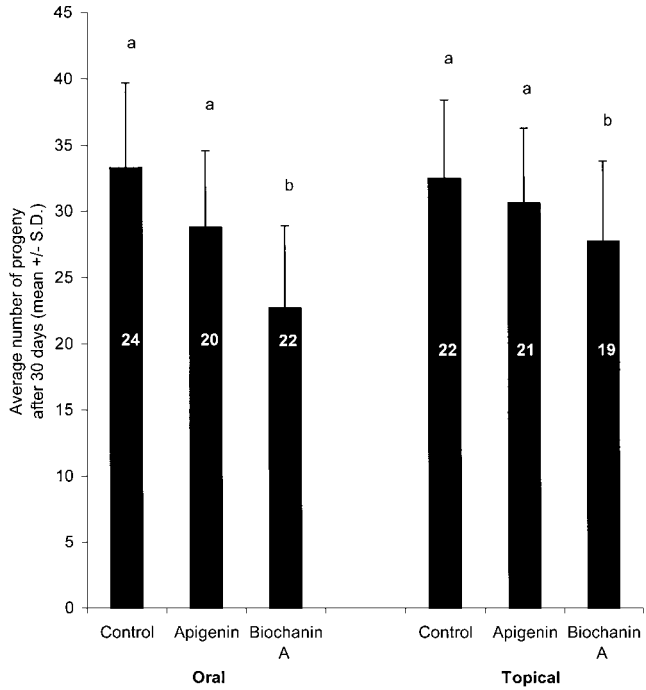

FIG. 3. Fecundity of primary reproductives of the Formosan subterranean termite 30 days following oral feeding (100 μg) or topical application (10 μg) with apigenin and biochanin A. Bars within a treatment method with different letters are significantly different ($P < 0.05$, Dunnett's Multiple comparison test). Numbers within bars indicate the sample size.

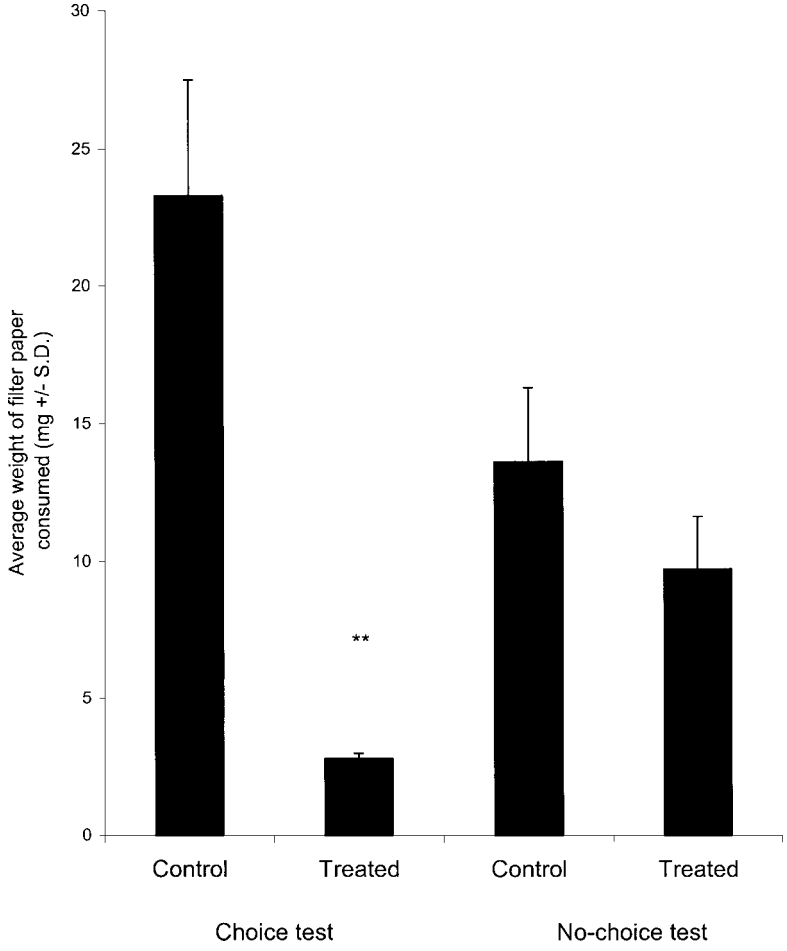

FIG. 4. Consumption of paper in no-choice and choice tests using biochanin A (50 μl). Means ($N = 3$) using 100 Formosan subterranean termite workers in no-choice test, and means ($N = 3$) using 200 workers in choice test. No significant difference versus acetone control was observed in no-choice test ($P > 0.05$), however in a choice test biochanin A was significantly different (**) versus control at $P < 0.01$ (Dunnett's Multiple comparison test).

the effect was lower (14.8% reduction) as compared to the oral method. Mortality caused by the two flavonoids ranged from 26.7 to 36.7%, which was not significantly higher than in the control groups (Table 1). The average consumption of paper in this experiment was 25% for the control group, 40% for the group treated with biochanin A, and 17.5% for the group treated with apigenin.

Preference Tests. Only biochanin A was tested in both no-choice and choice tests using *C. formosanus* workers. In the choice test, a significantly higher amount (88%) of the control paper was consumed in 72 hr, when compared to the consumption of biochanin-A-treated paper (Figure 4). Even though the workers were initially highly attracted to the flavonoid-treated paper when no choice was provided, consumption was not different between control paper as compared to biochanin-A-treated paper (Figure 4).

DISCUSSION

Flavonoids are numerous and widespread among natural plant constituents and serve many functions, including plant defense, pigmentation, and many diverse host–plant interactions. Most plants contain an array of flavonoids, and evidence has been presented suggesting that insects are able to discriminate among plants with varying flavonoid profiles (Schoonhoven, 1982; Feeny et al., 1988; Simmonds, 2001). Certain flavonoids either stimulate insect feeding (Bernays et al., 1991) or act as feeding deterrents (Morimoto et al., 2000). They can act as endocrine disruptors in mammalian systems, having high binding affinities for estrogen receptors, and have recently been shown to bind the ecdysone receptor of insects (Oberdorster et al., 2001). Although several studies have examined the effects of natural bioactive products on feeding, behavior, mortality, and fecundity of termites (Chang et al., 2001; Tellez et al., 2001; Zhu et al., 2001), only one study (Reyes-Chilpa et al., 1995) has examined the effects of flavonoids on termite behavior.

Of the flavonoids tested at 50 μg, only the isoflavones genistein and biochanin A resulted in a significant reduction in the number of progeny produced within 30 days following treatment. The flavones apigenin and quercetin, in contrast, had no significant effect on fecundity. However, the flavones caused higher mortality among the primary reproductives. In endocrine disruptor studies, isoflavones are more active when compared to flavones, particularly in their ability to bind to the estrogen receptor in mammalian systems and have been shown to disrupt animal reproduction (Shutt, 1976; Setchell et al., 1987). The isoflavone glyceollin is synthesized in soybean during pathogen attack and has antifungal properties, but is a weakly active endocrine disruptor (Burow et al., 2001). It showed little activity at inhibiting fecundity or increasing mortality among primary reproductives.

In the experiments that focused on the effects of biochanin A and apigenin on fecundity and mortality following oral feeding at 100 μg/pair and topical application at 10 μg/individual, biochanin A was again more active at decreasing egg-laying ability of the primary reproductives in both oral and topical treatments. After confirming the ability of biochanin A to lower fecundity in the primary reproductives, no-choice and choice tests were conducted to examine the flavone's

attractant and phagostimulant activities. There is a paucity of research on the effects of flavonoids on insect, particularly termite, feeding and toxicity. Several flavones are phagostimulants to many polyphagous insects (Bernays et al., 1991; Harborne and Grayer, 1994). Choice feeding tests with castillen D and E, isolated from the heartwood of the tropical tree *Lochocarpus castilloi*, showed concentration-dependent feeding deterrent activity, but they were not toxic to *Cryptotermes brevis* (Reyes-Chilpa et al., 1995). Our results showed that in a choice test, although termite workers were initially attracted to the filter paper treated with biochanin A, within 1 hr they dispersed evenly between the treated and control papers. After 72 hr, termites had consumed less of the treated filter paper. Consequently, no phagostimulant activity was observed. In order for biochanin A to be incorporated into bait matrix, a phagostimulant must be added to enhance the effectiveness of the bait matrix.

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LEAF ONTOGENY INFLUENCES LEAF PHENOLICS AND THE EFFICACY OF GENETICALLY EXPRESSED *Bacillus thuringiensis cry1A(a) d*-ENDOTOXIN IN HYBRID POPLAR AGAINST GYPSY MOTH

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Abstract—We tested the hypothesis that ontogenetic variation in leaf chemistry could affect the efficacy of genetically expressed *Bacillus thuringiensis cry1A*(*a*) *d*-endotoxin, and thus provide spatial variation in (1) foliage protection and (2) selective pressures that could delay the resistance of folivores. Our model consisted of clonal hybrid *Populus* plants (NC5339). Consumption of foliage and relative growth rates of gypsy moth, *Lymantria dispar* (L.) increased, and phenolic glycoside concentrations decreased, as leaves from transformed plants containing the *cry1A*(*a*) *d*-endotoxin and nontransformed plants matured from leaf plastochron index (LPI) 1–6. Feeding and growth rates were negatively correlated with phenolic glycosides in both transformed and nontransformed foliage. The presence of the *B. thuringiensis d*-endotoxin was at most, additive to the effect of the phenolic glycosides. Feeding and growth rates were positively correlated with condensed tannins in transformed foliage, but there was no relationship with condensed tannins in nontransformed foliage. The results indicate that the presence of foliar allelochemicals of poplar can enhance the effectiveness of genetically expressed *B. thuringiensis d*-endotoxin against gypsy moth larvae. However, the spatial variation in gypsy moth performance in response to the combination of foliar allelochemicals and *d*-endotoxin was not greater

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than the effect of ontogenetic variation in foliar allelochemicals alone. These results suggest that for this important pest, foliage protection may be obtained without genetically engineered defenses, and instead, by relying on ontogenetic and clonal variation in allelochemicals. The benefits of combining novel resistance mechanisms with natural ones will depend upon the specific folivore's adaptation to natural resistance mechanisms, such as allelochemicals. Moreover, some of the greatest benefits from transgenic resistance may arise from the need to protect trees from multiple pests, some of which may not be deterred by, or may even prefer, allelochemicals that confer protection from a few species.

Key Words—*Bacillus thuringiensis*, *Lymantria dispar*, *Populus*, transgenic resistance, allelochemicals.

INTRODUCTION

The evolution of resistant insect biotypes poses the greatest threat to the deployment of plants genetically engineered with insecticidal proteins (Gould, 1988; Raffa, 1989; Brattsten, 1991; Bauer, 1995). Resistance to toxins can develop when the selection pressure on an insect population is strong and consistent through time (Brattsten et al., 1986; Brattsten, 1991; Denholm and Rowland, 1992). The discovery of the soil-born bacterium, *Bacillus thuringiensis*, over 40 years ago, and the more recent discovery of additional *B. thuringiensis* strains has provided renewed promise for the development of environmentally safe and effective insecticides. Not only is the toxicity of *B. thuringiensis* specific to particular taxa of invertebrates, but early deployment was accompanied by optimism that resistance to a naturally evolved pathogen that produced multiple endotoxins would pose relatively few problems (Höfte and Whiteley, 1989; Gill et al., 1992). However, resistance to *B. thuringiensis* has emerged in both laboratory (McGaughey and Beeman, 1988; Stone et al., 1989; Bauer et al., 1994) and field (Shelton et al., 1993; Tabashnik, 1994a) populations, thus raising concerns about the long-term efficacy of transgenic plants expressing *B. thuringiensis* toxins.

The application of genetic engineering for pest control on trees has some economical and logistically attractive properties relative to traditional control tactics, but it must be managed wisely. The long-lived nature of trees combined with the constant genetic expression of insect resistance can provide conditions conducive for the development of biotype resistance in insects (Raffa, 1989). Strategies to reduce biotype evolution rely primarily on varying the pattern and predictability of any single selective force (Gould, 1988; Raffa, 1989; Brattsten, 1991). These have been summarized by Bauer (1995, 1997) to include (1) mixtures of toxins, (2) synergists to increase toxicity, (3) rotations of toxins, (4) temporal and spatial refuges, (5) sublethal toxins to enhance natural controls, (6) ultrahigh doses to kill resistant individuals, and (7) gene regulation of temporal and spatial toxin expression. Some of these same tactics resemble the naturally evolved chemical

defense systems of long-lived trees (Feeny, 1970; Schultz, 1983a,b; Whitham, 1983; Berenbaum, 1988; Hoy et al., 1998).

One way that plants can decrease damage due to insect herbivores and delay the resistance of insects to natural defense mechanisms is through temporal and spatial variation in the expression of allelochemicals (Schultz et al., 1983b; Coleman, 1986; Gould, 1988; Karban et al., 1997; Kleiner et al., 1999). In addition, the presence of more than one class of secondary compounds in a plant may confer a greater degree of resistance to herbivore damage than a single compound (Berenbaum and Neal, 1985; Berenbaum and Zangerl, 1988; Gould, 1988; Hay et al., 1994). These natural processes of reducing damage and delaying herbivore resistance suggest mechanisms that may be used to enhance and prolong the efficacy of genetically engineered insect resistance (but see discussions by Gould, 1988; Iwao and Rausher, 1997; Onstad and Gould, 1998a,b on how interactions among multiple selection pressures sometimes can increase the development rate of insect resistance to pesticides or, in natural systems, plant resistance to insects).

The natural variation in the allelochemistry of *Populus* foliage provides a potential mechanism for introducing variation in the deployment of short-rotation plants with genetically engineered insect resistance. *Populus* is characterized by indeterminate growth, as leaves are produced continually from budbreak to senescence. Therefore, a gradient of leaf development stages is present at all times. Concentrations of phenolic glycosides are highest in the youngest leaves, and decrease as they expand and mature (Lindroth et al., 1987; Bingaman and Hart, 1993). Since the toxicity of *B. thuringiensis* to Lepidoptera is enhanced by phenolic glycosides (Arteel and Lindroth, 1992; Hwang et al., 1995), the effect of the genetically expressed *B. thuringiensis d*-endotoxin should be greater in immature foliage than in mature foliage. Condensed tannin concentrations increase as leaves age (Meyer and Montgomery, 1987; Kleiner et al., 1998). Because condensed tannins have an inhibitory effect on the efficacy of *B. thuringiensis* to Lepidoptera (Navon et al., 1993; Hwang et al., 1995), larvae feeding on older leaves should be less affected than those feeding on younger leaves. Independently, variation in each class of allelochemicals contributes to greater *B. thuringiensis* efficacy in young foliage than in older foliage. In general, insect damage of young tissues is more detrimental to growth and yield than damage to older tissues (Reich et al., 1993), hence protection of these tissues would be more desirable. This natural array of allelochemicals could in effect, provide some of the spatial variation necessary to (1) increase the efficacy of genetically expressed *d*-endotoxin in young plant tissue and (2) reduce biotype evolution from a toxic selection pressure.

In this paper, we report on the efficacy of the *B. thuringiensis cry1A*(*a*) *d*-endotoxin genetically expressed in the foliage of hybrid *Populus* clone NC5339 against gypsy moth larvae, *Lymantria dispar* (L.) with the allelochemical concentration of leaves at different ontogenetic stages.

METHODS AND MATERIALS

Tree Culture. Transformed trees of *Populus* clone NC5339, *P. alba* L. × *P. grandidentata* Michx., "Crandon," (a cross between the Leuce and Tachamahaca sections of the genus) were produced by electrical discharge particle acceleration gene insertion, as described in McCown et al. (1991), and are from the same genetic stock as Transclone Bt-11 (Robison et al., 1994; Kleiner et al., 1995). The plasmid DNA (pTVBTGUS) contained three chimeric genes: (1) a neomycin phosphortransferase (NPT) gene enabling kanamycin resistance selection, (2) a chimeric gene for B-glucuronidase (GUS) for histochemical detection of gene expression, and (3) the gene encoding the *B. thuringiensis kurstaki* insecticidal *cry1A*(*a*) *d*-endotoxin protein, (Barton et al., 1987; McCabe et al., 1988). The *cry1A*(*a*) gene was derived from the HD-1-Dipel strain (Abbott Laboratories, Chicago, IL) and has moderate toxicity to gypsy moth larvae when added to artificial diet (van Frankenhuyzen et al., 1991, 1992) or genetically expressed in *Populus* clone NC5339 (Robison et al., 1994; Kleiner et al., 1995). The amount of *B. thuringiensis* protein in transgenic plant tissue is extremely low (0.001–0.1% of the total protein), so insect bioassays are the most sensitive method for detecting its presence (Fischhoff et al., 1987**;** Delannay et al., 1989; Fuchs et al., 1990; Cheng et al., 1992). The *cry1A*(*a*) gene used in this study is expressed at too low a level for the quantification of the *d*-endotoxin protein using Western blot analysis.

Transformed and nontransformed control plants were propagated from microcultured shoots. The top 3–4 cm of 5–8 cm shoots were excised and rooted *ex vitro* (Sellmer and McCown, 1989), acclimated, and transferred to 15-l pots (20 × 20 × 46 cm) containing a 2:2:1 mix of peat:sand:field soil and 114 g of Osmocote 13-13-13 fertilizer (Sierra Chemical Corp., Milpitas, CA, USA). Trees were grown under glasshouse conditions at ambient light and temperature conditions during June–September, 1994.

Insect Culture. Gypsy moth larvae were reared from eggs (laboratory colony NJSS F41) provided by the USDA Animal and Plant Health Inspection Service, Otis, MA. Eggs masses were surface sterilized in an aqueous solution containing 2% sodium hypochlorite and 1% polyoxyethylene sorbitan monooleate (Tween 80) for 5 min and rinsed with water. Neonates were transferred to 50-cell plastic jelly trays (Hopple Plastics, Florence, KY) containing a low protein (no casein) wheat germ-based diet (ODell and Rollinson, 1966). All larvae were reared in an environmental chamber at 27°C and a photoperiod of 16 L:8 D.

Insect Bioassays. A no-choice feeding assay was conducted using methods described by Kleiner et al. (1995). The ontogenetic stage of leaf development was standardized by sampling leaf plastochron index (LPI) leaves, 1–6. An apical leaf that has attained a lamina length of 2 cm is designated LPI 0 and the next oldest leaf designated as LPI 1 and so on down the stem. The use of the plastochron index adjusts plants of different sizes or developmental stages to a standardized

morphological time scale (Larson and Isebrands, 1971). On the morning of the assay (between 0700–0800 hours), leaves were excised at the base of the petiole from each of 10 transformed and 5 nontransformed control trees, sealed in plastic sandwich bags, placed on ice and brought to the University of Wisconsin Department of Entomology Gypsy Moth Quarantine Facility. Leaves were surface sterilized with a 0.53% sodium hypochlorite solution and rinsed in two washes of water. A #8 cork borer was used to remove leaf disks (169.7 mm^2) from the leaves. Five leaf disks and larvae were used for each leaf from LPIs 3–6. Leaves from LPIs 1 and 2 were small, and, therefore, only one and two leaf disks, respectively, were removed from these leaves for assays. The mean value of within leaf replicates was used to represent leaf level responses for comparisons among leaves.

The day before the assay, late second-instars were removed from diet and allowed to molt to third-instar. On the day of the assay, individual preweighed larvae were placed into each cell of a jelly tray along with a leaf disk inserted into an agar block. Initial larval weights did not differ ($P > 0.05$) among foliage treatments or leaf positions After feeding on leaf disks for 3 d, larvae were weighed, transferred to jelly trays containing low protein diet, and allowed to feed an additional 5 d, and weighed again. Transferring larvae to diet allowed us to check for mortality daily. Monitoring growth while on diet allowed us to evaluate whether surviving larvae avoided feeding or ingested a sublethal dose (see Kleiner et al., 1995). Relative growth rate (RGR) for individual larvae feeding on leaf disks and on diet was calculated as $(\text{weight}_{t2} - \text{weight}_{t1}/\text{weight}_{t1}/\Delta t)$, where Δt is the time interval between measurements at $t1$ and $t2$. Total wet weight gain was calculated as the difference between the initial weight and the weight after feeding on diet. Leaf consumption was calculated for each larva on a dry weight basis. One reference leaf disk was removed from each leaf and placed in a glass vial and oven dried (45°C) for an estimate of initial dry leaf disk weight. Portions of leaf disk remaining from the assay were oven-dried and weighed and subtracted from the initial estimate to calculate leaf consumption. In some instances where larval feeding was negligible, the reference disk weighed less than the assay disk, resulting in negative consumption values. Negative consumption values were recorded as zero.

Foliar Chemistry. Leaf material not used for bioassays was frozen on dry ice, freeze-dried, and ground to a 40 mesh with a Wiley mill. Leaf extracts for the analysis of condensed tannins were prepared by exhaustively (4x) extracting 45–55 mg of dry tissue in 1 ml ice cold 70% acetone with 10 mM ascorbic acid and sonicating for 30 min in ice water. The hydrolytic conversion (BuOH:HCL) of proanthocyanidins to anthocyanidins was used to measure condensed tannin content using a modification of the method of Porter et al. (1986). Condensed tannins from *P. alba* × *P. grandidentata* "Crandon" foliage were isolated by absorption chromatography, using a Sephadex LH-20 column and ethanol and acetone as solvents (Hagerman and Butler, 1980), and used to construct standard curves.

We also assayed the foliage for salicortin and tremulacin, the two most biologically active phenolic glycosides to Lepidoptera (Lindroth et al., 1988; Lindroth and Peterson, 1988). Salicortin and tremulacin were quantified using high performance liquid chromatography (HPLC) as described by Nichols-Orians et al. (1992). HPLC was performed on a Shimadzu LC-10AS with a SPD-10A UV–vis detector (Wood Dale, IL). We extracted 30–34 mg of leaf tissue in 2 ml of methanol with 1.5 mg/ml catechol as an internal standard. Samples were sonicated in ice water for 15 min, centrifuged, and the extracts filtered (0.2 um) for injection onto a reverse-phase C18 column (Spherisorb SS ODS-2, Supelco Inc., Bellefonte, PA) with a gradient of distilled H_20 and methanol and a flow rate of 1.0 ml/min. The detector was set at 274 nm. Standard curves were constructed from purified salicortin and tremulacin.

Statistical Analyses. To examine the effect of leaf position and foliage treatment on foliage consumption or larval growth, we used the PROC MIXED program in SAS (SAS Institute, 1999). Leaf position and foliage treatment were designated as fixed effects, and tree nested within foliage treatment was specified as the random effect. The weighting factor was the number of larvae used for each leaf position. To test for autocorrelation of the residuals among the leaf positions for each dependent variable, we conducted a repeated measures analysis with the AR(1) covariance structure and leaf position nested within tree as the subject effect. Models with and without the repeated statement were compared using the null model likelihood ratio test (SAS Institute, 1999). The difference in the -2 Res Log Likelihoods between the two models has a χ^2 distribution with $q - 1$ degrees of freedom. Repeated measure models for each of the four dependent variables (foliage consumption, RGR on test foliage, RGR on diet, and total weight gain) were not significantly different than models without the repeated statement ($P > 0.05$ and 1 df), indicating that each leaf position could be considered as an independent response.

A slopes analysis was conducted to test whether changes in larval performance across leaf positions were the same for both foliage treatments. We used PROC MIXED as described above, designating both foliage treatment and leaf position as fixed effects, but considered leaf position (lp) as a continuous variable. The weighting factor was the number of larvae used for each leaf position. PROC MIXED does not provide r^2 values, therefore, weighted linear regression (PROC REG) was used to examine the relationship between the independent variable, leaf position, and the dependent variable, larval performance, for transformed and nontransformed trees separately. The weighting factor was the number of larvae used for each leaf position.

Simple linear regression (PROC REG) was also used to examine putative relationships between leaf position and leaf chemistry. Weighted, multiple linear regression methods (PROC REG) were used to evaluate the relationship of individual measures of foliar chemistry from both foliage treatments on foliage

consumption and growth rates of larvae while on foliage (Freund and Littell, 1991). For these models, foliage consumption or relative growth rates were the dependent variables and foliage treatment, foliar chemistry, and the interaction of treatment by foliar chemistry, were the independent variables. If the interaction is not significant, and the slopes of both lines are negative, then the effect of the *d*-endotoxin and foliar chemistry are additive. If the interaction is significant, and the slope of the line between foliar chemistry and larval performance is more negative for transformed than nontransformed foliage, then there is a synergistic effect. Simple linear regression was used to examine the relationship of leaf chemistry with larval performance on each type of foliage.

RESULTS

Foliage consumption increased from LPI 1 to LPI 6 for larvae feeding on transformed and nontransformed foliage (*leaf position*, $F = 7.97$; df $= 4, 52$; $P < 0.001$) (Figure 1A). Less transformed foliage was consumed than nontransformed foliage (*treatment*, $F = 11.77$; df $= 1, 13$; $P < 0.005$). There was no significant

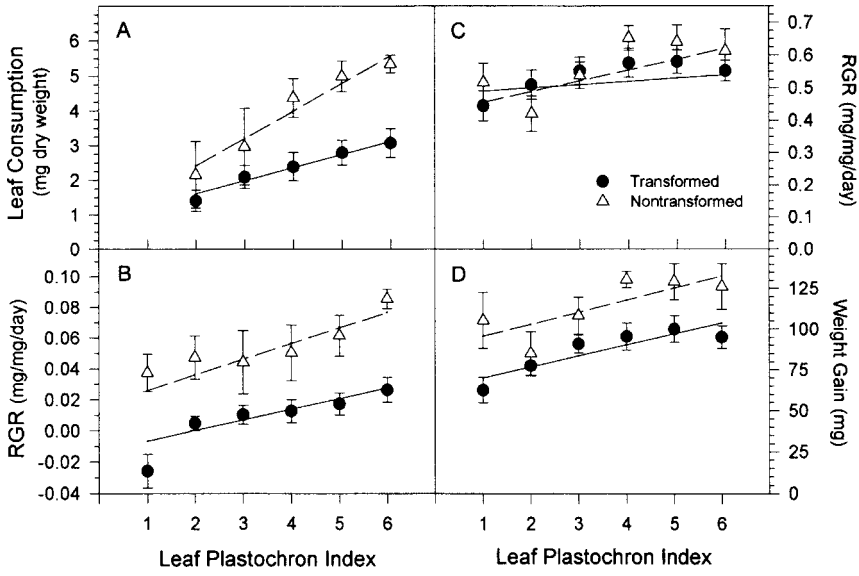

FIG. 1. Simple weighted regressions of gypsy moth (A) foliage consumption, (B) relative growth rate on foliage, (C) relative growth rate on artificial diet following foliage assay, and (D) total weight gain following leaf and artificial diet assays, on leaf plastochron index for transformed cry1A(a) *d*-endotoxin and nontransformed foliage.

Leaf position × *Treatment* interaction for the amount of foliage consumed during the bioassay ($F = 1.21$; df = 4, 52; $P = 0.32$).

Growth rates on foliage during the bioassay increased from LPI 1 to LPI 6 (*leaf position*, $F = 6.18$; df = 5, 65; $P < 0.001$) (Figure 1B). Larvae feeding on transformed foliage had lower relative growth rates than larvae feeding on nontransformed foliage (*treatment*, $F = 19.08$; df = 1, 13; $P < 0.001$). There was no significant *Leaf position* × *Treatment* interaction for relative growth rates of larvae feeding on foliage ($F = 1.03$; df = 5, 65; $P = 0.40$).

Growth rates on diet following the bioassay were positively related to the position of the leaf that was consumed (*leaf position*, $F = 3.76$; df = 5, 65; $P \leq 0.005$) (Figure 1C). Growth rates on diet did not differ between larvae that had fed on transformed versus nontransformed foliage (*treatment*; $F = 0.43$; df = 1, 13; $P = 0.52$), indicating that larvae were able to recover from consuming transformed foliage. There was no significant *Leaf position* × *Treatment* interaction for relative growth rates of larvae after transfer to artificial diet ($F = 0.93$; df = 5, 65; $P = 0.46$).

Although larval growth rates recovered once gypsy moths were transferred to unamended artificial diet, leaf position still affected total weight gained during the bioassay. Larvae gained more weight overall after feeding on older leaves (*leaf position*, $F = 3.80$; df = 5, 65; $P < 0.005$) (Figure 1D). After feeding on diet, larvae did not fully recover from the effects of feeding on transformed foliage. Total larval weight gain was, over the course of feeding on both transformed foliage and diet, lower than for control larvae (*treatment*, $F = 12.46$; df = 1, 13; $P < 0.004$). There was no significant *Leaf position* × *Treatment* interaction for total larval weight gain (foliage + diet) during the assay ($F = 0.67$; df = 5, 65; $P = 0.65$). One larva died after feeding on LPI 1 from a transformed plant.

Larvae feeding on nontransformed leaves consumed more foliage as leaf position increased as compared to larvae feeding on transformed leaves (see Table 1 for regression slopes) (*lp* × *Treament*, $F = 4.04$; df = 1, 58; $P \leq 0.05$). The slopes

TABLE 1. REGRESSION FROM COEFFICIENTS SIMPLE REGRESSIONS OF FOLIAGE CONSUMPTION OR LARVAL PERFORMANCE BY GYPSY MOTHS ON *Populus* LEAF POSITION

	Transformed				Nontransformed			
	Slope	Intercept	r	P	Slope	Intercept	r	P
Leaf consumption (mg dry weight)	0.375	0.504	0.41	0.003	0.79	0.061	0.57	0.002
RGR on foliage (mg/mg/day)	0.006	−0.021	0.41	0.001	0.01	0.006	0.42	0.022
RGR on diet (mg/mg/day)	0.015	0.479	0.17	0.177	0.033	0.422	0.39	0.035
Total weight gain on foliage + diet (mg)	0.005	0.066	0.32	0.012	0.007	0.083	0.38	0.037

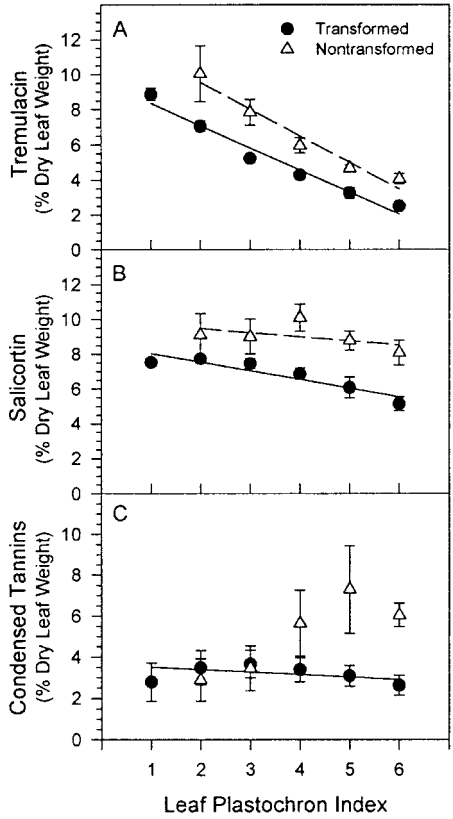

FIG. 2. Simple regressions of tremulacin, salicortin, and condensed tannins on leaf plastochron index for transformed and nontransformed foliage.

did not differ between transformed and untransformed trees for the relationships between leaf position and relative growth rate on foliage (*lp* × *Treatment*, $F = 1.05$; df = 1, 73; $P = 0.30$), leaf position and relative growth rate on diet (*lp* × *treatment*, $F = 1.25$, df = 1, 73; $P = 0.26$), and leaf position and total weight gain (foliage + diet) (*lp* × *Treatment*, $F = 0.30$; df = 1, 73; $P = 0.58$).

Concentrations of the phenolic glycosides tremulacin (Figure 2A) and salicortin (Figure 2B) were greatest in leaves at LPI 1 and decreased as leaf position increased to LPI 6 (Table 2; Figure 2A and B). Tremulacin concentrations decreased by up to 5-fold and salicortin concentrations decreased up to 2.4-fold over this ontogenetic gradient. Tremulacin concentrations were negatively related to leaf position for both nontransformed and transformed plants. Salicortin concentrations were negatively related to leaf position in transformed plants only.

TABLE 2. REGRESSION COEFFICIENTS FROM SIMPLE REGRESSIONS OF FOLIAR CHEMISTRY ON LEAF POSITION IN HYBRID POPLAR CLONE NC5339

	Transformed				Nontransformed			
	Slope	Intercept	*r*	*P*	Slope	Intercept	*r*	*P*
Tremulacin	−1.25	10.79	0.92	<0.001	1.49	13.96	−0.81	<0.001
Salicortin	−0.51	9.14	−0.60	<0.001	−0.25	10.29	−0.19	0.362
Condensed tannins	−0.11	3.75	−0.10	0.485	0.96	0.22	0.42	0.058

Condensed tannin concentrations were not related to leaf position in transformed plants and showed a marginally significant relationship (Table 3) in control plants (Figure 2C).

Larval consumption of transformed and nontransformed foliage was negatively correlated with phenolic glycosides (Figure 3A and B). Separate multiple linear regressions indicated that foliage consumption was negatively correlated with *tremulacin* ($P < 0.001$) and *salicortin* ($P < 0.001$). Reductions in consumption by phenolic glycosides and foliage expressing the *d*-endotoxin were additive, as indicated by the lack of significant *tremulacin* × *Treatment* ($P = 0.10$) or *Salicortin* × *Treatment* ($P = 0.14$) interactions (see Table 3 for regression slopes). *Condensed tannins* ($P = 0.60$) did not account for significant variation in feeding, but there was a significant *Condensed tannins* × *Treatment* interaction ($P < 0.02$). Condensed tannins were positively correlated with consumption of transformed foliage, but there was no relationship for nontransformed foliage (Table 3; Figure 3C).

Phenolic glycosides were negatively correlated with the relative growth rates of larvae feeding on foliage (Figure 3 D and E). *Tremulacin* ($P < 0.001$) was

TABLE 3. REGRESSION COEFFICIENTS FROM SIMPLE WEIGHTED REGRESSIONS OF LARVAL FOLIAGE CONSUMPTION AND RELATIVE GROWTH RATE BY GYPSY MOTH LARVAE (DURING LEAF ASSAY) ON CHEMISTRY OF *Populus* FOLIAGE

	Transformed				Nontransformed			
	Slope	Intercept	*r*	*P*	Slope	Intercept	*r*	*P*
Foliar consumption								
Tremulacin	−0.31	3.76	0.41	0.004	−0.56	7.65	0.71	<0.001
Salicortin	−0.24	4.07	0.30	0.03	−0.53	9.10	0.54	0.006
Condensed tannins	0.29	1.48	0.45	0.001	−0.05	4.64	0.09	0.69
Relative growth rate								
Tremulacin	−0.005	0.04	0.39	0.003	−0.009	0.12	0.60	0.002
Salicortin	−0.003	0.04	0.24	0.07	−0.013	0.18	0.67	0.001
Condensed tannins	0.003	0.003	0.28	0.04	−0.004	0.08	0.35	0.13

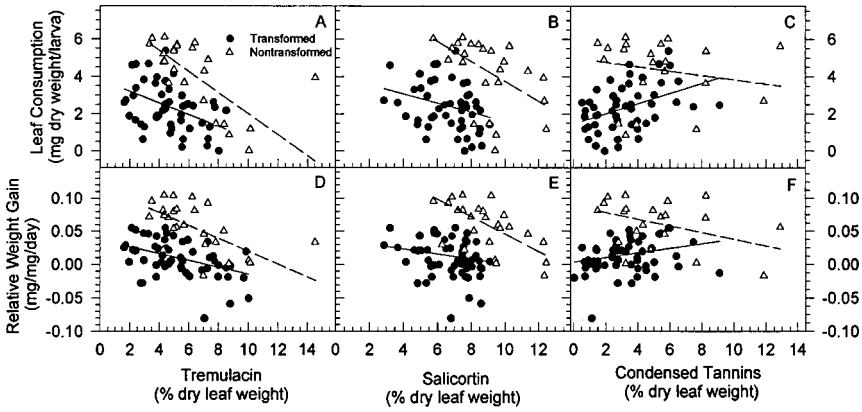

FIG. 3. Simple weighted regressions of gypsy moth foliage consumption and relative growth rate on tremulacin and condensed tannins in transformed and nontransformed foliage.

correlated with reduced relative growth rates, but the relationship between tremulacin and relative growth rate did not differ between foliage treatments (*Tremulacin* × *Treatment*, $P = 0.11$). This indicates that the effects of the *d*-endotoxin in transformed foliage and tremulacin were additive (see Table 3 for regression slopes). *Salicortin* ($P < 0.001$) was also correlated with reduced relative growth rates. The relationship between salicortin and relative growth rate differed between foliage treatments (*Salicortin* × *Treatment*, $P < 0.01$). Simple regressions indicated that salicortin had a greater negative effect on larval growth rates in nontransformed than transformed foliage (Table 2). In the full multiple regression model, *condensed tannins* ($P < 0.03$) were negatively correlated with relative growth rates. However, the relationship between condensed tannins and growth rate differed between foliage treatments (*Condensed Tannin* × *Treatment*, $P < 0.007$). Simple linear regressions indicated that condensed tannins were positively correlated with relative growth rates of larvae feeding on transformed foliage, but negatively associated for larvae feeding on nontransformed foliage (Table 3; Figure 3F).

DISCUSSION

We observed differential feeding and growth of gypsy moth larvae on nontransformed foliage that was related to ontogenetic variation in foliar allelochemicals. The amount of foliage consumed and larval weight gain reported here are similar to previous studies (Kleiner et al., 1995, 1998). Consistent with our expectations and the results of Meyer and Montgomery (1987), phenolic glycoside

concentrations decreased and condensed tannins increased as leaves matured. These trends in foliar chemistry were concurrent with increased feeding and growth rates of gypsy moth larvae on older leaves.

The effects of tremulacin and *B. thuringiensis d*-endotoxin in transformed foliage on larval feeding and relative growth rates were additive. These results agree with Arteel and Lindroth (1992) who observed additive negative effects of formulated *B. thuringiensis* suspensions and total phenolic glycosides in artificial diet, but differ from those of Hwang et al. (1995) who reported a synergistic effect between formulated *B. thuringiensis* suspensions and total phenolic glycosides in aspen leaves. Condensed tannins were positively correlated with larval feeding and growth on transformed foliage, but were not correlated with larval performance on nontransformed foliage. Our results are consistent with reports that condensed tannins can ameliorate the effects of *B. thuringiensis* (Navon et al., 1993; Hwang et al., 1995), but are not associated with negative effects on gypsy moth in the absence of this bacterium (Rossiter et al., 1988; Kleiner and Montgomery, 1994; Hemming and Lindroth, 1995).

In this study, three of the seven strategies outlined by Bauer (1995, 1997) to minimize biotype evolution were demonstrated by the combination of foliar allelochemical variation and the genetically expressed *d*-endotoxin: 1) a mixture of toxins provided by *d-endotoxin* and phenolic glycosides, (2) sublethal effects, and (3) variation in space due to leaf ontogeny. However, the efficacy of each tactic in reducing insect resistance remains unknown, and under certain conditions may even be counterproductive (Gould et al., 1991; Johnson and Gould, 1992; Caprio, 2001; Tang et al., 2001). For example, the production of herbivore induced chemical defenses could alter the relationship between leaf ontogeny and larval performance demonstrated in this study.

In contrast to other forms of refugia, ontogenic variation introduces interactions between spatial separation and other biotype management strategies such as chemical mixtures. Ontogenic variation also differs from most refugia approaches in that individual larvae can avoid transgenes by moving to other foliage categories (Gould, 1988). Most standard designs provide untreated and treated plants with varying degrees of spatial separation, which poses the risk of allowing isolated subpopulations to evolve, which can even accelerate biotype evolution under certain conditions (Tabashnik,1994b; Onstad and Gould, 1998b; Caprio, 2001; Tang et al., 2001). However, we currently lack the understanding to recommend this approach. For example, simulation modeling of transgenic maize with silk and kernels as refugia did not generate reduced rates of insect resistance (Onstad and Gould, 1998b). Moreover, in a greenhouse study, Tang et al. (2001) observed that touching between resistant and nonresistant plants accelerated dominance of the resistant allele in *Plutella xylostella*, and that the rate at which this dominance arose increased with the degree of contact among plants. Likewise, changes in efficacy of a transgene introduced by natural ontogenic variation may be analogous

to the seasonal decline of *d*-endotoxin in cotton (Fitt, 1998; Greenplate, 1999; Holt, 1998; Sachs et al., 1998; Adamczyk et al., 2001), which according to simulation models could accelerate insect resistance (Onstad and Gould, 1998a,b; Onstad and Guse, 1999). However, we were not able to measure or control the amount of *d*−endotoxin protein at each leaf position and, thus, cannot eliminate the possibility that some decline might be associated with increasing leaf age. Nonetheless, these results raise the caution that variation in either the expression or efficacy of *d*-endotoxin among leaves along a branch could theoretically increase biotype resistance.

Whether the benefits of genetically expressed *B. thuringiensis d*-endotoxin in short rotation hybrid poplar plantations outweigh the risks may depend additionally on the degree of specialization of the herbivore. Polyphagous species, such as the gypsy moth, can overcome the general defenses of older leaves, but have difficulty detoxifying phenolic glycosides (Lindroth and Hemming, 1990; Hwang and Lindroth, 1997), which are more abundant in younger leaves. Gypsy moth survival and feeding is sensitive to clonal variation in the phenolic glycosides concentrations and inducible defenses of *Populus* (Hwang and Lindroth, 1997; Havill and Raffa, 1999), suggesting a mixture of nontransformed poplar clones in a plantation could provide the necessary level of natural protection in the absence of genetically expressed *B. thuringiensis d*-endotoxin. This deployment strategy could reduce wood production among some *Populus* species. However, Hwang and Lindroth (1997) demonstrated a negative genetic relationship between tree size and foliar allelochemicals of 13 quaking aspen clones, *Populus tremuloides* Michx. In contrast, Robison and Raffa (1994) found no correlation between the growth of 15 hybrid poplar clones and either their defoliation tolerance or preference by forest tent caterpillars.

In contrast to polyphagous species, monophagous (e.g., cottonwood leaf beetle, *Chrysomela scripta*) and oligophagous (e.g., forest tent caterpillar, *Malacosoma disstria* Hübner) insects are adapted to the qualitative defenses in young leaves (Cates, 1980), such as phenolic glycosides, and may even use them as feeding cues (Bingaman and Hart, 1992, 1993; Hemming and Lindroth, 1995; Hwang and Lindroth, 1997). For example, forest tent caterpillars feed on a wide range of leaf positions (Robison and Raffa, 1997), and are less susceptible to phenolic glycosides than gypsy moth (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997). However, they are more sensitive to the c*ry1A* (*a*) *d*-endotoxin than gypsy moth (van Frankenhuyzen et al., 1991; Robison et al., 1994; Kleiner et al., 1995). In such cases, protection of short rotation poplar plantations may benefit from combining different deployment strategies in which mixtures of toxins are introduced and varied at both the between-plant and within-plant levels (Robison et al., 1994; Robison and Raffa, 1997). Delaying the resistance of insects to genetically expressed *B. thuringiensis d*-endotoxin will require judicious deployment based upon an understanding of interactions with foliar chemistry and microbial

associates (Broderick et al., 2000), the guild of insects capable of injuring various clones (Robison and Raffa, 1994), and the feeding breadth and dispersal capabilities of each target insect (Hemming and Lindroth, 1995).

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ARE INSECT-SYNTHESIZED RETRONECINE ESTERS (CREATONOTINES) THE PRECURSORS OF THE MALE COURTSHIP PHEROMONE IN THE ARCTIID MOTH *Estigmene acrea?*

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Abstract—The pyrrolizidine alkaloid (PA) profiles were determined for adults of the polyphagous arctiid *Estigmene acrea*, which as larvae had fed on artificial diet supplemented with *Crotalaria-pumila* powder with known concentrations of PAs. The larvae always had a free choice between alkaloid-containing and plain diets. The alkaloid profiles of adults revealed a striking sexual dimorphism. Both sexes contained macrocyclic PAs of the monocrotaline type sequestered from the diet and, in addition, a substantial proportion of supinidine and retronecine esters synthesized by the insects from necine bases derived from the dietary alkaloids and necic acids of insect origin. These insect alkaloids accounted for 35% and 55% of total PAs in males and females, respectively. The difference was that in females the retronecine esters (creatonotines) made up 58 μg (43% of total PAs), while males contained a fivefold lower proportion, 12 μg (13%). Four of the ten male individuals analyzed were found devoid of creatonotines. Based on the experimental data in combination with evidence from the literature, it is suggested that the creatonotines are direct pheromone precursors in *E. acrea*. It is hypothesized that this may represent a general mechanism of hydroxydanaidal formation from diverse macrocyclic PAs in arctiids.

Key Words—Arctiidae, *Estigmene acrea*, pyrrolizidine alkaloid, alkaloid sequestration, insect alkaloid, sexual dimorphism, creatonotine, courtship pheromone, hydroxydanaidal, pheromone biosynthesis.

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INTRODUCTION

Hydroxydanaidal is an important courtship pheromone of adult males in many species of arctiid moths. It is synthesized in the insect from plant-acquired, toxic pyrrolizidine alkaloids (PAs). The pheromone plays a decisive role in sexual selection and in the parental investment of PAs for chemical protection of their offspring (Nishida, 2002).

Little is known about the mechanisms by which the various structural types of PAs sequestered by arctiids are transformed into the pheromone. The only detailed study refers to the conversion of the monoester heliotrine to hydroxydanaidal in the Asian arctiid *Creatonotos transiens* (Schulz et al., 1993). This transformation encompasses epimerization at C-7 to attain the correct (*R*)configuration, aromatization of the dihydropyrrole ring, followed by ester hydrolysis, and oxidation of the resulting primary alcohol to the final aldehyde. Most arctiids sequester macrocyclic PAs of the senecionine and monocrotaline types (Hartmann and Witte, 1995). These diester alkaloids are known to be pheromone precursors, but the mode by which they are processed remains obscure.

In the course of current studies on sensory recognition, sequestration, and insect-specific processing of plant-acquired PAs in the North American arctiid *Estigmene acrea* (Bernays et al., 2002a,b; Hartmann et al., 2003), the origin of PA-derived pheromone attracted our attention. Larvae of *E. acrea* are highly polyphagous; they feed on a great variety of plants, among them various PA-containing species. In southern Arizona, *Senecio longilobus* and *Crotalaria pumila* are the principal PA sources. Male adults of *E. acrea* possess androconial organs (coremata) that emit hydroxydanaidal formed from plant-acquired PAs (Krasnoff and Roelofs, 1989). High PA levels in the larval diet increase the size of the coremata (Davenport and Conner, 2003). Larvae reared on artificial diet containing *C. pumila* sequester the plant's alkaloids; they also synthesize insect-specific PAs by esterification of necine bases of plant origin with their own necic acids. These so called insect alkaloids (Hartmann et al., 1990) account for 40–70% of total PAs found in larvae or adults of *E. acrea* (Hartmann et al., 2003).

Here we report on a striking sexual dimorphism concerning the insect PAs recovered from *E. acrea* adults. Arguments are presented that the significantly smaller amounts of retronecine O^9-esters (creatonotines) in males are apparently caused by their utilization as pheromone precursors.

METHODS AND MATERIALS

Caterpillars and adults of *Estigmene acrea* were obtained from cultures reared in the laboratory in the presence of artificial diet with PAs (Bernays et al., 2002a,b). Larvae were given the plain wheat-germ-based diet and, as a choice, the same diet

mixed with dry *Crotalaria pumila* powder at 10% of the dry weight, giving a final PA concentration in the diet of 0.018%. The PA composition (relative abundance) was: supinidine (19%), subulacine (13%), pumiline A (22%), pumiline B (31%), pumiline C (13%), and a fourth unknown monocrotaline type PA, PA-X (2%) (Figure 1). Individuals could eat from both foods *ad libitum*, but ate more of the PA-containing diet. After the PA treatment, larvae to be analyzed were confined with plain diet for 24 hr to ensure that PAs in the gut were evacuated. Other larvae were allowed to complete development with the choice of diet, and the adults that finally emerged were analyzed. Both larvae and adults were killed by freezing and kept at -20°C. All insect samples allotted to PA analysis were lyophilized and kept in closed vials until analysis.

Alkaloid extraction, analysis and identification by GC and GC-MS were performed as described previously (Hartmann et al., 2003).

RESULTS AND DISCUSSION

Alkaloid analysis of early and late instar larvae of *Estigmene acrea* revealed the presence of all *C. pumila* PAs except the simple necine epoxide subulacine (Figure 1). In addition to the sequestered *C. pumila* PAs, substantial amounts of insect PAs were detected; they accounted for about half of total PAs recovered from the insects. These insect PAs are synthesized by the caterpillars through esterification of supinidine and retronecine of plant origin, with 2-hydroxy-3-methylbutyric acid and 2-hydroxy-3-pentanoic acid of insect origin. The respective esters of supinidine are estigmine A and B, the respective O^9-esters of retronecine are creatonotine A and B (Figure 1) (Hartmann et al., 2003). Supinidine, the substrate for the formation of the estigmines occurs, as free necine base in substantial amounts (19% of total PAs) in the *C. pumila* material. By contrast, free retronecine is absent from the diet; it is only present as the common necine base moiety in the pumilines A–C. Thus, all the retronecine utilized by the insects for the formation of the creatonotines must be derived from the pumilines by hydrolysis (Figure 1).

The stored alkaloids are retained through pupation to adulthood. Alkaloid analysis of adults revealed a surprising sexual dimorphism. Among ten male adults analyzed, four were found to be completely devoid of the creatonotines, the other six contained small amounts, and in a few cases levels were comparable to those evaluated for females. The overall mean is about five times lower than that evaluated for females ($N = 10$), which all contained substantial amounts of creatonotines (Figure 2A). The difference between males and females is highly significant (Wilcoxon's two-sample test, $P \leq 0.001$). By contrast, the difference between estigmines, which appear to be even a little higher in males, is not significant ($P = 0.445$), indicating an unimpaired capability of males to synthesize insect PAs. The low level of creatonotines reduces the total amount of insect PAs

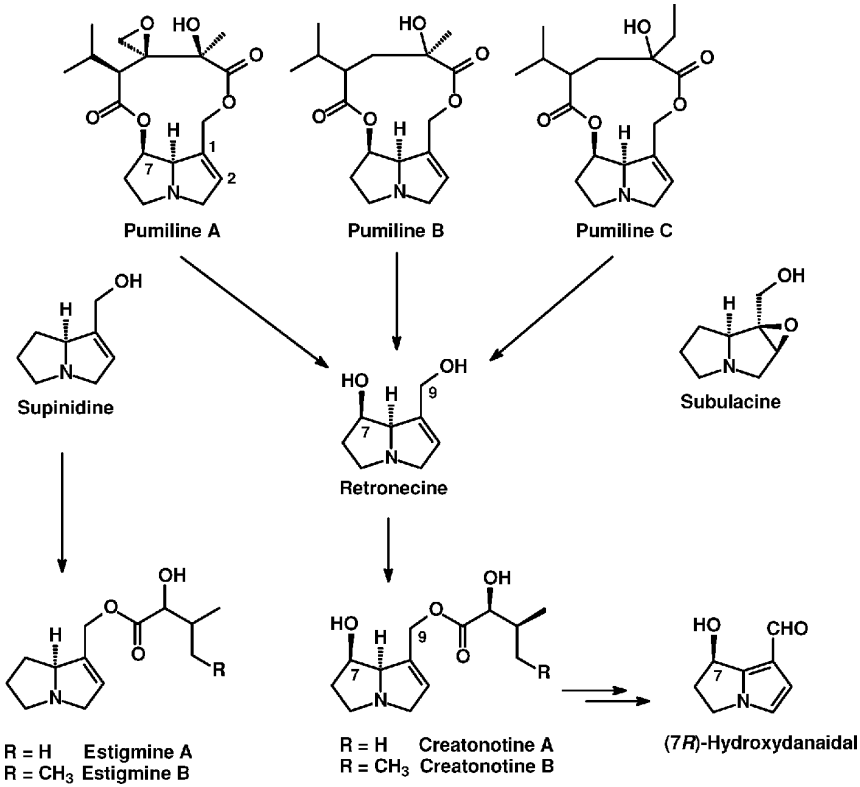

FIG. 1. Pyrrolizidine alkaloids of *Crotalaria pumila* one of the principal alkaloid sources of *Estigmene acrea* and their assumed metabolic fate in the insect. The pumilines A–C, supinidine and subulacine comprise the PA profile of *C. pumila*. With the exception of subulacine, all PAs are sequestered by *E. acrea* larvae. The estigmines and creatonotines are synthesized in the insect by esterification of the plant-derived necine bases with necic acids of insect origin. The creatonotines are suggested to be the direct biosynthetic precursors of hydroxydanaidal.

in males to about 31 μg in comparison to 66 μg in females; the difference is significant ($P = 0.025$). The total amount of PAs recovered from males and females was 85.2 ± 13.9 μg ($N = 10$) and 134.3 ± 27.3 μg ($N = 10$), respectively; the difference is not significant ($P = 0.162$). The total amounts of plant acquired PAs (i.e., pumilines) revealed similar figures, i.e., 54 μg for males and 66 μg for females; the difference is not significant ($P = 0.791$), implying that the lower level of total PAs in males is mainly due to the lower level of the creatonotines. This becomes even more evident if one compares the relative proportions of the alkaloid fractions recovered from the two sexes (Figure 2B).

