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DISTURBANCE AND REATTACHMENT BEHAVIOR OF SEA ANEMONES *Calliactis tricolor* (LE SUEUR): TEMPORAL, TEXTURAL AND CHEMICAL MEDIATION

WILLIAM R. BROOKS,[1] LEAH CEPERLEY,[2] and DAN RITTSCHOF[3,*]

[1]*Department of Biological Sciences*
Florida Atlantic University
Boca Raton, Florida

[2]*Kenyon College*
Gambier, Ohio

[3]*Duke University Marine Laboratory*
Beaufort, North Carolina

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Abstract—Two hermit crabs, *Dardanus venosus* (Milne-Edwards) and *Pagurus pollicaris* Say, have evolved mutualistic symbiotic relationships with sea anemones, *Calliactis tricolor* (Le Sueur). Anemones live on gastropod shells occupied by the crabs. Both partners are thought to participate in the transfer of the anemone to the crab's shell. However, neither species invariably exhibits transfer behavior when in contact with the other symbiont. Factors influencing transfer activity of *C. tricolor* were studied. Anemone attachment is stimulated by disturbance and is time dependent. Anemones attached to shells more frequently immediately after the anemone was disturbed. Assays testing surface texture and surface composition showed that attachment activity of the sea anemone is dependent upon the organic matrix of the shell and is enhanced by texture. Attachment by anemones to shells with organic matrix removed is reduced, but is stimulated by adsorption of complex odor mixtures. Anemones respond to odor mixtures that signal food to crabs and to odor mixtures that stimulate shell investigation and shell switching in crabs. Thus, *C. tricolor* participates in shell transfer activity after disturbance. Behavior of the anemone is cued by texture and chemicals, including chemical mixtures that cause hermit crabs to change shells.

Key Words—Anemones, *Calliactis tricolor*, chemical mediation, coevolution, *Dardanus venosus*, hermit crabs, *Pagurus pollicaris*, symbiosis.

*To whom correspondence should be addressed.

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INTRODUCTION

Communication and recognition are important factors in initiating and maintaining symbiotic associations. Information flow between symbionts can involve chemical, physical, and visual cues (for review, see Davenport, 1966; Ache, 1974). We chose the symbioses between hermit crabs and sea anemones to study information flow that mediates behavior. The hermit crabs *Pagurus pollicaris* and *Dardanus venosus* have the anemone *Calliactis tricolor* as a symbiont. *P. pollicaris* usually hosts no more than one or two anemones (Brooks and Mariscal, 1986). *Dardanus venosus* are often found with numerous *C. tricolor* attached to their gastropod shells (Brooks, 1991a). The relationship between crabs and anemones is mutualistic; both partners deter some of the other partner's predators (Ross, 1971; Brooks, 1988, 1989; Brooks and Gwaltney, 1993).

How crabs and anemones initiate their symbiosis in the field is unknown. Laboratory studies show that crabs can locate anemones from a distance using chemical cues (Brooks, 1991b). In the laboratory, *D. venosus* regularly transfer anemones to their shells from other shells or surfaces (Ross, 1960, 1970, 1979; Ross and Sutton, 1961; Cutress and Ross, 1969; Cutress et al., 1970; Brooks and Mariscal, 1986; Brooks, 1988, 1989). To transfer an anemone, the crab pokes the column of the anemone with its dactyls, which triggers the release of the pedal disc from the substrate. The crab then manipulates the anemone onto its shell where the anemone reattaches to this new surface.

Transferring of *C. tricolor* from shell to shell requires active participation by the anemone (Cutress and Ross, 1969; Cutress et al., 1970; Brooks, 1991a). First, *C. tricolor* clings to the shell with its tentacles, then, in a slow-motion somersaulting action (flipping), it attaches its pedal disc. Early studies indicated that tentacle adhesion and subsequent somersaulting by *C. tricolor* and the European species *C. parasitica* are responses to factors associated with molluscan shells (Ross and Sutton, 1961; Cutress and Ross, 1969; Ross, 1967).

In the present study, we examine factors influencing attachment of *C. tricolor*. Assays were developed to determine the effect of temporal, textural, and chemical factors on anemone attachment behavior. We show anemone attachment is directly related to time since disturbance. Additionally chemical mixtures that signal food and shells stimulate anemone attachment.

METHODS AND MATERIALS

Two species of anemones, *C. tricolor* and *Aiptasia pallida* Verrill, were used in studies of unassisted attachment. *C. tricolor* were removed from the shells of *Dardanus venosus* collected off-shore near Beaufort, North Carolina. *A. pallida*, nonsymbiotic and locally common anemones on rocks and pilings, were collected from flow-through seawater tables at the laboratory. Anemones

were maintained in monospecies groups in 25-liter aquaria at 23–26°C in the laboratory. Light cycles were uncontrolled, and anemones were fed *Artemia* spp. nauplii daily. In addition to *D. venosus*, the hermit crabs *Clibanarius vittatus* (Bosc) and *Pagurus pollicaris*, clam shells (of *Mercenaria mercenaria*), and the gastropods *Polinices duplicatus* were collected from the immediate vicinity of the Duke University Marine Laboratory and used in experiments. Shrimp (*Penaeus* sp.) used in the study were purchased frozen from local sources.

We disturbed anemones by dislodging them and observed their subsequent behavior. We determined the frequency of anemone reattachment to different objects after dislodgement. During the assays, anemones were kept in 12-liter plastic buckets containing 2 liters of filtered seawater. After each trial, the buckets were rinsed and filled with fresh seawater.

Time Assay. To simulate *D. venosus* disturbance of *C. tricolor*, 10 anemones in each of six buckets were dislodged from the bucket surface. A nonsymbiotic intertidal hermit crab, *Clibanarius vittatus*, was placed in each bucket with the anemones. In the first experiment, all buckets received a crab immediately (0 time); in the next experiment, anemones were disturbed and crabs added after 15 min. The process was continued to include additions at 45, 90, and 180 min and at 48 hr. Because *D. venosus* actively gather anemones, *C. vittatus* was used so that just the effects of crab movement on anemone attachment could be studied. The number of anemones that attached to the crab's shell after 3 min for each of the time regimes was scored as positive transfer behavior. *A. pallida* was used for controls in this attachment assay.

Object Assay. The specificity of anemone transferring behavior was investigated by presenting each of 60 *C. tricolor* 17 objects of various textures and composition (Table 1 below). The objects, one at a time per anemone, were placed in the assay buckets 15 min after the anemones were detached from the bucket surface. The response was scored as a positive if, after 3 min, the anemone remained attached when the object was moved.

Surface Treatments. Different techniques were used to prepare each surface. *Mercenaria mercenaria* (Linnaeus) shells used in texture and chemical adsorption studies were first baked at 500°C for 4 hr to remove native organic material. To obtain surfaces with *D. venosus* odor, bare clam shells were soaked for 45 min in water from a 10-liter aquarium containing five live *D. venosus*. All other objects were soaked in filtered seawater for 45 min. To prepare a coating of *Balanus amphitrite* Darwin odor on glass slides, slides were placed for 15 min in an 8-liter aquarium containing 100 *B. amphitrite*. Bovine serum albumin (BSA, Sigma Chemical, St. Louis, Missouri, #A4378) was applied to baked shells at a concentration of 0.5 mg/ml (10^{-5} M). Treated shells were dried in an oven overnight at 70°C to create a coating.

Responses to Complex Odors. We hypothesized that it would be adaptive if anemones responded to the same complex odor mixtures that signaled shell

searching and switching by their symbiotic hermit crabs. This would ensure the anemone gets on the new shell. Thus, we chose complex odors that are known to signal food (shrimp extract) (Rittschof, personal observation) and available shells (snail extract) (Rittschof, 1980; Rittschof et al., 1992) to many hermit crabs. Because these odors had not been previously tested in either *D. venosus* or *C. tricolor*, we tested the odor mixtures on both species of symbiotic hermit crabs and the anemone. The two complex mixtures tested were extracts from the snail *Polinices duplicatus* and the shrimp *Penaeus* sp. Both extracts were generated by thawing approximately 3 g of frozen flesh in 5 ml seawater for approximately 30 min before use (Rittschof et al., 1992).

Snail and shrimp extracts were applied separately to baked shells and dried overnight at 70°C. *C. tricolor* attachment behavior was recorded as previously outlined in the Object Assay section.

Snail and shrimp extracts were tested separately on *P. pollicaris* and *D. venosus* in modified bucket assays (Rittschof et al., 1992) to determine if these crabs were also responsive. In the modified assays, approximately 30 hermit crabs were allowed to adjust for several days to confinement in a 70-cm × 120-cm fiberglass tank filled to a depth of 10 cm with running ambient seawater. To perform an assay, the water flow was turned off and after 30 min 200 ml of seawater was added directly to the group of quiescent crabs. The number of crabs investigating other shells in a 2-min interval was recorded. Next, 200 ml of odor solution was added, and the number of crabs investigating other shells was recorded.

Previously, Katz and Rittschof (1992) showed that reducing the size of the shell (by clipping) occupied by *Clibanarius vittatus* will increase its rate of shell investigative behavior. Preliminary observations in our study showed that *D. venosus* was very responsive to snail extract, but the response of *P. pollicaris* was less dramatic (although still higher than control conditions). Thus, *P. pollicaris* was tested twice, once in unmodified shells and next in shells that were reduced approximately one fifth in size by clipping with wire cutters. After clipping, these crabs were tested the next day to determine whether their activity level increased.

Statistical analysis tested frequencies using contingency analysis (Sokal and Rohlf, 1981) and the proportions test (Walpole, 1974) when appropriate.

RESULTS

Time Assay. None of the *A. pallida* attached to shells of *C. vittatus*. The frequency with which *C. tricolor* transferred to the new surface was highest immediately after disturbance. At 0 min after removal from the bucket bottom, 48% of *C. tricolor* attached to the shell of *Clibanarius* as the crab moved around

the assay bucket. This transfer behavior onto shells occupied by *C. vittatus* decreased with time after disturbance, with a significant decrease to 10% after 90 min (Figure 1). After 180 min and 48 hr, the transferring was negligible. By then, most anemones were firmly attached to the bucket and remained there.

Object Assays. Responses to surface features were measured by recording the frequency of reattachment to new surfaces. Results are summarized in Table 1. Anemone transferring behavior was tested with *M. mercenaria* shells with a proteinaceous organic matrix and with the matrix removed by combustion of the shell at 500°C for 6–10 hr. Transferring was markedly reduced onto shells with the organic matrix removed ($Z > 2.33$, $P < 0.01$ compared to shells with a matrix). Transferring onto baked shells was significantly ($Z > 1.65$, $P < 0.05$) greater than onto smooth glass and unlike transferring tests with calcium carbonate (limestone), slate, and rough (sintered) glass, in which no anemones transferred. Odor of living, intact *D. venosus* and *Balanus amphitrite*, bovine serum albumin, and calcium carbonate did not stimulate transferring.

Responses to Complex Odors. Extracts of *Polinices duplicatus* (Figure 2)

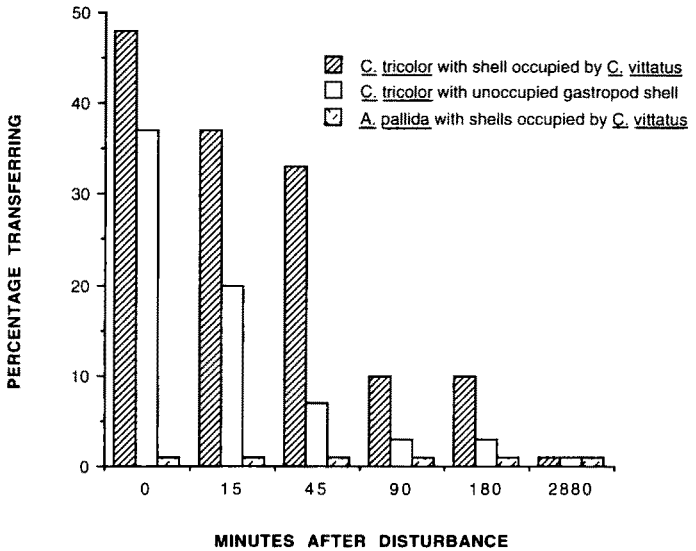

FIG. 1. Attachment of disturbed anemones to gastropod shells. Sixty anemones for each time interval and data test were disturbed. Total anemones tested = 1080. Immediately after disturbance, either empty shells or shells occupied by *C. vittatus*, a hermit crab that does not participate in shell transferring behavior, were added. Anemones that transferred within 3 min were recorded. The procedure was repeated at each time interval noted. With empty shells, one shell was provided to each anemone. With occupied shells, one shell was provided for each 10 anemones.

TABLE 1. RESPONSE OF 60 ISOLATED *Calliactis tricolor* TO SELECTED OBJECTS

Object	Response (%)	Significance[a] vs. slide	Significance[a] vs. shell
Clibanarius vittatus occupied shell	48	<0.01	NS
Glass slide	0		<0.01
Mercenaria shell	42	<0.01	
Baked *Mercenaria* shell	10	<0.05	<0.01
Baked *Mercenaria* shell + *Dardanus* odor	4	NS	<0.01
Baked *Mercenaria* shell + *Polinices* odor	48	<0.01	NS
Baked *Mercenaria* shell + shrimp odor	48	<0.01	NS
Baked *Mercenaria* shell + BSA[b] coat	4	NS	<0.01
Limestone (calcium carbonate)	0	NS	<0.01
Chalk	0	NS	<0.01
Acid-washed slate	0	NS	<0.01
Glass vial	0	NS	<0.01
Glass vial + *D. venosus* odor	0	NS	<0.01
Glass slide + *Balanus* odor	0	NS	<0.01
Glass slide with shrimp odor	0	NS	<0.01
Clean sintered glass filter	0	NS	<0.01
Sintered glass filter + 15 mM CaCl	0	NS	<0.01

[a]Significant tests are vs. glass slide (first column) and vs. *Mercenaria* shell (second column).
[b]BSA = bovine serum albumin.

and *Penaeus* sp. shrimp stimulated transferring in *C. tricolor* (Table 1). Modified bucket assays were used to test shell investigation behavior of *P. pollicaris* and *D. venosus* in response to *Polinices duplicatus* odor. Responses of hermit crabs to seawater addition were compared to responses to *P. duplicatus* odor. *P. pollicaris* were tested in unmodified shells and after shells were reduced approximately one fifth in size by clipping. *D. venosus* were tested in unmodified shells. *P. pollicaris* showed increases in shell investigation behavior and a doubling of responses when shells were reduced in size (Figure 3A). *D. venosus* also dramatically increased shell investigation activity in response to *P. duplicatus* odor (Figure 3B). Although feeding movements and activity increased, crabs did not increase shell investigation activity in response to shrimp odor ($Z < 1.0$, $P > 0.05$).

DISCUSSION

Attachment by Calliactis tricolor. Results from the present study support the conclusions by Cutress and Ross (1969) that chemical cues stimulate *C. tricolor* to transfer to gastropod shells. *Aiptasia pallida*, a nonsymbiotic species,

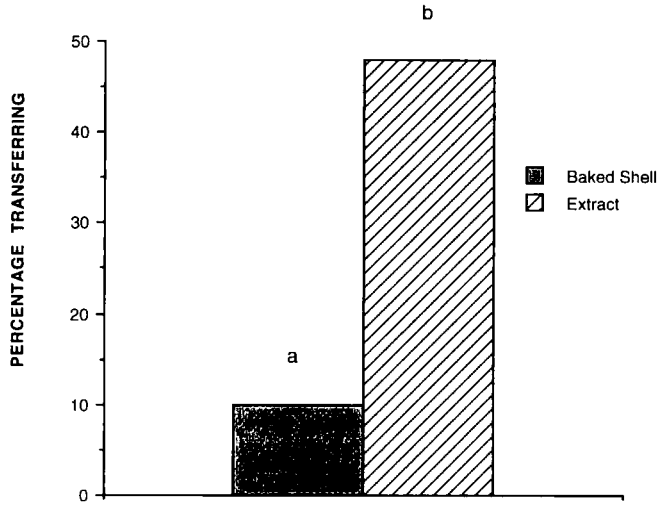

FIG. 2. Attachment of anemones to baked shells and to baked shells soaked in *Polinices duplicatus* odor mixture. *Mercenaria mercenaria* shells with organic matrix removed by baking at 500°C for 6–12 hr were soaked in seawater on *P. duplicatus* extract for 30 min and dried at 70°C overnight. Sixty anemones were tested in each condition. A Z test showed significantly ($P < 0.01$) more anemones attached to shells with the snail extract. Letters over the bars group them into significant subsets.

failed to respond to mollusk shells. This suggests that attachment behavior mediated by textural and chemical cues from mollusk shells is not a general actinian response. Cuing is not to hermit crab odor but to chemical components of shells. The shell components are organic rather than inorganic. We reach this conclusion because baking at 500°C destroys organic cues without substantially altering inorganic composition.

Hermit crabs prod and stroke the columns of *C. tricolor* until the anemone is detached from its surface. This behavior by the crab stimulates anemone transfer and has been simulated to induce anemone detachment (Cutress and Ross, 1969). Reattachment of anemones is highest immediately after disturbance and decreases with time after disturbance until, after 3 and 48 hr, anemones attached to shells at an extremely low frequency. Thus, without disturbance, anemones are unlikely to attach to a new surface. Upon disturbance, the availability of a shell substrate in the vicinity of the anemone is central to transferring behavior. Shells occupied by *C. vittatus* hermit crabs that do not participate in anemone transferring behavior were rapidly colonized by detached anemones. Observation showed the crab occupant ensured, through its movement, that

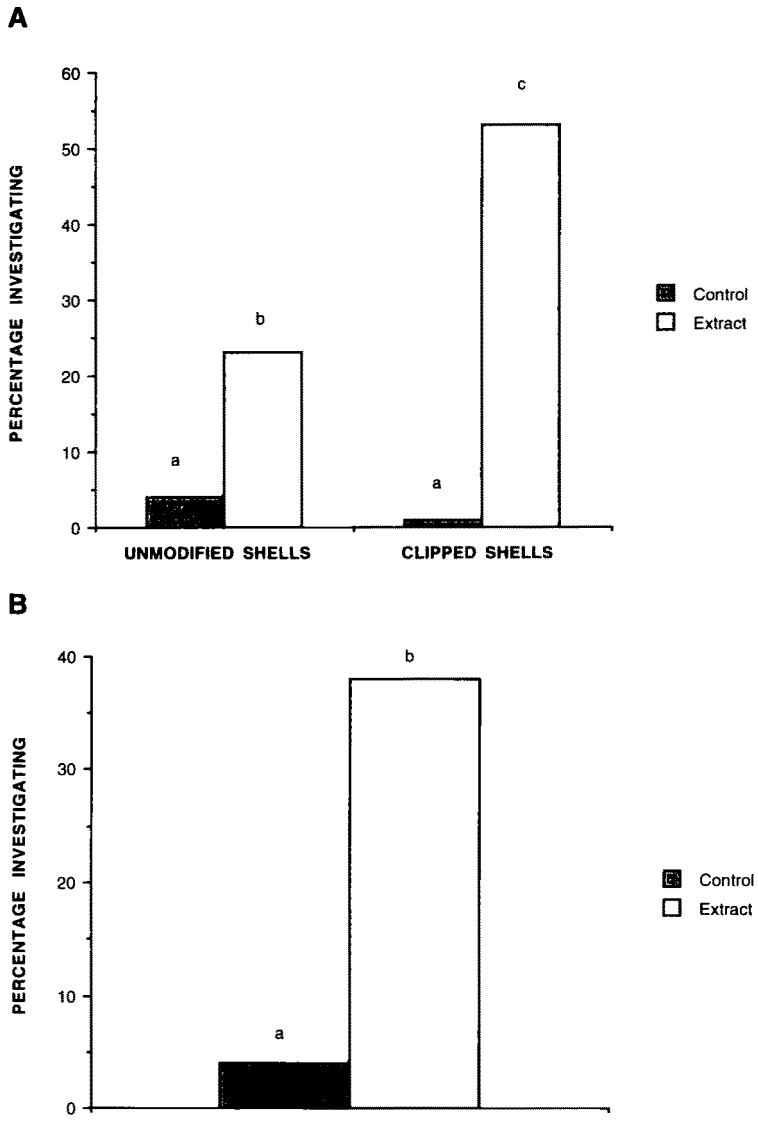

FIG. 3. Chemically stimulated shell investigation by hermit crabs symbiotic with *Calliactis tricolor*. (A) Responses of *P. pollicaris* to seawater control and *Polinices duplicatus* odor. Crabs were first tested in the shells in which they were collected. Next shells were reduced one fifth in size by clipping and the animals retested the next day. Different letters over the bars indicate where Z tests showed groups were different at $P < 0.05$. (B) Responses of *D. venosus* to seawater control and *Polinices duplicatus* odor. Crabs were tested without modifying shells. Letters over the bars group them into significant subsets.

anemones would contact the shell. Symbiotic hermit crabs use disturbance to accumulate anemones.

These experiments provide evidence about the nature of chemicals stimulating anemone attachment. In addition to responding to the organic shell matrix, we found that *C. tricolor* responds to the cue mixture that stimulates shell investigation and switching in hermit crabs. However, shrimp extract, a complex mixture that does not stimulate crabs to switch shells, stimulates anemone attachment. Thus, anemones probably use different cues in the mixture that signal hermit crabs to the availability of a new shell and stimulate shell switching. Natural sources of shell cues (Rittschof et al., 1990) typically are complex mixtures, and each partner could be responding to a different component in the mixture. The organic material in mollusk shells may emit a specific cue that resembles that in extracts (see Kratt and Rittschof, 1991) or the response may be a totally separate chemical cue. Thus, the potential exists for three or more chemical cues to guide anemone attachment.

Both species of symbiotic hermit crabs respond to gastropod extract by shell investigation behavior, as does the nonsymbiotic crab *C. vittatus* (Rittschof et al., 1992). At least for *P. pollicaris*, making shells smaller increases shell investigation activity. Shell investigation activity in response to volatile odors has been reported in terrestrial crabs (Small and Thacker, 1994). Thus, all major groups of hermit crabs display this response.

Although surface texture alone does not seem to be of great importance to anemone transfer, the combination of surface and chemical cues enhances *C. tricolor* attachment. The low or zero percentage of *C. tricolor* transferring to glass surfaces is not surprising if a chemical cue is needed to stimulate anemone attachment. It is likely that the tentacles of *C. tricolor* adhere more readily to rougher surfaces than to the smooth surface of the glass slide (see Mariscal, 1972).

We envision the following scenario in nature: (1) a gastropod is killed and gives off odors; (2) crabs carrying anemones are attracted to the predation site by odors from the flesh of gastropods (Rittschof, 1980) and switch into new shells; (3) anemones on the unoccupied shell are located by a crab; (4) anemones are disturbed by crabs and, with the aid of surface-associated chemical cues and texture attach to occupied shells.

Although symbiotic crabs initiate the transfer of *C. tricolor* by disturbance, it is the anemone's behavior that completes the transfer. Crabs can remove anemones from rocks or shells, but it is the anemone that finalizes the attachment. With the proper organic cue and a rough surface, disturbed anemones will readily attach to any surface.

Transferring Patterns in Related Species. Anemones, including swimming sea anemones of the genera *Actinostola* and *Stomphia*, respond to mollusk shells (Ross, 1965; Ross and Sutton, 1967). There are also two other common species

of *Calliactis* that are associated with hermit crabs in different regions of the world: *C. parasitica* and *C. polypus*.

Apparently, these three *Calliactis* species represent a continuum of behavioral responses towards shells. *C. parasitica* has the strongest responses to mollusk shells (Ross, 1960, 1967; Ross and Sutton, 1961). In fact, in some associations, the hermit crab has very little to do with anemone transfers (Ross, 1960). At the other extreme is *C. polypus*, which relies almost exclusively on the hermit crab's behavior during transfers (Ross, 1970, 1975). *C. tricolor* behavior is somewhere between *C. parasitica* and *C. polypus*, with a distinct response to shells but normally requiring disturbance by the hermit crab for initiation of transfers.

The differences in responses by the three anemones may reflect differences in evolutionary status of their respective associations with hermit crabs. Ross (1983) has argued that the most likely scenario for the development of these associations initially involved anemones living on live snails, which is true of several extant species (Hand, 1975; Ross and Kikuchi, 1976). For these anemones, a strong response to a molluscan shell factor would be a requisite, because the snail would not participate in the transfer. *C. parasitica* shows characteristics of this early stage. Because of its strong shell response, Ross (1960) points out that *C. parasitica* responds readily to shells of live snails in the laboratory, but it is rarely found on snails in the field. The scarcity of *C. parasitica* and other symbiotic anemones living on live snails could be explained by the development and subsequent prevalence of anemone-gathering hermit crabs, which could easily take anemones from these snails. Indeed, *D. venosus* individuals can be characterized as anemone hogs. They will gather up anemones in rapid succession until no room remains on the shell (Brooks, 1991a).

The scenario above suggests that participants in *C. tricolor*–hermit crab associations might be likely to coevolve similar responses to cues that signal shell switching because both organisms are active in the transfer to new shells. This could explain why both *D. venosus* and *C. tricolor* respond to *Polinices* odor. By the same reasoning, crab and anemone symbionts with unequal roles in anemone transfers might be less likely to respond to the same chemical cues.

Finally, associations involving *C. polypus* may represent the most evolutionarily advanced associations, with a further relative loss of response to shells by the anemone and a greater dependence on the crabs for transfer.

Concomitant with these different strategies, one would expect physiological differences between anemone species (and crabs) to account for their behavioral differences. Comparative studies of symbiotic anemones and crabs, as well as further efforts to identify the specific chemical nature of shell factors, would provide clues regarding the validity of Ross' hypothesis on the evolution of hermit crab–anemone symbioses.

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NOVEL TYPE OF SEX PHEROMONE STRUCTURE IDENTIFIED FROM *Stigmella malella* (STAINTON) (LEPIDOPTERA: NEPTICULIDAE)

MIKLÓS TÓTH,[1,*] GÁBOR SZÖCS,[1] ERIK J. VAN NIEUKERKEN,[2] PETER PHILIPP,[3] FRANK SCHMIDT,[3] and WITTKO FRANCKE[3]

[1]*Plant Protection Institute*
Hungarian Academy of Sciences
Budapest, Pf 102, H-1525, Hungary

[2]*National Museum of Natural History*
P.O.B. 9517, 2300 RA Leiden, The Netherlands

[3]*Institut für Organische Chemie der Universität Hamburg*
Martin-Luther-King-Platz 6, D-20146 Hamburg 13, Germany

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Abstract—Short-chain unsaturated chiral methyl carbinols are identified as a new class of lepidopteran pheromone components. The natural female-produced pheromone of the banded apple pigmy *Stigmella malella* (= *Nepticula malella*) (Stainton) (Lepidoptera: Nepticulidae) was identified to be a mixture of (*S*)-(*E*)-6,8-nonadien-2-ol and (*S*)-(*Z*)-6,8-nonadien-2-ol. For monitoring traps, a 10:3 *E*:*Z* blend at 100–1000 μg is recommended. It is suggested that pheromones with similar structures may be specific to Nepticulidae and other related microlepidopteran families.

Key Words—*Stigmella malella*, *Stigmella crataegella*, *Trifurcula (Glaucolepis) melanoptera*, Lepidoptera, Nepticulidae, sex pheromone, (*S*)-(*E*)-6,8-nonadien-2-ol, (*S*)-(*Z*)-6,8-nonadien-2-ol, (*R*)-(*E*)-6,8-nonadien-2-ol, (*R*)-(*Z*)-6,8-nonadien-2-ol.

INTRODUCTION

The vast majority of lepidopterous pheromone components were identified from species of only a handful of families such as Tortricidae, Noctuidae, Pyralidae, Gelechiidae, etc., where mono- or diunsaturated aliphatic primary alcohols,

*To whom correspondence should be addressed.

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acetates, or aldehydes are most abundant (Arn et al., 1992). Another group of pheromone components is represented by polyunsaturated hydrocarbons and oxygenated derivatives found predominantly in Geometridae, Arctiidae, and some noctuid subfamilies (Arn et al., 1992). The study of species belonging to taxonomic groups clearly distinct from and more primitive than the above families can still offer many surprises with respect to pheromone structures, as evidenced by scattered examples from the microlepidopteran families of Lyonetiidae (Sato et al., 1985, Francke et al., 1988), and more recently, of Bucculatricidae (Hall et al., 1992).

In the course of our studies on leafminers, our interest was strongly evoked by the banded apple pigmy *Stigmella malella* (= *Nepticula malella*) (Stainton) (Lepidoptera: Nepticulidae), which caused severe defoliation in apple orchards in the eastern part of Hungary in the late 1980s (Balázs, 1993). The family Nepticulidae includes some of the smallest moth species known, with a wingspan of only about 2–12 mm. The large suborder Ditrysia comprises more than 95% of the lepidopteran species, while the Nepticulidae belong to the lower Heteroneura and form the suborder Monotrysia, together with the superfamily Incurvaroidea and some smaller groups. In Europe only the families Micropterygidae, Eriocraniidae, and Hepialidae are placed lower in the phylogenetic tree of Lepidoptera (van Niekukerken, 1986). Apart from the life history of the larvae, most feeding as leafminers, some as miners in stems or bark, and a few gallmakers, an interesting aspect of this family is the presence of a unique antennal sensillum type, the sensillum vesiculocladum, unknown outside this family (van Nieukerken and Dop, 1987). This sensillum almost certainly plays an important role in pheromone detection. Knowledge of pheromones of the more primitive lepidopteran families is important for better understanding the evolution of pheromones within Lepidoptera and may add important phylogenetic information.

S. malella is known to be present all over Europe (Johansson and Nielsen, 1990) and is counted among the most important leafminer pests in France, Italy, Switzerland, Great Britain, and the Ukraine (Réal, 1966, Balázs, 1993). In Hungary, it has several flight periods (generations) per year (Molnárné, 1981), and the detection of the rise of the moth population, which could easily be accomplished by the use of pheromone-baited traps, could be very advantageous in timing control measures. In the present paper we describe the identification of a novel type of Lepidopteran sex pheromone structure from *S. malella*.

METHODS AND MATERIALS

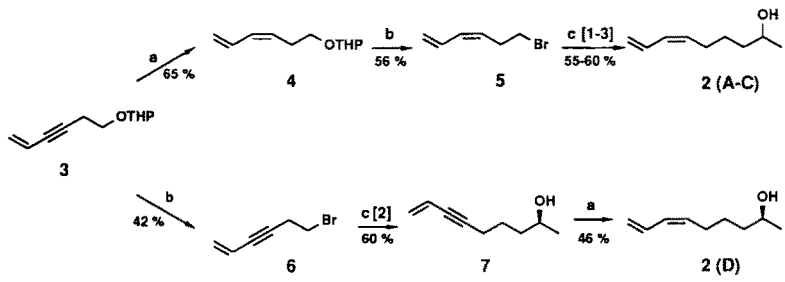
Insects. Insects were collected as pupae from the soil of heavily infested apple orchards near Sárospatak (Borsod-Abaúj county, Hungary). Pupae were held in outdoor conditions, and the emerging adults were collected each day.

Females and males were held in separate glass containers (2 liters) and were supplied with water on cotton wool.

For extraction of the female-produced pheromone several methods were tried. Intact bodies of 2- to 3-day-old female moths collected between 8:00 and 9:00 AM were extracted in a minimal amount of pentane (Merck pro Analysi) for 15 min. Headspace collections were carried out according to described methods (Francke et al., 1987). In the present experiments, which were run for several days, the collections were started on 1-day-old females.

Chemical Analysis. Preliminary gas chromatographic analyses of crude natural extracts were performed on 30-m × 0.25-mm-ID FFAP- or DB5-fused silica columns under various conditions. Structure elucidation of target compounds was carried out by combined gas chromatography–mass spectroscopy (GC-MS) under the same conditions as described earlier (Hansson et al., 1990). Mass spectra and gas chromatographic retention times (coinjection) of synthetic samples served as references. Microreactions such as hydrogenation and ozonolysis were conducted according to known methods (Huwyler, 1973; Beroza, 1975). Enantiomeric separation of chiral compounds was achieved by gas chromatography using a 1:2 mixture of octakis-(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin and OV-1701 as the stationary phase and hydrogen (4 ml/min) as the carrier gas. Separation conditions were as follows: 30-m × 0.2-mm-ID fused silica column, kept for 24 min at 90°C then programmed to 110°C at a rate of 2°C/min. Separation of geometrical isomers was carried out on a 20-m × 0.2-mm-ID fused silica column coated with a 1:1 mixture of hexakis-(2-methyl-3,6-di-*O*-pentyl)-α-cyclodextrin and OV-1701; hydrogen (4 ml/min) served as the carrier gas. The column was operated 9 min at 60°C then programmed to 120°C at a rate of 2°C/min. Synthesis of the cyclodextrins as well as preparation of our tailor-made columns have been described in detail (König et al., 1992, Pietruszka et al., 1992). Separation conditions were carefully optimized. Assignment of absolute configurations of naturally occurring compounds and determination of proportions of geometrical isomers was performed using synthetic optically active, geometrically defined reference compounds.

Synthesis. Syntheses of samples used for structure confirmation and bioassays are outlined in Figures 1 and 2. Purification of synthetic products was carried out by column chromatography using 10% $AgNO_3$ on Silicagel 60 (Merck 240-400 mesh) and a 5:1 mixture of ether–pentane as the mobile phase. Purities of samples used for bioassays are shown in Table 1. NMR spectra (400.1 MHz for ^1H and 100.6 for ^{13}C) of synthetic samples were run on a Bruker AMX 400; $\delta TMS = 0$; $\delta CDCl_3 = 7.26$ (bs = broad singlet, d = doublet, dd = doublet of a doublet, dddd = doublet of a doublet of a doublet of a doublet, bdd = broad doublet of a doublet, bt = broad triplet, dt = doublet of a triplet, ddt = doublet of a doublet of a triplet, bq = broad quartet, dq doublet of a quartet, hex = hexet, m = multiplet. Attribution of ^{13}C signals was made according to



a: Zn (Cu) / EtOH; b: Ph_3PBr_2 / CH_2Cl_2; c: Mg, rac-methyloxirane [c1] or (2*S*)-methyloxirane [c2] or (2*R*)-methyloxirane [c3]

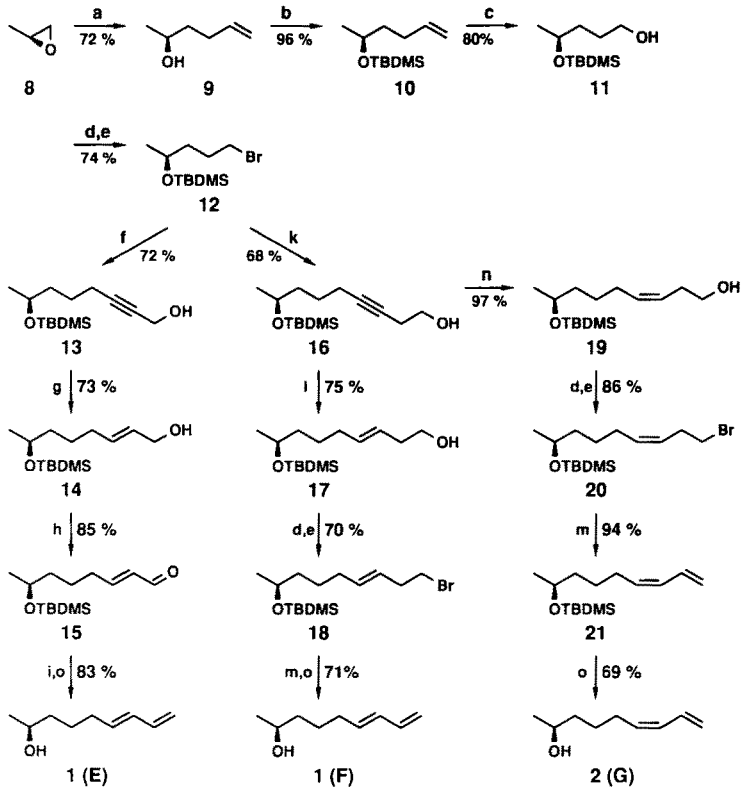
FIG. 1. Syntheses of samples A–D. Nonoptimized yields are shown; for enantiomeric, geometric, and chemical purity of samples, see Table 1.

DEPT spectroscopy (CH_3 and CH = +, CH_2 = −). Assignment of protons and carbon atoms are based on COSY experiments.

The syntheses of the first samples of (*Z*)-6,8-nonadien-2-ol are outlined in Figure 1. The key compound (**3**) was obtained by coupling protected 3-butyn-1-ol to vinyl bromide according to Andreini et al. (1987). Reduction of the triple bond by a Zn/Cu pair (Aerssens and Brandsma, 1984) and transformation of the protected (*Z*)-3,5-hexadien-2-ol (**4**) into the corresponding bromide (**5**) (Wijnberg et al., 1981), followed by Grignard reaction (Baker et al., 1991) with epoxypropane (Merck) completed the synthesis of the racemic sample A. Following the same route, but using optically active epoxypropane (Merck) (Johnston and Slessor, 1979), we synthetized sample B showing the *S* configuration and sample C showing the *R* configuration (for enantiomerical, geometrical, and chemical purity of samples see Table 1).

Starting from (**3**) in an analogous synthesis for the *S* enantiomer, we carried out the reduction of the en–yne system at a later stage. The alcohol (**3**) was transformed into the corresponding bromide (**6**), which was chain elongated with (2*S*)-epoxypropane to yield (**7**). Reduction with activated zinc (Aerssens and Brandsma, 1984) completed the synthesis of sample D.

Our first synthesis of (*S*)-(*E*)-6,8-nonadien-2-ol is shown in Figure 2. Reaction of (2*S*)-epoxypropane (**8**), prepared according to Ghirardelli (1973), with allyl magnesium chloride followed by protection of the alcohol moiety of the obtained (*S*)-5-hexen-2-ol, furnished (**10**). Chain shortening of (**10**) by ozonolysis and reductive work up yielded the monoprotected diol (**11**), which was converted into the corresponding bromide (**12**). This compound was coupled to propargyl alcohol (Brandsma, 1988), followed by Birch reduction (Campbell and Eby, 1941) to form the *trans*-configured allylic alcohol (**14**). Oxidation



a: $ClMgCH_2$-CH=CH_2 / THF, -30°C; b: TBDMSCl / DMF / imidazole, rt; c: O_3, $LiAlH_4$ / Et_2O
d: TsCl / pyridine, 5°C; e: LiBr / acetone, reflux; f: $LiNH_2$, HC≡CH-CH_2OH / NH_3, -78°C
g: $LiAlH_4$ / Et_2O, reflux; h: MnO_2 / MeOH; i: Ph_3P=CH_2 / THF-DMSO
k: $LiNH_2$ / HC≡CH-CH_2-CH_2OH, NH_3, -78°C; l: Na / NH_3-THF, -78°C
m: 18-crown-6, KO^{t}Bu, hexane, -20°C-0°C; n: Lindlar-cat / hexane, quinoline, H_2
o: HF / acetonitrile

FIG. 2. Syntheses of samples E–G. Nonoptimized yields are given; for enantiomeric, geometric, and chemical purity of samples see Table 1.

(Gritter and Wallace, 1959) and Wittig reaction with triphenyl methylphosphonium bromide followed by deprotection furnished the target compound (sample E).

In a highly efficient route to pure (*S*)-(**1**) and (*S*)-(**2**) the key bromide (**12**) was coupled to 3-butyn-1-ol (Figure 2). The resulting **16** could be hydrogenated

TABLE 1. ENANTIOMERIC, GEOMETRIC, AND CHEMICAL PURITY OF SYNTHETIC SAMPLES OF 6,8-NONADIEN-2-OL USED IN BIOASSAYS

Sample	Enantiomeric purity	*E*/*Z* ratio	Chemical purity (%)
A	racemic	ca. 1:1	ca. 90
B	*S* 95% ee	ca. 1:1	ca. 90
C	*R* 95% ee	ca. 1:1	ca. 90
D	*S* 95% ee	ca. 6:94	>96
E	*S* 98% ee	ca. 93:7	>96
F	*S* 98% ee	>99:1	>99
G	*S* 98% ee	<1:99	>99

[a]For synthesis and separation conditions see text.

to the 8-TBDMS-derivative of either (*S*)-(*E*)-3-nonen-1,8-diol (17) or to that of (*S*)-(*Z*)-3-nonen-1,8-diol (**19**). Transformation of these alcohols to the corresponding bromides, (**18**) and (**20**), followed by elimination (Dehmlow and Lissel, 1979) established the terminal conjugated double bond systems yielding pure (*S*)-(**1**) (sample F) and pure (*S*)-(**2**) (sample G). Sample F showed a rotation value of $\alpha_D^{22} = +10.4$ (C = 1.8 CH_2Cl_2), while sample G showed $\alpha_D^{22} = +9.2$ (C = 2.2 CH_2Cl_2).

The set of NMR data for compound 1, (2*S*)-(*E*)-6,8-nonadien-2-ol was registered as follows: ^{1}H NMR: 6.31 (dt, 1H, $J_{8,9} = 17.2$ Hz, $J_{8,7} = J_{8,9} = 10.4$ Hz, H-8); 6.06 (bdd, 1H, $J_{7,6} = 15.0$ Hz, H-7); 5.70 (dt, 1H, $J_{6,5} = 7.2$ Hz, H-6); 5.09 (dd, 1H, $J_{9,9'} = 1.6$ Hz, H-9); 4.97 (dd, 1H, H-9′); 3.80 (hex, 1H, $J_{2,1} = J_{2,3} = 6.1$ Hz, H-2); 2.11 (bq, 2H, $J_{5,6} = J_{5,4} = 7.2$ Hz, H-5); 1.39–1.56 (m, 4H, H-3, H-4); 1.30 (bs, 1H, OH); 1.19 (d, 3H, H-1). ^{13}C-NMR: 137.23 (+, C8); 124.94 (+, C6); 131.28 (+, C7); 114.90 (−, C9); 68.03 (+, C2); 38.80 (−, C3); 32.45 (−, C5); 25.33 (−, C4); 23.55 (+, C1).

The set of NMR data for compound **2**, (2*S*)-(*Z*)-6,8-nonadien-2-ol was registered as follows: ^{1}H NMR: 6.62 (dddd, 1H, $J_{8,9} = 16.8$ Hz, $J_{8,7} = 11.0$ Hz, $J_{8,9'} = 10.4$ Hz, $J_{8,6} = 1.2$ Hz, H-8); 6.00 (bt, 1H, $J_{7,6} = J_{7,8} = 11.0$ Hz, H-7); 5.44 (ddt, 1H, $J_{6,5} = 7.6$ Hz, H-6); 5.18 (dd, 1H, $J_{9,9'} = 2.0$ Hz, H-9); 5.08 (bdd, 1H, H-9′); 3.79 (hex, 1H, $J_{2,1} = J_{2,3} = 6.1$ Hz, H-2); 2.21 (dq, 2H, $J_{5,6} = J_{5,4} = 7.6$ Hz, $J_{5,7} = 1.4$ Hz, H-5); 1.36–1.54 (m, 5H, H-3, H-4, OH); 1.18 (d, 3H, H-1). ^{13}C NMR − 132.40 (+, C6); 132.19 (+, C8); 129.50 (+, C7); 116.97 (−, C9); 67.94 (+. C2); 38.76 (−, C3); 27.59 (−, C5); 25.70 (−, C4); 23.50 (+, C1).