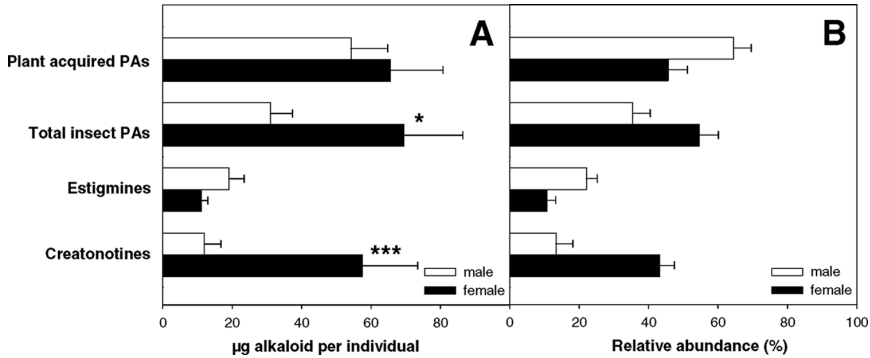

FIG. 2. Amount (A) and relative proportion (B) of insect PAs and plant acquired PAs (pumilines) recovered from adult males and females of *Estigmene acrea* (mean ± standard error). Creatonotines and estigmines together make up the insect PAs. Wilcoxon's two-sample test on males vs females: Plant-acquired PAs, $Z = 0.265$, $P = 0.791$; total insect PAs *, $Z = 2.229$, $P = 0.025$; estigmines $Z = 0.756$, $P = 0.449$; creatonotines $^{***}Z = 3.249$, $P = 0.0006$.

How can one explain the low level of creatonotines in males? Does there exist a relation between loss of creatonotine and production of male pheromone? The formation of both hydroxydanaidal and creatonotine requires the degradation of the pumilines to provide the retronecine moiety. Krasnoff and Roelofs (1989) found 18.4 μg hydroxydanaidal per individual in the coremata of *E. acrea* males, reared under conditions similar to those described here. In field-collected *E. acrea* males, the same authors found pheromone titers ranging from 0–150 μg hydroxydanaidal per individual. We found that females on average contain 45 μg more creatonotines than males (Figure 2B). This is in the order of magnitude one would expect if males were to convert creatonotines into hydroxydanaidal.

There are two possibilities for how pheromone biosynthesis may be linked to creatonotine formation: (i) competition of two independent processes for a common substrate, i.e., the retronecine moiety; (ii) the creatonotines are the direct pheromone precursors. The first possibility appears unlikely since it has been demonstrated for the conversion of the monoester heliotrine to hydroxydanaidal in *Creatonotos transiens* (see Introduction) that the aromatization of the dihydropyrrole ring occurs at the stage of the retronecine ester. The cleavage of the ester group takes place after aromatization yielding dehydroretronecine; free retronecine is not an intermediate (Schulz et al., 1993; Schulz, 1998). Interestingly the same authors described as a side-reaction the formation of creatonotine from retronecine, which in turn acted as precursor for hydroxydanaidal. Thus, it appears reasonable to assume that the low level of creatonotine in *E. acrea* males reflects its role as pheromone precursor. We hypothesize that this process represents a general

mechanism by which arctiids access macrocyclic PAs for pheromone production. Most of the highly diverse macrocyclic diesters of the senecionine and monocrotaline types possess the retronecine moiety as a common building block. Thus, hydrolysis of the macrocyclic alkaloids by an unspecific esterase and a specific, insect controlled re-esterification of the retronecine would provide a reasonable mechanism to obtain the unique pheromone, hydroxydanaidal, from a wide array of diverse structures.

Experimental proof with different alkaloids and various courtship pheromone producing arctiids is necessary to verify this suggestion. These studies are currently in progress.

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SPIDER MITES ASSESS PREDATION RISK BY USING THE ODOR OF INJURED CONSPECIFICS

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Abstract—When a predatory mite, *Amblyseius womersleyi* is present, *Tetranychus kanzawai* takes refuge on its webs during a quiescent stage. To examine the factors responsible for the antipredator behavior of *T. kanzawai*, we exposed *T. kanzawai* to the odor of conspecifics, *A. womersleyi*, and both *T. kanzawai* and *A. womersleyi*. The proportion of *T. kanzawai* females that took refuge was significantly higher when they were exposed to the odor of 'both *T. kanzawai* and *A. womersleyi*.' Furthermore, the proportion of *T. kanzawai* females that took refuge was significantly higher when they were exposed to injured conspecifics than to intact conspecifics. These results suggest that *T. kanzawai* assesses predation risk, at least in part, by using the odor of injured conspecifics.

Key Words—Antipredator behavior, injured conspecifics, odor, predation risk, quiescent stage, spider mites.

INTRODUCTION

Chemical cues resulting from predator activity provide important information for prey organisms to assess predation risk. Prey use direct chemical cues from predators (e.g., Chivers and Smith, 1994; Barnes et al., 2002) and indirect cues from intact or injured conspecifics (e.g., Janssen et al., 1997; McCarthy and Fisher, 2000).

The development of the herbivorous mite *Tetranychus kanzawai* (Acari: Tetranychidae) consists of active (larva, protonymph, and deutonymph) and quiescent (egg, protochrysalis, deutochrysalis, and teleiochrysalis) stages. *T. kanzawai*

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constructs highly complicated webs over leaf surfaces (Saito, 1983). In the presence of the native predatory mite, *Amblyseius womersleyi* (Acari: Phytoseiidae), *T. kanzawai* females enter a quiescent stage on the webs rather than on the leaf surface under the web. Furthermore, quiescent females on webs are less likely to be attacked by the predator, while they are also less likely to be guarded by conspecific males on the leaf (Oku et al., 2003). Therefore, *T. kanzawai* females may take refuge on the webs only when the benefit outweighs the cost of predator avoidance, i.e., when predation risk is high. Therefore, we asked what cues *T. kanzawai* uses to assess predation risk before entering a quiescent stage?

Spider mites can use chemicals to perceive distant information (e.g., Sabelis and van de Baan, 1983). Grostal and Dicke (1999) and Pallini et al. (1999) reported that adult female *T. urticae* avoid plants with predators, and this seems to be mediated by volatile or non-volatile chemicals that are produced by the predators. By contrast, Janssen et al. (1997) reported an odor produced by *T. urticae* adults in the presence of a predatory mite, *Phyotoseius persimilis*, which could be interpreted as an alarm pheromone to warn conspecifics of the predation risk. Here, we report that *T. kanzawai* appears to evaluate predation risk by using the odor of conspecifics attacked by *A. womersleyi*.

METHODS AND MATERIALS

Mites. The study population of *T. kanzawai* was collected from a strawberry patch in Kyoto, Japan, and maintained on discs of kidney bean (*Phaseolus vulgaris*) pressed onto water-saturated cotton in Petri dishes (90 mm diam, 14 mm depth). *A. womersleyi* were also collected in Kyoto, and reared on bean leaf discs that were heavily infested with *T. urticae* as prey (30–50 female adults and individuals of other stages per leaf). All of the dishes were placed together in a transparent plastic container and kept at 25°C, at 50% relative humidity, with a L16: D8 photoperiod.

Response of T. kanzawai Deutonymphs to Predator Odors. To examine whether *T. kanzawai* takes refuge in response to the odor of *A. womersleyi*, we cut a bean leaf in half and pressed the two halves onto water-saturated cotton in a Petri dish, 10 mm apart (Figure 1a). To prevent the mites (see below) from escaping, we placed tanglefoot (The Tanglefoot Company, Grand Rapids, MI) around the edge of one of the leaf halves. We replicated the procedure with two additional leaves. We then transferred 10 mated female *T. kanzawai* onto two leaves that were surrounded by tanglefoot, and onto three leaves that were not surrounded by tanglefoot. We allowed them to oviposit for one day, and then removed them. We kept the discs at 25°C: 16L:8D. After 6 days, when the female offspring of *T. kanzawai* had developed to the deutochrysalis stage, we performed three odor treatments on

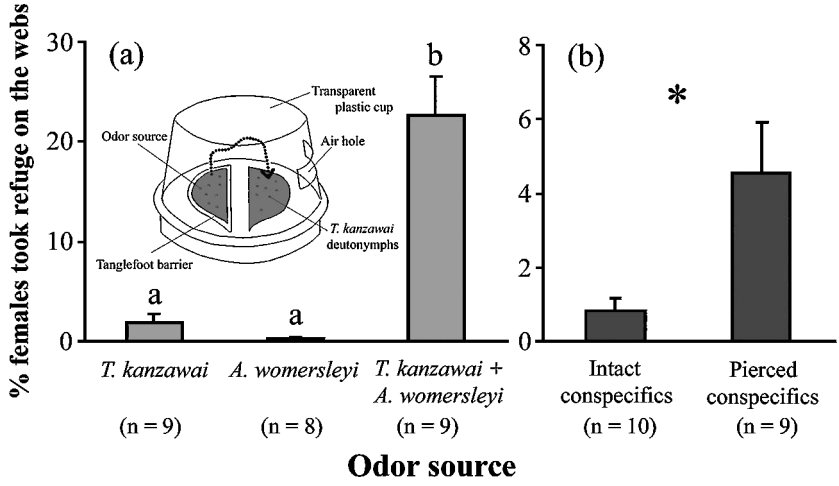

FIG. 1. Response of *T. kanzawai* deutonymphs to the odor source on a bean leaf. There was a significant difference between treatments followed by different letters ($P < 0.001$, Scheffé's test on arc-sine transformed data). (b) Response of *T. kanzawai* deutonymphs to an odor source on a paraffin sheet. $^{*}P < 0.05$ (*t*-test on arc-sine transformed data). The error bars are the means + SE.

the leaves surrounded by tanglefoot. We introduced ten adult female *A. womersleyi* each onto one leaf with *T. kanzawai* offspring ('*T. kanzawai* and *A. womersleyi*') and onto the leaf without *T. kanzawai* ('*A womersleyi*'). The remaining leaf with *T. kanzawai* offspring was used as a control ('*T. kanzawai*'). Then, we covered each leaf disc with a 200-ml transparent plastic cup with an air hole (10 × 30 mm; see Figure 1a). After two days, we recorded whether the *T. kanzawai* females on the leaves without tanglefoot entered the teleiochrysalis stage on the leaf or on their webs.

Response of T. kanzawai Deutonymphs to the Odor of Injured Conspecifics. To examine whether *T. kanzawai* responded to the odor of injured conspecifics, we cut a bean leaf in half and pressed two halves onto separate discs. We transferred 10 mated female *T. kanzawai* onto each leaf, and prepared deutonymphs on the leaves using the procedure described above. To simulate predation on *T. kanzawai* without damaging the leaf, we placed a 20 × 30-mm paraffin-sheet (Parafilm "M," American National Can) 10 mm from each leaf. We then introduced 50 deutonymph females onto each piece of Parafilm. We pierced all the females on one sheet with a fine needle (pierced conspecifics), while the others remained as a control (intact conspecifics). We covered each leaf disc with a transparent cup, as described above. After two days, we recorded whether *T. kanzawai* females on the leaves entered the teleiochrysalis stage on the leaf or on their webs.

RESULTS AND DISCUSSION

The proportion of *T. kanzawai* deutonymphs that entered the teleiochrysalis stage on their webs was significantly higher when they were exposed to the odors of both *T. kanzawai* and *A. womersleyi* ($P < 0.001$, Scheffé's test on arc-sine transformed data; Figure 1a). This suggests that *T. kanzawai* responds to predation. The observation that the proportion of *T. kanzawai* entering the teleiochrysalis stage on the webs was significantly higher when they had been exposed to the odor of pierced conspecifics than when they had been exposed to intact conspecifics further supports this ($P < 0.05$, *t*-test on arc-sine transformed data; Figure 1b). Since no live individuals were used in the latter experiment, an alarm pheromone is not likely the cause. The results suggest that *T. kanzawai* assesses predation risk, at least in part, by using the odor of injured conspecifics, while the different proportions of females on the webs in the two sets observations (Figure 1a and 1b) imply that infochemicals produced by conspecifics (Janssen et al., 1997; Pallini et al., 1999) may also be involved in predation risk assessment by *T. kanzawai*.

To evaluate the risk of predation by using infochemicals, a prey should rely on infochemicals that are readily detectable from a distance, rather than on those that might be more accurate but less detectable, which might be a fatal option for the prey. *T. kanzawai* did not respond to a direct cue from *A. womersleyi*, possibly because the predator has undergone natural selection to conceal itself from its prey. However, the predator cannot so easily conceal the odor of injured prey, which is an inevitable consequence of predation. Therefore, the prey could use such an odor as a reliable cue to assess predation risk.

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AGE AND SEASON AFFECT CHEMICAL DISCRIMINATION OF *Liolaemus bellii* OWN SPACE

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Abstract—We explored chemical discrimination of own vs. novel space by different age classes (neonates, juveniles, and adults) of the lizard *Liolaemus bellii*, during pre- and post-hibernation seasons. We recorded the number of tongue flicks (TF) lizards produced during 10 min in their own or a novel enclosure. Age class and season affected chemical discrimination. Only adults and neonates discriminated their own space, albeit using different strategies: while adults made fewer TF in their own enclosure, neonates made more TF in their own enclosure. This difference was interpreted in terms of different requirements for discrimination of individuals during their lives. Increased chemical exploration by juveniles and adults at the onset of the post-hibernation season was associated with food-searching and reproductive behaviors.

Key Words—Semiochemicals, *Liolaemus*, lizards, tongue-flicks, chemical discrimination, age, season.

INTRODUCTION

The vomeronasal organ is one of the main chemoreceptors of squamata reptiles, allowing the reception of semiochemicals from conspecifics and heterospecifics (i.e., prey, predators, congeners). Thus, chemical discernment ultimately determines vital aspects of squamate behavior. Animals collect molecules from the environment mainly using their tongue, and discrimination of semiochemicals may be inferred from differences in the number of tongue-flicks (chemical exploratory behavior), triggered by different signals. Most studies of chemical discrimination

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have been performed with adults (Cooper, 1994), although chemoreception is functional from the first stages of development (e.g., Font and Desfilis, 2002). The number of comparative studies analyzing whether discrimination of different semiochemicals changes through the life or seasons is much lower, even though animals experience changes in ecological, physiological, and morphological features during their lives, as well as seasonally. We investigated in *Liolaemus bellii* (Tropiduridae), a viviparous lizard species inhabiting rocky mountainous areas of central Chile (Donoso-Barros, 1966), if chemical discrimination of their own space differs between age classes and seasons. This species is active from approximately October to April, and hibernates the rest of the year (Donoso-Barros, 1966). Adults show seasonal changes of home range overlap (Fox and Shipman, 2003), and of conspecific and self-chemical discrimination (Labra et al., 2001).

METHODS AND MATERIALS

Lizards were collected in Farellones (east of Santiago, Chile), during February and March 2000 (pre-hibernation) and in October 2000 (post-hibernation). Sample size is indicated in Figure 1A. In the laboratory, lizards were kept in a vivarium exposed to sunlight, and maintained at thermal and photoperiod conditions approaching values recorded in the different seasons (pre-hibernation: 11–32°C and 10:14 L:D; post-hibernation: 13–37°C and 13:11 L:D). They were assigned to different age classes based on the snout-vent length (SVL): neonates (3 to 4.8 cm), juveniles (4.4 to 6.7 cm), and adults (6 to 8.8 cm). Coloration, and thickening of the tail base (associated with the development of genitalia) were used as complementary criteria to establish age classes (Donoso-Barros, 1966).

Lizards were housed individually in sand-filled plastic enclosures ($37 \times 30 \times 15$ cm), covered with plastic mesh. Enclosures contained a bowl for water and a rock for shelter and basking. Water was supplied *ad libitum* and food (mealworms) was supplied every other day (dusted with vitamins once per week). Animals remained in their enclosures for one week without disturbance. Experiments were performed in a room without natural light, outside the vivarium. A lizard was removed from its enclosure and maintained in a cloth bag for 30 min; thereafter, the bag was opened allowing the animal to move freely into an experimental enclosure, which was selected randomly: (1) Own: the enclosure of the test animal; (2) Novel: an unused and clean enclosure, containing only sand. During trials, the stone and the bowl of the own enclosure were removed. Novel enclosures were cleaned, and the sand was replaced before each new trial. Lizards were tested only once per day, with two resting days between testing days. Behavior was video-recorded for ten min; tape playback allowed the determination of the number of tongue-flicks (TF), i.e., when the lizard protrudes and rapidly retracts the tongue, regardless of whether it touches the substrate or is waved in the air. *L. bellii* rarely performs visual displays other than tongue-flicking (Fox and Shipman, 2003); hence, no other

behaviors were studied. In pilot experiments, neonates showed no differences in TF between novel, conspecific and own enclosures (one-way ANOVA $F_{(2,12)} = 0.468$; $P = 0.64$), while adults had similar TF in conspecific and novel enclosures (Labra et al., 2001). Hence, conspecific enclosures were not included in the experimental design. Once all experiments were performed, animals were released in the area where they were collected.

The effect of treatment, age class, and season on TF was assessed with a three-way ANOVA with repeated measures for treatments, followed by *a posteriori* Fisher LSD tests. Partial correlation was used to determine the relationship between SVL (a rough estimator of age) and the difference between TF performed in the novel and own enclosures, thus correcting for inter-individual behavioral variability.

RESULTS AND DISCUSSION

Liolaemus bellii chemically discriminated its own vs. a novel space. However, age class and season affected discrimination in an interrelated way (Table 1 and Figure 1A): while juveniles made no distinction between enclosures in either season, adults made fewer TF in their own than in a novel enclosure during pre ($P = 0.005$) and post-hibernation ($P < 0.001$), and neonates made more TF in their own than in a novel enclosure only during post-hibernation ($P = 0.006$). This suggests that own semiochemicals produce different effects on neonates and adults, as seen also from the relationship between the differences in number of TF between enclosures and SVL, during post-hibernation ($r = 0.61$, $P < 0.001$) (Figure 1B). The correlation remained significant after removing the two extreme points. A similar trend, albeit marginally non-significant, was observed during pre-hibernation ($r = 0.26$, $P = 0.057$).

How and why there is a change in the way in which age classes achieve discrimination, is unclear. Possibly, age classes have different requirements to

TABLE 1. ANOVA RESULTS OF THE EFFECTS OF SEASON (PRE-HIBERNATION VS. POST-HIBERNATION), AGE CLASS (NEONATES, JUVENILES OR ADULTS), TREATMENT (OWN VS. NOVEL ENCLOSURE), AND THEIR INTERACTIONS, ON THE NUMBER OF TONGUE FLICKS

Source of variation	*F*	*df*	*P*
Season	39.98	1,74	<0.001
Treatment	10.01	1,74	0.002
Age class	4.87	2,74	0.010
Season × Age class	4.24	2,74	0.018
Season × Treatment	0.29	1,74	0.591
Age class × Treatment	14.14	2,74	<0.001
Season × Age class × Treatment	7.38	2,74	0.001

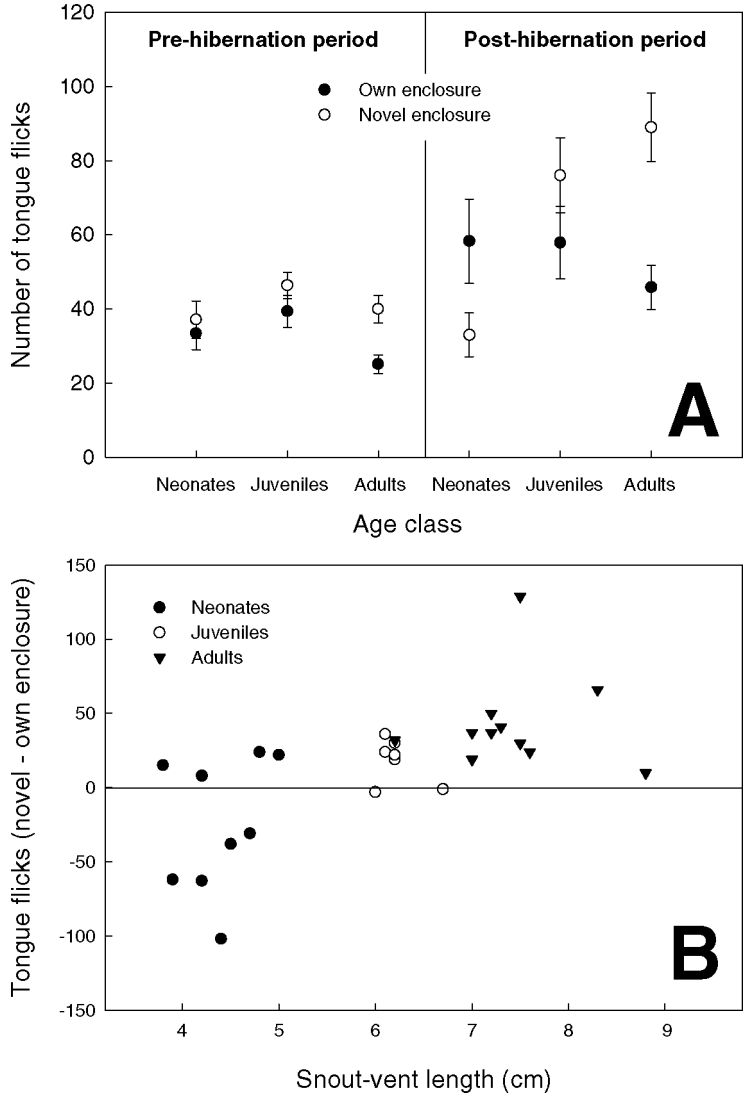

FIG. 1. A. Mean number of tongue flicks (±SE) performed by three age classes of *Liolaemus bellii*, during pre and post-hibernation periods, in own and novel enclosures. Sample sizes for pre and post-hibernation, respectively: adults (13, 9), juveniles (13, 7) and neonates (27, 11). B. Relationship between snout-vent length of *Liolaemus bellii* and the difference between the numbers of tongue flicks performed in novel minus those made in own enclosures during post-hibernation. The horizontal line indicates equal number of tongue flicks in both enclosures.

discriminate their own space. If *L. bellii* requires a familiarization period with its own semiochemicals in order to discriminate other conspecifics in the future ("self-referent matching," Wyatt, 2003), neonates would be the appropriate age for such familiarization, and, thus, their own semiochemicals would trigger higher exploration. Additionally, neonates are usually observed in association with an adult female (probably the mother), so they would not have strong requirements to explore novel semiochemicals. In fact, neonates showed fewer TF in the novel enclosure than juveniles and adults ($P < 0.001$ for both comparisons). Juveniles are frequently observed moving in the field, and being chased by adults when entering their defended areas. Hence, they probably still lack their own space and constitute the age class that disperses. Active exploration of all semiochemicals is, thus, expected from juveniles. This is supported by the higher TF shown by juveniles compared with neonates ($P = 0.01$) or adults ($P = 0.04$). Juveniles may have a low requirement, if any at all, to discriminate own from novel space. Finally, adults have preferred sites for activities; they are frequently observed using the same rocks (unpub. obs.). This necessarily requires discriminating their own semiochemicals. However, since adults are likely to be already familiarized with their own semiochemicals, exploration of new ones seems to acquire priority.

The season effect was manifested by a higher mean chemical exploration of juveniles and adults during post-hibernation ($P < 0.001$ for both comparisons). In addition, from pre to post-hibernation there was a significant increase in TF performed by neonates and adults in their own enclosure ($P = 0.009$ and 0.008, respectively), and by juveniles and adults in the novel enclosure ($P = 0.004$ and $P < 0.001$, respectively). Following hibernation, many species from temperate zones, such as *L. bellii*, search food, establish territories, and/or start their reproductive period (Gregory, 1982). Additionally, reproduction of *L. bellii* appears to occur during spring and early summer, i.e., during post-hibernation (Leyton and Valencia, 1992). All these behaviors require increased chemical exploration. In the case of neonates, familiarization with their semiochemicals would explain the increased exploration of their own space.

Results suggest that animals experience different requirements during their lives that affect the way they discriminate their own semiochemicals from those in the environment.

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KANGAROOS AVOID EATING SEEDLINGS WITH OR NEAR OTHERS WITH VOLATILE ESSENTIAL OILS

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Abstract—Preliminary studies indicate that western grey kangaroos browse seedlings of non-Myrtaceae species rather than Myrtaceae. Seven morphologically-matched species pairs of Myrtaceae/non-Myrtaceae placed at three field sites showed that kangaroos avoided the essential-oil-containing Myrtaceae, but readily consumed the matched essential-oil-lacking non-Myrtaceae. The one exception (Pittosporaceae) had limited herbivory and was later found to possess two essential oils in its leaves. Gas chromatography and mass-spectra showed the seven Myrtaceae plants contain between 2 and 9 essential oils in their leaves, particularly the highly volatile monoterpene, 2,5-dimethyl-3-methylene-1,5-heptadine. Three of the above species pairs were used to gauge their effectiveness as nurse plants for a highly palatable legume. Plants placed beside Myrtaceae nurse plants were less browsed than those placed beside non-Myrtaceae nurse plants. We conclude that western grey kangaroos use olfactory cues to avoid foliage containing potentially toxic essential oils, and that this also has implications for seedling recruitment patterns in regenerating communities.

Key Words—Essential oils, seedling herbivory, nurse plants, Myrtaceae, western grey kangaroo.

INTRODUCTION

Western grey kangaroos (*Macropus fuliginosus*) are the major indigenous herbivores in southwestern Australia (Short et al., 1983). They are members of the Macropodidae (kangaroos and wallabies) that evolved in the Miocene and Pliocene with the spread of grasslands (Hume, 1978; Short et al., 1983). They inhabit the

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dry-sclerophyll forests and open woodlands that are dominated by the Myrtaceae, a family with over 3500 species in Australia. Myrtaceae produce essential oils that are contained in glands located in leaves and young stems (Baker and Smith, 1920; Hay and Waterman, 1993). Essential oils are monoterpenoid or sesquiterpenoid in nature, and are strongly volatile and aromatic (Penfold and Willis, 1961; Seigler, 1979; Specht and Specht, 1999). Essential oils are known to limit the food choices of insects (Cork and Foley, 1991; Ganzhorn, 1992) by blocking the stimulatory effects of sugars on chemosensory receptor cells of the mouth parts (Janzen and de Groot, 1991) and inducing premature molting (Hay and Waterman, 1993). Mammalian herbivory is influenced positively by the nutrient value of the foliage and negatively by the presence of secondary metabolites (Palo and Robbins, 1991; Provenza et al., 1998). Therefore, the essential-oil-containing status of species might be relevant to the food choice of the western grey kangaroo.

All Macropods are foregut fermenters, a feature common in regions where forage of only poor nutritive value is available (Hume, 1978). The foregut is believed to have evolved for obtaining the energy from cellulose (Main, 1981), and maximizing the use of nitrogen in low quality diets (Kinnear et al., 1979). It is thought that herbivores are sensitive to essential oils because the antibacterial activity may have an adverse effect on cellulose-digesting bacteria in the rumen or foregut (Scholl et al., 1977). Some components of essential oils, such as linalool and α-terpineol, are known to reduce the digestive efficiency in ruminants by their antibacterial activity (Oh et al., 1967). Hume (1999) suggests that essential oils are toxic only to foregut bacteria, as hindgut fermenters like the koala (*Phascolarctos cinereus*) are unaffected by the antibacterial effects of essential oils. Lawler et al. (1998) noted that the koala could eat a larger amount of *Eucalyptus ovata* and *E. viminalis* than the common ringtail possum (*Pseudocheirus peregrinus*). They showed that it was the terpene content of the eucalypts as well as the phenolic compound, diformylphloroglucinol, that limited herbivory by the possum.

Western grey kangaroos tend to avoid eating mature plants of species with oil glands (Wann and Bell, 1997). It is thought that kangaroos can detect species with essential oils by smell alone, as Macropods are known to have a prominent olfactory bulb and vomero-nasal organ. Hunt et al. (1999) showed how the red kangaroo (*Macropus rufus*) learned to use an odor cue to avoid solutions of the bitter quinine monohydrochloride dihydrate. Other studies have shown that mammals can learn to associate distinctive flavors with the presence of plant secondary metabolites (Provenza et al., 1990; Kyriazakis et al., 1991) through a feedback from nausea or gastrointestinal illness (Provenza et al., 1994).

Plants may indirectly influence one another via their effects on herbivores, parasites, pathogens, and decomposers. This type of associational defense can provide a mechanism for maintaining species richness within a community. One competitor may have a strong positive effect on another if the first significantly reduces the probability that the second will be excluded from the community

through high rates of predation (Hay, 1986). An analysis of the evolutionary consequences of the indirect effects of plant unpalatability demonstrate that a plant that is susceptible to herbivory gains protection from herbivory when associated with an unpalatable plant. This type of defense can lead to associational refuges (Tuomi and Augner, 1993), and the term "nurse plant" can be used to describe the plant that protects a neighboring plant from negative environmental effects on plant growth, such as herbivory. The effectiveness of a particular nurse plant is influenced by the balance between competitive and facilitative processes under their crowns (Tewksbury and Lloyd, 2001), and the age and size of the protected plant, as younger plants are more vulnerable to harsh environmental conditions (Callaway and Walker, 1997).

There are numerous hypotheses to explain the protection of palatable plants provided by association with unpalatable plants. Tuomi (1992) suggests that these associational refuges reduce local herbivore density and consumption rates of individual herbivores within a refuge. Atsatt and O'Dowd (1976) note that some plants might act as repellent plants, directly or indirectly causing the herbivore to fail to locate or reject its normal target, while others might serve as attractant decoy plants, causing the herbivore to feed on alternative species. Since Myrtaceae often overlap or overtop the crowns of non-Myrtaceae species in plant communities, by acting as nurse plants, they might deter herbivory on these as well.

Preliminary observations at Whiteman Park and in the Darling Range (near Perth, Western Australia) showed that adult plants of Myrtaceae species were rarely eaten by western grey kangaroos (Table 1) and non-Myrtaceae species located beside Myrtaceae were often protected from herbivory. There have been no studies on the relative susceptibility of Myrtaceae and non-Myrtaceae seedlings. This is important as this region is prone to fire after which there is simultaneous germination and seedling establishment of all species, and selective herbivory could affect the way the community regenerates (Bell and Koch, 1980; Christensen, 1980;

TABLE 1. NUMBER OF SPECIES BROWSED BY WESTERN GREY KANGAROOS OVER 12 MONTHS

Family	Site location	No. of species examined	No. of species browsed	Reference
Myrtaceae/	Woodland[a]	9/1	2/0	C. Rafferty (unpublished)
Rutaceae	Woodland[a]	6/1	0/0	Wann and Bell (1997)
(aromatic)	Forest[b]	2/0	0/0	M. Fairbanks (unpublished)
Other	Woodland[a]	36	12	C. Rafferty (unpublished)
(non-aromatic)	Woodland[a]	66	25	Wann and Bell (1997)
	Forest[b]	35	29	M. Fairbanks (unpublished)

[a] *Banksia* woodland (Whiteman Park).
[b] *Eucalyptus marginata* (jarrah) forest (Dwellingup and Boddington).

Hanley and Lamont, 2001). Selective herbivory at the seedling stage could have a profound effect on the composition and structure of the post-fire community. We proposed that: 1) if essential oils deter kangaroo herbivory, they will be effective at the seedling stage; 2) western grey kangaroos are deterred from feeding on essential-oil-containing species by smell and not by taste; 3) Myrtaceae species are effective nurse plants.

METHODS AND MATERIALS

Study Sites. Our studies were conducted at Whiteman Park, a 2600 hectare area of remnant banksia and eucalypt woodland 20 km northeast of the Perth CBD. Three study sites in the northern part of Whiteman Park were used to erect 25 × 25-m kangaroo-proof exclosures. Site 1 (31° 48′ 9.7″ S, 115° 56′ 17.1″ E) was located in an area of low woodland of *Banksia attenuata*, *B. menziesii*, and *B. ilicifolia* on deep sands. Nomenclature throughout follows Paczkowska and Chapman (2000). Site 2 (31° 48′ 3.0″ S, 115° 56′ 6.5″ E) was in open woodland of *Corymbia calophylla* on moister, humic soils in a flatter, lower area than site 1. Site 3 (31° 48′ 47.2″ S, 115° 55′ 6.5″ E) was in an area of low woodland of *Banksia attenuata*, *B. menziesii*, and *Eucalyptus todtiana* on dry sands. The three sites were burnt in a wildfire on 14 February 2001. The population of western grey kangaroos at Whiteman Park in 2000 was estimated to be near 500 (Bamford and Bamford, 2000). Mattiske Consulting (2000) reported to the Whiteman Park Authority in 2000 that the western grey kangaroos were reducing the cover of certain native species, especially seedlings establishing after fire.

Experiment 1—Pilot Study. The pilot study looked at the susceptibility to herbivory of two representative plant species from each of five families. *Acacia saligna* and *A. iteaphylla* (Mimosaceae) were chosen as the leaves appeared palatable, although *A. iteaphylla* is known to contain saponin, a herbivore deterrent (Gardner and Bennetts, 1956). *Banksia grandis* and *B. menziesii* (Proteaceae) are sclerophyllous and may contain cyanogenic glycosides (Gardner and Bennetts, 1956), which can depress intake of food high in protein (Villabla et al., 2002). *Hypocalymma robustum* and *H. angustifolium* (Myrtaceae) appear palatable but produce essential oils. *Hardenbergia comptoniana* and *Kennedia prostrata* (Fabaceae) and *Lechenaultia biloba* and *L. formosa* (Goodeniaceae) appear palatable and are not known to produce antiherbivore compounds. Five plants of each species, in five groups of 10 species, were exposed to kangaroos at Whiteman, and another five were located in an exclosure 5 m away in randomaly allocated positions by using a random number table (Ott, 1993). The experiment was stopped after 6 days when the majority of plants outside the exclosure appeared to have been eaten. We examined all plants for signs of herbivory, and the plots for evidence of kangaroo visitation (tracks, scats).

TABLE 2. SPECIES USED IN EXPERIMENT 2— EACH MYRTACEAE SPECIES WAS MATCHED MORPHOLOGICALLY WITH A NON-MYRTACEAE SPECIES

Morphology type	Myrtaceae	Non-Myrtaceae (family)
1	*Agonis linearifolia*	*Sollya heterophylla* (Pittosporaceae)
2	*Hypocalymma robustum*	*Hovea pungens* (Fabaceae)
3	*Regelia inops*	*Lechenaultia floribunda* (Goodeniaceae)
4	*Astartea fascicularis*	*Gompholobium tomentosum* (Fabaceae)
5	*Calothamnus quadrifidus*	*Nuytsia floribunda* (Loranthaceae)
6	*Eremaea pauciflora*	*Euchilopsis linearis* (Fabaceae)
7	*Corymbia calophylla*	*Hardenbergia comptoniana* (Fabaceae)

Experiment 2—Matched Morphology Study. All species selected for this experiment were native to Whiteman Park. Seven Myrtaceae species were selected on the basis of availability, and non-Myrtaceae were selected on the basis of similar appearance to a chosen Myrtaceae species (Table 2). Seedlings were purchased from APACE nursery in Fremantle, Muchea Tree Farm in Muchea, and Men of the Trees in Hazelmere, Western Australia and grown in 50% washed river sand and 50% pinebark/jarrah sawdust, fertilized with a slow release low P fertilizer, in full sun for 6 months. The seedlings' appearance was typical of seedlings recruiting naturally after fire at Whiteman Park. Morphological data were collected on 10 seedlings of each species in order to confirm the visual similarities that were the basis of the pairings. Del image analysis system (DIAS II, Delta-T Devices, Cambridge, UK) was used for leaf area, length, and width measurements by ruler, and digital callipers (Mitutoyo, Japan) for leaf thickness. The 10 leaves were then oven-dried at 60°C, and their dry weights taken. Leaf properties measured included the angle of leaves to the node, presence of hairs, and number of leaves along a 50-mm section of mature stem. Shoot dimensions were recorded: these consisted of height and width in two dimensions, the widest and perpendicular to the widest. The data were converted to volume using the formula $\pi/6$ [height $\times$ width 1 (longest) $\times$ width 2 (at right angles to width 1)], assuming the shoot is ellipsoid. An average for each attribute per species was calculated. Data were normalized as necessary. In order to compare within/between pairs, canonical variates analysis with vectors was performed, on the quantitative attributes, using species pairs as groups (Podani, 1995).

The experiment was conducted at the three sites with 10 replicates per species inside and 10 outside the exclosures at each site, except for pairs 3 and 5 with eight replicates (Table 2). The pairs of seedlings were randomly allocated to seven positions in each of 10 rows by using a random number table. Seedlings per pair were planted 10 cm apart with 2 m between each pair. Seedlings outside the exclosures were placed no closer than 5 m to the fence to avoid any possible interfering effects the fence may have had on kangaroo behavior. Seedlings were

planted in their containers, level with the ground. The experiment was begun on 21 May 2001, and observations on shoot damage, kangaroo tracks and scats were made daily. The experiment concluded at each site when about 50% of seedlings outside the exclosures had signs of browsing. The experiment concluded at site 3 on 23 May, site 2 on 25 May, and site 1 on 27 May. Since there was no difference in patterns among sites by χ^2-test, the data were combined. The numbers of seedlings with/lacking signs of kangaroo herbivory were compared within species pairs by contingency χ^2-test (Zar, 1999).

Essential Oil Content. Seedlings used in experiment 2 were analyzed for the presence of essential oils. One (large) or two (small) plants of the 14 species was divided into leaves and stem(s), ground with mortar and pestle, and 200 mg of each were extracted in 5 ml of analytical grade dichloromethane. The mixtures were allowed to stand for 2 hr during which they were placed in a Branson ultrasonic bath (Connecticut, USA) for two periods of 5 min, and then filtered through a short plug of celite. The process was repeated on nine species to check for consistency of results. Heptadecane was chosen as the internal standard from a number of non-terpenes of similar molecular mass because its retention time seemed to interfere least with compounds of interest in test extracts of *Calothamnus quadrifidus* and *Regelia inops.* A solution of heptadecane in ethyl acetate at 0.2 mg/ml was made up based on these trials. Aliquots of 100 μl of the heptadecane solution were placed in vials and then made up to 2 ml with the plant extracts.

Gas chromatography was performed with a 5890 series II gas chromatograph (Hewlett Packard, Pennsylvania, USA) with a 15 m $\times$ 0.25 mm id fused silica column with a 0.25 μm film (HP-5MS). The oven temperature was programmed from 40 to 150°C at 10°C per min and 150–300°C at 20°C per min with an initial hold time of 4 min. The carrier gas was helium. Mass spectra were recorded using a 5972 series MSD (Hewlett Packard, Pennsylvania, USA) operating in scan mode. Wiley and NBS library spectra were used for compound identification. The mass spectra were examined for the presence of essential-oil-related compounds. Mass spectra of peaks with an abundance of over 100,000 units, identified as a monoterpenoid or sesquiterpenoid and with a library match above 75%, were recorded. Peak heights relative to the heptadecane peak were calculated, and their relative concentrations were ranked. The numbers of species of Myrtaceae and non-Myrtaceae containing essential oils were compared using χ^2- tests. Statistical analyses were performed with Minitab 13.1 (Minitab Statistical Software, Pennsylvania). The numbers of volatile compounds in the leaves and stems of Myrtaceae and non-Myrtaceae were compared by one-tailed Mann–Whitney tests.

Experiment 3—Nurse Plant Study. This experiment was conducted at sites 1 and 2. Nurse plants assessed were three species of Myrtaceae and three matched species of non-Myrtaceae as used in experiment 2. The Myrtaceae/non-Myrtaceae pairs consisted of *Hypocalymma robustum/Hovea pungens, Astartea fascicularis/ Gompholobium tomentosum*, and *Corymbia calophylla/Hardenbergia*

comptoniana. Each nurse plant was planted 1–2 cm from the base of a single *H. comptoniana* target. This target species was chosen as it was heavily browsed in the previous two experiments. The effectiveness of each nurse plant was assessed by measuring the amount of the target plant remaining.

There were four treatment groups. These were the Myrtaceae and non-Myrtaceae nurse–target plant treatments and two control groups of *H. comptoniana*, one placed inside and the other outside the exclosure. Each of the three morphology-type pairs had its own controls. The two nurse-plant treatments and one control treatment (nine pots) were arranged randomly in a 3×3 m square, 80–100 cm apart. There were 10 replicates of these groups at each site. The pots were buried such that they were flush with the soil surface. The experiment was begun on 5 August 2001 and ended on 11 August 2001 when about 50% of plants showed signs of herbivory at each site. The plants were then harvested, placed in sealed plastic bags, kept cold in a container with ice during transport, and fresh weights taken. The plots were examined for evidence of kangaroo visitations.

A nested ANOVA was performed on normalized weights for morphology types, sites, and treatments within morphology types. The term "morphology type" in this case is used to refer to the matched species plus outside control. Morphology type and site were not significant ($P = 0.869$, 0.198, respectively), so they were removed as variables, and the ANOVA was repeated. The control inside group was then included as a treatment to determine if herbivory had occurred outside the exclosure, and a further ANOVA was performed. Sequential Bonferroni multiple-range tests (Rice, 1989) were performed on the treatment effects, as the ANOVA revealed no interaction effects. A final ANOVA was performed on the data of only the Myrtaceae and non-Myrtaceae nurse plant treatments to determine if the ratio of nurse plant volume to target plant volume was a significant covariate. All statistical analyses were performed by using Minitab 13.1.

RESULTS

Experiment 1. All members of the Mimosaceae, Fabaceae, and Goodeniaceae were browsed, but the Myrtaceae and Proteaceae species were not. No plants of any species were browsed in the fenced areas. Clear kangaroo tracks were made through the study plots, kangaroo scats were present, and some pots were dug out and moved.

Experiment 2. Figure 1 shows that the species pairs were much more closely matched morphologically within the pairs than they were between. The only exception was one species in pairs 3 and 4 that were similar, although pair 3 had glabrous leaves, while pair 4 was hairy (not included in the ordination). Pairs 3 and 4 had a large number of small leaves per unit stem length, 1 and 6 were intermediate with thin leaves, 2 and 5 were intermediate with thick leaves, and 7 had a few large leaves at a wide angle to the stem.

Fresh tracks and scats of adult kangaroos were observed among the plants at all sites and on all visits. All (seven) non-Myrtaceae species showed signs of herbivory (mean per species 66% of plants), while five Myrtaceae showed no signs of herbivory (mean per species 3% of plants) (Table 3). Exceptions were the non-Myrtaceae, *Sollya heterophylla*, that had a few signs of herbivory (17% of plants), and in several cases broken pieces of seedlings remained on the ground nearby, and the Myrtaceae, *Eremaea pauciflora*, with 17% of plants showing herbivory. Statistically, all non-Myrtaceae within each pair were significantly more browsed than Myrtaceae within each pair (Table 3).

Essential Oil Content. Replicate chromatograms gave almost identical results. The heptadecane had a retention time of 15.7 min. The number of peaks eluting before this with an abundance > 100,000 units are given in Table 4. Leaves of all Myrtaceae contained essential oils, while *Sollya heterophylla* was the only non-Myrtaceae to contain essential oils ($P = 0.007$). The number of volatile compounds in the leaves of Myrtaceae (2–9) was significantly greater than in the non-Myrtaceae (0–2) ($P = 0.007$, χ^2 test), while the number in the stems of Myrtaceae (0–4) was not significantly different from non-Myrtaceae (0–4) ($P = 0.233$, Mann-Whitney test). Only two compounds were common among the essential-oil-containing species. These eluted at 13.03–13.04 and 13.23–13.24. The former was identified from the libraries as Germacrene D, α–cubebene epi-bicyclosesquiphellandrene or octohydro-7-methyl-3-methylene-4-(1-methylethyl)-1H-cyclopenta-[1,3]-cyclopropa-[1,2]-benzene. The latter was identified as 2,5-dimethyl-3-methylene-1,5-heptadiene or octahydro-tetramethyl-1H-cycloprop[e]azulene. The quality of the match for 2,5-dimethyl-3-methylene-1,5-heptadiene was usually above 90%.

TABLE 3. PERCENTAGE OF MYRTACEAE AND MORPHOLOGICALLY-MATCHED NON-MYRTACEAE SEEDLINGS SHOWING SIGNS OF HERBIVORY AFTER 2–6 DAYS AT WHITEMAN PARK, WESTERN AUSTRALIA (EXPERIMENT 2)

Morphology type (as in Table 2)	Herbivory (%)		P^a
	Myrtaceae	Non-Myrtaceae	
1	0.0	16.7	$P = 0.020$
2	0.0	93.3	$P < 0.001$
3	4.2	100.0	$P < 0.001$
4	0.0	86.7	$P < 0.001$
5	0.0	79.2	$P < 0.001$
6	16.7	83.3	$P < 0.001$
7	0.0	93.3	$P < 0.001$

Note. $N = 30$ seedlings except for morphology types 3 and 5 where $N = 24$.
[a] Significant difference, contingency table χ^2 tests.

TABLE 4. SUMMARY OF THE GC-MS RESULTS FOR THE SEVEN MYRTACEAE AND ONE NON-MYRTACEAE SPECIES (EXPERIMENT 2)

Species	No. of oils[a]		Conc. rank[b]	Origin	Compound	Chemical formula	Quality of match (%)
	leaf	stem					
Myrtaceae							
1. *Agonis linearifolia*	9	4	1	leaf	3,7-dimethyl-1,6-octadien-3-ol[c]	$C_{10}H_{18}O$	96
			2	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene[c]	$C_{10}H_{16}$	86
			3	stem	2,5-dimethyl-3-methylene-1,5-heptadiene ϕ	$C_{10}H_{16}$	86
2. *Hypocalymma robustum*	3	1	1	leaf	1-methyl-4-(1-methylethyl)-1,4-cylohexadiene[c]	$C_{10}H_{16}$	90
			2	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene[c]	$C_{10}H_{16}$	92
			3	stem	2,5-dimethyl-3-methylene-1,5-heptadiene[c]	$C_{10}H_{16}$	91
3. *Regelia inops*	4	0	1	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene[c]	$C_{10}H_{16}$	92
			2	leaf	alpha-cubebene ϕ	$C_{15}H_{24}$	89
4. *Astartea fascicularis*	5	2	1	leaf	6,6-dimethyl-bicyclo[3.1.3]hept-2-ene-2-carboxaldehyde[c]	$C_{10}H_{14}O$	93
			2	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene ϕ	$C_{10}H_{16}$	89
			3	leaf	epi-bicyclosesquiphellandrene ϕ	$C_{15}H_{24}$	78
5. *Calothamnus quadrifidus*	3	0	1	leaf	eucalyptol[c]	$C_{10}H_{18}O$	94
			2	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene[c]	$C_{10}H_{16}$	92
			3	leaf	epi-bicyclosesquiphellandrene ϕ	$C_{15}H_{24}$	78
6. *Eremaea pauciflora*	2	0	1	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene ϕ	$C_{10}H_{16}$	87
			2	leaf	epi-bicyclosesquiphellandrene ϕ	$C_{15}H_{24}$	89
7. *Corymbia calophylla*	4	0	1	leaf	globulol ϕ	$C_{15}H_{26}O$	95
			2	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene ϕ	$C_{10}H_{16}$	74
Non-Myrtaceae							
Sollya heterophylla	2	4	1	leaf	2,5-dimethyl-3-methylene-1,5-heptadiene[c]	$C_{10}H_{16}$	90
			2	stem	pentadeconal[c]	$C_{15}H_{30}O$	91
			3	stem	2,5-dimethyl-3-methylene-1,5-heptadiene[c]	$C_{10}H_{16}$	91

[a] Number of essential oils detected in the leaf or stem.
[b] Conc. rank—Identity of the three most concentrated terpenoids in the of the leaf or stem ranked 1 to 3 provided match $\geq$74%
[c] Indicates NBS library, ϕ indicates Wiley library.

TABLE 5. MEAN (± SE) REMAINING MASS (MG) OF *Hardenbergia comptoniana* LOCATED NEAR A NURSE PLANT AND EXPOSED TO KANGAROOS OR ISOLATED IN A FENCED AREA

	With Myrtaceae nurse plant	With non-Myrtaceae nurse plant	Unfenced *H. comptoniana* without nurse	Fenced *H. comptoniana* without nurse
Morphology type 7	477.8 (133.3)	267.3 (48.0)	216.5 (43.5)	579.2 (127.4)
Morphology type 4	281.3 (54.2)	188.4 (31.8)	318.9 (38.8)	481.5 (91.2)
Morphology type 2	296.4 (54.8)	225.5 (46.7)	401.5 (159.6)	567.0 (82.2)
Total mean ($N = 60$)[a]	351.8 (65.9)$^{b}_{b}$	227.1 (24.1)$_{c}$	312.3 (53.5)$_{bc}$	542.6 (30.7)$_{a}$

Note. Nurse plants were either a Myrtaceae or non-Myrtaceae plant, one of three morphology types from Experiment 2. See Table 2 for species list.

[a] Overall means for *H. comptoniana* from all four treatments.

[b] Subscripts denote significant difference ($P < 0.05$) between all four treatments with the sequential Bonferroni test. $N = 20$.

Experiment 3. All non-Myrtaceae plants were browsed, while no Myrtaceae were. Fresh tracks and scats of kangaroos were evident during each inspection. For shoot mass of the target species, *Hardenbergia comptoniana*, there were no significant differences between morphology types ($P = 0.869$) and sites ($P = 0.198$), and no significant interaction between them ($P = 0.266$). There were significant differences when treatment (Myrtaceae, non-Myrtaceae, and inside and outside enclosure controls) was nested with morphology type ($P = 0.007$). The Bonferroni simultaneous tests showed that all plants were smaller outside than inside the exclosures (Table 5). *H. comptoniana* beside a non-Myrtaceae was more severely browsed than when beside a Myrtaceae for the three individual cases and overall. The unfenced *H. comptoniana* without nurse plants were intermediate between the plants with Myrtaceae and non-Myrtaceae protection.

DISCUSSION

The pilot study showed that Myrtaceae seedlings were not browsed in contrast to seedlings from four other families. The fact that species in a fifth family (Proteaceae) were also not browsed indicates that Myrtaceae is not the only family in the Australian flora with attributes that deter herbivory. The role of physical and chemical (phenolic) deterrents in the Proteaceae has been perused elsewhere (Hanley and Lamont, 2002; C. Rafferty unpublished). These results agree with incidental observations (Table 1) that Myrtaceae and other aromatic families tend not to be eaten by western grey kangaroos.

Browsing on Myrtaceae. There was little or no browsing of Myrtaceae species in the three experiments. This is despite the fact that they represented seven genera

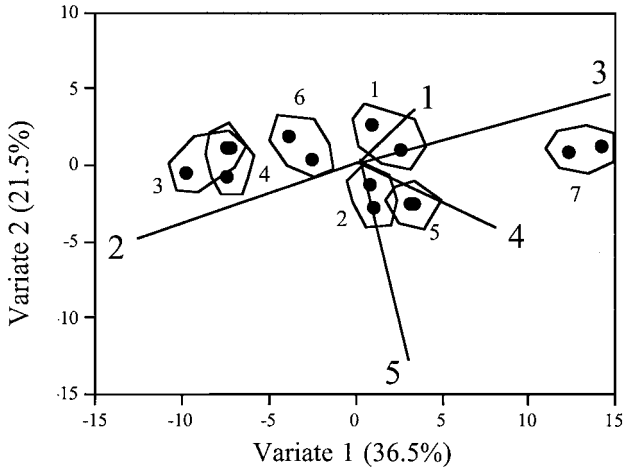

FIG. 1. Canonical variates analysis of the seven species pairs (morphological types) used in Experiment 2. Percentage of total variance accounted for by each variate is shown. Each species pair is enclosed and marked (Table 2). Vectors: 1 = shoot volume (log), 2 = number of leaves (log), 3 = leaf area (log), 4 = leaf angle of stem (log), 5 = leaf thickness.

and varied greatly in morphology (Figure 1). Most uneaten plants were not even tasted to gauge their palatability—despite morphological matching with highly palatable species placed just 10 cm away. Together with the GC-MS analyses and behavioral observations on kangaroos (C. Rafferty unpublished), we conclude that the volatile essential oils act as an effective odor cue in deterring these herbivores. This is an alternative chemical defense mechanism to the presence of alkaloids and tannins that are nonvolatile and require preliminary tasting to be detected (Hunt et al., 1999).

All Myrtaceae species studied contained volatile compounds. The presence of essential oils in these seedlings was enough to deter kangaroo herbivory. However, whether the type, concentration, or distribution of the essential oils in the plant influences avoidance is not known. *Eremaea pauciflora*, with only two oils detected, was the most eaten, while there was negligible herbivory in the other species, with 3–9 oils. The only compound universal in the leaves and of relatively high concentration was the monoterpenoid, 2,5-dimethyl-3-methylene-1,5-heptadiene (Table 4). As the lightest molecule, it may have been the most volatile. The oils were concentrated in the leaves rather than stems, providing a large surface area for volatilization. Oil volatility and leaf location may enhance their detection by kangaroos, obviating the need for preliminary tasting.

A number of the terpenes occurring in the Myrtaceae species studied, such as 1,8 cineole and α-terpineol, are known to be strongly antibacterial agents (Jedlickova et al., 1992). Assuming that it is the antibacterial effect of some

essential oil components that is deterring herbivory by the western grey kangaroo, then their effectiveness as feeding deterrents would be proportional to their potency, not only their concentration. Further complicating this is the fact that many essential oils are thought to act synergistically (Soderberg et al., 1996; Lis-Balchin and Deans, 1997; Hummelbrunner and Isman, 2001). The evidence of synergistic effects of terpenes on bacteria may indicate that the odors of these monoterpenes and sesquiterpenes also act synergistically in deterring herbivory. Kangaroos may in fact be sensing combinations of essential oils, some in low quantity. Associating the volatile oils with poor digestibility is probably a learned response passed between generations, bypassing the need to taste the foliage first.

Browsing on Non-Myrtaceae. Apart from morphology type 1, which was different due to the lesser extent of browsing on *Sollya heterophylla*, the other non-Myrtaceae morphology types were all heavily browsed. This is despite the fact that they varied greatly in morphology (Figure 1). Twenty-eight of the 30 *Hovea pungens* (Fabaceae) seedlings were browsed despite having spiny leaves. Most studies of Macropods have concluded that physical deterrents have no apparent impact on their dietary preferences (Shepherd et al., 1997; Wann and Bell, 1997; C. Rafferty unpublished). Shepherd et al. (1997) concluded that the western grey kangaroos are not deterred by physiological or morphological defense characteristics and noted that they consume species with sharp stem spines, such as *Acacia pulchella,* and leaf spines, such as *Hakea lissocarpha*.

That kangaroos did not significantly browse *Sollya heterophylla* was also of interest. *S. heterophylla* is a member of the Pittosporaceae, renowned for its high resin content (Willis, 1966). Resins belong to the same chemical group as essential oils. Essential oils commonly contain monoterpenes and sesquiterpenes, while resins may contain these, as well as diterpenes and triterpenes (Croteau et al., 2000). The volatile component of the resin would be considerably less than that of essential oils. Kangaroos may have had to taste the *S. heterophylla* in some cases in order to detect its terpene content. This could provide an explanation for the Myrtaceae having no signs of herbivory but *S. heterophylla* having broken unconsumed pieces on the ground near some of the plants. This, and the browsing of plants similar in morphology to the Myrtaceae species, suggests that terpenoid-avoiding behavior by western grey kangaroos is based primarily on odor, then taste, but not visual cues. Hunt et al. (1999) also showed that kangaroos learn to avoid chemical solutions of quinine monohydrochloride dihydrate by smell alone.

Myrtaceae as Nurse Plants. All the exposed *Hardenbergia comptoniana* plants experienced some degree of browsing. *H. comptoniana* that had a Myrtaceae nurse plant showed low levels of herbivory, while those with non-Myrtaceae nurse plants were browsed the heaviest. This pattern agrees with Frid and Turkington (2001) who observed that arctic ground squirrels (*Spermophilius parryii plesius*) were more likely to browse plants that had palatable neighbors than those that did not. The Myrtaceae nurse plants provided some protection for

H. comptoniana by deterring kangaroo herbivory, while the non-Myrtaceae nurse plants attracted greater herbivory through their own palatability. Differing nurse plant morphology had no significant impact on levels of herbivory. Physical characteristics of plants were not associated with deterring kangaroo herbivory. The presence of the spiny *Hovea pungens* actually increased browsing on its *H. comptoniana* neighbors.

In our study, the size of the nurse plant did not equate with the level of herbivory of the protected plant. However, the least eaten protected plant was located with the largest Myrtaceae used (type 7, Figure 1). The nurse seedlings used were small and narrow compared with the creeping target, *H. comptoniana*. Had the target plant been beneath or interspersed with the Myrtaceae species, the tendency for it to be less eaten than the controls may have been much stronger. Further experiments that use Myrtaceae of different size classes as treatments would be required in order to determine if nurse plant size is a significant factor in deterring kangaroo herbivory.

Our recent studies at Curtin University have continued to examine the potential of essential oils in deterring western grey kangaroo herbivory. Preliminary results showed that essential oils are quite toxic to the applied foliage, and that they quickly evaporate. However, by adding a wetting agent, the longevity of the essential oils increases. Tannins and essential oils are also to be added to pellets in food choice experiments, both at Whiteman Park and at Perth Zoo, thus determining any food choice problems that may occur by interfering secondary plant metabolites.

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SOUR-TASTE TOLERANCE IN FOUR SPECIES OF NONHUMAN PRIMATES

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Abstract—The taste of most fruits is characterized by a mixture of sensations termed sweet and sour by humans, and the food selection behavior of primates suggests that they may use the relative salience of sweetness and sourness to assess palatability of potential food items. Therefore, taste responses of six squirrel monkeys, five pigtail macaques, four olive baboons, and four spider monkeys to sweet–sour taste mixtures were assessed in two-bottle preference tests of brief duration (2 min). Monkeys were given the choice between a reference solution of 50 mM sucrose and mixtures containing 10, 30, or 50 mM citric acid plus 10, 20, 50, 100, 200, 400, 800, or 1000 mM sucrose. We found that the four species differed markedly in their acceptance of physiological concentrations of sour-tasting citric acid. Whereas olive baboons showed the highest degree of sour-taste tolerance and actually preferred most of the sweet–sour taste mixtures over sweet-tasting reference solutions, squirrel monkeys showed the lowest degree of sour-taste tolerance and rejected most of the sweet–sour taste mixtures even when they contained considerably more sucrose than the reference solutions. Additional tests demonstrated that the preference for sweet–sour taste mixtures was not based on masking effects. Rather, the animals perceived both the sweetness and the sourness of the taste mixtures and made a trade-off between the attractive and aversive properties of the two taste qualities. The results of this study suggest that the proximate reason for the marked differences in acceptance of sweet–sour taste mixtures are differences among species in the hedonic evaluation of the sour taste of citric acid. Possible ultimate reasons, which do not necessarily exclude, but may complement each other, include evolutionary

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adaptation to dietary specialization, avoidance of competition pressure, and phylogenetic relatedness.

Key Words—Sour-taste tolerance, sweet–sour taste mixtures, *Saimiri, Ateles, Macaca, Papio*, food preference.

INTRODUCTION

The sense of taste has been investigated anatomically (e.g., Pritchard, 1991; Rolls, 1995), behaviorally (e.g., Glaser, 1986; Laska, 1996, 1999, 2000), and electrophysiologically (e.g., Plata-Salaman et al., 1993, 1995; Hellekant and Roberts, 1995; Scott and Plata-Salaman, 1999) in a number of nonhuman primate species. However, most studies so far have concentrated on detectability of the four basic taste qualities, usually using sucrose, sodium chloride, hydrochloric acid, and quinine hydrochloride as the only, prototypic stimuli, or have assessed the properties of artificial tastants such as high-potency sweeteners or taste modifiers (e.g., Glaser, 1993; Nofre et al., 1996). There is sparse information as to taste perception of nonhuman primates for heterogenous taste mixtures (Plata-Salaman et al., 1996). This is remarkable given that the taste of almost any food is composed of more than one taste quality (Galef, 1996). The taste of most fruits, for example, is characterized by a mixture of sensations termed sweet and sour by humans (Nagy and Shaw, 1980), and the food selection behavior of primates suggests that they may use gustatory cues, and the relative salience of sweetness and sourness in particular, to assess palatability of a potential food item (Clutton-Brock, 1977; Laska, 2001). This appears to make sense in terms of optimal foraging (Stephens and Krebs, 1986), as the ratio between sour-tasting acids and sweet-tasting carbohydrates has been shown to change systematically as a function of the degree of ripeness in a large variety of fruits (Hulme, 1970) and thus is a good indicator of their nutritional value (Souci et al., 1989).

Animals that include fruits into their diet have to decide frequently whether a potential food item is already sweet enough to be consumed despite the presence of sourness, or whether it is still too sour to be consumed despite the presence of sweetness. Thus, it was the aim of this study to assess sour-taste tolerance in four species of nonhuman primates. To this end, we employed two-bottle preference tests of brief duration (2 min). This method makes it possible to directly measure preferences for or aversions to tastants, and largely rules out the influence of postingestive factors on the animal's ingestive behavior. More specifically, the monkeys were given a choice between a moderately sweet-tasting sucrose solution as a reference and mixtures containing variable concentrations of sour-tasting citric acid plus variable concentrations of sucrose.

It is important to note that, theoretically, such an approach could yield three different results: (A) The animal always prefers the solution containing no

sour-tasting citric acid, irrespective of the concentration of sucrose in the mixture. This would reflect an extremely low sour-taste tolerance. (B) The animal always prefers the solution containing the higher concentration of sucrose, irrespective of the concentration of citric acid in the mixture. This would reflect an extremely high sour-taste tolerance. (C) The animal displays a choice behavior that lies between the extremes outlined in (A) and (B), and shows preferences for one alternative or the other taking into consideration both the concentrations of the sour-tasting citric acid and the sweet-tasting sucrose.

The four primate species employed here, squirrel monkeys, spider monkeys, olive baboons, and pigtail macaques, are known to differ—at least to some degree—in their dietary habits (Clutton-Brock, 1977; Caldecott, 1986; Chapman, 1987; Ross, 1992) allowing us to address the question whether possible differences in sour-taste tolerance may reflect an evolutionary adaptation to dietary specialization. The possibility to include two New World primate species and two Old World primate species allowed us to additionally address the question whether the degree of phylogenetic relatedness rather than dietary specialization may affect the sour-taste tolerance of nonhuman primates.

METHODS AND MATERIALS

Animals. Testing was carried out using six male adult squirrel monkeys (*Saimiri sciureus*), two male and three female adult pigtail macaques (*Macaca nemestrina*), two male and two female adult olive baboons (*Papio hamadryas anubis*), and one male and three female adult spider monkeys (*Ateles geoffroyi*). Animals of all four species were housed as social groups in enclosures with adjacent single cages, which could be closed by sliding doors to allow temporary separation of animals for individual testing (for details of maintenance see Laska, 1996, 2000; Laska et al., 1996, 1999). Animals were fed commercial monkey chow, fresh fruit, and vegetables *ad libitum*, but were deprived of water overnight before testing on the following morning. The amount of food offered daily to the animals was such that leftovers were still present on the floor the next morning, and it was unlikely that ravenous appetite affected the animals' ingestive behavior in the tests.

Procedure. Sour-taste tolerance to three concentrations of citric acid was assessed by using a two-bottle preference test of short duration (Richter and Campbell, 1940). Tests were performed daily, and twice each day, approximately 2 and 1 hr before feeding, animals were separated and allowed 2 min to drink from a pair of simultaneously presented graduated cylinders (100 ml volume for *Saimiri* and *Ateles*, and 700 ml for *Papio* and *Macaca*) with metal drinking spouts.

In experiment 1, the monkeys were given a choice between a 50 mM sucrose solution and mixtures containing 10 mM citric acid plus variable concentrations

of sucrose (10, 20, 50, 100, 200, 400, 800, and 1000 mM). In experiments 2 and 3, the same combinations were tested except for the fact that the mixtures contained 30 and 50 mM citric acid, respectively, rather than 10 mM citric acid. The rationale for choosing these concentrations of citric acid was that they represent the range of concentrations present in a large variety of tropical fruits (Nagy and Shaw, 1980). Furthermore, they are detectable for all four primate species, as earlier studies have found taste preference thresholds for citric acid to be lower than 10 mM (Laska, 1999, 2000; Laska et al., 2000a,b; Laska et al., unpublished data). The rationale for choosing a 50 mM sucrose solution as the reference was that this concentration is also clearly detectable and attractive for all four primate species, but is only a factor of five (respectively, 10 for the spider monkeys) above their taste preference threshold (Laska, 1996, 2000; Laska et al., 1996, 1999) and, thus, represents a moderately sweet taste stimulus. In experiment 4, monkeys were given a choice between those mixtures of citric acid plus sucrose that were preferred over the 50 mM sucrose solution in experiments 1–3 and a sucrose solution containing the same concentration as present in the preferred mixture. This was done to get an indication of whether the preferences for the sweet–sour taste mixtures observed in experiments 1–3 were based on masking of the sour taste by the sweet taste or whether the animals still perceived the sourness and made a trade-off taking both taste qualities into consideration.

To keep up the animals' motivation and willingness to cooperate, testing did not follow a strict order of increasing or decreasing sucrose concentrations in the mixtures, but followed a pseudo-randomized scheme in which trials with taste mixtures containing high and, thus, presumably attractive concentrations of sucrose were alternated with mixtures containing low and, thus, presumably less attractive concentrations of sucrose. In all four experiments, each pair of stimuli was presented 10 times, and the position of the stimuli was pseudorandomized to counterbalance possible position preferences. All animals had served in previous studies using the same method (Laska, 1994, 1996, 1997, 1999, 2000; Laska et al., 1996, 1998, 1999b, 2000a,b), they were trained to enter the single cages voluntarily and were completely accustomed to the procedure.

The experiments reported here comply with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 86-23, revised 1985) and also with current German and Mexican laws.

Data Analysis. For each animal, the amount of liquid consumed from each bottle was recorded, summed for the 10 test trials with a given stimulus combination, converted to percentages (relative to the total amount of liquid consumed from both bottles), and 66.7% (i.e., 2/3 of the total amount of liquid consumed) was taken as criterion of preference. This rather conservative criterion was chosen for purposes of comparability of data, as the same criterion had been used in previous studies using the same method with the same primate species (Laska, 1994, 1996, 1997, 1999, 2000; Laska et al., 1996, 1998,

1999a,b, 2000a,b, 2001), and to avoid misinterpretation of data due to a too liberal criterion.

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

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

RESULTS

Figure 1 shows the mean taste responses of the four primate species given a choice between a 50 mM sucrose solution and mixtures of 10, 30, and 50 mM citric acid, respectively, plus various concentrations of sucrose. The squirrel monkeys needed a concentration of 200 and 800 mM sucrose to prefer mixtures with 10 and 30 mM citric acid, respectively, over a reference solution of 50 mM sucrose. With lower sucrose concentrations, *Saimiri sciureus* either rejected the sweet–sour mixtures (with 10 and 20 mM sucrose in a mixture with 10 mM citric acid, and with 10–100 mM sucrose in a mixture with 30 mM citric acid) or consumed approximately equal amounts from both alternatives (with 50 and 100 mM sucrose in a mixture with 10 mM citric acid, and with 200 and 400 mM sucrose in a mixture with 30 mM acetic acid). With 50 mM citric acid, even a concentration of 1000 mM sucrose was not sufficient for the squirrel monkeys to prefer a mixture over a reference solution of 50 mM sucrose. Sweet-sour mixtures containing up to 400 mM sucrose were rejected, and only mixtures with 800 and 1000 mM sucrose were about equally attractive for *Saimiri sciureus* as the reference solution.

The spider monkeys needed only a concentration of 100 mM sucrose to prefer sweet–sour taste mixtures with 10 and 30 mM citric acid over a sweet-tasting reference solution of 50 mM sucrose. With lower concentrations of sucrose in the mixture, *Ateles geoffroyi* generally rejected the sweet–sour mixtures, except for the mixture of 10 mM citric acid and 50 mM sucrose, which was consumed at approximatley equal amounts compared to the reference solution. With 50 mM citric acid, the spider monkeys needed a concentration of 800 mM sucrose to

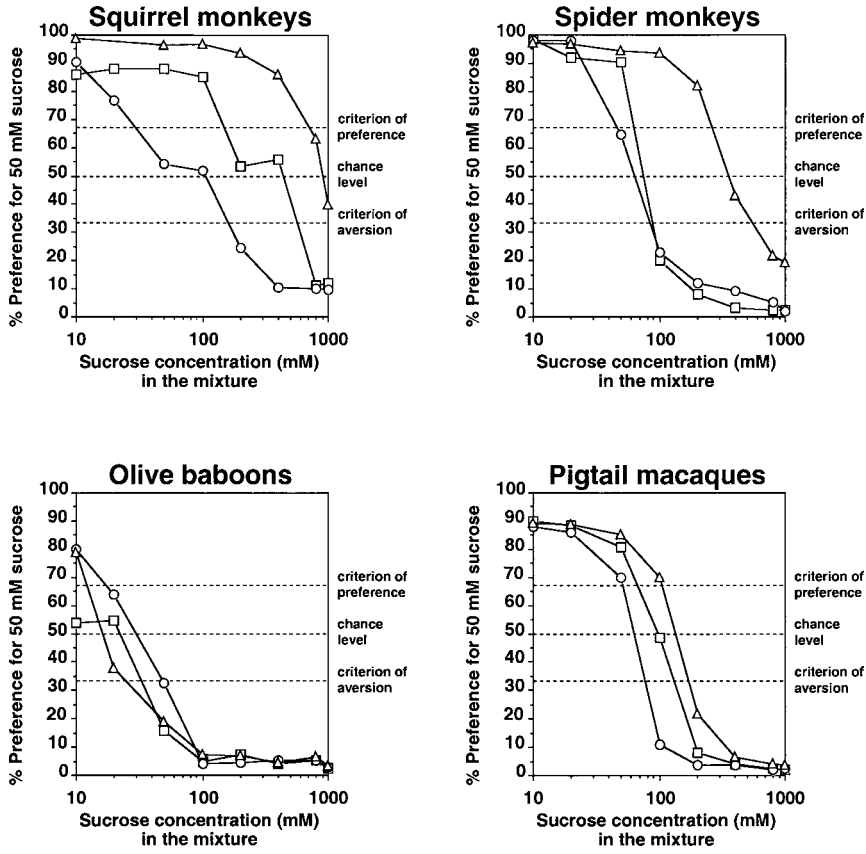

FIG. 1. Taste responses of six squirrel monkeys, four spider monkeys, four olive baboons, and five pigtail macaques given a choice between a 50 mM sucrose solution and mixtures of 10 mM (circles), 30 mM (squares), and 50 mM (triangles) citric acid plus various concentrations (10–1000 mM) of sucrose. Each data point represents the mean value of 10 test trials of 2 min per animal.

prefer a sweet–sour taste mixture over a sweet-tasting reference solution of 50 mM sucrose. A mixture containing 400 mM sucrose was as attractive for *Ateles geoffroyi* as the reference, and mixtures with 200 mM sucrose or less were rejected.

The olive baboons needed only a concentration of 50 mM sucrose to prefer sweet–sour taste mixtures with 10, 30, and 50 mM citric acid, respectively, over a sweet-tasting reference solution of 50 mM sucrose. Only with mixtures containing the lowest sucrose concentration of 10 mM plus 10 and 50 mM citric acid, respectively, *Papio hamadryas anubis* showed a preference for the reference solution.

TABLE 1. CONCENTRATIONS OF SUCROSE (MM) NEEDED IN MIXTURES WITH CITRIC ACID TO INDUCE A SIGNIFICANT PREFERENCE OVER A SWEET-TASTING REFERENCE SOLUTION OF 50 MM SUCROSE

	Concentration of citric acid (mM) in the mixture		
Species	10	30	50
Squirrel monkeys	200	800	>1000
Spider monkeys	100	100	800
Olive baboons	50	50	50
Pigtail macaques	100	200	200

Sweet-sour mixtures containing 20 mM sucrose were as attractive as the 50 mM sucrose reference at all three concentrations of citric acid tested.

The pigtail macaques needed a concentration of 100 mM sucrose to prefer a sweet–sour taste mixture with 10 mM citric acid over a sweet-tasting reference solution of 50 mM sucrose. With 30 and 50 mM citric acid, *Macaca nemestrina* needed 200 mM sucrose to show a preference for the mixtures when a 50 mM sucrose solution was presented as the alternative. Sweet–sour mixtures containing 10–50 mM sucrose were rejected at all three concentrations of citric acid tested. A mixture of 100 mM sucrose plus 30 mM citric acid was as attractive for the pigtail macaques as the reference solution.

With all four species, interindividual variability in the percentage of preference for a given solution was generally low. Standard deviations across all test combinations averaged 6.8%, and in the majority of cases were smaller than 5%.

Table 1 summarizes the lowest concentrations of sucrose needed in mixtures with citric acid to induce a preference over a moderately sweet-tasting reference solution of 50 mM sucrose. Marked differences among the species tested are apparent. Whereas the olive baboons needed the lowest sucrose concentrations to make mixtures with citric acid more attractive than the reference solution, the squirrel monkeys needed the highest sucrose concentrations. More specifically, olive baboons actually preferred a sweet–sour mixture of citric acid plus sucrose over a sweet solution containing the same concentration of sucrose only. Squirrel monkeys, on the other hand, needed sucrose concentrations that were 4- and 16-fold higher than that of the sweet-tasting reference solution to make sweet–sour mixtures with 10 and 30 mM citric acid attractive, and with a mixture containing 50 mM citric acid even a sucrose concentration 20 times that of the reference solution was not sufficient to make it preferable over the reference. The pigtail macaques and spider monkeys fell in between these extremes and needed sucrose concentrations 2- to 4-fold (*Macaca nemestrina*) and 2- to 16-fold (*Ateles geoffroyi*)

higher than that of the sweet-tasting reference solution to show a preference for sweet–sour taste mixtures containing 10, 30, and 50 mM citric acid, respectively.

To get an indication of whether these preferences for sweet–sour taste mixtures were based on masking of the sour taste by the sweet taste or whether the animals still perceived the sourness and made a trade-off taking both taste qualities into consideration, an additional experiment was performed. Figure 2 shows the mean taste responses of the four primate species given a choice between mixtures of citric acid plus sucrose that were preferred over the 50 mM sucrose solution in the experiments described above, and a sucrose solution containing the same concentration as present in the preferred mixture. In all 11 cases (the 12th case, a

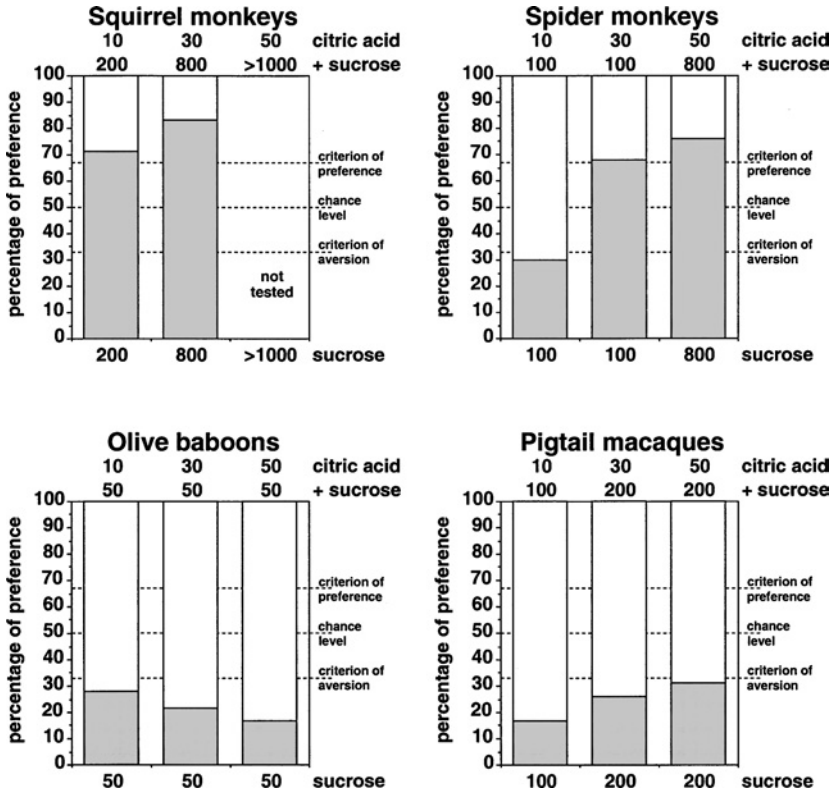

FIG. 2. Taste responses of six squirrel monkeys, four spider monkeys, four olive baboons, and five pigtail macaques given a choice between mixtures of citric acid plus sucrose that were preferred over the 50 mM sucrose solution in experiments 1–3, and a sucrose solution containing the same concentration as present in the preferred mixture. Each bar represents the mean value of 10 test trials of 2 min per animal.

mixture containing 50 mM citric acid with the squirrel monkeys was not tested as the animals failed to show a preference for any of the mixtures tested, cf. Figure 1), the animals showed marked preferences for one of the alternatives. Whereas the olive baboons and the pigtail macaques preferred the sweet–sour mixtures over a sweet-tasting alternative containing the same concentration of sucrose as present in the mixture, the squirrel monkeys, in contrast, preferred the sweet-tasting solution over the sweet–sour mixtures. Spider monkeys showed a preference for the sweet–sour mixture when the concentration of citric acid was low (10 mM), and rejected it when the concentration was medium or high (30 and 50 mM, respectively). These results indicate that all four primate species tested perceived the citric acid in the mixtures and, thus, suggest that the preferences shown in experiments 1–3 were not based on masking of the sour taste by the sweet taste, but rather on a trade-off between the attractive and aversive properties of the two taste qualities.

DISCUSSION

The results demonstrate that squirrel monkeys, spider monkeys, olive baboons, and pigtail macaques differ markedly in their tolerance to physiological concentrations of sour-tasting citric acid. Whereas olive baboons showed the highest degree of sour-taste tolerance among the four species and actually preferred most of the sweet–sour taste mixtures over sweet-tasting reference solutions, squirrel monkeys showed the lowest degree of sour-taste tolerance and rejected most of the sweet–sour taste mixtures even when they contained considerably more sucrose than the reference solutions. Additional tests demonstrated that the preference for sweet–sour taste mixtures shown by all four species was not based on masking effects. Rather, the animals perceived both the sweetness and the sourness of the preferred taste mixtures and made a trade-off between the attractive and aversive properties of the two taste qualities.

What are the possible reasons that underlie the observed differences in sour-taste tolerance among the primate species tested? The possibility that differences in sensitivity for citric acid and/or sucrose may account for the differing acceptance of sweet–sour taste mixtures found here can largely be ruled out, as all four species tested have been shown to share the same taste preference threshold value for citric acid (Laska, 1999, 2000; Laska et al., 2000a,b) and three out of four species have been reported to share the same taste preference threshold value for sucrose (Laska, 1996, 2000; Laska et al., 1999a), with the fourth species (*Ateles geoffroyi*) displaying a value that is only a factor of 3 lower (Laska et al., 1996).

It is well established that masking effects, i.e., complete suppression of one taste by another, qualitatively different taste, or other types of qualitative

or quantitave interactions such as enhancement or blending may occur when using heterogenous taste mixtures (Schifferstein and Frijters, 1993; Halpern, 1997). Although the possibility that species differences in such interactions between the tastes of citric acid and sucrose might have been responsible for the differing sour-taste tolerance cannot be ruled out completely, this seems unlikely as there is no reason to believe that the primate species tested here differ fundamentally from each other with regard to possible taste interactions such as masking. In a comprehensive human psychophysical study on detection of tastes in heterogenous binary mixtures, Stevens (1996) showed that sucrose concentrations of a factor of 10 or 100, respectively, above threshold, elevated the detection threshold for citric acid in a mixture only by a factor of 2 or 4, respectively. Further, he reported that tastants termed sweet and sour by humans mask each other much less efficiently compared to other combinations of tastants. This latter finding is supported by findings from electrophysiological recordings in the gustatory cortex of *Macaca fascicularis*, which have shown that in binary taste mixtures sourness induced by HCl is suppressed least among the four basic taste qualities (Plata-Salaman et al., 1996). The authors concluded that sour-best cells in primates are less susceptible to mixture suppression than other taste cell subtypes. Thus, although interaction effects are likely to have occurred with some of the taste mixtures employed in this study, their extent is unlikely to explain the marked differences among species that we found.

Possible differences in the hedonic evaluation (evaluation of the dimension "pleasant–unpleasant") of the sweet taste of sucrose can also be excluded as an explanation as all four species have been shown to display preferences of similar strength for suprathreshold concentrations of sucrose (Laska, 1996, 2000; Laska et al., 1996, 1999a). The most likely explanation for our finding is that the species differed in their hedonic evaluation of the sour taste of citric acid. This supposition is supported by the finding that squirrel monkeys generally reject suprathreshold concentrations of citric acid (Laska, 1999), whereas spider monkeys and pigtail macaques prefer low, but detectable, concentrations of this acid and reject only concentrations $\geq$20 and 50 mM, respectively (Laska, 2000; Laska et al., 2000a,b), and olive baboons even prefer concentrations up to 200 mM of this sour tastant (Laska, unpublished data).

Human and nonhuman primates have repeatedly been shown to be capable of learning to accept and even prefer tastes that they initially reject (Rozin and Schiller, 1980; Rozin and Kennel, 1983). Nevertheless, it is commonly agreed that hedonic responses of primates to tastants are generally innate and the result of an evolutionary adaptation to a species' dietary habits (Steiner et al., 2001).

Differences in dietary habits have repeatedly explained differences in taste performance among species (Kare, 1971; Pfaffmann, 1977; Spector, 2000). Among New World primates, for example, the degree of frugivory correlates positively with sensitivity for food-associated mono- and disaccharides (Laska, 1996; Laska et al.,

1996). Although considerable information on the dietary habits of the four primate species used in the present study are available (Caldecott, 1986; Chapman, 1987; Clutton-Brock, 1977; Ross, 1992), no definitive conclusions can be drawn whether differences in feeding specializations may account for the observed differences in sour-taste tolerance as, unfortunately, only few studies reported the proportion of unripe versus ripe fruits in their diet. It is interesting to note, however, that squirrel monkeys—the species showing the lowest degree of acceptance for sweet–sour taste mixture in this study—have also been reported to include the lowest proportion of fruits and seeds in their diet (26% of total intake) among the four species tested, whereas olive baboons (63%), pigtail macaques (72%), and spider monkeys (80%) include considerably higher proportions of fruit (Clutton-Brock, 1977).

An alternative explanation for the observed differences may be that preferring or at least accepting levels of acidic tastants that other species reject could help to avoid competition between sympatric frugivores for carbohydrate-rich fruits or might even give a species an advantage over competitors in the quest for fruits that are nutritionally valuable but still unripe and low in abundance (Gallina and Ausman, 1979). In their natural habitat, pigtail macaques compete for food with white-handed gibbons *Hylobates lar* and with Indonesian leaf monkeys *Presbytis potenziani* (Rowe, 1996). Whereas the latter two species do not include unripe fruits into their diets, pigtail macaques readily consume unripe *Ficus* fruits (Caldecott, 1986). As it is difficult to quantify competition pressure between species on a comparative basis, this explanation needs further verification.

Old World primates and New World primates differ in their ability to perceive substances such as aspartame and thaumatin, which both taste sweet to humans, suggesting that phylogenetic relatedness might also account for differences or correspondences in taste perception among species (Glaser, 1993). Indeed, in the present study, olive baboons and pigtail macaques, Old World primate species, accepted sweet–sour taste mixtures more readily than did squirrel monkeys and spider monkeys, New World primate species. Thus, the possibility of phylogenetic relatednes as an explanation for sour-taste tolerance also warrants further investigation.

In summary, the results of this study suggest that the major reason for the marked differences in acceptance of sweet–sour taste mixtures is that the primate species differed in their hedonic evaluation of the sour taste of citric acid. Other explanations that do not necessarily exclude, but may complement each other, include evolutionary adaptation to dietary specialization, avoidance of competition pressure, and phylogenetic relatedness.

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INDUCTION OF DIRECT AND INDIRECT PLANT RESPONSES BY JASMONIC ACID, LOW SPIDER MITE DENSITIES, OR A COMBINATION OF JASMONIC ACID TREATMENT AND SPIDER MITE INFESTATION

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Abstract—Jasmonic acid (JA) and the octadecanoid pathway are involved in both induced direct and induced indirect plant responses. In this study, the herbivorous mite, *Tetranychus urticae*, and its predator, *Phytoseiulus persimilis*, were given a choice between Lima bean plants induced by JA or spider mites and uninduced control plants. Infestation densities resulting in the induction of predator attractants were much lower than thus far assumed, i.e., predatory mites were significantly attracted to plants that were infested for 2 days with only one or four spider mites per plant. *Phytoseiulus persimilis* showed a density-dependent response to volatiles from plants that were infested with different numbers of spider mites. Similarly, treating plants with increasing concentrations of JA also led to increased attraction of *P. persimilis*. Moreover, the duration of spider mite infestation was positively correlated with the proportion of predators that were attracted to mite-infested plants. A pretreatment of the plants with JA followed by a spider mite infestation enhanced the attraction of *P. persimilis* to plant volatiles compared to attraction to volatiles from plants that were only infested with spider mites and did not receive a pretreatment with JA. The herbivore, *T. urticae* preferred leaf tissue that previously had been infested with conspecifics to uninfested leaf tissue. In the case of choice tests with JA-induced and control leaf tissue, spider mites slightly preferred control leaf tissue. When spider mites were given a choice between leaf discs induced by JA and leaf discs damaged by spider mite feeding, they preferred the latter. The presence of herbivore induced chemicals and/or spider mite products enhanced settlement of the mites, whereas treatment with JA seemed to impede settlement.

Key Words—*Tetranychus urticae*, *Phytoseiulus persimilis*, plant volatiles, carnivore attraction, spider mite, jasmonic acid, induced plant responses.

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INTRODUCTION

Jasmonic acid (JA) is a major component of signal-transduction pathways that mediate tritrophic interactions between plants, arthropod herbivores, and carnivores after herbivore wounding of the plant. Jasmonic acid is a ubiquitous plant hormone that is synthesized through the octadecanoid pathway (Vick and Zimmerman, 1984; Farmer and Ryan, 1992; Schaller and Weiler, 2002; Turner et al., 2002). Endogenous concentrations of JA increase after herbivore feeding damage, followed by an increase in gene activity (Reinbothe et al., 1994; Blechert et al., 1995). Expression of these genes results in the accumulation of secondary plant metabolites, such as nicotine in tobacco (Baldwin, 2001) and defense proteins, such as proteinase inhibitors and polyphenol oxidase in tomato (Farmer and Ryan, 1992; Stout et al., 1994; Constabel et al., 1995). Both secondary plant metabolites and defense proteins can hamper the performance of herbivores that feed on these plants (reviewed by Karban and Baldwin, 1997), which is referred to as induced resistance.

To locate their prey, many arthropod carnivores use cues associated with their herbivorous victims or the microhabitat. Numerous studies have demonstrated that plants produce volatiles in response to herbivore feeding damage, which are used by predators and parasitoids (for reviews see Tumlinson et al., 1992; Vet and Dicke, 1992; Turlings et al., 1995; Takabayashi and Dicke, 1996; Dicke, 1999). Jasmonic acid and the octadecanoid pathway play a major role in the induction of carnivore attractants that are produced in response to herbivore feeding damage (Hopke et al., 1994; Arimura et al., 2000; Dicke and van Poecke, 2002; Thaler et al., 2002; Schmelz et al., 2003). Plant responses to herbivore damage that result in the attraction of members of the third trophic level are known as induced indirect responses.

Exogenous treatment of plants with JA or its volatile derivative methyl jasmonate (MeJA) results in induced direct effects similar to plant responses induced by herbivore feeding (Bodnaryk, 1994; Thaler et al., 1996, 2002; Avdiushko et al., 1997; Bartlet et al., 1999; van Dam et al., 2000). Likewise, exogenous application of JA or MeJA induces indirect responses (Hopke et al., 1994; Dicke et al., 1999; Gols et al., 1999; Arimura et al., 2000; van Poecke and Dicke, 2002; Schmelz et al., 2003). Headspace analyses have demonstrated that volatile blends of JA-induced plants greatly overlap with volatile blends produced in response to herbivore damage (Dicke et al., 1999; Gols et al., 1999). Field studies have shown that the application of JA to plants leads to higher plant fitness, increased parasitism, and reduced abundance and performance of herbivores (Baldwin, 1998; Thaler, 1999; Kessler and Baldwin, 2001).

Plants can acquire enhanced resistance to a broad spectrum of pathogens after contact with necrotizing pathogens or nonpathogenetic root-colonizing pseudomonads or treatment with elicitors of plant resistance (reviewed by Conrath

et al., 2002). This induced resistance is often associated with an enhanced capacity to mobilize infection-induced defense responses (Conrath et al., 2002). Since JA is involved in the induction of plant responses against arthropod herbivory, it would be interesting to investigate whether treatment with it results in a more rapid and effective activation of direct and indirect plant responses against herbivores. Baldwin and Schmelz (1996) demonstrated that tobacco plants that were treated with MeJA increased their whole-plant nicotine pools, a direct plant response, more rapidly after consecutive applications of MeJA than did untreated control plants. In this study, we report the effect of a pretreatment with JA followed by a spider mite infestation on indirect plant responses.

The predatory mite *Phytoseiulus persimilis* Athias-Henriot is a specialist enemy of the polyphagous spider mite, *Tetranychus urticae* Koch, and exterminates local prey populations. Attraction of *P. persimilis* to spider mite induced volatiles has been demonstrated for many different plant species (Sabelis and Van de Baan, 1983; Dicke et al., 1998; van den Boom et al., 2002). Furthermore, plants treated with JA release volatiles that are attractive to *P. persimilis* (Dicke et al., 1999; Gols et al., 1999). *Tetranychus urticae* also responds to volatiles emitted by plants (Dicke, 1986; Pallini et al., 1997). The spider mite tends to move away from plants that are infested with conspecifics (Bernstein, 1984; Dicke, 1986). Application of JA to cotton plants reduces spider mite oviposition rates by more than 75% (Omer et al., 2001), indicating that JA also affects direct plant responses against spider mites. However, it remains unknown whether JA treatment affects the behavior of the spider mites.

Since spider mite colonies are usually founded by a single or only a few adult mite females, *P. persimilis* may encounter prey patches with very low densities of mites. In studies that investigate the response of *P. persimils* to plant volatiles induced by spider mites, infestation densities of 100 mites per plant or more are common (e.g., Sabelis and Van de Baan, 1983; Janssen et al., 1997; Dicke et al., 1999; Arimura et al., 2000). The aims of the present study were to investigate (1) how infestation with different densities of spider mites and application of different concentrations of JA affect the response of *P. persimilis* to plant volatiles, (2) how the duration of the mite infestation affects the induction of predator attractants, (3) how induction by JA affects behavior of both *P. persimilis* and *T. urticae* compared to induction by spider mite infestation, and (4) whether a pretreatment with JA followed by a spider mite infestation can increase the induction of predator attractants.

METHODS AND MATERIALS

Plants and Mites. Lima bean plants (*Phaseolus lunatus*) cv. Sieva (Kellog Seed Co., Crows Landing, California) were reared in a greenhouse at 20–30°C,

50–70% RH, and a photoperiod of at least 16 hr. If the light intensity dropped below 500 μmol photons/m^2/sec, supplementary illumination was applied by high-pressure mercury lamps. Plants were grown in steam-sterilized soil. Plants used in the experiments were 9–17 days old.

Tetranychus urticae (Acarina, Tetranychidae) was reared on Lima bean plants in a separate greenhouse compartment under the same conditions as the Lima bean plants. Adult spider mites used in the experiments were collected randomly from infested leaves.

Phytoseiulus persimilis (Acarina, Phytoseiidae) was reared in Petri dishes (14.5 cm in diam) on detached Lima bean leaves infested with spider mites. Adult females were allowed to oviposit for 24 hr and were subsequently discarded. Leaves without adults were kept in a climate cabinet (23 $\pm$ 2°C, L16:D8 photoperiod) for another 6–7 days until the eggs developed into adults. Additional food was added every 2–3 days. In the experiments, we used female predatory mites, 1–3 days after the final molt.

Bioassay with Predatory Mites. We tested the attractiveness of volatiles emitted by induced plants to *P. persimilis* in an olfactometer. This equipment consisted of a Y-shaped glass tube with a metal wire in the center. Each arm was connected to a 5-l glass vessel. Both vessels contained an equal number of differently treated plants, which were carefully removed from the pot soil. Roots were gently rinsed with tap water and wrapped in aluminum foil. Pressurized air was filtered over activated charcoal and was fed into the two vessels at a rate of 4 l/min. Air was extracted at the base of the Y-tube at 8 l/min (for details see Takabayashi and Dicke, 1992). Experiments were conducted at 23 $\pm$ 2°C and a light intensity of 30–35 μmol photons/m^2/sec (high frequency fluorescent lights). To enhance responsiveness to plant volatiles, all predators had been starved for 2–4 hr prior to release in the olfactometer, by containing them individually in Eppendorf tubes. The experiment started with the release of an individual adult female at the base of the wire. When the mite reached the end of either arm, we recorded its choice and discarded it. Predators that had not reached the end of an arm within 5 min, which occurred for less than 7% of the predatory mites, were excluded from the statistical analysis. After testing five mites, we exchanged the odor containers to correct for unforeseen asymmetry in the set-up. The system was purged for 5 min prior to release of the first predatory mite, and at each time the odor sources were exchanged.

Dose–Response Curves with Predatory Mites. To determine how predator responses to volatiles from JA-induced plants relate to the responses to volatiles emitted by spider-mite-induced plants, we investigated the dose–response relationship for both modes of induction. We tested the response of *P. persimilis* to JA-induced plants for the following concentrations, 0.1, 0.32, 0.56, and 1 mM JA. Jasmonic acid (Sigma–Aldrich) was dissolved in tap water by vigorously shaking. Plants were sprayed with a solution of JA (2–2.5 ml per plant) on both upper and

lower sides of the leaves. Plants treated with tap water served as controls. Each concentration was tested 10–16 times with 20 predatory mites in each test. To assess the effect of mite density, we infested plants with 1, 2, 4, 20, or 50 spider mites per plant. Mites were evenly distributed over the two primary leaves. For the one spider mite treatment, we placed one mite on just one of the two leaves. Plants without spider mites served as a control. Each mite density was tested 8–10 times with 20 predatory mites in each test. All plants were incubated for 2 days in a climate room (23 ± 2°C, L16:D8 photoperiod, 60–70% RH). Treatments and different doses were kept apart by placing plants in plastic cages (66 × 66 × 100 cm) that were individually connected to a house vacuum. In the Y-tube olfactometer (see bioassay), we tested the response of *P. persimilis* to induced plants versus noninduced control plants. We used 4–5 intact plants per odor source in each test.

Effect of Infestation Duration on Predator Attraction. In this experiment, we determined whether the duration of spider mite infestation has an effect on the response of *P. persimilis* toward volatiles emitted by infested plants. Ten sets of two plants each were infested with four spider mite females per plant, divided over the two primary leaves. Olfactometer tests were performed at 1, 2, 3, 7, 8, 14, 15, 21, 22, and 28 days after infestation. Plants were kept in a greenhouse compartment under the same conditions as mentioned in the *Plants and Mites* section. An equal number of uninfested control plants was placed in a separate greenhouse compartment on the same day. All plants were watered regularly. In the olfactometer, we tested two infested plants against two control plants with 20 predatory mites. Two trials of this experiment were conducted.

Combined Effect of JA and Spider Mite Induction on Attraction of Predatory Mites. To investigate whether a pretreatment of Lima bean plants with JA can enhance the attraction of predatory mites when the plant becomes subsequently infested with spider mites, we applied JA and subsequently infested the plants with spider mites, four adult females per plant. On the basis of the results of the dose response curve, a JA concentration (0.1 mM) was applied that did not induce plant volatiles to a level that leads to a significant attraction of the predatory mites. To determine whether a time lapse between JA-treatment and spider mite infestation had an effect on the level of induction, plants were either infested with spider mites directly after the JA-sprayed plants had dried or after a 7-day incubation period. Control plants were treated identically using tap water only. We infested each plant, both JA-treated and control, with four spider mites evenly distributed over the two primary leaves. After another 2 days of incubation, the attractiveness of these plants was tested in the Y-tube olfactometer with four–five intact plants per odor source. Plants were sprayed and incubated as described in the dose–response section. For both incubation periods, eight replicates were performed with 20 predatory mites in each test.

Choice Tests with Spider Mites. To determine whether JA-induced plants and herbivore-induced plants affect settling behavior of spider mites, spider mite females were offered the choice between plants that had been induced by JA or spider mites and control plants. Plants with recently unfolded primary leaves were cut just above the soil. The cut stems were immediately placed into water to regain turgor after which they were transferred into a vial containing 6 ml of a JA solution (0.1 or 1 mM) or tap water. Plants receiving spider mite treatments were infested with 25 mites per primary leaf. All plants were incubated for 2 days, unless stated differently, in a climate room (23 ± 2°C, L16:D8 photoperiod, 60–70% RH). Differently treated plants were kept apart by placing them in plastic cages (66 × 66 × 100 cm) that were individually connected to a house vacuum.

The experimental set-up consisted of two leaf disc (diam 2.4 cm) halves cut from differently treated plants (for the experimental set-up, see also Dicke and Dijkman, 1992). Discs were cut into two halves with a razor blade using secondary veins as dividing lines. We removed all spider mites and mite products (eggs, exuviae, feces, and silk) from the herbivore-infested leaf discs with a fine brush, and refer to this as "previously infested plants." Leaf disc halves were put onto a styrofoam plate covered with humidified filter paper. The position of the treatment of the two halves was alternated. A pin placed between the two leaf disc halves served as the release point for the spider mite females. We placed an individual female mite on top of the pin using a fine brush. An observation started when the mite left the pin and entered a leaf disc half ($t = 0$). We recorded the position of the spider mites at $t = 0$, 5, 15, 30, and 60 min. The following seven combinations of treatments were tested:

1–3: uninfested control plants vs. plants previously infested with spider mites after three different incubation periods, i.e., 2, 3, and 7 days,
4: uninfested control vs. spider-mite-infested plants from which the females had been removed but not the spider mite products, such as eggs, exuviae, silk, and feces,
5–6: untreated control vs. JA-treated plants with respectively 0.1 and 1 mM JA, and
7: JA-treated (1 mM) vs. previously infested plants.

For each combination of leaf disc halves, 96–150 spider mite females were tested individually. We used 6–12 leaf disc halves taken from the same plant, and we used a minimum of 12 different plants for each treatment in a combination. Spider mites that had left the leaf disc halves before the end of the observation period were discarded, which occurred for less than 8% of the tested mites.

Statistical Analysis. Predator responses are presented as proportions of total number of predators that chose induced plants. Statistics were performed on arcsine-transformed predator responses to normalize distributions (Sokal and Rohlf, 1994). The relationship between log-transformed JA concentrations and

arcsine-transformed predator responses was established using least-squares linear regression. We did the same for log-transformed spider mite densities and arcsine-transformed predator responses and spider mite infestation periods and arsine-transformed predator responses. Z tests (two-tailed, $\alpha = 0.05$) were performed to determine whether predator responses were significantly different from a no-preference situation ($\mu_0 = \text{arcsine}\, 0.5$). t Tests (two-tailed, $\alpha = 0.05$) were performed to determine whether a time lapse between JA-treatment and spider mite infestation had a significant effect on attraction of predators.

We used a binomial test to determine whether spider mite preferences were significantly different from a no-preference situation ($p = q = 0.5$, two-tailed, $\alpha = 0.05$).

RESULTS

Dose Response Curves with Predatory Mites. Odors emitted by plants infested with 1, 4, 20, and 50 spider mites per plants are significantly more attractive to *P. persimilis* than odors produced by uninfested plants (Figure 1A). Increased spider mite densities resulted in increased levels of attraction of the predatory mite. Using least square regression of log-transformed spider mite densities on arcsine-transformed predator responses, we found a significant positive relationship (arcsine $Y = 0.316 \log X + 0.602$, where Y is proportion of predators preferring induced plant volatiles and X is spider mite density in number of mites per plant, $F(0.05, 1, 67)$, $14(F(0.05, 1, 34))$, $25(F(0.05, 1, 18))$, $r^2 = 0.27$, $P < 0.001$, Figure 1A). In the case of JA application, a less profound, but still significant, positive relationship was found between log-transformed JA concentrations and arcsine-transformed predator response (arcsine $Y = 0.164 \log X + 0.749$, where Y is proportion of predators preferring induced plant volatiles and X is JA-concentration in mM, $F(0.05, 1.34) = 5.37$, $r^2 = 0.14$, $P = 0.027$, Figure 1B). The application of concentrations of 0.32 mM JA and higher resulted in a significant attraction of *P. persimilis* (Figure 1B).

Effect of Infestation Duration on Predator Attraction. Attraction of the predators to plant volatiles gradually increased with the duration of the spider mite infestation (Figure 2). Plants that had been infested with four spider mites for only 1 day were already attractive to *P. persimilis* ($P = 0.052$, binomial test, two-tailed), and attraction persisted even after an infestation period of 30 days. A significantly positive linear relationship was found between the duration of mite infestation in days and arcsine-transformed predator response (arcsine $Y = 0.0157\, X + 0.783$, where Y is proportion of predators preferring induced plant volatiles and X is duration of spider mite infestation in days, $F(0.05, 1.18) = 20.6$, $r^2 = 0.52$, $P < 0.001$, Figure 2).