Trapping Tests. Dispensers for the trapping tests were prepared by using 1 × 0.5-cm pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ

9691/6; extracted three times in boiling ethanol for 10 min, then three times in methylene chloride overnight, prior to usage). For making up the baits, the required amounts of compounds were administered to the surface of the dispensers in hexane (Merck, pro Analysi) solutions. Prepared dispensers were stored at −65°C until use.

For female-baited traps, 1-day-old unmated female *S. malella* were placed in 2 × 2 × 4-cm plastic screen cages, and the cages were suspended into traps the same way as the dispensers with synthetic compounds. The females in the cage were supplied with water in a 2-ml glass vial plugged by cottonwool.

Field tests were conducted at several apple orchards in Hungary. Traps similar in shape and size to those described by Arn et al. (1979), but made from polyethylene sheets, were used. Traps were suspended from the branches of orchard trees at a height of 1.5 m. Traps containing different baits were set up in rectangular blocks. The distance between traps within a block was 4–5 m. The distance between blocks ranged between 100 and 1000 m. Traps were moved one position forward within a block at each occasion when the traps were checked. At the same time, captured males were recorded and sticky inserts changed to new ones. Capture data were transformed to log $(x + 1)$, and differences between means were tested for significance by ANOVA followed by Duncan's new multiple range test (DNMRT).

Identification of males of species captured was confirmed by checking the genitalia of some trapped specimens belonging to groups, provisionally sorted on the basis of external characters. Samples of the species and genitalia slides are deposited in the collection of the National Museum of Natural History (Leiden, The Netherlands).

RESULTS AND DISCUSSION

Adult *S. malella* mated readily from the second day on after emergence, in the morning hours (7:00–11:00 AM). Surprisingly, there was no indication of calling behavior on the part of the females prior to mating (Tóth, unpublished).

Gas chromatographic investigations of extracts separately obtained from 2-day-old females or males as well as from headspace collections showed the constant presence of a female-specific compound (Figure 3), which was estimated to range in the upper nanogram level per individual. The mass spectrum of the substance (Figure 4A) showed a molecular mass at *m/z* 140, intense signals at *m/z* 79 and *m/z* 80, and a characteristic fragment at *m/z* 45. Microhydrogenation yielded a mixture of nonan-2-ol and some nonan-2-one, the mass spectra of which were known (McLafferty and Stauffer, 1989). Ozonolysis yielded 2-methyl-3,4-dihydro-2H-pyrane (Figure 4B), the mass spectrum of

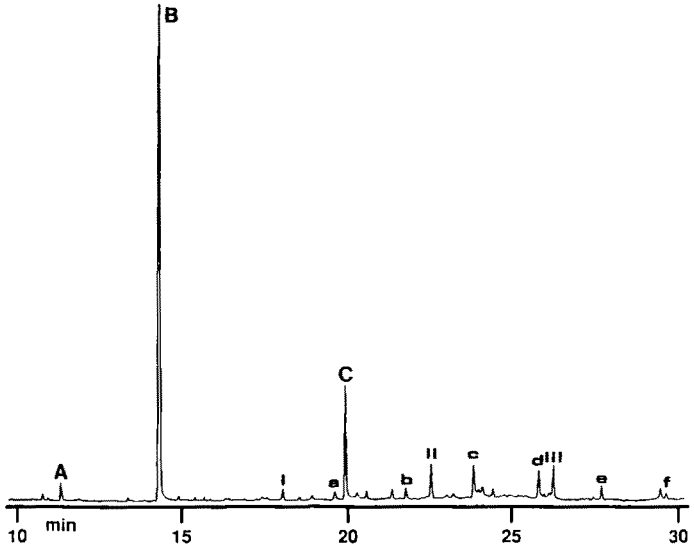


FIG. 3. Gas chromatogram of a pentane extract from 2-day-old female *S. malella*. A = (Z)-6-nonen-2-ol: female specific, not consistently present; B = 6,8-nonadien-2-ol: female specific, consistently present; C = unknown; female specific, not consistently present; I, II, III = alkenes C_{19}, C_{21}, C_{23}: not sex specific; a–f = straight chain carboxylic acids: C_7–C_{12}, not sex specific.

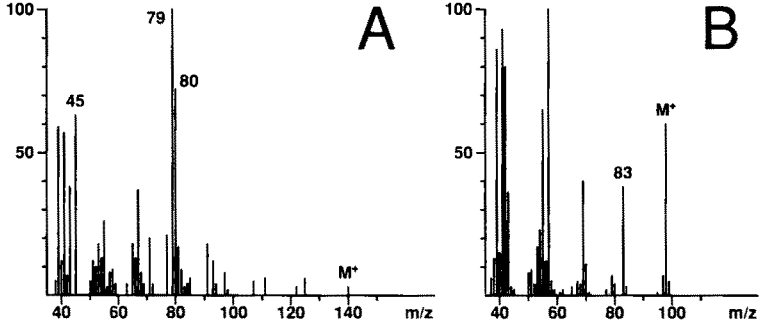


FIG. 4. Mass spectra of main pheromone component in *S. malella* female extracts (A) and of 2-methyl-3,4-dihydro-2H-pyrane (B).

which was known to us from earlier work (Hübener, 1988). The results of our microreactions indicated the structure of an unbranched doubly unsaturated methyl-carbinol, showing at least one double bond at position 6. Among the possible structures of 6,8-nonadien-2-ol, the allenic 6,7-nonadien-2-ol, and 6-nonyn-2-ol, we regarded the conjugated dienol as the most likely one, and we therefore decided to synthesize the corresponding *E* and *Z* isomers (see Figures 1 and 2).

Mass spectra and gas chromatographic retention times of synthetic samples obtained according to Figure 1 and run on FFAP and DB5 columns were identical with the corresponding data of the natural product. Although it became obvious that on the columns used the *E/Z* isomers (and of course the enantiomers) could not be distinguished, we carried out preliminary field trapping tests with samples A, B, and C (see Tables 2 and 3).

On the test site with very high population density, 10 times higher numbers of male *S. malella* were attracted into traps baited with sample B (*S* enantiomers) at both doses tested, as compared to sample C (*R* enantiomers) (Table 2; Sárospatak). The capture by sample B did not differ significantly from the capture in traps baited with three unmated female *S. malella* (Table 2).

At the site with low population level, the only traps that caught males were baited with sample B (*S* enantiomers) (Table 2; Julianna major). There was no significant difference between the doses of 1000 or 100 μg on any of the sites. These results suggested the *S* enantiomer to be the active compound. However,

TABLE 2. CAPTURES OF MALE *S. malella* IN TRAPS BAITED WITH THE (*S*)-ENANTIOMERS OF A 1 : 1 (*E*):(*Z*)-MIXTURE OF 6,8-NONADIEN-2-OL (SAMPLE B) OR THE CORRESPONDING MIXTURE OF (*R*)-ENANTIOMERS (SAMPLE C)[a]

Bait (μg), enantiomers of 1 : 1 *E/Z* 6,8-nonadien-2-ol		Males captured (mean *N*/trap/inspection)	
S (sample B)	*R* (sample C)	Sárospatak	Julianna major
1000		159.8 a	9.6 a
100		222.0 a	4.7 a
	1000	19.2 b	0.0
	100	9.9 b	0.0
3 unmated female *S. malella*		267.8 a	not tested

[a]Sárospatak, Abaúj-Zemplén county, May 4–5, 1991, apple orchard, inspected daily, seven traps of each treatment were used; Julianna major, Budapest, May 2–31, 1991, apple orchard, inspected each second day, three traps of each treatment were used. Means followed by same letter within one column are not significantly different, $P > 0.05$.

TABLE 3. CAPTURE OF RACEMIC 1 : 1 *E/Z* MIXTURE OF 6,8-NONADIEN-2-OL (SAMPLE A) AND INFLUENCE OF ADDITION OF *R* ENANTIOMERS OF 1 : 1 *E/Z* MIXTURE OF 6,8-NONADIEN-2-OL (SAMPLE C) ON CAPTURES OF MALE *S. malella* IN TRAPS BAITED WITH 1 : 1 *E/Z* MIXTURE OF (*S*)-6,8-NONADIEN-2-OL AND SAMPLE B[a]

Bait (μg), enantiomers of 1 : 1 *E/Z*-6,8-nonadien-2-ol			Males captured, *N* (mean/trap/ inspection)
Racemic (sample A)	*S* (sample B)	*R* (sample C)	
	100		15.33 a
		100	0.03 b
	100	100	0.70 c
100			0.47 bc

[a]Julianna major, Budapest, July 1–8, 1991, inspected every second day; 10 traps of each treatment were used. Means followed by same letter within one column are not significantly different, $P > 0.05$.

at this point of our investigations it was not clear whether the natural product was a pure enantiomer or a mixture. The addition of *R* enantiomers of 6,8-nonadien-2-ol (sample C) to the *S* enantiomers (sample B) dramatically reduced catches (Table 3). This corresponded well with the inactivity of the racemic sample A (Table 3) and also suggested the natural product to have the *S* configuration.

Gas chromatographic separation of the enantiomers of nonan-2-ol could be achieved on the γ-cyclodextrin phase under the conditions mentioned above. The obtained retention value of $\alpha = 1.04$ [$R_t(R) \div R_t(S)$] represented a difference of retention times of about 1 min during a running time of 25 min. A hydrogenated sample of the natural product coeluted with (*S*)-nonan-2-ol and showed no traces of the *R* enantiomer. Under these conditions, the four stereoisomers of 6,8-nonadien-2-ol could only be partially resolved, producing three peaks. The *E/Z* isomers showing *S* configuration furnished one peak at $R_t = 33.4$ min only, while the *R* enantiomers were well resolved with (*R*)-(*E*)-6,8-nonadien-2-ol eluting at $R_t = 33.8$ min and the *Z* isomer appearing at $R_t = 34.1$ min. Again, the natural product coeluted with the *S* enantiomers. Our synthetic samples B–D showed enantiomeric purities of 95% ee (see Table 1), while samples E and F were of higher purity. Consequently, as a result of our analyses and field tests, the structure of the target compound was established to be (*S*)-6,8-nonadien-2-ol.

With respect to the configuration of the double bond in position 6, we were puzzled by the fact that in further trapping tests (Table 4) sample B [1 : 1 *E/Z* mixture of enantiomerically pure (*S*)-6,8-nonadien-2-ol; Table 1] caught better

TABLE 4. CAPTURES OF MALE *S. malella* IN TRAPS BAITED WITH DIFFERENT *E/Z* MIXTURES OF (*S*)-6,8-NONADIEN-2-OL (SAMPLES B, D, AND *E*)[a]

Bait (μg), (*S*)-6,8-nonadien-2-ol			Males captured, *N* (mean/trap/inspection)	
1:1 *E/Z* (sample B)	6:94 *E/Z* (sample D)	93:7 *E/Z* (sample E)	Test 1	Test 2
	100		2.6 b	not tested
		100	not tested	5.9 b
100			15.5 a	13.6 a

[a]Test 1: August 12–21, 1991; test 2: August 8–19, 1992; inspected twice weekly; five traps of each treatment were used. Means followed by same letter within one column are not significantly different, $P > 0.05$.

than sample D [(*S*)-(*Z*)-6,8-nonadien-2-ol containing 6% of the *E* isomer; Table 1] or sample E [(*S*)-(*E*)-6,8-nonadien-2-ol containing 7% of the *Z* isomer; Table 1]. We, therefore, had to establish unequivocally the geometry of the crucial double bond in the natural product and the isomeric purities of our samples. For the separation of the geometrical isomers, we successfully used a mixture of OV-1701 and a derivative of α-cyclodextrin, which, however, left the corresponding enantiomers unresolved. Under the conditions mentioned above, the obtained retention values were $\alpha = 1.02$ [$R_t(E) \div R_t(Z)$]. A careful reinvestigation with respect to the *E/Z* ratio revealed insufficient purities in some of our samples (Table 1). We found that during the syntheses of samples A–C (Figure 1) isomerization of the double bond system had occurred and that even after extensive chromatographic purification only ca. 1:1 mixture of the geometrical isomers of 6,8-nonadien-2-ol had been obtained. Samples D and E contained 6–7% of the corresponding *E* or *Z* isomer, respectively. Samples F and G proved to be highly pure, containing only traces of the geometrical isomer. Analyses of natural pheromone samples showed *E/Z* ratios of 10:1 to 10:3.

On the basis of these results, we established the natural pheromone to be a mixture of pure *S* enantiomers of 6,8-nonadien-2-ol in an *E/Z* ratio of 10:1 to 10:3 and continued our bioassays using the samples F and G. When these were tested in different ratios, maximal catches were recorded in traps baited with the 10:3 *E/Z* ratio, followed by the 10:1 and 1:1 blends (Figure 5). (*S*)-(*E*)-6,8-Nonadien-2-ol on its own captured some males, while (*S*)-(*Z*)-6,8-nonadien-2-ol yielded no catches.

In a test with different doses of the optimal 10:3 blend of (*S*)-(*E*)-6,8-nonadien-2-ol and (*S*)-(*Z*)-6,8-nonadien-2-ol, catches increased in parallel with doses, the higher doses catching significantly higher numbers as compared to

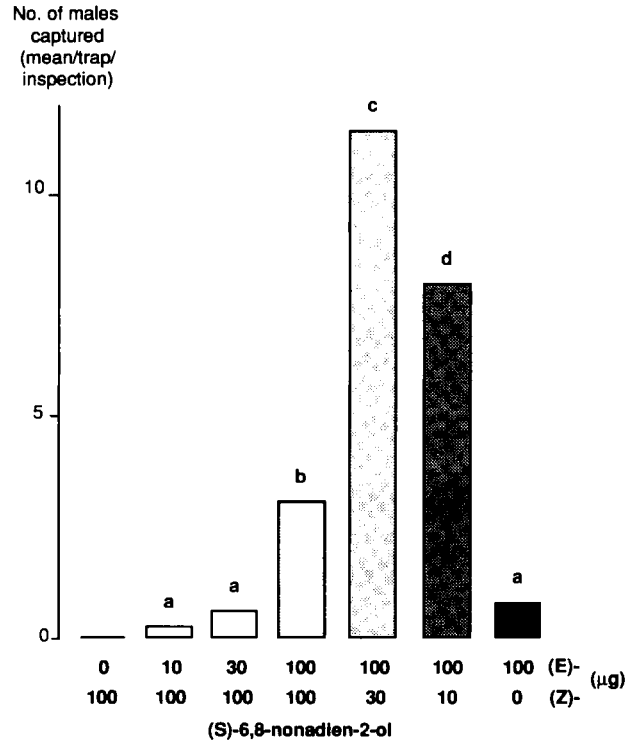


FIG. 5. Captures of male *S. malella* in traps baited with (*S*)-(*Z*)-6,8-nonadien-2-ol and (*S*)-(*E*)-6,8-nonadien-2-ol and their mixtures in different ratios. Julianna major, Budapest, June 10–16, 1993, inspected every second day; 10 traps of each bait variation were used. Columns with same letter are not significantly different by DNMRT, $P > 0.05$.

lower ones (Table 5). Best activity was recorded at the 1000-μg level, the highest dose tested.

Results of the field tests corroborate results of the analyses of extracts and demonstrate that the main pheromone components of *S. malella* are (*S*)-(*E*)-6,8-nonadien-2-ol and (*S*)-(*Z*)-6,8-nonadien-2-ol. For monitoring traps, a 10:3 *E*/*Z* blend at 100–1000 μg can be recommended. Synthetic samples used for the preparation of monitoring baits should be cross-checked for the presence of the *R* enantiomer, as it may interfere with the activity of the sample.

In season-long monitoring trials, the only species other than *S. malella* captured by a 1:1 mixture of *E*/*Z* isomers showing *S* configuration (sample B), was *Stigmella crataegella* (Klimesch) (Lepidoptera: Nepticulidae), on sites with *Crataegus* bushes (Table 6). It is interesting to note that in these tests large

TABLE 5. CAPTURES OF MALE *S. malella* IN TRAPS BAITED WITH DIFFERENT DOSES OF 10:3 MIXTURE OF (*S*)-6(*E*),8-NONADIEN-2-OL AND (*S*)-6(*Z*),8-NONADIEN-2-OL (SAMPLES F AND G)[a]

Bait (μg) (*S*)-6,8-nonadien-2-ol		
>99% *E* (sample F)	>99% *Z* (sample G)	Males captured, *N* (mean/trap/inspection)
1000	300	36.60 a
100	30	18.95 b
10	3	9.30 c
1	0.3	4.05 d

[a]Julianna major, Budapest, June 16–29, 1993, inspected twice weekly; four traps of each treatment were used. Means followed by same letter within one column are not significantly different, $P > 0.05$.

TABLE 6. SPECIES CAPTURED IN SEASON-LONG MONITORING TRIALS IN TRAPS BAITED WITH 100-μg DOSE OF ENANTIOMERS (1:1 *E*/*Z*) OF 6,8-NONADIEN-2-OL (SAMPLES B AND C)[a]

Bait (μg), 6,8-nonadien-2-ol (1:1 *E*/*Z*)	Males captured (*N*)					
	Stigmella malella		*Stigmella crataegella*		*Trifurcula melanoptera*	
	1992	1993	1992	1993	1992	1993
S (sample B)	881	1144	55	232		
R (sample C)		1			216	296

[a]Julianna major, Budapest, April 5–November 7, 1992; April 6–August 30, 1993; inspected twice weekly; baits were renewed monthly; two traps of each treatment were used.

numbers of a recently described species, *Trifurcula (Glaucolepis) melanoptera* (van Nieukerken and Puplesis, 1991) of the same family were attracted to the mixture of the *R* enantiomers (sample C) (Table 6).

The identification of a secondary alcohol is novel in moth pheromone chemistry. Pheromone components with similar structures may be specific to Nepticulidae and other related microlepidopteran families. In screening tests with compounds similar in structure to the pheromone components of *S. malella*, several Nepticulidae spp. and some Eriocraniidae spp. were captured (Tóth et al., to be published in detail elsewhere).

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IDENTIFICATION OF A NOVEL MOTH SEX PHEROMONE IN *Eriocrania cicatricella* (Zett.) (LEPIDOPTERA: ERIOCRANIIDAE) AND ITS PHYLOGENETIC IMPLICATIONS

JUNWEI ZHU,[1,*] MIKHAIL V. KOZLOV,[2,4] PETER PHILIPP,[3] WITTKO FRANCKE,[3] and CHRISTER LÖFSTEDT[1]

[1]*Department of Ecology*
Lund University
S-223 62 Lund, Sweden

[2]*Laboratory of Ecological Zoology, Biological Faculty*
University of Turku
Turku FIN-20500, Finland

[3]*Institute of Organic Chemistry*
University of Hamburg
Martin-Luther-King-Platz 6, D-20146, Hamburg, Germany

[4]*Institute for Plant Protection*
Pushkin 8, St. Petersburg 189620, Russia

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Abstract—Extracts from different body parts of adult female *Eriocrania cicatricella* (Zett.) were tested for electrophysiological activity on conspecific male antennae. Extracts from the Vth abdominal segment, containing a pair of exocrine glands, elicited the largest electroantennographic response when compared to extracts of other body parts. Female extracts were analyzed by gas chromatography with simultaneous flame ionization and electroantennographic detection (EAD). The EAD active peaks were identified as (*Z*)-4-hepten-2-one, (2*R*)-heptane-2-ol, and (2*R*)-(*Z*)-4-hepten-2-ol by coinjection on a gas chromatography and by comparison of mass spectra with those of synthetic standards. In field tests, a blend of these three pheromone components was highly attractive to conspecific males, and a subtractive assay confirmed that the unsaturated alcohol is the major pheromone component, whereas no definite behavioral activity could be assigned to the ketone or the saturated alcohol. A bait containing the two alcohols with *S*-configuration was attractive to male *E. sparrmannella* (Bosc), whereas no males of *E. cicatricella* were found in these traps. The sex pheromone compounds in *E. cicatricella* are

*To whom correspondence should be addressed.

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chemically similar to pheromones reported in Trichoptera and they are produced in homologous glands.

Key Words—*Eriocrania cicatricella*, *Eriocrania sparrmannella*, Eriocraniidae, Lepidoptera, sex pheromone, EAG, GC-EAD, mass spectrometry, synthesis, evolution, (*Z*)-4-hepten-2-one, (2*R*)-heptan-2-ol, (2*R*)-(*Z*)-4-hepten-2-ol.

INTRODUCTION

Although sex pheromone communication systems in moths are generally well investigated, there is still a lack of data on the pheromones of the first evolutionary lineages of the order Lepidoptera. Absence of sex pheromones has been suggested for *Micropterix calthella* (L.) (Pirngruber, 1944). However, male attraction by synthetic lures and by conspecific females was reported for three eriocraniid species (Kozlov, 1985, 1988; Wagner and Rosovsky, 1991). The only examination of a nonditrysian species reported so far, *Lampronia capitella* (Clerck) (Prodoxidae), suggested that it may have the same type of sex pheromone as most of the ditrysian lepidopterans (Löfstedt, 1991).

Within the Trichoptera, the sister group of Lepidoptera, sex pheromone components are short-chain alcohols and ketones, which are different from the olefinic compounds that are widely used in Lepidoptera. Based on the accumulated data on the pheromones identified in Lepidoptera (Arn et al., 1992) and some previous studies on pheromone production in Trichoptera, independent evolution of sex pheromones of Trichoptera and Lepidoptera has been hypothesized (Löfstedt, 1991). An investigation of archaic Lepidoptera is needed to state whether the chemical structure of lepidopteran pheromone components (which generally include products of Δ11-desaturation of long-chain fatty acid precursors) has evolved within the Lepidoptera or whether it should be considered a synapomorphy[5] of the whole order.

Trichoptera differ from ditrysian Lepidoptera not only in the structure of the sex pheromones, but also in the location of the pheromone-producing glands, which are situated in the Vth abdominal segment (Nielsen, 1980). Similar glands have been described in some archaic Lepidoptera (for a list of taxa possessing these glands, see Davis, 1975), whereas most females of ditrysian moths possess specialized pheromone glands at the ovipositor (Percy-Cunningham and MacDonald, 1987). The function of the abdominal glands in the primitive Lepidoptera is still unclear, although it has been repeatedly hypothesized that they release some kind of pheromone (Razowski, 1975; Kristensen,

[5]Synapomorphy: a homologous character shared by two or more taxa and inferred to have been present in the nearest common ancestor but not in earlier ancestors nor in taxa outside this group.

1984a). Bioassay and chemical elucidation of components produced by abdominal glands is necessary to prove this hypothesis.

Since the presence of sex pheromone communication has already been demonstrated in Eriocraniidae (Kozlov, 1985, 1988; Wagner and Rosovsky, 1991), this family was chosen as a target group for a detailed investigation of pheromone communication in archaic Lepidoptera. Our aims in the present study were: (1) to associate the pheromone production with some morphological structures of the female's body, (2) to identify the sex pheromone components produced by female *Eriocrania cicatricella*, and (3) to contribute to an understanding of the evolutionary trend in location of pheromone-producing glands and pheromone composition in the Trichoptera and primitive Lepidoptera.

METHODS AND MATERIALS

Insects. Eriocranic cicatricella Zett. were collected from a young birch forest on May 1–6, 1992 (Turku, Finland) and on April 21–30, 1993 (Lund, Sweden). Adults were sexed immediately after capture, kept in plastic vials containing moist filter paper, and transported to the laboratory in Lund for electrophysiological tests and chemical analyses.

Preparation of extracts. Extracts of the whole abdomen, the Vth abdominal segment, and the ovipositor from adult females and the whole abdomen from adult males of *E. cicatricella* were used in electroantennogram (EAG) experiments. Extracts from the presumed pheromone gland located at the Vth abdominal segment of females were used for gas chromatography with electroantennographic detection (GC-EAD). The insects were anesthesized by cooling them to −20°C, and they were then dissected by use of different scalpels for different body parts to avoid contamination. The dissected segments were extracted for at least 30 min in pointed tubes containing 50 μl dichloromethane or hexane. These extracts were subsequently used for both electrophysiological tests and chemical identification.

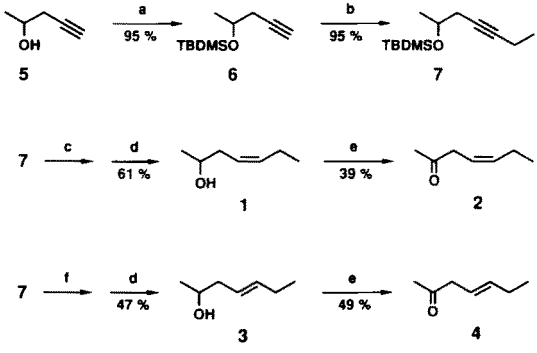
Electrophysiological and Chemical Analysis. To examine male antennal sensitivity to different body extracts of females, the tip of the male antenna of *E. cicatricella* was cut off and the antenna was mounted between two pipet electrodes containing Beadle-Ephrussi Ringer, and grounded via an Ag–AgCl wire. The tip electrode was connected to a high-impedance DC amplifier with automatic baseline drift compensation. One female equivalent of the different body extracts, deposited in the tip of a Pasteur pipet, was used as the odor source. One milliliter of air was puffed through the pipet and injected into the airstream flowing over the antenna (0.5 m/sec). The two different solvents used for the extraction, dichloromethane and hexane, were also tested for male EAG responses.

A Hewlett Packard 5830 GC equipped with a DB-wax column (30 m × 0.25 mm ID, J & W Scientific, Folsom, California) and an effluent split allowed simultaneous flame ionization (FID) and electroantennographic detection (EAD) of the separated pheromone components. Hydrogen was used as carrier gas, and the effluent split ratio was approximately 1:1. Samples were injected splitless. The injector temperature was 250°C and the split valve was opened 1 min after injection. The column temperature was maintained at 40°C for 3 min following the injection and then linearly increased to 230°C at a rate of 5°C/min. The outlet for the EAD was placed in a purified airstream flowing over the antennal preparation at a speed of 0.5 m/sec. Five microliters of extracts containing 5–10 female equivalents were injected into the column for the analysis. The equivalent chain lengths of active pheromone compounds relative to a homologous series of straight-chain acetates (7–22:OAc) were established.

Gas chromatographic–mass spectrometric analyses of GC-EAD active compounds were performed by using a fused-silica capillary (50 m × 0.32 mm ID) coated with FFAP, programmed from 60 to 220°C at a rate of 5°C/min. Mass spectra were recorded with a double focusing instrument VG 70-250 SE at 70 eV. Microhydrogenation was carried out according to Huwyler (1973). Enantiomeric separation was achieved by using a fused silica capillary (25 m × 0.32 mm ID) coated with a mixture of 6-*O*-methyl-2,3-di-*O*-pentyl-γ-cyclodextrin and OV-1701 (1:4) operated under the following conditions: 12 min at 60°C, then programmed to 120°C at a rate of 3°C/min. NMR spectra (400.1 MHz for ^{1}H and 100.6 for ^{13}C) of synthetic samples were run on a Bruker AMX 400; δ TMS = 0, δ $CDCl_3$ = 7.26 (s = singlet, bs = broad singlet, d = doublet, t = triplet, qu = quintet); attribution of ^{13}C signals was made according to DEPT spectroscopy (CH_3 and CH = +; CH_2 = −; quart C = 0).

Field Tests. Field-trapping was conducted in a young birch forest close to Lund (Sweden) and at a similar site in Turku (Finland). Synthetic blends were prepared in hexane. Red rubber septa (Arthur H. Thomas Co. Catalog No. 1780-J07) were used as dispensers, and the traps used were Lund II sticky traps (Anderbrant et al., 1989). All traps were hung on the branches of young birch trees 1–2 m above ground. Within a replicate (N = 5), each trap was set at least 5 m apart. The traps were checked every day, and trap positions within a series were randomized to minimize the effects of habitat heterogenities. Subtractive assays were first carried out in Lund, April 23–27, 1993, to determine the attractiveness to males of blends containing compounds isolated from the female pheromone gland extracts. The second set of field trapping tests was designed the same way and carried out in Turku, May 3–8, 1993. These experiments included a dose response test and an examination of the *R* and *S*-enantiomers of two alcohols.

Chemical Synthesis. The synthesis of the compounds used in the field tests is shown in Scheme 1 and is described as follows.



SCHEME 1. Syntheses of 4-hepten-2-ols (**1**) and (**3**) and 4-hepten-2-ones (**2**) and (**4**).

rac.-2-(t-Butyldimethylsiloxy)-4-heptyne **(7).** To a stirred suspension of 0.6 g (26 mmol) lithium amide in 30 ml liquid ammonia, 1.0 g (5.04 mmol) 3-(*t*-butyl-dimethylsiloxy)-1-pentyne **(6),** synthesized by alkynylation of methyloxirane according to Jacobson *et al.* (1982), followed by protection of the produced 4-pentyn-2-ol **(5),** was added during 15 min. Subsequently, 7.2 g (66 mmol) of ethylbromide was added, and the mixture was warmed to 0°C. After the addition of 50 ml ether and 20 ml saturated aqueous (aq.) ammonium chloride, the organic layer was separated, and the aqueous phase was extracted twice with 50 ml of ether. The combined organic solutions were dried over anhydrous magnesium sulfate and concentrated. The crude product (yield 1.05 g = 92%) was used for the next step without further purification.

rac.(Z)-4-Hepten-2-ol **(1).** According to the general procedure for the production of P-2 nickel (Brown and Ahuja, 1973), 260 mg (1.02 mmol) nickel acetate tetrahydrate dissolved in 10 ml of ethanol was reduced with 1.02 ml of a 1 M solution of sodium borohydride in ethanol. After hydrogen production ceased, 137 μl (2.04 mmol) ethylene diamine was added. To the black suspension 1.05 g (4.64 mmol) of **(7)** dissolved in 1 ml of ethanol was added. The stirred mixture was hydrogenated under standard conditions (1m H_2, room temperature). The mixture was filtered through a pad of celite, the solvent was evaporated, and then 10 ml of water and 10 ml ether were added. The organic layer was separated, the aqueous layer was extracted twice with 10 ml of ether, and the ethereal extracts were dried over anhydrous $MgSO_4$. The solvent was evaporated and the crude product was dissolved in 15 ml of acetonitrile and stirred with 10 drops of 40% aq. hydrogen fluoride. After 1 hr, the mixture was poured onto brine and extracted twice with 20 ml of ether. The filtered organic

layer was washed with sodium bicarbonate, dried over magnesium sulfate, and concentrated. The residue was chromatographed on silica Merck 60/240–400 mesh impregnated with 10% $AgNO_3$ using a 2:1 mixture of pentane and diethylether, which, upon TLC, had been previously known to give an R_f value of 0.25, yielding 490 mg (61%) of **(1)**.

^{1}H NMR (400.1 MHz, $CDCl_3$, attribution of carbon atoms according to COSY experiment): δ = 5.57 (dtt, $J_{5,4}$ = 10.8 Hz, $J_{5,6}$ = 7.2 Hz, $J_{5,3}$ = 1.5 Hz, 1H, H-5), 5.32–5.41 (m, 1H, H-4), 2.77–3.82 (m, 1H, H-2), 2.14–2.29 (m, 2H, H-3), 2.08 (dqu, $J_{6,5}$ = $J_{6,7}$ = 7.4 Hz, 2H, H-6), 1.55 (bs, 1H, OH), 1.21 (d, $J_{1,2}$ = 6.2 Hz, 3H, H-1), 0.97 (t, 3H, H-7). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 135.12 (+, C 5), 124.46 (+, C 4), 67.70 (+, C 2), 37.04 (−, C 3), 22.71 (+, C 1), 20.70 (−, C 6), 14.25 (+, C 7).

rac.(E)-4-Hepten-2-ol **(3)**. To a solution of 1.9 g (8.4 mmol) of **(7)** in a mixture of 50 ml liquid ammonia and 20 ml tetrahydrofuran 0.6 g (25 mmol) sodium was added in small portions at −40°C. The dark blue solution was stirred for 1 h and subsequently warmed to room temperature. After the addition of 50 ml ether and 50 ml saturated aq. ammonium chloride, the organic layer was separated and the aqueous phase was extracted twice with 20 ml of ether. The combined organic solution was washed with 20 ml of water, dried with magnesium sulfate, and concentrated in vacuo. The crude product was diluted with 20 ml of acetonitrile. After the addition of 15 drops of 40% aq. hydrogen fluoride, the product was stirred for 1 hr, and worked up. The final purification was carried out by column chromatography (Merck 60 impregnated with 10% $AgNO_3$ using a 5:2 mixture of pentane and diethylether, which, upon TLC, had been previously known to give an R_f value of 0.22), yielding 460 mg (47%) of **(3)**.

^{1}H NMR (400, 1 MHz, $CDCl_3$): δ = 5.59 (dtt, $J_{5,4}$ = 15.2 Hz, $J_{5,6}$ = 6.4 Hz, $J_{5,3}$ = 1.3 Hz, 1H, H-5), 5.36–5.45 (m, 1H, H-4), 3.74–3.81 (m, 1H, H-2), 2.16–2.23 (m, 1H, H-3), 2.00–2.13 (m, 3H, H-3, H-6), 1.65 (bs, 1H, OH), 1.18 (d, $J_{1,2}$ = 6.1 Hz, 3H, H-1), 0.99(t, $J_{7,6}$ = 7.5 Hz, 3H, H-7). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 136.24 (+, C5), 124.82 (+, C4), 67.23 (+, C 2), 42.54 (−, C 3), 25.68 (−, C 6), 22.65 (+, C 1), 13.82 (+, C 7).

(Z)-4-Hepten-2-one **(2)**. To a solution of 0.4 g (3.6 mmol) of **(1)** in 20 ml dichloromethane, 1.4 g (3.6 mmol) pyridine dichromate and 1.4 g pulverized molsieve 4 Å (Herscovici et al., 1982) was added and vigorously stirred for 4 hr. The mixture was filtered through 10 g of silica (Merck 60), which was subsequently washed with 80 ml of ether. The organic solution was concentrated and chromatographed on silica (Merck 60 impregnated with 10% $AgNO_3$ using a 10:1 mixture of pentane and diethylether, which, upon TLC, had been previously known to give an R_f value of 0.25), yielding 150 mg (39%) of **(2)**.

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.61 (dtt, $J_{5,4}$ = 10.7 Hz, $J_{5,6}$ = 7.1 Hz, $J_{5,3}$ = 1.5 Hz, 1H, H-5), 5.51 (dtt, $J_{4,3}$ = 7.1 Hz, $J_{4,6}$ = 1.4 Hz, 1H,

H-4), 3.17 (bd, 2H, H-3), 2.16 (s, 3H, H-1), 2.05 (qu, $J_{6,7}$ = 7.6 Hz, 2H, H-6), 0.99 (t, 3H, H-7). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 207.55 (o, C 2), 136.86 (+, C 5), 120.83 (+, C 4), 47.67 (−, C 3), 29.32 (+, C 1), 25.63 (−, C 6), 13.52 (+, C 7).

(E)-4-Hepten-2-one **(4).** The procedure was essentially the same as for the synthesis of **(2).** Purification was carried out by column chromatography on Merck 60 using a 8:1 mixture of pentane and diethylether as the eluent. The same yield was obtained as with (*Z*)-4-Hepten-2-one.

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.47–5.63 (m, 2H, H-4), 3.11 (bd, $J_{3,4}$ = 6.8 Hz, 2H, H-3), 2.14 (s, 3H, H-1), 2.02–2.10 (m, 2H, H-6), 0.99 (t, $J_{7,6}$ = 7.4 Hz, 3H, H-7). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 207.55 (o, C 2), 136.86 (+, C 5), 120.83 (+, C 4), 47.67 (−, C 3), 29.32 (+, C 1), 25.63 (−, C 6), 13.52 (+, C 7).

(2R)-(Z)-4-Hepten-2-ol **(1a).** Using (2*R*)-methyloxirane (Merck) in the synthesis of **(5),** the procedure was essentially the same as for the synthesis of **(1):** $[\alpha]_D^{22}$ = −4.9 (c = 2.2 CH_2Cl_2). The optical purity was 98% ee (enantiomeric excess) as determined by chiral gas chromatography.

(2S)-(Z)-4-Hepten-2-ol **(1b).** Using (2*S*)-methyloxirane in the synthesis of **(5),** the procedure was essentially the same as for the synthesis of 1: $[\alpha]_D^{22}$ = +4.8 (c = 1.8 CH_2Cl_2). The optical purity was 96% ee as determined by chiral gas chromatography.

Heptan-2-ol. While racemic 2-heptanol was commercially available (Merck), optically active products were synthesized from optically active methyloxirane by Grignard reaction (Gaylord and Becker, 1951).

RESULTS

Courtship Behavior. Eriocrania cicatricella is a day-flying insect. Males have an unusual type of search behavior. Starting from a height of 0.5–1.5 m, they fly slowly upwards, along a wave-form trajectory, up to 2–3 m height; then they fly downwards quickly. This cycle is usually repeated two to five times. Virgin females do not demonstrate any special pose when "calling." Males flew around the female (which usually was sitting on the thin branch of a birch), then landed 10–15 cm away from the female and ran to her displaying a typical wing-fanning behavior. If the population density was high, up to five males "dancing" around a female were observed. The moths took position with their heads in opposite directions 1–2 sec after copulation. During the first 20–40 sec, the male had his wings raised, then he folded them slowly. If more than one male was attracted, the other males tried to copulate with the pair during 1–2 min, and then flew away. Copulation usually took 10–30 min. Copulating pairs were most frequently observed from 14:00–19:00 hr, when the air temperature usually reached the daily maximum.

EAGs of Different Body Parts. Male EAG response to the extract from the Vth abdominal segment of female *E. cicatricella* was as high as to the extract of the whole abdomen (Figure 1), indicating that the gland contained in this segment produced the active compounds. The extract from female ovipositor elicited a low response compared to extracts from the rest of the abdomen. There were no significant male EAG responses to the two solvents, dichloromethane and hexane, used for the female extraction. The reaction of female antennae to the extracts of the abdomen of both sexes was also tested, but no responses were observed. Thus, we found no EAG evidence for a male-produced pheromone in this species.

GC-EAD of Female Extracts and Chemical Identification. Analysis of the extract from female Vth abdominal segment of *E. cicatricella* indicated that three compounds elicited strong responses from a conspecific male antenna (Figure 2). On a DB-wax column, the equivalent chain lengths of three pheromone candidates were 1236, 1310, and 1330, respectively. The mass spectra of these three GC-EAD active compounds suggested two methylcarbinols (*m/z* 45) and a methyl ketone (*m/z* 43), each having seven carbon atoms. According to its fragmentation pattern, one of them (II) could easily be identified as heptan-2-ol (McLafferty and Stauffer, 1989). A minor component (I) showed a mass spectrum almost identical to (*E*)-4-hepten-2-one (McLafferty and Stauffer, 1989). The mass spectrum of the main component (III) is shown in Figure 3. Upon microhydrogenation (Huwyler, 1973), the compound was converted to heptan-2-ol, proving the natural product to be an unbranched hepten-2-ol. Provided that this unsaturated methylcarbinol and the above mentioned methylketone, 4-hepten-2-one, are biogenetically related, the main component would most probably

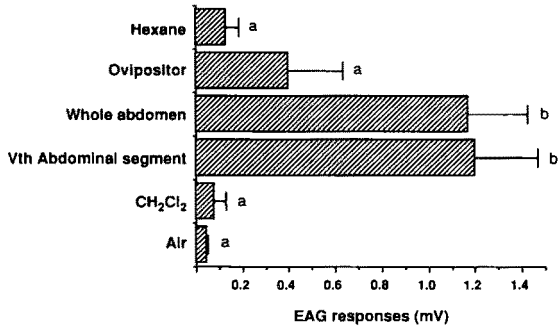


FIG. 1. EAG responses (mV ± SE) of male *Eriocrania cicatricella* to extracts of different body parts of conspecific females. Bars followed by the same letter are not statistically different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using a Mann-Whitney U test ($P > 0.05$).

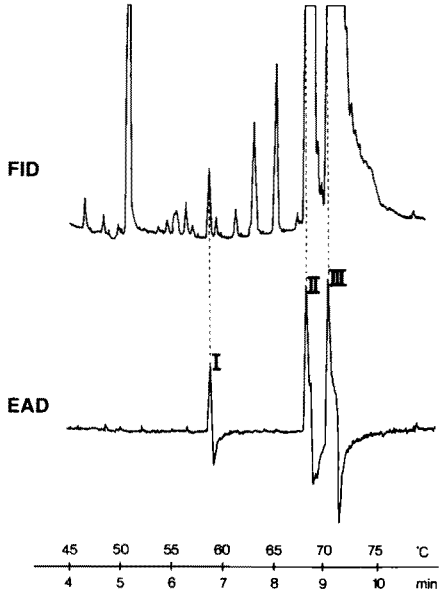


FIG. 2. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) responses using antennae of male *Eriocrania cicatricella* in response to pheromone gland extracts from conspecific females.

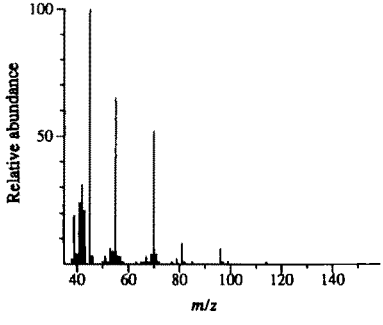


FIG. 3. Mass spectrum of the most abundant pheromone component, (2*R*)-(*Z*)-4-hepten-2-ol from female pheromone extracts of *Eriocrania cicatricella*.

show the double bond in position 4. Gas chromatographic investigations showed that the geometrical isomers of both 4-hepten-2-one and 4-hepten-2-ol could be very well separated on a FFAP-fused silica column under the conditions mentioned above. For the ketone, a separation value of $\alpha = 1.04$ [$R_t(Z)/R_t(E)$] was found, while the alcohol showed $\alpha = 1.10$ [$R_t(Z)/R_t(E)$]. Synthetic reference samples of (*Z*)-4-hepten-2-one and (*Z*)-4-hepten-2-ol coeluted with the natural products and showed the identical mass spectra. The absolute configuration of the natural alcohols could be determined by chiral gas chromatography on a cyclodextrin derivative. As shown with synthetic samples, the two racemates were well separated under the conditions mentioned above (Figure 4). The results revealed that this species produced pure enantiomers that coeluted with (2*R*)-(*Z*)-4-hepten-2-ol and (2*R*)-heptan-2-ol. The EAD active compounds in the extracts could thus be identified as (2*R*)-(*Z*)-4-hepten-2-ol, (2*R*)-heptan-2-ol, and (*Z*)-4-hepten-2-one in a ratio of 110:20:1, and an average female contained approximately 126 ng of (2*R*)-(*Z*)-4-hepten-2-ol, 23 ng of (2*R*)-heptan-2-ol and 1.1 ng of (*Z*)-4-hepten-2-one.

Field Tests. A synthetic mixture of these three compounds identified from *E. cicatricella* trapped large numbers of conspecific males in a field experiment (Table 1). Subtraction of either (*Z*)-4-hepten-2-one or (2*R*)-heptan-2-ol from the mixture did not significantly reduce attractiveness of the synthetic lure. Subtraction of (2*R*)-(*Z*)-4-hepten-2-ol caused complete loss of the attractivity. The importance of the enantiomeric composition of the two alcohols was elucidated

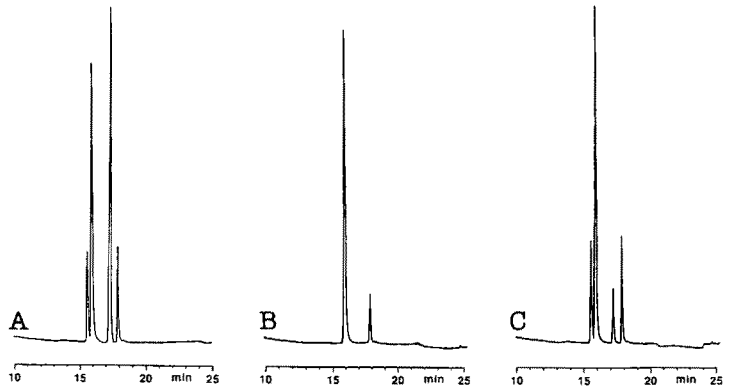


FIG. 4. Determination of the absolute configuration of the female sex pheromone components of *Eriocrania cicatricella* through chiral gas chromatography. Mixture of racemic (*Z*)-4-hepten-2-ol spiked with (2*R*)-(*Z*)-4-hepten-2-ol (first pair of peaks) and racemic 2-heptanol spiked with (2*S*)-heptanol (second pair of peaks) (A), natural gland extract (B), and mixture of racemic (*Z*)-4-hepten-2-ol, racemic 2-heptanol and natural gland extract (C).

in a field test in Turku, Finland (Table 2), which showed that the traps with baits containing the (*R*)-enantiomer of the major component, (*Z*)-4-hepten-2-ol, caught the highest number of males of *E. cicatricella*. Neither (2*R*)- nor (2*S*)-heptan-2-ol showed any effect on male attractivity. (2*S*)-(*Z*)-4-hepten-2-ol seemed to act as an inhibitor on the attraction of male *E. cicatricella*, but a large number of male *E. sparrmannella* (Bosc) were caught in the traps by the bait with this compound. When the dose of baits containing the two alcohols in (*R*)-configuration was decreased to half, the number of moths attracted to the

TABLE 1. SUBTRACTIVE ASSAY OF SYNTHETIC PHEROMONE CANDIDATES FOR *Eriocrania cicatricella* IN LUND, SWEDEN, APRIL 23–27, 1993

Treatment (μg)			Catch per trap (mean ± SE)[a]
(*Z*)-4-Hepten-2-one	(2*R*)-Heptan-2-ol	(2*R*)-(*Z*)-4-Hepten-2-ol	
0.5	15	100	33.4 ± 9.2 a
—	15	100	28.2 ± 8.3 a
0.5	15	—	0.6 ± 0.4 b
0.5	—	100	16.2 ± 5.0 a
—	—	—	0.2 ± 0.2 b

[a]Means followed by the same letter are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P > 0.05$), $N = 5$.

TABLE 2. FIELD TRAPPING OF MALE *Eriocrania cicatricella* WITH DIFFERENT ENANTIOMERS OF TWO PHEROMONE COMPOUNDS IN TURKU, FINLAND, MAY 3–8, 1993

Heptan-2-ol (μg)		(*Z*)-4-hepten-2-ol (μg)		Catch per trap (mean ± SE)[a]	
2*S*	2*R*	2*S*	2*R*	*E. cicatricella*	*E. sparrmannella*
—	15	—	100	131.0 ± 32.5 a	0 ± 0 b′
15	—	—	100	148.0 ± 30.6 a	0 ± 0 b′
—	15	100	—	0.2 ± 0.2 d	8.6 ± 1.6 a′
15	—	100	—	1.3 ± 0.5 d	13.3 ± 3.5 a′
—	—	—	100	102.8 ± 18.8 a	0 ± 0 b′
—	15	—	—	0.4 ± 0.5 d	0 ± 0 b′
7.5	7.5	50	50	13.8 ± 2.9 c	0 ± 0 b′
—	7.5	—	50	68.0 ± 16.7 b	0 ± 0 b′

[a]Means within each column followed by the same letter are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P > 0.05$), $N = 5$.

TABLE 3. FIELD TRAPPING OF MALE *Eriocrania cicatricella* WITH DIFFERENT DOSES OF PHEROMONE COMPOUNDS IN TURKU, FINLAND, MAY 3–8, 1993

Compounds (μg)			Catch per trap (mean ± SE)[a]
(*Z*)-4-hepten-2-one	(2*R*)-Heptan-2-ol	(2*R*)-(*Z*)-4-Hepten-2-ol	
0.05	1.5	10	6.2 ± 2.2 c
0.5	15	100	32.8 ± 14.3 b
5	150	1000	123.2 ± 31.1 a
—	1.5	10	4.4 ± 1.9 c
—	15	100	20.4 ± 7.5 b
—	150	1000	70.4 ± 31.8 ab

[a]Means followed by the same letter are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P > 0.05$), $N = 5$.

traps was significantly reduced (Table 2). A mixture of the two alcohols with (*R*)–configuration and this mixture plus (*Z*)-4-hepten-2-one were also tested in different doses. The highest dose of the three-component mixture was the most attractive. The attraction of males to the highest dose of the two-component mixture was not different from that of the medium dose of the three-component or the highest dose of the three-component mixture (Table 3).

DISCUSSION

Little is known about sex pheromones in archaic lepidopteran groups. The present investigation of the pheromone of *E. cicatricella* demonstrates that this primitive lineage of Lepidoptera (suborder Eriocraniina = Dacnonypha) is more similar to Trichoptera than to the well-investigated ditrysian lepidopterans, when it comes to pheromone communication. *E. cicatricella* produces short-chain ketones and alcohols as pheromone components, which are related to compounds earlier identified from Trichoptera; heptan-2-ol is a female-produced pheromone component in *Rhyacophila fasciata* (Mclachlan), and (*Z*)-6-nonen-2-one was identified in sternal gland extracts from male *Hydropsyche angustipennis* (Curtis) (Löfstedt et al., 1994). Our findings agree with the hypothesis of Wagner and Rosovsky (1991), which states that the presence of female-produced sex pheromones should be considered a synapomorphy of Trichoptera and Lepidoptera.

The pheromone components identified from *E. cicatricella*, (*Z*)-4-hepten-2-one, (2*R*)-(*Z*)-4-hepten-2-ol, and (2*R*)-heptan-2-ol are chemically very similar and most likely have a common biosynthetic origin. Within Lepidoptera, such

chemical structures have not been reported as pheromone components in any ditrysian family, but in the monotrysian family Nepticulidae similar chemical structures have been found (Tóth et al., 1995). Thus, the typical "lepidopteran" composition of sex pheromones has evolved within the order Lepidoptera. If we look at the phylogenetic tree of the order (Kristensen, 1984b), we can suppose that this event might be attributed a synapomorphy within nonditrysian Heteroneura, since one of the first lineages of the last taxa includes incurvarioid moths, which probably possess the "typical" lepidopteran pheromone (Löfstedt, 1991).

The site of pheromone synthesis and release may prove to be of special phylogenetic significance. A pair of glands opening on the Vth abdominal segment are considered an autapomorphy[6] of Amphiesmenoptera (Trichoptera + Lepidoptera), but these glands have been reduced independently in many lineages in both orders (Kristensen, 1984b). The ultrastructure of these glands (Kristensen, 1984a) is rather uniform within archaic Lepidoptera; the walls of the reservoirs are covered with secretory cells that belong to "type 3" according to the scheme by Noirot and Quennedy (1974). In most species in the family Eriocraniidae, the Vth abdominal segment is variously modified in association with the presence of the glands. The glands usually occur in both sexes (Davis, 1978), but in the genus *Eriocrania* we observed that the glands occur in the females only. In Trichoptera, female sex pheromones evidently are released from the Vth sternite (Wood and Resh, 1984; Löfstedt et al., 1994). The EAG test of different body parts showed that in Eriocraniidae, the pheromone production is associated with Vth abdominal segments. We were unable to isolate the gland specifically for EAG examination, but since no other secretory structures are known from this segment, we believe this is enough to associate the identified pheromones with the Vth abdominal glands. More advanced Lepidoptera, as summarized by Davis (1975), do not possess glands of the type mentioned above, and pheromone production, if present, is provided by other morphological structures. A large variety of pheromone-producing glands has been described, but generally females of ditrysian moths possess pheromone glands in the intersegmental region between abdominal segments VIII and XI (see references in Percy-Cunningham and McDonald, 1987).

Our field trapping experiments indicate that there is no significant reduction in the male attraction when (*Z*)-4-hepten-2-one or (2*R*)-heptan-2-ol was subtracted from the 3-component pheromone bait (Table 1). The chiral alcohols identified in *E. cicatricella* are pure (*R*)-enantiomers and (2*R*)-(*Z*)-4-hepten-2-ol is very attractive to conspecific males. When testing the attractivity of both (*R*)- and (*S*)-enantiomeric forms of these pheromone components in the field, we found that the *S* antipodes of the alcohols caught almost no *E. cicatricella*,

[6] Autapomorphy: The derived character state found in only one of two sister groups.

but large numbers of *E. sparrmannella*. Enantiomeric composition of the chiral components may contribute to the species specificity of this kind of pheromone. Geometrid moths have earlier been reported to use different combinations of chiral epoxides in their pheromone communication systems (Szöcs et al., 1993). Eriocranniidae provide another example within Lepidoptera of species where pheromone specificity is based on the chirality of the sex pheromone components.

Thus, both chemical and morphological data on the sex pheromone in this primitive moth, *E. cicatricella*, demonstrate the great similarities between the chemical communication system of the basic lineages of Trichoptera and Lepidoptera. This similarity can be explained by the common origin of these sister groups and therefore should be considered as a symplesiomorphy.[7] Sex pheromones used by most ditrysian species in Lepidoptera are derived from palmitic acid acted upon by various combinations of desaturases, β-oxidases, reductases, and acetyltransferases. The $\Delta 11$ desaturase seems to account for the largest number of unsaturated pheromone components, although several unusual desaturases such as $\Delta 10$, $\Delta 9$, and $\Delta 14$ are also involved in some species (see references in Löfstedt, 1991). Studies of pheromone biosynthesis in *E. cicatricella* will allow a comparison of biosynthetic routes to pheromones employed by primitive and more advanced lepidoptera.

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COMPARISON OF THREE METHODS FOR QUANTIFYING BROWN ALGAL POLYPHENOLIC COMPOUNDS

KATHRYN L. VAN ALSTYNE

Department of Biology
Kenyon College
Gambier, Ohio 43022

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Abstract—Three methods of quantifying total phenolic compounds in marine brown algae were compared for their ability to measure accurately known concentrations of phloroglucinol in the presence of a protein, bovine serum albumin (BSA). These methods were: (1) a Folin-Ciocalteu assay for compounds dissolved in 80% methanol, (2) a Folin-Ciocalteu assay for compounds dissolved in 75% methanol–25% trichloroacetic acid, and (3) an assay in which polyvinylpolypyrrolidone (PVPP) was used to remove phenolic compounds in order to quantify nonphenolic, Folin-Ciocalteu-reactive materials. The Folin-Ciocalteu assay used with compounds dissolved in 80% methanol was the method that produced the most consistent results. It was also the assay in which the presence of proteins interfered least with the measurements of phloroglucinol and the assay that was easiest to conduct. The presence of TCA decreased the pH of the extracting solution and increased the amounts of proteins in solution. These proteins later interfered with the Folin-Ciocalteu reaction. TCA effectively bound proteins when protein concentrations were low but not when protein concentrations were high. The amount of phloroglucinol removed by PVPP varied widely and was dependent on phloroglucinol concentration, making this an unreliable method for quantifying phenolic concentrations. The Folin-Ciocalteu assay will measure some nonphenolic substances when brown algae are extracted in 80% methanol; however, in *Fucus gardneri*, nonphenolic substances were at most 5% of the material quantified by the assay.

Key Words—Brown alga, polyphenolics, phenols, *Fucus gardneri*.

INTRODUCTION

Marine brown algae (Division Phaeophyta) frequently contain large quantities (up to 20% dry wt, K. Van Alstyne, unpublished data) of phenolic compounds

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(phlorotannins). These compounds are polymers of phloroglucinol (Ragan and Glombitza, 1986) and are structurally different from the hydrolyzable and condensed tannins of terrestrial plants. Phlorotannins have been hypothesized to serve a variety of functions in phaeophytes including deterring grazers from feeding on the algae (Geiselman, 1980; Geiselman and McConnell, 1981; Anderson and Velimirov, 1982; Steinberg, 1985, 1988; Van Alstyne and Paul, 1990), preventing bacterial infections (Conover and Sieburth, 1964; Sieburth, 1968), and serving as allelopathic agents (Conover and Sieburth, 1966; McLachlan and Craigie, 1966; Fletcher, 1975).

Two groups of methods are commonly used to quantify concentrations of tannins and phenolic compounds (Mole and Waterman, 1987; Bernays et al., 1989; Hagerman and Butler, 1989, 1991). The first group, chemical assays (*sensu* Hagerman and Butler, 1989), include redox assays, metal binding assays, and assays based on specific chemical reactivity (Hagerman and Butler, 1989). The second group, protein-binding assays, are used to determine the tanning ability of phenolic compounds (Mole and Waterman, 1987). The binding of proteins is one of the putative modes of action of phenolic compounds. Plant phenolic compounds that are ingested by an herbivore could potentially bind to plant proteins and herbivore digestive proteins, reducing the assimilation efficiency of the herbivore (Boettcher and Targett, 1993). Therefore, assays that measure protein binding may have biological relevance.

The Folin-Denis assay is one of the most common chemical assays used for ecological research (Folin and Denis, 1912). This assay is one of several that quantifies concentrations of easily oxidized compounds, such as phenols, by color changes accompanying a redox reaction (Hagerman and Butler, 1989). To conduct the assay, known quantities of algal tissue are extracted in aqueous methanol. The extracts are diluted and reacted with a phosphotungsten–polymolybdic acid that reacts with hydroxylated aromatic compounds. In alkaline solutions, the reagents produce a blue color that can be quantified spectrophotometrically. The Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927) provides an improvement over the Folin-Denis reagent with the addition of lithium sulfate to the reagent. The lithium salts reduce the amounts of precipitate that can form when high concentrations of reagent are used to increase the assay's reactivity. Both the Folin-Denis and Folin-Ciocalteu assays have been widely used by marine chemical ecologists to quantify concentrations of brown algal phlorotannins (e.g., Boettcher and Targett, 1993; Carlson and Carlson, 1984; Pfister, 1992; Ragan and Glombitza, 1986; Steinberg, 1984, 1985, 1986, 1989; Steinberg and van Altena, 1992; Targett et al., 1992; Van Alstyne, 1988, 1989; Van Alstyne and Paul, 1990).

The Folin-Denis and Folin-Ciocalteu assays share a common problem; both reagents react with many oxidizable compounds. In brown algae, the most abundant reactive compounds are phenols; however, brown algae may also contain

other reactive compounds, such as tyrosine, tryptophan, and ascorbic acid. To address this problem, Hatch et al. (1993) proposed extracting organisms in an acetone–trichloroacetic acid (TCA) solution. TCA, like other strong acids, precipitates proteins. Hence, it should eliminate the tyrosine and tryptophan residues from solution, reducing the nonphenolic, reactive compounds quantified in the Folin-Denis and Folin-Ciocalteu assays.

Even if all proteins are eliminated from the extracts, there will still be some nonphenolic substances measured by the Folin-Denis and Folin-Ciocalteu assays. One means of alleviating this interference is to remove selectively phenolic compounds with polyvinylpolypyrrolidone (PVPP) (e.g., Yates and Peckol, 1993). Phenolic compounds bind to nonsoluble PVPP under acidic conditions and can then be removed from solution by filtering or centrifuging. The remaining oxidizable species are then quantified with a Folin-Ciocalteu or Folin-Denis assay and subtracted from the results of the assay prior to the addition of PVPP. However, if all the phenolics are not removed by the PVPP, phenolic concentrations are underestimated. The binding efficiency of PVPP for brown algal phlorotannins has not been explored.

The purpose of this study is to examine the efficiencies of these three methods for quantifying phenolic compounds in the presence and absence of a protein, bovine serum albumin (BSA), and known concentrations of phloroglucinol. In the first part of this study, the Folin-Ciocalteu assay was used to quantify the interference of proteins dissolved in 80% aqueous methanol or in 75% methanol–25% TCA. A second set of assays was used to examine the effectiveness of PVPP in removing phenolic compounds from the solutions. The final part of this study addressed the question of whether these three methods for extracting phenolics and preparing extracts yielded significantly different results when used to measure concentrations of phenolics in a marine brown alga, *Fucus gardneri*.

METHODS AND MATERIALS

Measurements of Known Concentrations of Phenols and Proteins. In the first set of assays, known mixtures of phloroglucinol (Sigma P-3502) and bovine serum albumin (BSA Sigma A-6918) were used to examine the effects of proteins on the Folin-Ciocalteu assay when compounds were dissolved in 80% methanol or 75% methanol–25% TCA. Sixteen solutions were made by dissolving various amounts of phloroglucinol (0–0.17%) and BSA (0–1.10%) in 10 ml of 80% aqueous methanol. A second set of 16 solutions was made by adding the same amounts of phloroglucinol and BSA to 75% methanol–25% TCA. The methanol–TCA solution was prepared by dissolving 100 g of TCA in 100 ml of distilled water. The TCA solution was then added to 300 ml of methanol.

The amounts of phloroglucinol that were used were chosen to be equivalent to the amounts of brown algal phenolics that would be present in an extract of a 1-g brown algal sample (dry–wet mass ratio of 0.25) containing 0%, 2%, 5%, or 10% phenolic compounds by dry weight. The BSA concentrations used were chosen to be equivalent to the amount of nitrogen that would be present in algal samples containing either 0%, 2%, 4%, or 10% nitrogen. Steinberg (1985) reported that nitrogen concentrations of 13 brown algal species from California ranged from 1.02% to 2.87% dry mass. Hence, the maximum amount of nitrogen in these assays will exceed the maximum nitrogen concentration measured by Steinberg (1985) by a factor of three.

The 32 solutions were filtered through Whatman No. 1 filters to remove precipitated compounds. Large amounts of precipitates formed in the 80% methanol solutions containing BSA, but no preceipitates were seen in the methanol–TCA solutions. An aliquot (0.050 ml) from each filtrate was added to 1.00 ml of distilled water and 1.00 ml of 40% Folin-Ciocalteu reagent. The flasks were stirred to mix the solutions thoroughly. Five minutes later the solutions were made alkaline with the addition of 1.00 ml of 2 N Na_2CO_3. The flasks were heated in a 50°C water bath for 30 min. The absorbance of the solutions was then measured at 765 nm after removal from the bath.

In the second series of experiments, the sixteen 10-ml aqueous methanol solutions from the previous experiments were acidified with 0.10 ml of concentrated hydrochloric acid to bring the pH to ~1. The sixteen 10 ml methanol–TCA solutions were already at pH 1.3 so it was not necessary to acidify them further. One gram of PVPP (Sigma P-6755) was added to each of the 32 solutions. This amount of PVPP, equivalent to 100 g/liter, is far more than is generally used to remove phenolic compounds from solutions. The slurry was vigorously mixed and allowed to react overnight in darkness at 4°C. The next morning, the slurries were mixed again and approximately 1 ml of each slurry was spun down in a microcentrifuge. Folin-Ciocalteu assays were then performed on the liquid fractions.

Simple linear regressions (MINITAB, Release 9) were used to obtain regression equations of phloroglucinol concentrations (x) and absorbance at 765 nm (y). The slopes and elevations (y intercepts) of regression equations for different concentrations of BSA were compared with analysis of covariance (Zar, 1984).

Measurements of Phenolic Compounds in Fucus gardneri. *Fucus gardneri* were collected from an exposed ($N = 7$) and a protected ($N = 10$) site at Fogarty Creek Point, Oregon. Approximately 0.3 g of blade and midrib tissue from each alga was weighed and extracted in 15 ml of 80% aqueous methanol. Another blade and midrib sample from each individual of approximately the same size was weighed and extracted in 15 ml of 75% methanol–25% TCA solution. A third sample from each individual was weighed and placed in a 60°C drying

oven for 48 hr to obtain a dry-to-wet mass ratio. The algal samples in 80% methanol and 75% methanol–25% TCA were extracted overnight in darkness at 4°C. A Folin-Ciocalteu assay was used to measure the concentrations of phlorotannins in the solution prior to homogenization. The samples were then homogenized with a Virtishear Tempest I.Q. homogenizer. After filtration, Folin-Ciocalteu assays were used to quantify phenolic concentrations in the extracts.

PVPP was used to remove phenolic compounds from the mixture to attempt to quantify the concentrations of nonphenolic, Folin-Ciocalteu-reactant materials. The 80% methanol extracts were acidified with 0.15 ml HCl and 1.5 g of PVPP was then added to each of the 15-ml aqueous methanol and methanol–TCA extracts. After reacting overnight in the darkness at 4°C, the slurries were stirred. A 1-ml sample was removed from each, spun in a microcentrifuge, and assayed again with a Folin-Ciocalteu assay. The amount of nonphenolic, Folin-Ciocalteu-reactant materials was determined by subtracting the concentrations of reactive materials after the addition of PVPP from the concentrations prior to the addition of PVPP.

Paired *t* tests (MINITAB, Release 9) were used to compare: (1) phenolic concentrations of algae prior to and after homogenization, and (2) algae extracted in 80% methanol and algae extracted in 75% methanol, 25% TCA. Correlation coefficients (MINITAB, Release 9) were calculated to determine if quantities of nonphenolic, Folin-Ciocalteu-reactive compounds were correlated with concentrations of phenolic compounds.

RESULTS

The addition of bovine serum albumin (BSA) did not affect the outcome of the Folin-Ciocalteu assays that were used to measure known concentrations of phloroglucinol when 80% methanol was used as a solvent (Figure 1A). When BSA was added to 80% methanol, a substantial portion of the protein precipitated from solution and was removed by filtering before the assays were conducted. Absorbance at 765 nm was tightly and positively correlated (Figure 1) with phloroglucinol concentration but was unaffected by the addition of BSA.

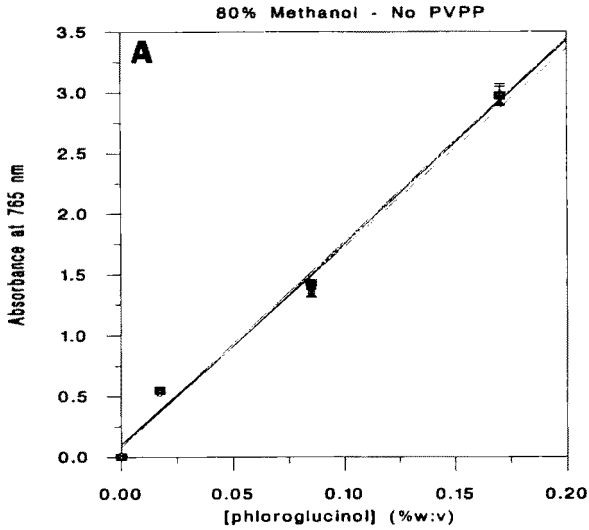
PVPP was not effective at removing all of the phloroglucinol from the acidified 80% methanol solutions (Figure 1B). The amount of phloroglucinol remaining in solution was significantly correlated with the initial phloroglucinol concentration (regression $r^2 = 0.988$; $P = 0.006$). The addition of BSA to the solution significantly decreased the amount of phloroglucinol remaining in solution (Figure 1B). This decrease may have occurred because phloroglucinol was more effectively absorbed by PVPP in the presence of BSA. Alternatively, some of the phloroglucinol may have complexed with the BSA and precipitated from solution in the presence of PVPP. The amount of phloroglucinol remaining in the solution was independent of BSA concentration (Figure 1B).

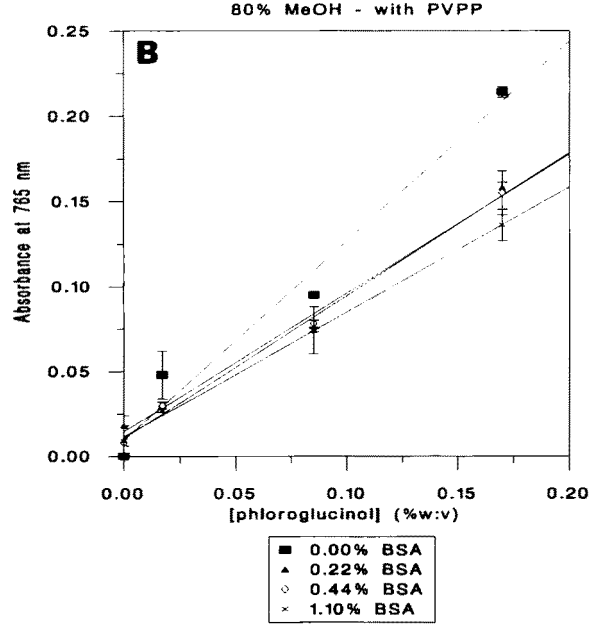
Measurements of phloroglucinol in 25% TCA–75% methanol yielded a tight correlation between the measured absorbance and the concentration of phloroglucinol in the solution in the absence of BSA (Figure 2A). The addition of BSA had little effect on the regression at concentrations comparable to those seen in most brown algae (2–4% N). However, at high protein concentrations (equivalent to 10% N), proteins affected the absorbance of phenols, causing an increase in the slope of the regression line. BSA also affected the uptake of phloroglucinol by PVPP in 25% TCA–75% methanol (Figure 2B). The addition of BSA to the methanol–TCA solutions appeared to have decreased the absorption of phloroglucinol by PVPP. In contrast, when BSA was added to the aqueous methanol solutions, phloroglucinol absorption appeared to increase (Figure 1B).

Both 80% methanol and 25% TCA–75% methanol work equally well as solvents for extracting *Fucus* for Folin-Ciocalteu analyses. There were no significant differences in the phenolic levels of algae extracted in 80% methanol versus 25% TCA–75% methanol (Table 1: paired t test: $P = 0.48$). However, it is important that the samples be ground prior to running the analysis. Grinding samples increased the mean measured phenolic concentrations by 54% to 210% (Table 1; paired t test: $P < 0.001$).

In *Fucus gardneri*, nonphenolic substances make up at most ~3–5% of

FIG 1. (A) The results of Folin-Ciocalteu assays of known mixtures of phloroglucinol and bovine serum albumin (BSA) dissolved in 80% methanol prior to the addition of PVPP. The four lines shown in each graph represent the best fit lines for phloroglucinol concentrations versus absorbance ($N = 3$) for each of the four BSA concentrations used. Vertical lines represent ±1 SE. The regression equations for the lines are: 0% BSA absorbance = 16.7 * [phloroglucinol] + 0.10 ($r^2 = 0.997$, $P < 0.001$), 0.22% BSA absorbance = 16.3 * [phloroglucinol] + 0.10 ($r^2 = 0.997$, $P < 0.001$), 0.44% BSA absorbance = 16.7 * [phloroglucinol] + 0.08 ($r^2 = 0.993$, $P < 0.001$), and 1.1% BSA absorbance = 16.7 * [phloroglucinol] + 0.08 ($r^2 = 0.996$, $P < 0.001$). The slopes and elevations of the regressions are not significantly different (ANCOVA: slope $F = 0.146$, $P > 0.5$, elevation $F = 0.557$, $P > 0.5$). (B) The results of Folin-Ciocalteu assays of known mixtures of phloroglucinol and bovine serum albumin (BSA) dissolved in 80% methanol after the addition of PVPP. The four lines shown in each graph represent the best fit lines for phloroglucinol concentrations versus absorbance ($N = 3$) for each of the four BSA concentrations used. Vertical lines represent ±1SE. The regression equations for the lines are: 0% BSA absorbance = 1.172 * [phloroglucinol] + 0.010 ($r^2 = 0.973$, $P < 0.001$), 0.22% BSA absorbance = 0.816 * [phloroglucinol] + 0.015, ($r^2 = 0.972$, $P < 0.001$), 0.44% BSA absorbance = 0.841 * [phloroglucinol] + 0.011 ($r^2 = 0.951$, $P < 0.001$), and 1.1% BSA absorbance = 0.733 * [phloroglucinol] + 0.012 ($r^2 = 0.917$, $P < 0.001$). The slopes of the regressions are significantly different (ANCOVA: $F = 12.67$, $P < 0.001$). However there are no significant differences among the elevations of the regression lines (ANCOVA: $F = 0.412$, > 0.5).


80% Methanol - No PVPP
A
Absorbance at 765 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00
0.05
0.10
0.15
0.20
[phloroglucinol] (%w:v)

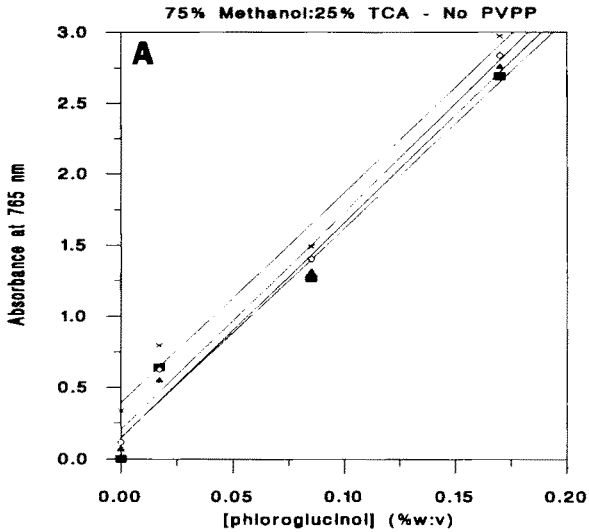

80% MeOH - with PVPP
B
Absorbance at 765 nm
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.05
0.10
0.15
0.20
[phloroglucinol] (%w:v)
0.00% BSA
0.22% BSA
0.44% BSA
1.10% BSA

the compounds measured by the Folin-Ciocalteu analysis (Table 2). It should be noted that these values are likely to be overestimates of the nonphenolic materials since PVPP does not remove 100% of the phenolics present in either solvent (Figures 1B and 2B). In *Fucus gardneri*, the amounts of nonphenolic materials quantified by the Folin-Ciocalteu analysis were not significantly correlated with the concentrations of phenolic compounds (80% methanol: $r = -0.242$, $P > 0.05$; 75% methanol, 25% TCA: $r = 0.038$, $P > 0.05$).

DISCUSSION

None of the three methods used for quantifying brown algal phenolic compounds were without problems. Extracting plants in 80% methanol results in some nonphenolic, Folin-Ciocalteu-reactive substances being included in the measurement of total phenolic compounds. However, it is unlikely that amino acid residues from proteins will be a substantial portion of these compounds because proteins are generally insoluble in high concentrations of organic solvents (Bollag and Edelstein, 1991). Extracting algae in 75% methanol–25% TCA does not improve the precision or accuracy of the Folin-Ciocalteu results

FIG. 2. (A) The results of Folin-Ciocalteu assays of known mixtures of phloroglucinol and bovine serum albumin (BSA) dissolved in 75% methanol–25% TCA prior to the addition of PVPP. The four lines shown in each graph represent the best fit lines for phloroglucinol concentrations versus absorbance ($N = 3$) for each of the four BSA concentrations used. Vertical lines represent ± 1 SE. The regression equations for the lines are: 0% BSA absorbance = 14.7 * [phloroglucinol] + 0.15 ($r^2 = 0.988$, $P = 0.006$), 0.22% BSA absorbance = 15.1 * [phloroglucinol] + 0.15 ($r^2 = 0.990$, $P = 0.005$), 0.44% BSA absorbance = 15.3 * [phloroglucinol] + 0.20 ($r^2 = 0.989$, $P = 0.006$), and 1.1% BSA absorbance = 14.8 * [phloroglucinol] + 0.39 ($r^2 = 0.987$, $P = 0.004$). The slopes of the regressions are not significantly different (ANCOVA: $F = 0.122$, $P > 0.5$). However, there are significant differences among the elevations of the regression lines (ANCOVA: $F = 10.20$, $P < 0.0005$). (B) The results of Folin-Ciocalteu assays of known mixtures of phloroglucinol and bovine serum albumin (BSA) dissolved in 75% methanol–25% TCA after the addition of PVPP. The four lines shown in each graph represent the best fit lines for phloroglucinol concentrations versus absorbance ($N = 3$) for each of the four BSA concentrations used. Vertical lines represent ± 1 SE. The regression equations for the lines are: 0% BSA absorbance = 0.052 * [phloroglucinol] − 0.008 ($r^2 = 0.995$, $P < 0.001$), 0.22% BSA absorbance = 0.050 * [phloroglucinol] + 0.057 ($r^2 = 0.999$, $P < 0.001$), 0.44% BSA absorbance = 0.046 * [phloroglucinol] + 0.095 ($r^2 = 0.998$, $P < 0.001$), and 1.1% BSA absorbance = 0.036 * [phloroglucinol] + 0.173 ($r^2 = 0.916$, $P < 0.001$). Both the slopes (ANCOVA: $F = 3.14$, $P < 0.05$) and elevations (ANCOVA: $F = 46.23$, $P < 0.001$) of the regression lines for the different levels of BSA are significantly different from one another.


75% Methanol:25% TCA - No PVPP
A
Absorbance at 765 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00
0.05
0.10
0.15
0.20
[phloroglucinol] (%w:v)

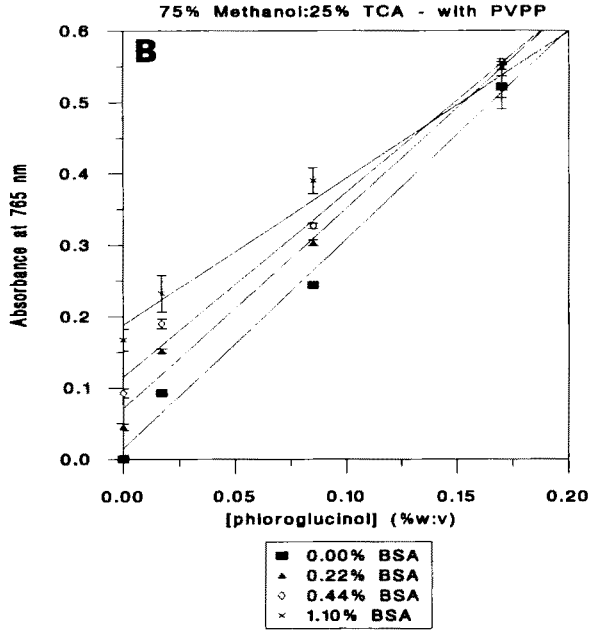

75% Methanol:25% TCA - with PVPP
B
Absorbance at 765 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.00
0.05
0.10
0.15
0.20
[phloroglucinol] (%w:v)
0.00% BSA
0.22% BSA
0.44% BSA
1.10% BSA

TABLE 1. CONCENTRATIONS OF PHLOROTANNINS IN BLADE AND MIDRIB TISSUE OF *Fucus gardneri* FROM FOGARTY CREEK POINT, OREGON[a]

	Phlorotannins as % dry wt (mean ± SE)					
	Extracted in 80% Methanol			Extracted in 75% Methanol:25% TCA		
	Before homogenization	Prior to PVPP addition	After PVPP addition	Before homogenization	Prior to PVPP addition	After PVPP addition
Protected site	3.25 ± 0.16 (N = 10)	7.60 ± 0.20 (N = 10)	7.22 ± 0.20 (N = 10)	4.85 ± 0.26 (N = 10)	7.47 ± 0.28 (N = 10)	7.13 ± 0.26 (N = 10)
Exposed site	2.56 ± 0.24 (N = 7)	7.99 ± 0.40 (N = 7)	7.64 ± 0.47 (N = 7)	4.94 ± 0.56 (N = 7)	7.84 ± 0.49 (N = 7)	7.38 ± 0.51 (N = 7)

[a]Concentrations after PVPP addition were calculated by subtracting the measured concentration of non-phenolic compounds measured by the Folin-Ciocalteu reaction from the concentrations of compounds prior to PVPP addition.

TABLE 2. NONPHENOLIC COMPOUNDS QUANTIFIED BY FOLIN-CIOCALTEU ASSAY[a]

	Extracted in 80% methanol		Extracted in 75% methanol–25% TCA	
	Amount (% dry weight)	Percent of total material measured by Folin-Ciocalteu assay prior to PVPP addition	Amount (% dry weight)	Percent of total material measured by Folin-Ciocalteu assay prior to PVPP addition
Protected Site	0.33 ± 0.03% (N = 10)	4.33 ± 0.40 (N = 10)	0.34 ± 0.03 (N = 10)	4.50 ± 1.13 (N = 10)
Exposed Site	0.25 ± 0.05 (N = 7)	2.94 ± 0.49 (N = 7)	0.41 ± 0.05 (N = 7)	5.39 ± 0.83 (N = 7)

[a]Two pieces of blade and midrib tissue from each of 17 individual *Fucus gardneri* were extracted. The first piece was extracted in 80% methanol–distilled water. The second piece was extracted in 75% methanol–25% TCA. The amounts of nonphenolic material were measured with a Folin-Ciocalteu assay after phenolic compounds were removed from the extracts with PVPP.

at low protein concentrations, and at high protein concentrations may make the assays less accurate. In my experiments, proteins stayed in solution in the methanol–TCA solutions, but readily precipitated out of the aqueous methanol solutions. The presence of high concentrations of proteins in the methanol–TCA solutions later interefered with the Folin-Ciocalteu analysis. Therefore, the use of aqueous methanol as a solvent is preferred over a methanol-TCA mixture.

The concentrations of nonphenolic materials measured with the Folin-Ciocalteu method appear to be a small fraction of the actual phenolic concentrations (Table 2). At least 95–97% of the Folin-Ciocalteu-reactive compounds were phenolic compounds in *Fucus gardneri*. The percentage of phenolic compounds removed by PVPP from *F. gardneri* was larger than the percentage removed from phloroglucinol–BSA mixtures. PVPP may have a greater affinity for complex phlorotannins, such as the polyphenols found in brown algae, than for low-molecular-weight compounds, such as phloroglucinol.

The small concentrations of nonphenolic materials in *Fucus gardneri* were well below the differences in phenolic levels that have been described to occur in brown algae over a variety of spatial and temporal scales (Geiselman, 1980; Steinberg, 1984; Johnson and Mann, 1986; Ilvessalo and Tuomi, 1987; Van Alstyne, 1988, 1989; Tugwell and Branch, 1989). Yates and Peckol (1993) measured concentrations of Folin-Denis-reactive compounds before and after the addition of PVPP in 70% aqueous methanol extracts of *Fucus vesiculosus* from MA. They report that concentrations of nonphenolic, Folin-Denis-reactive

compounds were about 70% of the concentrations of phenolics removed by PVPP. This is substantially higher than the 3–5% concentrations of nonphenolic, Folin-Ciocalteu-reactive compounds reported in *Fucus gardneri* in this study. These differences could be due to species-specific differences in oxidizable, nonphenolic compounds, but they are more likely due to the incomplete binding of phenolics to PVPP, resulting in an underestimate of phenolic concentrations. In my study, there was a high degree of variation in the amounts of phloroglucinol removed by PVPP, suggesting that this method is not able to give consistent results and should be avoided.

Several other methods are routinely used to quantify phenolic compounds and their activity in terrestrial plants. The Prussian blue assay (Price and Butler, 1987) is a chemical assay for quantifying total phenolic compounds that has been used by terrestrial chemical ecologists, but not by marine chemical ecologists. Several protein binding assays (Mole and Waterman, 1987) are also routinely used, although their relevance in marine systems is, at this point, unknown. Brown algal polyphenolic compounds do complex with proteins (Tugwell and Branch, 1992); however, there is currently little evidence to support or refute protein binding as the primary functional role of brown algal polyphenolic compounds.

Measurements of total phenolic levels with chemical methods such as the Folin-Denis or Folin-Ciocalteu assays appear to provide ecologically relevant information about defensive abilities of algal tissue. Measurements of total phenolic compounds with chemical assays have provided strong negative correlations between phenolic levels and herbivore food preferences (Steinberg, 1984, 1985; Johnson and Mann, 1986; Van Alstyne, 1988, 1989; Van Alstyne and Paul, 1990), even though the biological effects of brown algal phenolics are often dependent on their molecular size (Geiselman and McConnell, 1981; Boettcher and Targett, 1993). The data from this study provide some guidelines for the use of the Folin-Denis and Folin-Ciocalteu chemical assays for quantifying levels of brown algal phenolic compounds.

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PRODUCTION OF PHEROMONE BY HAIRPENCIL GLANDS OF MALES OBTAINED FROM INTERSPECIFIC HYBRIDIZATION BETWEEN *Heliothis virescens* AND *H. subflexa* (LEPIDOPTERA: NOCTUIDAE)

PETER E.A. TEAL,* and ANDREA OOSTENDORP

Insect Attractants Behavior and Basic Biology Research Laboratory, USDA PO Box 14565, Gainesville, Florida, 32604

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Abstract—Pheromone produced by the hairpencil glands of interspecific hybrid- and backcross-generation males from crosses between *Heliothis virescens* (F.) with *H. subflexa* (Gn.) was studied. Males of reciprocal F_1 hybrids, all of which had hairpencil glands morphologically similar to those of *H. virescens*, produced neither the same pheromone blend nor amounts of pheromone that were produced by males of *H. virescens*. Instead, these hybrid males produced pheromone that was quantitatively and qualitatively similar to that produced by *H. subflexa*. Hairpencil gland extracts from males obtained from backcrossing F_1 females of either cross to males of *H. subflexa* were the same as those of *H. subflexa*. However, extracts from backcross males of crosses between F_1 females and *H. virescens* were variable. Some extracts from these backcross males were like those of *H. virescens* while others were either like *H. subflexa* or were intermediate between those of the parent species. These results showed that the production of pheromone by the hairpencil glands of hybrid and backcross males is under the dominant regulation of autosomal genes of the *H. subflexa* genome.

Key Words—Hairpencils, sex pheromone, *Heliothis*, autosomes, sex chromosomes.

INTRODUCTION

Heliothis virescens (F.) and *H. subflexa* (Gn.) are closely related species of noctuid moths that can be induced to hybridize in no-choice laboratory condi-

*To whom correspondence should be addressed.