Interaction between JA-Treatment and Spider Mite Infestation on Predator Attraction. Lima bean plants treated with JA followed by a spider mite infestation

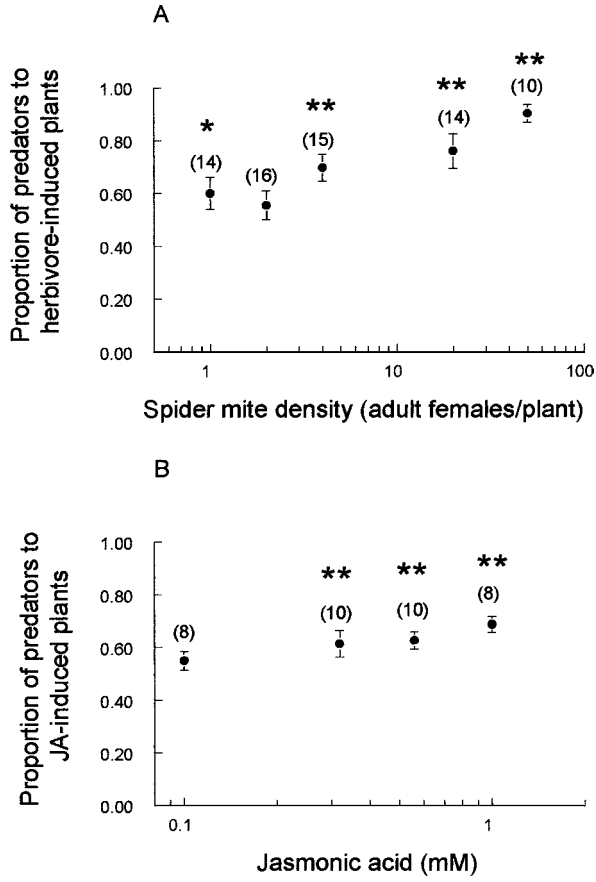

FIG. 1. Attraction of *Phytoseiulus persimilis* to volatiles emitted by plants that were induced with different spider mite densities (A) and different concentrations of JA (B). Predators were given the choice between volatiles from induced and noninduced control plants in a Y-tube olfactometer. Predator responses are presented as numbers of predators that chose the induced plants divided by the total number of responding predators. Each data point represents the mean ± SE with the number of olfactometer bioassays in brackets. Z tests were performed on arcsine transformed predator responses with $\mu_0 = \text{arcsine}(0.5)$ ($^{*}0.05 > P > 0.01$, $^{**}P < 0.01$).

were significantly more attractive to *P. persimilis* than plants that had received spider mite damage only (Figure 3). A time lapse of 1 week between JA treatment and spider mite damage resulted in a predator response that was not significantly different from predator responses to plants that had received JA and spider damage at the same time (t test, $t_{14} = 0.71$, $P = 0.49$).

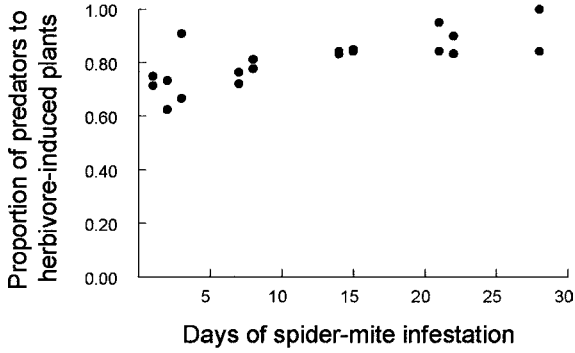

FIG. 2. The effect of the infestation period with spider mites on the attraction of *Phytoseiulus persimilis* toward volatiles emitted by infested plants. In a Y-tube olfactometer, predators were given the choice between volatiles from plants that had been incubated with spider mites and uninfested control plants that had been incubated for the same period. Predator responses are presented as numbers of predators that chose spider-mite-infested plants divided by the total number of responding predators. Each data point represents an olfactometer test with 20 predators. The standard regression line (not shown) for the data is arcsine $Y = 0.0157X + 0.783 (F(0.05, 1, 18) = 20.6, P < 0.001, r^2 = 0.52)$.

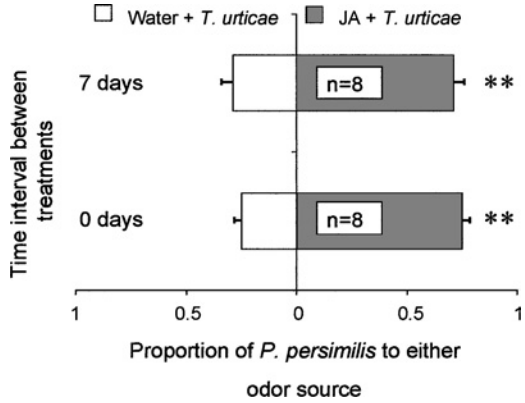

FIG. 3. Respones of *Phytoseiulus persimilis* females tested in an *y*-tube olfactometer set-up with odors of plants that were treated with both JA and spider mites (grey bars) and plants that were only infested with spider mites (white bars). Plants were either infested with spider mites directly after the plants were sprayed with JA (upper bar) or after a 7-day JA-induction period (lower bar). After introduction of the spider mites, the plants were incubated for 2 days. *Z* tests were performed on arcsine transformed predator responses with $\mu_0 = \text{arcsine } (0.5)$ ($^{**}P < 0.01$).

Spider Mite Responses. When spider mites were given a choice between uninfested leaf tissue and leaf tissue that previously had been infested with conspecifics, they always preferred the latter (Figure 4A). Infestation periods of 2 and 3 days had similar effects on mite preferences. Preference for infested leaf tissue

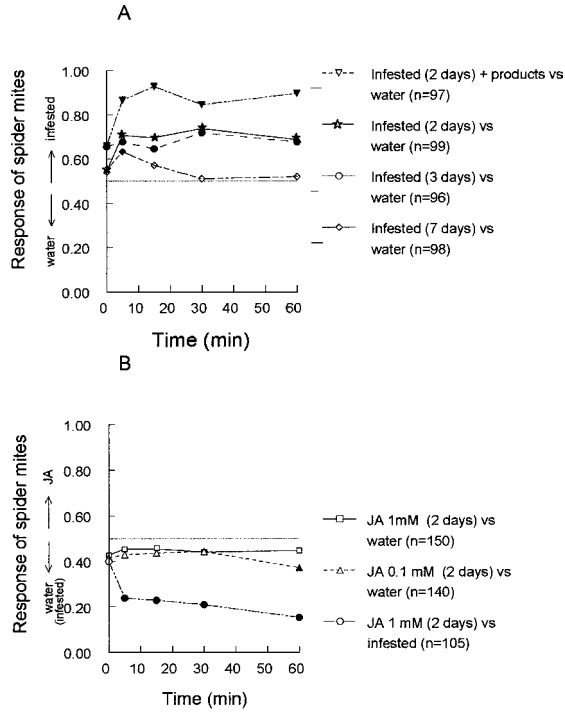

FIG. 4. Spider mites responses to leaf disc halves previously infested with conspecifics or JA at different intervals during a 1-hr observation period. Individual spider mites were given a choice between a leaf disc half cut from a plant that had been infested with conspecifics and a leaf disc half from an uninfested control plant (A) or from a JA-treated and a water-treated plant (B). In (A) plants were infested for 2 days (stars), 3 days (circles), or 7 days (diamonds) and all spider mites and their products were removed from the herbivore-infested leaf discs prior to the release of the spider mites. In one experiment (triangles) only, the spider mites were removed but not their products. In (B), plants were treated with 0.1 mM (triangles) or 1 mM JA (squares). Graph B includes the response of spider mites to plants treated with JA vs. plants previously infested with spider mites (circles). Each data point represents the number of spider mites on spider-mites-infested leaf disc halves (A) or JA-treated leaf disc halves (B) divided by the total number of tested spider mites that remained on the leaf discs throughout the observation period (denoted by n). Filled black symbols indicate significant differences from a 50:50 distribution (binomial test $P = 0.5, \alpha = 0.05$).

was statistically significant for both incubation periods for the total observation period (binomial tests, $P < 0.01$), except at $t = 0$ with plants that had been infested for 2 days ($P = 0.42$). In the experiments mentioned above, spider mites and their products had been removed just prior to the release of the mite. When only the spider mites were removed, but not the eggs, webbing and feces that had been produced during the incubation, preference for mite-infested sites was even stronger (Figure 4A). When plants had been infested with conspecifics for 7 days, the proportion of mites choosing the previously infested sites was much lower (Figure 4A). Proportions of mites that were found on infested leaf disc halves were only significantly different from a 50:50 distribution at $t = 5$ min ($P = 0.011$).

Of the spider mites that were given a choice between JA-treated (0.1 and 1 mM) and water-treated leaf disc halves (Figure 4B), more than 50% of the mites went to the leaf disc halves treated with water and stayed there throughout the observation period of 60 min. There was no apparent difference between the two tested concentrations of 0.1 and 1 mM. Preference for water-treated leaf disc halves was statistically significant only at $t = 60$ min at 0.1 mM JA ($P < 0.01$). When spider mites were given a choice between leaf disc halves that had previously been infested with spider mites for 2 days and JA-treated (1 mM) leaf disc halves, the mites preferred leaf disc halves previously infested with conspecifics (Figure 4B). This preference was highly significant from $t = 5$ to $t = 60$ min ($P < 0.001$).

DISCUSSION

Jasmonic acid and the octadecanoid pathway are involved in induction of both direct and indirect plant responses to herbivore feeding damage. This study showed that both the herbivorous spider mite, *T. urticae*, and its predator, *P. persimilis*, prefer spider-mite-infested plants to uninfested plants, whereas only the predators also prefer JA-induced plants to uninduced control plants. A clear correlation was found between spider mite density and the degree of attraction of *P. persimilis*. Not only is the emission of plant volatiles affected by spider mite densities, the duration of the infestation affects the quantity and quality of the released plant volatiles as well (Maeda and Takabayashi, 2001). In this study, we found a linear relationship between the infestation duration and the degree of attraction of *P. persimilis* to plant volatiles. An infestation density of 100 mites per plant or more is quite common in induction experiments (e.g., Sabelis and Van de Baan, 1983; Janssen et al., 1997; Dicke et al., 1999; Arimura et al., 2000). The present study shows that a spider mite density of only a few mites per plant and an infestation period of 2 days already results in a significant attraction of *P. persimilis.* Furthermore, infestation with 50 spider mites per plant resulted in a very high level of attraction of predatory mites, i.e., on average more than 90% of the predators preferred the prey-infested plants. In the literature, response levels up to 90% of the predators choosing

herbivore-induced plants or leaves have been recorded (e.g., Sabelis et al., 1984; Janssen et al., 1997; Dicke et al., 1999). This study shows that infestation densities leading to the induction of predator attractants are much lower than assumed and that maximal levels of attraction, i.e., 90% of the predators preferring volatiles from mite-induced plants, are reached at a density of 50 spider mites per plant.

Inducing Lima bean plants with increasing concentrations of JA also resulted in increased attraction of *P. persimilis* to JA-induced plant volatiles. However, the highest applied concentration of JA (1 mM) resulted in a response level comparable to a spider mite density of only four mites per plant. Increasing the JA concentration above 1 mM would likely lead to phytotoxic effects (10 mM JA is phytotoxic to Lima bean, personal observation). Hence, it seems impossible to reach predator response levels as high as with spider mite infestations through the application of JA. Furthermore, Dicke et al. (1999) demonstrated that predatory mites preferred plants infested with spider mites to JA-treated plants when tested in an olfactometer. Headspace analysis revealed that there is a large degree of resemblance between the blend emitted by plants induced with JA and plants induced with spider mite damage (Dicke et al., 1999; Gols et al., 1999). In the study by Dicke et al. (1999), the headspace of plants infested with 100 spider mites per plant was compared with the headspace of plants that were treated with 1 mM JA. They found that the total amount of emitted volatiles is similar, although quantitative and qualitative differences exist. A major difference is that JA-application does not induce methyl salicylate, which attracts *P. persimilis* (Dicke et al., 1990, 1999). The absence of methyl salicylate in the odor blend is a major determinant of the difference in predator attraction to spider-mite-infested and JA-induced plants (de Boer and Dicke, in press).

Plants can respond more rapidly after being induced repeatedly. Tobacco plants that were treated with MeJA increased their whole-plant nicotine pools more rapidly after consecutive inductions with MeJA than did untreated control plants (Baldwin and Schmelz, 1996). We found that plants that were treated with a JA concentration that does not induce attraction of the predators (0.1 mM), followed by a low-density spider mite infestation, were more attractive to *P. persimilis* than plants that were damaged by spider mites without a JA-treatment. The same degree of predator attraction was found when JA application and spider mite infestation were temporally separated. Plants can acquire enhanced resistance to plant pathogens after being exposed to pathogens or elicitors of plant resistance (Conrath et al., 2002). This study shows that exposure of plants to JA can enhance induced indirect responses to spider mite damage. This offers exciting options for improving biological control. Plant breeding strategies are mainly focused on enhancing plant resistance to herbivore attack, whereas manipulation of plant characteristics that facilitate the performance of natural enemies have been less studied (Cortesero et al., 2000). Application of elicitors such as JA that induce plant defense traits may enhance the effectiveness of biological control agents by releasing higher

amounts of attractive volatile compounds (Stout et al., 2002). Field studies have shown that the application of JA to plants led to reduced herbivore abundance and performance (Thaler, 1999) and increased plant fitness (Baldwin, 1998).

We also studied how spider mite infestation and JA-treatment affect settling behavior of newly arrived spider mites. Spider mites preferred leaf tissue that previously had been infested with conspecifics to uninfested leaf tissue. The herbivores preferred these sites even when all conspecifics and their products had been removed. Removing only the spider mites but not their products resulted in a much stronger preference for spider-mite-infested leaf material, which indicates that spider mite products themselves are at least partly responsible for the mite response. Spider mites may also assess the previous presence of conspecifics either through the changed chemical constitution of leaf tissue or by volatiles emitted by the damaged leaf. It is possible that brushing off spider mite products did not remove all cues left by the mites.

Spider mites also respond to volatiles emitted by plants. They are attracted to volatiles emitted by undamaged Lima bean leaves but tend to disperse when exposed to volatiles emitted by Lima bean leaves infested with spider mites (Dicke, 1986). On the other hand, Pallini et al. (1997) reported that spider mites preferred volatiles from cucumber plants infested with conspecifics to volatiles from uninfested control plants. In this study, spider mites preferred sites that had been occupied by conspecifics for 2 days. An infestation period of 7 days led to a reduction in preference for these infested sites. Bernstein (1984) demonstrated that spider mite emigration is positively correlated to the number of days of infestation, i.e., increased herbivore feeding damage resulted in increased emigration from these infested plants. Spider mites may benefit from sites where conspecifics are already present through reduced predation risk and lower costs involved with the production of webbing (Gerson, 1985). Extensive feeding will reduce the nutritious value of plant tissue, and this may outweigh the benefits of moving to sites that are already occupied by conspecifics and lead to reduced attractiveness and eventually to avoidance of already infested sites.

The response of spider mites to leaf discs taken from plants treated with JA was clearly not the same as the response to leaf discs taken from plants that had been infested with conspecifics. Spider mites marginally preferred water-treated leaf tissue to JA-treated leaf tissue. When previously infested leaf disc halves were tested against JA-treated leaf discs, spider mites had a strong preference for the former. Storms (1971) demonstrated that *T. urticae* injects saliva during feeding. By using radioatively labelled mites, Storms (1971) showed that the injected material is transported through the plant. Herbivore-derived elicitors may be needed to modify or induce the complete cascade of biosynthetic pathways involved in induced plant response to herbivore wounding. Other studies have shown that active agents in oral secretions from the herbivore induce the plant to produce volatile blends that resemble chemical emissions that are produced in response to feeding damage

(Mattiacci et al., 1995; Alborn et al., 1997; Halitschke et al., 2001). However, it cannot be excluded that mite products, which are not present on JA-induced plants, play a role in the behavioral response of conspecifics that have been recorded here. The absence of a herbivore-derived elicitor may also explain the difference between attraction of the predator to JA-induced and spider-mite-induced plant volatiles.

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INDUCTION OF HYPERICINS AND HYPERFORINS IN *Hypericum perforatum* IN RESPONSE TO DAMAGE BY HERBIVORES

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Abstract—Plants respond to herbivore and pathogen attack by a variety of direct and indirect mechanisms that include the induction of secondary metabolites. The phytomedicinal plant *Hypericum perforatum* L. produces two different classes of secondary metabolites:hyperforins, a family of antimicrobial acylphloroglucinols; and hypericins, a family of phototoxic anthraquinones exhibiting antimicrobial, antiviral, and antiherbivore properties *in vitro*. To determine whether these compounds are part of the herbivore-specific inducible plant defense system, we used an *in vitro* detached assay to assess the effects of specialist and generalist herbivore damage on the levels of hypericins and hyperforin. Greenhouse-grown *H. perforatum* plant sections were challenged with the specialist, *Chrysolina quadrigemina*, or with one of the following generalist feeders: *Spilosoma virginica, Spilosoma congrua,* or *Spodoptera exigua*. Feeding by the specialist beetle or mechanical wounding caused little change in phytochemical levels in plant tissue, whereas the small amount of feeding by the generalists caused 30–100% increases in hypericins and hyperforin as compared to control levels. Although the leaf damage index of the specialist feeding was 2.7 times greater, *C. quadrigemina* had little effect on *H. perforatum* chemical defenses in response to feeding damage in comparison to generalist feeding.

Key Words—*Hypericum perforatum*, induced resistance, *Chrysolina quadrigemina*, hypericins, hyperforin, herbivory, defense response, plant–herbivore interactions.

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INTRODUCTION

Plants possess a variety of biochemical and physical defense mechanisms that can deter, poison, or starve herbivores that feed on them (Hammond-Kosack and Jones, 1996; Karban et al., 1997; Constabel, 1999; Karban and Kúc, 1999; Stout and Bostock, 1999). Many plant secondary metabolites have toxic effects on a variety of herbivores and pathogens, known from studies *in vitro*, while other plant defenses appear to have indirect effects upon pests and pathogens, such as in attracting predators and in inhibiting insect oviposition. The induction of secondary metabolites, such as phytoalexins, has been documented in response to biotic challenges in a number of plant species (Creelman and Mullet, 1997; Constabel, 1999; Tebayashi et al., 2000), but it is still unclear whether secondary metabolites that are typically present are affected. Constitutive levels of phytochemicals have been correlated positively with inducibility in two studies (Zangerl and Berenbaum, 1990; Mitchell-Olds and Pedersen, 1998), but no relationship was reported in a third study (Brody and Karban, 1992).

The phytomedicinal plant *H. perforatum* L. (family Hypericaceae) is an herbaceous perennial species that grows well on disturbed sites especially throughout the Western United States, and is commonly called St. John's wort (Upton et al., 1997). The use of *H. perforatum* for over-the-counter treatment of mild depression has significantly increased in the last few years with a market size for St. John's wort over $400 million in 1998, and it continues in popularity (Grünwald, 1999). *Hypericum perforatum* L. contains phototoxic anthraquinones, the hypericins, as well as antimicrobial phloroglucinols. Both groups of compounds could be involved in the chemical defense arsenal of this species against herbivores and plant pathogens (Arnason et al., 1983). Since the purported health effects and market value of this plant are linked to the amounts of hypericins and hyperforins present, it is of interest to determine what factors may be responsible for inducing high levels of the phytochemicals in *H. perforatum*. Several studies have noted the variability of hypericin levels in the wild, including those *H. perforatum* populations in Australia (Southwell and Campbell, 1991; Campbell et al., 1997; Southwell and Bourke, 2001;), Nova Scotia (Jensen et al., 1995), Switzerland (Büter et al., 1998), Armenia (Melikian et al., 1998; Kirakosyan et al., in press), and the United States (Walker et al., 2001; Sirvent et al., 2002). In our previous study, we observed that two population sites differed from all other sites in that the levels of hypericins were higher in the leaf material as compared to the reproductive plant material. These two sites had a high incidence of either herbivory or an increased exposure to herbivory pressures. We speculated that the elevation of hypericins in leaf samples from these sites might represent a plant defense response, and demonstrated that plantlet tissues had higher levels of both hypericins and hyperforins when treated with the plant elicitors, methyl jasmonate or salicyclic acid, in comparison to control plantlets (Sirvent and Gibson, 2002).

To further determine whether hypericins and hyperforin are components of an inducible plant defense response in St. John's wort, we assessed the effects of both specialist and generalist herbivore damage on secondary metabolite production. For a specialist feeder, we chose the biocontrol agent, *Chrysolina quadrigemina*, and three generalist feeders not known to preferentially attack *H. perforatum*, *Spilosoma virginica* (ginger bear), *Spilosoma congrua,* and *Spodoptera exigua* (fall armyworm). In 1944, *C. quadrigemina* was initially imported into the United States in California from Australia where it had been used as a successful biocontrol agent for *H. perforatum* in rangelands (Campbell, 1988); additional releases also occurred in Northern Idaho (Campbell, 1988) and Montana (Clayton, 1978). *C. quadrigemina* has no preference for crop plants such as sugar beet, flax, hemp, sweet potato, tobacco, and cotton (Clayton, 1978), and has been considered an effective biological control for range populations of *H. perforatum* in the United States.

METHODS AND MATERIALS

Plant Material. The New Stem and Anthos cultivars were established from seed in the greenhouse; these two cultivars have similar gland densities and levels of phytochemicals in leaf foliage (Kirakosyan et al., in press). Seeds were germinated in flats filled with metro-mix soil that were kept moist under a 16:8 hr; L:D cycle at a light intensity of 234 μE/m/sec^2. Newly emerged seedlings were transferred to 2" pots and watered three times a wk. Once seedlings were approximately 6" tall, plants were transplanted to 4" pots and watered three days a wk. Mature plants were transplanted to 12" pots and watered twice a wk.

A detached leaf assay was used in order to assess effects of herbivory on foliage in controlled environmental conditions. The top 3 inches of vegetative plant material was randomly harvested from five plants that were grown from seeds obtained from the same seed lot. Cut material was placed into arenas. An individual arena was created by using a 20 × 15-mm petri plate that was predrilled with a 2.0-mm hole on the side, which was then permanently attached to the inside of a 250 × 15-mm petri plate. A cut stem containing leaf foliage was placed through the hole in the side of the smaller petri plate that was then filled with water-soaked cotton. One piece of filter paper no. 1 (Whatman®) was placed under the plant tissue and lightly sprayed with deionized water. The lid of the larger petri dish was placed on top as a cover to the entire arena. Deionized water was added daily into the 20 × 15-mm petri dishes.

Herbivores C. quadrigemina (Biological Control of Weeds, Inc.; Bozeman, Montana) were shipped overnight via FEDEX. Upon receipt, *C. quadrigemina* beetles were transferred to a *H. perforatum* plant growing in a 6" pot placed inside a mesh container housed in an incubator set at 28°C under a 16:8 hr L:D

(138 μE/m/sec^2). Two adult females of *S. virginica* and *S. congrua* were captured near Ithaca, New York and allowed to oviposit on paper; the eggs were kept separately at 25°C until hatching occurred. Newly emerged caterpillars were placed in sealed containers with either foliage of *H. perforatum* or wild-harvested dandelion. Caterpillars were typically maintained on dandelion in an incubator set at 24°C under a 16:8 hr, L:D cycle. A small screen containing *S. exigua* (fall armyworm) eggs (provided by Lee French) was placed on fall armyworm (FAW) diet (Bioserve®) and kept in an incubator set at 24°C under a 16:8 hr, L:D cycle. Egg hatch typically occurred at 3–4 days, and larvae were then transferred to fresh FAW media at one wk. Larvae at 3rd and 4th instar stages were used for herbivory challenge experiments.

Herbivore Challenge Treatments. Thirteen replicates of one, two, or three *C. quadrigemina* beetles were added per arena as described above in an incubator set at 24°C under a 16:8 hr, L:D cycle, and allowed to feed on *H. perforatum* foliage for 24 hr, and then removed. Plants were kept hydrated in the incubator for an additional 48 hr. Experiments were independently replicated three times, and the data were pooled for statistical analysis.

Ten *S. virginica* or thirteen *S. congrua* 3rd instars per treatment were transferred to individual arenas containing 4" samples of *H. perforatum* cultivar New Stem. Caterpillars were kept in the arenas inside an incubator set at 24°C under a 16:8 hr, L:D cycle for of 24 or 48 hr. After caterpillar removal, the plant material was left in the incubator under the same conditions for an additional 48 hr (96 hr total), at which time the plant material was scored for herbivory by assessing the percent leaf consumption of each leaf for the entire plant sample using a damage index. The index quantitatively describes the amount of damage: 0 indicates that no herbivory occurred, 1 represents that less than 10% herbivory, 2 denotes that more than 10% but less than 25% damage was sustained by the leaf, 3 signifies that at least 25% but less than 45% damage was incurred, 4 represents at least 45% but less than 75% damage, and 5 represents damage that was greater than 75% of the leaf area. The damage index for each leaf was recorded, and an average damage index number was obtained for the entire plant cutting by adding the individual damage index values for the cutting, then dividing by the number of leaves present. The number of attacks per leaf was also recorded.

Leaf material was harvested and stems were removed prior to fresh weight measurement, and then placed into 50 ml Erlenmeyer flasks, lyophilized overnight, and the dried material weighed. Samples were crushed using a tissue homogenizer, and extracted with 15 ml acetone by constant shaking at 150 rpm overnight on an orbital shaker. The crude plant extract was collected via filtration through Whatman no. 1 paper on a Büchner funnel, evaporated on a rotary evaporator, reconstituted in 100% MeOH, and analyzed by HPLC (Sirvent and Gibson, 2000).

For *S. exigua*, 2-week-old 3rd instars larvae were placed on Anthos *H. perforatum* foliage placed within the arenas, and placed inside an incubator set at 24°C under a 16:8 hr, L:D cycle. Fall armyworms were allowed to feed on foliage for 48 hr at which time they were removed, and the plants were kept hydrated in the incubator for an additional 48 hr. Samples were scored for damage, harvested, extracted, and analyzed as described above.

Mechanical Wounding. In order to mimic the damage caused by herbivory by artificial means, leaves were mechanically cut with a pair of scissors. Two different treatments of wounding were used to obtain two subsequent levels of damage. In one treatment, 25% of the leaf area on four leaves was cut. In the second category of wounding, 50% of the leaf area (along one side of the midrib) on five leaves was removed. The wounded plant portion was placed into the specialized arena and kept moist for a period of 24 hr, at which time, the plants were scored for damage, harvested, and extracted as described.

HPLC Analysis. HPLC analysis of hypericins was carried out as described. (Sirvent and Gibson, 2000), except using a micro Diazemphenyl™ column (Metachem®, 5 μm, 50 $\times$ 4.6 mm), in tandem with a guard column of the same material. Retention times and absorption spectra were compared to authentic standards to confirm presence of hypericins and hyperforin.

Statistical Analysis. Data was analyzed using JMP® statistical software; Dunnett's post-hoc test ($P < 0.05$) was used for sample sets with untreated controls, while Tükey–Kramer's post-hoc test ($P < 0.05$) was used for comparisons within treatments.

RESULTS

Damage Index of Generalist and Specialist Herbivores on St. John's Wort. Preliminary assessments of foliar changes in whole plants due to herbivory were complicated by the use of greenhouse conditions and plant-to-plant variability, so a detached leaf assay system allowing multiple replicates per treatment under controlled environmental conditions was developed, similar to those typically employed for plant resistance screening and gene expression studies (Fitches et al., 1997; Wegulo et al., 1998; Bieri et al., 2003). *C. quadrigemina* is a specialist beetle that feeds on several *Hypericum* species, and can cause significant defoliation on *H. perforatum*. The adults of *C. quadrigemina* are dark colored, and their testa prevents UV radiation from entering their body and photoactivating ingested hypericin (Fields et al., 1990). In this study, *C. quadrigemina* were able to feed on plants in light, similar to previous observations in the field (Campbell, 1988; Fields et al., 1990). *C. quadrigemina* displayed no feeding preference for either young or old leaves of *H. perforatum* and did not avoid or show attraction to the hypericin-containing glands located along the leaf margins. They did, however,

TABLE 1. LEVEL OF HERBIVORY DAMAGE INFLICTED ON *H. perforatum* BY SPECIALIST AND GENERALIST HERBIVORES OR BY WOUNDING

Herbivore	# of Attacks	Damage index[a]
C. quadrigemina		
1 beetles	0.25 ± 0.10b	0.53 ± 0.13b
2 beetles	0.43 ± 0.15a	1.16 ± 0.62a
3 beetles	0.48 ± 0.03a	1.38 ± 0.16a
Mechanical Wounding		
4 leaves at 25%	0.22 ± 0.22	0.44 ± 0.05b
5 leaves at 50%	0.21 ± 0.03	0.85 ± 0.11a
S.virginica		
24 hours	0.15 ± 0.02a	0.020 ± 0.001
48 hours	0.25 ± 0.19b	0.023 ± 0.002
S. congrua		
24 hours	0.36 ± 0.02b	0.022 ± 0.002
48 hours	0.49 ± 0.02a	0.026 ± 0.002

Note. Values represent $N = 10 \pm$ SE, different letters represents a significant difference between treatments using Tükey–Kramer's post hoc test ($P < 0.05$).

[a] Damage index indicates the severity of the herbivory damage: 0 = *no damage*, 1 = 10% *or less of the leaf consumed*, 2 = *greater than* 10% *but less than* 25%.

exhibit unusual feeding behavior in that over 60% of the time, individual feeding would occur on one leaf until longitudinally 50% of the leaf was consumed, ie., either the leaf tissue on one side of the midrib or the other. This behavior has not been described previously in the literature, although *C. quadrigemina* larvae have been observed to feed on leaf margins as a possible concealment from irradiation by curling up under the leaf (Fields et al., 1990). Consumption of hypericin-containing tissue does not appear to harm the specialist beetle since hypericins are presumably eliminated via the digestive tract (Duffey and Pasteels, 1993).

C. quadrigemina fed on the leaf tissues of the plant at random. The more beetles present per arena, the greater the number of attacks per plant observed ($df = 2$, $F = 9.37$, $P < 0.001$). In general, the beetles had a large area of damage per attack ($df = 2$, $F = 7.29$, $P < 0.001$) as measured by the damage index (Table 1). This is in contrast to the *Spilosoma* generalist species that mounted several attacks but caused little leaf damage per attack (*S. virginica* $df = 1$, $F = 13.73$, $P < 0.001$; *S. congrua* $df = 1$, $F = 52.30$, $P < 0.001$). On average, the specialist caused up to 69 times more damage than either of the *Spilosoma* generalists even though the number of attacks either stayed the same (for the *S. virginica* species) or increased by a factor of two (*S. congrua*).

We also conducted mechanical wounding in a manner to mimic the damage as a result of the herbivory caused by *C. quadrigemina*. The number of wounds

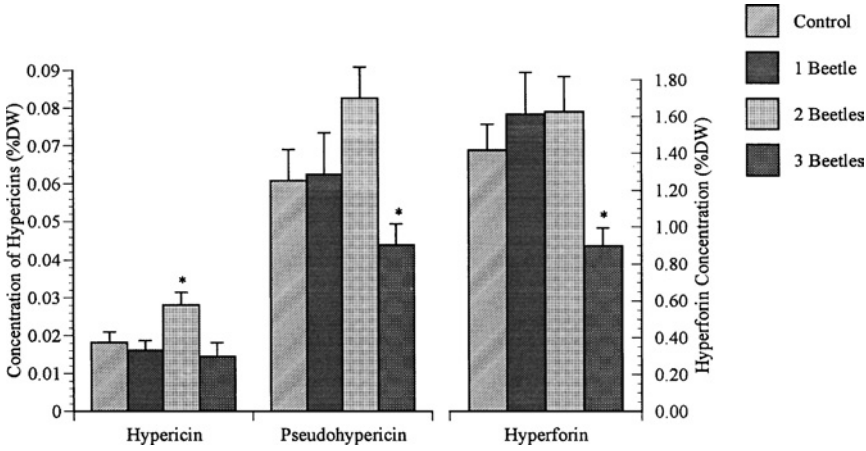

FIG. 1. Specialist herbivory effects on the phytochemical composition of *H. perforatum in planta*. Values represent $N = 13 \pm$ SE, and * denotes those values that are significantly different from the control using Dunnett's post-hoc statistical test, $P < 0.05$.

remained statistically the same between the two treatments, but the area of damage per wound increased in the treatments (df $= 1$, $F = 13.59$, $P < 0.002$) (Table 1). Overall, the amount of damage caused by the mechanical wounding was within the range of damage caused by the specialist herbivore.

Specialist Effects on the Concentrations of Hyperforin and Hypericins. Since the amount of damage to leaf tissue varied between specialists and generalists, stem tissue that constituted a large amount of biomass that could bias the analysis was removed so that only leaf tissue was used for extraction and assessment of phytochemicals. The extensive damage caused by *C. quadrigemina* did not induce significant levels of hypericins (df $= 3$, $F = 3.11$, $P = 0.041$) in the remaining foliage as compared to controls when challenged with 1 or 3 beetles per arena, although there was an increase in hypericin levels with the two beetles per arena treatment (Figure 1). Also, changes in pseudohypericin (df $= 3$, $F = 1.67$, $P = 0.194$) and hyperforin (df $= 3$, $F = 1.62$, $P = 0.206$) levels were not increased in comparison to controls, although there was a tendency to increased levels with the one- and two-beetle treatments. At the highest herbivory treatment using three beetles per arena, all phytochemical levels were lower than the control. When mechanical wounding was used to damage plant tissue to the same degree as that caused by herbivory, there were also no increases in the concentration of the phytochemicals but decreases were seen in hypericin (df $= 2$, $F = 3.28$, $P = 0.055$), pseudohypericin (df $= 2$, $F = 2.59$, $P = 0.096$), and hyperforin (df $= 2$, $F = 4.79$, $P = 0.019$) *in planta* (Figure 2).

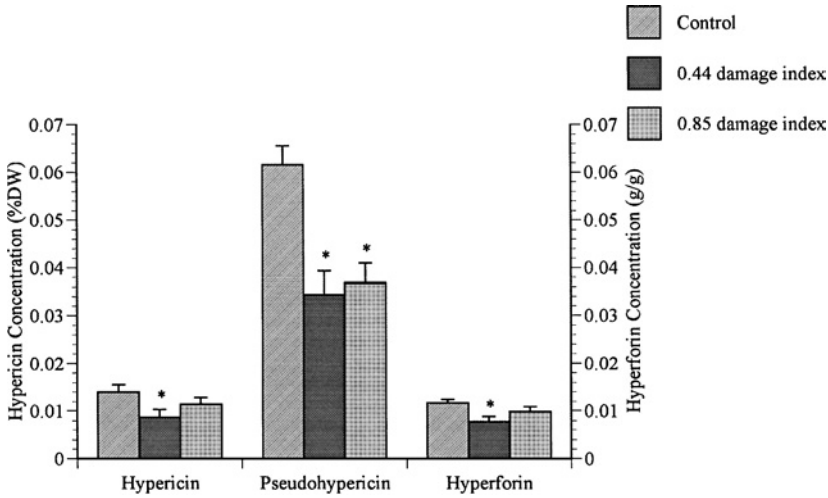

FIG. 2. Effect of artificial wounding (either from a 25% or 50% wounding treatment—which corresponds to a damage index of 0.44 ± 0.05 and 0.85 ± 0.11, respectively) on the phytochemical composition of *H. perforatum in planta*. Values represent $N = 10 \pm$ SE, and * denotes those values that are significantly different from the control using Dunnett's post-hoc statistical test, $P < 0.05$.

Biotic Challenges with Generalist Herbivores. Three generalists were chosen to determine the effect that generalist herbivores might have on hypericin, pseudohypericin, and hyperforin levels in *H. perforatum*. *S. virginica* was reared on dandelion or *H. perforatum* from hatching. Those feeding on dandelion were healthier and grew faster than those on *H. perforatum*. After 4 weeks, all *S. virginica* individuals fed on *H perforatum* foliage were dead. Observations of *S. virginica* feeding patterns on *H. perforatum* indicated that the caterpillars avoided the hypericin-containing glands on leaf tissue and sporadically fed along the leaf margins, causing minimal damage. This generalist feeding behavior was found in contrast to the *C. quadrigemina*'s feeding behavior where leaf tissue was consumed in whole on either side of the midrib.

Fourth instars of *S. virginica* and *S. congrua* reared on dandelion from hatching were starved overnight before they were placed on *H. perforatum* plants to feed for a period of 24 or 48 hr, then removed, and the plants harvested after an additional 24 and 48 hr. The average combined herbivory damage index for the two *Spilosoma* species was 0.021 and 0.024 for the 24 and 48 hr treatments, respectively. Both caterpillars caused similar inductions of hypericin (*S, virginica* $\text{df} = 2, F = 2.18, P = 0.038$; *S. congrua* ($\text{df} = 2F = 3.85, P = 0.032$), and pseudohypericin (*S, virginica* $\text{df} = 2, F = 3.31, P = 0.050$; *S. congrua* df =

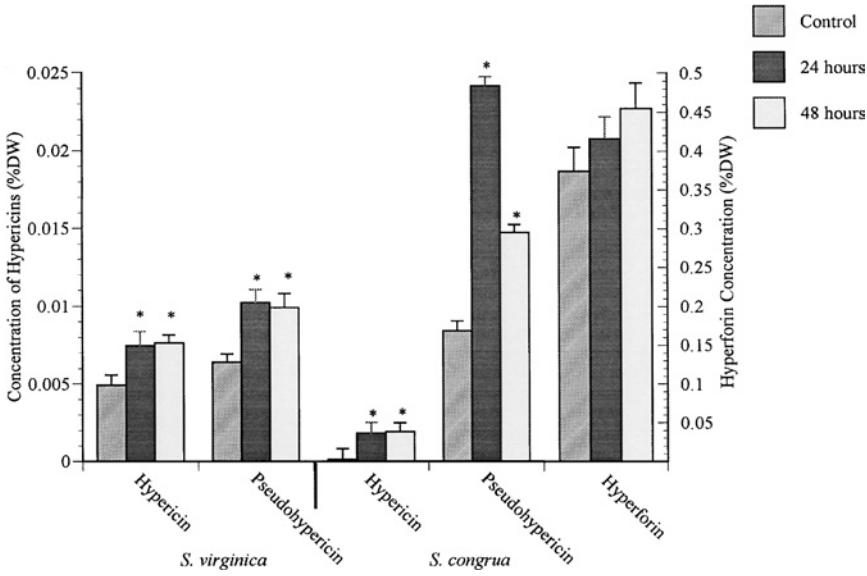

FIG. 3. Levels of hypericin, pseudohypericin, and hyperforin in *H. perforatum* plant cuttings after being challenged by two different *Spilosoma* species (generalist herbivores). Values represent $N = 13 \pm$ SE, and * denotes those values that are significantly different from the unchallenged control using Dunnett's post-hoc test, ($P < 0.05$).

$2F = 5.38$, $P = 0.009$) in foliage tissues after 24 and 48 hr of feeding (Figure 3). Significant increases in the levels of hypericins and pseudohypericins were observed with respect to the control levels of the phytochemicals in nonchallenged *H. perforatum* plant cuttings. Levels of hyperforin, although increased to some degree, were not significantly higher (df $= 3$, $F = 1.629$, $P = 0.212$) than control plants for *S. congrua* (Figure 3).

Another generalist herbivore was also tested to determine if the increase in hypericins was a generalized response to herbivore attack regardless of species. *S. exigua* was chosen because of its diverse dietary habits and its dissimilarity to *S. virginica*. *S. exigua* did not feed on *H. perforatum* plant material from hatching, so *S. exigua* was reared on fall armyworm (FAW) diet prior to testing on *H. perforatum* foliage. Individual caterpillars were transferred to separate arenas containing *H. perforatum* plant material and allowed to feed for a period of 48 hr. *S. exigua* did not cause as much damage as *S. virginica*. In order to achieve approximately the same levels of herbivory damage as recorded by the average damage index (0.024) in the previous experiment with *S. virginica* and *S. congrua*, the caterpillars were allowed to feed for 48 hr. *S. exigua* fed along the margins of the leaf similar in behavior to the previous two *Spilosoma* species.

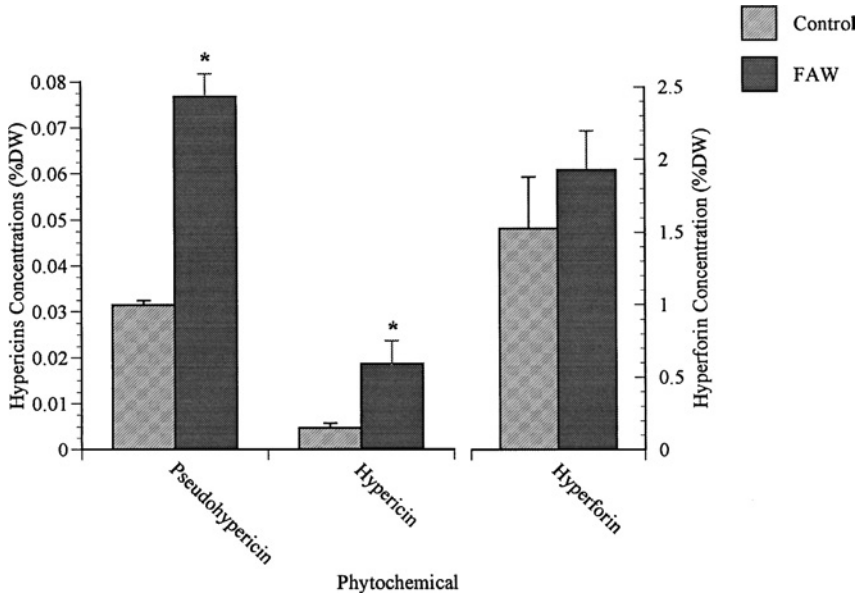

FIG. 4. Levels of hypericin, pseudohypericin, and hyperforin in *H. perforatum* plant cuttings after being challenged by *S. exguia* herbivores. Values represent $N = 13 \pm$ SE, and * denotes those values that are significantly different from the unchallenged control using Dunnett's post-hoc test, ($P < 0.05$). FAW = Fall armyworm.

Challenges by *S. exigua* resulted in increased levels of hypericin (df $= 1$, $F = 5.93$, $P = 0.030$) and pseudohypericin (df $= 1$, $F = 11.59$, $P = 0.004$), as compared to nonchallenged controls, similar to the results obtained with *S. virginica* and *S. congrua* (Figure 4). Levels of hyperforin were not significantly increased (df $= 2$, $F = 0.97$, $P = 0.343$) (Figure 4). In response to all generalist herbivore challenges, levels of hypericin increased in the range of 1.55 to 19 times that of control plant cuttings. Challenge of *S. virginica* to *H. perforatum* resulted in the greatest increase. Pseudohypericin increases were in the range of 1.6–2.4 times that of control plants. The greatest increase in levels was observed with those cuttings challenged with *S. exigua*.

DISCUSSION

Toxic proteins and secondary metabolites are commonplace in diverse plant families, suggesting that their abundance may be due in some part to the selective pressure of herbivory (Ehrlich and Raven, 1964; Berenbaum, 1983, 1990). Various phytochemicals such as phenolics (Dixon and Paiva, 1995), terpenoids

(Gershenzon and Croteau, 1991), and alkaloids (Hartmann, 1991), proteins (Bowles, 1990), protease inhibitors (Ryan, 1990), oxidative enzymes (Duffey and Stout, 1996), and even physical barriers such as trichomes and hairs (Agrawal, 1999) can all be defense responses that are induced in response to herbivore attack as well as plant wounding (Bennett and Wallsgrove, 1994). What has been unclear in the literature to date, however, is whether constitutively expressed secondary metabolites can be induced to higher levels under herbivore or pathogen attack and whether those levels serve defensive roles. Total herbivory damage caused by only one type of herbivore on leaves has been reported to be unrelated to the phytotoxic activity of host plant tissues (Guillet, 1997; Schappert and Shore, 1999).

Even though a high damage index was present in treatments with one or two beetles per arena, we observed no changes in either pseudohypericin or hyperforin concentrations relative to control, although there was an increase in hypericin levels in the two-beetle treatments. Since *C. quadrigemina* neither avoided nor sought out gland-containing regions on the leaf tissue, the remaining tissue that was undamaged represents a net reduction in total dry weight having a relatively constant percentage of phytochemicals present. Only in treatments with three beetles per arena did we observe reduced phytochemical concentrations, possibly due to the large loss of leaf tissue. At this higher level of herbivory, we observed leaf sections with remaining midribs, a tissue type that has no glands, constituting a higher percentage of the sample mass and, thus, a possible dilution of chemical concentration.

This evidence suggests that *H. perforatum* does not possess any feeding deterrents for the specialist herbivore (Guillet, 1997). These results differ from those reported by Guillet et al. (2000) where three specialist herbivores (including two beetle species)—*Galeruca tanaceti, Cloantha perspicillaris*, and *Chrysolina geminata*—were compared to three generalist herbivores, *Ruspolisa nitidula, Conocephalus discolor*, and *Tettigonia viridissima*, in terms of their feeding behaviors. In that particular study, the selective ingestion index was the same for all species tested, except for the generalist *R. nitidula* whose selective ingestion was five times higher than the pooled value of all the other insect species. In our study, we used generalists that were never observed to feed on *H. perforatum*, whereas the generalist species in the Guillet study had been observed to feed occasionally on *H. perforatum* plants in southern France. As a result, the generalists in the Guillet study may have developed at least a partial mechanism for protection against hypericins and/or hyperforins.

In this study, damage done to *H. perforatum* by the specialist, *C. quadrigemina* was up to 69 times greater than damage caused by *S. virginica* caterpillars or by *S. exigua*. However, the generalists differed in their herbivory patterns and their effects on the phytochemicals, hypericin and hyperforin. Levels of both hypericins and hyperforin increased following attack with generalist herbivores, whereas there

was little change or decrease by treatment with artificial wounding or challenge by the specialist herbivore, *C. quadrigemin*a. In this study, the leaves that suffered herbivory damage as a result of generalist treatments began to look chlorotic when harvested. Although we did not investigate this phenomenon, lower transcripts of photosynthetic genes have been reported for interactions of *Manduca sexta* and its host *Nicotiana attenuata* (Hersmeier et al., 2001).

It is possible that *C. quadrigemina*, as a specialist on *H. perforatum*, like other chrysomelid beetles that feed on *Hypericum* species, does not elicit chemical defenses due to herbivory damage. Specialist feeders have been theorized to have a higher fitness on plants with higher amounts of chemical defenses because they have circumvented the specific defense system. Although some studies have reported that specialist herbivores can induce secondary metabolite production in herbivorized plant material (Halitschke et al., 2001), the phytochemical coevolution theory would predict that specialists have co-evolved with their respective hosts so that they are able to evade or cope effectively with the plant's defenses (Ehrlich and Raven, 1964), thus not potentially triggering any phytochemical induction or other plant defensive mechanisms (Cornell and Hawkins, 2003). In a recent study using black mustard and two specialist herbivores, *Pieris rapae* or *Phyllotreta cruciferae*, no increase in glucosinolate concentrations or trichome densities in leaf tissue occurred after damage by *P. cruciferae*, while substantial increases were seen in tissue damaged by *P. rapae* (Traw and Dawson, 2002).

The generalist herbivores used in this study were poor feeders on *H. perforatum* foliage. This result was expected since many generalists do not perform well on plants that contain high levels of phytochemicals or toxins; rather, they typically show increased fitness on plants lacking or having low concentrations of chemical defenses (van der Meijden, 1988). Although in many cases attack by generalist herbivores can stimulate defense responses in plants (Baldwin and Preston, 1999; Reymond et al., 2000), it is not known whether the induction of those defense responses can cause decreased fitness of the generalist. There is one report noting that two generalists, *T. viridissima* and *C. discolor* (which have been known to feed occasionally on *H. perforatum*), are well adapted to avoid the toxic effects of hypericins by having a higher constitutive level for glutathione reductase and glutathione S-transferase as compared to two chrysomelid specialists, *G. tanaceti* and *C. geminata* (Guillet et al., 2000). In our study, however, *H. perforatum* foliage did not serve as a sustainable food source for at least two generalists since the larvae died within 2 weeks of rearing, and the other generalist could not be reared on the foliage. As a result, the overall fitness of *S. virginica, S. congrua*, or *S. exigua* may be lower than those generalists included in the Guillet study.

We have shown in previous work that *H. perforatum* responds defensively to salicylic acid, methyl jasmonate, and a plant pathogen by increasing phytochemical levels as a defense response (Sirvent and Gibson, 2002). In this current study, we have found that generalists, but not a specialist, herbivores can elicit defense

responses. How *C. quadrigemina* disguises the herbivore-specific cues to silence plant defense responses is, as yet, unknown.

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ANALYSIS OF A CHEMICAL DEFENSE IN SAWFLY LARVAE: EASY BLEEDING TARGETS PREDATORY WASPS IN LATE SUMMER

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Abstract—Contact of certain sawfly larvae with predators frequently elicits an easy bleeding behavior involving local disruption of the integument and release of hemolymph droplets. The efficacy of this putative antipredator defense was investigated in the turnip sawfly, *Athalia rosae*, by using common garden experiments in which particular guilds of predators were excluded, and through manipulations of the chemical defense of larval prey. After 3 d of exposure to walking and/or flying arthropod predators, the proportion of surviving sawfly larvae remained quite high at 71.3% in midsummer, but dropped to 37.5% in late summer. However, *Pieris rapae* caterpillars without the bleeding defense were killed within hours by predatory wasps (*Vespula vulgaris*). Topical treatment of *P. rapae* caterpillars with sawfly hemolymph or sinalbin, a glucosinolate sequestered by the sawflies, revealed that the easy bleeding of *A. rosae* is an efficient defense against wasps, partially due to this compound. These experiments are the first to demonstrate the efficacy of this chemical defense mechanism in a natural setting.

Key Words—*Athalia rosae*, bleeding defense, field experiment, glucosinolate, hemolymph, *Pieris rapae*, predation, sinalbin, *Vespula vulgaris.*

INTRODUCTION

Many insects when disturbed release fluids that are thought to play a defensive role (Kay et al., 1969; Eisner et al., 1974; Smedley et al., 2002) but whose effectiveness has seldom been demonstrated in field experiments (e.g., Heads and Lawton, 1985;

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Stamp, 1992; Bowers and Stamp, 1997). Some of these attempts have failed to show deterrence in the field (Peck, 2000). The easy bleeding phenomenon found in several sawfly species belonging to the Tenthredinidae (Hymenoptera) is characterized by a low mechanical resistance of the integument with damage to any part of the body, provoking the release of hemolymph at the site of damage (Boevé and Schaffner, 2003). The efficiency of this mechanism as a defense against predators has been demonstrated by offering larvae in containers to potential predators or testing the effect of hemolymph or its extracts in the laboratory (e.g., Ohara et al., 1993; Schaffner et al., 1994; Boevé and Schaffner, 2003). Birds and shrews have been examined in addition to arthropod predators such as ants, spiders, and bushcrickets. In the easy bleeding species *Athalia rosae* L. (Hymenoptera: Tenthredinidae) (Figure 1a), the larval hemolymph contains glucosinolates sequestered from cruciferous host plants (Müller et al., 2001). Feeding inhibition activity of

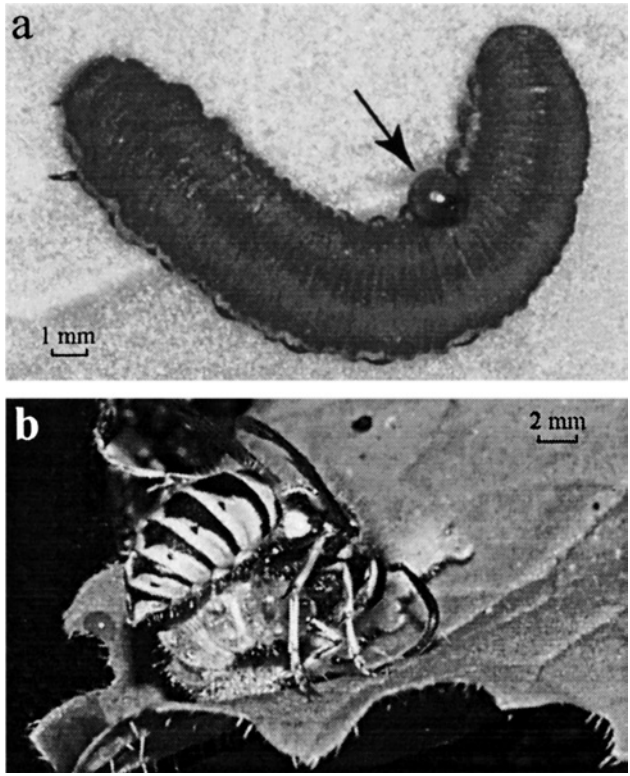

FIG. 1. (*a*) Easy bleeding of a disturbed *Athalia rosae* larva (head to left) with arrow indicating hemolymph released. (*b*) Attack on a *Pieris rapae* caterpillar with hemolymph exuding from a cut in its ventral fore end which is curled upwards below the wasp.

the hemolymph against the ant *Myrmica rubra* L. was demonstrated to be partly due to these secondary plant compounds (Müller et al., 2002) that are known for their deterrent effects on various generalists (Li et al., 2000; Potter et al., 2000).

However, the efficiency of the sawfly defense *via* easy bleeding, and the impact of different natural predators has not been studied in the field. In this study, we used a series of common garden experiments with wild predators to examine the survival of released larvae when excluding different guilds of arthropod predators (flying or walking). The studies were done at two different seasons of activity of *A. rosae*. In late summer, *Pieris rapae* L. (Lepidoptera: Pieridae) butterfly caterpillars were also released as a control species that does not show easy bleeding. These caterpillars feed on the same host plant, *Sinapis alba* L., but do not sequester the glucosinolate 4-hydroxybenzylglucosinolate (sinalbin) (Müller et al., 2003a). Common predatory wasps, *Vespula vulgaris* L. (Hymenoptera: Vespidae), were found to have a high impact, especially on the caterpillars. Therefore, we tested the role of the sawflies' hemolymph and of the glucosinolate sinalbin in the defense against wasps by using manipulation assays. The hemolymph released by easy bleeding and its chemical components proved to be in part responsible for the efficient defense of the sawfly against wasps.

METHODS AND MATERIALS

Insects and Plants. Athalia rosae larvae were obtained from a laboratory culture kept on 4–5-wk old, preflowering plants of *Sinapis alba* (cultivar: SALVO, seeds from Advanta Seeds B.V., The Netherlands) at 20°C, 70% R. H. and L16:D8 photoperiod. Eggs of *Pieris rapae* were obtained from a culture at Wageningen University, and hatching caterpillars were transferred to *S. alba* plants under the same conditions as for *A. rosae.*

Predator–Exclusion Experiments. To investigate the impact of different arthropod predator guilds on the survival of larvae in a natural setting, experiments were conducted in an experimental garden on the edge of the city of Leiden, The Netherlands. Eight plots of bare ground (1 × 1 m) were chosen in a single 10 × 20 m area of mixed herbs and shrubs. In each plot, seven (experiment 1) or eight (experiment 2) pots (diam: 11 cm × height:10 cm) were placed in a regular pattern (30 cm apart) in the ground. Each pot contained three *S. alba* plants, and each was surrounded by a cage (100 × 100 × 95 cm, $l \times w \times h$) made of hexagonal mesh iron wire (mesh width: 2.5 cm) to exclude larger animals such as birds or rabbits. To exclude the access of walking predators, several plant pots were surrounded by plastic cylinders (diam: 15 cm) that were partly dug into the soil, leaving 5 cm of the cylinder above ground. The upper edges of the cylinders were treated with sticky band (4.5 cm, Aeroxon, Germany) and vaseline so that walking arthropod predators could not cross the cylinders and, thus, had no access to the plants. Differently treated pots were distributed at random both over and within plots.

When the plants were 4-wk old (about 40-cm height, not flowering), 15 larvae (14 d old, 2nd–3rd instar) were placed on each plant. This density is comparable to those observed in infested *Sinapis*-fields in the Rhine Valley (Germany), which ranged from 0 to 25 larvae per plant (personal observation). After 3 d, surviving larvae were collected, counted, and brought into the laboratory. They were reared to the adult stage to detect any parasitization. To assay potential predators present in the field, pit-fall traps (250 ml) were placed in the four corners of every plot and filled with 50-ml salt-saturated water. One blue (32 × 7 cm) and one yellow (13 × 15 cm) sticky flying insect trap were attached at a height of 50 cm at each wire cage. On the second experimental day, each plant was screened for the presence of predators at hourly intervals, from 7.30 until 17.30 hr.

Experiment 1. Influence of Different Predator Guilds on Sawfly Larvae in Mid-Summer. The experiment was carried out in late June 2002, when the first generation of *A. rosae* larvae was found in the wild. Three plants were surrounded by plastic cylinders to exclude walking arthropods. One of these, together with two additional plants, was covered by a fine gauze cage of 90 × 35 × 70 cm ($l \times w \times h$) that was open up to 2 cm above the ground to exclude flying predators but allow access by walking. Thus, each experimental block consisted in total of seven plants, two plants accessible to walking predators only, two plants accessible to flying predators only, two plants accessible to all predators, and one control plant (exclusion of all predators) (Figure 2A).

Experiment 2. Influence of Predators on Sawfly Larvae Compared to Caterpillars in Late Summer. This experiment was carried out in mid-September 2002, when the last generation of the year of *A. rosae* larvae was active. Because of the lack of any comparison of predation-rate in experiment 1 relative to control herbivores, we decided to introduce another larval species that showed no easy bleeding. To do this we included only one treatment aimed at a single predator guild, as only small differences had been detected between these treatments in the earlier experiment. Four pots in each plot were surrounded by plastic cylinders to exclude the access of walking predators, while all predators had access to another four pots. To compare the easy bleeding species with a nonbleeder, two plants of each type were hosts to *A. rosae* larvae, while the other two were hosts to *P. rapae* caterpillars of similar size (Figure 2C).

Experiment 3. Impact of Wasps. During experiment 2, many wasps were observed visiting the plants (see below). Thus, the following additional experiments were designed to study their specific role as predators on *A. rosae* and *P. rapae*.

Experiment 3a. Predation Pressure From Wasps. Ten uninfested *S. alba* plants were spread among the plots in the experimental garden. Each plant received either seven *A. rosae* (4th–5th instar) larvae or seven *P. rapae* (3rd–5th instar) caterpillars. Observations began immediately and continued for 3 hr. After 24 hr, the number of surviving individuals was counted.

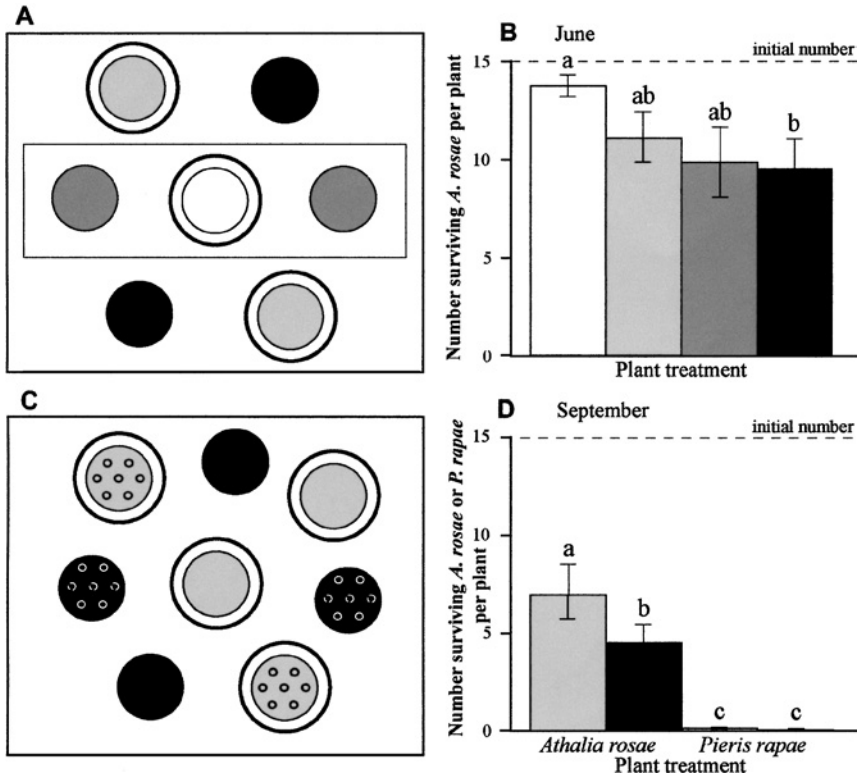

FIG. 2. June (A, B) and September (C, D) experiment examining survival of *Athalia rosae* sawfly larvae and of *Pieris rapae* caterpillars. Maps (A, C) show experimental plots with potted *Sinapis alba* plants receiving sawfly larvae only (A) or sawfly larvae (*blank circles*) and caterpillars (*spotted circles*) (C). From half of the pots, walking predators were excluded (*outer black circles*), from three pots flying predators were excluded (*circles inside rectangular block, A*). Differently treated pots were distributed at random over and within plots. Histograms (B, D) show mean number ($\pm$ SD) of sawfly larvae and caterpillars remaining after three days on the plant pots, with exclusion of all (*white*), walking (*light grey*), flying (*dark grey*), and no (*black*) predators (in D exclusion of walking or no predators only). Letters above bars indicate significant differences (ANOVA, Scheffé-test: $P < 0.05$).

Experiment 3b. Role of Sawfly hemolymph in the Defense Against Wasps. Some sawfly larvae did not succumb to wasps. Therefore, to test whether the hemolymph of *A. rosae* was responsible for the defense, *P. rapae* caterpillars (5th instar) were treated with 8 μl of freshly collected hemolymph of either *A. rosae* (*A*-caterpillars) *or P. rapae* (*P*-caterpillars) in the laboratory. Hemolymph treatment was done by carefully piercing the integument of a larva with a needle

without damaging the gut, collecting exuding hemolymph in a capillary, and applying it in a line dorsally on the thorax and abdomen of a *P. rapae* caterpillar. Caterpillars were transferred to the experimental garden and, about 30 min later, five caterpillars of one or the other treatment were offered per *S. alba* plant, on four plants in total (10 caterpillars/treatment). The distance between the plants was 30 cm, with plants arranged in a square. Observation started at about 9.30 A.M. and ended when all caterpillars had been touched by at least one wasp.

Experiment 3c. Role of the Plant–Derived Glucosinolate Sinalbin in the Defense Against Wasps. To test the effect of a glucosinolate on the wasps, sinalbin was applied to *P. rapae* caterpillars (5th instar) in an amount equivalent to that present in 8 μl of *A. rosae* hemolymph (i.e., 0.08 μmol according to Müller et al., 2001, dissolved in 3-μl ethanol). Control caterpillars were treated with ethanol only. Caterpillars were offered as in experiment 3b.

RESULTS

Experiment 1. Influence of Different Predator Guilds on Sawfly Larvae in Mid-Summer. In total, 71.3% of the released sawfly larvae were recovered after 3 d of exposure to naturally occurring predators. In 3 of the 8 plots, all 15 larvae were still present on the control plants. No differences were found in survival rate among the plots ($F_{7,24} = 1.06$; $P = 0.42$; two-factorial randomized block ANOVA), and there was also no interaction between plot and predator-exclusion treatment ($F_{21,24} = 0.71$; $P = 0.79$). The number of larvae differed significantly between treatments ($F_{3,24} = 3.76$; $P = 0.02$) and was significantly higher on the control plants than on those accessible to all predators. However, the number of larvae on plants accessible to different predator groups did not differ between treatments ($P < 0.05$, Scheffé-test) (Figure 2B). Only 1 out of 599 surviving larvae was successfully parasitized. This individual was from a plant with open access to predators and had been attacked by a tachinid fly.

During the second day, an ant (*Lasius niger* L.) was observed touching an *A. rosae* larva. The larva started to wave its abdomen in response and fended the ant off. Fresh feces of larvae seemed to repel ants, as they would retreat and start cleaning their mouthparts, while dried feces had no visible influence on the ants' walking behavior. Ants, earwigs, and spiders were found on plants and trapped in the pit-fall traps, while five wasps were trapped on the blue sticky traps. In addition, carabid beetles were found on the ground and in the traps. We did not identify the trapped individuals at the species level, as no direct interactions with the sawflies were observed.

Experiment 2. Influence of Predators on Sawfly Larvae Compared to Caterpillars in Late Summer. The proportion of *A. rosae* larvae that survived in September was only about one-half of that in midsummer. In total, 37.5% of the released *A. rosae* larvae and less than 1% of *P. rapae* were recovered after 3 d.

Thus, plant treatment had a highly significant effect ($F_{3,32} = 50.28$; $P < 0.001$; two-factorial randomized block ANOVA). No difference existed in survival rate among the 8 plots ($F_{7,32} = 0.88$; $P = 0.53$), and there was also no interaction between plot and plant treatment ($F_{3,32} = 0.65$; $P = 0.85$). The number of surviving *A. rosae* larvae was significantly higher on plants from which walking predators were excluded than on those to which all predators had access ($P < 0.05$, Scheffé-test) (Figure 2D). No parasitoids emerged in the laboratory from recovered larvae.

On day 2 of the experiment, almost all *P. rapae* caterpillars had disappeared. Wasps were highly active from 7.30 A.M. onwards. Ant activity on the ground in the plots started around noon. No interactions between predators and herbivore larvae were observed during the 10-hr observation period. Ants and spiders were found on plants and trapped in the pit-fall traps, and wasps were observed visiting the plants. In addition, carabid beetles were found on the ground and in the traps.

Experiment 3a. Predation Pressure From Wasps. After 3 hr, all *P. rapae* ($N = 35$) caterpillars had been taken by wasps. Smaller *P. rapae* (3rd instar) were carried away in one piece. Larger caterpillars (4–5th instar) were serially sectioned (Figure 1b), (processed), and the pieces then carried away (label = *foraged*). Wasps would return to take away the remaining pieces. Wasps also contacted sawfly larvae but typically rejected them quickly. One larva was mortally injured but not carried away. Easy bleeding was elicited in two others causing the wasps to retreat rapidly in an apparently startled manner. Both larvae survived and developed normally. Four larvae had even shorter contacts with wasps with no obvious easy bleeding.

Experiment 3b. Role of Sawfly Hemolymph in the Defense Against Wasps. Eight *P*-treated, and two *A*-caterpillars were all *foraged* by wasps at first contact. In contrast, six *A*-caterpillars were mortally injured but *dropped* by the wasps after 10–30 sec followed by mouthpart-cleaning (Table 1). Three of these six caterpillars (and two more at initial contact) were subsequently reattacked, but the

TABLE 1. NUMBERS OF *Pieris rapae* CATERPILLARS DROPPED BY WASPS AT INITIAL CONTACT OR FORAGED BY WASPS IN A STANDARD MANNER (IMMEDIATELY DISSECTED AND PROCESSED)

Type of encounter with wasp	Treatment with hemolymph of		*P*
	A. rosae	*P. rapae*	
Dropped at initial contact	6	0	0.005
Foraged in "standard" manner	2	8	0.02

Note. Caterpillars were treated with 8 μl of freshly collected hemolymph from either *Athalia rosae* or *P. rapae* and then exposed to predation on their host plant in the field. Differences were tested by a two-tailed Fisher's exact test.

wasps *avoided* the treated, dorsal integument by turning the body and cutting away ventral portions. The proportion of caterpillars attacked in a standard manner (i.e., immediately dissected and processed) differed significantly between treatments (Table 1).

Experiment 3c. Role of the Plant-Derived Glucosinolate Sinalbin in the Defense Against Wasps. All 10 control caterpillars were *foraged* by wasps. Five of the 10 sinalbin-treated caterpillars were likewise *foraged*, but one was *dropped* and two *avoided* (the other two were contacted but fell from the plant with the wasp flying away). The difference between the proportion of control and sinalbin-treated caterpillars *foraged* was significant (two-tailed Fisher's exact test: $P < 0.05$).

The wasps did move between the plants and contacted differently treated caterpillars and sawfly larvae in mixed order in experiments 3a–c. We therefore interpret the number of prey items offered as independent samples.

DISCUSSION

Predatory wasps are an important target of easy bleeding in sawflies; caterpillars and other herbivores lacking such chemical protection can be decimated by predators (Figure 2D; Reveret Richter, 2000; Schenk and Bacher, 2002). Many sawfly larvae in our experiment survived 3 days of exposure. The efficacy of the bleeding phenomenon as a defense against wasps was clearly demonstrated in experiment 3. While the first attack on an individual *A. rosae* ended in death of the larva (although it was not carried away), later attacks resulted in easy bleeding only or in no visible damage to the sawfly larva at all. The wasps possibly learned that *A. rosae* was unpalatable, but we cannot be certain whether the same or different individuals were involved in the attacks on larvae (although wasps were at a high local density in the garden). The observation that some larvae were released without any visible harm suggests that something on the surface of the integument was deterrent, even before easy bleeding was elicited. This property could be chemical or physical. The rejection by wasps of the usually acceptable prey, *P. rapae*, when treated topically with *A. rosae* hemolymph, implicated one or more of the compounds within the sawfly hemolymph to act as deterrents. The repulsive effect on wasps was not due to a physical change of the caterpillar surface by hemolymph treatment, as control *P*-caterpillars treated with (caterpillar) hemolymph in the same way were killed and eaten in the typical manner. Wasps still preyed on treated *A*-caterpillars by skilfully excising ventral parts that were not treated with *A. rosae* hemolymph, and transporting only these parts to their nests.

The manipulation assays with sinalbin-treated caterpillars suggest that the deterrent effect of sawfly hemolymph is in part due to this sequestered glucosinolate. Several wasps *dropped* or *avoided* such prey. Thus, glucosinolates are not only a feeding deterrent for this plant against generalist herbivores (Chew, 1988;

Halkier, 1999; Wittstock et al., 2003), but can also be used by specialist herbivores for their own defense. Other social wasps, such as *Mischocyttarus flavitarsis*, are similarly repelled by quinolizidine alkaloids that are sequestered by caterpillars from their host plants (Montillor et al., 1991).

In plants, glucosinolates are hydrolyzed by myrosinases after tissue disruption to form isothiocyanates, nitriles, and other products, known for their greater biological activity than the intact compounds (Halkier, 1999; Wittstock and Halkier, 2002). Some aphid species contain their own myrosinase, probably to release isothiocyanates when attacked (Bridges et al., 2002). However, myrosinase activity is not known for either *A. rosae* or *P. rapae*. Thus, it seems unlikely that hydrolysis of glucosinolates is involved in the defensive effect of the easy-bleeding (Müller, unpublished data). As half of the sinalbin-treated caterpillars were *foraged* in a regular way, the glucosinolate on its own is not a complete defense, and other compounds in the hemolymph might have synergistic or additive repulsive effects. The defensive efficacy of the sawfly hemolymph might be associated with host plant quality, particularly as the concentration of glucosinolates can vary widely in *A. rosae* (Müller et al., 2003b). An influence of host plant genotype on predators has been demonstrated in iridoid glycoside sequestering herbivores (Bowers and Stamp, 1997).

The easy bleeding phenomenon is efficient throughout all free-living instars of *A. rosae.* Both 2nd–3rd instar (experiment 2) and 4th–5th instar (experiment 3) larvae survived when activity of wasps was high. However, the defense mechanism of *A. rosae* is not entirely protective against all predators, as survival of larvae was significantly reduced on plants with access to arthropod predators (Figure 2B). Ants, earwigs, spiders, and carabid beetles were representative potential predators found in pit-fall traps. In June, there was no evidence that one particular predator group might have higher impact on *A. rosae* than the other. Surprisingly, the number of larvae recovered on plants where both walking and flying predators had access was not significantly lower than on plants where either one of these guilds was excluded (we expected that loss of larvae would be about the sum of losses due to flying and walking predators). This result may be due to chance or to an unidentified interaction between flying and walking predators that favors the survival of the *A. rosae* larvae.

Predation pressure was much higher later in the season. In September, walking predators reduced the number of sawfly larvae significantly (Figure 2D). Although the hemolymph of *A. rosae* has been shown to have an inhibitory effect on the ant, *Myrmica rubra* (Schaffner et al., 1994; Müller et al., 2002), other ant species or walking predators such as spiders with a high activity late in the season may not be deterred. However, we did not observe any candidate species *in flagranti*.

The observed repulsive effect of fresh feces on ants in June is not due to glucosinolates, as these are not present in feces of *A. rosae* (Müller et al., 2001). Rather it might be due to volatile compounds that evaporate from wet feces. Any

possible influence of walking predators on caterpillars of *P. rapae* could not be studied as they were decimated so quickly by the wasps. Different responses of walking predators are known toward this caterpillar. Although hemolymph from this species is not deterrent against the ant *M. rubra* (Müller et al., 2002), glandular hairs arranged in rows along the body secrete minute droplets, which were shown to produce a potent deterrent activity against the ant *Crematogaster lineolata* in a North-American study (Smedley et al., 2002). These tiny droplets, if produced by our stock, clearly offered no protection against predation by wasps. In cabbage, predation rates of *P. rapae* ranged from 23 to 80% (Schmaedick and Shelton, 1999). Wasps were not observed as predators in the study of Schmaedick and Shelton. It is likely that at places where activity of *V. vulgaris* is high, *P. rapae* has no chance to survive.

In summary, our study demonstrates the importance of field experiments with replicates at different seasons combined with manipulation assays to more fully understand the mechanism and efficiency of an herbivores' defensive property; our later series of experiments was crucial to detecting the effective defense of the sawfly larvae against wasps. Although *A. rosae* larvae are not perfectly protected against all arthropod predator guilds *via* their easy bleeding, they are more effectively protected than other herbivorous insects such as *P. rapae*. The bleeding mechanism, involving the release of a deterrent compound sequestered from the host plant, might have evolved as a specific response to carnivorous foraging by wasps in *A. rosae*. However, these predators are unlikely to interact with those species of tenthredinid sawfly with easy bleeding behavior that lack late summer larval generations.

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STRUCTURE–ACTIVITY OF VALENCENOID DERIVATIVES AND THEIR REPELLENCE TO THE FORMOSAN SUBTERRANEAN TERMITE

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Abstract—Eight valencenoid derivatives were evaluated for their repelling activity against Formosan subterranean termites, *Coptotermes formosanus* Shiraki. Among them, 1,10-dihydronootkatone was the strongest repellent, and valencene was the weakest. Results of the structure–repellency relationships indicated (1) reduction of the ketone group to the alcohol on position 2 of nootkatone curtailed the activity; (2) because of the low activity of valencene relative to nootkatone that the ketone group was essential for repellent activity; (3) reduction of the 1,10 double bond (1,10-dihydronootkatone and tetrahydronootkatone) produced compounds more repellent than nootkatone; (4) the isopropenyl group probably does not participate in binding as evidenced by no significant difference in the repellent activity among nootkatone (double bond between position 11 and 12), isonootkatone (double bond between position 7 and 11), and 11,12-dihydronootkatone.

Key Words—*Coptotermes formosanus*, valencenoids, chemical structure, repellence, pharmacophore.

INTRODUCTION

Recent research on a number of plant-derived natural products suggests that components of essential oils may have potential as insect repellents, fumigants, and

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feeding deterrents (Shaaya et al., 1991; Sharma et al., 1994; Franzios et al., 1997; Ngoh et al., 1998; Isman, 1999; Zhu et al., 2001a). The volatile and semivolatile components of essential oils can be classified into four major groups: terpenes, benzene derivatives, hydrocarbons, and miscellaneous compounds (Ngoh et al., 1998). Among them, the mono- and sesquiterpene derivatives predominate (Rice, 1983; Rice and Coats, 1994a).

Most insecticidal terpenes that have been tested are monoterpenoids, such as thymol, eugenol, geraniol, nerol, and citral (Rice and Coats, 1994b; Ngoh et al., 1998; Zhu et al., 2001b, 2003). Some monoterpenoids are termiticides (Nagnan and Clement, 1990; Sharma et al., 1994; Cornelius et al., 1997). Rice and Coats (1994c) evaluated the topical, fumigant, and ovicidal activity of 14 monoterpenoids and 31 monoterpenoid derivatives against the house fly, *Musca domestica* (L). They reported that minor structural variations can elicit major differences in toxicity, suggesting a precise fit between the receptor and the ligand.

We reported that nootkatone, a sesquiterpene ketone, is repellent and a feeding deterrent to Formosan subterranean termites (Maistrello et al., 2001; Zhu et al., 2001a). Recently, Ibrahim et al. (unpublished) also studied two derivatives of nootkatone, namely 1,10-dihydro- and tetrahydronootkatone, and found that they were all toxic to Formosan subterranean termites, among them, tetrahydronootkatone exhibited the highest toxicity.

Generally, insect repellents act on the chemoreceptors responsible for olfaction to cause avoidance movements (Ahmad et al., 1995), but their mode of action is not understood. Studying structure–activity relationships may help in understanding their mode of action.

In this report, we evaluated the repellence of eight valencenoid derivatives against Formosan subterranean termites. We found that increased saturation of the naphthalene rings, especially with the ketone group at the 2-position of nootkatone, is an important determinant of termite repellency.

METHODS AND MATERIALS

Termites. Two carton nests (colonies C and D) of Formosan subterranean termites were collected from infested trees. Colony C was collected from a cypress tree in Lake Charles, LA, in 2001 and colony D was collected in New Orleans in 2000 from a tree limb of an undetermined species. Termites were held in 250-l cans with pine as a food source and kept at 24–26°C. Moistened corrugated cardboard rolls were used to retrieve termites from the cans (La Fage et al., 1983). Termites were gently knocked from the cardboard rolls into clean plastic trays (40 × 50 cm), and isolated from debris by allowing them to climb on moistened paper towels.

Materials. Palladium (Pd) and platinum (Pt) used for synthesis of valencenoid derivatives were purchased from Aldrich Chem. Co. (Saint Louis, MO). Nootkatone (>97% crystalline) was purchased from Lancaster Synthesis

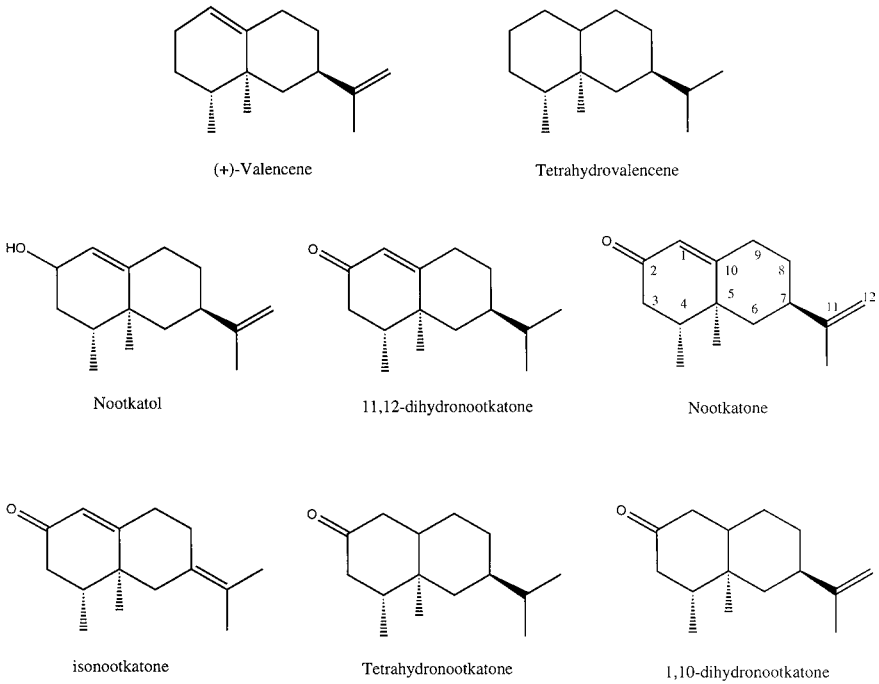

FIG. 1. Structure of the eight valencenoids.

(Windham, NH). 1,10-Dihydronootkatone (85%) and tetrahydronootkatone (85%) were obtained from Subcon Products, Inc. (Totowa, NJ). Valencene (>95%) was purchased from Fluka (Milwaukee, WI). Isonootkatone, 11,12-dihydronootkatone, nootkatol, and tetrahydrovalencene were synthesized in our laboratory, and isonootkatone standard was a gift from Prof Ekkhard Winterfeldt (Institut für Organische Chemie,Technische Universität, Berlin). The chemical structures of eight valencenoid derivatives are shown (Figure 1).

Purification of 1,10-Dihydronootkatone and Tetrahydronootkatone. One gram of 1,10-di- or tetrahydronootkatone was dissolved in 5-ml ethend, mixed with 5 g of silica gel, and air-dried for 15 min. The silica gel with sample was placed in the bottom of a 2 × 30 cm glass the column which was then filled with silica gel. Chloroform was used to develop the samples in ascending mode. The silica gel was forced out of the column after chloroform reached the top. Every 2.5-cm section of silica gel was collected and extracted with 40-ml ethyl alcohol. The purity of fractions was assessed by thin layer chromatography (TLC) and gas chromatography–mass spectroscopy (GC–MS). The final purity of 1,10-dihydronootkatone and tetrahydronootkatone was >95% (yields ~ 60%).

Synthesis of Derivatives.

(1) Isonootkatone and tetrahydrovalencene were prepared by the procedure of Stevens et al. (1970). The crude isonootkatone was purified by HPLC (Chromeleon; Dionex Co., Sunnyvale, CA) eluting with acetonitrile: H_2O (50:50). Two major peaks (isonootkatone and nootkatone) appeared upon 6c and the isonootkatone peak was identified by its mass spectrum. The purity of isonootkatone was >95% after HPLC.

Tetrahydrovalencene (95% purify) was obtained as a colorless oil; and used without further purification.

(2) 11,12-Dihydronootkatone was synthesized according to the method of Odom and Pinder (1972). The crude product was purified by chromatograph on silica gel, eluting with 90:10 hexane: ethyl acetate. ($R_f = 0.31$). 11,12-Dihydronootkatone was obtained as a colorless liquid (>95% pure by GC) ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.80–0.91 (3d and m obscured, 10H), 0.99 (s, 3H), 1.0–1.12 (m, 1H), 1.40 (m, 2H), 1.78–2.0 (m, 3H), 2.10–2.47 (m, 4H), 5.50 (s, 1H); (13)C NMR ($CDCl_3$): δ 14.84, 16.85, 19.34, 19.87, 29.50, 32.36, 33.06, 38.80, 39.09, 40.41, 41.97, 42.34, 124.18, 171.49, 199.60.

(3) Nootkatone was reduced with —OH by $NaBH_4$ according to Spiro (1964) to form Nootkatol. Nootkatol was identified by GC–MS as the trimethylsilyl derivative, and the crude sample was further purified by preparative TLC on silicagel using chloroform as the developing solvent, then reverse phone HPLC eluting with acetonitrile: H_2O = 60:40. The purity of the resulting nootkatol (>95%) was determined by GC–MS. The trimethylsilyl derivative of nootkatol was made by mixing 10-μg nootkatol in 100-μl ethyl acetate mixed with 100-μl *N*-methyl-trimethylsilyl trifluoroacetamide (MSTFA). The mixture was incubated at room temperature for 15 min before analysis by GC–MS (Laine and Sweeley, 1971).

Repellency Test of Valencenoid Derivatives Against Formosan Subterranean Termites. The procedure for the repellency test was described by Lewis et al. (1978) and modified by Zhu et al. (2001b). A 5 cm diam × and 1 cm high Petri dish was used for the assay. One milliliter of hot agar solution (1.5-g agar in 100-ml H_2O) was spread evenly in the bottom of each dish and allowed to cool. This ensured adequate moisture for the termites and helped to hold the sand that was layered above it in place. Blasting sand (fine, #4) was autoclaved for 30 min and oven-dried. One half of the top of the agar layer was covered with 1 g of treated sand. The other half was covered with 1 g of untreated sand. The chemicals were dissolved in ethyl alcohol and prepared in a series of dilutions for testing. Seven concentrations (2.5, 5, 12.5, 25, 50, 100, and 200 μg/dish) of each chemical were used for two colonies (C and D) to evaluate the repellent activity.

To record termite behavior, 10 worker termites were added to each Petri dish, and the number of termites observed on the untreated sand was recorded every hour for 3 h. Five replicates were used for each test including the control (untreated sand). A chi-square test indicated that when 32 or more of the 50 termites (a sum of the 5 replicates) were observed on the untreated sand, there was a significant difference in the repellence between the treated and control groups ($\chi^2 = 6.15$, df $= 1$, $P = 0.048$) (Anonymous, 1998). The repellency threshold was defined as the lowest concentration of the chemicals which showed a repellent effect.

RESULTS AND DISCUSSION

Repellence of valencenoid derivatives to Formosan subterranean termites is shown in Table 1 and Figure 1. Among the eight valencenoids tested, 1,10-dihydronootkatone was most repellent having a repellency threshold of 5 μg/dish in both colonies. The level of repellent activity of tetrahydronootkatone was similar (12.5 μg/dish for colony C and 5 μg/dish for colony D). The repellency of valencene and tetrahydrovalencene was much weaker than nootkatone and its derivatives, with thresholds of $\geq$200 μg/dish for both colonies, indicating the importance of the 2-ketone group in otherwise similar structure (Figure 1).

The observation that nootkatone (OH group at the 2-position) showed weaker repellence than nootkatone (thresholds 50–100 μg/dish) also supported this conclusion. Nootkatone and its derivatives, isonootkatone, 1,10-dihydronootkatone, 11,12-dihydronootkatone, and tetrahydronootkatone, showed different activities with respect to their level of unsaturation, and among them, 1,10-dihydronootkatone

TABLE 1. REPELLENCE OF EIGHT VALENCENOIDS AGAINST FORMOSAN SUBTERRANEAN TERMITES

Compound	Repellency threshold (μg/dish)	
	Colony c	Colony d
1,10-Dihydronootkatone	5	5
Tetrahydronootkatone	12.5	5
Issonootkatone (α-vetivone)	25	12.5
Nootkatone	25	25
11,12-Dihydronootkatone	12.5	25
Nootkatol	100	50
Tetrahydrovalencene	>200	200
Valencene	>200	200

Note. Repellence of the components on Formosan subterranean termites was based on chi-square analysis. Threshold value is the lowest effective concentration (μg/dish) for repellence and the threshold value was calculated at the 3rd h of exposure.

and tetrahydronookatone were more repellent than when the double bond between carbons 1 and 10 was hydrogenated (nootkatone, isonootkatone, and 11,12-dihydronootkatone). The lack of significant differences in activity between 1,10-dihydronootkatone and tetrahydronootkatone suggested that hydrogenation of the 11, 12 alkene was not necessary for enhancement of the repellency. The repellency of nootkatone and isonootkatone was similar, with thresholds between 12.5 and 25 μg/dish in two colonies suggesting that the double bond at position 7 and 11 or at 11 and 12 was not critical. Comparing the repellency of valencene and tetrahydrovalencene, hydrogenation of the 1, 10 unsaturated bonds had no discernable effect at the doses tested.

There are many more sesquiterpenoids known in nature than there are monoterpenoids (Harborne, 1991). The biological activities associated with sesquiterpenes are many and varied, such as plant growth regulation (Harborne, 1991), insect antifeedant activity (Wakabayashi et al., 1988; Garlaschelli et al., 1994), and insecticidal effects (Miyazawa et al., 2000; Maistrello et al., 2001; Zhu et al., 2001a). In addition to the low apparent mammalian toxicity of sesquiterpenoids, another advantage they have as repellents is their persistence when compared to monoterpenes (Zhu et al., 2001b). Ibrahim et al. (unpublished data) recently found that acetylcholinesterase (AChE) activity of the Formosan subterranean termite is stimulated by toxic doses of nootkatone and tetrahydronootkatone. We also found that tetrahydronootkatone, a more repellent derivative of nootkatone, is more potent in stimulating AChE activity. We believe that this effect may lead to cholinergic neurotransmission dysfunction in termites.

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ENHANCED INVERTASE ACTIVITIES IN THE GALLS OF *Hormaphis hamamelidis*

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Abstract—Invertases are sucrose hydrolyzing enzymes often associated with plant tissues acting as physiological sinks, and plant galls are physiological sinks. We investigated several types of invertase and their potential benefits in galls of the aphid *Hormaphis hamamelidis*. Invertase activities in galls differed from those in leaves throughout the growing season. Vacuolar invertase activities (per g FW) were always greater in galls than leaves. In contrast, cell wall invertase activities per g FW started low in galls, but increased with time and were greater than those in leaves after 1 month. Gall growth was most closely related to vacuolar invertase activity, whereas leaf growth was correlated with both vacuolar and cell wall invertase activities. In separate correlational studies of aphid fecundity and invertase activities, cell wall invertase activity per gall accounted for 15–21% of the variation in offspring per gall. Gall dry weight explained more of the variation in offspring per gall (34.2%) than did gall volume (17.8%), a likely indication of the importance of sink strength over the life of the gall. Increased invertase activity probably underlies the enhanced sink strength commonly observed in galls. *Hormaphis hamamelidis* fundatrices appear to maximize reproductive output by eliciting multiple beneficial responses from their hosts. Initial gall growth correlated positively with high vacuolar invertase activity, while later in the season aphid fecundity correlated positively with cell wall invertase activity.

Key Words—Source–sink relations, invertase, induced responses, resource manipulation, herbivore offense.

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INTRODUCTION

Galls are sinks for photoassimilates and mineral nutrients (Jankiewicz et al., 1970; Abrahamson and McCrea, 1986; Andersen and Mizell, 1987; Larson and Whitham, 1991; Bagatto and Shorthouse, 1994; Burstein et al., 1994; Bagatto et al., 1996). By altering patterns of allocation, gall formers can cause major changes in the architecture (Abrahamson and McCrea, 1986; Raman and Abrahamson, 1995; Fay et al., 1996a) and ecology (Dennill, 1988; Erasmus et al., 1992; Fay et al., 1996b) of their hosts. In extreme cases, galls can be such strong sinks that they cause the death of other plant parts (Stiling et al., 1992; Burstein et al., 1994; Price and Louw, 1996; De Souza et al., 1998) and may alter plant population structure (Abrahamson and McCrea, 1986). The sink strength of gall tissue affects the success of gallers, both among galls as well as between galls and host plant sinks (Whitham, 1980; Inbar et al., 1995; Larson and Whitham, 1997; B. Rehill and J. Schultz, unpublished data). The mechanisms that galling insects use to enhance physiological sinks and thereby acquire resources from the host plant are unknown.

Sink strength in both vegetative and reproductive plant tissues depends on the activity of sucrose hydrolyzing enzymes and sucrose transporters (Ho, 1988; Truernit et al., 1996; Sturm, 1999; Sturm and Tang, 1999; Williams et al., 2000; Winter and Huber, 2000). Invertases are sucrose-hydrolyzing enzymes with multiple physiological functions that vary among tissues as well as with developmental state. Different forms of invertases are localized to particular cellular compartments. Vacuolar invertases, often termed soluble acid invertases, function in the expansion growth of plant cells as well as carbon partitioning. Cell wall invertases play a major role in carbon partitioning in many plant tissues, but also function in phloem loading and unloading. Both vacuolar and cell wall invertases can be induced by wounding and other stimuli (Sturm and Chrispeels, 1990; Ehness et al., 1997; Ohyama et al., 1998; Kefi et al., 2000). Cytosolic (soluble neutral or alkaline) invertases appear to play little or no role in carbon partitioning, but do function in respiration and the biosynthesis of primary and secondary compounds (Sturm and Tang, 1999). In general, cytosolic invertase activities are not useful as measures of sink strength, in contrast to those of vacuolar and cell wall invertases (Sung et al., 1989).

The Nutrition Hypothesis posits that galls provide enhanced nutrition for their inhabitants (Price et al., 1987), and is supported by the specialized structure of some galls that include highly nutritious tissue for consumption by cynipid wasp, cecidomyiid fly, and nematine sawfly gallers (Larew, 1982; Meyer, 1987; Nyman and Julkunen-Tiitto, 2000). Sucking insects such as aphids also enhance nutrition (Kennedy, 1951; Forrest, 1971; Llewelyn, 1982) by stimulating phloem proliferation (Lewis and Walton, 1958; Wool et al., 1999) and increasing the sink strength of the gall (Larson and Whitham, 1991; Inbar et al., 1995).

Given the importance of sink status in the ecology of galls, we investigated invertase activities as a potential mechanism of increased sink strength and nutritional

modification in galls. We attempted to identify the mechanistic basis of increased sink strength in galls by testing the hypothesis that galls formed by the aphid *Hormaphis hamamelidis* on witch hazel (*Hamamelis virginiana*) have enhanced invertase activities in the gall and/or surrounding leaf. We predicted that vacuolar invertase activities in galls would be greater than those in leaves and that cell wall invertase activities would be enhanced relative to ungalled leaf tissue. The relative growth rates of tissues were tracked to investigate which sink enzyme was most related to growth. Next, we tested the potential adaptive value of invertase activities. Also, because gall size is related positively to fecundity in this system (Rehill and Schultz, 2001), we clarified its role by comparing the relative importance of gall volume versus that of gall dry weight. Our findings supported the hypotheses that altered invertase activities are a mechanism of increased sink strength in galls, and that fundatrices induce beneficial biochemical responses in the host plant.

METHODS AND MATERIALS

Research System. Witch hazel *Hamamelis virginiana* L. (Hamamelidaceae) is a common understory tree or shrub in eastern deciduous forests of North America, ranging from Nova Scotia to Florida (Grimm, 1983). The witch hazel cone gall aphid, *Hormaphis hamamelidis* (Fitch) (Homoptera: Hormaphididae) forms conical galls on the leaves of witch hazel.

Hormaphis hamamelidis has a monoecious life cycle of three generations, beginning with the fundatrix that hatches in the spring from a winter egg and initiates a gall just after bud break (Lewis and Walton, 1958; von Dohlen and Gill, 1987). The fundatrix chooses a site on the abaxial side of the leaf and initiates gall formation with repeated brief insertions of her stylet into the leaf. The gall grows from this area of intense probing, and eventually a red cone-shaped gall 7–10 mm tall forms on the adaxial side of the leaf. The fundatrix bears the second generation by parthenogenesis within the gall; all the aphids feed from phloem in the interior walls of the gall. The alate second-generation adults leave the gall and bear the third, sexual generation by parthenogenesis. After maturation, sexuals mate and the female deposits 5–10 winter eggs on the bark of the host. The number of second-generation aphids in the gall provides a measure of the fecundity of each fundatrix, and is the response variable in all of the studies of fecundity.

Study Site. All experiments were performed at a site within Rothrock State Forest in Pennsylvania, USA (centered approximately at 40°45′22″ N latitude and 77°44′10″ W longitude) that contained large stands of witch hazel trees. The entire canopy of most study trees was accessible using a 2-m ladder.

Invertase Time Course. Leaves and galls were collected on dates corresponding to important phases in gall and fundatrix development: May 17, May 28, June 6, and July 8, 1997 (see Table 1). Six to twelve galled leaves and 6–12 ungalled

TABLE 1. FUNDATRIX, GALL, AND LEAF STATUS ON THE DATES THAT LEAVES AND GALLS WERE COLLECTED FOR THE INVERTASE TIME COURSE

Date	Fundatrix status	Gall status	Leaf status
May 17, 1997	1st instar nymph	Open depression	Expansion started
May 28, 1997	2nd instar nymph	Closed, 1–2 mm tall	Still expanding
June 6, 1997	3rd instar nymph	Cone 3–4 mm tall	Fully expanded, light green
July 8, 1997	Adult: First offspring appearing in gall	Cone 8–10 mm tall	Fully expanded, dark green

leaves were taken from each of six trees. Each gall was opened with a sterile scalpel and the fundatrix removed. Leaves and galls were flash frozen in liquid nitrogen and kept on dry ice until storage at −80°C.

Leaves and galls were pooled within trees and tissue type (gall, galled leaf, or ungalled leaf) to provide adequate mass for extraction. Each pooled sample (consisting of three to six galls or leaves) was homogenized in a mortar and pestle with 6–10 volumes of ice-cold extraction buffer that contained: 50 mM MES pH 6.5, 20 mM DTT, 5 mM EDTA, 5% (v/v) PVPP (insoluble), 2% (v/v) Tween 20, 2% (v/v) glycerol, 2.5 mM benzamidine, and 0.1 mM PMSF. (The extraction buffer was optimized for witch hazel tissues prior to the experiment.) Samples were centrifuged at 30,000 *g* RCF and 4°C for 20 min, and the supernatant removed for measurements of soluble invertase activities (vacuolar invertase, VI, and cytoplasmic invertase, CI). The pellet was twice suspended in 10 volumes of water, centrifuged as above, and the supernatant discarded. The pellet was suspended in five volumes of a modified extraction buffer (as above except without PVPP) with 50 mM NaCl, centrifuged as above, and the supernatant discarded. The pellet was then extracted overnight at 4°C by suspension in 10 volumes of the modified extraction buffer with 1 mM NaCl, centrifuged as above, and the supernatant removed for measurements of ionically bound cell wall invertase activity (CWIi). This method yields only wall-bound solutes free of any cytoplasmic contaminants (Fahrendorf and Beck, 1990). The pellet was twice suspended in 10 volumes of water, centrifuged as above, and the supernatant discarded. The remaining pellet was used for measurements of covalently bound cell wall invertase activity (CWIc).

Extracts were assayed for invertase activities using a modification of the method of Arnold (1965). Each reaction (soluble invertases or ionically bound cell wall invertase) contained 5–20 μl sample extract, 60–75 μl reaction buffer (see below), and 20 μl 1.0 M sucrose substrate in a total reaction volume of 100 μl. The reaction buffer provided constant ionic strength at each pH (Ellis and Morrison, 1982) and contained 0.05 M acetic acid, 0.05 M MES, and 0.1 M Tris HCL as buffering agents. Assays for soluble invertase activity were run at pH 4.0, 4.5, 5.0, 5.5, 6.5, 7.0, and 7.5 to identify the pH optima of vacuolar and cytoplasmic invertases, and run at pH 4.0, 4.5, 5.0, and 5.5 to identify the pH

optimum of ionically bound invertase. Each assay was incubated for 20–40 min at 30°C; enzyme kinetics were linear, and there was no substrate limitation. Reactions were stopped by addition of 100 μl of a modified Sumner's reagent that contained 1% DNSA, 0.5% phenol, 2.5% NaOH, and 0.05% Na_2SO_3. The reactions were heated at 100°C for 10 min, 33 μl of 40% Rochelle salt solution was added to each reaction, and absorbance was read at 560 nm. Reducing sugar concentrations were computed using a standard curve of glucose in 0.2 M sucrose. Blanks consisted of complete reaction mixtures with the reaction stopped immediately.

Assays of covalently bound cell wall invertase were performed directly on the prepared sample pellets. The pellet was suspended in eight volumes of pH 4.5 50 mM acetate buffer and the reaction was started by addition of two volumes of 1.0 M sucrose substrate solution. Samples were reacted for 20–40 min at 30°C, placed on ice for 15 min to halt enzyme activity, and then centrifuged at 30,000 *g* RCF and 4°C for 20 min. The supernatant was assayed for reducing sugars using Sumner's reagent and compared to a glucose standard containing 0.2 M sucrose in pH 4.5 50 mM acetate buffer.

Enzyme activities are reported as units (U) per g fresh weight (FW). A unit equals 1 μmol sucrose hydrolyzed per min. Invertase activities reported are: vacuolar (VI: soluble activity at pH 4.5), cytoplasmic (CI: soluble activity at pH 7.0), ionically bound cell wall (CWIi: measured at pH 4.5), and covalently bound cell wall (CWIc).

The relative growth rates of leaves and galls were calculated for the enzyme assay samples; each pooled sample was a replicate. To standardize between sampling dates the mean FW per organ (gall or leaf) was computed as: FW = total fresh weight/# of galls or leaves. Relative growth rate was computed as: RGR = $(FW_2 - FW_1)/FW_1$/# days between sampling.

Fecundity and Invertase Study I. This study was performed to test the hypothesis that the invertase activities of galls were positively related to the numbers of offspring per gall. Eight leaves with a single gall and eight leaves with multiple galls were collected from each of five trees along a transect on August 5, 1998. Gall height (highest point above the top of the leaf to the lowest point on the underside of the leaf) and gall diameter (widest point on top of the leaf) were measured to the nearest 0.1 mm with digital calipers. Galled leaves were collected by cleanly breaking the petiole off at the stem, flash freezing in liquid N_2, and holding on dry ice until storage at −80°C. Galls were dissected at 4°C, the inhabitants removed, and leaves refrozen in liquid N_2 and stored at −80°C for later analysis of invertase activities. Aphids were counted under 80× with a dissecting microscope. Only galls with no sign of attack by natural enemies were used for invertase assays. In all cases, given the conical shape, gall volume was calculated as the volume of a cone (volume = $\{\pi r^2 h/3\}$, where r = radius and h = height).

Fecundity and Invertase Study II. Eighty galls were sampled haphazardly on July 23, 1998, eight each from 10 trees on a transect. At this time, the mean number

of offspring per gall was at or near its peak and the first alates were appearing within galls, as determined by a weekly census of galls from other trees within the field site (data not shown). Each gall was alone on the leaf, and samples were processed as described above. Galls were classified as occupied (all aphid inhabitants alive and no sign of attack by natural enemies), invaded (some of the inhabitants alive, a natural enemy present), or unoccupied (none of the aphid inhabitants alive, and obvious signs of attack by a natural enemy). Mortality was high among the collected galls: of the 80 galls, 30 were occupied, 46 were unoccupied, and 4 were invaded. Several natural enemies attacked gall inhabitants, including fungi and several types of insect larvae. Only undamaged galls, with no caterpillar herbivory, were used for invertase measurements, and only galls that showed no sign of attack from natural enemies were used in analyses of fecundity.

Fecundity and Invertase Studies: Invertase Assays. The extraction scheme used in the chemistry time course of 1997 was modified to measure total cell wall invertase in a more expedient manner. This total cell wall invertase (CWI) equals the sum of the two types of cell wall invertase (CWIi and CWIc) measured in the time course. Each gall was weighed to the nearest 0.1 mg, ground under liquid N_2 in a mortar and pestle, and the recovered portion reweighed. Galls were extracted by 30 min of sonication in ice with 10 volumes of ice-cold extraction buffer (formulated as in the chemistry time course study). After sonication, the homogenate was centrifuged at 20,000 *g* RCF and 4°C for 20 min and the supernatant was removed for assay of vacuolar invertase activity. The residue was resuspended in distilled water, centrifuged as above, and the supernatant discarded to remove any residual vacuolar invertase activity. This cycle was performed a total of three times, and the washed pellet was used for assay of cell wall invertase activity. Leaves were processed analogously, except after homogenization only 20 mg of tissue was used for extraction.

Extracts were assayed for vacuolar invertase activity and pellets for total cell wall invertase activity as in the chemistry time course. For these studies, invertase activities for galls are expressed in two ways: per g FW as in the time course and per gall (activity per g FW times the FW of the gall).

Gall Size and Fecundity Study. This study defined the relationships among gall size, gall weight, and fecundity. Previous studies indicated that gall size, calculated as the volume of the gall from exterior measurements, was positively correlated with fecundity. We did this study to ascertain if gall dry mass (one indication of sink strength over the life of the gall) was a better predictor of fecundity than gall volume. On July 25, 1999, 50 galls were collected from each of four trees for a total of 200 galls. At this time, the mean number of offspring per gall was at its peak, and the first alates were appearing within galls, as determined by a weekly census of galls from other trees within the field site (Rehill and Schultz, 2001). Samples were processed as described above. Analyses of gall size and fecundity included only galls that showed no sign of attack from

natural enemies ($N = 116$). Galls were lyophilized before measurements of dry weight.

Statistical Analyses. All statistical analyses were performed with SAS Version 8 (SAS Institute, Cary, NC). The invertase time course data were analyzed with repeated measures ANOVA of date by tissue with trees as subjects. Pairwise comparisons were made among least squares means for all date by tissue combinations, allowing comparisons between and among all dates and tissues. On all graphs, each bar represents the least squares mean plus one standard error of the mean, and bars with different letters indicate least squares means significantly different at $\alpha = 0.05$, unless otherwise specified. For the 1998 and 1999 studies, analysis of variance and linear regression were performed using PROC GLM, and all correlation coefficients are Pearson product moments.