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tions (Laster, 1972; Proshold and LaChance, 1974). However, the two species are reproductively isolated, and there is no evidence for interspecific hybridization in nature. Reproductive isolation between the two species is maintained by both pre- and postmating mechanisms. The principal means by which species integrity and reproductive isolation are maintained is through the use of highly specific blends of female produced pheromones (Tingle et al., 1978; Heath et al., 1991). These pheromones attract males from varying distances and are the first step in the mating ritual. However, the success of mating depends also upon female acceptance of courting males.

Among many moth species, acceptance of males by females is mediated chemically by pheromones released from male hairpencil glands during courtship (Birch and Hefetz, 1987). Males of *H. virescens* partially expose genital hairpencil glands just prior to landing near a female that is releasing pheromone and fully expose these structures just prior to copulation (Teal et al., 1981). Exposure of the hairpencils results in a sudden release of a blend of pheromone components in which hexadecan-1-ol and hexadecan-1-ol acetate are present in greatest amounts (Teal and Tumlinson, 1989). Studies have indicated that effective hairpencil exposure is important for reproductive success (Teal et al., 1981; Teal and Tumlinson, 1989). Similarly, males of *H. subflexa* expose the genital segments prior to attempting to copulate with conspecifics (Cibrian-Tovar and Mitchell, 1991).

Recent studies have shown that inheritance of the hairpencil glands by interspecific hybrid and backcross males is controlled by a dominant gene(s) on the male sex chromosome (Z chromosome) of *H. virescens* (Teal and Oostendorp, 1993). Thus, all hybrid and any backcross males that obtain one of the two Z chromosomes from *H. virescens* have the same type of hairpencil glands as males of *H. virescens* because males of Lepidoptera are homogametic and have two sex chromosomes (denoted ZZ). Given that the hairpencil pheromone is sex specific and that the structure of the hairpencil glands was regulated by the chromosomes, we were interested in knowing if the production of pheromone by these male glands was regulated by sex-linked inheritance or autosomal genes as reported for pheromone production by females of these hybrids (Klun et al. 1982). The following reports the results of studies in which the amounts and ratios of hairpencil pheromone present in extracts from hybrid and backcross males were compared to those present in extracts from males of the parent species.

METHODS AND MATERIALS

Insect Cultures. Both *H. virescens* and *H. subflexa* parent stocks were obtained as pupae from colonies maintained at our laboratory. The founding colonies of both species were begun from larvae collected in fields in Alachua

County, Florida, in 1986. Interspecific hybridizations were conducted by combining five males of one species with five females of the other species in 500-ml cardboard cartons with screen tops. Three cartons were set up each time a cross was conducted. Cartons were inspected daily for the presence of larvae. Approximately equal numbers of larvae from each of the three cartons were reared individually as described by Teal and Oostendorp (1993). All insects were held in environmental chambers having a reversed 14:10 (light–dark) photoperiod at 26°C and 55% relative humidity. Four separate crossing experiments, conducted at different times, were undertaken for each interspecific cross. Female hybrids from each rearing were backcrossed to males of the parent species in a similar manner. Thus, all experiments were replicated four times and hybrid and backcross insects from each crossing experiment were the progeny of at least three matings between different parents. After adult emergence, males were aged for two to four days prior to use. No males from hybrid or backcross lines could be used for crossing experiments because of sterility (Laster, 1972; Proshold and LaChance, 1974; Karpenko and Proshold, 1977).

Hairpencil Gland Excision and Extraction of Pheromone. Studies on the pheromone produced by males of hybrid and backcross insects were conducted using insects sampled during the fifth to sixth hours of the second to fourth scotophase after adult emergence. This time period corresponded to the peak periods of pheromone production by females of *H. virescens* and *H. subflexa* (Heath et al., 1991; Teal et al., 1993).

Tissue used to obtain extracts included the terminal abdominal segments, which are normally retracted into the abdomen. These segments, bearing the hairpencil glands, were exposed by application of pressure to the anterior abdominal segments. The exposed genital segments were then excised and placed into 60 μl of hexane containing 20 ng each of octadecane and nonadecane, internal standards used for quantitation and calculation of relative retention indices. After ca. 10 min, the solvent was removed and stored in a clean microvial at −60°C until analysis. Prior to chemical analysis, the volume of the extracts was reduced to ca. 5 μl under a stream of nitrogen.

Chemical and Data Analysis. Routine chemical analyses were conducted by capillary gas chromatography (GC) using Hewlett Packard 5890 GCs equipped with split/splitless injectors and flame ionization detectors. Capillary columns used for analysis included both apolar phase (SPB1) and polar phase (Supelcowax 10) columns. The fused silica columns were 30 m × 0.25 mm ID and had a film thickness of 0.25 μm. Conditions of chromatography were as follows: initial oven temperature 60°C, oven temperature increased after 2 min at 30°/min, final temperature 200°C. Helium was used as the carrier gas at a linear flow velocity of 18 cm/sec. Individual extracts were injected in the splitless mode with the injector being purged after 0.5 min. Data were acquired with a Nelson 3000 data acquisition system and analyzed by using Nelson 2600 soft-

ware. Quantitative data on the two principal components of the pheromone of *H. virescens*, hexadecan-1-ol (16:OH) and hexadecan-1-ol acetate (16:Ac), were transferred to Lotus 1-2-3 spreadsheets and ratios were calculated. Statistical analyses were performed on these data using NCSS statistical software. For the purposes of this study, we considered the ratio of components in extracts obtained from hybrid and backcross males to be like *H. virescens* if the ratio of 16:AC to 16:OH was 3.5:1 or greater or to be like *H. subflexa* if the ratio was 1:1 or less. Extracts containing more than a total of 125 ng of pheromone were considered to be like *H. virescens* and those containing less than 10 ng were considered to be like *H. subflexa*. All other values were considered to be intermediate between the two species.

Mass spectral analyses were used to confirm the identities of compounds present in extracts obtained from hairpencil glands of the parent species and in samples selected at random from hybrid and backcross insects. A Nermag R1010 MS operated in the chemical ionization mode (methane reagent gas) and interfaced to a Hewlett Packard 5790 GC was used. The SPB1 column used for GC-MS studies was operated as described above.

RESULTS AND DISCUSSION

Parent Species. Extracts of the hairpencil glands from individual males of *H. virescens* analyzed during the present study showed that 16:Ac and 16:OH were the principal components of the pheromone, confirming previous findings (Teal and Tumlinson, 1989). The mean amount of 16:Ac was 286.1 ng (SE = 24.84, N = 15, max = 430.5 ng, min = 128.4 ng) and for 16:OH was 70.4 ng (SE = 8.89, N = 15, max = 127.64 ng, min = 17.5 ng, mean ratio Ac:OH = 4:1). These values were also in agreement with those of earlier work (Teal and Tumlinson, 1989).

Analysis of extracts of the terminal abdominal segments of males of *H. subflexa* did not reveal the presence of any compounds that were unique to this species. Minute amounts of 16:OH (mean = 1.36 ng, SE = 0.36) were found in 18 of the 50 extracts analyzed by GC. Data supporting the presence of 16:OH was obtained from GC-MS analysis of an extract of 15 glands on the basis of retention time and selected ion monitoring using major ions determined from analysis of synthetic 16:OH (m/z 242 = M, 241 = M-1 (parent ion), 225 = (M+1)-18, 224 = M-18). Only six of the 50 samples contained detectable amounts of 16:Ac (mean = 0.39 ng, SE = 0.28) and the presence of the acetate could not be confirmed by GC-MS. The maximum amount of 16:AC plus 16:OH found in an individual extract was 7.3 ng (3.1 ng 16:Ac plus 4.2 ng 16:OH).

F_1 Hybrids. Extracts of the hairpencil glands from both of the hybrid lines

contained small amounts of pheromone. The mean amounts of 16:Ac and 16:OH for *H. subflexa* female × *H. virescens* male (S line) males were 0.8 ng (SE = 0.13, N = 125), and 3.17 ng (SE = 0.29, N = 125) and for *H. virescens* female × *H. subflexa* male (V line) males were 1.01 ng (SE = 0.20, N = 102) and 3.76 ng (SE = 0.35, N = 102), respectively (Figure 1). Neither the mean total amounts of 16:OH plus 16:Ac nor the mean amounts of the individual compounds present in extracts from either hybrid line were greater than that of *H. subflexa* (Fisher's LSD, P = 0.05). Similarly, the mean ratios of 16:OH to 16:Ac for the hybrid groups were the same as that of *H. subflexa*. Although four extracts from V line males and six extracts from S line males contained more 16:Ac than 16:OH, the ratios from these individual extracts were not different from the highest ratio of 16:Ac to 16:OH (3.1 to 4.2, 16:Ac to 16:OH) found in an individual extract from *H. subflexa* (χ^2, P = 0.05).

These data indicate that both the blend and amounts of 16:Ac and 16:OH present in hairpencil glands were the same as that present in extracts of *H. subflexa*. Therefore, although the presence and structure of the hairpencil glands are regulated by dominant genes on the Z chromosome of *H. virescens* (Teal and Oostendorp, 1993), the male sex chromosome (Z chromosome) of this species does not exert a dominant effect on pheromone production. Although the data implied that regulation of both the amount and ratio of pheromone components produced by hybrids is controlled by dominant genes in the *H. subflexa* genome, we could not determine if the genes were sex linked or auto-

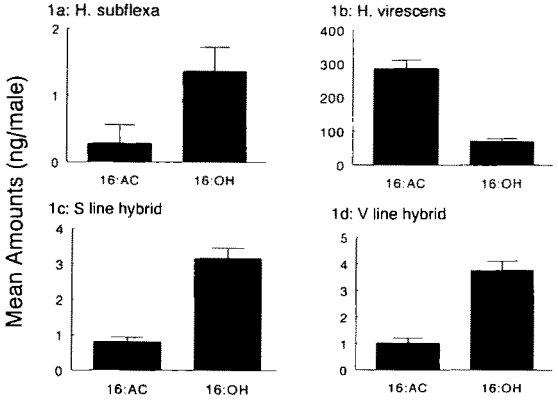

FIG. 1. Comparison of mean amounts (±SE) and ratios of 16:Ac and 16:OH present in extracts obtained from hairpencil glands of males of S line F_1 (N = 125) and V line F_1 hybrids (N = 102) with those found in extracts from males of *H. virescens* and *H. subflexa*.

somal because males of Lepidoptera are homogametic and the hybrid males obtained a Z chromosome from each of the parent species (Table 1).

Backcross 1 Generations. Extracts of males obtained from backcrossing hybrid V line F_1 females to *H. subflexa* (V line female × *H. subflexa* male = VA line) or S line F_1 females to *H. subflexa* (S line female × *H. subflexa* male = SD line) contained the same amounts and ratios of 16:OH and 16:Ac as those obtained from *H. subflexa* males (Figure 2, Table 1). These results were expected because the chromosomal make up of both hybrid lines was either the same as that of the F_1 males or males of *H. subflexa* (Table 1). Although these results confirmed that production of 16:OH and 16:Ac was regulated by dominant genes in the *H. subflexa* genome, they did not indicate if the genes were sex linked.

Extracts obtained from males of backcrosses between V line females and *H. virescens* males (VB line) contained variable amounts of 16:Ac and 16:OH (N = 75) (Figure 2, Table 1). Only six of the extracts contained combined amounts of the two compounds that were greater than 100 ng. These males were considered to be able to produce as much pheromone as males of *H. virescens*. Seven of the extracts contained amounts equivalent to those from *H. subflexa*.

TABLE 1. EFFECT OF HYBRIDIZATION AND BACKCROSSING ON RATIO AND TOTAL AMOUNT OF PHEROMONE IN EXTRACTS OF HAIRPENCIL GLANDS OF MALES[a]

Insect line	Male sex chromosomes[b]	Phenotypes if sex linked, *H. subflexa* dominant	Observed phenotypes for total amount	Observed phenotypes for ratio of 16:Ac and 16:OH
Parent, *H. virescens*	ZvZv	all V type	all V type	all V type
Parent, *H. subflexa*	ZsZs	all S type	all S type	all S type
S line hybrid	ZvZs	all S type	125 S type	125 S type
V line hybrid	ZvZs	all S type	102 S type	102 S type
BC1 SC line	ZvZv	all V type	17 V type[c]	12 V type[c]
			39 I type	34 I type
			6 S type	16 S type
BC1 SD line	ZvZs	all S type	35 S type	35 S type
BC1 VA line	ZsZs	all S type	30 S type	30 S type
BC1 VB line	ZvZs	all S type	6 V type[c]	13 V type[c]
			62 I type	36 I type
			7 S type	26 S type

[a]Phenotype designations are: V = *H. virescens* type, S = *H. subflexa* type, I = intermediate between parental types.

[b]Zv = *H. virescens* Z chromosome, Zs = *H. subflexa* Z chromosome.

[c]There is a difference in these cells between the observed frequency and the frequency expected if the trait were linked to the Z sex chromosome.

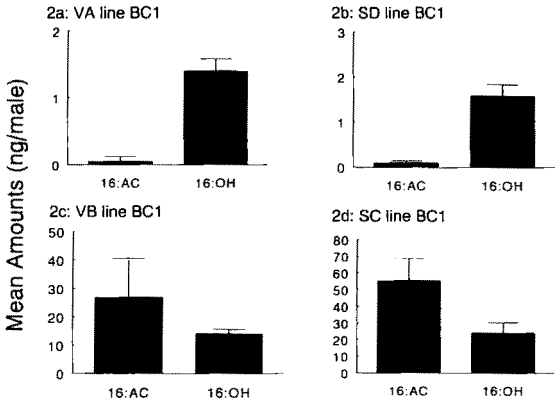

FIG. 2. Mean amounts (±SE) and ratios of 16:Ac and 16:OH present in extracts obtained from hairpencil glands of VA line (N = 30), SD line (N = 35), VB line (N = 75), and SC line (N = 62) backcross 1 generation males.

The remaining 62 extracts contained amounts of these two components that were intermediate between those of *H. subflexa* and *H. virescens*.

Thirteen of the extracts from VB line males contained a 16:Ac to 16:OH ratio similar to that of *H. virescens* (i.e., 4:1) and of these, five contained total amounts greater than 100 ng, while the remaining eight had intermediate amounts of pheromone. Twenty-six extracts contained the same ratio of 16:Ac to 16:OH that was found in *H. subflexa*. The remaining 36 extracts contained ratios of 16:Ac to 16:OH that were intermediate between those of the parent species.

The data obtained from VB line backcross males indicated that genes responsible for the regulation of production of both amounts of 16:Ac and 16:OH and ratios of these compounds were not sex linked. The V line hybrid females obtained their Z chromosome from *H. subflexa*. Thus, backcrossing these females to *H. virescens* males resulted in male progeny that obtained one Z chromosome from *H. virescens* and the other from *H. subflexa* (Table 1). These backcross males had the same sex chromosome makeup as V line hybrid males. If the genes were sex linked, then all of the VB line males should have produced the same amount and ratio of 16:Ac to 16:OH that was produced by males of V line hybrids and *H. subflexa*. However, a significant number of extracts had amounts and ratios of 16:Ac and 16:OH that were intermediate between those of the parent species and others were like *H. virescens*.

Backcrossing S line hybrid females to *H. virescens* (SC line) resulted in males (N = 62) that produced both 16:Ac and 16:OH. However, both the amounts and ratios of the two compounds were variable (Figure 2, Table 1). Seventeen of the extracts contained combined amounts of 16:Ac and 16:OH

equivalent to that of *H. virescens*. Six extracts contained amounts as low as those present in *H. subflexa* extracts, and the remaining 39 contained intermediate amounts of pheromone.

Extracts of 12 SC line males contained the same ratio of 16:Ac to 16:OH as was present in males of *H. virescens* and, of these, seven had as much pheromone as *H. virescens* (Table 1). Another group of 16 extracts contained a ratio of 16:Ac to 16:OH that was like *H. subflexa*. The remaining 34 were intermediate between the two parental ratios.

If the genes regulating the ratio and amount of pheromone were sex linked, then all SC line males should have been identical to *H. virescens* because SC line males obtained both of their Z chromosomes from *H. virescens* (Table 1). However, as occurred with extracts from VB line males, a large number of extracts contained intermediate amounts and ratios of the two components. Additionally other extracts were the same as extracts from males of *H. subflexa*. Therefore, the regulation of both the ratio and amounts of 16:Ac and 16:OH produced by the hairpencil glands of interspecific hybrid and backcross males is controlled by autosomal genes. Additionally, the data from analyses of extracts of both the VB and SC line backcross males show that more than one gene is responsible for regulation of each trait. If single genes were responsible for regulating either trait, then 50% of the populations of males from backcrossing females of either hybrid line to *H. virescens* should have been like this species and the other half should have been like *H. subflexa*. However, approximately 50% of the males from both VB and SC backcross lines were intermediate for both traits (Table 1).

These results, coupled with those from other studies on the regulation of the pheromone-mediated biology of interspecific hybrids between *H. virescens* and *H. subflexa*, indicate that different aspects of the communication system are regulated in different ways. For example, studies on the sex pheromones produced by female hybrids from crosses between *H. subflexa* females and *H. virescens* males showed that these females produced the same blend of pheromone components as do females of *H. virescens* (Klun et al., 1982). This indicates that the regulation of pheromone production by females of this hybrid line appears to be under the dominant regulation of the *H. virescens* genome (Klun et al., 1982). Similarly, both the presence and structure of the male *H. virescens*-type hairpencil glands are dominantly regulated by the *H. virescens* genome (Teal and Oostendorp, 1993). However, in this case, the genes reside on the Z sex chromosome, whereas genes controlling production of the female pheromone are autosomal. The results of this study show that the production of the male hairpencil pheromone in hybrids is under the dominant regulation of autosomal genes of *H. subflexa*. These results, in conjunction with previously published work on *Heliothis*, demonstrate that control of the pheromone communication system in *Heliothis* moths is regulated by a complex genetic system

and support the hypothesis that genes controlling different aspects of the system are not closely linked and are inherited independently in Lepidoptera (Lofstedt et al., 1989).

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STRUCTURE–ACTIVITY RELATIONSHIP OBSERVATIONS OF *trans*-TRIMEDLURE ENANTIOMERS

J.D. WARTHEN, JR.,[1]* W.F. SCHMIDT,[2] R.E. DOOLITTLE,[3] and R.T. CUNNINGHAM[4]

United States Department of Agriculture, Agricultural Research Service

[1]*Insect Chemical Ecology Laboratory, BARC-W*
Beltsville, Maryland 20705

[2]*Environmental Chemistry Laboratory, BARC-W*
Beltsville, Maryland 20705

[3]*Insect Attractants, Behavior, and Basic Biology Research Laboratory*
Gainesville, Florida 32604

[4]*Tropical Fruit & Vegetable Research Laboratory*
Hilo, Hawaii 96720

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Abstract—*tert*-Butyl 4- (and 5-) chloro-*trans*-2-methylcyclohexane-1-carboxylate (TML), a mixture of four major *trans* and four minor *cis* isomers, is used as an attractant for detecting and monitoring the male Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann). The eight isomers (racemic mixtures) were isolated by HPLC, and their relative attractiveness in the field was determined. A quantitative structure–activity relationship (QSAR) was proposed that related a molecular measurement ($Å^3$) of the TML structure to male medfly captures. More recently, the *trans*-TML enantiomers were synthesized in sufficient quantities for field testing. This paper reports the computer-aided molecular modeling, via Chem-X, of the *trans*-TML enantiomers and the staggered and superimposed fitting with the most attractive isomer, (1*S*,2*S*,4*R*)-TML-C, to determine common volumes and surface areas from Van der Waals (VdW) maps. Observations of structure–activity relationships (SAR) are reported for the staggered fittings.

Key Words—Insecta, *Ceratitus capitata*, medfly, trimedlure, enantiomer, attractant, lure, SAR, VdW, Chem-X.

*To whom correspondence should be addressed.

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INTRODUCTION

tert-Butyl 4- (and 5-) chloro-*trans*-2-methylcyclohexane-1-carboxylate (TML), is a synthetic mixture of 90–95% of four *trans* isomers (Beroza et al., 1961; McGovern et al., 1986). Each of these isomers exists as two enantiomers (Sonnet et al., 1984; Doolittle et al., 1991); the racemic isomers are designated A, B1, B2, and C (McGovern and Beroza, 1966). TML is used as an attractant for detecting and monitoring the male medfly, *Ceratitis capitata* (Wiedemann), which is a worldwide pest of fruits, nuts, and vegetables (Hagen et al., 1981; Jackson and Lee, 1985).

The stereochemical structural assignments of each of the *trans* (McGovern and Beroza, 1966) and *cis* racemic isomers (McGovern et al., 1986) and the GLC separation of these eight racemic isomers (Leonhardt et al., 1982; Warthen and McGovern, 1966; Beroza and Sarmiento, 1964) paved the way for their semipreparative HPLC separation (Warthen and McGovern, 1988) in sufficient quantities to determine relative medfly attractiveness (McGovern et al., 1990) in the field. The biological data permitted the postulation of a structure/medfly-attractiveness relationship via computer molecular modeling that related a molecular measurement ($Å^3$) to medfly catch (Warthen et al., 1993):

$$\text{Medfly catch} = (\text{molecular volume/molecular surface area}) \times (\cos abcd + 0.895)^2 \times \{[(t\underline{C}H_3\text{-}6C) + (Cl\text{-}2\underline{C}H_3)/2] - 5.221\}^2$$

Essentially, this equation states that optimal medfly catch of the eight-isomer racemic TML series is determined by an optimal ratio of molecular volume to surface area, but the molecular surface area is modulated by an optimal torsion angle (*abcd*) and an optimal average of two interatomic distances. The index or independent variable in terms of the molecular measurement determines the dependent variable of medfly catch; a linear best-fit line results ($R^2 = 0.939$, $N = 8$, $P < 0.01$).

It has been known for some time that the most attractive of the eight racemic TML isomers was *trans*-TML-C (McGovern et al., 1966, 1987), or more specifically the (1*S*,2*S*,4*R*)-TML-C enantiomer (Sonnet et al., 1984). The recent report by Doolittle et al. (1991), involving the stereospecific chiral synthesis of the eight *trans*-TML enantiomers, made possible the determination of the relative field attractiveness of these substances. It has also afforded an opportunity to study the SAR of the *trans*-TML enantiomers in light of our previous work (Warthen et al., 1993) with TML racemic isomers.

Therefore, this paper reports an examination of the eight *trans*-TML enantiomers via computer molecular modeling so as to relate structural characteristics to medfly attractiveness.

METHODS AND MATERIALS

Lowest energy conformations of *trans*-TML enantiomers were determined using molecular mechanics (Burkert and Allinger, 1982; Clark, 1985; Warthen et al., 1993) with modified force-field parameters (including Gasteiger charges) of the computer program Chem-X (January 1991 VAX version, Chemical Design Ltd, Oxford, England).

The mirror images of the lower-energy molecular mechanics (MM) minimized *trans*-TML enantiomers (Warthen et al., 1993) were constructed via the Crystal Symmetry mode in Chem-X. All enantiomers were then compared to (1*S*,2*S*,4*R*)-TML-C, which was the most attractive TML enantiomer for medflies. Comparison of each *trans*-TML enantiomer with (1*S*,2*S*,4R)-TML-C was accomplished in a staggered and a superimposed fitting so that carbon 1 of the cyclohexane ring was directly below the carbon 1 of (1*S*,2*S*,4*R*)-TML-C or superimposed with carbon 1 of (1*S*,2*S*,4*R*)-TML-C, respectively.

The staggered fit was achieved by placing ring carbons of the isomer directly above or below the ring carbons of (1*S*,2*S*,4*R*)-TML-C so that the substituted axial groups would be parallel (Figure 1). Dummy planes with accompanying dummy atoms (P1 and P2) were placed in both enantiomers utilizing carbons 6, 2, and 4 in (1*S*,2*S*,4*R*)-TML-C and carbons 3, 5, and 1 in (1*R*,2*R*,4*S*)-TML-C. A view was then set through P1 and carbon 4 for (1*S*,2*S*,4*R*)-TML-C; and a view was set through P2 and carbon 1 for (1*R*,2*R*,4*S*)-TML-C.

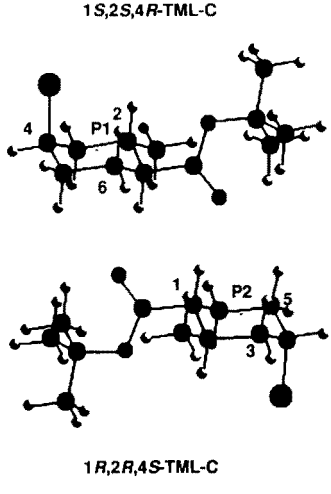

FIG. 1. Calculated lowest energy MM minimized (1*S*,2*S*,4*R*)-TML-C and (1*R*,2*R*,4*S*)-TML-C with dummy atoms P1 and P2.

Another view was set through carbons 6 and 2 for (1*S*,2*S*,4*R*)-TML-C; and a view was set through carbons 3 and 5 for (1*R*,2*R*,4*S*)-TML-C. (1*R*,2*R*,4*S*)-TML-C was then rotated about the *y* axis 180°. In this manner, the dummy planes were oriented perpendicular to the *y* axis and parallel to the *x* axis. A rigid fit between the two enantiomers was then performed by specifying connection between P1 and P2 (Figure 2). The superimposed dummy atoms were then deleted. The remaining six enantiomers were each fitted to (1*S*,2*S*,4*R*)-TML-C in a similar fashion.

The superimposed fitting was implemented by specifying carbons 5, 3, and 4 of (1*S*,2*S*,4*R*)-TML-C and carbons 3, 5, and 4 of (1*R*,2*R*,4*S*)-TML-C (Figure 3) and performing a rigid fit (Figure 4). The remaining six enantiomers were each fitted to (1*S*,2*S*,4*R*)-TML-C in a similar fashion.

A Gasteiger charge was then placed upon the staggered, fitted pair (Figure 2). After a contour density of 3 contours/Å was selected, a Van der Waals (VdW) map was constructed for (1*S*,2*S*,4*R*)-TML-C as map 1; then, a similar map was drawn for (1*R*,2*R*,4*S*)-TML-C as map 2. The two maps were combined after setting the logical cutoff at 0.95 with a Chem-X ".AND. A MAP" selection to create map 3 (Figure 5). After redrawing maps 1 and 2, the volumes and surface areas were displayed and recorded (Table 1). A similar procedure was used to construct the VdW maps of the superimposed fitted pair of the same enantiomers. Similar maps were constructed for the remaining six staggered and six superimposed, fitting pairs of the other enantiomers and (1*S*,2*S*,4*R*)-TML-C.

Simple linear equations were used to fit the data points from the scatter plots via the linear least-squares method (sum of the square of the errors was minimized) to determine the best fit. These were performed on medfly catch

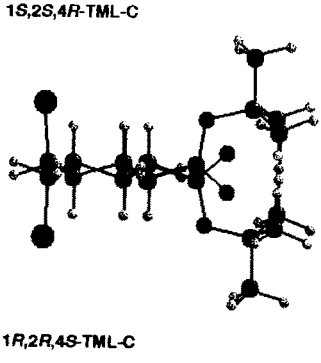

FIG. 2. Staggered fitting of calculated lowest energy MM minimized (1*S*,2*S*,4*R*)-TML-C and (1*R*,2*R*,4*S*)-TML-C.

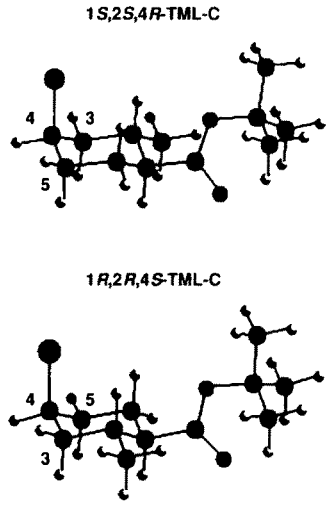

FIG. 3. Calculated lowest energy MM minimized (1*S*,2*S*,4*R*)-TML-C and (1*R*,2*R*,4*S*)-TML-C for three-point superimposed fitting.

(Doolittle et al., 1991) vs six observations consisting of common volume, common surface area, and common volume/common surface area ratio for the staggered and superimposed fittings with (1*S*,2*S*,4*R*)-TML-C (Table 1).

RESULTS AND DISCUSSION

The previous work (Warthen et al., 1993) on the medfly quantitative structure–activity relationship (QSAR) of the eight racemic TML isomers revealed that the molecular volume of each isomer was extremely important to the relative medfly attractancy of the eight isomers. This total molecular volume was modulated by the surface area, a torsion angle, and an average of two interatomic distances in order to relate it to medfly catch (Warthen et al., 1993). Since the *trans*-TML enantiomers were synthesized and their medfly field attractancy was determined (Doolittle et al., 1991), a more specific measure of molecular volume characteristic for each enantiomer was permissible, namely, that volume of each enantiomer that was common to the most active enantiomer, (1*S*,2*S*,4*R*)-TML-C, when compared in a specific type of fitting. That fitting was determined by considering the stereochemistry of the most active enantiomer, (1*S*,2*S*,4*R*)-TML-C, which was used as a standard for optimal stereochemistry, atomic, and electrostatic potential for attractancy.

The importance of the stereochemical position of the equatorial 1-*t*-butyl

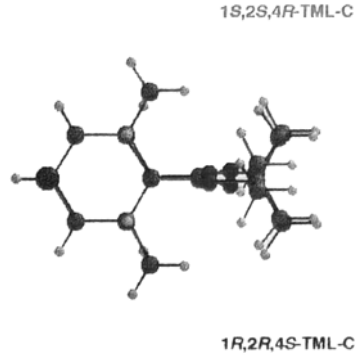

FIG. 4. Superimposed fitting of calculated lowest energy MM minimized (1*S*,2*S*,4*R*)-TML-C and (1*R*,2*R*,4*S*)-TML-C.

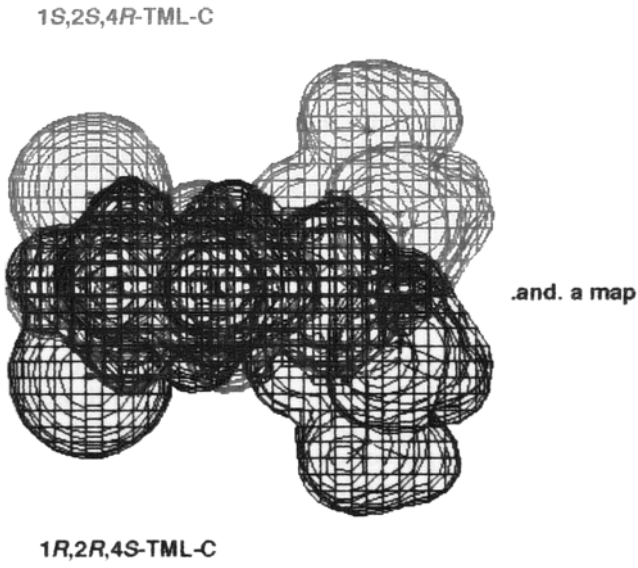

FIG. 5. VdW map of staggered fitting of calculated lowest energy MM minimized (1*S*,2*S*,4*R*)-TML-C and (1*R*,2*R*,4*S*)-TML-C.

carboxy, the equatorial 2-methyl, and the axial 4-chloro of (1*S*,2*S*,4*R*)-TML-C for attractancy is obvious. When the orientation of the calculated lowest energy MM minimized (1*S*,2*S*,4*R*)-TML-C is viewed as in Figure 1, then the upper face of the molecule would have to approach a medfly antennal receptor in order

TABLE 1. CHANGE IN VOLUME AND SURFACE AREA MEASUREMENTS OF CALCULATED LOWEST ENERGY MM MINIMIZED *trans*-TML ENANTIOMERS DETERMINED BY COMPARISON WITH (1*S*,2*S*,4*R*)-TML-C[a,b] VIA STAGGERED/SUPERIMPOSED FITTINGS AND VdW MAPS

		Staggered fitting				Superimposed fitting		
TML enantiomer[b]	MFC[c]	ΔVol (Å^3)	ΔSA (Å^2)	ΔVol/ ΔSA	100 × ΔVol / Vol (1*S*,2*S*,4*R*)-C[a,b]	ΔVol (Å^3)	ΔSA (Å^2)	ΔVol/ ΔSA
(1*R*,2*R*,5*S*)-A	172.45c	119.04	127.87	0.9309	63.906	159.28	158.06	1.0077
(1*S*,2*S*,5*R*)-A	78.25d	111.56	122.15	0.9133	59.892	165.55	164.47	1.0066
(1*R*,2*R*,5*R*)-B1	50.01de	112.37	121.83	0.9224	60.327	154.08	154.97	0.9942
(1*R*,2*R*,4*S*)-C	46.79de	113.22	123.29	0.9183	60.785	169.87	167.08	1.0167
(1*R*,2*R*,4*R*)-B2	29.64de	110.58	120.64	0.9166	59.364	151.35	151.72	0.9976
(1*S*,2*S*,5*S*)-B1	23.66e	104.09	115.71	0.8995	55.882	171.62	169.44	1.0129
(1*S*,2*S*,4*S*)-B2	23.37e	104.44	115.58	0.9036	56.067	162.72	161.55	1.0072

[a] Comparison with the most medfly-attractive enantiomer: (1*S*,2*S*,4*R*)-TML-C[b], MFC = 502.57_a[c], Vol = 186.27, SA = 184.92.
[b] Doolittle et al. (1991).
[c] Medfly catches in this column and in (1*S*,2*S*,4*R*)-TML-C[b] followed by the same letter are not significantly different (Doolittle et al., 1991).

to optimize the stereochemical fit of these three groups. The chlorine atom and the majority of the *t*-butyl carboxy are above the plane of the cyclohexane ring, and the methyl group is accessible for binding in the equatorial position.

Since the *t*-butyl carboxy group is the bulkiest, we compared each *trans*-TML enantiomer with the standard in a staggered (Figure 2) and a superimposed fitting (Figure 4), so that carbon 1 of the cyclohexane ring was directly below the carbon 1 of (1*S*,2*S*,4*R*)-TML-C or superimposed with carbon 1 of (1*S*,2*S*,4*R*)-TML-C, respectively. The staggered fitting was chosen as a comparison to the superimposed fitting, but also so that the puckered cyclohexane rings would be in the same plane with their axial groups parallel to one another.

Those volumes, surface areas, and volume–surface area ratios common to (1*S*,2*S*,4*R*)-TML-C from the staggered and superimposed fittings of the *trans*-TML enantiomers are reported in Table 1. Simple linear regression (Kilpatrick, 1987; Freund and Walpole, 1980; Hayslett, 1968) for seven data points of the six observations revealed regression lines for Δ volume ($R^2 = 0.693$, $N = 7$, $P < 0.05$), Δ surface area ($R^2 = 0.710$), and Δ volume/Δ surface area ($R^2 = 0.567$) from staggered fittings and $R^2 < 0.02$ for the same three observations from superimposed fittings. It is obvious that the staggered fitting produces the better regression lines. The regression lines obtained from Δ volume and Δ surface area from staggered fittings are each more important than the regression line from Δ volume/Δ surface area with its lower R^2. A factor that may be involved is that the common surface area is actually a measurement of the surface of map 3 and not "available" surface area as related to attractancy.

Simple linear regression of the log weighted mean male medfly catch versus Δ volume or Δ surface area from staggered fittings (Figure 6) revealed $R^2 = 0.815$ or $R^2 = 0.840$, respectively. The log scale of the male medfly catch was more linear with Δ volume or Δ surface area than the numerical male medfly catch scale. Thus, the sensitivity of the male medfly catch to small changes in Δ volume or Δ surface area at a molecular level was even more significant than with a numerical scale.

The best information on the attractancy of any one isomer comes from the most active isomer. Attractancy of less active isomers reinforces the structural basis of this activity and determines what structurally hinders attractiveness. Comparing the attractiveness of the isomers makes it possible to see the direct effects of Δ volume and Δ surface area from staggered fittings with (1*S*,2*S*,4*R*)-TML-C without modulating with angles and distances. The logical input of these descriptors based on chemistry (Warthen et al., 1993) was used to determine the independent variable for the *x* axis (Figure 6), resulting in a type of principal components analysis. Therefore, since stepwise multiple regression (Topliss and Edwards, 1979; Dunn et al., 1984) was not specifically used, it was not possible (Hansch, 1991) to evaluate the probability for the chance correlations. We believe that each of the observed R^2 (Figure 6), without the

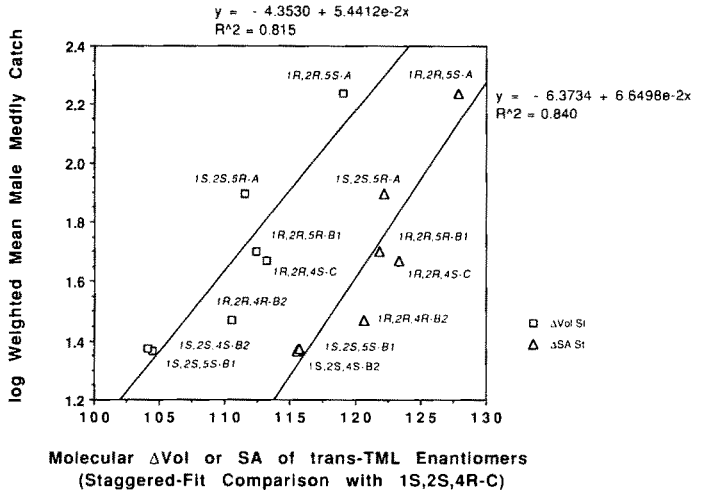

FIG. 6. Simple linear regression of log weighted mean male medfly catch versus common molecular volume ($Å^3$) or surface area ($Å^2$) of calculated lowest energy MM minimized *trans*-TML enantiomers determined by staggered-fit comparison with calculated lowest energy MM minimized (1*S*,2*S*,4*R*)-TML-C.

angle and distance modulators (Warthen et al., 1993), only substantiates the observed relationship between male medfly catch and Δ volume or Δ surface area, rather than indicating chance correlations. The Δ volume and Δ surface area are not proposed as absolute keys to activity, but they are variables that reflect the atoms, the charge on the atoms, the torsion angles, the interatomic distances, the stereochemical nature, etc., in that volume or on that surface.

CONCLUSIONS

Chirality is a factor in SAR that is usually addressed by comparing related isomers of a series by superimposed fitting. However, in the case of the *trans*-TML enantiomers, it was necessary to compare them via staggered fitting of the cyclohexane carbons with the carbon 1 being directly above and below one another to show the SAR. Computer-aided molecular modeling, via Chem-X, of *trans*-TML enantiomers with staggered fitting with (1*S*,2*S*,4*R*)-TML-C and determination of common volumes from VdW maps and subsequent simple linear regression of data resulted in the observation that Δ volume and Δ surface area were directly proportional to the log of the medfly catch if one related the statistical significance of the catches to these molecular measurements. There was a sharp decrease in medfly attactiveness when the Δ volume of a *trans*

enantiomer is $<64\%$. Small changes in the optimal Δ volume or Δ surface area had a much greater effect when male medfly catch was high than when male medfly catch was low (non-optimal Δ volume or Δ surface area). The chirality of (1*S*,2*S*,4*R*)-TML-C was important for optimal activity in this series of enantiomers, but it does not explain why there is activity with the other enantiomers. It is proposed that changes in Δ volume or Δ surface area among the enantiomers are the basis for the differences in their biological activity.

This work and previous research (Warthen et al., 1993), it is hoped, are establishing the groundwork for the use of computer molecular modeling in explaining SAR of agricultural compounds that exhibit behavioral responses, similar to the well-established work that has been done in the pharmaceutical industry. The goal of this tool in agriculture is to be predictive in the design of behavioral substances. Future molecular modeling is planned for possible further elaboration of the TML medfly SAR when *cis*-TML enantiomers are synthesized and field attractancy data become available. Other on-going research is being carried out on other behavioral substances also.

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EFFECTS OF FERTILITY ON BIOMASS, PHYTOTOXICITY, AND ALLELOCHEMICAL CONTENT OF CEREAL RYE

VASEY N. MWAJA,[1] JOHN B. MASIUNAS,[1,*] and LESLIE A. WESTON[2]

[1]*Department of Horticulture*
University of Illinois at Urbana-Champaign
Urbana, Illinois 61801

[2]*Department of Horticulture and Landscape Architecture*
University of Kentucky
Lexington, Kentucky 40546

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Abstract—Studies were conducted to evaluate biomass production, tissue phytotoxicity, and allelochemical content of rye (*Secale cereale* L.) shoots grown in three fertility regimes (low, medium or high) in the greenhouse. Wheeler rye or a polyculture of rye and hairy vetch (*Vicia villosa* Roth) grown with high fertility produced the greatest biomass (78.7 and 82.7 g), with the lowest root-to-shoot ratio (0.22 and 0.43) produced in the high-fertility treatments. The polyculture treatment grown with low fertility had a greater proportion of hairy vetch (18%) than when grown with high fertility (6%). Rye shoot residue phototoxicity was affected by fertility regime. Radicle elongation of cress (*Lepidium sativum* L.) and barnyardgrass [*Enchinochloa crus-galli* (L.) Beauv.] was inhibited by rye shoot residues in a modified Parker bioassay. Rye shoot residue grown with low fertility was less inhibitory to cress radicle elongation than rye residue from the medium- or high-fertility regimes. Extracts of dried rye shoots grown with high fertility were less inhibitory than extracts from other fertility regimes. The concentrations of ether extracts of rye causing 50% inhibition (I_{50}) of cress radicle elongation were between 125 and 276 μg/ml for greenhouse-grown and 60 and 138 μg/ml for the field-grown rye shoots. The major phytotoxic compounds in the rye shoot extracts were identified as DIBOA and BOA. The concentration of DIBOA in the greenhouse-grown rye shoots ranged between 128 and 423 μg/g while BOA concentration ranged between 2.5 and 31 μg/g. DIBOA and BOA levels were lowest in rye shoots grown with high fertility. Correlations between rye shoot biomass,

*To whom correspondence should be addressed.

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DIBOA and BOA concentration, and cress barnyardgrass radicle length were significant.

Key Words—*Secale cereale* L., *Vicia villosa* Roth, allelopathy, phytotoxicity, fertility regime, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one, 2(3H)-benzoxazoline, reduced tillage, weed suppression

INTRODUCTION

Rye (*Secale cereale* L.) is a winter-hardy, erect, herbaceous plant that grows well on marginal soils (Bushuk, 1976; Nuttonson, 1958). It is a long-day plant capable of developing an extensive root system that reduces erosion and nutrient loss (Nuttonson, 1958). In the Midwestern United States, rye is widely grown as a cereal, interseeded with legumes in winter pastures, or as a green manure crop (Amador and Gliessman, 1990).

Rye and its residues reduce weed and crop growth by modifying the microenvironment and releasing allelochemicals (Putnam et al., 1983; Rice, 1984). Rye produces a dense canopy, which is more competitive than weeds for light, moisture, and nutrients. In addition, rye residues on the soil surface reduce weed germination and growth by shading, lowering soil temperatures, moderating diurnal temperature fluctuations, and acting as a physical barrier (Barnes and Putnam, 1986; Putnam et al., 1983; Rice, 1984; Vidal et al., 1994). Rye and its residues can release allelochemicals that accumulate near the soil surface inhibiting seed germination and seedling growth (Barnes and Putnam, 1983; Barnes et al., 1987).