RESULTS

Vacuolar Invertase (VI). VI activities per FW decreased over time although not equally among the different tissues (effect of date: $F_{3,6} = 139.15$, $P < 0.001$; tissue: $F_{2,4} = 101.57$, $P < 0.001$; and tissue by date; $F_{6,11} = 9.96$, $P < 0.001$). The VI activity per FW in galls was always significantly higher than that in both galled and ungalled leaves on any date, and galled leaves also had significantly greater activity than ungalled leaves on May 17, 1997 (Figure 1A). The pH optimum for all samples was 4.5, thus, vacuolar invertase measurements in subsequent work were made at pH 4.5 only.

Cytosolic Invertase (CI). CI activities per FW in leaves remained constant, except for the decrease in activity in both types of leaves on June 6, 1997. Only the effect of date was significant (date: $F_{3,6} = 4.95$, $P = 0.046$; tissue: $F_{2,4} = 1.81$, $P = 0.276$; tissue by date: $F_{5,10} = 1.73$, $P = 0.215$), although activities in galls increased over time (Figure 1B). The pH optimum for CI activity was 7.0 in all tissues.

Ionically Bound Cell Wall Invertase (CWIi). CWIi activities per FW in leaves decreased over time, whereas they increased in galls (Figure 1C). Thus, there were significant effects of date ($F_{3,6} = 23.64$, $P = 0.001$) and tissue by date ($F_{5,10} = 6.16$, $P = 0.007$), but not of tissue ($F_{2,4} = 3.86$, $P = 0.116$). The pH optimum ranged between 4.0 and 4.5.

Covalently Bound Cell Wall Invertase (CWIc). As with the CWIi, activities per FW of the CWIc decreased over time in leaves but increased in galls (Figure 1D; effect of date: $F_{3,6} = 14.71$, $P = 0.004$; tissue: $F_{2,4} = 36.36$, $P = 0.003$; and tissue by date: $F_{5,10} = 24.19$, $P < 0.001$).

Relative Growth Rate. Relative growth rate (RGR) of all tissues decreased over time (Figure 2). For all tissues analyzed together, RGR correlated significantly with VI activity, but not with any other invertase activity (Table 2). However,

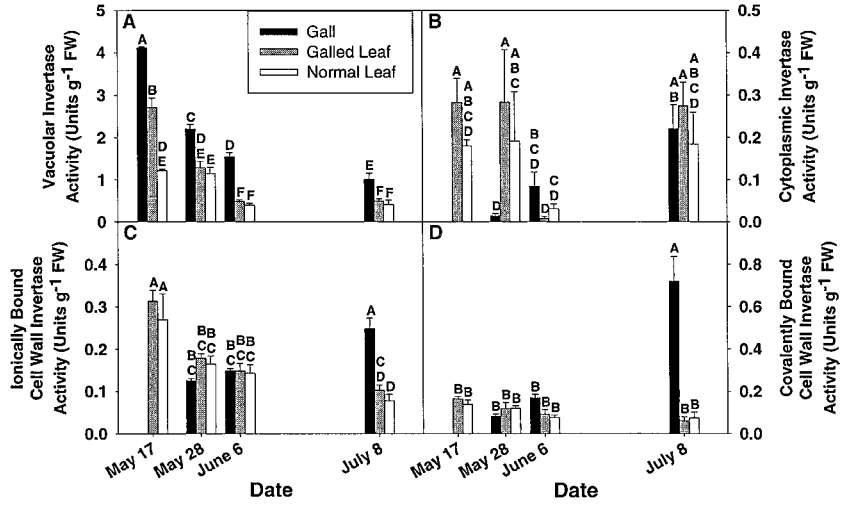

FIG. 1. Time course of invertase activities in witch hazel tissues on four dates in 1997. $N = 6$ for all bars except where noted. (A) Vacuolar (soluble acid) invertase. $N = 3$ for galls on May 17. (B) Cytosolic (soluble neutral) invertase. (C) Ionically bound cell wall invertase. (D) Covalently bound cell wall invertase. For B, C, and D, activity in galls was not measured on May 17 samples because of lack of tissue for extraction. Within each graph, bars with different letters indicate least squares means significantly different at $\alpha = 0.05$ using a Tukey adjustment.

tissues differed in the relationships between RGR and invertase activities. Gall RGR correlated strongly and positively with VI activity (Figure 3), but correlated negatively with all other invertase activities. In contrast, the RGR of leaves, both galled and ungalled, correlated positively with both VI and CWIi activities.

Fecundity and Invertase Study I. Invertase activities varied widely among individual galls, but activities per FW were not related to gall size (Table 3). Enzyme activities per FW had no relationships with either gall volume or FW; however, activities per gall related positively to gall size as expected. Also, VI and CWI activities per FW were not related ($r_p = 0.314$; $P = 0.086$).

However, invertase activities had a positive relationship with fecundity. In linear regressions (both: $P < 0.05$), CWI activity per gall accounted for 20.5% of the variation in offspring per gall (Figure 4A), whereas VI activity per gall accounted for 14.7% of the variation (Figure 4B). Together in a multiple regression, they accounted for 22.5% of the variation.

Vacuolar invertase activity had a weaker relationship than cell wall invertase with the number of offspring per gall. Twenty-eight of 31 galls had a VI activity less than 0.05 U per gall, and the three galls with the highest activities appeared somewhat anomalous relative to the other galls (Figure 4B). In the linear regression,

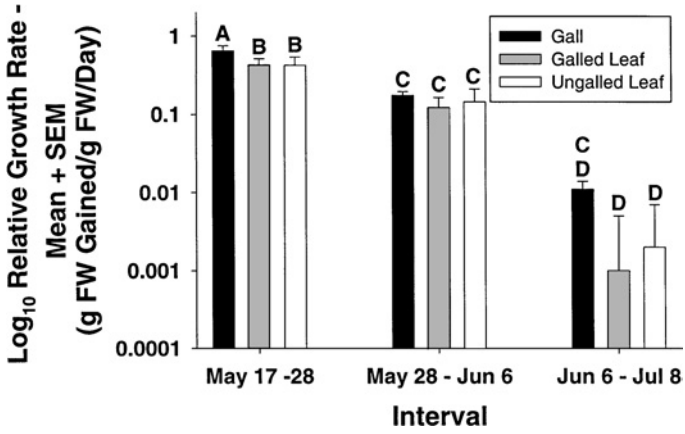

FIG. 2. Relative growth rates of galls, galled leaves, and ungalled leaves during the intervals between the four sampling dates in 1997. There was a significant effect of interval (repeated measures ANOVA of date × tissue of log +1 transformed data − interval: $F_{2,4} = 59.88$; $P = 0.001$; tissue: $F_{2,4} = 2.54$; $P = 0.194$; intervals × tissue: $F_{4,7} = 1.18$; $P = 0.397$). Also, galls had a higher relative growth rate than leaves during the first interval. Letters indicate pairwise comparisons of least squares means that are significant at $\alpha = 0.05$.

these three points had leverages (0.480, 0.176, 0.132) far higher than others (median leverage = 0.039). Removing the point with the highest leverage made the relationship insignificant ($P = 0.083$). In contrast, the relationship between CWI activity per gall and offspring per gall was more robust (Figure 4A). Only one point in the regression had relatively high leverage (0.221 versus a median of 0.048), but removing this point from the regression did not make the relationship insignificant ($P = 0.027$). Therefore, subsequent work focused on cell wall invertase.

TABLE 2. PEARSON CORRELATION COEFFICIENTS BETWEEN RELATIVE GROWTH RATE AND ENZYME ACTIVITIES IN THE INVERTASE TIME COURSE

Tissue	Vacuolar invertase	Cytosolic invertase	Ionically bound cell wall invertase	Covalently bound cell wall invertase
All	0.55***	−0.08‡	0.14‡	−0.21†
Galls	0.86***	−0.57*	−0.60*	−0.62*
Galled leaves	0.66**	0.14‡	0.59**	0.32†
Ungalled leaves	0.47*	−0.02‡	0.61**	0.47*

Note. Replicate numbers: all samples ($N = 51$), galls alone ($N = 15$), galled leaves ($N = 18$), and ungalled leaves ($N = 18$).
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; † $P > 0.1$; ‡ $P > 0.2$.

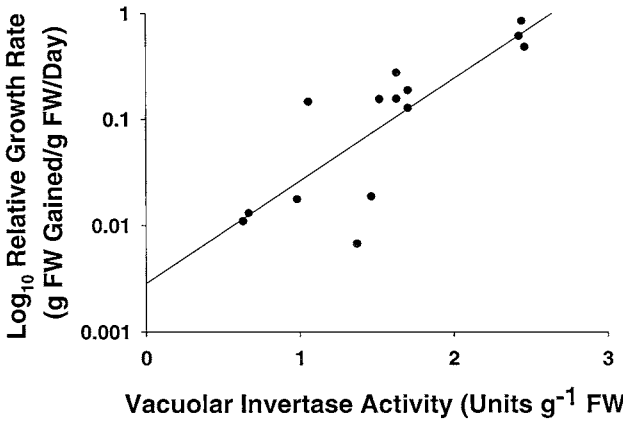

FIG. 3. Relative growth rate of galls regressed against their vacuolar invertase activities. There was a strong, significant relationship (linear regression using untransformed growth rates: $r^2 = 0.74$; $P < 0.001$).

Fecundity and Invertase Study II. Cell wall invertase activity per FW was greater in occupied than in unoccupied galls ($P < 0.05$; mean ± SEM in U/g FW: 4.18 ± 0.47 versus 2.57 ± 0.37, respectively). Activities of galled and ungalled leaves were the same (Student's t test: $t = 0.42$; df = 6; $P = 0.689$), and the mean CWI activity per FW of all leaves did not differ from those of unoccupied or invaded galls (mean ± SEM in U/g FW: 2.46 ± 0.57 versus 2.57 ± 0.37 and 2.69 ± 0.83, respectively).

Cell wall invertase activity related positively to fecundity. CWI activity per gall accounted for 14.5% of the variation in the number of offspring per gall (linear regression: $F_{1,27} = 4.58$; $P = 0.042$). A multiple regression model that used gall volume ($P = 0.123$), CWI activity per FW ($P = 0.126$), and their interaction ($P = 0.044$) as predictor variables explained 66.7% of the variation in

TABLE 3. SUMMARY DATA AND RELATIONSHIPS OF INVERTASE ACTIVITIES PER GALL AND PER GRAM FRESH WEIGHT WITH GALL VOLUME AND FRESH WEIGHT IN THE FIRST FECUNDITY AND INVERTASE STUDY

Invertase activity	Mean ± SEM (U)	Maximum (U)	Minimum (U)	Correlation with Volume	Correlation with FW
Vacuolar per gall	0.027 ± 0.005	0.120	0.000	0.511^{***}	0.660^{***}
Cell Wall per gall	0.076 ± 0.007	0.165	0.027	0.671^{***}	0.837^{***}
Vacuolar per FW	0.676 ± 0.089	1.778	0.000	$0.303^{\dagger}$	$0.316^{\dagger}$
Cell Wall per FW	2.025 ± 0.097	1.107	2.929	$0.182^{\ddagger}$	$0.088^{\ddagger}$

*** $P < 0.001$; † $P > 0.05$; ‡ $P > 0.20$.

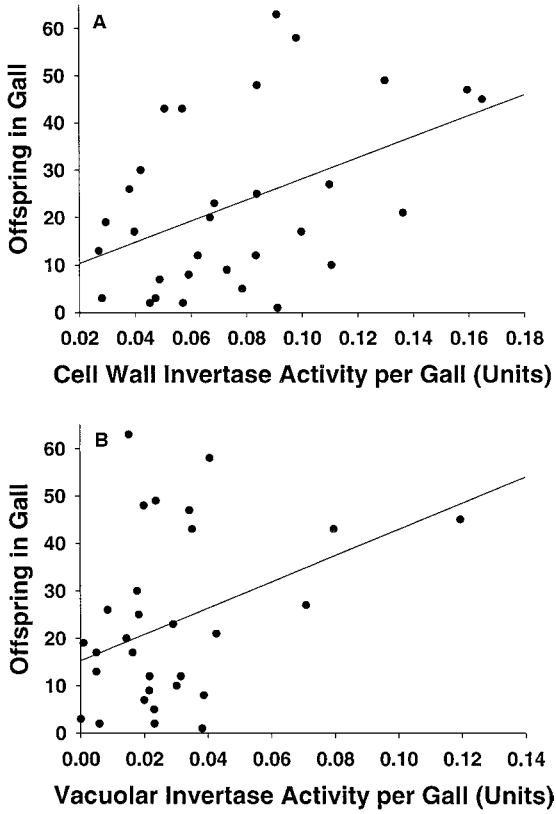

FIG. 4. The number of offspring in each gall regressed against (A) cell wall invertase activity of the gall and (B) vacuolar invertase activity of the gall in the first fecundity versus invertase study.

offspring per gall ($F_{3,25} = 16.69$; $P < 0.001$), and demonstrated that the interaction between gall size and CWI activity affected the number of offspring per gall. The interaction was a reinforcement, i.e., larger galls benefited more from a given increase in CWI activity per FW, as modeled by a conditional effects plot (Figure 5).

Gall Size and Aphid Fecundity. Gall dry weight predicted the number of offspring per gall better than gall volume. The correlation between gall volume and offspring per gall, and that between gall dry weight and offspring per gall, were both positive and significant (Figure 6). However, in a stepwise regression of offspring per gall on gall volume and dry weight, dry weight alone explained 34.2% of the variation, but gall volume failed to explain a significant amount of variation beyond that accounted for by dry weight ($P = 0.601$). Dry weight and

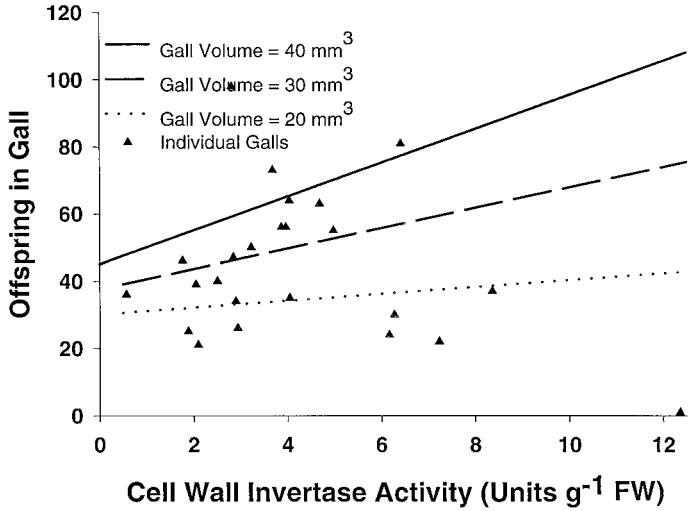

FIG. 5. Conditional effects plot of cell wall invertase activity per fresh weight and gall volume on the number of offspring per gall, with gall volume held constant at 20, 30, and 40 mm^3. The equation plotted is offspring $= 15.15 + 0.747$ (gall volume) $- 3.00$ (cell wall invertase activity) $+ 0.20$ (gall volume $\times$ cell wall invertase activity) for the three fixed gall volumes indicated.

volume were moderately and significantly correlated ($r_p = 0.687$; $P < 0.001$), thus, the explanatory power of gall volume in this and other studies likely stems from its correlation with gall dry weight.

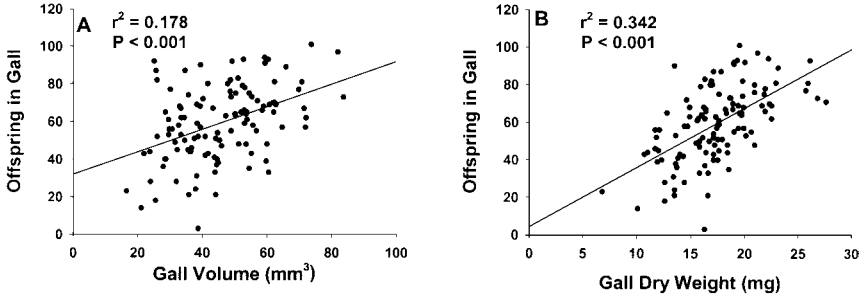

FIG. 6. Relationships among gall size and fecundity: offspring per gall regressed against (A) gall volume and (B) gall dry weight.

DISCUSSION

The patterns observed in the invertase time course were consistent with induced plant responses to the galling aphid. These patterns differed among all the types of invertase or tissues assayed, and so cannot be attributed to differential extraction efficiencies.

Galls always had greater vacuolar invertase activity than leaves, but all activities decreased over time, consistent with the enzyme's role in growth and development (Sturm and Tang, 1999). Gall growth correlated strongly with the activity of vacuolar invertase, consistent with this enzyme's role in growth and expansion of many plant tissues, whereas both vacuolar and ionically bound cell wall invertase correlated with leaf growth. Fundatrices could have chosen particular locations on leaves with higher invertase activity as sites for gall formation, but galls exhibited elevated activities throughout the course of gall growth, suggesting that the plant cells responded to the insect. This pattern is consistent with the fact that the fundatrix' presence is required to sustain gall growth (Rehill and Schultz, 2001). Also, we subdivided single leaves into small (ca. 6 mm) diameter portions and found considerably less variation ($<20\%$) in vacuolar invertase activities within individual leaves than the differences between galls and leaves (data not shown).

Cytosolic invertase activities showed no early enhancement, but the activities in galls subsequently varied independently of activity in leaves. Activities in galls indicated a later induction, steadily increasing over the last three sampling dates. The different patterns of VI versus CI activities in galls indicate that the CI activity was due to cytosolic invertase, not simply an artifact of vacuolar invertase activity at pH 7.0.

The patterns in cell wall invertase activity per FW provided the strongest evidence of enhanced enzyme activities in the gall. Activities of both the ionically and covalently bound cell wall invertases decreased over time in leaves, consistent with normal sink to source transition during leaf development (Schaffer et al., 1987; Ruffner et al., 1990), whereas the activities in galls increased over time. Active, nonphotosynthetic sinks such as corn kernels (Bulant et al., 2000) and potatoes (Kefi et al., 2000) often have high cell wall invertase activities, especially later in development as they accumulate mass after attaining nearly full size. Hence, the pattern observed in galls is consistent with the gall maintaining and/or increasing its status as a strong sink for photoassimilates, similar to other plant tissues. Also, these increases in cell wall invertases occurred *before* any offspring were found in galls, thus, it is highly likely that they constitute plant responses to the fundatrix only.

Increased invertase activities in *H. hamamelidis* galls are consistent with invertases acting as a mechanism of increased sink strength in galls, but we have not yet identified any mechanisms of induction. Both vacuolar and cell wall invertases are wound-inducible (Sturm and Chrispeels, 1990; Ehness et al., 1997; Ohyama

et al., 1998), thus, the repeated wounding of the leaf by the fundatrix at the time of gall elicitation could explain the early increase in vacuolar invertase activity found in galls and galled leaves. However, the later increases of cytosolic and cell wall invertase activities occur at a time when the females remain in a single feeding site and only move after molting (Lewis and Walton, 1958). Substances transmitted via the aphid's saliva during probing and feeding could have caused the observed reactions; however, no aphid investigated contains any invertase among its complement of salivary enzymes (Miles, 1999). Increased invertase activities likely play a role in increasing sink strength in other insect galls, but may be induced by a variety of stimuli.

Observations of individual galls revealed a wide range of cell wall invertase activity per FW and per gall. The positive relationship between cell wall invertase activity per gall and the number of offspring per gall was consistent with the hypothesis that increased sink enzyme activity benefits the fundatrix. A conditional effects plot (Figure 5) shows that fundatrices that create larger galls benefit more from later increases in cell wall invertase activity per FW. Expansion growth in plants is driven in part by vacuolar invertase (Sturm and Tang, 1999), and this appears to be the case for galls of *H. hamamelidis*. These galls have greater vacuolar invertase activities than leaves perhaps caused (at least initially) by the stereotypical wounding behavior performed by fundatrices during gall initiation, and gall size is related to vacuolar invertase activity. Thus, it appears that fundatrices that create larger galls by inducing greater initial vacuolar invertase activities are better positioned to benefit from later increases in cell wall invertase activities.

Unlike vacuolar invertase, cell wall invertase was not linked with gall size; larger galls did not have greater cell wall invertase activities per FW. Indeed, in one study, the smallest gall had the greatest cell wall invertase activity per FW, yet had the lowest number of offspring per gall. In contrast, the largest gall had the most offspring and average cell wall invertase activity per FW. This suggests that multiple gall traits arising at different times influence fecundity, including early vacuolar invertase activity and later the induction of greater cell wall invertase activity. A more detailed study of temporal variation in such traits would be needed to sort out these effects. For example, fundatrix ovaries and the first *H. hamamelidis* embryos are formed before maturation (Lewis and Walton, 1958). Thus, enhanced nutrition before and during this time would likely improve reproductive performance, and impacts of increased invertase activities could propagate through time. Sampling for invertase activities and offspring are both destructive, so these hypotheses cannot be tested directly in this system.

That gall dry matter accumulation predicts aphid fecundity better than gall volume further illustrates the impact of sink strength on aphid performance. For most of its life, the gall has little or no apparent chlorophyll and so little or no ability to photosynthesize. Thus, gall dry weight indicates the average sink strength of the gall over its lifetime, since it results from sink activity over time (Bulant

et al., 2000; Kefi et al., 2000; Keutgen et al., 2002). Fundatrices appear to control gall size (Rehill and Schultz, 2001) and induce vascular proliferation in their galls (Lewis and Walton, 1958), as is the case for other galling aphids (Wool et al., 1999). Space and feeding sites for the second generation aphids in *Hormaphis* galls both appear to be adequate, and our results suggest that sink strength and gall mass are at least as important.

We suggest that *H. hamamelidis* is one of possibly many sessile insects that enhance resources by manipulating the host plant (Danks, 2002; Karban and Agrawal, 2002). For example, some nongalling aphids induce beneficial changes in susceptible hosts (Dorschner et al., 1987; Formusoh et al., 1992; Miller et al., 1994), but not resistant hosts (Telang et al., 1999). The widespread relationship between gall size (under insect control to some extent) and fecundity is another example of host plant improvement (Weis and Abrahamson, 1986; Abrahamson et al., 1989; Stiling and Rossi, 1997; McKinnon et al., 1999; Bjorkman, 2000; Rehill and Schultz, 2001). It seems likely that success of many herbivores such as *H. hamamelidis* is related to their ability to manipulate plant responses for their own benefit.

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THE VENOM ALKALOIDS FROM SOME AFRICAN *Monomorium* SPECIES

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Abstract—A comparative analysis of the venom alkaloids of 13 species of ants in the genus *Monomorium* collected in Africa was undertaken. Ants were collected in Kenya and South Africa. Most species produced 2,5-dialkylpyrrolidines and some of their analogous 1-pyrrolines. No alkaloids were detected in two species, *M. robustior* and *M. macrops*. Additionally, a novel 2-alkyl-1-pyrroline, whose structure was established by synthesis, was detected in *M. notulum*. This compound and others like it may serve as biosynthetic precursors for the formation of the 2,5-dialkylpyrrolidines found in many Myrmicine ant species.

Key Words—*Monomorium* spp., Hymenoptera, Formicidae, 2,5-dialkylpyrrolidines, pyrrolines, ant venom alkaloids, chemotaxonomy.

INTRODUCTION

Saturated nitrogen heterocycles are known components of the venoms of ants in the genera *Monomorium* and *Solenopsis* (Jones et al., 1982b). These alkaloids play a well-documented defensive role in *Monomorium* species (Andersen et al., 1991). While different species of ants may have the same alkaloids, the alkaloid composition of a particular species seems to be characteristic, varying only with the age of the ants (Deslippe and Guo, 2000). Comparisons of the alkaloid composition in *Solenopsis* species have been made a number of times (Brand et al., 1972; MacConnell et al., 1976; Vander Meer and Lofgren, 1988). Conservatively, there

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are ca. 300 species of *Monomorium* worldwide (Heterick, 2001), and the chemistry of a number of individual species has been reported (Jones et al., 1982b, 1989, 1990, 2003; Andersen et al., 1991; Don et al., 2001). Although indolizidines, piperidines, and pyrrolizidines have been found in *Monomorium* species, 2,5-dialkylpyrrolidines are the most commonly detected alkaloids in this genus. There have been comparative studies of the alkaloids of some groups of *Monomorium* species in the United States and New Zealand (Jones et al., 1982a, 1988) made with some interest in their taxonomic value.

In the New Zealand *Monomorium "antarcticum"* species, a variety of 17- and 19-carbon 2,5-dialkylpyrrolidines are present with their bicyclic pyrrolizidine analogs. The structural variations in the alkaloids from the New Zealand ants also include the presence or absence of side chain double bonds and the position of cyclization in the bicyclic compounds. Analysis of the mixtures of alkaloids from nearly 40 collections indicates the presence of at least four "species" in this complex. In contrast with this complexity, the set of previously described U.S. *Monomorium* species studied in 1982 contained only 19-carbon 2,5-dialkylpyrrolidines with six- and nine-carbon side chains. Variations among species were the differing numbers of double bonds in the side chains. The presence or absence and relative amounts of these compounds were consistent for several collections.

In this paper, we describe the venom alkaloids of 13 African species of *Monomorium* collected in Kenya and South Africa along with two species that do not produce alkaloids, *M. macrops* and *M. robustior*. In all but one case, the alkaloids are mixtures of previously reported 2,5-dialklypyrrolidines or their 1-pyrrolines. However, in one South African species, the sole nitrogen containing component was 2-pentyl-1-pyrroline, **12**, the first example of a monosubstituted pyrroline to be detected in an ant. The structure of **12** was suggested by its GC/MS and FT-IR data and confirmed by synthesis.

METHODS AND MATERIALS

Chemical Analysis. Gas chromatography/mass spectrometry was carried out in the EI mode using a Shimadzu QP-5000 GC/MS equipped with a RTX-5, 30 m × 0.32-mm i.d. column. The instrument was programmed from 60 to 250°C at 10°/min. Vapor phase FT-IR spectra were obtained using an Hewlett-Packard model 5965B detector interfaced with an Hewlett-Packard 5890 gas chromatograph fitted with a 30 m × 0.25 mm Rtx-5 amine column. High resolution mass spectrometry was performed on a JEOL SX102 instrument. Identification of the alkaloids was confirmed by direct comparison of their mass spectra and retention times with those of synthetic samples available from previous studies.

Ants. Ants (workers) were collected and immediately placed in small vials containing methylene chloride or absolute methanol. The alkaloid components

TABLE 1. ALKALOIDS IDENTIFIED FROM AFRICAN *Monomorium* SPECIES

Monomorium species (# collections)	Alkaloids												Species groups
	1	2	3	4	5	6	7	8	9	10	11	12	
M. fridae (5)	−	+	−	−	o	*	o	−	o	−	−	−	*salomonis*
M. australe (5)	−	+	−	−	−	+	−	−	*	−	−	−	*salomonis*
M. australe complex (3)	−	−	−	−	−	−	−	−	−	−	*	−	*salomonis*
M. macrops (3)	−	−	−	−	−	−	−	−	−	−	−	−	*setuliferum*
M. albopilosum (1)	−	−	−	−	−	o	−	−	*	−	−	−	*salomonis*
M. rufulum (1)	−	*	−	−	−	−	−	−	−	−	−	−	*salomonis*
M. rhopalocerum (1)	−	−	−	−	−	o	o	−	*	−	−	−	*monomorium*
M. notulum (1)	−	−	−	−	−	−	−	−	−	−	−	*	*setuliferum*
M. mediocre (1)	−	−	−	−	−	−	−	−	*	−	−	−	*salomonis*
M. afrum (1)	o	*	+	o	−	+	o	o	−	−	−	−	*salomonis*
M. subopacum (2)	−	−	o	o	−	+	*	−	−	−	−	−	*salomonis*
M. robustior (5)	−	−	−	−	−	−	−	−	−	−	−	−	*destructor*
M. cf *pallidipes* (1)	−	−	−	−	−	−	−	−	−	*	−	−	*monomorium*

Note. * = major component (>20%); + = minor component (5–20%); o = trace (<1%); − = not detected.

of the extracts can be distinguished from the lipid components by their mass spectra, which generally have odd-mass molecular ions and even-mass fragments (for alkaloids with one nitrogen). Those ants containing alkaloids are listed in Table 1. Collections of *M. fridae* Arnold were obtained from near Witelsbos, South Africa (34.12S, 24.12E), *M. australe* Emery from Wuppertal, South Africa (32.16S, 19.13E, 8/89), *M. australe* complex and *M. macrops* Arnold from Kromrivier Farm, Cedarberg, South Africa (32.32S, 19.17E) 8/89, *M. albopilosum* Emery from near Mafikeng, South Africa (25.93S, 25.83E), *M. rufulum* Stitz from 32.1 km NW of Disaneng Dam, South Africa (25.53S, 24.97E), *M. rhopalocerum* Emery from Kleinmond Nature Reserve, South Africa (34.33S, 19.00E), *M. notulum* Forel from 32.1 km NW of Disaneng Dam, South Africa, *M. mediocre* Santschi form West Cape, South Africa (32.294S, 23.01E), *M. afrum* André from Laikipia District, Kenya (0.30N, 36.91E), *M. subopacum* Smith and *M. robustior* Forel from Ewaso Ng'iro near Mpala Research Centre, Laikipia District, Kenya (0.29N, 36.90E), and *M.* cf *pallidipes* Forel from Mpala Research Centre, Laikipia District, Kenya. A collection of *Monomorium pharaonis* Linné also was obtained from Kakamega District, Isecheno, Kakamega Forest, Kenya (0.02N, 34.87E, 10/99). Voucher specimens of all Kenyan samples are deposited in the collection of the Los Angeles County Museum of Natural History, Los Angeles, CA.

M. notulum. GC/MS analysis of the extracts of *M. notulum* showed a single nitrogen containing major component with a mass spectrum: MS m/z (rel%): 138(M−1, 1), 124(1), 110(10), 96(30), 84(6), 83(10), 82(27), 68(7), 55(20), and 41(40), and GC/FT-IR 1645 cm^{-1}. Four other minor nitrogen-containing

components were detected comprising ca. 1% of the mixture. A small portion of the extract was treated with $NaCNBH_3$ to provide a single component MS m/z (rel%): 141(M+, 1), 126(1), 112(1), 82(1), and 70(100).

*Preparation of 2-Pentyl-1-Pyrroline (**12**).* A solution containing 2.3 g (15 mmol) of 4-oxononylnitrile (Stetter et al., 1980), 10 ml of ethylene glycol, and 0.5 g of *p*-toluenesulfonic acid in 100 ml of benzene were heated to reflux under a Dean–Stark trap until all separation of water ceased. The mixture was cooled, washed with saturated $NaHCO_3$, dried over $MgSO_4$, and the solvent was removed to provide 4-oxononylnitrile ethylene ketal: MS m/z (rel%): 197(1), 152(1), 143(20), 126(100), 82(79), 71(15), 55(30), 54(52), and 43(44). This product was taken up in 150 mL of anhydrous ether, and treated with 1.5 g of $LiAlH_4$. The mixture was stirred overnight and treated with 1.5 ml of H_2O, 1.5 ml of 10%NaOH, and 4 ml of H_2O. After the addition of anhydrous K_2CO_3, the mixture was filtered, and the solvent was removed to provide nearly pure 2-aminoethyl-2-pentyl-1, 3-dioxolane: MS m/z (rel%): 201(M+, 1), 171(1), 159(2), 143(73), 130(35), 113(81), 99(11), 86(15), 69(34), 55(25), 43(100), and 41(50). This product was stirred overnight in a mixture of 20 ml of THF and 70 ml of 10% HCl containing 1 ml of 70% $HClO_4$. The mixture was extracted with ether and neutralized with K_2CO_3. The basic solution was extracted with 3×50 ml of ether, and the ether layers were dried over anhydrous K_2CO_3, filtered, and solvent was removed to provide 2.1 g of 2-pentyl-1-pyrroline, whose GC-MS retention time and GC/FT-IR spectrum were identical to those of the major component in *M. notulum*: MS m/z (rel%): 138(M−1, 1), 124(1), 110(10), 96(30), 84(6), 83(10), 82(27), 68(7), 55(20), and 41(40); HRMS: Calculated for $C_9H_{17}N$, 139.1361; observed 139.1353. Calculated for $C_9H_{16}N$ (M−1), 138.1283; observed 138.1280.

RESULTS

The alkaloids detected in most African *Monomorium* species examined were *trans*-2,5-dialkylpyrrolidines (Table 1, and Figure 1). Their structures were suggested by their mass spectra and were confirmed along with their stereochemistry by direct comparison with synthetic samples available from a previous study (Jones et al., 1989). In two cases, *M*. nr *drapenum* and *M*. cf *pallidipes*, known 2,5-dialkyl-1-pyrrolines were detected (Pedder et al., 1976), the *N*-methyl derivatives of the pyrrolidines were detected in *M. fridae* and in *M. australe*, and in *M. macrops* and *M. robustior*, no alkaloids were detected. *Monomorium pharaonis* contained alkaloids previously reported for this species (Ritter and Persoons, 1975).

The extracts of *M. notulum* showed a single nitrogen-containing component with a mass of 139, and an intense ion at $m/z = 83$, suggesting a rearrangement. This was confirmed by an infrared band at 1645 cm^{-1}, indicating a C=N bond in a five-membered ring. Treatment of a small portion of the extract with $NaCNBH_3$

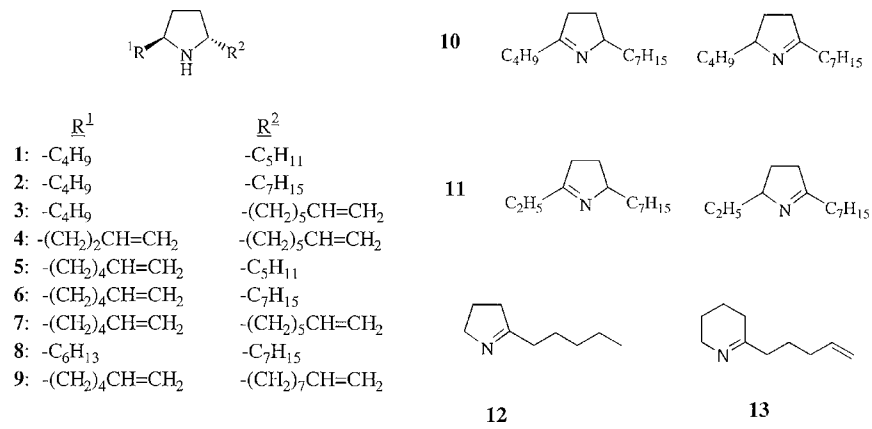


FIG. 1. Alkaloids (**1–12)** detected in African *Monomorium* species.

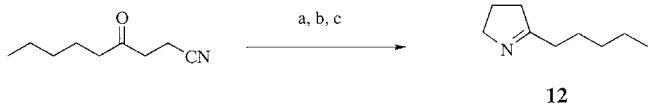
converted this compound to a single component with a molecular weight of 141 and a base peak in its mass spectrum at $m/z = 70$ indicative of 2-pentyl pyrrolidine.

The synthesis of 2-pentyl-1-pyrroline (**12**) was achieved in a straightforward manner from 4-oxononylnitrile (Stetter et al., 1980). After protection of the ketone, the nitrile was reduced to the amine with lithium aluminum hydride, and acidic deprotection provided the pyrroline **12** nearly quantitatively (Scheme 1).

DISCUSSION

Prior to this study, the venom alkaloids of only one other African *Monomorium* species, *M. delagoense*, had been investigated, and in that case, a unique set of 2,6-dialkylpiperidines were found (Jones et al., 1990). In contrast, in these *Monomorium* collections from Kenya and South Africa, the alkaloids all have five-membered rings, although in multiple collections of two species, no alkaloids were detected (Table 1).

One of the primary differences observed in previous comparisons from different ant species is the length of the carbon chain. The presence of the 19-carbon



a = $(HOCH_2)_2$, ptsa; b = $LiAlH_4$; c = HCl/ $HClO_4$

SCHEME. 1. Synthesis of 2-pentyl-1-pyrroline (**12**).

trans-2-(5-hexenyl)-5-(8-nonenyl)-pyrrolidine (**9**) in *M. australe*, *M albopilosum*, and *M. rhopalocerum* is reminiscent of *Monomorium* species from North America and New Zealand. Pyrrolidine **9** and its bicyclic homologues predominate in the venom of some New Zealand species, while **9** and its saturated homologues are the only detectable alkaloids in a number of North American species (Jones et al., 1982a, 1988). The 17-carbon alkaloids **6**, **7**, and **8** occur in more complex mixtures in *M. fridae* and *M. subopacum,* somewhat like the alkaloid mixture from *M. indicum* (Jones et al., 1989) where they are major components in a complex mixture. A previous investigation of *M. subopacum* collected in Spain revealed the presence of the 15-carbon pyrrolidine **2** as the major component (Jones et al., 1982b). The disparity between the venom chemistry of the collection from Spain and the present results suggests that two sibling species may be involved. The 15-carbon alkaloids of *M. australe* complex, *M. rufulum*, *M. afrum,* and *M*. cf *pallidipes* are well known from a number of *Solenopsis (Diplorhoptrum)* species (Jones et al., 1982b).

The 1-pyrrolines, **11**, in *M. australe* complex are major components in the South African ant *Solenopsis punctaticeps* where they are present with the corresponding pyrrolidines and **1** and **2** (Pedder et al., 1976). To find them, as well as pyrroline **10**, in *M*. cf *pallidipes* from Kenya, as the only detectable alkaloids in these species is surprising. Since it is likely that reduction of a carbon nitrogen double bond is the final step in the formation of naturally occurring, saturated nitrogen heterocycles, it may be that these 1-pyrrolines are the result of an incomplete biosynthetic pathway.

The occurrence of 2-pentyl-1-pyrroline (**12**) in *M. notulum* is the first report of a monosubstituted 1-pyrroline from ant venom. This structure may be analogous to the monosubstituted 1-piperideine **13**, which is the only alkaloid found in the workers of the Caribbean ant *Solenopsis azteca* (Spande et al., 1999). In that case, the queens contain a series of decahydroquinolines for which **13** may be the precursor. Little is known about the biosynthesis of the 2,5-dialkylpyrrolidines in ants, and monosubstituted 1-pyrrolines like **12** could be precursors to this large group of alkaloids. It would be worth investigating the queens of this species to see there are any caste specific differences in alkaloids, as have been observed in a number of *Solenopsis* species (Jones et al., 1996; Spande et al., 1999; Torres et al., 2001).

Nearly 145 species of afrotropical *Monomorium* have been listed (Bolton, 1987). Since the comparison of venom alkaloids of *Monomorium* species from New Zealand and the United States may have taxonomic utility, the present results should serve as a future basis for a comparison with other African *Monomorium* species.

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VOLATILE COMPONENTS IN METATARSAL GLANDS OF SIKA DEER, *Cervus nippon*

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Abstract—GC-MS analysis of the metatarsal gland secretion from a female sika deer, *Cervus nippon*, showed 35 major volatile compounds that included 13 straight-chain carboxylic acids, a single branched-chain carboxylic acid, 9 straight-chain aldehydes, 3 monounsaturated aldehydes, 5 long-chain alcohols, a ketone, and cholesterol. The four most abundant compounds were heptanal, nonanal, octanoic acid, and 6-methyl-2-heptanone. Many of the compounds have previously been found in cervid secretions, but the unsaturated aldehydes, (*E*)-2-octenal, (*E*)-2-nonenal, and (*E*)-2-decenal, have not previously been reported in the glands of any cervid. The compounds in this gland may be pheromones, since metatarsal gland odor has been implicated in chemical communication among conspecifics of other cervids.

Key Words—Cervidae, *Cervus nippon*, sika deer, metatarsal gland secretion, fatty acids, long-chain alcohols, straight-chain aldehydes, cholesterol.

INTRODUCTION

Skin glands are a prominent feature of many species of deer (Cervidae) and the identification of the chemicals in these secretions has been the focus of a number of studies. These investigations include studies of the foot or interdigital glands of reindeer, *Rangifer tarandus* (Sokolov et al., 1974; Brundin et al., 1978; Andersson et al., 1979; Brundin and Andersson, 1979), whitetail deer, *Odocoileus virginianus* (Gassett et al., 1996; Wood, 1999), and black-tailed deer, *O. hemionus columbianus* (Wood et al., 1995a,b), the forehead gland of whitetail deer (Gassett et al., 1997), the tarsal gland of black-tailed deer (Müller-Schwarze et al., 1978) and reindeer (Andersson et al., 1975), the preorbital gland of reindeer (Sokolov et al., 1977; Andersson, 1979), the caudal gland of reindeer (Müller-Schwarze

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et al., 1977), the nasal gland of the marsh deer, (*O. Dorcelaphus*) *dichotomus* (Jacob and Von Lehmann, 1976), and the tail gland of red deer, *Cervus elaphus* (Bakke and Figenschou, 1983).

Many deer have a prominent metatarsal gland on each lower hind leg. The metatarsal gland components of impala, *Aepyceros melampus*, the only African antelope that has metatarsal glands (Estes, 1991) have been analyzed (Wood, 1997). Behavioral studies with American black-tailed deer (Müller-Schwarze et al., 1984) and European roe deer (Broom and Johnson, 1980) indicate the glandular secretion is important in intraspecies communication, but the contents of these glands have not been reported for these or any other cervid species. This communication reports the major volatile components from the metatarsal gland of sika deer, *Cervus nippon.* Sika are medium-sized deer that were originally found in Eastern Siberia, Japan, Manchuria, Formosa, and China. Numerous introductions have been made throughout the world, and in the United States free-ranging populations are found in Texas, Maryland, Wisconsin, and Virginia (Feldhamer, 1980).

METHODS AND MATERIALS

The metatarsal secretion from a single, free-ranging, adult sika doe from Kerr County, Texas, USA, was collected during the month of March 2003. The secretion was obtained from the metatarsal glands by cutting a sample of the hair from inside the gland and placing it in glass vials with Teflon-lined stoppers containing 2.0 ml of CH_2Cl_2. A control sample of hair was collected from the leg about 30 cm above the gland and similarly preserved in CH_2Cl_2. A sample of the solvent was saved for later analysis. The samples were carried at ambient temperature to Arcata, California, USA, and within 3 days of collection were analyzed by gas chromatography–mass spectrometry (GC-MS).

The GC-MS analyses were performed on the CH_2Cl_2 extracts in splitless mode (0.5 min), using an Hewlett-Packard GCD Plus fitted with a 30 m $\times$ 0.25 mm cross-linked phenyl methyl silicone capillary column (HP-5MS). The gas chromatograph was programmed so the oven temperature was kept at 40°C for 4 min, then increased to a final temperature of 325°C at a rate of 30°C/min, and kept at this temperature for 5 min. Mass spectral fragments below $m/z = 39$ were not recorded. The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average of both glands ($\pm$standard deviation). Impurities identified in the solvent and/or the nonglandular hair control samples are not reported.

Initial identification of secretion components was done by comparison of mass spectra in the NIST 1998 computerized mass spectral library. All identifications were confirmed by comparison of spectra and retention times with those of authentic standards. Authentic samples were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, and Fisher Scientific (Acros), Pittsburgh, Pennsylvania,

except for 6-methyl-2-heptanone. 6-Methyl-2-heptanone was prepared by catalytic hydrogenation of 6-methyl-5-hepten-2-one (Acros) with PtO_2 and H_2.

RESULTS AND DISCUSSION

The GC-MS analyses of the sika deer metatarsal secretion showed 35 major volatile compounds, of which 14 carboxylic acids, 12 aldehydes, 6 alcohols, and 1 ketone were identified (Table 1). The four most abundant compounds were

TABLE 1. VOLATILE COMPOUNDS IN SIKA METATARSAL SECRETION

Retention time	Compound	Average (%)[a]
4.54	Hexanal	2.0 ± 0.1
4.63	Pentanoic acid	0.8 ± 0.5
5.94	Heptanal	5.3 ± 0.6
6.11	Hexanoic acid	1.8 ± 0.2
6.48	6-Methyl-2-heptanone	5.6 ± 0.4
6.88	Octanal	2.2 ± 0.2
7.29	(*E*)-2-Octenal	2.3 ± 0.1
7.52	Heptanoic acid	4.0 ± 0.5
7.58	Nonanal	9.0 ± 0.7
7.91	(*E*)-2-Nonenal	3.8 ± 0.5
8.08	Octanoic acid	7.9 ± 0.1
8.16	Decanal	2.2 ± 0.4
8.45	(*E*)-2-Decenal	2.0 ± 0.2
8.53	Nonanoic acid	4.4 ± 0.1
8.66	Undecanal	1.7 ± 0.2
8.97	Decanoic acid	4.6 ± 0.4
9.13	Dodecanal	2.2 ± 0.1
9.39	Undecanoic acid	3.4 ± 1.0
9.41	1-Dodecanol	1.0 ± 0.5
9.56	Tridecanal	1.1 ± 0.3
9.79	Dodecanoic acid	2.8 ± 0.1
9.83	1-Tridecanol	1.6 ± 0.1
9.97	Tetradecanal	1.0 ± 0.3
10.17	Tridecanoic acid	2.5 ± 0.3
10.22	1-Tetradecanol	2.2 ± 0.1
10.41	12-Methyltridecanoic acid	1.9 ± 0.1
10.54	Tetradecanoic acid	3.2 ± 0.2
10.60	1-Pentadecanol	2.0 ± 1.0
10.73	Unidentified	1.7 ± 0.5
10.89	Pentadecanoic acid	2.7 ± 0.5
10.95	1-Hexadecanol	2.4 ± 0.1
11.28	Hexadecanoic acid	3.0 ± 0.9
11.63	Unidentified	1.4 ± 0.2
11.84	Octadecanoic acid	1.0 ± 0.7
14.61	Cholesterol	3.3 ± 0.1

[a] Average of two glands ± standard deviation.

heptanal, nonanal, octanoic acid, and 6-methyl-2-heptanone. Except for 6-methyl-2-heptanone, 12-methyltridecanoic acid, and cholesterol, all of these compounds have straight chains. The 13 straight-chain carboxylic acids ranged sequentially from pentanoic acid to octadecanoic acid with the exception of heptadecanoic acid. All saturated aldehydes from C_6 to C_{14} were present, along with (*E*)-2-octenal, (*E*)-2-nonenal, and (*E*)-2-decenal. The straight-chain alcohols range in length from C_{12} to C_{16}.

The unsaturated aldehydes, (*E*)-2-octenal, (*E*)-2-nonenal, and (*E*)-2-decenal, have not previously been found in the glands of any deer species. The only ketone in the secretion, 6-methyl-2-heptanone, has been identified from other cervids. It is found in reindeer interdigital gland secretion (Andersson et al., 1979) and among urinary volatiles excreted by whitetail deer (Jemiolo et al., 1995; Miller et al., 1998). Recently this compound was identified in the ear secretion of the African elephant (Riddle et al., 2000).

The metatarsal gland odor has been implicated in chemical intraspecific communication in cervids. Female black-tailed deer exhibit increased alertness response when presented with metatarsal odor from either male or female glands (Müller-Schwarze et al., 1984). In roe deer, males respond to conspecific metatarsal secretion (Broom and Johnson, 1980). Response to female secretion was stronger than to metatarsal scent from males. In this latter study, females were not tested for their response to either male or female scent.

The tarsal gland, located above the metatarsal gland and on the inside of the leg of black-tailed deer, also is involved in the chemical communication in this species (Müller-Schwarze et al., 1978). Chemicals in the urine, especially (*Z*)-4-hydoxy-6-dodecenoic acid lactone, are deposited on the tarsal gland tufts during rub-urination. These deer respond to the tarsal gland of conspecifics by sniffing and licking and also to synthetic samples of this lactone. The compounds in reindeer tarsal glands have also been investigated (Andersson et al., 1975). Several straight-chain aldehydes (C_7 to C_{10}) and straight-chain alcohols (C_{12}, C_{14}, and C_{16}) were identified from this gland, but no behavioral tests were done with these compounds. All of these compounds from the reindeer tarsal glands were present in the metatarsal gland of the sika deer.

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FIELD EVALUATION OF PLANT ODOR AND PHEROMONAL COMBINATIONS FOR ATTRACTING PLUM CURCULIOS

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Abstract—The attractiveness of different synthetic host odors and a synthetic aggregation pheromone (grandisoic acid [GA]) to overwintered adult plum curculios (PCs), *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), was examined using two types of traps (sticky panels and black pyramids) placed in border areas surrounding an unsprayed section of an apple orchard in Massachusetts. In 2001, we evaluated the response of PCs to three synthetic fruit volatiles (benzaldehyde [BEN], ethyl isovalerate [EIV], and limonene [LIM]) assessed alone and in combination with GA, as well as the response to GA alone and a no-odor (control) treatment. BEN was the only host volatile that synergized the response of PCs to GA for both trap types. For both trap types, GA was as attractive to PCs as a single component as when in combination with either EIV or LIM. In 2002, four release rates of BEN (0, 2.5, 10, and 40 mg/day) and two release rates of GA (1 and 2 mg/day) were evaluated for attractiveness to PCs using panel and pyramid traps. For panel traps, an increase in amount of GA released (from 1 to 2 mg/day) was associated with a 35% increase in captures. However, PC captures by pyramid traps were similar regardless of the amount of GA released. For panel traps, 10 and 40 mg/day of BEN were the most attractive release rates regardless of the amount of GA released. For pyramid traps baited with GA, PC captures were enhanced by the presence of BEN, regardless of release rate. In 2003, GA at 1 mg/day + BEN at 80 mg/day of release did not enhance PC captures by panel traps relative to lower release rates of BEN. Pyramid traps releasing GA at 1 mg/day performed best when baited with BEN at 10 mg/day of release; a release rate of 80 mg/day of BEN decreased the attractiveness of the binary combination of BEN + GA. Combined results suggest that BEN at 10 mg/day + GA at 1 mg/day of release constitutes an attractive lure that may improve the effectiveness of monitoring traps for PCs.

Key Words—Plum curculio, *Conotrachelus nenuphar*, host–plant volatiles, response, aggregation pheromone, benzaldehyde, synergism, monitoring traps.

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INTRODUCTION

Aggregation via behavior-modifying chemicals occurs in a wide range of insects, but predominantly in the Coleoptera (Borden, 1984; Landolt, 1997). This behavioral feature has allowed researchers and integrated pest management practitioners to develop and implement semiochemical-based trapping of adults of a number of beetle species that are pests in both agroecosystems and forests (Borden, 1993). For instance, the discovery that aggregation pheromones and natural host-produced volatiles mediate host selection behavior in weevils has led to the development of monitoring and mass trapping systems for several species of beetles. Thus, bark beetles are attracted by synthetic aggregation pheromones in association with live or dead host trees (Borden, 1984; Byers, 1989; Landolt and Phillips, 1997). Analogously, for species of weevils for which mass trapping is used as a control tactic, such as *Rhynchophorus palmarum* L. (Alpizar et al., 2002; Oehlschlager et al., 2002a), *R. ferrugineus* (Olivier) (Hallett et al., 1999), and *Metamasius hemipterus* L. (Ramirez-Lucas et al., 1996; Alpizar et al., 2002; Oehlschlager et al., 2002b), the most powerful attractants are composed of aggregation pheromones, synthetic host plant volatiles, and host plant material. Recent investigations by Phillips et al. (1993), Gries et al. (1994), Perez et al. (1994), Weissling et al. (1994), Ramirez-Lucas et al. (1996), Perez et al. (1997), and Hallet et al. (1999) have shown that either natural host tissue or synthetic host plant volatiles synergistically enhance weevil response to aggregation pheromone.

The plum curculio (PC), *Conotrachelus nenuphar* (Herbst), is a key pest of pome and stone fruit in eastern and central North America (Racette et al., 1992). In the fall, most adults migrate from apple orchards to the edges of adjacent forested areas, where they hibernate (LaFleur et al., 1987). In spring, overwintered adults immigrate into apple orchards, where they cause injury to apple buds and developing fruit as a result of adult feeding and oviposition activity, the latter commencing shortly after petal fall (Lafleur and Hill, 1987; Chouinard et al., 1993). Despite its status as a key pest, no truly effective traps have yet been developed for tracking the seasonal course of PC immigration into apple orchards (reviewed in Piñero et al., 2001; Prokopy et al., 2003) although in the southern USA, baited pyramid traps have been proposed for sampling PC in peach orchards (Johnson et al., 2002).

Plum curculios use chemical cues to locate potential hosts (Butkewich and Prokopy, 1993). Based upon screening more than 30 individual compounds, recent investigations have revealed that some volatiles such as benzaldehyde (BEN), ethyl isovalerate (EIV), and limonene (LIM), released from developing host fruit, are attractive to PCs (Leskey and Prokopy, 2000; Leskey et al., 2001; Prokopy et al., 2001). In particular, BEN, when used in combination with the PC aggregation pheromone grandisoic acid (GA) (Eller and Bartelt, 1996), is a highly attractive lure for PCs (Piñero et al., 2001; Prokopy et al., 2003). Relative to other combinations

of synthetic fruit odor and GA, this odor combination significantly enhanced the performance of both clear sticky Plexiglas panels and black pyramid traps (two promising PC monitoring devices) deployed near woods adjacent to apple trees (Piñero et al., 2001).

Given the promise of these compounds as synthetic lures for PC, it becomes important to determine potential additive or synergistic relationships between synthetic fruit odors and GA, as well as to determine the optimal dose of fruit odor and GA for use with panel and pyramid traps when deployed in apple orchards. We report here the results of a 2001 study that evaluated the attractiveness of three different synthetic host volatiles for PC (BEN, EIV, and LIM) alone and in combination with GA. Specifically, our objective was to determine the extent to which each synthetic host volatile enhanced PC response to GA. In a follow-up experiment in 2002, we evaluated PC response to four different release rates of BEN (the most attractive host odor) and two release rates of GA to determine the amount of each odor needed to maximize the performance of monitoring devices. In a final study in 2003, our objective was to determine the maximum release rate at which BEN in combination with GA was attractive to PCs, relative to release rates of BEN evaluated in 2002.

METHODS AND MATERIALS

Studies were carried out in 2001, 2002, and 2003 in an unsprayed section of apple trees at the University of Massachusetts Cold Spring Orchard in Belchertown, MA. The perimeter of the unsprayed section was about 500 m long and bordered almost entirely by woods.

Traps. For each of the 3 years, two trap types were used: (a) clear Plexiglas panels (60 × 60 cm, with the side facing the woods coated with Tangletrap), intended to capture PCs in flight (particularly during warm days), and (b) black pyramid traps (61 cm wide at base × 122 cm tall, modified after Tedders and Wood, 1994), intended primarily to capture crawling PCs, mostly during cool days or at night. Trap design is described in detail in Piñero et al. (2001).

Synthetic Host Volatiles in Association with GA. The host volatiles tested in 2001 were benzaldehyde (BEN) (99.5%), ethyl isovalerate (EIV) (98%), and limonene (LIM) ([*R*]-[+]-limonene, 97%), all obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). These three synthetic odors had previously been shown to be attractive to PCs (Leskey et al., 2001; Prokopy et al., 2001), particularly when in association with GA (Piñero et al., 2001). Three different groups of odor treatments were arranged, each involving a single host volatile alone, GA alone, a combination of host volatile and GA, and a no-odor (control) treatment. There were four replicates for each trap type and group of treatments, arranged in a randomized complete block design.

BEN was released from 1-ml low-density white polyethylene vials (Wheaton W242830 Millville NJ) to prevent polymerization of this chemical by UV light, as may have occurred in a previous field study (Piñero et al., 2001) that involved use of clear polyethylene vials. Because this shortcoming was not found for EIV and LIM, these two chemicals were evaluated using 400 μl high-density clear polyethylene vials (VWR Scientific Products, 20170-328 Boston, MA). Two 400 μl vials containing LIM (in a 50:50 ratio of LIM and mineral oil to slow the release rate) and three vials containing EIV (each filled with 400 μl of EIV) were needed per trap to accomplish a release rate of $\sim$10 mg/day. A white vial filled with 1 ml of BEN released $\sim$2.5 mg/day. Four such vials per trap were used, giving a release rate of $\sim$10 mg/day/trap. Each permeable membrane-type pheromone dispenser (ChemTica International, S.A., San Jose, Costa Rica) released $\sim$1 mg/day of racemic GA. Release rates of GA and synthetic fruit volatiles were determined outdoors by estimating the daily mean weight loss of each chemical using an analytical balance ($\pm$0.01 mg).

Vials containing host volatiles were attached to the lower edge of panels with steel binder clips. For pyramid traps, vials were placed inside a screen funnel (boll weevil trap top; Gempler's, Mt. Horeb, WI) that capped each trap. A single GA-releasing dispenser was attached to the upper edge of a Plexiglas panel with a binder clip, or was positioned inside the boll weevil trap top. Vials containing BEN were replaced approximately every 2 weeks during May and June to ensure consistent release. Vials containing EIV and LIM, along with GA dispensers, were replaced twice, once in mid-May and once in mid-June.

Evaluation of Different Release Rates of BEN and GA. In 2002, eight different odor treatments, dictated by a combination of four different release rates of BEN (0, 2.5, 10, and 40 mg/day) and two release rates of GA (1 and 2 mg/day), were evaluated for attractiveness to PCs. The low release rate of BEN ($\sim$2.5 mg/day) was achieved by using a 1-ml low-density white polyethylene vial filled with 1 ml of BEN per trap, as in our 2001 study. The medium release rate ($\sim$10 mg/day) was achieved by using one 15-ml low-density white polyethylene vial (Wheaton #W242835) filled with 15 ml of BEN, per trap. The high release rate ($\sim$40 mg/day) was achieved by using four such vials per trap. Because each pheromone dispenser released on average $\sim$1 mg of GA per day, the high release rate of GA (2 mg/day) was achieved by using two GA dispensers per trap. There were six replicates for each trap type and odor combination, following a randomized complete block design.

Each vial containing BEN (either a 1-ml or a 15-ml vial, depending on the treatment) was hung by its neck from a wire and placed inside an inverted green 266-ml plastic cup (CVS, Woonsocket, RI) to provide additional protection for this chemical against rainfall and polymerization by UV light. Cups holding BEN-dispensing vials were attached to the bottom edge of panels with wire and steel binder clips. Cups were hung from the end of a wooden pole (buried in the ground

at a 45° angle) in such a way that bases of cups were ~10 cm above pyramid trap tops. Either one or four cups were attached to each pole, depending on the treatment. GA dispensers were attached to the upper edge of panels with binder clips, or were placed inside the inverted screen funnel capping pyramid traps. BEN-releasing vials and GA dispensers were replaced once, 4 weeks after trap deployment.

2003 Evaluation of BEN. In 2003, we evaluated BEN at 0, 10, 40, and 80 mg/day of release, in association with GA at 1 mg/day of release, with the aim of determining whether a further increase of BEN (relative to the amounts of BEN evaluated in 2002) was associated with an increase in PC captures by traps. The first three treatments (i.e., BEN at 0, 10, and 40 mg/day of release) evaluated in 2003 were considered as baseline. BEN was dispensed as in the 2002 study with the difference that (1) BEN was mixed in a 9:1 ratio with 1,2,4-trichlorobenzene (99 + %) (Sigma-Aldrich Chemical Co., Milwaukee, WI) as a stabilizing chemical (S. E. Wright, personal communication), and (2) we used vials containing 7.5 ml of the mixture, releasing on average ~10 mg of BEN per day. Vials releasing BEN were not replaced throughout the study. GA dispensers were replaced once, 5 weeks after trap deployment. There were four replicates for each trap type and odor treatment, arranged in a randomized complete block design.

Trap Deployment. In 2001 and 2002, traps were deployed in pairs (one of each type spaced 1 m apart) along the periphery of the orchard, in close proximity (~1 m) to woods. Each pair of traps was baited with the same odor treatment and spaced 10 m from other trap pairs on either side. In 2003, panel and pyramid traps were spaced 10 m apart to ensure that adult response was solely to intended release rate and not to double the intended release rate that in 2001 and 2002 may have occurred as a consequence of a panel and a pyramid trap having been deployed in close proximity (i.e., 1 m apart).

Trap deployment and baiting took place during the tight cluster stage of bud development (Piñero et al., 2001) (on April 30, 16, and 28, in 2001, 2002, and 2003, respectively). Traps were inspected for PC captures on a daily basis, although results for each year show PC captures by panel or pyramid traps across the entire period of immigration. To minimize the influence of trap location on adult captures we rotated, within each replicate, the bait-containing portion of each trap by one position. This procedure was performed clockwise 12 times in 2001, and eight times in 2002 and 2003.

Statistical Analysis. For all analyses, the response variable was the number of adult PCs captured by traps of each type across the total number of days comprising the period of PC immigration (62 days in 2001, 82 days in 2002, and 77 days in 2003). Results present total PC captures (males and females combined), as preliminary analyses showed that for all 3 years, males and females responded similarly to the odor treatments and trap types. Data were subjected to analysis in a nontransformed fashion as the homogeneity-of-variances assumption was met

in all cases. For all ANOVAS performed, block was used as random factor. For each analysis performed, Fisher-protected LSD separations of treatment means were performed when appropriate. All statistical analyses were performed using Statistica (StatSoft, 1999).

In 2001, in addition to ANOVA, we performed comparisons of ratios of interaction (ROIs) (Hammack, 1996) to examine type of interaction (inhibitory, additive, or synergistic) among single vs. 2-component odor treatments. In our case, a ROI = [(A + GA) + control]/[(A) + (GA)], where (A) represents PC captures by traps baited with a particular fruit volatile alone, (GA) is the capture of PCs by traps baited with GA alone, (A + GA) denotes PC captures by traps baited with a particular fruit volatile in association with GA, and control represents PC captures by unbaited traps. ROI values significantly inferior, equal, or superior to 1 offer evidence for inhibitory, additive (= neutral effect), or synergistic interactions, respectively, between chemicals (Hammack, 1996). Each ROI value obtained corresponded to one of the four replicates performed for each odor group and trap type. Subsequently, a two-tailed Student's t test using such ROI values was performed to test the null hypothesis of ROI = 1 for each fruit odor evaluated (BEN, EIV, and LIM). Further, to verify the possible occurrence of synergism in the particular case of BEN, after performing the ANOVA we compared PC response to traps baited with BEN+GA against the combined response of PCs to traps baited with either BEN or GA alone using linear orthogonal contrast analysis, which allows for specific comparisons of means of the treatments of interest by use of sets of contrast weights (StatSoft, 1999).

RESULTS

Attractiveness of Synthetic Host Volatiles in Association with GA. In 2001, BEN in combination with GA was the most attractive odor combination for PCs for both panel (ANOVA df = 3, 9; $F = 24.39$; $P < 0.001$) and pyramid (ANOVA df = 3, 9; $F = 5.36$; $P = 0.02$) traps. Figure 1A shows that panel traps baited with BEN + GA captured significantly more PCs than did BEN-baited, GA-baited, or unbaited panel traps. PC response to GA alone was greater than response to traps baited with BEN alone or to unbaited traps. Likewise, pyramid traps baited with BEN + GA captured more PCs than did BEN-baited, GA-baited, or unbaited pyramid traps (Figure 1B). When baited with BEN alone or GA alone, pyramid traps captured more PCs than did unbaited pyramid traps. Comparisons of ROI values (Table 1), as well as contrast analysis, provided evidence that for both trap types BEN synergistically increased the response of PCs to GA.

For both trap types, the addition of EIV did not increase the attractiveness of GA to PCs (Figure 1A and B). For both trap types, more PCs were captured by

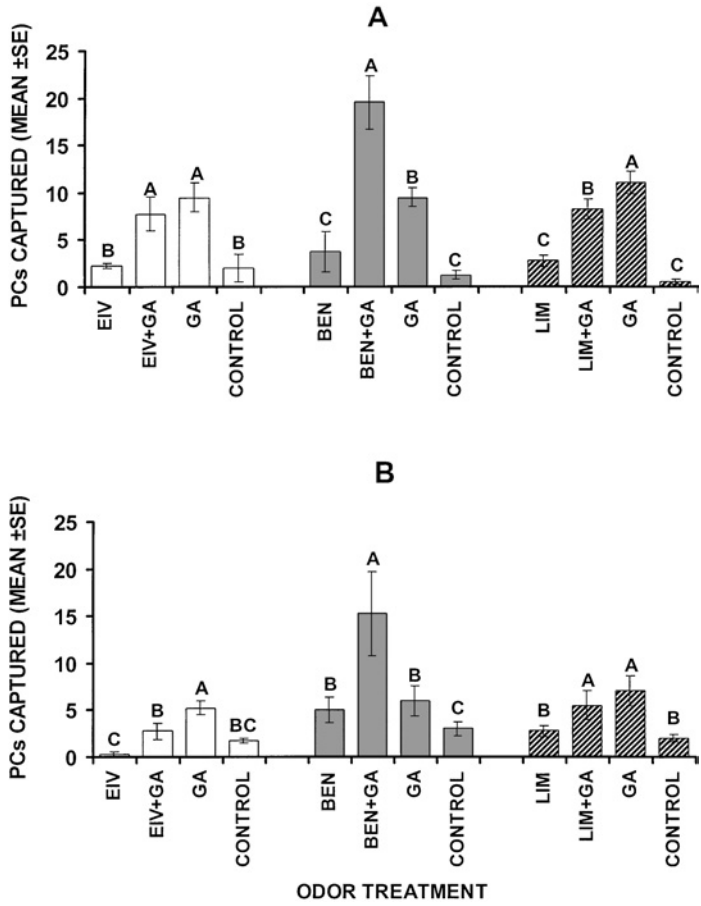

FIG. 1. Plum curculio captures (mean ± SE) by (A) panel traps and (B) pyramid traps baited with three different synthetic fruit volatiles alone or in combination with GA (grandisoic acid) in 2001. EIV = Ethyl isovalerate; BEN = benzaldehyde; LIM = Limonene. For each graph, bars in each group not superscribed by the same letter are significantly different from each other according to ANOVA ($P < 0.05$) and LSD tests.

traps baited with GA alone than by traps baited with EIV alone or by control traps (ANOVA df = 3, 9; $F = 10.57$; $P < 0.01$ and df = 3, 9; $F = 12.72$; $P < 0.001$ for panel and pyramid traps, respectively). In no case did EIV alone enhance adult response above that of control traps. Comparisons of ROI values provided evidence that EIV interacted with GA in an additive manner (Table 1).

For both trap types, the presence of LIM had no positive effect on the attractiveness of GA to PCs. Relative to LIM-baited traps or control traps, panel

TABLE 1. TYPE OF INTERACTION FOUND BETWEEN SINGLE ATTRACTANTS (SYNTHETIC FRUIT ODOR OR GA) AND COMBINED ATTRACTANTS (SYNTHETIC FRUIT ODOR + GA) WHEN EVALUATED IN ASSOCIATION WITH PANEL AND PYRAMID TRAPS FOR CAPTURING PCS IN 2001

Trap type	Chemical	Ratio of interaction (ROI)[a]	Type of interaction
Panel	BEN + GA	1.58 ± 0.17*	Synergistic[b]
Pyramid	BEN + GA	1.65 ± 0.27*	Synergistic[b]
Panel	EIV + GA	0.88 ± 0.18	Additive
Pyramid	EIV + GA	0.90 ± 0.21	Additive
Panel	LIM + GA	0.66 ± 0.13*	Inhibitory
Pyramid	LIM + GA	0.90 ± 0.28	Additive

Note. BEN = Benzaldehyde; GA = Grandisoic acid; EIV = Ethyl isovalerate; LIM = Limonene.

[a] Mean ± SE.

[b] As further verified by orthogonal contrast analysis ($P < 0.001$ and $P < 0.01$ for panel and pyramid traps, respectively).

* $P < 0.05$ for the null hypothesis that ratio of interaction (ROI) = 1 (Hammack, 1996), by a two-tailed t-test (df = 3) .

and pyramid traps baited with GA alone or with GA + LIM captured more PCs (ANOVA df = 3, 9; $F = 26.57$; $P < 0.001$ and df = 3, 9; $F = 4.14$; $P = 0.04$ for panel and pyramid traps, respectively). For panel traps, however, GA attracted more PCs when alone than when in combination with LIM, whereas for pyramid traps both treatments attracted similar numbers of PCs. Captures by traps of either type baited with LIM alone did not differ from captures by unbaited traps. For panel traps, a ROI value of 0.66 indicated an inhibitory effect when LIM (at the release rate evaluated) was added to GA. For pyramid traps, a ROI value of 0.90 denoted that the interaction between LIM and GA was additive (Table 1).

Evaluation of Different Release Rates of BEN and GA. In 2002, we found that panel traps baited with two GA dispensers, releasing 2 mg/day of GA on average, captured about 35% more PCs than did panel traps baited with only one GA dispenser (releasing, on average, 1 mg/day of GA) (ANOVA df = 1, 5; $F = 6.95$; $P = 0.04$). In contrast, pyramid traps captured similar numbers of PCs regardless of the amount of GA released (ANOVA df = 1, 5; $F = 2.31$; $P = 0.18$) (Figure 2).

Figure 3A reveals that, for panel traps baited with only one GA dispenser (1 mg/day of release), BEN at 40 mg/day of release attracted the most PCs, followed by BEN at 10 mg/day of release (ANOVA df = 3, 15; $F = 4.41$; $P = 0.02$). For panel traps baited with two GA dispensers (2 mg/day of release), differences among BEN treatments were not statistically significant (ANOVA df = 3, 15; $F = 2.77$; $P = 0.08$).

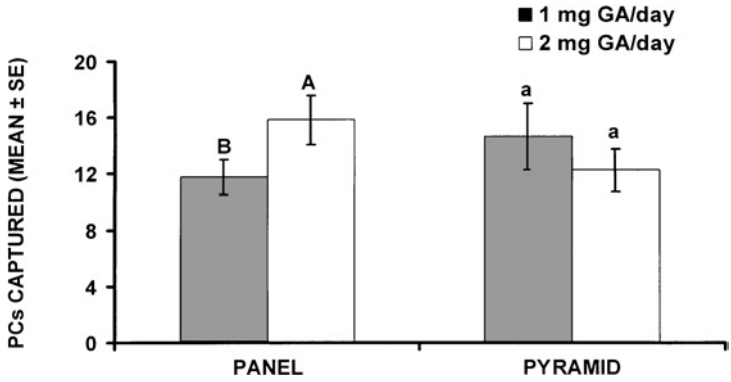

FIG. 2. Plum curculio captures (mean ± SE) by panel and pyramid traps as a function of amounts of grandisoic acid (GA) released per day in 2002. Data were pooled across all amounts of BEN evaluated. For each trap type, bars not superscribed by the same letter are significantly different according to ANOVA ($P < 0.05$) and LSD tests.

Figure 3B shows that for pyramid traps baited with the low release rate of GA (1 mg/day), addition of BEN (regardless of amount released) did not increase PC captures relative to traps baited with GA alone (ANOVA df = 3, 15; $F = 2.16$; $P = 0.13$). For pyramid traps baited with the high release rate of GA (2 mg/day), addition of BEN at either 2.5 or 10 mg/day (but not 40 mg/day) did enhance PC response relative to GA alone (ANOVA df = 3, 15; $F = 4.68$; $P = 0.02$).

2003 Evaluation of BEN. Results from this follow-up study (involving GA released at 1 mg/day) showed that, for panel traps, PC captures were enhanced by addition of BEN to GA, irrespective of the amount of BEN released (ANOVA df = 3, 9; $F = 4.38$; $P = 0.04$). For pyramid traps, the most attractive release rate of BEN was 10 mg/day; additional amounts of BEN decreased the attractiveness of traps to PCs (ANOVA df = 3, 9; $F = 6.95$; $P = 0.01$) (Figure 4).

DISCUSSION

Our 2001 results provided evidence that benzaldehyde (BEN), naturally present in both apple flowers (Buchbauer et al., 1993) and developing fruit (Leskey et al., 2001), synergistically enhanced the response of PCs to the male-produced pheromone grandisoic acid (GA). This contrasted with results for EIV and LIM, which indicated that addition of either fruit volatile to GA did not improve (at the dose evaluated) the attractiveness of GA. In the 2002 study, BEN at 10 mg/day of release (particularly when in association with GA at 1 mg/day) was the most attractive bait combination for PCs for both panel and pyramid traps. The 2003

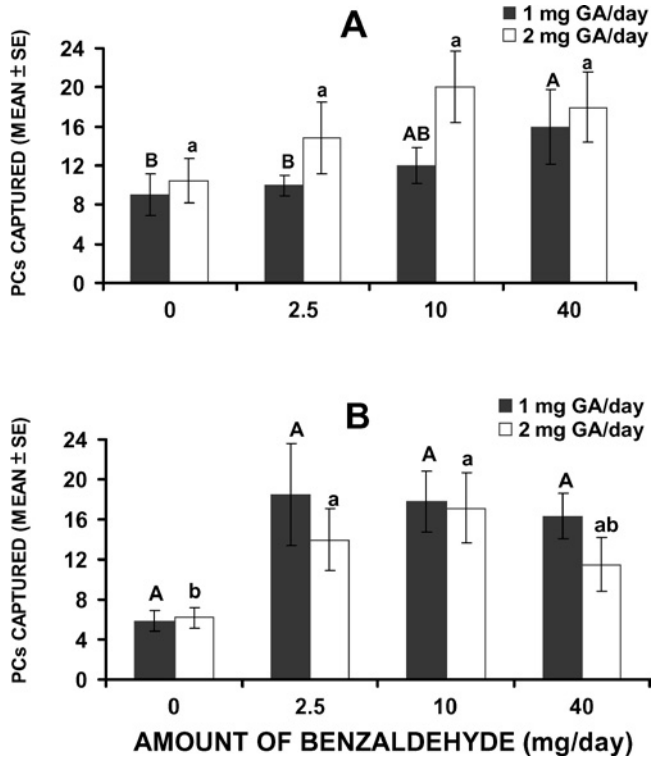

FIG. 3. Plum curculio captures (mean ± SE) by (A) panel traps and (B) pyramid traps as a function of amounts of benzaldehyde (BEN) released per day in 2002 in association with GA at either 1 or 2 mg/day of release. For each trap type, bars not superscribed by the same letter are significantly different according to ANOVA ($P < 0.05$) and LSD tests.

study confirmed results of 2002 and additionally showed that release of BEN at 80 mg/day with GA (1 mg/day) did not further enhance (for panel traps) and actually decreased (for pyramid traps) PC captures.

This study is one of the few showing that a single synthetic component of host odor synergizes the attractiveness of an aggregation pheromone to a species of weevil without using supplementary sources (natural or synthetic) of host plant odor. Most other reports (e.g., Dowd and Bartelt, 1991; Bartelt et al., 1992; Lin et al., 1992; Weissling et al., 1994; Giblin-Davis et al., 1996; Hammack, 2001) show that a synthetic aggregation pheromone in combination with blends of synthetic and/or natural food odor is needed to synergistically increase attraction in beetles.

Our 2001 findings indicate that (1) among all baits evaluated here, BEN + GA was the most attractive lure for PCs, whereas GA alone or BEN alone were less

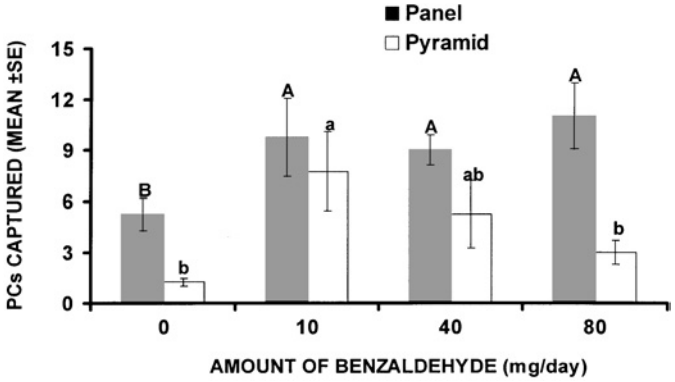

FIG. 4. Plum curculio captures (mean ± SE) by panel and pyramid traps as a function of amounts of benzaldehyde (BEN) released per day in 2003 in association with GA at 1 mg/day of release. For each trap type, bars not superscribed by the same letter are significantly different according to ANOVA ($P < 0.05$) and LSD tests.

attractive, (2) the response of PCs to the combination of BEN + GA was similar for panel and pyramid traps, which suggests that PCs are similarly attracted to this lure regardless of their mode of immigration (i.e., by flight or by crawling) into an apple orchard, and (3) BEN + GA is attractive to PCs of both sexes (data not shown).

The type of interaction found between BEN and GA in our 2001 study differs from that found in West Virginia for the bivoltine "southern strain" PC (T. C. Leskey and S. E. Wright, unpublished data). In their study, involving the same kind of traps and odors, Leskey and Wright found that PCs responded equally to BEN alone and BEN in combination with GA, and that GA alone ranked third in attractiveness. Such contrasting results might be explained by the fact that adult PCs used in Leskey and Wright's study were dislodged from host trees (i.e., they had already immigrated into host trees) before testing, and, therefore may have been in a different physiological state. Alternatively, there might be variation in responsiveness to these types of volatiles between the northern (e.g., Massachusetts) and southern (e.g., West Virginia) strains of PC. More detailed information about the role of BEN and GA in host-finding, feeding, mating, and oviposition for both PC strains is needed.

Results from our 2002 experiment suggest that a low release rate of GA (1 mg/day) is more cost-effective for use in both panel and pyramid traps than a higher release rate of GA (2 mg/day). For panel traps, BEN performed best at the high release rates (10 or 40 mg/day) regardless of the amount of GA released from panel traps. For pyramid traps, however, the attractiveness of BEN decreased somewhat at the highest release rate, particularly when in association with GA at the high release rate (2 mg/day), suggesting some degree of close-range repellency.

Results from our 2003 study indicated that for panel traps, BEN at 80 mg/day of release in association with GA released at 1 mg/day did not enhance captures relative to lower amounts of BEN. Moreover, for pyramid traps BEN at high release rates decreased the attractiveness of the binary combination of BEN + GA, corroborating the close-range repellency found in our 2002 study.

Our collective findings, together with an assessment of the cost of BEN (as formulated by us) and GA (a commercial formulation), suggest that BEN at a release rate of 10 mg/day/trap, in association with GA at a release rate of 1 mg/day/trap, seems to be the most cost-effective bait combination for use with both panel and pyramid traps to monitor adult PCs. An alternative use of this odor combination, at a release rate of BEN of 40 mg/day in association with GA at 1 mg/day of release, has been put forward under a "trap-tree" approach, which has proven useful for simplifying the monitoring of injury inflicted by PC to fruit (Prokopy et al., 2003, R. J. Prokopy, unpublished data).

In conclusion, use of panel and pyramid traps baited with BEN+GA at proposed release rates could be helpful to apple growers for several reasons: (1) when deployed adjacent to forested areas of unsprayed orchards, such traps have proven useful in tracking the seasonal course of immigration of overwintered PCs for four consecutive years (J. C. Piñero and R. J. Prokopy, unpublished data), (2) such traps intercept immigrant PCs before they reach host trees, and thus prior to their engagement in feeding, mating, and oviposition activities, which could change the degree of PC responsiveness to lures, and (3) BEN + GA remains attractive as a lure to PCs throughout the entire period of immigration (62 days in 2001, 82 days in 2002, 77 days in 2003) regardless of the stage of bud and fruit development, sex of adults, female sexual maturity stage, or mating status (Piñero et al., 2001; J. C. Piñero and R. J. Prokopy, unpublished data).

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PHEROMONE ANALYSIS OF WILD FEMALE MOTHS WITH A PBAN C-TERMINAL PEPTIDE INJECTION FOR AN ESTIMATION OF ASSORTATIVE MATING IN ADZUKI BEAN BORER, *Ostrinia scapulalis*

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Abstract—The adzuki bean borer, *Ostrinia scapulalis*, has distinct genetic variation in the blend of two sex pheromone components, (*E*)- and (*Z*)-11-tetradecenyl acetates. This variation is largely controlled by a single autosomal locus with two alleles, *E* and *Z*. *E*-type (*EE*) females produce a pheromone with a mean *E*:*Z* ratio at 99:1 whereas *Z*-type (*ZZ*) and *I*-type (*ZE*) produce pheromones with mean of 3:97 and 64:36, respectively. Interestingly, in many natural populations of *O. scapulalis* in Japan, this pheromone polymorphism appears to be stably maintained. We tried to predict the changes in relative abundance of each pheromone type by estimating the pheromone production genotype of wild females and their male mates. The pheromone titer in the wild, mated females was increased without changing the blend ratio by an injection of a peptide with pheromone biosynthesis activating activity (TKYFSPRL-NH_2). The frequencies of *E*-, *I*-, and *Z*-types at Matsudo were 15, 52, and 33%, respectively, and did not deviate from the Hardy–Weinberg expectations. The estimated mating patterns were concordant with the assumption that no assortative mating was occurring in this population, and this is suggested as a cause of sustained polymorphism at Matsudo.

Key Words—*Ostrinia scapulalis*, *Ostrinia nubilalis*, assortative mating, sex pheromone, polymorphism, PBAN.

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INTRODUCTION

The moth sex pheromone plays an important role in mating behavior, and thus is linked with premating reproductive isolation and speciation (for a review, see Löfstedt, 1990). Sexual communication systems such as this are subject to stabilizing selection pressure (Löfstedt, 1993; Butlin and Trickett, 1997), and intraspecific variations in sex pheromone production in females and responses of males are expected to be low (Zhu et al., 1996a; Laforest et al., 1997). Nevertheless, large variations in sex pheromone production and response have been found in some species (e.g., Klun et al., 1975; Laforest et al., 1997; Zhu et al., 1997; Emelianov et al., 2001; Huang et al., 2002). The European corn borer, *Ostrinia nubilalis*, is one such species, and served as a model insect for population genetic studies, because the genetic basis of variations in pheromone production and male response is well understood (Roelofs et al., 1987; Klun and Huettel, 1988; Löfstedt et al., 1989; Bengtsson and Löfstedt, 1990; Glover et al., 1991; Zhu et al., 1996a; Butlin and Trickett, 1997).

Adzuki bean borer, *Ostrinia scapulalis* (Walker) (Lepidoptera: Crambidae), a congener of *O. nubilalis*, is another example of a species with a variable pheromone similar to that in *O. nubilalis*. The pheromone was identified as a mixture of *E* and *Z* isomers of 11-tetradecenyl acetate (E11-14:Ac and Z11-14:Ac), and there are three pheromone types that vary in the blend of the two isomers, i.e., *E* type (96–100% of E11-14:Ac; mean in a typical laboratory culture, 99%), *I* type (33–95% E mean 64%), and *Z* type (0–31% E; mean 3%) (Huang et al., 1997, 2002; Ishikawa et al., 1999; Takanashi, 2001). These three types are mainly determined by one autosomal locus with two alleles, *E* and *Z*. *E*-type, *Z*-type, and *I*-type females have *EE*, *ZZ*, and *ZE* genotype, respectively (Takanashi, 2001). The behavioral response of *O. scapulalis* males to the pheromone, on the other hand, is controlled by a gene on the Z chromosome and is not linked to the gene for pheromone production (Takanashi, 2001).

Interestingly, during an extensive survey of geographic variation of pheromone types in *O. scapulalis*, we found that the three types stably coexist in many local populations in Japan (Takanashi, 2001; Huang et al., 2002). For a detailed analysis of this phenomenon, it is essential to estimate natural mating frequencies among the pheromone genotypes. However, it has been difficult to determine the pheromone type of field-collected, gravid females after allowing them to lay eggs because sex pheromone titers are greatly decreased in mated females (Raina and Stadelbacher, 1990) and not sufficient for individual analysis in some species.

Pheromone biosynthesis activating neuropeptide (PBAN) is a key factor in the biosynthesis of sex pheromones in many lepidopteran species (for a review, see Rafaeli, 2002) including *O. nubilalis* (Ma and Roelofs, 1995). Synthesis, or secretion, of PBAN is repressed in the mated female moth, causing termination of sex pheromone production (Jurenka et al., 1993; Foster and Roelofs, 1994; Ando

et al., 1996). In the present study, we tried to reactivate *de novo* biosynthesis of sex pheromones in the mated female moth by injecting a synthetic PBAN C-terminal octapeptide (Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2; TKYFSPRL-NH_2; *Helicoverpa zea*) (Ozawa and Matsumoto, 1996; Fonagy et al., 1999). If PBAN C-terminal peptide injection could increase the pheromone titer without changing its blend ratio, it would be possible to determine the pheromone type of mated females. By applying this method to wild *O. scapulalis* females collected from a local population where three pheromone types (E, I, Z) appeared to coexist stably, we also attempted to estimate the frequency of assortative mating in terms of pheromone production genotype ($EE \times EE$, and $ZZ \times ZZ$) in this population.

METHODS AND MATERIALS

Insect Cultures. Cultures of *Ostrinia scapulalis*, homozygous for *ZZ* or *EE* sex pheromone production genes, were established as described below in our laboratory from females collected with sweep nets in a field at Matsudo (35.8°N, 139.9°E), Japan in September 2001. Wild females were separately housed in 40-ml plastic cups with a cotton pad soaked with water, and allowed to lay eggs on the inner surface of the cups. Eggs from a single female were reared on an artificial diet (Silk mate®, Nihon Nosan K.K., Yokohama, Japan) in a plastic bottle (8-cm diam, 12-cm height) maintained in a rearing room (60% relative humidity; 16:8, L:D, 23°C). After pupation, the pupae were sexed, and females and males were separately maintained in 430-ml plastic cups. One- to 3-day-old adults from single broods were transferred to screen cages (20 × 20 × 20 cm) for 3 d with water-soaked cotton for free mating. Concomitantly, the pheromone type of at least 10 virgin females from each brood was determined by gas chromatography (GC), and those broods that were monomorphic with regard to the pheromone type were selected. As a consequence, three Z-type cultures, and one E-type culture were established.

Injection of TKFYSPRL-NH_2. A synthetic C-terminal peptide of *H. zea* PBAN (TKYFSPRL-NH_2; QIAGEN K.K., Tokyo) was dissolved in distilled water at a concentration of 10 pmol/μl, and 2 μl each was injected into the abdomen of females using a microsyringe. Seven-day-old mated E-type females ($N = 8$) and Z-type females ($N = 10$) were injected with the peptide and allowed to rest for 4 hr in a dark room (60% relative humidity; 23°C). As controls, 7-day-old mated and 3-day-old virgin E-type females ($N = 9$ and $N = 6$, respectively) and corresponding Z-type females ($N = 7$ and $N = 9$, respectively) were subjected to pheromone extraction without the peptide injection.

Extraction of Sex Pheromone. The abdominal tip, which includes a sex pheromone gland, was excised from females, and tips were immersed individually in hexane (10 μl/female; residual pesticide analysis grade, Wako Pure Chemicals,

Kyoto). The tip was removed after 30 min, and the extract was kept at −20°C until analysis by GC.

Analysis of Sex Pheromone by Gas Chromatography. Extracts were analyzed with a gas chromatograph GC-14B (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, a split/splitless injector, an automatic injector (AOC-20, Shimadzu), and a DB-Wax column (30 m × 0.25 mm i.d., J&W Scientific, CA). Nitrogen was used as the carrier gas. Aliquots (2 μl) of samples were injected into the GC with a splitless time of 2 min. The injector and detector temperatures were 230 and 270°C, respectively. The column oven temperature was maintained at 120°C for 2 min, raised to 150°C at a rate of 5°C/min, to 200°C at 3°C/min, and finally to 240°C at 5°C/min. The pheromone component ratio in a female was obtained by comparing the peak height of the components. The authentic compounds, E11- and Z11-14:Ac, were purchased from Pherobank (Wageningen, The Netherlands).

Analysis of Wild Females' Pheromone Type and Their Mating Pattern. Females of *O. scapulalis* were collected with sweep nets in a field at Matsudo between May 26 and June 8, 2002. After obtaining eggs as described above, the pheromone type of each female was analyzed after injection of TKYFSPRL-NH_2. Then, females were dissected, and the number of spermatophores in the bursa copulatrix was checked for single mating. Only those females that had one spermatophore were used for further analysis. The eggs laid by a total of 52 wild females were reared by the method described above, and the pheromone type of female progenies (11–32 females/brood; 1140 females in total) was analyzed 3 days postemergence. To reduce excess labor, the number of females analyzed per brood was varied depending on the extent of pheromone variation. The pheromone production genotype of the male parent was estimated from the phenotypes of a field-collected female (mother) and her daughters. For example, when a mother was *E* type and all her daughters were *I* type, the pheromone production genotype of the male parent was estimated as *ZZ* (see Figure 3 for further examples).

Statistical Test of Assortative Mating. Exact test of goodness of fit (StatXact, Cytel Software Corp., MA) was applied to test the occurrence of assortative mating in a natural population of *O. scapulalis*. Exact test provides exact probability of the null hypothesis (Mehta and Patel, 1997). The more common chi-square test was not applicable because of the presence of a sample with expected frequency smaller than 1.

RESULTS

Effect of TKYFSPRL-NH_2 Injection on Sex Pheromone Biosynthesis. Injection of TKYFSPRL-NH_2 to 7-day-old mated females elicited a significant increase in the pheromone titer in females of both E and Z cultures (Figure 1). Moreover, the blend ratio in the peptide-injected females was similar to that of the corresponding

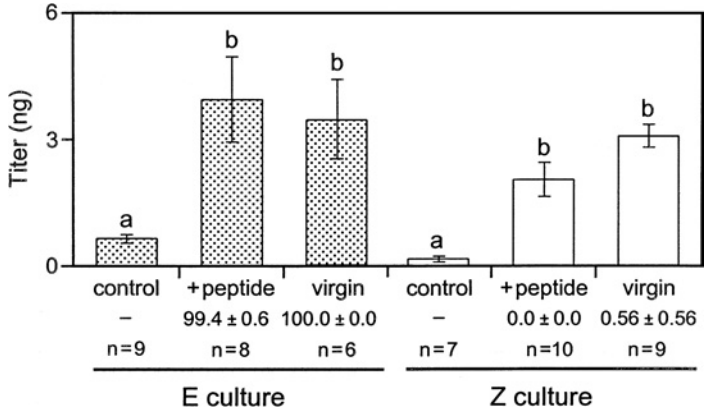

FIG. 1. The effect of TKTFSPRL-NH_2 injection on *de novo* biosynthesis of pheromones in *O. scapulalis*. The upper graph shows the sum of the two pheromone components, E11- and Z11-14:Ac. The blend ratios (% of E11-14:Ac ± SE) are shown below the graph. The bars with the same letter are not significantly different at the level of 5% (Tukey–Kramer HSD test). –: Unable to determine because the presence of minor components below the detection limit (about 0.15 ng in the present experiment) cannot be excluded.

3-day-old virgin females in both E and Z cultures (Figure 1). It was demonstrated, therefore, that TKYFSPRL-NH_2 injection into a mated *O. scapulalis* female reactivated biosynthesis of sex pheromones without changing the blend ratio.

The Pheromone Types of Wild O. scapulalis Females at Matsudo. The pheromone blend of field-collected females ($N = 52$) was determined after TKYFSPRL-NH_2 injection (Figure 2). A few females showed a pheromone blend near the borders (≈30% E and ≈95% E), and it was difficult to determine the pheromone genotype from the blend ratio alone. However, analysis of the variation of their daughters' pheromone enabled determination of their own and their male mate's pheromone production genotypes unequivocally (for example, see Figure 3d). The numbers (percentages) of *E*-type, *I*-type, and *Z*-type females were 8 (15%), 27 (52%), and 17 (33%), respectively. Three types were found in this single local population, and *I* type was most prevalent. The numbers of *E*-, *I*-, and *Z*-type females expected from the Hardy–Weinberg equilibrium were 8.9, 25.2, and 17.9, respectively, and not significantly different from the actual values ($\chi^2 = 0.26$, df = 1, $P = 0.61$).

Estimated Mating Patterns Among Three Genotypes in the Field. Because we dealt with females that mated only once (with one spermatophore), no wild females produced female progenies with very complex pheromone blend variation. Every variation pattern could be explained by Mendelian inheritance, along with an unambiguous estimation of the pheromone production genotype of the male

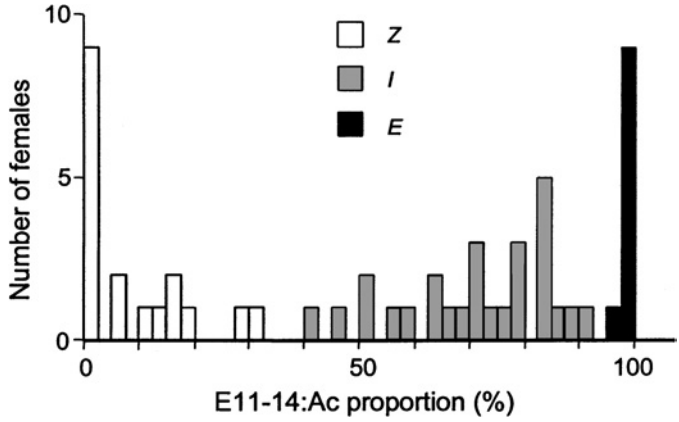

FIG. 2. Distribution of the pheromone types of females collected with sweep nets in a field at Matsudo (35.8°N, 139.9°E), Japan, between May 26 and June 8, 2002.

mate (Figure 3). The pheromone production genotypes of 52 male parents were estimated as *ZZ*:18, *EE*:4, and *ZE*: 30 (Table 1). Mating between heterozygous genotypes (*ZE* × *ZE*) was predominant. Although the frequency was low, there were mating patterns in which a male has selected a female with a pheromone type opposite his own pheromone production genotype (a male with pheromone production genotype *ZZ* mated with an *EE* female, and *vice versa*). These findings suggested a random mating in terms of pheromone production genotype. The number of pairs in each mating pattern, expected on the assumption of random mating, was calculated using a formula, total sample number × frequency of i genotype in females × frequency of j genotype in males (i, $j \in EE$, ZE, ZZ). The observed mating patterns were not significantly different from that estimated on the assumption of random mating in terms of pheromone production genotype (Table 1; exact test of goodness of fit, exact $P = 0.457$). In summary, there was no indication of assortative mating in the Matsudo population of *O. scapulalis*.

DISCUSSION

The Effect of PBAN C-Terminal Peptide on Pheromone Biosynthesis in Mated Females. PBANs have been isolated and their amino acid sequence identified in *H. zea* (Hez-PBAN; Raina et al., 1989), *Bombyx mori* (Bom-PBAN; Kitamura et al., 1989, 1990), and *Lymantria dispar* (Lyd-PBAN; Masler et al., 1994). In some other moths, PBAN sequences have been deduced from cDNA (Rafaeli, 2002). The primary structures of PBANs from these species share high homology (66–82%) (Raina and Menn, 1993; Masler et al., 1994). The C-terminal pentapeptide (FSPRL), in particular, is identical and has pheromonotropic activity (Raina and

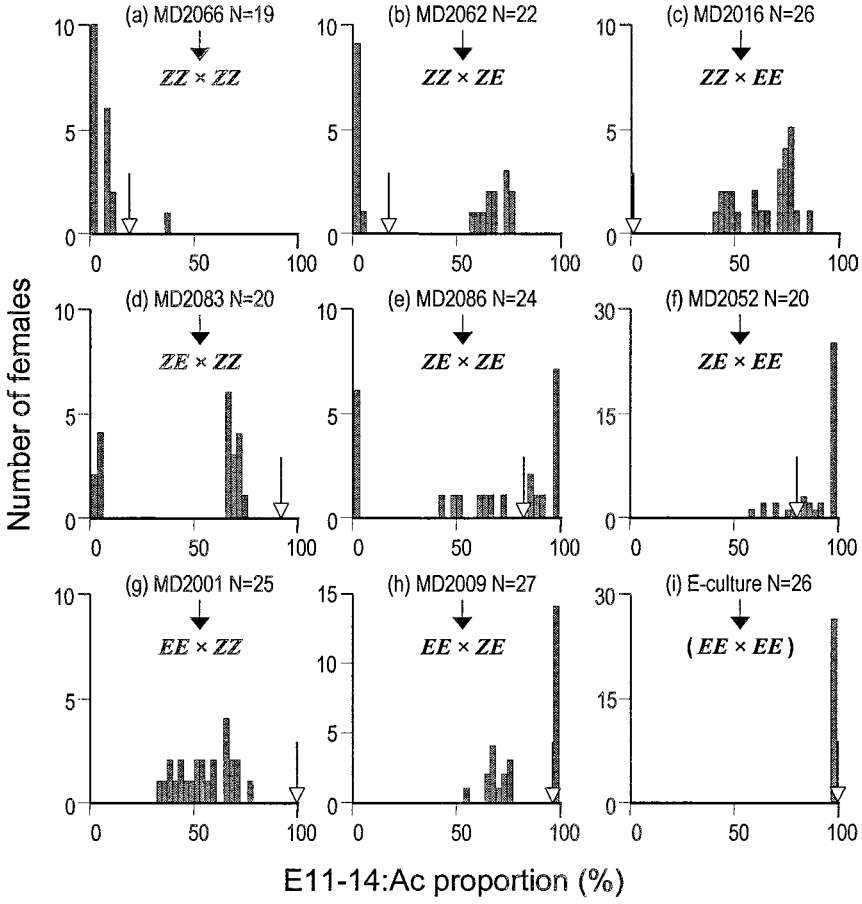

FIG. 3. Examples of how to estimate the pheromone genotype of male parents based on the variations of pheromone blend in the female progenies and the mother's blend. The mother's blend is indicated on each graph by an arrow. The estimated cross is shown as female × male. The variation in female progenies of the other 44 wild females (11–32 females/brood) was analyzed in a similar manner. *A combination *EE* × *EE* was not found from the natural population (i).

Kempe, 1990). The PBAN C-terminal peptide injection, therefore, is likely to be applicable to many moth species. The method employed in the present study would be a valuable tool for population genetic studies on the sex pheromone communication systems in moths, because it enables direct estimation of mating patterns in terms of pheromone production genotypes, from previously mated females that would not normally produce pheromone.

TABLE 1. OBSERVED FREQUENCY OF PHEROMONE GENOTYPES, AND EXPECTED AND OBSERVED FREQUENCY OF MATING COMBINATIONS

	Frequency of genotype[a]		
Sex	*EE*	*ZE*	*ZZ*
Female	0.15 (8/52)	0.52 (27/52)	0.33 (17/52)
Male	0.08 (4/52)	0.60 (31/52)	0.33 (17/52)

Observed and expected frequency of mating combination		
Mating combination[b]	Observed	Expected[c]
EE* × *EE	**0**	**0.6**
ZE* × *ZE	**18**	**16.1**
ZZ* × *ZZ	**9**	**5.6**
EE × *ZE*	7	4.8
EE × *ZZ*	1	2.6
ZE × *EE*	2	2.1
ZE × *ZZ*	7	8.8
ZZ × *EE*	2	1.3
ZZ × *ZE*	6	10.1

[a] In parentheses are shown the numbers of each genotype out of 52 cases examined.
[b] Female × male. The bold letters indicate mating between the same genotypes.
[c] Expected frequency of a mating combination was calculated from the frequencies of genotypes of wild females and their male mates (see text). The observed and expected frequency of mating combination is not significantly different ($P = 0.457$) by the exact test of goodness of fit (Mehta and Patel, 1997).

Biochemical Basis of the Control of Pheromone Blend. Although the mechanism that controls pheromone blend is not clarified in *O. scapulalis*, knowledge from *O. nubilalis*, which shows very similar pheromone polymorphism, allows inferences to be made with *O. scapulalis*. In *O. nubilalis*, both Δ11-desaturase and acetyltransferase, which convert 14:Acyl to E11/Z11-14:Acyl, and E11/Z11-14:OH to E11/Z11-14:OAc, respectively, are not stereoselective; the E/Z ratio is regulated by a stereospecific reductase complex, which converts E11/Z11-14:Acyl to E11/Z11-14:OH (Zhu et al., 1996b). Thus, the two alleles of a pheromone production gene, E and Z, are likely to be involved in the control of the stereospecificity of the reductase complex.

Pheromone Polymorphism and No Indication of Assortative Mating in O. scapulalis at Matsudo. In this study, pheromone polymorphism was observed within a local population of *O. scapulalis* at Matsudo, and no evidence for deviation of genotypic frequency from the Hardy–Weinberg expectations at equilibrium was obtained (Figure 2). Moreover, there was no indication of positive assortative mating in terms of pheromone production genotype in this population (Table 1). These findings may partly explain the apparently stable pheromone

polymorphism in this *O. scapulalis* population. However, sex pheromone polymorphism in a local population like this may be a rare case, because sexual communication systems, including sex pheromone systems, are subject to sexual selection pressure strong enough to generate a rapid shift to a monomorphic state (Löfstedt, 1990, 1993; Butlin and Trickett, 1997). At present, the reason why this selection pressure appears not to be working on this population is unknown. The relatively wide response window of *O. scapulalis* males, irrespective of their response genotypes (Takanashi, 2001), may possibly contribute to the sustained polymorphism.

Although *O. scapulalis* and *O. nubilalis* share similar sex pheromone communication systems, the present findings clearly contrast with the findings in the study of sympatric pheromone type polymorphism in *O. nubilalis*. The heterozygous *I* type was prevalent in the Matsudo population of *O. scapulalis*, whereas the homozygous *Z* type was predominant in the Beltsville population of *O. nubilalis* (Klun and Huettel, 1988). This may be a reflection of the absence or presence of assortative mating, and difference in *E* and *Z* gene frequencies ($E{:}Z = 0.41{:}0.59$ in *O. scapulalis* at Matsudo, $E{:}Z = 0.22{:}0.78$ in *O. nubilalis* at Beltsville) (the present study; Klun and Huettel, 1988; Bengtsson and Löfstedt, 1990). In the Beltsville population of *O. nubilalis*, positive assortative mating among the pheromone production genotypes was demonstrated (Klun and Huettel, 1988), although no evidence for sexual selection was obtained (Bengtsson and Löfstedt, 1990). Therefore, despite apparent genetic similarity, sustained sex pheromone polymorphism in *O. scapulalis* and *O. nubilalis* may depend on different mechanisms.

Continuing studies on various local *O. scapulalis* populations in Japan using the method employed in this study will provide further information on pheromone polymorphism in this species, because positive assortative mating appears to be occurring in some populations of *O. scapulalis* in northern Japan (Takanashi, 2001).

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ALLELOCHEMICAL POTENTIAL OF *Callicarpa acuminata*

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Abstract—The allelochemical potential of *Callicarpa acuminata* (Verbenaceae) was investigated by using a biodirected fractionation study as part of a long-term project to search for bioactive compounds among the rich biodiversity of plant communities in the Ecological Reserve El Eden, Quintana Roo, Mexico. Aqueous leachate, chloroform-methanol extract, and chromatographic fractions of the leaves of *C. acuminata* inhibited the root growth of test plants (23–70%). Some of these treatments caused a moderate inhibition of the radial growth of two phytopathogenic fungi, *Helminthosporium longirostratum* and *Alternaria solani* (18–31%). The chloroform-methanol (1:1) extract prepared from the leaves rendered five compounds: isopimaric acid (**1**), a mixture of two diterpenols [sandaracopimaradien-19-ol (**3**) and akhdarenol (**4**)], α-amyrin (**5**), and the flavone salvigenin (**6**)]. The phytotoxicity exhibited by several fractions and the full extract almost disappeared when pure compounds were evaluated on the test plants, suggesting a synergistic or additive effect. Compounds (**4**), (**5**), and the semisynthetic derivative isopimaric acid methyl ether (**2**) had antifeedant effects on *Leptinotarsa decemlineata.* Compound **5** was most toxic to this insect, followed by (**2**), (**4**), and (**6**) with moderate to low toxicity. No correlation was found between antifeedant and toxic effects on this insect, suggesting that different modes of action were involved. All the test compounds were cytotoxic to insect Sf9 cells while (**6**), (**4**), and (**1**) also affected mammalian Chinese Hamster

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Ovary (CHO) cells. Compound **5** showed the strongest selectivity against insect cells. This study contributes to the knowledge of the defensive chemistry and added value of *C. acuminata.*

Key Words—*Callicarpa acuminata,* Verbenaceae, allelochemicals, isopimaric acid, α-amyrin, sandaracopimaradien-19-ol, akhdarenol, salvigenin, bioactivity.