Environmental factors influence allelochemical production in higher plants and also related biological activity (Putnam, 1988; Rice, 1984; Tukey, 1969). For example, increased air temperatures enhanced allelochemical concentration and consequent inhibition of plant growth (Putnam and Tang, 1986; Rizvi and Rizvi, 1992). Stowe and Osborne (1980) reported reduced plant growth and increased allelochemical concentrations due to limited nutrient availability. Fast-growing plants in fertile environments contained lower allelochemical concentrations than slow growing species in areas of limited ferility. Besides affecting allelochemical concentration, allelopathy was enhanced in soils of low fertility (Stowe and Osborne, 1980).

The phytotoxicity of rye and its residues has been attributed to cyclic hydroxamic acids, phenylacetic acid, 4-phenylbutryic acid, and a complex of benzoic and cinnamic acids (Barnes and Putnam, 1986; Einhellig and Leather, 1988; Putnam and Tang, 1986; Rizvi and Rizvi, 1992; Shilling et al., 1985). Two benzoxazinones, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and its decomposition product, 2(3H)-benzoxazolinone (BOA), have been identified in extracts of rye shoots and their allelopathic activity characterized using cress

radicle elongation (*Lepidium sativum* L.) as a bioassay (Barnes and Putnam, 1987; Barnes, et al., 1987; Gagliardo and Chilton, 1992; Nair et al., 1990). DIBOA and BOA were more toxic to seedling growth than were other allelochemicals from rye residues (Barnes et al., 1987). In intact plant tissues, DIBOA exists as a glycoside (Hofman and Hofmanova, 1971). β-Glycosidases released upon injury to the plants rapidly hydrolyze the glycoside to release DIBOA, which decomposes in water to form BOA. Additional allelochemicals may arise by microbial transformation of compounds from rye residues. Nair et al. (1990) isolated and characterized a compound [2,2'-oxo-1,1'-azobenzene (AZOB)] produced by microorganisms in the presence of rye residues. AZOB was more toxic to curly cress and barnyardgrass than either DIBOA or BOA. Although allelochemicals have been identified, their significance and role in the inhibition of weed growth by rye residues still remain unclear.

Rye was seeded alone or interseeded with hairy vetch (*Vicia villosa* Roth) in field and greenhouse experiments in an effort to determine how fertility influences allelopathy. We hypothesized that allelochemicals from rye residues are partially responsible for reductions in weed growth observed in previous studies with monocultures and polycultures (Masiunas, et al., 1994; Mwaja, 1994). Our objectives in these experiments were to: (1) determine the effect of low-, medium-, or high-fertility regimes on biomass production and rye allelopathic activity; and (2) isolate, identify, and quantify the allelochemicals in rye shoots grown under selected fertility regimes.

METHODS AND MATERIALS

Rye and Hairy Vetch Biomass Production. A greenhouse experiment was conducted to investigate factors that influenced biomass production of winter rye and hairy vetch. The cropping systems evaluated were: (1) a monoculture of rye, (2) a monoculture of hairy vetch, and (3) a polyculture of rye and hairy vetch. Wheeler rye and hairy vetch were seeded into sterilized silica quartz sand in 38-liter plastic containers at rates equivalent to 112 and 34 kg/ha, respectively, for monoculture treatments and similar rates for polyculture treatments. Prior to planting, the silica sand was prewashed first with deionized water and then with twice the volume of the appropriate nutrient solution (Osborne and Wellington, 1981).

Eight days after seeding (two days after germination), three fertility regimes were initiated. The nutrient solutions utilized were dilutions of Hoagland and Arnon (1950) solution, which contained either: (1) low fertility, 5 mM NO_3^-, 0.5 mM P, and 3 mM K; (2) medium ferility, 10 mM NO_3^-, 1 mM P, and 6 mM K; or (3) high ferility, 20 mM NO_3^-, 2 mM P, and 12 mM K. All nitrogen was in the form of nitrate. Other nutrients, including S, Ca, Mg, and micro-

nutrients (i.e., Fe, Cl, Mn) were added in similar proportions as N, P, and K. The pots were fertilized every other day with 250 ml (from 8 to 37 days) or 500 ml (from 38 to 74 days) of the nutrient solutions, and watered daily with deionized water to prevent wilting. Once a week, an excess of deionized water (approximately 1 liter) was applied to flush salts from the sand cultures.

After 75 days, when rye was 45–50 cm tall, the experiment was terminated. Rye and hairy vetch shoots were harvested separately. Roots were washed with running water to remove any adhering sand. Shoot and root biomass was determined after drying at 45°C for seven days. Dried shoots were retained for bioassay and extraction of allelochemicals. The experiment was arranged as a randomized complete block with four replications and was repeated once.

Winter Rye Shoot Bioassay. Experiments were designed to determine the influence of rye fertility regime on rye residue phytotoxicity. Hairy vetch tissue was not evaluated in these bioassays or for allelochemical content because it represented a small (<20%) portion of the polyculture biomass, and its residues are not generally considered to be allelopathic or weed suppressive (White et al., 1989). A modified Parker bioassay was used to measure the allelopathic potential of rye shoots (Parker, 1966; Weston et al., 1989). Flanagan silt loam soil was sifted through a 3- × 3-mm^2 mesh screen and combined with fine quartz sand (1 : 1 v/v). The soil–sand mixture was dried at 45°C for seven days and 100 g was placed in 95- × 95-mm^2 plastic Petri dishes. Next, either 0.5 or 1.0 g of chopped, dried rye from each fertility regime or paper toweling cut into 1.5- × 1.5-cm^2 pieces (control) was placed on the soil. Another 50 g of soil was spread over the chopped residue or toweling. A sheet of filter paper (Whatman No. 1) was placed on the soil surface. A 35-ml aliquot of distilled water was then added to each dish.

Ten seeds each of cress (*Lepidium sativum* L. cv. Extra Fine Curled) and barnyardgrass [*Enchinochloa crus-galli* (L.) Beau.] were placed in two parallel rows (approximately 3.5 cm apart) on the surface of the filter paper. The Petri dishes were taped shut and randomly arranged by treatment in a 28 ± 2°C germination chamber in the dark. The plates were maintained in a vertical position to encourage downward root growth of seedlings. Radicle length was measured after 72 hr. The experiment was arranged as a completely randomized design with four replications and was repeated once. Cress and barnyardgrass indicators were analyzed separately.

Rye Extract Bioassay. This study evaluated the activity and quantity of phytoinhibitors present in extracts of rye shoot residues grown under selected fertility regimes (low, medium, or high fertility). Dried rye shoot residues from each treatment were ground separately in a Wiley mill (mesh screen size of 1 mm). Powdered material was stored in tightly closed glass containers until used for extraction of phytoinhibitors. For each treatment, 40 g of powdered shoot residue were placed in 720 ml distilled water and shaken at 4°C on an orbital

shaker at 200 rpm. The aqueous rye extracts were filtered through four layers of cheesecloth and centrifuged at 9500*g* for 15 min. After centrifugation, extracts were filtered under vacuum using a series of filter papers (Whatman Nos. 4, 1, and 42). The filtered extracts were maintained at 4°C.

Filtered extracts were sequentially partitioned six times each with 150 ml of hexane and 150 ml of ethyl ether. The extracts were rotary evaporated at 35°C to dryness. Dried extracts were weighed and dissolved in the appropriate amount of chloroform to develop a dose–response curve for bioassaying. Six concentrations (666, 333, 166, 83, 42, and 21 μg/ml) of each treatment extract were formulated, and 0.5 ml solution was added to individual glass Petri dishes lined with Whatman No. 1 filter paper. Chloroform was evaporated completely from the dishes and 1.5 ml distilled water was added. Controls consisted of dishes to which 0.5 ml chloroform was added, allowed to evaporate, and followed by addition of 1.5 ml distilled water. Twenty cress seeds were then placed on each moistened filter paper. Dishes were closed, sealed with parafilm, and placed in the dark at 26 ± 2°C. Radicle length was measured after 72 hr of incubation. Treatments were arranged in a completely randomized design with three replications. Sixty seeds were used per treatment, and results were expressed as a percentage of the untreated controls.

Identification and Quantification of Allelochemicals. Major phytotoxic components present in ether extracts of rye shoots were examined using thin-layer chromatography (TLC) and reverse-phase high-pressure liquid chromatography (HPLC). For TLC characterization, 50 μl of the ether extracts of each rye shoot treatment were loaded onto a silica K_6F plate (Whatman). The plates were developed using a mobile phase of 5 : 1 chloroform–methanol (v/v). TLC plates were sprayed with a mixture of 5% vanillin–H_2SO_4, and compounds were visualized under UV (254 nm). The two major components were identified by comparing their migration on TLC plates with that of pure standards of DIBOA and BOA (Barnes et al., 1987). BOA was provided by Dr. M. Nair (Department of Horticulture, Michigan State University) and DIBOA was purified by the laboratory of L. Weston from corn seedlings using the methodology of Nair et al. (1990). The two components and DIBOA and BOA standards had identical R_f values, exhibited fluorescence under UV (254 nm) and absorption maxima in methanol above 254 nm (Chou and Patrick, 1976; Nair et al., 1990). Bands at R_f = 0.44–0.50 and R_f = 0.51–0.57 were identified as DIBOA and BOA, respectively (Nair et al., 1990).

For HPLC characterization, each sample extract was partially purified by eluting through an octadecyl (C_{18}) Sep-Pack (Baker bond, 40 μm APD, 60 Å) with approximately 2 ml of methanol, and then twice filtering through a disposable nylon 66 filter (25 mm syringe filter, 0.2 μm). Samples were rotary evaporated to dryness and redissolved in chloroform at a 1 mg/ml concentration. Twenty microliters of each sample was injected into a HPLC equipped with a

C_{18} column (Water's Nova Pak, Water's, Millpore, Milford, Massachusetts) (3.9 × 150 cm^2). Absorbance at 254 nm was measured with an UV detector (Water's Lambda model 481). The mobile phase consisted of 75% acidified water, adjusted to pH 3.0 with addition of acetic acid and 25% acetonitrile at a flow rate of 1 ml/min. Identities of unknown compounds were confirmed by coelution with standards of DIBOA and BOA. The concentrations of the two benzoxazinones in each sample extract were estimated using standard concentration curves generated for DIBOA and BOA (integration of peak area). All samples were injected twice and results were averaged.

Data from the greenhouse biomass experiment and the laboratory bioassays were subjected to analysis of variance using the general linear model procedure of SAS (SAS Institute, Cary, North Carolina). When effects were significant, means were separated using Fisher's protected least significant difference test (LSD, 0.05). The relationships between rye shoot extract concentration and cress radicle elongation were analyzed using linear regression after logarithmic transformation (base 10). Correlation analysis between rye shoot biomass production, extract concentration, bioassay results, and allelochemical content was performed using SAS.

RESULTS AND DISCUSSION

Rye and Hairy Vetch Biomass Production. Rye and hairy vetch biomass production was affected by both fertility regime and cover crop production system (polyculture vs. monoculture). Shoot biomass of rye grown in monoculture and in polyculture with hairy vetch was significantly increased under high fertility by up to 15% in comparison to low-fertility treatments (Table 1). Hairy vetch biomass was significantly less than rye biomass in all treatments. In general, root dry weight in the three cover crop systems (rye monoculture, rye–vetch polyculture, and hairy vetch monoculture) was highest in the medium-fertility regime. This is similar to the results obtained by Osborne and Whittington (1981), who reported maximum dry weights of *Agrostis* species with 10 mM NO_3^- with a similar sand culture. In our experiments, root dry weights were lower in the hairy vetch monoculture than in either the rye monoculture or rye–vetch polyculture. Root biomass of the hairy vetch monoculture was not affected by fertility (Table 1). However, the root biomass of the rye–vetch polyculture was significantly greater under medium fertility in comparison to the low fertility regime.

Fertility regime and cover crop system also interacted to affect the root-to-shoot ratio. The root-to-shoot ratio ranged from 0.22 for the hairy vetch monoculture under high fertility to 0.63 for the rye monoculture under moderate fertility. Rye root biomass was generally only 50% of the corresponding shoot

TABLE 1. EFFECT OF FERTILITY REGIME ON RYE AND HAIRY VETCH DRY WEIGHT, ROOT-TO-SHOOT RATIOS, AND PROPORTION OF EACH SPECIES IN POLYCULTURE, 75 DAYS AFTER PLANTING

	Dry mass (g/pot)									Proportion of species in rye-HV polyculture (g/pot)			
	Shoots cover crop[b]			Roots cover crops			Root-to-shoot ratio			Rye		HV	
Fertility regime[a]	Rye	HV	Rye + HV	Rye	HV	Rye + HV	Rye	HV	Rye + HV	Wt[c]	%	Wt	%
Low	64.2	25.2	57.7	35.9	8.9	24.7	0.56	0.30	0.49	47.3	82	10.4	18
Medium	63.3	33.8	71.6	40.8	9.2	43.5	0.63	0.35	0.48	62.3	87	9.3	13
High	78.7	35.9	82.7	33.4	8.2	36.2	0.43	0.22	0.43	77.7	94	5.0	6
LSD (5%)[d]		12.3			14.1			0.22		6.5	NS	NS	3

[a] Fertility regimes were: low, medium, and high nutrient levels applied in Hoagland's solution.
[b] Cover crops are: Rye, winter rye in a monoculture; HV, hairy vetch in a monoculture; Rye + HV, winter rye and hairy vetch polyculture.
[c] Shoot dry mass of each species.
[d] Mean separation within fertility regime and cover crop using Fisher's LSD at the 5% level.

biomass (Table 1). This was also observed by Barnes and Putnam (1986) and Nuttonson (1958) in rye field and greenhouse studies.

Increasing the fertility regime reduced the proportion of hairy vetch in the rye–hairy vetch polyculture from 18% of the total shoot biomass in low fertility to 6% in high fertility. This decrease may have occurred because higher fertility levels favored greater rye shoot growth. Since rye grows and tillers at a rapid rate, it may quickly utilize nutrients and water, making these resources less available to hairy vetch. However, the levels of nitrogen and other elements such as P, K, Ca, Mg, and Mn were not significantly influenced by fertility regime or cropping system (data not shown). Since the amounts of nutrients, water, and light provided were closely controlled in this study, it is possible that differences observed in biomass production and root-to-shoot ratios between monoculture or polyculture cropping systems were due to plant–plant interactions. For example, rye plants may exude from living roots allelochemicals that inhibit hairy vetch growth (Barnes and Putnam, 1983; Perez and Ormeno-Nunez, 1993).

Winter Rye Shoot Bioassay. Radicle elongation of cress and barnyardgrass

TABLE 2. EFFECTS OF RYE RESIDUES ON CRESS AND BARNYARDGRASS (ECHCG) RADICLE GROWTH AFTER 72 HOURS OF GROWTH

		% of control			
		Cress residue (g)		ECHCG residue (g)	
Crop system[a]	Fertility regime[b]	0.5	1.0	0.5	1.0
Greenhouse					
Monoculture	Low	81	22	128	24
Monoculture	Medium	53	26	63	24
Monoculture	High	68	33	89	25
Polyculture	Low	77	33	49	14
Polyculture	Medium	51	23	34	23
Polyculture	High	63	25	58	23
LSD (0.05)		14	NS	40	NS
Field[c]					
Polycul (Spring)		46	32	78	20
Polycul (Fall)		74	32	40	20
LSD (5%)[d]		NS	NS	32	NS

[a]Crop systems are: Monoculture, winter rye in a monoculture; and Polyculture, winter rye in a rye and hairy vetch polyculture.
[b]Fertility regimes were: low, medium, and high nutrient levels applied in Hoagland's solution.
[c]Polycul, polyculture; spring- and fall-planted rye harvested after 75 and 240 days of growth, respectively.
[d]Mean separation using Fisher's LSD at the 5% level.

(ECHCG) was reduced in the presence of moistened rye residues (Table 2). In all treatments, 1 g of rye residue was more inhibitory to radicle elongation than 0.5 g of residue. In the presence of 1.0 g of rye residue, cress seeds imbibed water, darkened in color; and subsequent radicle elongation was inhibited. Injury symptoms were similar to those observed by Barnes and Putnam (1983) and Chou and Patrick (1976) when lettuce seeds were germinated in the presence of rye residues.

The cover crop system (polyculture versus monoculture) and fertility regime affected cress and barnyardgrass radicle growth in the presence of 0.5 g of rye shoot residue, but there were no treatment differences at 1.0-g residue rates. At 1.0-g residue rates all treatments were quite inhibitory. Cress radicle length ranged from 51 to 81% of the control with 0.5 g residue, and the monoculture did not differ from the polyculture. The effect of rye residue treatments was dependent upon the indicator species evaluated. Rye residues grown under moderate- or high-fertility regimes were more inhibitory to cress radicle elongation than residues grown under low-fertility regimes. At 0.5 g residue, barnyardgrass radicle growth ranged from 63 to 128% and from 34 to 58% of the control when exposed to rye residues from the monoculture and polyculture, respectively (Table 2). Rye produced in a polyculture was more inhibitory to radicle elongation than rye produced in a monoculture. Residues of fall-seeded, field-grown rye were equally toxic in comparison to greenhouse-grown rye. However, toxicity of spring-seeded, field-grown rye differed from fall-seeded rye. Spring-seeded, field-grown rye was more inhibitory to cress radicle growth, while fall-seeded rye was more inhibitory to barnyardgrass growth. Barnes and Putnam (1983) also observed greater phytotoxicity to curly cress from spring-seeded versus fall-seeded, field-grown rye. The inhibition of cress and barnyardgrass radicle elongation indicates that small germinating seeds are vulnerable to chemicals released from rye residues, but cultural practices used to produce the rye cover crop residues may influence tissue toxicity. It is possible that practices used to produce residues could be manipulated to better manage weeds in no-till cropping systems.

Rye Extract Bioassay. The recovery of crude ether extracts of dried rye shoots differed between fertility regimes. For example, rye produced at moderate to high fertility levels in monoculture yielded two to five times more extract than rye produced at low fertility levels (Table 3). Rye produced at moderate fertility in the polyculture yielded almost two times more extract than did rye at low fertility.

The toxicity of the crude ether extracts of rye shoots was influenced by cropping system and fertility regimes in which the rye was grown (Table 4). Ether extracts of rye shoots grown in a polyculture with hairy vetch were generally less inhibitory than those treatments grown in monoculture. Regression analysis indicated that for the rye monoculture, cress radicle elongation was

TABLE 3. MATERIAL RECOVERED FROM ETHER EXTRACT AND LEVELS OF DIBOA AND BOA FROM RYE RESIDUES

Crop system[a]	Fertility regime[b]	Recovery ether extract (mg/g)	Concentration μg/g	
			DIBOA	BOA
Greenhouse				
Monoculture	Low	2.58	210 ± 8[c]	21.5 ± 8.5
Monoculture	Medium	12.08	319.3 ± 18.5	15 ± 14
Monoculture	High	5.10	128.8 ± 5.5	2.5 ± 2.3
Polyculture	Low	3.90	423.8 ± 22.3	20 ± 19
Polyculture	Medium	6.38	335.3 ± 3.5	31.3 ± 25
Polyculture	High	3.78	138.5 ± 9	2.5 ± 1.3
Field[d]				
Polycul (spring)		2.65	532.8 ± 83	16.8 ± 12
Polycul (fall)		4.03	138 ± 21.5	23.5 ± 22

[a]Crop systems were: Monoculture, winter rye in a monoculture; and Polyculture, winter rye in a rye and hairy vetch polyculture.
[b]Fertility regimes were: low, medium, and high nutrient levels applied in a Hoagland's solution.
[c]Mean ± standard error.
[d]Spring- and fall-planted rye harvested after 75 and 240 days of growth, respectively. The greenhouse-grown rye was harvested after 75 days of growth.

linearly related to extract concentration. In rye monoculture, extracts of rye shoots grown under low fertilty (I_{50} = 60 μg/ml) were more toxic than those grown at higher fertility (I_{50} = 120 μg/ml). In polyculture treatments, there was a quadratic effect of extract concentration on radicle elongation. Fertility treatments had little effect on toxicity of rye shoot extracts grown in polyculture.

Extracts from stressed plants tend to be more inhibitory to cress radicle elongation than those from plants not under stress (Barnes and Putnam, 1986; Barnes et al., 1987; Rice, 1984). The monocultures of rye grown in the low-fertility regimes may be more stressed than the polyculture treatments of rye grown with hairy vetch. The quadratic equations for the polycultures suggest that there may be a critical concentration below which the allelochemicals are tolerated (Barnes and Putnam, 1983). The range of concentrations we found to be inhibitory to cress radicle elongation (Table 5) were similar to the range reported to be inhibitory by Barnes et al. (1987).

Identification and Quantification of Allelochemicals. Phytotoxic compounds were isolated from extracts of winter rye shoots. The HPLC and TLC data (Table 5) indicated that DIBOA and BOA were the major substituents of ether extracts of rye shoots. In all rye treatments, DIBOA was the major component present in the filtered ether extracts (69–86%), with considerably less BOA present (0.6–5.8%). Following separation by TLC, a strong inhibitory

TABLE 4. EFFECT OF CRUDE ETHER EXTRACT FROM RYE SHOOT RESIDUES ON CURLY CRESS RADICLE ELONGATION AFTER 72 HOURS OF GROWTH

Crop system[a]	Fertility regime[b]	Regression equations[c]	Correlation coefficient (r^2)[d]
Greenhouse			
Monoculture	Low	$Y = 67.9 - 0.101X$	0.86**
Monoculture	Medium	$Y = 74.9 - 0.118X$	0.75
Monoculture	High	$Y = 86.9 - 0.134X$	0.90*
Polyculture	Low	$Y = 104 - 0.459X + 0.00047X^2$	0.93*
Polyculture	Medium	$Y = 104 - 0.496X + 0.000528X^2$	0.90**
Polyculture	High	$Y = 80.8 - 0.126X$	0.92**
Field[e]			
Polycul (spring)		$Y = 111 - 0.508X + 0.00051X^2$	0.89*
Polycul (fall)		$Y = 89 - 0.437X + 0.000468X^2$	0.92*

[a] Crop systems were: monoculture, winter rye in a monoculture; and polyculture, winter rye in a rye and hairy vetch polyculture.
[b] Fertility regimes were: low, medium, and high nutrient levels applied in Hoagland's solution.
[c] Y is percent of control and X is ether extract concentration in micrograms per milliliter.
[d] * ** Significant at 5 or 1% levels, respectively.
[e] Spring- and fall-planted rye harvested after 75 and 240 days of growth, respectively. The greenhouse grown rye was harvested after 75 days of growth.

activity was associated with two zones having R_f = 0.44–0.48 and 0.51–0.54 (data not presented). These compounds cochromatographed with DIBOA and BOA standards, suggesting that the extracts contained the two benzoxazinones. Furthermore, similar color development, fluorescence in UV radiation, and UV absorption maximum were observed for the unknowns and standards on TLC plates. IN HPLC analysis, each of the unknown samples contained two predominant constituents that coeluted with DIBOA and BOA, respectively, at 2.08 and 3.11 min. In addition, the extracts from the unknown samples showed two other minor constituents that eluted at approximately 2.50 and 2.80 min. These constituents were most likely 2,4-dihydroxy-7-methoxy-1,4-benzoxazinone (DIMBOA) and 2-*O*-glucosyl-1,4-benzoxazin-3-one (GDIBOA), compounds which are structurally related to DIBOA and BOA (Hofman and Hofmanova, 1971; Nair et al., 1990).

Ether extracts of rye shoot tissues varied in their concentrations of DIBOA and BOA. BOA concentrations ranged from 2.5 to 31.3 μg/g (Table 3). DIBOA concentrations ranged from 138 to 532 μg/g, with the greatest quantity detected in spring-seeded, field-grown rye. This is not surprising, since plant tissues grown under high light intensity generally produce greater levels of extractable allelochemicals (Rice, 1984). Our results differed from the findings of Barnes and Putnam (1987), who reported greater quantities of DIBOA in greenhouse-

TABLE 5. IDENTIFICATION OF DIBOA AND PHYTOTOXINS IN RYE TISSUES USING HPLC AND TLC PROCEDURES AND I_{50} VALUES OF EXTRACTS

Crop system[a]	Fertility regime[b]	HPLC analysis[c] DIBOA Min	DIBOA %	BOA Min	BOA %	Cress bioassay I_{50} value (μg/ml)[d]
Greenhouse						
Monoculture	Low	2.07	82.7	3.13	3.2	177.2
Monoculture	Medium	1.96	74.7	3.16	2.5	211.0
Monoculture	High	1.99	69.7	3.09	0.4	275.4
Polyculture	Low	2.00	86.4	3.10	1.6	125.0
Polyculture	Medium	1.98	83.5	3.20	1.5	136.0
Polyculture	High	1.98	72.1	3.30	0.6	244.4
Significance[e]		NS	*	NS	NS	*
Field[f]						
Polyculture (spring)		1.97	86.0	3.02	2.8	138.0
Polyculture (fall)		2.02	71.0	3.20	5.8	60.0
Significance[e]		NS	*	NS	NS	*
Standards[g]						
DIBOA		2.01	98.0			100.0
BOA				3.13	99.0	212.0

[a]Crop systems were: Monoculture, winter rye in a monoculture; and Polyculture, winter rye in a rye and hairy vetch polyculture.

[b]Fertility regimes were: low, medium, and high nutrient levels applied in Hoagland's solution.

[c]HPLC parameters: the mobile phase was based on the Weston et al. (1989) method; 75% acidified water, 25% acetonitrile; adjusted to pH 3.0 with addition of acetic acid; Min is the peak retention time in minutes, and percent is component compound present in the filtered ether extracts of rye shoots detected at the peak retention time.

[d]Cress bioassay: I_{50} values represent the point at which 50% inhibition of cress radicle growth occurred. A dose-response curve was calculated on the basis of 60 seeds at six concentrations of the ether extract. The I_{50} values were estimated using the regression equations shown in Table 4.

[e]*, NS significant at 5% and not significant, respectively.

[f]Spring- and fall-planted rye harvested after 75 and 240 days of growth, respectively.

[g]The identity of the benzoxazinones was based on comparison with the values obtained in DIBOA and BOA standards in our laboratory analyses and values reported by Barnes et al. (1987).

grown rye shoots as compared to field-grown rye shoots. Fall-planted, field-grown rye shoot extracts contained lower levels of DIBOA and higher levels of BOA then extracts from spring-planted, field-grown rye. Fall-seeded rye was also more inhibitory to barnyardgrass than were other treatments, such as spring-seeded rye. Rye grown in the high-fertility regime had lower levels of DIBOA and BOA than rye grown in moderate or low fertility. Fertility regime and cropping systems effects on DIBOA and BOA are not unexpected. Two major differences between greenhouse-grown and field-grown (spring- or fall-planted)

rye are the age of tissue at harvest and the environmental conditions encountered during growth. Tissue age influences biosynthesis of secondary plant products, and plants growing in unfavorable conditions produce more secondary metabolites than those growing in favorable environments (Barnes and Putnam, 1987; Bushuk, 1976; Putnam et al., 1983; Rice, 1984). Plant age can be managed in the field by timing the rye kill, potentially maximizing DIBOA and BOA concentration and increasing field-grown rye's weed-suppressing ability.

In our study, ether extracts of rye shoots contained primarily DIBOA. The estimated concentrations of DIBOA and BOA in shoot tissues ranged between 2.28 and 18.94 kg/ha for DIBOA and 0.001 and 1.90 kg/ha for BOA (data not presented). These estimates agree with the 13.5 kg/ha of DIBOA and BOA reported by Barnes and Putnam (1987) and Barnes et al., (1986). Based on our field studies (Masiunas et al., 1994; Mwaja, 1994), rye residues and their allelochemicals can effectively control redroot pigweed (*Amaranthus retroflexus*) for four to eight weeks, depending on weather conditions.

Biomass production, extract concentrations, bioassay results, and allelochemical content were correlated (Table 6). As shoot dry matter accumulation increased, crude ether extract ($r = -0.73$), DIBOA concentration ($r = -0.81$) decreased while BOA concentration ($r = 0.62$) increased. A modified Parker bioassay examining toxicity of rye shoot residues gave low correlations with DIBOA and BOA concentrations in ether extracts of rye shoot residues. This suggests that the field soil bioassay using cress as a seedling indicator is limited in its ability to predict allelochemical concentration. Barnyardgrass radicle growth had a correlation coefficient of -0.53 for DIBOA but only -0.18 for BOA. These results are in agreement with those of Barnes and Putnam (1987) and Barnes et al. (1986, 1987), showing DIBOA as most active to monocots and BOA as more active to dicots. The concentration of DIBOA and BOA in ether extracts was most strongly correlated with the results obtained from the crude ether extract bioassay using 333 μg/g. Simple extraction of rye residues followed by bioassay may be a relatively quick and accurate method to estimate DIBOA and BOA concentrations in allelopathic residues.

In conclusion, although high-fertility regimes may produce large amounts of rye biomass, the allelochemical content and biological activity of these residues may not be increased. The negative correlation of rye shoot dry weight with filtered extract content or DIBOA concentration (Table 6) suggests that cultural practices (i.e., fertility) that increased growth of winter cover crops may not optimize phytotoxicity of residues or allelochemical content. Our bioassays implicated chemical interference by rye residues with suppression of seedling growth. We also observed strong weed suppressive activity by rye residues in field experiments. DIBOA and BOA concentrations may be associated with weed suppressive activity of rye residues in the field. Hence, an integral approach

TABLE 6. MATRIX OF CORRELATION COEFFICIENTS BETWEEN BIOMASS PRODUCTION, EXTRACT CONCENTRATIONS, BIOASSAY RESULTS, AND ALLELOCHEMICAL CONTENT

Variable[a]	Shoot dry wt	Extract Conc.		Soil bioassay		Extract bioassay conc. (μg/ml)		Conc. (μg/g)	
		Crude	Filtered	Cress	ECHCG	42	333	DIBOA	BOA
Shoot dry wt (g)		−0.19	−0.74	−0.32	−0.04	0.04	0.73	−0.81	0.62
Crude extract			0.73	−0.74	−0.35	−0.58	−0.40	0.30	0.07
Filtered				−0.25	−0.39	−0.13	−0.75	0.78	0.32
Cress bioassay					0.62	0.44	0.27	−0.08	−0.10
ECHCG bioassay						−0.37	0.56	−0.53	−0.18
42 μg/ml assay							0.00	0.16	−0.22
333 μg/ml assay								−0.97	−0.88
DIBOA									0.72
BOA									

[a]The variables were: shoot dry wt in grams per pot, Crude, crude extract recovered from 40 g of dry shoot tissue in milligrams per gram; filtered, filtered extract recovered in milligram per gram; Cress bioassay, cress radicle length after 72 hr as a percent control; ECHCG bioassay, barnyardgrass radicle length after 72 hr as a percent control; 42 μg/ml, cress radicle length as a percent control after exposure to 42 μg/ml of crude extract; 333 μg/ml, cress radicle length as a percent control after exposure to 333 μg/ml of crude extract; DIBOA, DIBOA concentration in micrograms per gram shoot dry weight, and BOA, BOA concentration in micrograms per gram shoot dry weight.

incorporating time of kill and fertility management could increase allelochemical concentrations and weed suppression from rye cover crops.

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RESPONSE OF *Np* MUTANT OF PEA (*Pisum sativum* L.) TO PEA WEEVIL (*Bruchus pisorum* L.) OVIPOSITION AND EXTRACTS

ROBERT P. DOSS,[1] WILLIAM M. PROEBSTING,[2,*] SANDRA W. POTTER,[3] and STEPHEN L. CLEMENT[4]

[1]*USDA-ARS*
Corvallis, Oregon 97331
[2]*Department of Horticulture*
[3]*Department of Zoology*
Oregon State University
Corvallis, Oregon 97331
[4]*USDA-ARS*
Pullman, Washington 99164

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Abstract—The *Np* mutant of pea (*Pisum sativum* L.) is characterized by two physiological responses: growth of callus under pea weevil (*Bruchus pisorum* L., Coleoptera: Bruchidae) oviposition on pods, and formation of neoplastic callus on pods of indoor-grown plants. Although these two responses are conditioned by *Np*, they are anatomically and physiologically distinguishable, based on sites of origin, distribution pattern, and sensitivity to plant hormones. Further characterization of the response to extracts of pea weevil showed that response of excised pods, measured by callus formation, was log-linear, and treatment with as little as 10^{-4} weevil equivalents produced a detectable response. Mated and unmated females contained similar amounts of callus-inducing compound(s), and immature females contained significantly less of the compound(s). Female vetch bruchids (*Bruchus brachialis* F., Coleoptera: Bruchidae), a related species, contained callus-inducing compound(s), but usually less than pea weevils on a per weevil basis. Males of both species contained less than 10% of the activity of the mature females. Extracts of female black vine weevils, a nonbruchid species, did not stimulate callus formation. Based on partitioning and TLC analysis, the biologically active constituent(s) was stable and nonpolar. Thus, the *Np* allele probably conditions sensitivity to a nonpolar component of pea weevil oviposition as a mechanism of resistance to the weevil.

*To whom correspondence should be addressed.

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Key Words—*Bruchus brachialis*, *Bruchus pisorum*, Coleoptera, Bruchidae, neoplastic pods, *Pisum sativum*, resistance.

INTRODUCTION

Expression of the dominant gene *Np* (neoplastic pod) in pea (*Pisum sativum* L.) is characterized by neoplastic growth, or "pustular outgrowths" of callus, on pods of greenhouse-grown plants (Nuttall and Lyall, 1964). Absence of ultraviolet light permits full development of this *Np* phenotype, where neoplasms form over the entire pod, often preventing normal seed development (Snoad and Matthews, 1969). Snoad and Matthews (1969) reported that subsidiary cells associated with stomata on pods, but not on leaves and stems, were the site of neoplasm formation. Seed removal reduced the occurrence of neoplasms, and the seed effect was replaced by GA_3 and auxins (Jones and Burgess, 1977).

Following a report by Vilkova et al. (cited in Berdnikov et al., 1992) that oviposition by pea weevil (*Bruchus pisorum* L., Coleoptera: Bruchidae), a major insect pest of pea (Clement, 1992), resulted in cell growth under the egg, Berdnikov et al. (1992) showed that aqueous extracts of pea weevil stimulated callus on pods of *Np* but not on *np* genotypes. They proposed that this response comprised a form of resistance to pea weevil, as callus dies and falls from the pod, with the weevil egg. More often, however, larvae hatched and burrowed into the pod before the callus died; thus, the resistance provided by *Np* appeared to be weak. Hardie (1990, 1993) also observed callus formation in response to pea weevil oviposition, although he proposed that the callus retarded entry of the weevil larvae into the pod, thereby reducing infestation of the seed.

These separate lines of investigation have converged on the involvement of *Np* in control of cell division, a question of fundamental importance to biology. In this case, a stimulus from the weevil is associated with a specific plant gene that also mediates a response to plant hormones. This interaction of pea weevil with a single genetic locus to elicit growth in a specific plant organ may yield new insights into regulation of cell division. The purpose of this study was to: (1) initiate characterization of the extractable weevil factor, and (2) explore physiological and anatomical responses of pea tissue to weevil extracts.

METHODS AND MATERIALS

Plant Material. Pea plants (*Pisum sativum* L.) were grown in a greenhouse as described previously (Proebsting et al., 1992). Two lines, C887-332 (*Np/Np*) and I_3 (*np/np*), a selection from cv. Alaska (both lines derived from material provided by the late Dr. G.A. Marx, New York Agricultural Experiment Station, Geneva, New York), were used for all studies. To prevent neo-

plasm formation, potted plants were transferred outdoors at the onset of flowering. Unless otherwise noted, pods selected for bioassay were one half to fully expanded in length.

Insects. Adult pea weevils (*Bruchus pisorum* L., Coleoptera: Bruchidae) were obtained from infested pea seed harvested the previous season from experimental plots in eastern Washington and stored at 4°C. Sufficient seed to yield the desired number of weevils was placed in a screen-covered container at 25°C. As the insects emerged from the seed, their sex was determined (Pesho and van Houten, 1982), and the females were placed individually in plastic Petri dishes containing a moist dental wick. The weevils were provided with fresh pea flowers two or three times per week to supply fresh pollen required for sexual maturation (Pesho and van Houten, 1982). Weevils were assumed to be sexually mature upon deposition of a few eggs on the Petri dish or pea flower. Maturation typically occurred 10–20 days after exposure to pollen.

Sexually mature vetch bruchids (*Bruchus brachialis* F., Coleoptera: Bruchidae) were field-collected from wild vetch plants (*Vicia villosa* Roth.) growing near Pullman, Washington. The sex of these insects was determined (Pinckney, 1937), and they were extracted as described below. Sexually mature black vine weevils (*Otiorhynchus sulcatus* F., Coleoptera: Curculionidae), a parthenogenetic species, were provided by Dr. C.H. Shanks, Jr. (Washington State University, Vancouver) from a colony maintained on strawberry plants.

Insect Extraction. Whole insects were homogenized either in water or in chloroform–methanol (2 : 1 v/v) (100 μl per insect) using a glass tissue homogenizer. After brief centrifugation, the supernatant was removed and used for bioassay. Organic extracts were partioned 3× against equal volumes of water and each fraction, unpartitioned chloroform–methanol, nonpolar, and polar, was dried in vacuo and reconstituted with 100 μl water per weevil.

Female weevils secrete an egg-covering fluid at oviposition that also is used to attach eggs to pea pods and is referred to as "accompanying fluid" (Annis and O'Keefe, 1984). Extracts were prepared from pea weevil eggs and the accompanying fluid by pressing the underside of the terminal segments of the abdomen of sexually mature female weevils to cause extrusion of the copulatory and ovipository organs and provide access to the eggs and accompanying fluid. Eggs were transferred to a microcentrifuge tube using a glass capillary tube drawn to a fine, sealed tip. The eggs were homogenized in water (100 μl per weevil) using a plastic pestle fitted to the microcentrifuge tube. After centrifugation, the supernatant was transferred to a fresh tube and used for bioassay.

The accompanying fluid was taken up in finely drawn glass capillaries. The capillaries containing the extract were placed in microcentrifuge tubes and broken with a plastic pestle. The fluid was extracted into water (chloroform, if using thin-layer chromatography), centrifuged, and the supernatant transferred to a fresh microcentrifuge tube.

Bioassay. Pods at mid- to late-flat pod phase (Meicenheimer and Muehlbauer, 1982) were split along the suture and the seeds removed. The half pods were placed with their inner side on moist filter paper in plastic Petri dishes (100 mm diameter). One-microliter droplets of test solutions were placed along the middle of the pods. Droplet order was random, except for TLC fractions. In some cases, the pod surface was marked adjacent to the droplet to indicate site of application. The droplets were air-dried, the dishes covered, and placed in an incubator [23 ± 2°C, continuous light, fluorescent tubes (Philips F36T12/CW/HO, 30–40 μmol photons/m^2/sec).

After one week in the growth chamber, callus that formed at the sites of application was removed and weighed. All bioassays were comprised of six half pods (treated as blocks), and the data analyzed by analysis of variance. In some cases, means were compared using Tukey's studentized range test. All bioassays were carried out at least two times with qualitatively similar results.

Thin-Layer Chromatography. Five weevil equivalents (WEq) of a chloroform extract of eggs and accompanying fluid were spotted and dried on reversed-phase TLC plates (Whatman $KC_{18}F$) and developed in ethanol–water (80:20 v/v) to 10 cm beyond the origin. Fractions 1 cm^2 were eluted from the adsorbent with chloroform–methanol (1:1 v/v). The 10 fractions were dried in vacuo and redissolved in water.

Microscopy. Light microscopy was conducted using material fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.3, dehydrated in an ethanol series, and embedded in paraffin. Sections 10 μm thick were deparaffinized and stained with hematoxylin and eosin. Specimens for scanning electron microscopy were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, dehydrated, and critical point dried before coating with gold–palladium (60:40) and examined using an Amray 1000A scanning electron microscope operated at 30 keV.

RESULTS AND DISCUSSION

Response of Np *to Weevil.* Callus developed under pea weevil eggs laid on *Np* pods (Figure 1A and B). These micrographs also show early development of neoplasms associated with stomata, as reported previously (Snoad and Matthews, 1969). Unlike the neoplasms, the response to weevil was not restricted to stomata (Figure 1B and C). Both oviposition and application of weevil extract stimulated callus formation throughout the site of contact.

Aqueous extracts prepared from sexually mature female pea weevils also induced callus development when applied to intact pods of *Np*. Callus growth was preceded by discoloration (browning) of the pod surface at the site of application within 3–6 hr after application of active extracts. This effect is

evident in Figure 1D, where the application site is clearly delineated by darkened epidermis. Darkening did not appear in response to inactive extracts from other insects, inactive chromatographic fractions of female pea weevils, or organic solvents. Browning was not gene-specific, however, appearing in *np* lines in response to active extracts. We considered this discoloration to be a rapid, but preliminary, index of biological activity.

Swelling at the site of extract application appeared within two days and expanded on subsequent days (Figure 1E and F). By day 7, a disordered mass of callus, still largely bounded by the epidermis, was evident (Figure 1G). These figures clearly show involvement of exocarp and mesocarp cells (Esau, 1977), rather than stomata, in response to weevil components, thereby distinguishing the weevil-generated response from the autogenous, neoplastic response. Cell growth on the inner surface of the pod was also apparent by day 2 (Figure 1D–G). This growth occurred on both *np* and *Np* pods, and was not stimulated by weevil extracts.

Response of the pod to pea weevil oviposition and extracts: (1) occurred both outdoors and in the greenhouse, (2) occurred on seeded and deseeded pods, and (3) was restricted to the site of contact with either weevil eggs or extract. In contrast, neoplasms were formed almost exclusively under low UV light in the greenhouse, and covered the entire pod, often preventing normal seed development. Limited neoplastic development was observed occasionally on shaded pods within the canopy of plants grown outdoors.

Neoplasm formation on *Np* reportedly occurs exclusively on pods (Snoad and Matthews, 1969). We routinely observed small neoplasms on stems and petioles of indoor-grown plants, and occasionally on field-grown plants of line C887-332. However, pea weevil extracts did not stimulate callus development on immature or mature stems, petioles, or leaves, whereas pods responded to extract at any time prior to senescence (data not shown). Pea weevil oviposition and extracts did not stimulate callus formation on *np* tissues. Thus, *Np* conditions an organ-specific response to pea weevil and to autogenous factors that stimulate neoplasm formation. Although these events depend on *Np*, they appear to be physiologically and anatomically distinguishable.

Bioassay. We observed a log-linear dose–response of *Np* half pods to weevil extract. Application of as little as 10^{-4} WE reliably produced a measurable response in the excised pods (Figure 2). In one experiment, visually detectable callus formed on two of six pods treated with 10^{-5} WE. The amount of callus varied from pod to pod, but the qualitative response to weevil extract was always very distinct. Browning of the epidermis occurred within a few hours of application, and callus development could be visually distinguished within two or three days.

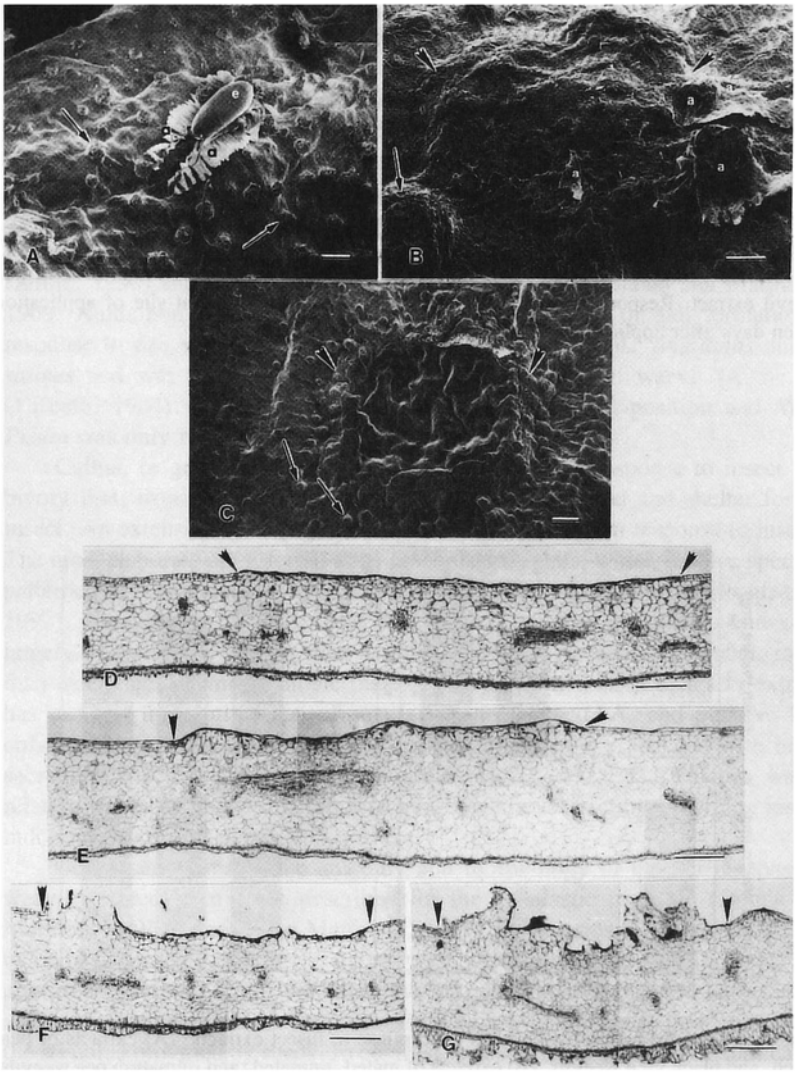
Berdnikov et al. (1992) emphasized the importance of using pea lines with weak expression of *Np* in order to distinguish neoplasms from weevil-induced

callus. We found only occasional neoplasm formation on excised, expanding, half pods from outdoor-grown plants. Furthermore, weevil-induced callus appeared compact and light-brown, whereas neoplasms were pustular in texture and nearly white in color. As a result of these characteristics, this simple bioassay was rapid and sensitive.

Sources of Callus-Inducing Activity. Female pea weevils and vetch bruchids, a closely related species, were the best source of callus-inducing compound (Figure 3A), whereas female black vine weevils, a distantly related coleopteran, contained no callus-inducing compound. Mated and unmated females contained roughly similar amounts of callus-inducing compound, whereas immature females contained significantly less compound (Figure 3B). Based on dose–response studies, mature females of both species contained at least 10-fold more activity than males (Figure 3C), and, in the case of pea weevil, than immature females (Figure 3B). Pea weevil eggs and the accompanying fluid both stimulated callus formation (Figure 3D). We could not distinguish whether the activity in the egg fraction was actually in the small amount of fluid adhering to the eggs.

Gibberellic acid (GA_3) and naphthaleneacetic acid (NAA) were inactive in the half-pod bioassay over the concentration range of 10^{-6}–10^{-2} M and within the seven-day assay period (data not shown). Benzylaminopurine occasionally, but not reproducibly, stimulated a small amount of callus. In contrast, GA_3 and NAA were reported to stimulate neoplasms on deseeded pods still attached to the plant, and on *Np* pods in sterile culture on medium containing MS salts plus GA_3, IBA, or IAA (Jones and Burgess, 1977). We reproduced both these responses, but found them to be variable and relatively weak compared to the response to pea weevil.

FIG. 1. Micrographs showing response of *Np* pods to pea weevil oviposition (A, B) and to application of 1 μl (2×10^{-2} WE) pea weevil extract on *Np* pods (C–G). Bar = 200 μm in all cases. (A) Effects of pea weevil oviposition (e, egg and a, egg adhesive) after two days. Slight swelling under the oviposition is apparent. Nascent neoplasms associated with stomata are also evident (arrow). (B) SEM with oviposition removed (a, egg adhesive) from pod and showing swelling (arrowheads) at site four days after oviposition. Neoplasm is indicated by arrow. (C) SEM of pod three days after treatment with pea weevil extract. Swelling is evident at site of application (arrowheads) and nascent neoplasms are evident (examples marked by arrows). (D–G) Light micrographs of pod cross sections. Arrowheads mark boundaries of extract application. (D) One day after application of pea weevil extract, discoloration of epidermis and swelling of exocarp cells are evident. (E–F) Progressive swelling of exocarp and mesocarp under application site is detectable two and four days after application of pea weevil extract. (G) Seven days after application, substantial, irregular callus development has broken through the discolored epidermis, but appears to be still largely bounded by the epidermis.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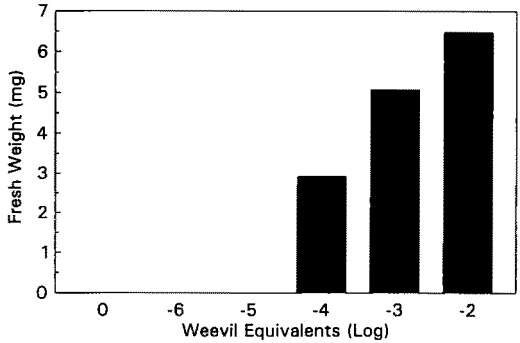

FIG. 2. Response of excised, expanding, deseeded *Np* pods to logarithmic dose of pea weevil extract. Response measured by weighing callus produced at site of application seven days after application.

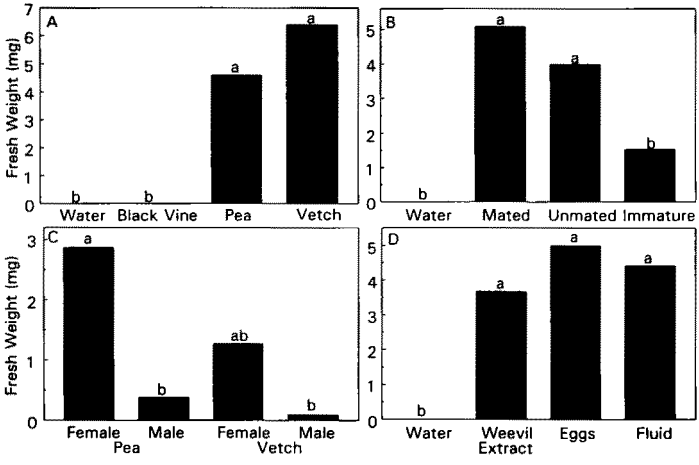

FIG. 3. Callus formation by *Np* pods in response to insect extracts. (A) extracts of pea, vetch, and black vine weevils. (B) extracts of mated, unmated, and immature pea weevils. (C) extracts of female and male pea and vetch weevils. (D) extracts of whole weevils, eggs and accompanying fluid. Response measured by weighing callus produced at site of application seven days after application. Letters over bars represent mean separation by Tukey's studentized range test at $P < 0.05$.

Characterization. Callus-inducing compound was extractable by both water and chloroform–methanol. When chloroform–methanol extracts were partitioned against water, however, the biological activity remained in the nonpolar fraction (data not shown). Reversed-phase, thin-layer chromatography of partitioned extracts resulted in recovery of activity from R_f 0.2 to 0.3 (data not shown). Retention of biological activity through these procedures indicated that the active constituent(s) were sufficiently stable to be purified by routine methods.

Np was originally characterized as an abnormal, and rather curious, growth response to seeds and plant hormones in specific environmental conditions. The tumor-like growths were described as neoplasms, defined as a new growth of tissue serving no physiological function. Neoplasm formation similar to that in *Pisum sativum* was described in pea subspecies (Snoad and Matthews, 1969; Hardie, 1990) and on two sweet pea (*Lathyrus*) species (Snoad and Matthews, 1969; Annis and O'Keefe, 1984; Hardie, 1993). Callus or gall formation in response to pea weevil oviposition was also reported on *L. tingitanus* and *L. sativus* and was proposed to play a role in resistance to weevil (Annis and O'Keefe, 1984). However, the relationship of weevil oviposition and *Np* in *Pisum* was only reported recently (Berdnikov et al., 1992).

Callus, or gall, formation is a common defensive response to insect herbivory that, ironically, usually results in high-quality food and shelter for the insect. An extensive variety of galls are formed by plants in response to insects. The most elaborate are referred to as prosoplasmic galls, which involve specific, patterned development and have not been mimicked experimentally (Rohfritsch, 1992). Kataplasmic galls, on the other hand, are less organized, consisting largely of calluslike growth such as that formed by *Np* peas. Chemical, rather than mechanical, stimuli initiate these galls. Characterization of insect extracts has resulted in identification of several amino acids, IAA, and putative IAA cofactors, a steroid, and a variety of unidentified factors associated with insect secretions and gall formation (reviewed by Hori, 1992). The *Np*–pea weevil relationship in pea is the first reported gene-specific interaction governing insect-induced growths on plants.

Our observations of the anatomy and morphology of the *Np* response to weevil differed from those described for the neoplastic response (Dodds and Matthews, 1966; Snoad and Matthews, 1969). Neoplasms formed a thick layer of cells covering virtually the entire pod, were eliminated by UV light, and appeared lighter in color and more pustular than weevil-induced callus. GA_3 and auxins could stimulate neoplasm formation over the entire pod, mimicking the stimulus provided by seeds. In contrast, pea weevil oviposition or application of pea weevil extracts caused callus formation only at the site of application and callus did not grow beyond this site. Perhaps more importantly, the response to weevil involves exocarp and mesocarp tissues and is not restricted to the stomatal apparatus, as neoplasms were reported to be (Snoad and Matthews, 1969).

It appeared, superficially, that the seed- and weevil-induced responses of *Np* pods were somewhat different manifestations of the same cellular response. Thus, based on earlier work (Berdnikov et al., 1992), as well as ours, cell proliferation on pods of *Np* now appears to have a function. We think the *Np* allele of pea regulates expression of a plant resistance trait (callus formation), albeit only after attack by an insect (pea weevil) that has a highly specific relationship with pea. The degree of weevil resistance conferred by *Np* has yet to be determined, however. The seed and hormone responses previously observed under low UV light conditions are believed to be related, but secondary.

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PHYTOTOXIC AND ANTIMICROBIAL ACTIVITY OF 5,7-DIHYDROXYCHROMONE FROM PEANUT SHELLS[1]

S.F. VAUGHN

Bioactive Constituents Research
USDA, Agricultural Research Service
National Center for Agricultural Utilization Research
1815 N. University St., Peoria, Illinois 61604

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Abstract—A flavonoid decomposition product that is present in peanut (*Arachis hypogaea*) shells, 5,7-dihydroxychromone (DHC), was found to inhibit the radial growth of cultures of the soil pathogenic fungi *Rhizoctonia solani* and *Sclerotium rolfsii* with I_{50} (the concentrations of DHC required to inhibit growth 50%) values of 18 and 26 μM, respectively. Radicle elongation of velvetleaf, corn, peanut, and wheat was inhibited by DHC with I_{50} values of 30, 50, 65 and 200 μM, respectively. DHC had no effect on the growth of *Bradyrhizobium* sp. at 10 μM in medium containing low (1.0 g/liter) mannitol as the carbon source, although the related flavones luteolin and chrysin each promoted bacterial growth at 10 μM 48 hr after inoculation. When tested in high (10.0 g/liter) mannitol medium, DHC initially inhibited growth of *Bradyrhizobium* sp., but 120 hr after inoculation the growth of all treatments were similar. These results suggest a role for DHC released from peanut shells in suppressing pathogenic fungal infection and competing plant growth but not for *Bradyrhizobium* growth promotion.

Key Words—Peanut, *Arachis hypogaea*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Bradyrhizobium*, 5,7-dihydroxychromone, chrysin, luteolin, flavonoids, allelopathy, growth inhibition.

INTRODUCTION

Flavonoids are a diverse class of compounds with over 1000 different flavonoids reported in higher plants (Harborne, 1982). Their physiological roles are varied,

[1] Mention of firms or products does not imply endorsement by the U.S. Department of Agriculture over other firms or products not mentioned.

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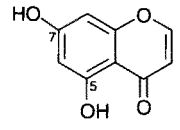
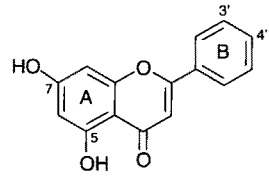
ranging from plant pigments to defensive substances and pollination attractants (Harborne, 1982). Flavones are a subgroup of flavonoids containing a ketone oxygen at the 4 position of the central ring. Certain flavones have been shown to function in the induction of nodulation genes in legumes (Hartwig et al., 1990; Maxwell et al., 1989; Peters and Long, 1988); in the growth promotion of *Rhizobia* bacteria (Hartwig et al., 1990); in the inhibition of growth of plants and fungi (Paszkowski and Kremer, 1988; Rivera-Vargas et al., 1993; Weston et al., 1987); in the stimulation of vesicular–arbuscular mycorrhizal fungi (Poulin et al., 1993); and as phytoalexins (Sharon et al., 1992).

The flavone decomposition product 5,7-dihydroxychromone (DHC; Figure 1) has been found at relatively high (0.02% by weight) levels in peanut shells (Pendse et al., 1973). DHC has been reported to inhibit velvetleaf (*Abutilon theophrasti*) seed germination (Spencer and Tjarks, 1985) and depress aflatoxin production by *Aspergillus parasiticus* (DeLucca et al., 1987). The high levels of DHC in the shells suggest a role for it in the chemical ecology of the soil environment surrounding the shells, as the shells generally remain under the surface of the soil for several months before the seeds break dormancy and are able to germinate. Because peanut shells are the primary structures encountered by soil microorganisms and competing plant seeds during the time period that the dormant seeds are in the soil, compounds present in the shells would likely have the greatest effect on these rhizosphere organisms. DHC may thus confer a competitive advantage to the peanut seeds by preventing soil pathogenic fungi from entering the shells and attacking the seeds prior to germination while also inhibiting the germination and growth of neighboring seeds.

The 5,7-dihydroxy substitution pattern may be critical for biological activity, as Lane et al. (1987) found that eight of nine isoflavonoids isolated from



DHC



3' = 4' = H; Chrysin

3' = 4' = OH; Luteolin

FIG. 1. Chemical structures of DHC, chrysin, and luteolin.

Lupinus angustifolius that had insect deterrent activity and antifungal activity contained a 5,7-dihydroxychromone moiety. The 5,7-dihydroxychromone group may also be critical for the attraction and growth promotion of *Bradyrhizobium* sp. associated with nitrogen fixation in peanut. Flavonoids released by alfalfa (*Medicago sativa*), which promoted nodulation (*nod*) gene expression and the growth of *Rhizobium meliloti*, contained this functional group (Hartwig et al., 1991). Lynn and Chang (1990) hypothesized that flavonoids released by plants could be catabolized by symbiotic *Rhizobia* species by removal of the B ring, thus becoming simple benzoquinones. They observed a similar phenomenon existing in the parasitic plant *Striga*, which converts the flavanone hesperitin to a benzoquinone. However, 5,7-dihydroxysubstituted flavones such as chrysin and luteolin (Figure 1), which have been shown to promote the growth of several *Rhizobium* species, would be converted to DHC rather than benzoquinones by removal of the B ring.

The purpose of this study was to examine the effects of DHC on the growth of *Bradyrhizobium* sp., two soil pathogenic fungi of peanut (*R. solani* and *S. rolfsii*), and several plant species in order to explore possible roles of DHC in regulating the soil microenvironment surrounding peanut shells.

METHODS AND MATERIALS

Synthesis of 5,7-Dihydroxychromone. DHC used in experiments was prepared by chemical synthesis by the method of Spencer (1991), and confirmed to be identical with DHC obtained from the extraction of peanut shells by the method of Spencer and Tjarks (1985). Synthesized rather than extracted DHC was used because of the difficulty in extracting sufficient amounts of naturally occurring DHC for experiments. The structure of naturally occurring and synthetic DHC was confirmed by gas chromatography–mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy.

Bradyrhizobium Bioassays. A bacterial culture of *Bradyrhizobium* sp. (NRRL #B-14192) symbiotic with peanut was obtained from the culture collection at the USDA, ARS, National Center for Agricultural Utilization Research, Peoria, Illinois. Bacteria were maintained on a solid maintenance medium and transferred to a minimal liquid medium for experiments. Both the maintenance and minimal media were slight modifications of those of Hartwig et al. (1991) and contained the following constituents (milligrams per liter): KH_2PO_4 (300); K_2HPO_4 (300); $MgSO_4 \cdot 7H_2O$ (100); $CaCl_2 \cdot 2H_2O$ (5); H_3BO_3 (15); $ZnSO_4 \cdot 7H_2O$ (2); $CuSO_4 \cdot 5H_2O$ (1); $MnCl_2 \cdot 4H_2O$ (0.5); $Na_2MoO_4 \cdot 2H_2O$ (0.1); $FeCl_3 \cdot 6H_2O$ (0.1); biotin (1); thiamine HCl (1); and calcium pantothenate (1). The solid maintenance medium additionally contained 2.5 g/liter yeast extract and 15 g/liter agar. The liquid minimal medium contained 2.5 g/liter NH_4NO_3

and lacked the yeast extract and agar. In both cases, the carbon source was mannitol, at either 1.0 or 10.0 g/liter. Growth experiments were conducted with 6 ml of minimal medium in 50 ml sidearm culture flasks kept at 28°C. Growth of the bacteria was monitored spectrophotometrically by absorbance at 600 nm (A_{600}). DHC, chrysin, and luteolin were added to autoclaved medium by diluting 60 μl of filter-sterilized methanol stock solutions into the medium after cooling to approximately 70°C, which prevented chemical degradation of the flavonoids. Sixty microliters of methanol without compounds was added to controls, and the open flasks were allowed to cool to room temperature in a laminar flow hood. Cells in early log growth phase were added to the medium to give A_{600} values between 0.050 and 0.060, corresponding to approximately 10^7 viable cells/ml for *Bradyrhizobium*. Experiments were conducted three times with three replicate flasks per treatment.

Fungal Bioassays. Cultures of *R. solani* and *S. rolfsii* isolated from and pathogenic on peanut were obtained from Dr. T. Brenneman, University of Georgia. Both fungi were maintained on, and experiments conducted with, ATCC medium 343 (Jong and Gantt, 1987), pH adjusted to 5.8. Appropriate amounts of stock solutions of DHC in methanol were added to cooled ATCC 343 medium in 9.0-cm plastic Petri dishes. Experiments were initiated by placing 7-mm plugs taken from the growing margins of week-old cultures mycelial side down on the appropriate medium. Radial growth of the cultures was measured by taking average values calculated from several points taken from the edge of the inoculum disk to the growing margin of each plate when the fastest-growing culture reached the edge of the culture plates. Treatments were reported as percentages of the untreated control. Each experiment consisted of 10 plates per treatment per fungus and was repeated. I_{50} values (the concentrations of DHC required to give 50% radial growth inhibition) were calculated from the graphed results.

Seedling Radicle Elongation Bioassay. Peanut (cv. Red Tennessee), corn (cv. DeKalb IL 645-786), wheat (cv. Cardinal), and velvetleaf (purchased from Valley Seed Service, Fresno, California) seed were surface disinfected with 10% Clorox for 15 min, after which they were rinsed with and then soaked in sterile distilled water for 2 hr. Seeds were wrapped in sterile paper towels saturated with water and incubated overnight in darkness at 25°C. DHC stock solutions in methanol were added to autoclaved water agar (0.6% w/v) at ~70°C (controls contained only methanol) and placed in 9.0-cm-square plastic Petri dishes. Five germinated seedlings were removed from the wet paper towels and placed on the agar in the Petri dishes, and the dishes were incubated in darkness at 25°C on 45° slants. The growth of the radicles was measured after 48 hr, and I_{50} values were calculated from the graphed results.

RESULTS AND DISCUSSION

Effect of DHC and Flavonoids on Bradyrhizobium Growth. Bradyrhizobium sp. growth was initially inhibited by 10 μM DHC when the bacteria were grown in the low (1.0 g/liter) mannitol medium (Figure 2A) but not in the high (10.0 g/liter) mannitol medium (Figure 2B). Chrysin and luteolin in both mannitol concentrations promoted bacterial growth after 48 hr (and after 96 hr in the high mannitol medium), but by 120 hr there were no significant differences between treatments. These results differ from those of Hartwig et al. (1991), who found that in 1.0 g/liter mannitol medium *R. meliloti* growth was promoted to a greater extent by the addition of flavonoids than with 10.0 g/liter mannitol medium. Perhaps with this *Bradyrhizobium* species DHC inhibited sugar utilization and hence produced the slower growth at the lower mannitol level. The lack of promotion of growth by DHC for *Bradyrhizobium* cultures compared to chrysin and luteolin is not surprising, as DHC has only been reported in peanut shells

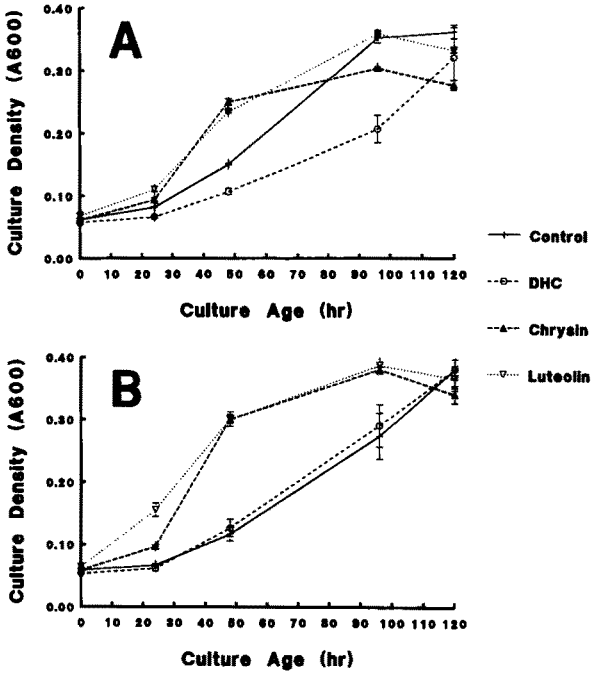


FIG. 2. *Bradyrhizobium* sp. growth with (A) 1.0 g/liter mannitol as the carbon source or (B) 10.0 g/liter mannitol. Vertical bars represent ± 1 SE.

and not in seeds or seed coats. Other growth-promoting flavonoids have been found in seed and seed coats of other legume species as well as closely related flavonoids such as 5,7-dimethoxyisoflavone (Daigle et al., 1985; Turner et al., 1975) and dihydroquercetin (Pratt and Miller, 1984).

Fungal Bioassays. DHC mildly suppressed both *R. solani* and *S. rolfsii* cultures at 5 and 10 μM concentrations and was fungicidal at 100 μM (Figure 3). I_{50} values for *R. solani* and *S. rolfsii*, calculated from the graphed results, were 18 and 26 μM, respectively. This fairly high toxicity towards both species is surprising when compared to the data of DeLucca et al. (1987), who found no significant decrease in the dry weights of mycelial mats of *Aspergillus parasiticus* after 14 days incubation on medium containing 61 μM DHC. However, these authors found that aflatoxin production was markedly decreased by DHC, to a greater extent than either luteolin or the related flavonoid eriodictyol, which are also found in peanut shells. Perhaps *A. parasiticus*, which is also a post-harvest pathogen, has a much higher tolerance to DHC than do either *R. solani* and *S. rolfsii*, which primarily attack the underground structures of the peanut plant prior to harvest. The reported I_{50} for the phytoalexin 2-(4-hydroxyphenoxy)-5,7-dihydroxychromone, found in *Cassia obtusifolia* leaves infected with *Alternaria cassiae*, was 300 μM, over 10 times the levels required to inhibit *R. solani* and *S. rolfsii* (Sharon et al., 1992). However, in this case the authors noted that levels of up to 3 mM were detected in infected leaves, so that sufficiently inhibitory concentrations were reached *in vivo*. The total amount of DHC reported in peanut shells by Pendse et al. (1973) of 0.02% and in the present study (0.023% w/w; data not shown) would correspond to roughly 1 mM based upon average fresh weights of the extracted shells. Although the long-term stability of DHC in the soil is not known, it is moderately water soluble, so that localized concentrations of DHC much higher that the I_{50}s found in these experiments could occur in the rhizosphere surrounding peanut shells.

Seedling Bioassays. DHC affected the elongation of seedling radicles in all

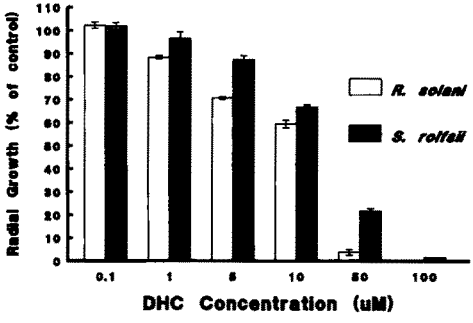


FIG. 3. Inhibition of fungal growth by DHC. Bars represent ± 1 SE.

species tested (Figure 4). Velvetleaf was the most sensitive species tested, with an I_{50} value of 30 μM, while wheat was the most tolerant, with an I_{50} of 200 μM. Corn and peanut had similar I_{50} values of 50 and 65 μM. Somewhat surprisingly, peanut radicle elongation was inhibited to a greater degree at 10^{-4} M DHC than wheat, even though wheat was the only species tested that was completely inhibited by 10^{-3} M DHC. Because DHC is present naturally in peanut, it would be expected that it would be fairly tolerant to the compound. Some aspect of lipid metabolism would appear to be a possible active site for toxicity to DHC, as peanut, corn, and velvetleaf are species that rely primarily on lipid metabolism during germination while wheat utilizes stored carbohydrate.

This study demonstrates that DHC possesses activity against certain soil fungal and plant species at concentrations lower than have been found in intact peanut shells. Because the main role of the peanut shell is to protect the seeds during development and dormancy under the soil, some antifungal activity by DHC, especially against soil pathogens, was expected. Siqueira et al. (1991) reported that flavonoids related chemically to DHC had a significant impact on plant-soil organism relationships. However, depending on the particular organism, similar concentrations of other 5,7-dihydroxysubstituted flavonoids have been shown to be either inhibitory or stimulatory. Quercetin and myricetin at 10 μM promoted hyphal growth of the mycorrhizal fungus *Gigaspora margarita*, while apigenin inhibited growth and luteolin had no effect (Poulin et al., 1993). This concentration of quercetin and luteolin slowed the growth of the alfalfa pathogens *Pythium irregulare* and *P. ultimum* (Hartwig et al., 1991).

The present data, coupled with related studies, furnish strong evidence for implicating DHC and other flavonoids in the regulation of rhizosphere ecology. From a more practical standpoint, the relatively high toxicity of DHC towards soil pathogens in our tests and in related research indicate that breeding for

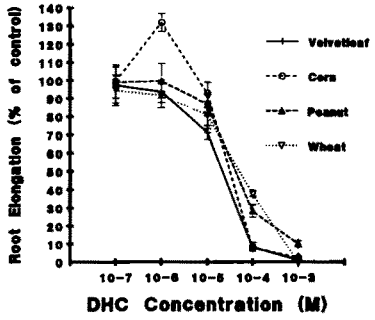


FIG. 4. Inhibition of seedling radicle elongation by DHC after 48 hr treatment. Vertical bars represent ± 1 SE.

increased DHC levels in peanut shells may be desirable for increased disease resistance and the reduction of mycotoxins during storage.

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EXTRACTION OF PHENOLIC COMPOUNDS FROM FRESH LEAVES: A COMPARISON OF METHODS

SYLVIA D. TORTI,* M. DENISE DEARING, and THOMAS A. KURSAR

201 South Biology
University of Utah
Salt Lake City, Utah 84112

(Received June 10, 1994; accepted October 17, 1994)

Abstract—The conventional sonicator/shaker bath method for phenolic extraction was compared with a less traditional one using a homogenizer. The homogenizer proved to be both more efficient and consistent in extracting phenolics from tender, as well as tough, leaves. We propose that adoption of the homogenizer technique will increase phenolic yield and efficiency.

Key Words—Extraction, phenolics, homogenizer, *Ouratea lucens*, *Acomastylis rossii*, tannin, sonicator.

INTRODUCTION

Phenolics are carbon-based compounds present in many plants. They are of general interest because of their wide-ranging ecological effects from the organism to ecosystem level (Appel, 1993). They are perhaps most noted for their ability to bind to proteins in vitro, forming soluble and insoluble complexes (Goldstein and Swain, 1965; Feeny, 1976; Hagerman and Butler, 1980; McManus et al., 1981; Hagerman and Klucher, 1986; Hagerman and Robbins, 1987). These phenolic–protein interactions are thought to be, in part, responsible for the putative function of phenolics as plant defense compounds (Feeny, 1976; Rhoades and Cates, 1976; Coley, 1983; Mole and Waterman, 1987).

To explore any of the several hypotheses in which phenolics play a prominent role, e.g., plant defense theory, phenolic quantification is often necessary. Numerous researchers have investigated various phenolic assays (Peri and Pom-

*To whom correspondence should be addressed.

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pei, 1971; Hagerman and Butler, 1980, 1989; Mole and Waterman, 1987; Wisdom et al., 1987; Mole et al., 1989), appropriate standards (Hagerman and Butler, 1989) and extraction solvents (Hagerman, 1988). To our knowledge, no studies have systematically investigated the extraction technique itself.

Conventional methods typically involve reducing the plant material into smaller particles using liquid nitrogen and a mortar and pestle. The pulverized plant material is then extracted in a solvent, and the extraction is enhanced by using a sonicator or shaker bath. This step is repeated several times (Hagerman, 1988; Hagerman and Butler, 1989). Our extraction method differs from the conventional method in the use of a homogenizer to facilitate the release of phenolics instead of several sonicator/shaker bath extractions. We propose that the homogenizer method is superior to that of the sonicator/shaker bath technique in both accuracy and efficiency of extraction. Moreover, we predict that this difference will be most pronounced in plants with tough, fibrous leaves, as the thicker cell walls should further hinder extraction by conventional methods.

METHODS AND MATERIALS

We chose two species of plants that differ in leaf toughness: *Acomastylis* (*Geum* Ser.) *rossii* (R.Br.) Greene (Rosaceae), a temperate alpine plant with relatively tender leaves, and *Ouratea lucens* Engl. (Ochnaceae), a tropical species with tough leaves. Toughness was estimated from the amount of mass required to force a 5-mm-diameter rod through leaf material and is correlated with fiber content (Coley, 1983). Toughness was measured directly on *O. lucens*, and is 500 g (Kursar, unpublished data). It was impossible to directly measure toughness on *A. rossii* leaves because they are highly dissected. However, we calculated *A. rossii*'s toughness from its acid detergent fiber (ADF) content (Dearing, unpublished data), using an equation obtained by Coley (1983), who regressed toughness on ADF content for 46 species. Her equation is $y = 11.95x + 77.5$, where y is toughness in grams and x is percent ADF content ($r^2 = 0.32$, $P < 0.01$). The estimated toughness of *A. rossii* is 220 g, which is representative of a very nontough species.

A. rossii leaves were harvested in August 1993 and kept at −70°C until the day of the experiment. *O. lucens* was freshly harvested from the University of Utah greenhouse and immediately put on Dry Ice.

General Leaf Preparation. Major veins were removed with a razor and the leaf fragments were cut into small pieces. The leaf fragments were further ground under liquid nitrogen using a mortar and pestle. Grinding in liquid nitrogen was necessary for both techniques. A subsample was weighed, dried at 50°C, and reweighed to obtain the dry weight/wet weight ratio.

Extraction. Samples of ground leaves were simultaneously subjected to both extraction techniques three separate times (trials 1–3). Wet weight samples of powdered leaves, approximating 0.1 g dry weight, were extracted first with 25 ml of 85% MeOH (extract A). Each sample was extracted three successive times with 10 ml 85% MeOH (extracts B, C, and D).

Phenolics were extracted by using either a Di Sontegrator (Ultrasonic Industries Inc.) sonicating bath followed by shaking in a standard shaker bath, or a Brinkmann Homogenizer Polytron 10/35, equipped with a PTA 10 TS generator with saw teeth (Brinkmann Instruments, Inc.). For the conventional extraction, test tubes with ground leaves and solvent were placed in a sonicator at 4°C. Tubes were removed after 30 min and centrifuged at 16,000*g* for 15 min at 4°C. The supernatant volume was measured, and 1 ml was saved for phenolic analysis (extract A). The pellet was resuspended in 10 ml solvent, vortexed and placed in a shaker bath at 4°C at high speed for 30 min. The sample was centrifuged as described above, the supernatant was measured, and again 1 ml was saved (extract B). This was repeated two more times, for a total of four extracts (A–D). Extracts were stored in a freezer (−5°C) in the dark until the phenolics analyses were conducted.

The plant material was extracted by using the homogenizer for 60 sec at maximum speed. Homogenization for 60 sec slightly warms but does not greatly raise the temperature of the solvent. The test tubes were then centrifuged at 16,000*g* for 15 min at 4°C, after which the supernatant was removed, measured, and 1 ml was saved for phenolic assaying (extract A). For the subsequent extracts (B–D), the pellet was resuspended by vortexing with 10 ml of 85% MeOH. The samples were centrifuged between extractions as described above.

To determine whether the homogenization extraction procedure was exhaustive, we passed samples through a French press (Aminco, SLM Instruments Inc.). Because the plant material must be ground extremely fine for use in the French press, more than that which is possible with a mortar and pestle, we first extracted the plant material by homogenization as described above. After the four extractions, the pellet was then resuspended in 15 ml of 85% MeOH and passed once through the French press at 21 MPa. This machine imparts a force of 21 MPa on sample material, which causes the rupture of all cells (Milner et al., 1950). The French press extracts were then analyzed for phenolics.

Phenolics Assay. Extracts were assayed within one week of extraction. Sonicator/shaker bath and homogenizer samples that had been extracted on the same day were assayed at the same time. Extracts A–D were assayed individually for total phenolics using the Folin-Ciocalteu method (Singleton and Rossi, 1965). The amount for each extract was combined (A + B + C + D) for an absolute quantity of phenolics/sample. Tannic acid was used as the standard (Sigma lot #1764 KCNT).

RESULTS

The homogenizer extracted significantly more phenolics for all extracts combined (A–D) than did the sonicator/shaker bath method. This difference was consistent for both plant species tested (Randomized complete block, blocking on trial: *A. rossii*, method F = 94.91, $P < 0.0001$, $df = 1, 20$; block F = 14.43, $P < 0.0001$, $df = 2, 20$; *O. lucens*, method $F = 30.53$, $P < 0.0001$, $df = 1, 20$; block $F = 8.61$; $P < 0.002$, $df = 2, 20$; Figure 1). The sonicator/shaker bath technique extracted only three quarters of the total amount of phenolics extracted by the homogenizer (*A. rossii*, 74.6% ± 5.2 and *O. lucens*, 68.0% ± 9.0, Figure 2). In addition, for both species we found three to four times less variability among samples using the homogenizer technique (Table 1).

Results from the analysis for absolute phenolic quantity in samples extracted after using the French press show zero phenolics for one sample of *A. rossii*, negligible phenolics for the other sample (0.06% of total), and no remaining phenolics for the two *O. lucens* samples. Hence, we are confident that the higher yields extracted using the homogenizer represent virtually all extractible plant phenolics detectable by the Folin-Ciocalteu assay.

Interfraction Comparison: Extracts A–D. The percent of the total extracted per extraction (A–D) for the two methods was compared. Because the homogenizer extracted all phenolics, this amount is used as the denominator for the sonicator extracts. We found that for both species, the homogenizer removed significantly more phenolics in the first extraction than did the sonicator and that this difference diminished, and even reversed, in successive extractions (Figure 2). The homogenizer extracted virtually all phenolics with only one extraction, while even with four extractions, the sonicator had not always extracted a comparable amount of phenolics. In the initial extraction of *A. rossii* leaves, the homogenizer extracted 93.8% of the total extractible phenolics, whereas the sonicator/shaker bath extracted only 38.7% (Figure 2). The difference in the extraction efficiency of the two techniques was similar in *O. lucens*; the homogenizer extracted 99.2% of the total phenolics in the initial extract and then negligible amounts in subsequent extractions. The sonicator/shaker bath extracted only 45.5% of the total in the initial extract. In both species, the homogenizer pellet additionally released only small amounts of phenolics in subsequent extractions (B–D), while the sonicator/shaker bath consistently yielded more phenolics in these extracts (Figure 2).

DISCUSSION

Our results show that the homogenizer technique extracts totally more phenolics, with less variability, than the sonicator/shaker bath technique. Moreover, extraction time for the same number of samples (up to eight) with four extrac-

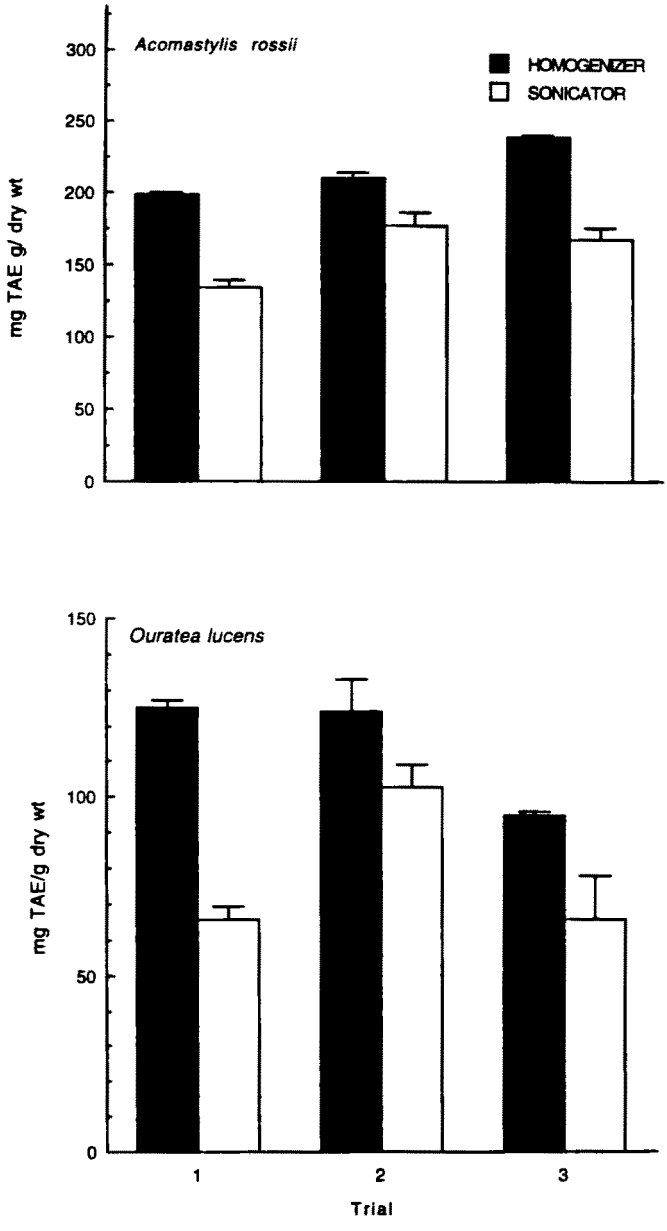

FIG. 1. Comparison of total phenolics extracted by the two methods. Bars represent means for each trial ($N = 3$) plus 1 standard error.

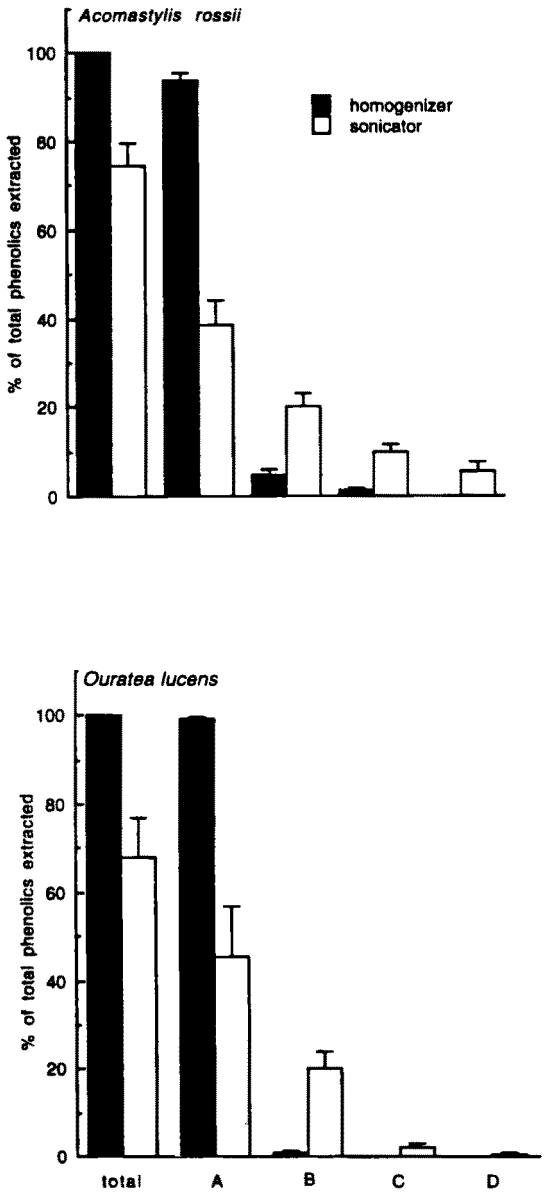

FIG. 2. Comparison of the percent of phenolics extracted by each of the two methods: overall and for each extraction (A-D). Bars are means for each trial (N = 3) plus 1 standard error.