INTRODUCTION

Callicarpa acuminata H. B. K. (Verbenaceae) commonly known as alahualte, elté, chachalaca herb, uvilla, patsumacáshil, and granadilla (Martínez, 1979), is distributed in Mexico from Tamaulipas, Veracruz, Chiapas, Oaxaca, to the Yucatan Peninsula. *C. acuminata* is a shrub or a small tree up to 6 m in height and abundant in secondary plant communities in Quintana Roo (Cabrera Cano et al., 1982). Economically, the family is perhaps most important for its teak (*Tectona grandis*) lumber. A number of genera, such as *Callicarpa* and *Lantana*, are important ornamental species.

This study on *Callicarpa acuminata* is a part of long-term research project on bioactive compounds from the rich biodiversity of different plant communities present in the Ecological Reserve El Eden, Quintana Roo, Mexico. El Eden is located in the Yalahau region in the north of Quintana Roo. This region includes more than 250,000 ha of well-preserved natural environments that represent the main ecosystems of the Yucatan Peninsula and the Caribbean. Chemical diversity studies have been performed on the plant communities in this area as have taxonomic and systematic studies in light of the importance of biological, ecological, and evolutionary information contained therein.

Some chemical studies have already been done on other species of the genus *Callicarpa*. *C. macrophylla* from Calcutta, for example, is used in traditional medicine to relieve stomach disorders and rheumatism. Talapatra et al. (1994) identified L(+)-α-amino-β-(p-metoxyphenyl)-propionic acid, calliterpenone and its monoacetate, β-sitosterol, β-sitosterol-β-D-glucoside, ursolic acid, 2α-hydroxy-ursolic acid, crategolic acid, 7-O-glucuronides of luteolin and apigenin, and 5,4′-dihydroxy-3,7,3′-trimethoxy-flavone from the leaves of *C. macrophylla*. In addition, calliphyllin, betulinic acid, and 5,4′-dihydroxy-3,7-dimethoxy-flavone were isolated and identified from the leaves of this species. Agrawal et al. (1996) described the complete spectral assignment of calliterpenone and related phyllocladane diterpenoids. Xu et al. (2000) isolated four new clerodane-type diterpenoids (pentandranoic acids A–C and pentandralactone) from the leaves of *C. pentandra.* Hu et al. (2002) described the isolation and identification of 14-α-hydroxy-7,15-isopimaradien-18-oic acid, 16-α-17-dihydroxy-3-oxophyllocladane, 8,11,13,15-abietatetraen-18-oic acid, and 6-α-hydroxy nidorellol from *C. pedunculata.* However, the biological activities of these compounds have yet

to be studied in detail, although Kawazu et al. (1967a,b) reported that the tricarbocyclic diterpene callicarpone from *C. candicans* was toxic to fish. In addition, Hayashi et al. (1997) observed that a flavone (5,6,7-trimethoxyflavone) isolated from *C. japonica* exhibited a relatively high inhibitory effect on herpes simplex virus type 1 (HSV-1), human cytomegalovirus, and poliovirus. Tellez et al. (2000) reported some bioactive compounds isolated from the essential oil of *C. americana.* This oil was selectively toxic to *Oscillatoria perornata*, a cyanobacterium, and was only slightly phytotoxic or antifungal.

Cruz-Ortega et al. (2002) examined the effect of the aqueous leachate (1%) of *C. acuminata* on protein expression, catalase activity, free radical production, and membrane lipid peroxidation in roots of bean, maize, and tomato. The results showed that the allelochemical stress produced by the mixture of compounds in *C. acuminata* aqueous leachate modified the expression of various proteins in the roots of all treated plants. Catalase activity, free radicals, and peroxidation were increased in some treated roots.

The first biodirected phytochemical study of *Callicarpa acuminata* was aimed at identifying its main bioactive and defensive secondary metabolites against plants, phytopathogenic fungi, *Artemia salina*, insects, and insect and mammalian cell lines.

METHODS AND MATERIALS

Plant Material. Leaves of *Callicarpa acuminata* (Verbenaceae) collected from El Eden Ecological Reserve in 1996 (L.M. Ortega-Torres 2641, Sept. 1996. UCR Herbarium) were air-dried and finely fragmented.

General Experimental Procedures ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian VXR-300 S or Varian VXR-500 S spectrometers. IR spectra were obtained using NaCl discs (oils films) on a Perkin-Elmer 599 B spectrophotometer. Melting points were determined via a Fisher Johns apparatus and are uncorrected. GC-MS analyses were conducted on an Hewlett-Packard Model 5890 gas chromatograph interfaced with a JEOL JMS AX50HA.

HPLC. A Waters binary gradient analytical HPLC consisting of 2 Model 6000A pumps, a Model 680 gradient module, and a Shimadzu SPD-MIOAVP photodiode array detector was used. Column: L-column ODS, 5 micron, 4.6 $\times$ 250 m. Gradient: 65 percent A-95 A, solvent A-acetonitrile/methanol (1:1/v:v); solvent B-water, with flow rate 1.5 ml/min. The effluent was monitored by a UV detector at 214 nm and retention times for compounds **3** and **4** were 21 and 21.7 min, respectively.

A Rainin auto preparative HPLC equipped with 3 Rabbit HP pumps, Erma ERC 3320 degasser, Erma ERC 7510 refractive index detector, Knauer 87 variable wavelength UV/Vis monitor, and MacRabbit controller was used for preparative

HPLC. Conditions for the HPLC were as follows: column Vydac HS-C18, 5 μm, 1×25 cm, solvent 70 percent (1:1 acetonitrile/methanol)/water, flow rate 5 ml/min, detection RI and UV (220 nm). Retention times for compounds **3** and **4** were 62 and 66 min, respectively.

Chemicals and Solvents. Analytical and preparative TLC was performed on precoated silica gel 60 F_{254} plates (Merck). Argentation TLC was carried out with silica gel plates impregnated with a 10% solution of $AgNO_3$. TLC spots were visualized by spraying with a 10% solution of $Ce(SO_4)_2$ in 2N H_2SO_4, followed by heating at 100°C. For open CC, silica gel 60 (70–230 mesh, Merck) was used.

Extraction Procedures. Three kilograms of dried leaves of *C. acuminata* were extracted with a total volume of 15 l of a $CHCl_3$/MeOH (1:1) mixture three times (72 hr each time). The extract was filtered and concentrated *in vacuo* to yield 415 g of dry extract. The extract was fractionated using an open chromatography column on silica gel (2.5 kg) and eluted with a gradient of hexane, chloroform, and methanol. A total of 250 primary fractions were obtained. Similar fractions were pooled using thin layer chromatography (TLC) to obtain 19 fractions (B–T).

Preparation of Aqueous Leachates. Two grams of leaves were soaked for 3 hr in 100 ml of distilled water. The leachates were filtered through Whatman paper No. 4 and a Millipore membrane. The osmotic pressure was measured with a freezing-point osmometer (Osmette A. Precision System, Inc.). The values ranged from 15 to 17 mosm/l.

Bioassays with Seeds. Aqueous leachates were mixed (1:1) with 2% pure agar to obtain a 1% w/v test solution. Pure agar (1%) and 2,4-dichlorophenylacetic acid (2,4-D, 100 μg/ml) were used as controls. Germination and seedling root growth bioassays for the aqueous leachates were carried out according to procedures published previously (Anaya et al., 1990) using *Amaranthus hypochondriacus* L. (Amaranthaceae), *Echinochloa crus-galli* (L.) Beauv. (Poaceae), and *Lycopersicon esculentum* Mill. (Solanaceae) as test species. *A. hypochondriacus* seeds were purchased from a local market in Tulyehualco, Mexico, D. F. Seeds of *E. crus-galli* were from Valley Seed Service, Fresno, California, USA, and *L. esculentum* seeds from Semillas Berentsen, Celaya, Guanajuato, Mexico. Bioassays were set up in 6-cm Petri dishes. Ten seeds of each test plant were sown directly on to the agar of each Petri dish following a completely random design with four replicates. Petri dishes were kept in darkness at 27°C. Root lengths were measured 24 hr after treatment for *A. hypochondriacus*; 48 hr for *E. crus-galli*, and 72 hr for *L. esculentum*. Data were analyzed by ANOVA and Tukey's tests (Mead et al., 2002). Bioassays with organic extracts and compounds were set up using the same conditions as for aqueous leachates, but with filter paper as a substrate. The filter paper (Whatman 42) was impregnated with 1.5 ml of the solutions at 100 μg/ml (or 2,4-D) and, after total evaporation of the solvents, it was moistened with 1.5 ml of distilled water. Test seeds and time of harvesting for these bioassays were the same.

Bioassays with Phytopathogenic Fungi. Organic extract and isolated compounds were tested on the growth of some phytopathogenic fungi with economic importance, such as *Alternaria solani* and *Helminthosporium longirostratum.* The bioassays were performed by incubating, in 6-cm Petri dishes, a fungal bud on potato-dextrose-agar (PDA) sterile medium. The medium contained the respective treatment. Extracts and fractions were evaluated at 100 μg/ml. Isopimaric acid and isopimaric acid methyl ether were evaluated at 25, 50, 150, 250, and 500 μg/ml, and Captan (50 *N*-(trimetil)tiol-4 ciclohexen, 1,2 dicarboximide), a commercial fungicide, was used at the same concentrations as a control. The bioassay was performed by following a completely random design with four replicates. Fungi were incubated in darkness at 27°C. The effects of treatments were determined by measuring the radial growth of the mycelium after 72 hr of incubation. Two perpendicular measurements of colony diameter were taken and the mean value was calculated. Results were analyzed by ANOVA and Tukey's statistical tests (Mead et al., 2002).

Brine Shrimp Lethality Test. Organic extracts and pure compounds were evaluated for lethality to brine shrimp (*Artemia salina*) larvae (BST). This crustacean is a good indicator to test for bioactive compounds that can be used as insecticides or cytotoxic substances (Meyer et al., 1982). Eggs of *A. salina* were hatched by incubating them in saline water during 48 hr at 30°C with light.

Twenty mg of each treatment were dissolved in 2 ml of an appropriate solvent. From each solution, aliquots of 500, 50, and 5 μl were added to vials in triplicate. Solvent was evaporated to dryness at room temperature or by vacuum. Five ml of saline water and 10 larvae were added to each vial. Vials were maintained at room temperature and light for 24 hr and lethality was subsequently measured. LC_{50} values of <1000 μg/ml were the activity standard for extracts or chromatographic fractions. LC_{50} values of <200 μg/ml were the activity standard for pure compounds (Anderson et al., 1991). In all bioassays, control treatments contained only saline water.

Insect Bioassays. Leptinotarsa decemlineata and *Spodoptera littoralis* colonies were reared on potato foliage, artificial diet (PoitOut and Bues, 1970) and bell pepper (*Capsicum annuum*) plants, respectively, and maintained in a growth chamber at 22 ± 1°C, >70% RH with a photoperiod of 16:8 (L:D).

Feeding Preference Assays. These experiments were conducted with adult *L. decemlineata*, newly emerged fifth-instar, and *S. littoralis* larvae. Percent feeding inhibition (% FI $= [1-(T/C)] \times 100$, where $T =$ surface of treated leaf disks and $C =$ surface of control leaf disks) was calculated as previously described (Reina et al., 2001). Compounds with FI > 70% were tested in a dose–response experiment to calculate their relative potency (EC_{50} values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (%FI on log dose).

Oral Cannulation. This experiment was performed with preweighed newly molted *S. littoralis* L6-larvae as previously described (Reina et al., 2001). At the end of the experiments (72 hr), the relative consumption rate (RCR) and the relative growth rate (RGR) were calculated on a dry weight basis according to Farrar et al. (1989). All dry larval weight measures were log-transformed prior to ANOVA to test treatment effects.

Hemolymph Injection. Twenty adult *L. decemlineata* beetles were injected with DMSO solutions of the test compounds (5 μg/insect) as described (Reina et al., 2001). Beetle mortality was recorded up to 3 days after injection. Percent mortality was analyzed with contingency tables and the data (Table 6) were corrected for the mortality of the control (Abbott, 1925).

Cytotoxicity Studies. Sf9 cells derived from *S. frugiperda* pupal ovarian tissue (European Collection of Cell Cultures, ECCC) were maintained in TC-100 insect cell medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin at 26°C. Mammalian Chinese hamster ovary cells (CHO) contributed by Dr Pajares, I.C. Biomédicas, CSIC, were grown in RPMI 1640 medium supplemented as above at 37°C under a humidified atmosphere of 5% CO_2/95% air. Cytotoxicity was measured by the MTT method (Mossman, 1983) as described in González-Coloma et al. (2000a). The active compounds were tested in a dose–response experiment to calculate their relative potency (LD_{50}values, the effective dose to give 50% cell viability), which was determined from linear regression analysis (% cell viability on log dose). A cytotoxic selectivity index (CSI = Sf9-LD_{50}/CHO-LD_{50}) was used to compare the value of the selective cytotoxic action between insect and mammal cells of a given compound.

Isolation and Identification of Compounds

Isopimaric Acid. From fractions H and K, isopimaric acid (**1**) spontaneously crystallized (1.011 g) as clear, plane square crystals, m.p. of 155–158°C. The spectral properties (IR, NMR, and MS) were identical to those previously described in the literature (Talapatra et al., 1994) (Figure 1).

Isopimaric Acid Methyl Ether. One-hundred-milligram isopimaric acid was dissolved in 20-ml methanol and treated with an excess of ethereal solution of diazomethane. After 24 hr, the excess diazomethane was eliminated and the solution evaporated to dryness. Clear crystals of isopimaric acid methyl ether (**2**) precipitated from the residue and were identified by IR, NMR, and MS (Figure 1).

α-Amyrin, Sandaracopimaradien-19-ol, and Akhdarenol. Fraction K (22 g) that was toxic against *A. salina*, *A. hypochondriacus*, and *E. crus-galli* was subjected to open column chromatography on silica gel (220 g). The initial elution system was hexane/$CHCl_3$ (1:1). A total of 239 fractions were obtained and similar fractions were pooled using TLC to yield 21 fractions (K1–K21). Further column chromatography (50 g of silica gel impregnated with 5 ml of 10% silver nitrate

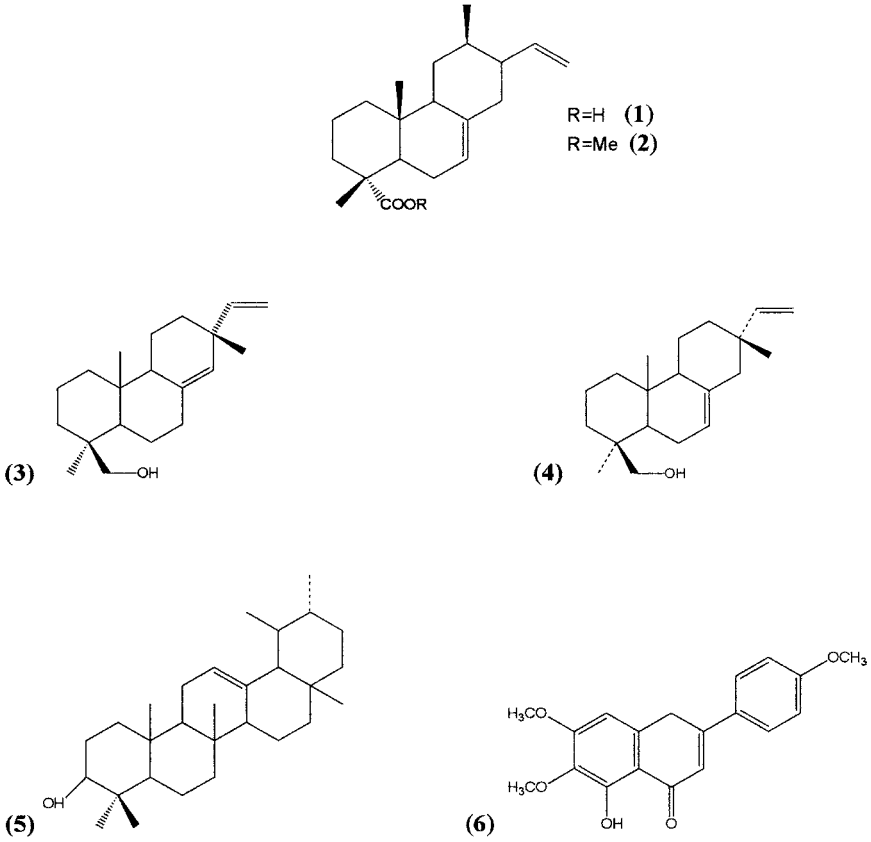

FIG. 1. Structures of compounds from *Callicarpa acuminata:* isopimaric acid (**1**), sandaracopimaradien-19-ol (**3**), akhdarenol (**4**), α-amyrin (**5**), salvigenin (**6**), and isopimaric acid methyl ether (**2**).

solution) of fraction K-9 (2 g) was performed. Altogether 110 fractions were obtained and pooled on the basis of their TLC profiles into four fractions (A–D). TLC from fraction C showed two spots (C1 and C2). The proton and carbon NMR spectra of C1 were very close matches for literature values for α-amyrin (Ahmad and Ur-Rahman, 1994). Compound **5** was positively identified as α-amyrin (Figure 1). Mixture of Fraction C2 was resolved via HPLC into two predominant components **3** and **4**, present in about a 1:2 ratio. The chromatography system utilized was 70% (1:1 methanol-acetonitrile) in water on a 25 $\times$ 1 cm Vydac HS-C18 column. At a flow rate of 5 ml/min, retention times were 62 min for **3** and 66 min for **4**. They were about 85% resolved, but apparently pure samples were obtained by peak shaving techniques.

Salvigenin, 5-Hydroxy-6,7,4'-Trimethoxyflavone. Fraction L (23 g) was further fractioned by CC using an elution system of $CHCl_3$ and $CHCl_3$:MeOH (100, 95:5, and 90:10). A total of 206 fractions were obtained and pooled using TLC to obtain 22 fractions (L1–L22). A total of 173 mg of a yellow compound crystallized from fraction L9 and was further purified by TLC using $CHCl_3$:MeOH (98:2). The pure compound, m.p. 193–195°C, MW 328.32 was identified as the known flavone 5-hydroxy-4',6,7-trimethoxyflavone (salvigenin) (**6**) by spectral means (Xaasan et al., 1980) (Figure 1).

RESULTS AND DISCUSSION

Activity of Aqueous Leachates and Organic Extracts. One percent aqueous leachate of *C. acuminata* leaves significantly inhibited the radical growth of *A. hypochondriacus* (48%), *E. crus-galli* (72%), and *L. esculentum* (42%). The effects of the chloroform:methanol extract (1:1) of the leaves of *Callicarpa,* and the most active primary fractions following column chromatography of the full extract (named by letters) on the root growth of the same three test species are shown in Table 1.

The chloroform-methanol (1:1) extract significantly inhibited the root growth of *A. hypochondriacus* (23.5%), *E. crus-galli* (59%), and *L. esculentum* (53.5%). The root growth of the latter species was strongly inhibited by fraction D (56.6%). Fractions B, C, K, L, M, and N also inhibited the root growth of the test plants. *A. hypochondriacus* and *E. crus-galli* were strongly inhibited by fractions O and P.

TABLE 1. EFFECT OF *Callicarpa acuminata* LEAF EXTRACT AND ACTIVE PRIMARY FRACTIONS FOLLOWING COLUMN CHROMATOGRAPHY ON ROOT GROWTH

	A. hypochondriacus	*E. crus-galli*	*L. esculentum*
Extract/Fraction (100 μ/ml)	% of root growth vs. control[a]		
A (Full extract)	76.5*	41*	46.5*
Active column fractions			
B	64*	67.8*	52*
C	89	57.6*	62.4*
D	62.6*	82.2	43.4*
I	114	89.7	74*
K	53.4*	61.8*	104
L	76.7*	59.4*	106
M	63.4*	61*	90
N	64.8*	59.7*	96.5
O	44*	30.4*	61*
P	46.6*	40.8*	77*

[a] Distilled water.
* $p < 0.05$.

TABLE 2. EFFECT OF *Callicarpa acuminata*, LEAF EXTRACT AND PRIMARY FRACTIONS ON RADIAL GROWTH OF PHYTOPATHOGENIC FUNGI

Treatments (100 $\mu g/ml$)	*H. longirostratum*	*A. solani*
	% of radial growth vs. control[a]	
A (Full extract)	109	106.04
Active column fractions		
B	68.7*	95.5
J	70.7*	111.6
K	76.7*	93.5
M	80*	105
O	111	82*

[a] PDA (potato–dextrose–agar).
* $P < 0.05$.

Biological tests during fractionation showed that both *Artemia salina* and the three test plants were negatively affected by the allelochemicals from *C. acuminata* indicating the presence of bioactive compounds. Fractions I (BST $LC_{50} = 213.79$ μg/ml), J (BST $LC_{50} = 311.17$ μg/mL), and K (BST $LC_{50} = 500$ μg/ml) were toxic to brine shrimp. In addition, biological tests with *A. salina* showed that fraction (K-9) was highly toxic to this crustacean (BST $LC_{50} = 2\mu$g/ml).

Because of the scarce quantities of fractions O and P, fractionation was continued with fraction K, which was more abudant.

Phytopathogenic fungi were less sensitive to the full extract and primary chromatographic fractions than the test plants or *Artemia salina* (Table 2). In general, *H. longirostratum* was more sensitive than *A. solani.* The chloroform extract had no effect on the radial growth of either fungal species, but fractions B, J, K, and M had a moderate inhibitory effect on *Helminthosporium*. Fraction M was the only fraction to inhibit *Alternaria* growth.

Isolation and Characterization of Sandaracopimaradien-19-ol and Akhdarenol. NMR data (1D and 2D proton and carbon correlations) indicated that **3** and **4** were isomers with the formula $C_{20}H_{32}0$. The NMR spectra of both compounds were too complex and too overlapped to completely assign, even with advanced 2D methods. Therefore, we employed the lanthanide shift reagent (LSR), europium (III) tris (1,1,1,2,2,3,3,)-heptafluoro-7,7,-dimethyl-d_6-octane-4,6-dione-d_3 ($Eu(fod)_3$), to resolve the resonances in the NMR spectra and deduce the complete stereochemistry and structure of these diterpenols. We used a combination of ^{1}H-^{1}H-COSY, CHCOR, LRCOR, INAPT, and NOESY 2D-experiments in combination with shift reagent to verify these structures (König and Wright, 1995).

Finally, the full relative stereochemistry of C2a was determined by NOESY experiments. The top face of the molecule could be defined by NOe's observed between CH_3-20 and H-1e, H-2a, H-6a, H-11a, CH_3-17, and H-19a and between CH_3-17 and H-11a. The bottom face was shown by NOe's between H-1a and H-5 and H-9, and between CH_3-18 and H-5 and H-6e. In conclusion, C2a was assigned the structure 4*S**, 5*R**, 9*S**, 10*R**, 13*R**-isopimara-8(14),15-dien-19-ol, also known as sandaracopimaradien-19-ol (**3**) (Grant et al., 1969) (Figure 1). The complete relative stereochemistry of C2b was determined by lanthanide-shifted NOESY experiments. The top face of the molecule was defined by NOe's between CH_3-20 and H-1e, H-11a, CH_3-17, and CH_2-19, and by NOe's between CH_3-17 and H-14e. The bottom face showed NOe's between H-9 and H-1a, H-5, and H-14a and between H-5 and CH_3-18. Thus, all stereocenters of the C2b molecule are defined. The CH_2OH must be axial as are the ring junction protons, H-5 and H-9, and the methyl groups at C-10 and C-13. In conclusion, C2b was identified as 4*S**, 5*R**, 9*S**, 10*R**, 13*S** isopimara-7,15-dien-19-ol. This compound is also known as akhdarenol (**4**) (Kitajima et al., 1982) (Figure 1).

Activity of Compounds. The phytotoxicity exhibited by several fractions and the full extract almost disappears when pure compounds were evaluated (Table 3). Only compounds **5** and **6** caused a significant inhibition on two of them, α-amyrin (**5**) inhibited the root growth of *Amaranthus* and *Echinochloa* (26.8 and 34%, respectively). In addition, **5** was toxic to *Artemia salina* (BST $LC_{50} = 200\ \mu$g/ml). α-Amyrin is an ursane type of triterpenoid widely prevalent in nature. It also occurs in the latex of many plants: *Ficus variegata* (Moraceae), rubber trees, *Erythroxylon coca* (Erythroxylaceae), and *Balanophora elongata* (Balanophoraceae), among others (Harborne and Baxter, 1993). Salvigenin (**6**) inhibited the root growth of *Amaranthus* by 26.2%. Miura et al. (2001) isolated salvigenin from the leaves of

TABLE 3. EFFECTS OF ISOPIMARIC ACID (**1**), METHYL ISOPIMARIC ACID (**2**), THE MIXTURE OF TWO ISOMERIC DITERPENOLS (**3, 4**), α-AMYRIN (**5**), AND SALVIGENIN (**6**) ON PLANT RADICLE GROWTH

Treatments (100 μg/ml)	*A. hypochondriacus*	*E. crus-galli*	*L. esculentum*
	% of radicle growth vs. control[a]		
Control	100	100	100
2,4-D	0	0	0
1	85.5	82.6	100
2	86	72.4	113.8
3+4	103.7	78.3	94.0
5	73.2*	66.0*	105.4
6	73.8*	100	110

[a] Distilled water.
* $P < 0.05$

Salvia officinalis together with some apianane terpenoids and other known compounds. These findings may suggest a defensive role played by both compounds (**5** and **6**) in all these plants, as in *C. acuminata*. Further studies on the allelochemical potential of *C. acuminata* could allow us to determine the compounds responsible for the phytotoxic effects. *L. esculentum* was not affected by any of the isolates, but 2,4-D, which served as control. This suggests a probable synergistic or additive effect of compounds in the mixtures, a common phenomenon observed in phytotoxic studies. In most cases, chemical inhibition is the result of the simultaneous action of several compounds, and often these include compounds whose chemistry is divergent (Einhellig, 2002).

Isopimaric acid (**1**) was toxic to *Artemia salina* (BST $LC_{50} = 10\,\mu$g/ml). Table 4 shows that (**1**) also produced a significant inhibition of the radial growth of *Helminthosporium longirostratum* at 150, 250, and 500 μg/ml (41, 50, and 47%, respectively) ($IC_{50} = 403\,\mu$g/ml). The effect of this compound on *H. longirostratum* was similar to the commercial fungicide captan ($IC_{50} = 355\,\mu$g/ml).

TABLE 4. RADIAL GROWTH OF TWO PHYTOPATHOGENIC FUNGI IN RESPONSE TO VARIOUS CONCENTRATIONS OF ISOPIMARIC ACID (**1**), ISOPIMARIC ACID METHYL ETHER (**2**), AND A COMMERCIAL FUNGICIDE (CAPTAN)

	H. longirostratum	*A. solani*
Treatments (μg/ml)	% of radial growth vs. control[a]	
Control	100	100
Isopimaric acid (1)		
25	94	84.5
50	88.2	80.2*
150	58.8*	77*
250	50.4*	80.8*
500	53*	78*
Isopimaric acid methyl ether (2)		
25	104.7	95.0
50	98.6	95.3
150	95.2	96.5
250	105.6	89.6
500	ND	ND
Captan		
25	60	82.3
50	57.3*	75.2*
150	55.3*	75.6*
250	54.2*	56.6*
500	46*	49.1*

[a] Potato-dextrose-agar (PDA).
* $P < 0.05$. ND = not determined.

TABLE 5. EFFECTIVE ANTIFEEDANT DOSES (EC_{50}) AND 95% CONFIDENCE LIMITS (LOWER, UPPER) OF THE TEST COMPOUNDS

Compounds	$EC_{50}(\mu g/cm^2)$	
	S. littoralis	*L. decemlineata*
1	>100	>100
2	>100	29.7 (21.2, 41.6)
3	>100	>60
4	>100	4.7 (1.7, 18.5)
5	>100	14.6 (4.8, 44.4)
6	>100	>60

Isopimaric acid (**1**) caused moderate, but significant, growth inhibition of *A. solani* from 50 to 500 μg/ml. Captan also inhibited the growth of *A. solani* from 50 to 500 μg/ml (25–51%) ($IC_{50} = 426.36\,\mu$g/ml). Isopimaric acid methyl ether (**2**) had no significant effect on the growth of either fungus, but was toxic to *Artemia salina* (BST $LC_{50} = 50\,\mu$g/ml). Isopimaric acid and other related resin acids such as pimaric, abietic, dehydroabietic, neoabietic, dehydroisopimaric, and sandaracopimaric acids are common compounds in oleoresins of Pinaceae (Harborne and Baxter, 1993). Their presence in *C. acuminata* suggests that they probably play an important role in defense against herbivores in plants from different taxa.

Table 5 shows the antifeedant effects of the test compounds on *S. littoralis* and *L. decemlineata*. None of them had any effect on the polyphagous *S. littoralis*, while the oliphagous *L. decemlineata* was sensitive to akhdarenol (**4**), followed by α-amyrin (**5**), and isopimaric acid methyl ether (**2**). These results are consistent with the model suggesting that differences in taste sensitivity to deterrent compounds could account for the difference in host range (Bernays and Chapman, 2000).

Previous results have shown that some diterpene acids and alcohols have antifeedant, repellant, and acaricidal effects against several insect species (Morita, 1989; Yamaaki et al., 1998; Yamada, 1998; Powell and Raffa, 1999), but this is the first report of the antifeedant properties for akhdarenol, α-amyrin, and isopimaric methyl ether acid.

Comparison of the activity of the diterpenes indicated that the unsaturation of the B ring (>12-fold increase in activity of **4** with respect to **3**) with a hydroxy (**4**) or methyl ester (**2**) in C-19 (>20 and >3-fold increase in activity of **4** and **2** in relation with **1**) play an important role in the antifeedant effect of this class of diterpenes.

Some pentacyclic triterpene derivatives have been described as insect antifeedants against *S. littoralis* and *L. decemlineata* (Lugemwa et al., 1990; Huang et al., 1995; Jagadeesh et al., 1998). Here, we have found that triterpene **5** (α-amyrin) had moderate antifeedant activity against *L. decemlineata*. Previous results

TABLE 6. EFFECT OF TEST COMPOUNDS ON INSECT LARVAL PERFORMANCE (20 μG INSECT), ADULT MORTALITY (5 μG/INSECT) FOLLOWING ORAL AND HEMOLYMPH INJECTION, AND EFFECTIVE CYTOTOXIC DOSES (IC_{50}) AND 95% CONFIDENCE LIMITS ON INSECT AND MAMMALIAN CHO CELLS

	S. littoralis		*L. decemlineata*	LD_{50} (μg/ml)		CSI[d]
Compounds	RCR[a] (% of control)	RGR[b] (% of control)	% Mortality (72 hr)[c]	Sf9	CHO	
1	144.4	108.9	0	4.1 (6.3,2.6)	16.31 (4.9,53.9)	0.25
2	87.8	78.1	27[e]	na	na	—
3	102.5	87.0	23[e]	14.3 (9.0,22.8)	>100	<0.14
4	103.3	104.7	11	4.3 (2.9,6.6)	6.9 (4.3,11.0)	0.60
5	91.9	80.7	60[e]	8.66 (6.8,11.09)	>100	<0.05
6	77.3	75.7	20[e]	5.74 (3.5,9.3)	5.8 (3.2,10.6)	0.9

[a] RCR = $I/(\mathrm{BI})\times T$, I = mg consumed diet, T = length (days), BI = initial insect weigth (mg).
[b] RGR = $\Delta B/(BI) \times T$, ΔB = change in insect body weight (mg).
[c] Corrected according to Abbot (1925).
[d] Cytotoxic Selective Index (CSI = IC_{50}Sf9: IC_{50}CHO).
[e] Significantly different from the control. Contingency table analysis ($p < 0.05$).

with triterpenes structurally-related to **5** have shown that the presence of a hydroxy (as in **5**) or a ketone substituent in C-3 is needed for the antifeedant effect against *L. decemlineata* in the absence of an unsaturation in the E ring (as in **5**) (Santana, 2000).

None of the compounds had detrimental, postingestive effects on *S. littoralis* (Table 6). However, on abdominal injection, compound **5** was toxic to *L. decemlineata,* followed by compounds **2**, **3**, and **6** with moderate-to-low toxicity. We did not find any correlation between antifeedant and toxic effects on this insect as previously shown for other terpenoid antifeedants (see González-Coloma et al., 2002b), suggesting different modes of action for the antifeedant and toxic effects.

Authors have also tested the cytotoxicity of the natural products on insect- and mammalian-derived cells (Sf9 and CHO, respectively) (Table 6). Overall, the compounds were cytotoxic to Sf9 cells, suggesting that the lack of postingestive effects is probably due to metabolic detoxification. Among the diterpene alcohols, **1** and **4** were similarly cytotoxic to Sf9 cells. CHO cells were sensitive to compounds **6**, **4**, and **1**. Compound **5** showed the strongest selectivity against insect cells (with the lowest CSI value), followed by compounds **3**, **1**, and **4**, with **6** being equally toxic to both cell lines.

Some diterpene acids, including isopimaric acid (**1**) and triterpenes structurally related to **5** have been described as being cytotoxic to mammalian and tumor cells (Montagnac et al., 1997; Chang et al., 2000). Furthermore, there is indirect evidence of the antitumor action of α-amyrin (**5**) (Akihisa et al., 1996)

and salvigenin (**6**) (Tezuka et al., 2000). However, this is the first report on the cytotoxic effects of these compounds on insect-derived cells. The selective cytotoxic action of the diterpene alcohol **3** and the triterpene **5** against Sf9 cells is probably due to membrane-related factors. The antibacterial and cytotoxic effects of some isopimaranes have been attributed to membrane-damaging effects (Woldemichael et al., 2003).

In the present study, we found diverse and selective bioactivity of an organic extract, chromatographic fractions, and pure compounds from *C. acuminata* on a variety of test organisms. In addition, we confirmed the ecological importance of terpenoids as infochemicals (compounds that carry some information in chemical interactions between two organisms), particularly showing the toxicity of some of them on fungi, insects, and mammalian and insect cells. The terpenoid pathways generate a great structural diversity and complexity of compounds, which have an enormous potential for mediating ecological interactions (Langenheim, 1994).

Current studies on plants and fungi of El Eden, Quintana Roo, Mexico will contribute to the knowledge of their allelochemical potential, and as a consequence, to the discovery of the added value of these organisms from the endangered dry tropical forests.

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BOOK REVIEW

Plant Resins: Chemistry, Evolution, Ecology and Ethnobotany. By Jean H. Langenheim. Timber Press, Portland, OR. US $50. ISBN 0-88192-574-8.

For those of us living in temperate Europe and North America, it is not easy to appreciate the enormous diversity of plant resins, their intricate chemical differences, their wide taxonomic distribution, and their fascinating ecological roles. Regular readers of this journal often learn about the ecological significance of conifer resin, for example, but hear little about the vast number of resins of angiosperm origin, most of which come from species native to the tropics or semiarid regions. Fortunately, this knowledge gap can be pleasantly filled by spending a few hours browsing an outstanding, new, single-author compendium on the botanical, chemical, ecological, and ethnobotanical aspects of plant resins. As the first book on this subject in over 50 years, it provides encyclopedic, but very readable coverage of virtually all facets of these intriguing plant products. Don't let the stylish, coffee-table format, and lush illustrations fool you. This is an authoritative scientific work overflowing with facts and over 1000 references (extending to 2002). The author, Jean Langenheim, is well known to chemical ecologists from her work on the ecological roles of terpenes in tropical legumes and temperate genera, such as *Sequoia* and *Umbellularia*. But her interests in resin terpenes also extend to their anatomy, paleobotany, and ethnobotany, and in this volume she has compiled information and insights gained over her entire scientific career in an attractive, readily accessible, and well-organized format.

The volume is divided into three somewhat independent parts dealing with (I) botanical and chemical, (II) geological and ecological, and (III) ethnobotanical and industrial aspects. These parts can be read in any order since the frequent cross-references allow the reader to stray easily from one aspect of the subject to another. Coverage in part I begins with a definition of resin: "a lipid-soluble mixture of volatile and nonvolatile terpene and/or phenolic secondary compounds that are usually secreted in specialized structures . . . and are of potential significance for ecological interactions." The author goes to considerable lengths to distinguish true resin from other plant exudates, such as gums, mucilages, oils, waxes, and latex. She then presents a brief, but lucid review of the chemistry and biochemistry of terpene and phenolic resin constituents. This is followed by an account of the plant species producing resin organized by their taxonomic affinities. One

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cannot fail to be overwhelmed by the vast number of resin-producing plant genera representing a wide variety of taxonomic backgrounds, life forms, and habitats. Whatever selection pressures led plants to produce resin, seem to have recurred again and again over the course of evolution. Also included in this part is a superb, detailed discussion of the different anatomical structures used in resin secretion and storage.

The geological and ecological facets of plant resins are addressed in part II. First, we are treated to the enthralling story of amber and other fossilized resins, and what they reveal about paleoecological conditions. The next section, covering the ecological roles of plant resins, will probably be the highlight for most readers of this journal. Here, Dr. Langenheim expertly summarizes the main currents of research in this area over the last 30–40 years, providing sufficient references to permit easy access to the primary literature. The emphasis is on plant–herbivore interactions with most space devoted to the interactions of insects with the resins of conifers and the tropical legumes, *Hymenaea* and *Copaifera* (the author's own primary experimental systems). But, mammalian herbivores are not ignored, nor are the resins of *Bursera*, *Dalechampia*, the Dipterocarpaceae, and those found on the surfaces of plants growing in semiarid habitats. Special attention is given to the effect of the physical properties of resin, the extensive variation in resin composition, and other such factors on biological interactions.

Finally, part III considers resins and their interactions with human affairs, including their historical, present day, and projected future roles. The subject encompasses many remarkable stories, such as those on Baltic amber and *Cannabis*. Newcomers to this area will appreciate the way the text uses explicit chemical, botanical, and physical criteria to distinguish among the many different types of resins, including oleoresins, balsams, lacquers, varnishes, etc.

Each reader will probably have his/her own favorite parts of the book. I most enjoyed the sections in which the author uses her broad background and lifetime of research on resins to make connections among topics such as secretory structures and resin function, or the taxonomic distribution of resin production and its evolution. Her own research on resin-producing *Hymenaea* and *Copaifera* is also well-covered here, but not disproportionately so compared to the research of other scientists. Dr. Langenheim is very good at summarizing the results of others in a careful, organized fashion, while resisting the temptation to generalize. As a habitually lazy reader, I would have preferred more simplified conclusions, but in the diverse world of resins, generalizations are hard to come by, and one walks away impressed with the enormous diversity of nature and the need for further study to find the major patterns.

The editing and production of this book were obviously done with lots of care. There are a few errors in the chemical structures, but almost no typographical or other mistakes of any other kind. The text is accompanied by many tables, maps, line drawings and photographs. Most notable are the beautiful, well-reproduced

color plates (nearly half of them taken by the author herself), which offer glimpses of a wonderful assortment of resins, amber, resin-containing plants, and resin-harvesting activities. The appendices include lists of resin types, resin uses, resin-producing plants, and amber deposits.

This volume can be recommended for any scientist interested in plant resins who wants a complete reference on the subject, a source of stimulating reading, or a choice list of questions for further study. For a scientific book of over 500 pages with many color illustrations, the cost is quite reasonable, thanks to the publishers' apparent attempt to market the book to a lay, as well as a scientific audience. While I have my doubts about its accessibility to a lay audience, I have no hesitation in recommending this volume to scientists, in general, and chemical ecologists, in particular. It is hard to imagine a more authoritative, aesthetic and well-written treatise in this area appearing for many years to come.

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A SIPHONOTID MILLIPEDE (*RHINOTUS*) AS THE SOURCE OF SPIROPYRROLIZIDINE OXIMES OF DENDROBATID FROGS

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Abstract—Poison frogs of the neotropical family Dendrobatidae contain a wide variety of lipophilic alkaloids, which are accumulated from alkaloid-containing arthropods. A small millipede, *Rhinotus purpureus* (Siphonotidae), occurs microsympatrically with the dendrobatid frog *Dendrobates pumilio* on Isla Bastimentos, Bocas del Toro Province, Panamá. Methanol extracts of this millipede contain the spiropyrrolizidine *O*-methyloxime **236**, an alkaloid previously known only from skin extracts of poison frogs, including populations of *D. pumilio*. Thus, *R. purpureus* represents a likely dietary source of such alkaloids in dendrobatid frogs.

Key Words—Alkaloids, arthropods, frogs, dendrobatids, millipedes, and pyrrolizidines.

INTRODUCTION

Millipedes produce a wide range of allomones, which presumably act to deter potential predators. Millipede allomones include benzoquinones, phenols, benzaldehyde/hydrogen cyanide, and alkaloids (Eisner et al., 1978 and references therein). Three groups of alkaloids are known from millipedes as follows: (1) the glomerins (Ia & b) from millipedes of the genera *Glomeris* and *Loboglomeris* (Order: Glomerida, Family: Glomeridae) (Schildknecht et al., 1967 and reference therein), (2) the terpenoid alkaloids polyzonimine (II) and nitropolyzonamine (III) from the

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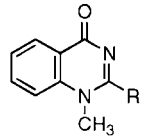
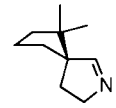
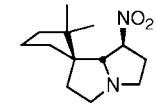


Ia R = CH_3 **Glomerin**

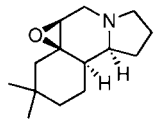
Ib R = C_2H_5 **Homoglomerin**



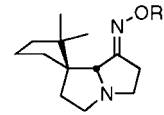
II **Polyzonimine**



III **Nitropolyzonamine**

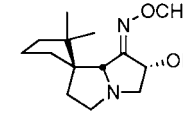


IV **Buzonamine**



Va R = H **222**

Vb R = CH_3 **236**



VI **252A**

FIG. 1. Millipede alkaloids.

millipede *Polyzonium rosalbum* [now *Petaserpes crytocephalus*] (Order: Polyzoniida, Family: Polyzoniidae) (Meinwald et al., 1975 and reference therein), and (3) the terpenoid alkaloid buzonamine (IV) from a millipede of the genus *Buzonium* (Order: Polyzoniida, Family: Polyzoniidae) (Wood et al., 2000) (Figure 1). Certain poison frogs/toads of the families Dendrobatidae, Mantellidae, and Bufonidae contain spiropyrrolizidine alkaloids (Figure 1; Va & b and VI)(Daly et al., 1994 and references therein) with carbon skeletons identical to those of the millipede alkaloid nitropolyzonamine. These spiropyrrolizidine oximes have been assigned 'bold type' code names (**222**, **236**, and **252A**), which consist of a boldfaced number corresponding to the nominal mass and a boldfaced letter for identification of individual alkaloids. Such frogs appear to obtain their skin alkaloids from dietary sources (Daly et al., 1994, 2000), and, therefore, it seemed likely that a polyzoniid millipede would prove to be a dietary source of spiropyrrolizidine oximes.

Spiropyrrolizidine **236** and nitropolyzonamine have been detected in extracts of leaf-litter arthropods collected with Berlese Funnels from Cerro Ancón, Panamá (Daly et al., 1994) and in mixed leaf-litter samples from Isla Bastimentos, Panamá (Daly et al., 2002), however, the specific arthropod source was not determined. In an effort to taxonomically identify arthropod sources for the frog skin alkaloids, a series of leaf-litter arthropod samples, separated by taxa (including millipedes), were again collected and analyzed from Isla Bastimentos, Panamá. From these collections, the spiropyrrolizidine oxime **236** was detected in samples containing only millipedes. We now report on the identification of a small millipede, *Rhinotus purpureus* (Pocock, 1894) (Order: Polyzoniida, Family: Siphonotidae), that

contains the spiropyrrolizidine oxime **236** and, thus, represents a dietary source for the same alkaloid found in the skin extracts of the dendrobatid frogs (*Dendrobates pumilio*) from the same location on Isla Bastimentos. In addition, trace amounts of nitropolyzonamine were detected in the millipede extract. Based on stomach content analysis, the diet of *D. pumilio* consists of a variety of small leaf-litter arthropods, including small millipedes (Donnelly unpublished data; Saporito et al., unpublished data).

METHODS AND MATERIALS

A variety of arthropods were collected from the leaf-litter and separated by taxa at several different sites on Isla Bastimentos, Bocas del Toro Province, Panamá. Arthropod collections were made from February 2–8, 2003, from the following 8 locations: Site 1: N 9°21.618′; W 82°12.074′. Site 2: N 9°21.250′; W 82°12.519′. Site 3: N 9°21.169′; W 82°12.627′. Site 4: N 9°21.123′; W 82°12.620′. Site 5: N 9°20.996′; W 82°12.726′. Site 6: N 9°20.364′; W 82°10.807′. Site 7: N 9°21.021′; W 82°12.704′. Site 8: N 9°20.490′; W 82°10.486′. Several of these sites were at the same location where mixed arthropod collections were previously collected (Daly et al., 2002). All arthropod specimens were collected with forceps, from leaf-litter placed on white cloth, and placed in plastic 150 ml vials according to individual taxa. Only small arthropods (less than 10 mm), suitable as prey for the dendrobatid frog *D. pumilio* (average snout-vent length 20 mm), were collected from these sites.

Gas chromatographic mass spectral analyses (GC-MS) were conducted with a Finnigan GCQ instrument with a 25 m × 0.25 mm i.d. Rtx-5 Amine fused silica column, using a temperature program from 100 to 280°C at a rate of 10°C/min. Arthropod extracts were screened for the presence of alkaloids by injecting a 4 μl mixture, which consisted of 1μl portions from each of 4 different vials (arthropod samples). All mixtures were based on specific taxonomic groups (i.e., millipede samples were only combined with other millipede samples, etc.). If an alkaloid was detected in a mixture, then each of the four vials was analyzed separately to determine the source of the alkaloid. Identification of alkaloids was based on gas chromatographic mass spectra and comparison to authentic spiropyrrolizidine **236** or nitropolyzonamine.

RESULTS AND DISCUSSION

The spiropyrrolizidine alkaloid **236** was detected in two separate millipede samples, both of which were from site 7 located on Isla Bastimentos. Both samples contained one individual of *Rhinotus purpureus* (Pocock, 1894). In addition, trace amounts of nitropolyzonamine were also found in these samples.

Alkaloids were not detected in extracts from the following other millipede samples: (1) Order: Polyxenida; 1 individual. (2) Order: Stemmiulidae; Family: Stemmiulidae; Species: *Prostemmiulus* cf. *cincinnatus* (Loomis); 3 individuals. (3) Order: Spirobolida; Family: Spirobolellidae; Species: *Spirobolellus trifasciatus* (Loomis); 3 individuals. (4) Order: Polydesmida; Family: Aphelidesmidae; Genus: *Aphelidesmus*; 1 adult and 1 immature. (5) Order: Polydesmida; Family: Chelodesmidae; Genus: *Trichomorpha*; 1 individual. (6) Order: Polydesmida; Family: Cyrtodesmidae; Genus: *Cyrtodesmus*; 1 individual. (7) Order: Polydesmida; Family: Fuhrmannodesmidae; 2 individuals. (8) Order: Polydesmida; 3 immature individuals.

Spiropyrrolizidine **236** was not detected in any of the other arthropod extracts examined in this study, however, a decahydroquinoline and a 5,6,8-trisubstituted indolizidine were detected in certain ant extracts. These results will be reported separately. Decahydroquinolines have been reported from myrmicine ants (Daly et al., 2000, 2002 and references therein). However, until now, there have been no known reports of branched chain izidine alkaloids from ant extracts, although a branched chain 5,8-disubstituted indolizidine was detected in extracts from mixed arthropod samples from Isla Bastimentos (Daly et al., 2002). In the present study, the frogs from the site at which the millipedes had the spiropyrrolizidine **236** were also shown to have that alkaloid in skin extracts (Daly et al., 2002 and unpublished data). Nitropolyzonamine was not detected in the skin extracts from *D. pumilio* on Isla Bastimentos. However, nitropolyzonamine, as well as the spiropyrrolzidine oximes **236** and **252B** (not shown, see below) have been detected as trace amounts in *Dendrobates auratus* from Cerro Ancon, Panamá (Daly et al., 1994).

The spiropyrrolizidine oxime **236** has been detected in frog skin extracts from several species of dendrobatid frogs from a variety of locations throughout Central and South America (Table 1). From these locations, it is interesting to note the absence of **236** from some of the frog populations in Panamá during initial visits (Table 1). The appearance of **236** in later visits to the same Panamanian sites may suggest an introduction and, therefore, availability of *R. purpureus* to frogs within these populations.

The millipede *R. purpureus* has a relatively wide distribution throughout the tropics; therefore, it seems probable that other *Rhinotus* species may serve as a source for spiropyrrolizidine oximes found in dendrobatid frogs of South America. Spiropyrrolizidine oxime **236** has been detected from an Argentinean bufonid toad (Garraffo et al., 1993) and from Madagascan mantellid frogs (Daly et al., 1996). In addition, one extract from an Australian myobatrachid frog had spiropyrrolizidine oxime **252B**, an isomer of **252A** in which the hydroxyl group is in a different position (Daly et al., 1990). It is likely that such spiropyrrolizidine oximes will be found to have a deterrent effect on potential millipede predators,

TABLE 1. OCCURRENCE OF SPIROPYRROLIZIDINE **236** IN FROG/TOAD SKIN EXTRACTS: AN INDICATION OF AVAILABILITY OF *Rhinotus* MILLIPEDES?

Family: *Species*	Location	Year	**236**
Dendrobatidae:			
Dendrobates pumilio	Rumpala, Bocas Province, Panamá	1981	Present
	Isla Bastimentos, Bocas Province, Panamá	1972	Absent†
		1980, 1981, 1987, 1993, 2002*	Present
	La Gruta, Isla Colon, Bocas Province, Panamá	1983, 1986	Absent†
		1992	Present
	Río Sand Box, Limón Province, Costa Rica	1989	Present
	Río Sarapiqui, Heredia Province, Costa Rica	1989	Present
	28 Millas, Limón Province, Costa Rica	1990, 1995	Present
	Siquirres, Limón Province, Costa Rica	1995	Present
	Pocora, Limón Province, Costa Rica	1995	Present
Dendrobates auratus	Isla Tobago, Panamá	1974	Absent†
		1993	Present
	Cerro Ancón, Panamá	1993	Present
Dendrobates tinctorius	Pet Trade	1985	Present
Epipedobates macero	Parque Nacional Manu, Perú	1989	Present
Epipedobates cf. pictus	Parque Nacional Manu, Perú	1989	Present
Epipedobates pulchripectus	Serra de Navio, Amapa, Brazil	1992	Present
Epipedobates tricolor	Pasaje, El Oro, Ecuador	1987	Present
Epipedobates trivittatus	Pet Trade	1985	Present
Mantellidae:			
Mantella baroni	Sahavondrana, Madagascar	1993	Present
Mantella betsileo	Mahambo, Madagascar	1998	Present
Bufonidae:			
Melanophryniscus stelzneri	Tanti, Cordoba, Argentina	1989	Present

*see Daly et al. 2002.
†not detected.

such as ants, however, such studies have yet to be conducted. The oxime **236** does exert potent blocking action at mammalian nicotinic receptors, particularly those of the ganglionic subtype (Badio et al., 1996).

Acknowledgments—We would like to thank the Autoridad Nacional del Ambiente and the República de Panamá for permission to collect and export the arthropod specimens used in this study (Permit: SE/A-45-03). We thank the Smithsonian Tropical Research Institute (STRI), especially Orelis Arosemena and Maria Leone, for assistance in obtaining these permits. This work is based on research supported by the Environmental Protection Agency, the Explorers Club, and a Courtesy Associate appointment given by the National Institute of Diabetes and Digestive and Kidney Diseases. This paper is contribution number 67 to the program in Tropical Biology at Florida International University.

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