TABLE 1. COEFFICIENTS OF VARIATION FOR TENDER AND TOUGH LEAVES EXTRACTED BY TWO METHODS[a]

	A. rossii (tender leaves)	*O. lucens* (tough leaves)
Homogenizer	1.9 (0.9)	6.7 (4.1)
Sonicator	9.2 (3.5)	20.4 (8.4)

[a] Average coefficient of variation for three trials plus one standard error.

tions per sample is much shorter using the homogenizer technique: 1 hr 10 min for the homogenizer vs. 3 hr for the sonicator/shaker bath. The number of extractions for the homogenizer technique can be reduced further to two extractions, with no decline in accuracy, as the third and fourth extractions are superfluous, yielding negligible phenolics. Thus, total extraction time using the homogenizer requires approximately 40 min, which represents a considerable time savings over the 3-hr sonicator/shaker bath technique.

Those familiar with the sonication method may initially conclude that the homogenizer is less efficient for large numbers of samples than the sonicator. It is important to note that the number of samples that can be processed using the sonication method is not infinite. The sonication method may be limited by either the sonicator or centrifuge size. Furthermore, even if neither the sonicator nor the centrifuge is restricting, measuring the supernatant after centrifugation can become tedious with sample sizes greater than 20. We suggest grinding be done in subsamples as numerous as the centrifuge will allow and that other subsamples are ground while the first set of subsamples are spinning in the centrifuge. Thus, large sample sizes, i.e., 20, can be managed using the homogenizer with little increase in total time.

The dramatic differences between the two techniques can be partly attributed to the different modes of action of the machines used in the extractions. Several compounding factors contribute to the variability and inefficiency of a sonicating bath. First, ultrasonic waves are effective at emulsifying lipids and are typically employed to break up the lipid membrane surrounding bacteria and animal cells. However, the lipid membrane of a plant cell is encased within a rigid, fibrous cell wall that is probably more resilient to ultrasonic waves. Thus, sonication effectiveness is likely to be dependent on the extent of cell wall damage during mortar and pestle grinding. Moreover, the ultrasonic waves in a sonicating bath are typically not focused or uniform; thus, some samples may receive more sonication than others. In practice, sonicating probes, not baths, are employed to disrupt cells, as probes better focus the sound waves within the sample, while sonicating baths are typically used for cleaning surfaces. Given these considerations, the appropriateness of the sonicating bath for the purpose

of rupturing plant cells is questionable. Cork and Krockenberger (1991) found no difference in extraction yield when a shaker bath was substituted for a sonicator. In addition to these technical drawbacks, the long extraction period (3 hr) may further reduce yield due to phenolic oxidation during the extraction itself.

The superiority of the homogenizer is partially due to its efficacy in breaking down cell walls. The joint action of the partial vacuum created by the homogenizer and the tearing by the saw tooth generator serve to break the cell walls of plant leaves in a way in which a sonicating bath cannot. This difference is obvious in the appearance of the ground leaves. The initial extractions prepared in the homogenizer are bright green in color, while first extractions from the sonicator are much lighter green.

Contrary to our prediction, there was no major difference in extraction efficiency with respect to leaf toughness. The sonicator/shaker bath extracted approximately 75% of all phenolics, regardless of leaf toughness. The homogenizer consistently extracted all detectable phenolics in both tender and tough leaves. Furthermore, the pattern of extraction was similar with both fresh and frozen leaves.

CONCLUSION

We propose that the efficiency and accuracy of phenolic extraction can be enhanced by the utilization of the homogenizer method. The adoption of this device may be beneficial for extractions of other types of phenolics as well and should facilitate investigations in the area of plant defense.

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DRIED LEAVES FROM ROCKY MOUNTAIN PLANTS DECREASE INFESTATION BY STORED-PRODUCT BEETLES[1]

DAVID K. WEAVER,[2,5,*] THOMAS W. PHILLIPS,[3] FLORENCE V. DUNKEL,[2] T. WEAVER,[4] ROBERT T. GRUBB,[2] and ELIZABETH L. NANCE[2]

[2]*Department of Entomology, Montana State University Bozeman, Montana 59717*

[3]*USDA-ARS Stored-Product Insects Research Unit Department of Entomology, University of Wisconsin 1630 Linden Drive, Madison, Wisconsin 53706*

[4]*Department of Biology, Montana State University Bozeman, Montana 59717*

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Abstract—Leaves of two highly aromatic plants, *Artemisia tridentata* (Nutt.) and *Monarda fistulosa* L., prepared according to a patented process, inhibited oviposition by the Mexican bean weevil, *Zabrotes subfasciatus* (Boheman), in beans at concentrations less than 1% w/w. Both plant species were less effective against the rice weevil, *Sitophilus oryzae* L., in wheat, with only *M. fistulosa* exhibiting any concentration-dependent activity. The maximal control achieved against this species was less than 50% at 3% w/w. Two less aromatic plant species, *Balsamorhiza sagittata* (Pursh.) Nutt. and *Geranium viscosissimum* Fisch. and Mey., caused only low levels of inhibition against both insect species. Volatiles probably caused the response to *A. tridentata* and *M. fistulosa*, while the asymptotic concentration dependence for the less volatile plant material was likely caused by behavioral factors related to the physical presence of foreign particulate matter in the foodstuff. Chemical analysis indicated that most of the volatile components from the dried leaf

*To whom correspondence should be addressed.

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[5]Current address: USDA-ARS Insect Attractants, Behavior, and Basic Biology Research Laboratory, 1700 S.W. 23rd Dr., Gainesville, FL 32608.

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material from all species were terpenoids, with camphor (9.7 mg/g) and 1,8-cineole (4.0 mg/g) being most abundant in *A. tridentata* and carvacrol (26.3 mg/g) being most abundant in *M. fistulosa*.

Key Words—Insecticide, fumigant, terpenoids, Coleoptera, Bruchidae, Curculionidae, camphor, carvacrol, *Monarda fistulosa*, *Artemisia tridentata*, *Zabrotes subfasciatus*, *Sitophilus oryzae*.

INTRODUCTION

Many plant species are known to contain phytochemicals that may protect them against herbivory, and farmers have historically used such plants to protect stored foodstuffs from insects. Aromatic plants, those yielding a pungent odor from essential oils after crushing, are noted for insecticidal activity (e.g., Duke, 1990). For example, the wild mint species *Ocimum canum* Sims has been used as a protectant for stored beans in Rwanda (Dunkel et al., 1986) and for groundnuts in the Congo (Delobel and Malonga, 1987). The volatile terpene alcohol linalool is predominant in *O. canum* leaves and acts as a potent insecticide against several species of stored-product insects (Shaaya et al., 1991; Weaver et al., 1991, 1994). Crude plant materials used as protectants against pest insects are usually locally available aromatic plants, and their use has a long history. Insecticidal activity of plants that lack a history of such use has been little tested.

Here we examine the possibility that Rocky Mountain aromatic plants might protect against two species of stored-product insects. We tested four plant species that were relatively free of damage by insect herbivores, but for which there was no prior knowledge about their protectant activity. Two highly aromatic species, wild bergamot, *Monarda fistulosa* L. (Lamiaceae), and big mountain sagebrush, *Artemisia tridentata* (Nutt.) (Compositae), and two less aromatic species, arrowleaf balsamroot, *Balsamorhiza sagittata* (Pursh.) Nutt. (Compositae), and sticky geranium, *Geranium viscosissimum* Fisch. and Mey. (Geraniaceae), were investigated. Specific objectives were: (1) to determine if an admixture of powdered material, prepared according to a patented process, from these species could protect stored beans and wheat from infestation by two species of stored-product beetles, and (2) to determine chemical composition of volatiles extracted from these plant species.

METHODS AND MATERIALS

Plant Material. Material was collected from plants growing in a foothill *Artemisia tridentata–Festuca idahoensis* community (Mueggler and Stewart, 1980) on June 27, 1991. All four species were collected at an altitude of 1550 m on a southwestern facing slope in T2S, R4SW, approximately 8 km northeast

of Bozeman, Montana, USA. Representative leaves were harvested in a random pattern from most individuals in the stand (dozens to hundreds of individual plants). The materials collected included petioles, but main stems were removed. Samples were dried <3 hr after harvest, at 52°C for 72 hr. Intact leaves were then stored under dry N_2 at −20°C in sealed glass containers until use. Voucher specimens were deposited in the Montana State University Herbarium on July 10, 1991 as: *Balsamorhiza sagittata*, voucher F. Dunkel #2, *Artemisia tridentata*, voucher F. Dunkel #3; *Monarda fistulosa*, Voucher F. Dunkel #4; and *Geranium viscosissimum*, Voucher F. Dunkel #5.

Insect Culture. Protectant material was tested against two insect species, a cosmopolitan pest of stored beans, *Zabrotes subfasciatus* (Coleoptera: Bruchidae) (*Boheman*) and a severe cosmopolitan pest of stored grains, *Sitophilus oryzae* L. (Coleoptera: Curculionidae). Both species develop within the seed kernel. *Z. subfasciatus* is short-lived and nonfeeding in the adult stage. Eggs are firmly attached to the bean surface and the larva bores directly into the bean from within the egg. *S. oryzae* is long-lived and feeds as an adult. Females chew a small hole in the host grain, deposit a single egg, and seal the hole with a mucilaginous plug. *Z. subfasciatus* was reared on a diet of dried pinto beans, and *S. oryzae* was reared on a diet of soft white wheat. Cultures were maintained in 0.95-liter jars containing 0.6 liters of commodity and held in 27 ± 1°C incubators maintained at 65 ± 5% relative humidity with a 12L:12D photoperiod. *Z. subfasciatus* cultures were inoculated with approximately 300 adult bruchids, and *S. oryzae* cultures were inoculated with approximately 100 adult weevils. Cultures were discarded after the emergence of two subsequent generations. Both species had been maintained in the laboratory for many years.

Bioassay. Leaves were prepared according to a patented process (Dunkel et al., 1994). This material was added to the commodity to yield concentrations of 0.1, 0.2, 0.3, 1.0, and 3.0% w/w. For *Z. subfasciatus*, the processed leaf material was mixed with 20 g of pinto beans (equilibrium moisture content 14.4%) in a washed 10-dram plastic shell vial, while for *S. oryzae*, the material was added to 1 g of equilibrated soft white wheat (moisture content 12.9%) in a similar vial. There were 10 replicates of each dosage and 12 of the control for *Z. subfasciatus*, while there were eight replicates of each dosage and the control for *S. oryzae*. Five males and five females were added to each vial. The *Z. subfasciatus* were zero to one day after adult emergence and the *S. oryzae* were four to nine days after adult emergence. Incubation conditions were similar to those for culture maintenance. Hatched and unhatched eggs of *Z. subfasciatus* were counted at 25 days and live and dead progeny adults were counted at 55 days. For *S. oryzae*, adults were removed at seven days, and the immature insect cohorts were incubated for a further seven days and then frozen. Wheat kernels were soaked in 70% ethanol (w/w in distilled H_2O) for 2 hr and then immersed in 15 mg/ml malachite green in absolute ethanol for 15 min. This

selectively stained the egg plugs, facilitating dissection of the wheat to determine egg viability. The adult *S. oryzae* were sexed according to the Halstead (1963) method. Postmortem sexing was superior to live determination and thus resulted in elimination of some replicates. The high densities of pests used in our experiments resulted in suboptimal reproduction. The staining and counting methods were limiting, which is reflected in the experimental design. Therefore, low level treatment effects may have been obscured if density-stressed females oviposited in available resources, even when marginally suitable.

Statistical Analyses. Data from the experiments were fitted to three equations of biological relevance. These were: (1) the logistic dose–response transition equation, $y = a + \{b/[1 + (x/c)^d]\}$ with a and b being constants for this data set, (2) an asymptotic equation, $y = a + b/x$, and (3) a linear equation $y = a + b(\ln x)$. For these experiments $x = \log_{10}$ (concentration) + 0.02. Analyses were performed with PROC NLIN or PROC REG (SAS Institute, 1988). Tests for a significant effect of plant species within asymptotic data sets for a particular insect (with the common control data deleted) were conducted with PROC GLM (SAS Institute, 1988), and evaluations of a significant concentration effect on the same data sets were conducted using PROC REG (SAS Institute, 1988).

The data collected from these experiments follow a "many y for each x" pattern. Thus, it is difficult to determine how well the regression equation describes the data with its innate variability. This is an important and measurable component of the percentage variation explained (r^2) and can be used to calculate the maximum r^2 possible, that is, the greatest amount of the total variation that can be explained by any model given the inherent variation, or pure error, in the data. The equation used is Max r^2 possible = $(SS_{\text{corrected total}} - SS_{\text{pure error}})/SS_{\text{corrected total}}$ (Draper and Smith, 1981). This equation has been suggested for the percentage variation explained for linear equations, for which it is exact, but it also provides useful descriptive information for the approximate percentage variation explained for nonlinear equations (Bates and Watts, 1988). The models were subjected to lack-of-fit tests ($\alpha = 0.1$) prior to evaluation of the significance of the regressions themselves (Draper and Smith, 1981; Bates and Watts, 1988).

Extraction and Analysis of Plant Volatiles. Five grams of the processed material from each plant were extracted separately in 50 ml of 1:4 isopropanol:hexane (both HPLC grade) and 1.33 mg of *n*-decane was added as an internal standard. The crude extracts were subjected to a purification procedure using flash column chromatography on a 1.9-cm-diameter × 10-cm column packed with TLC grade MgO using 125 ml of the extraction solvent. Samples were then concentrated under cool N_2 to approximately 3 ml and then were subjected to a second flash chromatography procedure on a 1.0-cm-diameter × 12-cm column packed with TLC grade MgO using 50 ml of the extraction solvent.

The samples were evaporated under cool N_2 to near dryness and then resolvated in 1.5 ml of HPLC-grade hexane.

Extracts were subjected to gas chromatographic (GC) analyses on two separate instruments. Initial inspection of the extracts and ultimate quantification of volatiles were done on a Hewlett Packard 5890 GC equipped with a flame ionization detector (FID). The column used was a 30-m × 0.252-mm-ID DB-1 with 0.25-μm film thickness (J & W Scientific, Folsom, California); He flow was 1 cm/sec. The injector was set at 225°C, the detector at 280°C, and the column oven programmed at 60°C for 30 sec, then increased to 270°C at 10°C/min, and held at 270°C for 20 min. The injector purge was operated in split mode throughout. Output went to a Hewlett Packard 3392A integrator recorder, and quantities of detected compounds were calculated by comparison of the area under the decane peak with areas under sample peaks. GC–mass spectrometric (GC-MS) analyses were made using a Shimadzu GC-14A coupled to a Finnigan model 800 series Ion Trap Detector with an ionizing voltage of 70 eV. The injector oven was set at 230°C and the heated transfer line to the MS was set at 265°C. The column used was also a 30-m × 0.252-mm DB-1 with 0.25-μm film thickness, temperature programmed at 40°C for 30 sec, then 20°C/min to 60°C, held for 1 min, then increased to 280°C at 10°C/min, and held at 280°C for 20 min. Injection was made with the splitter closed initially, but then opened at 30 sec. The MS was operated in the full-scan mode, ranging from 35 to 350 amu at 1 scan/sec. Tentative identifications were made by matching background-subtracted sample spectra with those in the National Bureau of Standards mass spectral library and with those in the terpene library of Adams (1989) using the Finnigan ITDS software. Confirmation of library matches was made when possible by matching retention time and spectra of authentic samples.

RESULTS

The bioactivity of the powdered material from all four plants varied against the two stored-product pests. *Z. subfasciatus* was more susceptible to the more aromatic plant species than *S. oryzae*. Both *M. fistulosa* and *A. tridentata* completely prevented reproduction of *Z. subfasciatus* at higher concentrations (Figures 1–3), with no oviposition occurring above 1.0% w/w. The activity of *M. fistulosa* was much higher, with 80% inhibition of oviposition occurring at the lowest concentration tested (0.1% w/w). The concentration-dependent activity of both more aromatic plant species was similar within each plant for all three biological variables, although the decrease in beans exploited (Figure 1) was lower at low doses than the decrease in eggs hatched (Figure 2) or the decrease in progeny adult emergence (Figure 3). In contrast, both *B. sagittata* and *G. viscosissimum* caused clear asymptotic inhibition of biological productivity, with

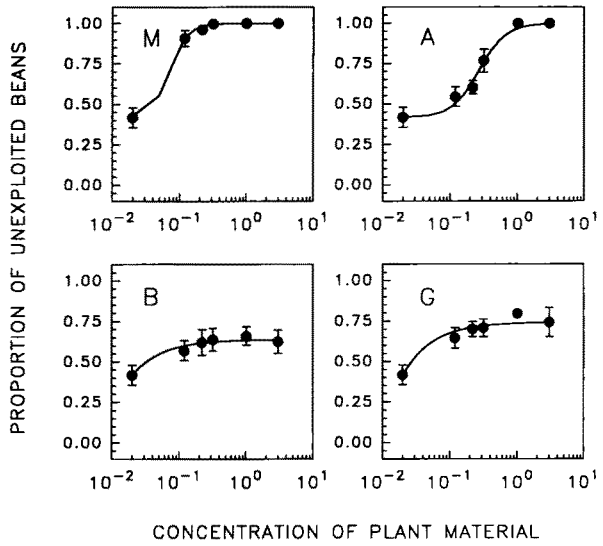

FIG. 1. Mean proportion (±SE) of beans unexploited by *Z. subfasciatus* as influenced by increasing concentration of plant material. Plant species: M, *Monarda fistulosa*; A, *Artemisia tridentata*; B, *Balsamorhiza sagittata*; G, *Geranium viscosissimum*. Concentration units are % (w/w) + 0.02. Regression equations: M, proportion unexploited = $a + b/1 + (\text{concentration}/c)^d$, $a = 0.42$, $b = 0.58$, $c = 0.07$, $d = -3.19$, lack-of-fit $F = 0.39$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.93$, percentage of maximum attainable $r^2 = 100\%$; A, proportion unexploited = $a + b/1 + (\text{concentration}/c)^d$, $a = 0.42$, $b = 0.58$, $c = 0.27$, $d = -2.19$, lack-of-fit $F = 1.65$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.88$, percentage of maximum attainable $r^2 = 99\%$; B, proportion unexploited = $a + b/(\text{concentration})$, $a = 0.64$, $b = -0.0045$, lack-of-fit $F = 0.42$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.39$, percentage of maximum attainable $r^2 = 95\%$; G, proportion unexploited = $a + b/\text{concentration}$, $a = 0.74$, $b = -0.0067$, lack-of-fit $F = 1.59$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.62$, percentage of maximum attainable $r^2 = 94\%$.

very limited concentration dependence at doses greater than the control (Figures 1–3). Deletion of the control data gave no significant slope for *B. sagittata* (proportion of unexploited beans: $F = 0.24$, $df = 1, 48$, $P = 0.63$; hatched eggs per female: $F = 2.26$, $df = 1, 48$, $P = 0.14$; progeny adults per female: $F = 0.37$, $df = 1, 48$, $P = 0.55$) or *G. viscosissimum* (proportion of unexploited beans: $F = 3.07$, $df = 1, 48$, $P = 0.09$; hatched eggs per female: $F = 2.50$, $df = 1, 48$, $P = 0.12$; progeny adults per female: $F = 1.04$, $df = 1, 30$, $P = 0.32$), but there was a significant effect of species when comparing the data for the two plants without the common control (proportion of unexploited beans: $F = 21.00$, $df = 1, 99$, $P < 0.001$; hatched eggs per female: $F = 8.77$, $df =$

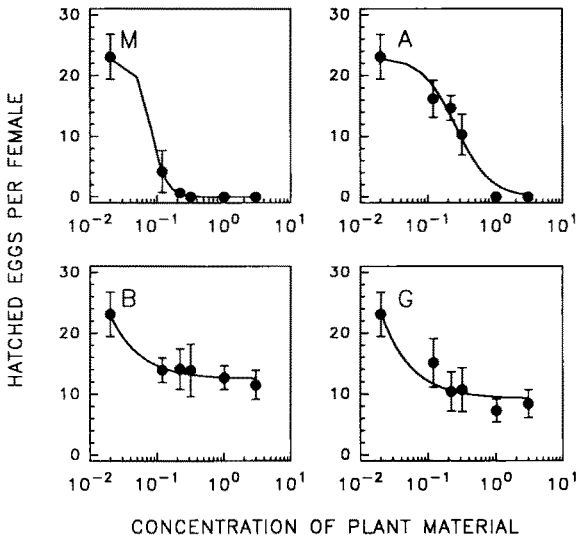

FIG. 2. Mean number (±SE) of hatched eggs per female *Z. subfasciatus* as influenced by increasing concentration of plant material. Plant species and concentration units as indicated in Figure 1. Regression equations: M, hatched eggs per female = $a + b/1 + (\text{concentration}/c)^d$, $a = 0.0$, $b = 23.10$, $c = 0.08$, $d = -3.74$, lack-of-fit $F = 0.012$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.87$, percentage of maximum attainable $r^2 = 100\%$; A, hatched eggs per female = $a + b/1 + (\text{concentration}/c)^d$, $a = 0.00$, $b = 23.10$, $c = 0.27$, $d = -1.71$, lack-of-fit $F = 1.63$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.80$, percentage of maximum attainable $r^2 = 98\%$; B, hatched eggs per female = $a + b/\text{concentration}$), $a = 12.53$, $b = 0.21$, lack-of-fit $F = 0.21$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.40$, percentage of maximum attainable $r^2 = 98\%$; G, hatched eggs per female = $a + b/\text{concentration}$, $a = 9.28$, $b = 0.29$, lack-of-fit $F = 1.76$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.51$, percentage of maximum attainable $r^2 = 92\%$.

1, 99, $P = 0.004$; progeny adults per female: $F = 3.96$, $df = 1, 30$, $P = 0.05$), indicating that the asymptotes are different for the two species.

M. fistulosa was the only plant species that caused concentration-dependent inhibition of oviposition by *S. oryzae* (Figures 5 and 6), although the proportion of grains exploited was asymptotic (Figure 4). In contrast, only asymptotic patterns were evident for the other three plant species. Deletion of the control data gave no significant slope for the remaining data for *A. tridentata* (proportion of unexploited grains: $F = 0.00$, $df = 1, 31$, $P = 1.00$; eggs laid per female: $F = 0.63$, $df = 1, 31$, $P = 0.43$; hatched eggs per female: $F = 0.87$, $df = 1, 31$, $P = 0.36$), for *B. sagittata* (proportion of unexploited grains: $F = 1.60$, $df = 1, 30$, $P = 0.22$; eggs laid per female: $F = 0.03$, $df = 1, 30$, $P = 0.86$;

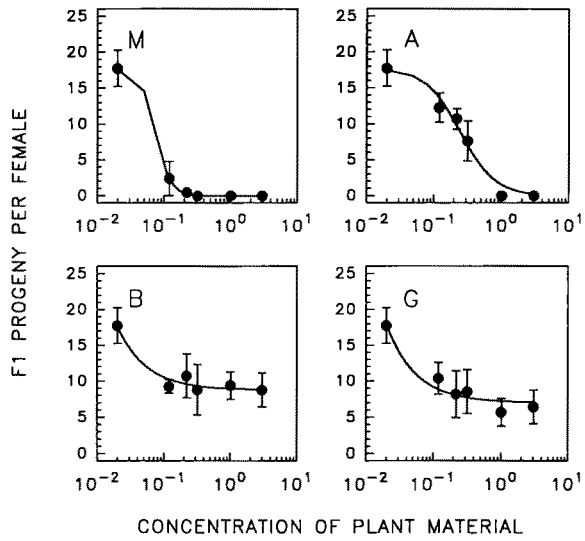

FIG. 3. Mean number (±SE) of progeny adults per female *Z. subfasciatus* as influenced by increasing concentration of plant material. Plant species and concentration units as indicated in Figure 1. Regression equations: M, progeny adults per female = $a + b/1 + (\text{concentration}/c)d$, $a = 0.00$, $b = 17.75$, $c = 0.07$, $d = -3.85$, lack-of-fit $F = 0.023$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.89$, percentage of maximum attainable $r^2 = 100\%$; A, progeny adults per female = $a + b/1 + (\text{concentration}/c)^d$, $a = 0.00$, $b = 17.75$, $c = 0.25$, $d = -1.67$, lack-of-fit $F = 1.53$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.82$, percentage of maximum attainable $r^2 = 98\%$; B, progeny adults per female = $a + b/\text{concentration})$, $a = 8.79$, $b = 0.18$, lack-of-fit $F = 0.42$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.42$, percentage of maximum attainable $r^2 = 98\%$; G, progeny adults per female = $a + b/\text{concentration}$, $a = 7.03$, $b = 0.22$, lack-of-fit $F = 0.87$, $df = 4, 56$, $P > 0.05$, $r^2 = 0.50$, percentage of maximum attainable $r^2 = 94\%$.

hatched eggs per female: $F = 0.65$, $df = 1, 30$, $P = 0.43$), or for *G. viscosissimum* (proportion of unexploited grains: $F = 2.90$, $df = 1, 27$, $P = 0.10$; eggs laid per female: $F = 0.89$, $df = 1, 27$, $P = 0.35$; hatched eggs per female: $F = 1.04$, $df = 1, 27$, $P = 0.32$), but there was a significant effect of species when comparing the data for the three plants without the common control (proportion of unexploited grains: $F = 7.63$, $df = 2, 88$, $P < 0.001$; eggs per female: $F = 21.29$, $df = 2, 88$, $P < 0.001$; hatched eggs per female: $F = 18.18$, $df = 2, 88$, $P < 0.001$), indicating that the asymptotes were different for at least two of the three species.

In general, the predetermined equations showed no significant lack of fit, although certain of the data patterns were quite variable, particularly for *S.*

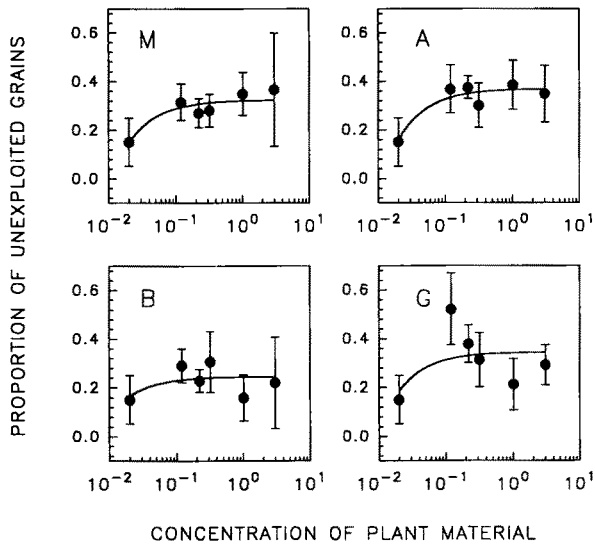

FIG. 4. Mean proportion (±SE) of grains unexploited by *S. oryzae* as influenced by increasing concentration of plant material. Plant species and concentration units as indicated in Figure 1. Regression equations: M, proportion unexploited = $a + b$(ln concentration), $a = 0.32$, $b = -0.0035$, lack-of-fit $F = 0.70$, $df = 4, 30$, $P > 0.05$, $r^2 = 0.24$, percentage of maximum attainable $r^2 = 80\%$; A, proportion unexploited = $a + b$/concentration, $a = 0.37$, $b = -0.0042$, lack-of-fit $F = 0.72$, $df = 4, 32$, $P > 0.05$, $r^2 = 0.25$, percentage of maximum attainable $r^2 = 78\%$; B, proportion unexploited = $a + b$/concentration), $a = 0.25$, $b = -0.0016$, lack-of-fit $F = 2.70$, $df = 4, 31$, $P = 0.05$, $r^2 = 0.05$, percentage of maximum attainable $r^2 = 30\%$; G, proportion unexploited = $a + b$/concentration, $a = 0.34$, $b = -0.0032$, lack-of-fit $F = 5.53$, $df = 4, 28$, $P < 0.005$, $r^2 = 0.11$, percentage of maximum attainable $r^2 = 22\%$.

oryzae. The only exception was the proportion of unexploited grains for *G. viscosissimum* with *S. oryzae*, in which there was a significant lack of fit (Figure 4). A systematic trend for the lower three doses is apparent when viewing the data residuals, but subsequent curve-fitting trials yielded no better fitting, biologically plausible equation. The same trend was not apparent for egg numbers (Figure 5) or hatch (Figure 6), so the equation was retained despite the poor fit, and the data for the lower doses can be treated as logically explainable anomalies (see Discussion).

Total ion current chromatograms of plant extracts are given in Figure 7, and identities and quantities of compounds are given in Table 1. *A. tridentata* contained the largest number of extractable volatiles, primarily terpenoids, with camphor and cineole being most abundant (Figure 7 and Table 1). *M. fistulosa*

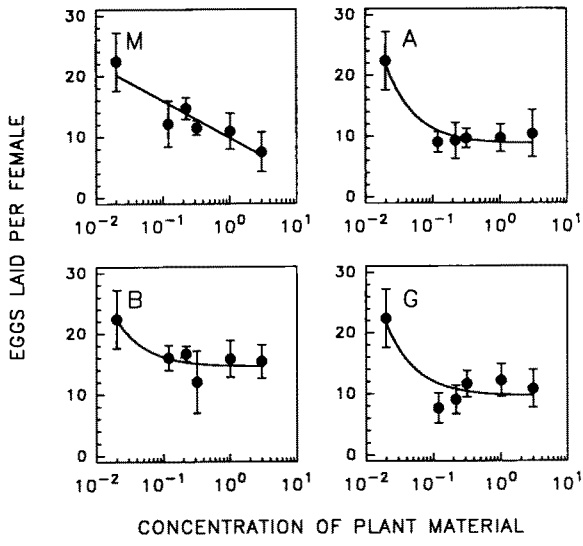

FIG. 5. Mean number (±SE) of eggs laid per female *S. oryzae* as influenced by increasing concentration of plant material. Plant species and concentration units as indicated in Figure 1. Regression equations: M, eggs laid per female = $a + b$(ln concentration), $a = 9.83$, $b = -2.64$, lack-of-fit $F = 1.63$, $df = 4, 30$, $P < 0.05$, $r^2 = 0.44$, percentage of maximum attainable $r^2 = 82\%$; A, eggs laid per female = $a + b$/concentration, $a = 8.73$, $b = 0.26$, lack-of-fit $F = 0.94$, $df = 4, 32$, $P > 0.05$, $r^2 = 0.60$, percentage of maximum attainable $r^2 = 94\%$; B, eggs laid per female = $a + b$/concentration), $a = 14.57$, $b = 0.16$, lack-of-fit $F = 1.06$, $df = 4, 31$, $P > 0.05$, $r^2 = 0.25$, percentage of maximum attainable $r^2 = 74\%$; G, eggs laid per female = $a + b$/concentration, $a = 9.64$, $b = 0.24$, lack-of-fit $F = 0.07$, $df = 4, 28$, $P > 0.05$, $r^2 = 0.52$, percentage of maximum attainable $r^2 = 79\%$.

also contained a substantial number of volatile compounds, with carvacrol being most abundant (Figure 7 and Table 1). The amount of carvacrol extracted from *M. fistulosa* is nearly three times greater than that for the next most abundant compound extracted, camphor from *A. tridentata*. Both *B. sagittata* and *G. viscosissimum* contained limited quantities of volatile material, with germacrene from *B. sagittata* being the only identified compound present in greater than 1 mg/g quantities (Figure 7 and Table 1).

DISCUSSION

The addition of small amounts of plant material from any of the test plant species inhibited the size of the resulting insect population. For the two less aromatic species, no concentration-dependent effects were seen for either pest

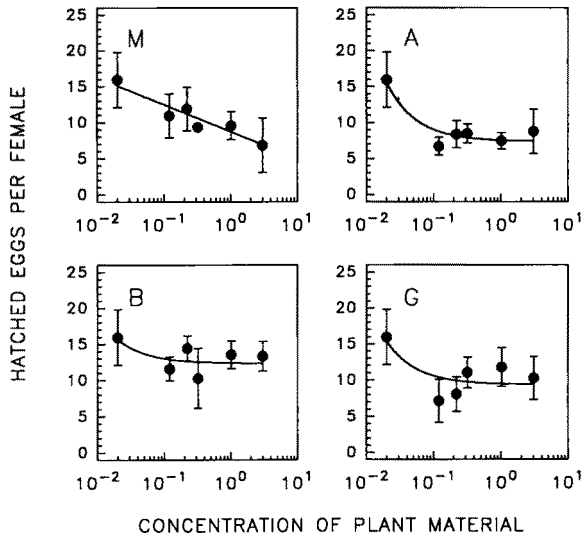

FIG. 6. Mean number (±SE) of hatched eggs per female *S. oryzae* as influenced by increasing concentration of plant material. Plant species and concentration units as indicated in Figure 1. Regression equations: M, hatched eggs per female = $a + b$(ln concentration), $a = 8.78$, $b = 1.63$, lack-of-fit $F = 0.67$, $df = 4, 30$, $P > 0.05$, $r^2 = 0.34$, percentage of maximum attainable $r^2 = 85\%$; A, hatched eggs per female = $a + b$/concentration, $a = 7.42$, $b = 0.17$, lack-of-fit $F = 1.58$, $df = 4, 32$, $P > 0.05$, $r^2 = 0.49$, percentage of maximum attainable $r^2 = 85\%$; B, hatched eggs per female = $a + b$/concentration), $a = 12.34$, $b = 0.067$, lack-of-fit $F = 1.68$, $df = 4, 31$, $P > 0.05$, $r^2 = 0.09$, percentage of maximum attainable $r^2 = 36\%$; G, hatched eggs per female = $a + b$/concentration, $a = 9.38$, $b = 0.12$, lack-of-fit $F = 2.54$, $df = 4, 28$, $P > 0.05$, $r^2 = 0.24$, percentage of maximum attainable $r^2 = 55\%$.

species. Rather, at the low concentrations used, some threshold was exceeded. This may be due to irritation of the insects when moving through the fine plant preparation. The threshold is especially evident in the data for the proportion of unexploited grains for *G. viscosissimum* against *S. oryzae* (Figure 4), in which the avoidance of grains in contact with any plant material seems to be a plausible explanation for the greater activity occurring for the lower doses (resulting in a significant lack of fit for the asymptotic equation). Very simply, the less material present, the greater the probability that more grains will be untreated during the mixing procedure. This seems probable because the resulting fecundity and fertility data show no such effect, i.e., the same proportion of eggs hatched in fewer grains, which is likely a physical/behavioral phenomenon influenced by clumped oviposition in grains that were free of plant preparation. This effect

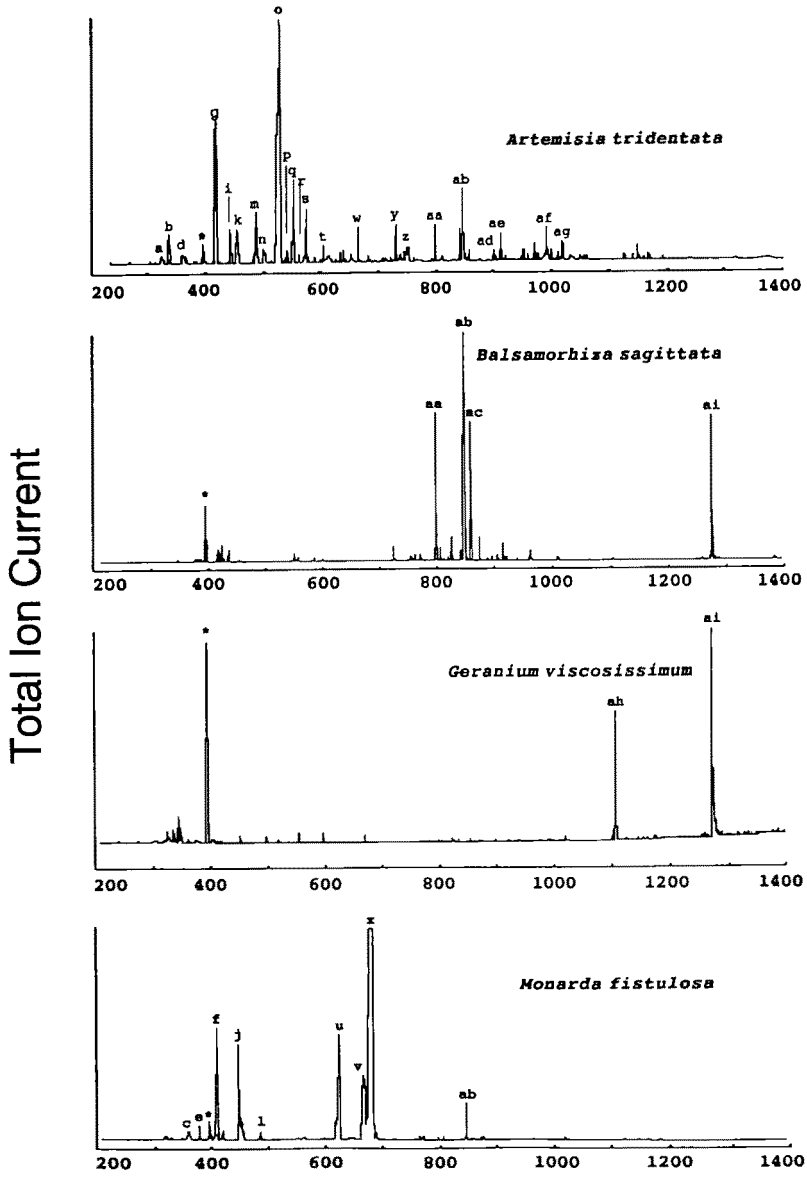

FIG. 7. Total ion current chromatograms of volatiles extracted from the four species of plants obtained on a DB-1 column. Retention times are given in seconds on the *x* axis. *Internal standard of n-decane added to each sample at 1.33 mg; identities and quantities of the labeled peaks are given in Table 1.

TABLE 1. IDENTITIES AND QUANTITIES OF VOLATILES EXTRACTED FROM FOUR SPECIES OF ROCKY MOUNTAIN PLANTS

Species	Peak letter[a]	Retention time (sec)[a]	Compound name	Evidence[b]	Quantity (mg/g)[c]
Artemisia tridentata					
	a	325	α-Pinene	C	0.264
	b	338	Camphene	C	0.821
	d	361	Sabinene	L	0.139
	g	418	1,8-Cineole	C	4.004
	i	441	Artemisole	L	0.495
	k	452	*cis-p*-menth-2-en-l-ol	L	0.704
	m	488	α-Thujone	C	0.629
	n	501	Myrtenal	L	0.562
	o	531	Camphor	C	9.708
	p	542	Pinocarvone	L	0.069
	q	554	Borneol	C	0.844
	r	564	*cis*-Sabinene hydrate	L	0.047
	s	576	α-Terpineol	C	0.567
	t	614	Undet. oxyg. terpenoid	S	0.154
	v	667	Isobornyl acetate	L	0.224
	y	732	Geranyl formate	L	0.234
	z	753	Ocimenone	L	0.185
	aa	799	*trans*-Caryophyllene	C	0.338
	ab	849	Germacrene	L	0.707
	ad	902	Nerolidol	L	0.069
	ae	914	Undet. sesquiterpene	S	0.218
	af	993	Undet. sesquiterpene	S	0.355
	ag	1021	Undet. sesquiterpene	S	0.208
Balsamorhiza sagittata					
	aa	799	*trans*-Caryophyllene	C	0.492
	ab	849	Germacrene	L	2.149
	ac	860	Bicyclogermacrene	L	0.457
	ai	1275	Phytol	C	0.405
Geranium viscosissimum					
	ah	1108	Undet. C_{19} hydrocarbon	S	0.150
	ai	1275	Phytol	C	0.303
Monarda fistulosa					
	c	359	l-Octen-3-ol	C	0.250
	e	379	Myrcene	C	0.219
	f	411	Para-cymene	C	2.079
	h	419	Limonene	C	0.136
	j	449	γ-Terpinene	C	1.329
	l	485	Linalool	C	0.132
	u	624	Thymoquinone	C	0.569
	v	666	Thymol	C	3.412
	x	684	Carvacrol	C	26.285
	ab	847	Germacrene	L	0.419

[a] Peak letters and retention times correspond to those in Figure 7.

[b] Identity of peaks determined in the following ways: C = confirmed by comparison with retention time and spectrum of authentic material; L = identity assigned based on match of sample spectrum to library spectrum; S = type of compound determined by direct interpretation of spectrum and retention information.

[c] Quantity per gram of dried plant material estimated by quantitative GC/FID on a DB-1 column with 1.33 mg decane as an internal standard in each sample.

was noted against *Z. subfasciatus* with the leaves of another plant species that did not contain many highly volatile compounds (Weaver et al., 1992).

The very significant activity of the two aromatic plant species against *Z. subfasciatus* suggests that highly volatile terpenoid compounds can be liberated from the site of biosynthesis, the glandular hairs (Heinrich et al., 1983; Duke, 1990), by powdering according to a patented process. This is a very simple insecticidal preparation for bruchid control, and control can be achieved at dramatically low doses of unconcentrated, unpurified material. However, these preparations are not candidates as broad-spectrum insecticides, as was evidenced by the low level of control against *S. oryzae* even by the carvacrol-rich *M. fistulosa*. This insect was found to be quite resistant to fumigation with volatile natural products by Shaaya et al. (1991). This indicates that careful screening of such simple natural product preparations is required to ensure that they have significant toxicity to the target species.

There is an enormous volume of literature on the insecticidal and repellent activity of terpenoids. The three most abundant compounds we identified, camphor, carvacrol, and 1,8-cineole, all have been evaluated by Shaaya et al. (1991) as fumigants against stored-product insects. The commercial insecticide toxaphene, which structurally is polychlorinated camphor, was marketed for use against several pests until its use was restricted (Duke, 1990). Extensive reviews of the terpenoid chemistry of *Artemisia* spp. have been written by Duke (1990) and Duke et al. (1988). Essential oil characterization has been conducted for *M. fistulosa* (Simon and Beliveau, 1986) and the site of oil biosynthesis elucidated (Heinrich et al., 1983). The flavonoids of *B. sagittata* (Bohm et al., 1989) have been studied, as have the sesquiterpene lactones (Bohlmann et al., 1985).

We believe that volatile plant preparations have significant potential as insecticides against short-lived stored-product pests. The volatile component exerts its controlling effect as it dissipates, thus reducing harmful residue in the foodstuff. Chemical characterization of the major components of the preparation are desirable to preclude the possibility of harmful residues, either volatile or nonvolatile. If a reliable source of plant material is established and used in stored commodities, the dry plant powder remaining after dissipation of volatiles can be removed mechanically. Such preparations may be very practical in low technology surroundings, particularly in subsistence agriculture.

Here, we have demonstrated that plant species that are aromatic and free of insect damage will likely protect stored foodstuffs. Such observations may have been the basis for the origination of some insect control practices in primitive times. However, every new material needs to be evaluated to ensure the safety and palatability of the end product to consumers.

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PHEROMONES IN WHITE PINE CONE BEETLE, *Conophthorus coniperda* (SCHWARZ) (COLEOPTERA: SCOLYTIDAE)

GÖRAN BIRGERSSON,[1,7] GARY L. DEBARR,[2,*] PETER DE GROOT,[3,4] MARK J. DALUSKY,[1] HAROLD D. PIERCE, JR.,[5] JOHN H. BORDEN,[4] HOLGER MEYER,[6] WITTKO FRANCKE,[6] KARL E. ESPELIE,[1] and C. WAYNE BERISFORD[1]

[1]*Department of Entomology, University of Georgia Athens, Georgia 30602*

[2]*USDA Forest Service, Southeastern Forest Experiment Station Forestry Sciences Laboratory Athens, Georgia 30602*

[3]*Canadian Forest Service, Forest Pest Management Institute Box 490, Sault Ste. Marie, Ontario, Canada P6A 5M7*

[4]*Centre for Pest Management, Department of Biological Sciences Simon Fraser University Burnaby, British Columbia, Canada V5A 1S6*

[5]*Department of Chemistry, Simon Fraser University Burnaby, British Columbia, Canada V5A 1S6*

[6]*Department of Organic Chemistry, Universität Hamburg Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany*

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Abstract—Female white pine cone beetles, *Conophthorus coniperda*, attacking second-year cones of eastern white pine, *Pinus strobus* L., produced a sex-specific pheromone that attracted conspecific males in laboratory bioassays and to field traps. Beetle response was enhanced by host monoterpenes. The female-produced compound was identified in volatiles collected on Porapak Q and in hindgut extracts as (+)-*trans*-pityol, (2*R*,5*S*)-(+)-2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran. Males and females produced and released the (*E*)-(−)-spiroacetal, (5*S*,7*S*)-(−)-7-methyl-1,6-dioxaspiro-[4.5]decane, which was not an attractant for either sex, but acted as a repellent

*To whom correspondence should be addressed.
[7]Present address: Chemical Ecology, Göteborg University, S-413 20 Göteborg, Sweden.

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for males. Porapak Q-trapped volatiles from both sexes contained (+)-*trans*-pinocarveol and (−)-myrtenol. In addition, hindgut extracts of females contained *trans*-verbenol, while males had pinocarvone and verbenone. Work in Georgia and Canada confirmed that the same isomers of pityol and spiroacetal are present in two distinct and widely separated populations of *C. coniperda*.

Key Words—*Conophthorus coniperda*, white pine cone beetle, Scolytidae, pheromone, pityol, 2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran, (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane, spiroacetal, chiral analysis, walking bioassay, traps.

INTRODUCTION

The white pine cone beetle (WPCB), *Conophthorus coniperda* (Schwarz) (Coleoptera: Scolytidae), occurs throughout the range of eastern white pine, *Pinus strobus* L., and is rarely found on other hosts (Wood, 1982). It is the most serious insect pest of eastern white pine cones in natural stands (Godwin and Odell, 1965; Hedlin et al., 1980) and seed orchards (DeBarr et al., 1982).

Semiochemicals are commonly used by scolytids for locating suitable hosts or mates (Borden, 1985). However, little is known about the responses of *Conophthorus* spp. to either pheromones or odors released by cones. Beetle-infested cones and their monoterpenes attracted male and female ponderosa pine cone beetles, *C. ponderosae* Hopkins, in laboratory bioassays (Kinzer et al., 1972; Kinzer and Reeves, 1976). However, Mattson et al. (1984) and Mattson and Strauss (1986) rejected the hypothesis of cone-directed alightment by the red pine cone beetle (RPCB), *C. resinosae* Hopkins, in response to cone-produced volatiles.

Recently, we reported evidence for a female-produced sex pheromone that attracts WPCB and RPCB males to their cone galleries (de Groot et al., 1991). Herein we describe the isolation and identification of both female- and male-produced compounds from airborne collections and hindguts of WPCB. Laboratory bioassays and field trapping were used to determine beetle response to host- and beetle-produced volatiles and to compounds identified as pheromone candidates. Volatiles from beetles and beetle-infested cones were analyzed independently in the United States and Canada to identify pheromone candidates. Analyses in Canada were also aimed at clarifying taxonomic relationships for three sympatric *Conophthorus* species (de Groot, 1992).

METHODS AND MATERIALS

Collection and Maintenance of Beetles. Eastern white pine cones containing overwintering WPCB were collected in February 1989 at the USDA Forest Service's Beech Creek seed orchard near Murphy, North Carolina. They were

kept in the dark at 5°C and 50–70% relative humidity until late March. Beetles were allowed to emerge naturally from the cones under a 16L:8D photoperiod at 29°C and 70% relative humidity. They were sexed by examining the seventh and eighth abdominal tergites (Herdy, 1959) and held in darkness on moistened filter paper in Petri dishes at 4–5°C and 70% relative humidity.

WPCB were also collected during September and October 1988, near Chalk River, Ontario, and handled as described by de Groot et al. (1991). Materials and methods used in Canada are described in detail in Pierce et al. (1995).

Collection of Beetle and Host Volatiles. Fresh second-year cones (20–30 mm long) were picked from eastern white pines at Beech Creek, North Carolina; their petioles were sealed with melted paraffin, and they were stored at 5°C. Emerged WPCB were placed individually with one cone in a 10-ml plastic cup. To stimulate beetle attack, a basal cone scale was removed. Cones that were not successfully attacked within 2 hr were discarded. For cones with paired beetles, a male was added 24 hr after the female. After attacks were initiated, cones with beetles were placed in a small desiccator jar (600 ml; 10 cm ID). Charcoal-filtered air was introduced into the bottom of the desiccators and drawn over the attacked cones. Volatiles were collected on Porapak Q (Supelco, Inc. Bellefonte, Pennsylvania) columns (ID 50 mm × 5 mm; 300 mg; 80–100 mesh) (Birgersson and Bergström, 1988; Dobson, 1991; Gries et al., 1988). In control aeration, undamaged cones were used to measure the turpentine background, while cones with a small hole (2 mm ID) drilled at the base were used to simulate beetle attack. Volatiles were collected for 24 hr at a flow rate of 500 ml/min. The Porapak Q columns were rinsed with 3 ml of redistilled diethyl ether (Baker p.a.) and the extracts were concentrated in 0.5-ml tip-vials prior to analysis.

To accumulate sufficient material for chiral analysis, several groups of ca. 100 single females attacking new cones were aerated for 24 hr, starting 24–48 hr after attack. The aeration extracts were combined and concentrated in a tip-vial to $< 100\ \mu l$. The concentrated extract was put on a short Sep-Pac (Millipore Corp., Bedford, Massachusetts) column filled with Florisil (Fisher Scientific Co., Pittsburgh, Pennsylvania) (8 mm × 20 mm ID; 500 mg), and eluted with 3 ml pentane (redistilled) + 2 × 1.5 ml pentane + 2 × 1 ml diethyl ether + 0.5 ml ether (Figure 1). The last 0.5-ml ether fraction contained material sufficiently pure for chiral analysis.

Extracts of Beetle Hindguts. After the aerations, WPCB remaining in cones were excised and stored on Dry Ice. To analyze their volatiles, they were thawed, and pentane extracts were made of hindguts. Wings, elytra, and legs were removed from the degutted beetles, and a second pentane extract was made of the carcass. During dissection, the sex of the beetles was verified by the median lobe (de Groot, 1992) or spermatheca.

Synthetic Compounds. Syntheses of the racemate and the (5*S*,7*S*)-enantiomer of 7-methyl-1,6-dioxaspiro[4.5]decane followed the method of Jacboson

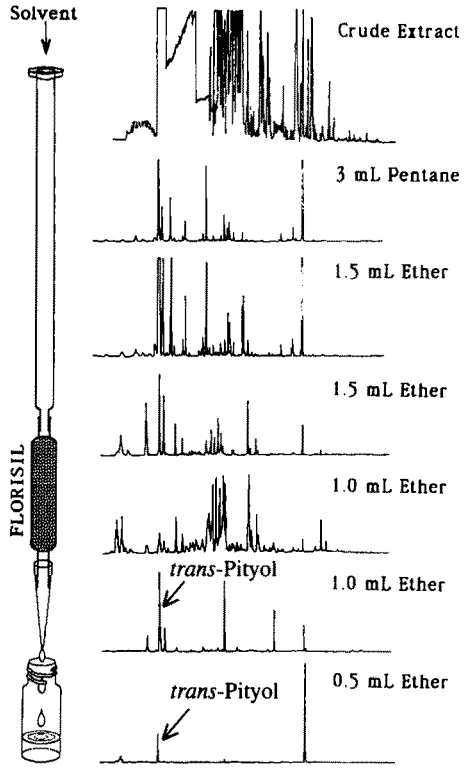

Fig. 1. Isolation of *trans*-pityol in Porapak Q-trapped volatiles from *C. coniperda* females in *P. strobus* cones.

et al. (1982). The enantiomers of 2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran (*trans*-pityol) were provided by K. Mori (for synthesis see Mori and Puapoomchareon, 1987). The (±)-*trans* and (+)-*trans*-pityol used in field bioassays were made by H.D. Pierce, Jr. (Pierce et al., 1995). The enantiomers of *trans*-pinocarveol were prepared from the enantiomers of β-pinene (Aldrich) according to Joshi (1968) by W. Francke; pinocarvone was obtained by oxidation of *trans*-pinocarveol. All other terpenes were commercially available (Aldrich, Milwaukee, Wisconsin).

Chemical Analyses. Porapak Q-trapped volatiles and hindgut extracts were analyzed on a Hewlett-Packard 5890-5970 coupled gas chromatograph and mass spectrometer (GC-MS). Each extract contained an internal standard of 250 ng heptyl acetate (C_7Ac) (Birgersson et al., 1984). The limit of reliable quantification was ca. 0.5 ng/beetle, and the limit of identification was <0.05 ng using

the extracted ion current profile (EICP). The fused silica capillary columns used were HP-1 (Hewlett-Packard Corp., Avondale, Pennsylvania) (25 m × 0.20 mm ID; 0.3 μm film thickness; temperature program: 50°C for 5 min then 8°/min to 200°C for 10 min) and HP-FFAP (Hewlett-Packard) (50 m × 0.20 mm; 0.3 μm film thickness; temperature program: 50°C for 5 min then 10°C/min to 200°C for 10 min). In both columns the flow rate of the helium carrier gas was 25 cm/sec.

The absolute configuration of the spiroacetal in pentane extracts of male hindguts was determined on a glass capillary (40 m × 0.25 mm ID) coated with hexakis-per-*O*-hexyl-α-cyclodextrin (Tengö et al., 1990), programmed at 2°C/min from 50 to 100°C. The same column at 60°C was used to separate the enantiomers of trifluoroacetylated *trans*-pityol from aerations, while underivatized *trans*-pityol was resolved on octakis-(3-*O*-acetyl-2,6-di-*O*-pentyl)-γ-cyclodextrin at 80°C (Grégoire et al., 1992). Trifluoroacetates of monoterpene alcohols were separated on a glass capillary column (40 m × 0.25 mm ID) coated with heptakis-(3-*O*-methyl-2,6-di-*O*-pentyl)-β-cyclodextrin (König et al., 1989). Volatiles produced by *C. coniperda* collected in Chalk River, Ontario, were analyzed as described by Pierce et al. (1995).

Laboratory Bioassays of Beetle- and Host-Produced Volatiles and Candidate Pheromones. Responses by male and female WPCB to beetle- and host-produced volatiles were measured in laboratory bioassays (Payne et al., 1976; de Groot et al., 1991) at Athens, Georgia, during April 1989 and 1990, coinciding with emergence of feral WPCB at the Beech Creek seed orchard. For the bioassays, volatile concentrations were expressed as cone-minutes (CM) or beetle-minutes (BM) where 1 CM or BM is the quantity of volatiles collected during 1 min of aeration (Birgersson et al., 1995). Groups of five beetles of the same sex were exposed to diluted diethyl ether formulations containing 1 CM or BM/μl equivalents dispensed from a 10-μl syringe at 1 μl/min for 10 min. Beetles were placed onto the center of an 18.5-cm-diam. filter paper arena; positive responders were those that walked up the airstream to within 1 cm of the syringe tip. A minimum of 15 replications per sex were run per treatment in bioassay 1. Fewer replications were run for bioassays 2 and 3 because of low populations of feral beetles.

Bioassay 1 tested the responses of WPCB to: (1) control (air only); (2) diethyl ether; (3) diethyl ether extracts containing 10 CM of Porapak Q-captured volatiles from aerations of wounded uninfested cones; (4–6) diethyl ether extracts containing 10 BM of Porapak Q-captured volatiles, respectively, from males, females, or pairs of beetles feeding in cones; and (7 and 8) (*E*)-(±)-spiroacetal alone or in combination with volatiles from cones in 10 BM equivalents.

Bioassays 2 and 3 compared responses to candidate pheromones. Volatiles from aerations or synthetic chemicals were formulated in redistilled diethyl ether in 10 BM or 10 CM equivalents (Birgersson et al., 1995). In bioassay 2 the

effects of combining various beetle and host-produced compounds were compared in nine treatments (Table 1). In bioassay 3, the effects of excluding single components from four- or five-component mixtures were determined in 11 treatments (Table 1).

Field Experiments. Experiments 1–3 and 5 were conducted in the Beech Creek seed orchard and experiment 4 was in a natural white pine forest in Pancake Bay Provincial Park, 80 km north of Sault Ste. Marie, Ontario. Experiments 1 and 2 compared beetle catches in traps baited with: (1) *P. strobus*

TABLE 1. TREATMENTS TESTED FOR OLFACTORY RESPONSE BY MALE AND FEMALE *C. coniperda* TO COMBINATIONS (BIOASSAY 2) AND SINGLE DELETION (= SUBTRACTIVE; BIOASSAY 3) OF BEETLE OR HOST-PRODUCED VOLATILES IN WALKING BIOASSAYS, ATHENS, GEORGIA, 1990[a]

Treatment	Aeration of females in cones	Female compounds				Host volatiles			Male compound
		Pit[1]	Pit[2]	tP	Mt	CV	MT	BorAc	Spiroacetal
Bioassay 2									
CT	–	–	–	–	–	–	–	–	–
1	–	+	–	–	–	–	–	–	–
2	–	–	+	–	–	–	–	–	–
3	–	+	–	+	+	–	–	–	–
4	–	–	+	+	+	–	–	–	–
5	–	–	+	+	+	–	–	–	+
6	–	–	+	+	+	+	–	–	–
7	–	–	+	+	+	–	+	–	–
FV	+	–	–	–	–	–	–	–	–
Bioassay 3									
1	–	–	–	+	+	+	–	–	–
2	–	–	+	–	+	+	–	–	–
3	–	–	+	+	–	+	–	–	–
4 (mixture 1)	–	–	+	+	+	+	–	–	–
5	–	–	+	+	+	–	+	–	–
6	–	–	+	+	+	–	–	+	–
7	–	–	–	+	+	–	+	+	–
8	–	–	+	–	+	–	+	+	–
9	–	–	+	+	–	–	+	+	–
10 (mixture 2)	–	–	+	+	+	–	+	+	–
FV	+	–	–	–	–	–	–	–	–

[a] Pit[1] = (±)-*trans*-pityol; Pit[2] = (+)-*trans*-pityol; tP = (+)-*trans*-pinocarveol; Mt = (−)-myrtenol; CV = volatiles from aerations of cones; MT = synthetic monoterpene hydrocarbons (mixture of α-pinene, β-pinene, myrcene, limonene 1:1:1:1; Aldrich); BorAc = bornyl acetate (Aldrich); Spiroacetal = (*E*)-(±)-7-methyl-1,6-dioxaspiro[4.5]-decane; CT = control (air only); FV = volatiles from aerations of cones infested with females.

cones that were artificially wounded; (2 and 3) cones infested with male or female WPCB (de Groot et al., 1991); (4 and 6) Porapak Q-trapped volatiles from wounded cones or cones infested with male or female beetles; or (7 and 8) (*E*)-(±)-spiroacetal alone or in combination with cone volatiles. Experiments 3 and 4 compared catches for (±)-*trans*-pityol, (+)-*trans*-pityol, and WP cone oils alone or in combinations. The volatile oil of *P. strobus* cones (WP cone oil) was prepared as described in Pierce et al. (1995) and dispensed from 2-ml polypropylene Eppendorf tubes (Brinkman Instruments, Rexdall, Ontario). No baits were placed in "unbaited" traps in Experiments 3 and 4. Experiment 5 compared catches to (±)-*trans*-pityol and α-pinene with and without (*E*)-(±)-spiroacetal.

Plexiglas barrier traps (de Groot et al., 1991) were used in experiment 1, 12-unit multiple-funnel traps (Lindgren, 1983) were used in experiment 2, and yellow Japanese beetle trap tops (Trécé Inc., Salinas, California) fitted with plastic Mason jar bottoms containing 25 ml of propylene glycol were used in experiments 3–5. Traps were hung ca. 10–15 m high in the upper third of the tree crown, one trap per tree, and were spaced 12–30 m apart. For experiments 1–3 and 5, treatment locations were randomized at weekly intervals. For all experiments, beetles were removed from the traps weekly and the traps were rebaited. Captured beetles were preserved in 70% alcohol, identified, counted, and the sex determined.

Screw-cap glass vials (2 ml) served as releasers for volatiles in experiments 1, 2, and 5. Cotton wicks pulled through 5-cm lengths of 1.6-mm-ID Teflon tubing were inserted through holes drilled in the plastic caps. For experiment 1, a wire was used to suspend a vial in the center of a 10-cm hole cut in the Plexiglas vanes of each barrier trap. For experiment 2, a spring clip was used to attach a vial to one of the plastic rods between funnels 6 and 7 on the multiple-funnel traps. For experiment 5, the vial was clipped to one of the vanes of the Japanese beetle trap. Each vial contained volatiles formulated at a final volume of 2 ml of *n*-octane (Aldrich) and released at a rate of 2 ml/week at 25 ± 2°C in the laboratory. Volatiles collected on Porapak Q were eluted with redistilled diethyl ether and formulated in *n*-octane to release 20 cone equivalents (CE), female equivalents (FE), or male equivalents (ME), where 1 CE, FE, or ME is the quantity of volatiles collected during aerations of one cone or beetle-infested cone for 1 hr (Birgersson et al., 1995). Capillary tubes (1.04 mm ID) were used to dispense 3 μl neat pityol or spiroacetal in experiments 3 and 4. The release rate was 4.2 mg of pityol/week at 24°C in the laboratory.

Data Analyses. Percent response data from the bioassays were analyzed by the GLM procedure and count data from the field experiments (except experiment 5) were analyzed using the RANK and GLM procedures (SAS Institute, 1987). A completely randomized, factorial design was used for the pedestrian bioassays. Factor one, with two levels, was gender of the beetles bioassayed

for olfactory response. Factor two was mixtures of volatiles offered, with levels referred to as treatments. The treatments differed for each bioassay experiment and were various combinations of host-, female-, and male-produced volatiles. Within each experiment these treatments had a structure planned to provide tests of specific hypotheses, so all possible pairwise comparisons of treatments was not a goal. Within each experiment, treatment and gender main effects, and treatment–gender interaction were tested at a 95% confidence level ($\alpha = 0.05$). In order to maintain this experiment-wise (α_E) confidence level and perform a group of planned, nonorthogonal contrasts of treatment–gender interaction effects using multiple t tests, Šidák's inequality was used to set a comparison-wise (α_C) confidence level used to declare significance of any specific comparison (Šidák, 1967; Games, 1977; Jones, 1984). Šidák's inequality relates α_E and α_C as $\alpha_C \leq 1 - (1 - \alpha_E)^{1/n}$, which sets an upper bound on the comparisonwise α_C for n, possibly dependent, comparisons that should be used to declare significance of any single comparison in a group and still maintain the experimentwise probability of a type-I error. In the five field experiments, traps were deployed in a randomized block design. Two response variables were analyzed separately. One response was the number of males caught in baited traps, the other was the number of females caught. In these experiments, planned treatment contrasts were made using t tests with significance levels controlled with Šidák's inequality.

RESULTS

Volatiles from Attacked Cones. All *P. strobus* cones with or without *C. coniperda* released large amounts of host terpenes (Figure 2A). Attacked cones released the four major monoterpene hydrocarbons α-pinene, β-pinene, myrcene, and limonene (Mirov, 1961) and the oxygenated monoterpene bornyl acetate.

Both female and male beetles released (+)-*trans*-pinocarveol and (−)-myrtenol (Figure 2B and C). Volatiles from feeding males contained a beetle-produced peak (Figure 2C) with a mass spectrum dominated by two large fragments, m/z 84 and 87, and a molecular ion at m/z 156. The compound was identified as 7-methyl-1,6-dioxaspiro[4.5]decane by comparison of the mass spectral data to that reported by Francke et al. (1979b). This spiroacetal has been named MDOS by Kohnle et al. (1992) and conophthorin by de Groot (1992), after the genus. Analysis of Porapak Q-trapped volatiles from females feeding in second-year cones in Canada also revealed presence of the (*E*)-spiroacetal (96% ee) (Figure 3). After the female-specific compound pityol had been found in the hindgut extracts (see following), a reexamination of the Porapak Q-trapped volatiles using an extracted ion current profile (EICP) (Garland

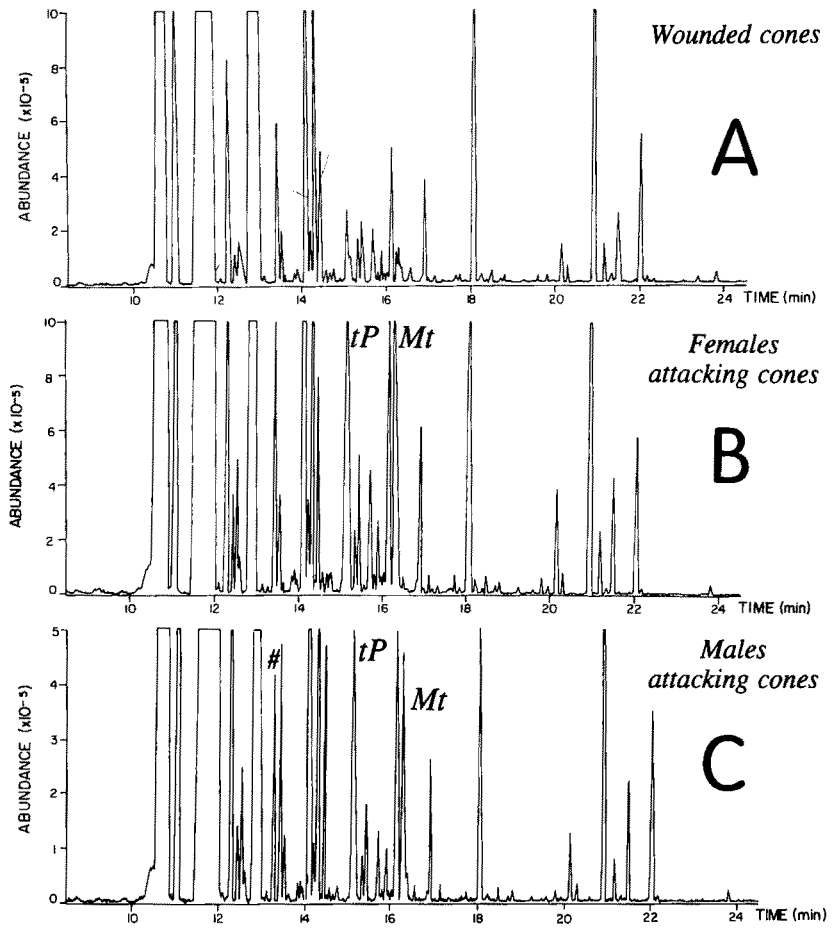

Fig. 2. Chromatograms from GC-MS analyses of Porapak Q-collected volatiles of *C. coniperda* attacking *P. strobus* cones in Georgia; fused silica column coated with HP-1. (A) Wounded cones, (B) single females in cones, (C) single males in cones. tP = *trans*-pinocarveol, Mt = myrtenol, # = spiroacetal.

and Powell, 1981) of *m/z* 59 revealed that it coeluted with, and was totally hidden by β-pinene in all aerations of female-infested cones (Figure 2B).

Analyses of Hindgut Extracts. In addition to the compounds identified in the aerations, analyses of hindguts showed that females produced *trans*-verbenol (Figure 4A) and males produced pinocarvone and verbenone (Figure 4B). Females also produced a female-specific compound (Figure 4A), with a mass

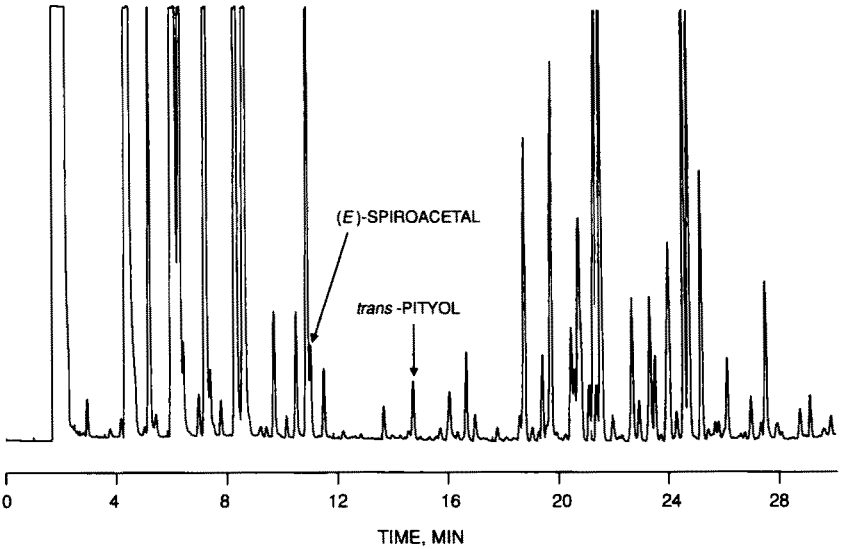

Fig. 3. Chromatogram of Porapak Q-collected volatiles of female *C. coniperda* attacking *P. strobus* cones in Ontario; glass capillary column coated with SP-1000 (30 m × 0.5 mm ID).

spectrum having a base peak of *m/z* 59 and a molecular ion at *m/z* 144, indicating a tertiary alcohol with the formula of either $C_8H_{16}O_2$ or $C_9H_{20}O$. The compound was identified as 2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran, first described as a pheromone of a *Pityophthorus* spp. and named pityol by Francke et al. (1987). Chiral analysis revealed the female-specific pityol to be the 2*R*,5*S*-isomer, and 1% of a *cis* isomer was also present. The same isomer of pityol was also present in the Porapak Q-captured volatiles of feeding *C. coniperda* from Canada (Figure 3).

The chromatogram for hindguts from males was dominated by a large peak of a spiroacetal. A smaller peak, with almost the same mass spectrum, eluted $1\frac{1}{2}$ min later (Figure 4B). Chiral analysis determined that the major peak was (5*S*,7*S*)-7-methyl-1,6-dioxaspiro[4.5]decane, and the minor peak, the 5*R*,7*S*-isomer. Traces of other spiroacetals were detected, but not identified. Pityol was never detected in any male hindgut sample. No additional host- or beetle-produced compounds were found in pentane extracts of degutted beetles, besides those identified in hindgut extracts or aerations. Pentane extracts of both female and male *C. coniperda* from Canada also contained both *E* and *Z* isomers of the spiroacetal (Figure 5).

Laboratory Bioassays. WPCB adults showed strong responses to Porapak

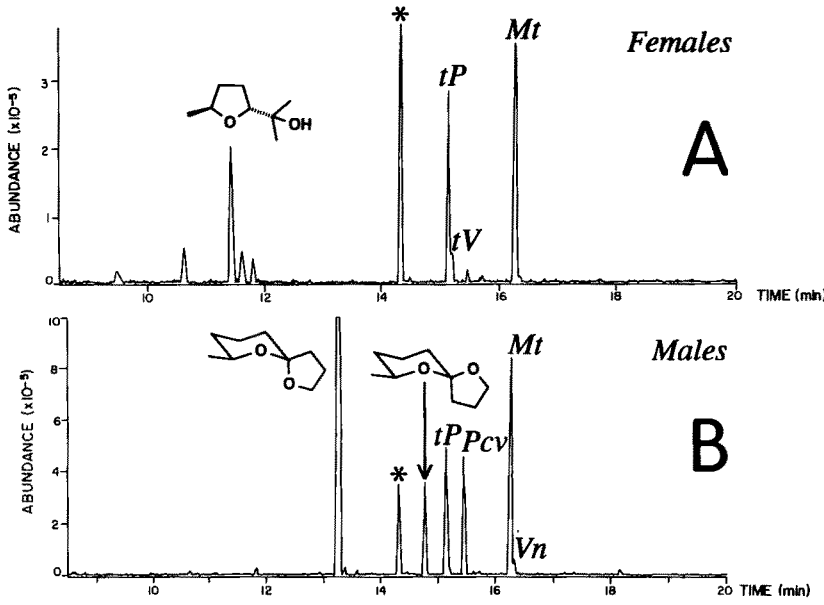

Fig. 4. Chromatograms from GC-MS analyses of volatiles of hindguts of *C. coniperda* attacking *P. strobus* cones in Georgia; fused silica column coated with HP-1. (A) Pityol from seventeen single females in cones 40 hr, (B) spiroacetal from 15 single males in cones 40 hr. tP = *trans*-pinocarveol; tV = *trans*-verbenol; Mt = myrtenol; Vn = verbenone; Pcv = pinocarvone; * 250 ng C_7Ac.

Q-trapped volatiles from infested cones in the walking bioassay (Figure 6, Table 2). Males responded more often than females. Significant response differences occurred among treatments ($F = 67.1$; $df = 7, 377$; $P = 0.0001$), between sexes ($F = 7.96$; $df = 1, 377$; $P = 0.0001$), and for the treatment-by-sex interaction ($F = 24.75$; $df = 7, 337$; $P = 0.0001$).

Only 4% of the beetles responded positively to air alone (Figure 6); adding ether to the airstream did not significantly increase the response (Table 2). However, both sexes responded significantly to Porapak Q-trapped volatiles containing host- and/or beetle-produced compounds, and males responded positively to cone volatiles. Significantly more beetles of both sexes responded to volatiles from males in cones than to cones alone. Over 90% of the test males responded to volatiles from cones with females, but females did not respond to this stimulus better than to cones alone. Females, but not males, responded significantly more to volatiles from cones containing pairs of beetles than to volatiles from cones containing either sex. Neither sex showed a significantly greater response to a combination of cone volatiles and spiroacetal than to cone

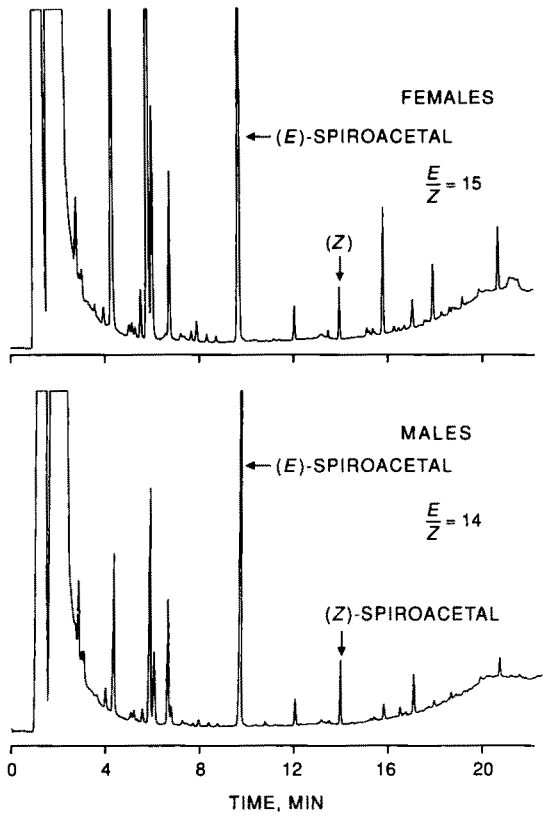

Fig. 5. Chromatograms of volatiles in pentane extracts of *C. coniperda*, excised from *P. strobus* cones in Ontario; glass capillary column coated with SP-1000.

volatiles or the spiroacetal alone. However, male response to a mixture of spiroacetal and cones volatiles was significantly less than their response to volatiles from male-infested cones.

Synthetic beetle and host-produced compounds also elicited strong positive responses by WPCB (Tables 3 and 4). Again, males responded more frequently than females. Responses differed significantly among treatments (bioassay 2: $F = 31.8$; $df = 8, 117$; $P = 0.0001$; bioassay 3: $F = 6.21$; $df = 10, 77$; $P = 0.001$) and between sexes ($F = 11.5$; $df = 1, 117$; $P = 0.0001$; bioassay 3: $F = 48.4$; $df = 1, 77$; $P = 0.0001$). Treatment-by-sex interactions were also significant (bioassay 2: $F = 5.46$; $df = 1, 117$; $P = 0.0001$; bioassay 3: $F = 2.96$; $df = 9, 77$; $P = 0.0045$).

Beetle responses to pityol alone or in combination with two other female-

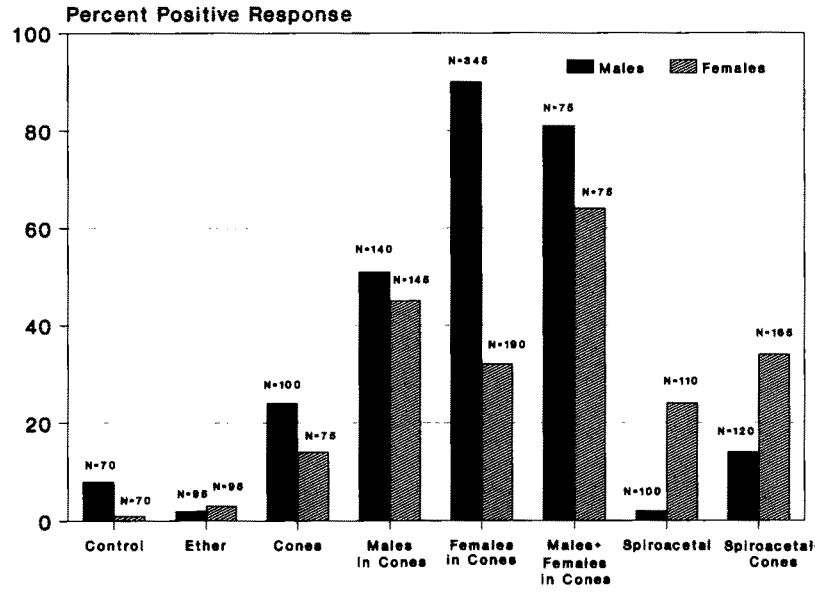

Fig. 6. Responses by female and male *C. coniperda* to volatiles in laboratory bioassay 1.

produced compounds, *trans*-pinocarveol and myrtenol, ranged from 18 to 33% (Table 3). Comparisons for the (±)-*trans*-pityol vs. control; (+)-*trans*-pityol vs. (±)-*trans*-pityol; and the (+)-*trans*-pityol, *trans*-pinocarveol, and myrtenol mixture vs. (+)-*trans*-pityol were not significant for either sex. Male response to (*E*)-(±)-spiroacetal plus the three-component mixture, was only 8%, while 33% responded to the mixture alone, but the differences were not significant for either sex.

Combining volatiles from cones with the three female compounds significantly increased male response to 91%, but did not increase female response (Table 3). Male response to a mixture of four host monoterpenes and the three female compounds was equal to their response to cone volatiles plus the three female compounds or Porapak Q-trapped volatiles from female-infested cones. In contrast, the female response of 82% to volatiles from females in cones was significantly greater than their 23% response to the three female compounds and monoterpenes.

Female responses were not significantly different for any component deletion from the complex mixtures of beetle- and host-produced compounds (Table 4). Male response was 91% for mixture 1 containing the three female-produced compounds plus cone volatiles and 81% for mixture 2 containing the three

TABLE 2. RESPONSE BY FEMALE AND MALE *C. coniperda* TO PORAPAK Q-TRAPPED VOLATILES FROM *P. strobus* CONES, CONES WITH BEETLES, AND (*E*)-(±)-SPIROACETAL IN WALKING BIOASSAY 1, ATHENS, GEORGIA 1989

	Females (%)			Males (%)		
Single df comparisons:	*N*	$\bar{X} \pm$ SE	*P*	*N*	$\bar{X} \pm$ SE	*P*
Air vs.	70	1 ± 1	0.8197 NS[a]	70	8 ± 8	0.4714 NS
ether	95	3 ± 2		95	2 ± 2	
All treatments except control vs.	760	36 ± 5	0.0001[b]	880	44 ± 3	0.0001[b]
ether	95	3 ± 2		95	2 ± 2	
Cones vs.	75	14 ± 3	0.1584 NS	100	24 ± 5	0.0018[b]
ether	95	3 ± 2		95	2 ± 2	
Males in cones vs.	145	45 ± 4	0.0001[b]	140	51 ± 3	0.0001[b]
cones	75	14 ± 3		100	24 ± 5	
Females in cones vs.	190	32 ± 5	0.0052 NS	345	90 ± 2	0.0001[b]
cones	75	14 ± 3		100	24 ± 5	
Males + females in cones vs.	75	64 ± 8	0.0001[b]	75	81 ± 5	0.0762 NS
males or females in cones	335	39 ± 5		485	71 ± 3	
Cones + (*E*)-(±)-spiroacetal vs.	165	34 ± 5	0.0030 NS	120	14 ± 3	0.1182 NS
cones	75	14 ± 3		100	24 ± 5	
Cones + (*E*)-(±)-spiroacetal vs.	165	34 ± 5	0.1177 NS	120	14 ± 3	0.0774 NS
(*E*)-(±)-spiroacetal	110	24 ± 7		100	2 ± 1	
Cones + (*E*)-(±)-spiroacetal vs.	165	34 ± 5	0.0449 NS	120	14 ± 3	0.0001[b]
males in cones	145	45 ± 4		140	51 ± 3	

[a]NS = not significant.
[b]$P \leq 0.0028$ is required for an experiment-wise error rate of $\alpha = 0.05$ (Šidák, 1967; Games, 1977) for each of the 18 comparisons.

female-produced compounds plus monoterpenes and bornyl acetate. These responses were significantly greater than to pityol alone. However, when pityol was removed from either mixture 1 or mixture 2, male response was significantly decreased to only 20%. Substituting bornyl acetate for the four monoterpene hydrocarbons significantly reduced male response.

Field Experiments. Catches of females in traps baited with beetle-infested cones or Porapak Q-trapped volatiles of infested cones were no greater than to uninfested cones, but significantly more males were attracted to female-infested cones than to uninfested cones or male-infested cones (Table 5, experiment 1). However, in experiment 2, more beetles of both sexes were both attracted to Porapak Q-trapped volatiles from female-infested cones than to volatiles from male-infested cones. The (*E*)-(±)-spiroacetal was not an attractant for either sex.

Females were not attracted to pityol in experiment 3, but in experiment 4 significantly more females were caught in traps baited with (±)-*trans*-pityol

TABLE 3. RESPONSE BY FEMALE AND MALE *C. coniperda* TO VARIOUS COMBINATIONS OF BEETLE- AND HOST-PRODUCED VOLATILES IN WALKING BIOASSAY 2, ATHENS, GEORGIA 1990

Single *df* comparisons	Females (%)			Males (%)		
	N	$\bar{X} \pm$ SE	*P*	*N*	$\bar{X} \pm$ SE	*P*
(±)-*trans*-Pityol vs. control[a]				29 13	22 ± 9 0 ± 0	0.1237 NS[a]
(+)-*trans*-Pityol vs. (±)-*trans*-pityol	29 15	18 ± 9 27 ± 18	0.5659 NS[b]	28 29	24 ± 8 22 ± 9	0.8429 NS
(+)-*trans*-Pityol + *trans*-pinocarveol + myrtenol vs. (+)-*trans*-pityol	24 29	25 ± 7 18 ± 9	0.9645 NS	26 28	33 ± 8 24 ± 8	0.4347 NS
(±)-*trans*-Pityol + *trans*-pinocarveol + myrtenol vs. (+)-*trans*-pityol + *trans*-pinocarveol + myrtenol	24 24	26 ± 9 25 ± 7	0.9386 NS	29 26	31 ± 8 33 ± 8	0.8328 NS
(+)-*trans*-Pityol + *trans*-pinocarveol + myrtenol + (*E*)-(±)-spiroacetal vs. (+)-*trans*-pityol + *trans*-pinocarveol + myrtenol	25 24	28 ± 10 25 ± 7	0.8172 NS	25 26	8 ± 5 33 ± 8	0.0432 NS
(+)-*trans*-Pityol + *trans*-pinocarveol + myrtenol + cone volatiles[c] vs. (+)-*trans*-pityol + *trans*-pinocarveol + myrtenol	41 24	49 ± 11 25 ± 7	0.0344 NS	50 26	91 ± 5 33 ± 8	0.0001[d]
(+)-*trans*-Pityol + *trans*-pinocarveol + myrtenol + monoterpenes[e] vs. (+)-*trans*-pityol + *trans*-pinocarveol + myrtenol + cone volatiles	37 41	23 ± 12 49 ± 11	0.0093 NS	36 50	84 ± 5 91 ± 5	0.4532 NS
(+)-*trans*-Pityol + *trans*-pinocarveol + myrtenol + monoterpenes vs. (+)-*trans*-pityol	37 29	23 ± 12 18 ± 9	0.0047 NS	36 28	84 ± 5 24 ± 8	0.0001[d]
(+)-*trans*-Pityol + *trans*-pinocarveol + myrtenol + monoterpenes vs. volatiles from *C. coniperda* females in cones[f]	37 51	23 ± 12 82 ± 7	0.0001[d]	36 124	84 ± 5 96 ± 2	0.1417 NS

[a]Control = air only; not tested for females.
[b]NS = not significant.
[c]Volatiles from aerations of cones.
[d]$P \leq 0.003$ is required for an experiment-wise error rate of $\alpha = 0.05$ (Šidák, 1967; Games, 1977) for each of the 17 comparisons.
[e]Four major monoterpenes from *P. strobus* cones (α-pinene, β-pinene, myrcene, limonene 1 : 1 : 1 : 1).
[f]Volatiles from aerations of cones infested with females.

TABLE 4. RESPONSE BY FEMALE AND MALE *C. coniperda* TO SINGLE DELETIONS FROM MIXTURES OF BEETLE OR HOST-PRODUCED VOLATILES IN BIOASSAY 3, ATHENS, GEORGIA 1990

Single *df* comparisons	Females (%)			Males (%)		
	N	$\bar{X} \pm$ SE	*P*	*N*	$\bar{X} \pm$ SE	*P*
Mixture 1[a] − *trans*-Pityol vs. mixture 1	13 41	23 ± 2 49 ± 11	0.0878 NS[b]	10 50	20 ± 0 91 ± 5	0.0001[c]
Mixture 1 − *trans*-pinocarveol vs. mixture 1	10 41	10 ± 10 49 ± 11	0.0288 NS	10 50	70 ± 3 91 ± 5	0.2335 NS
Mixture 1 − myrtenol vs. mixture 1	24 41	13 ± 8 49 ± 11	0.0051 NS	24 50	100 ± 0 91 ± 5	0.4591 NS
Mixture 2[d] − bornyl acetate vs. mixture 1	37 41	23 ± 12 49 ± 11	0.0192 NS	36 50	84 ± 5 91 ± 5	0.4984 NS
Mixture 2 − monoterpenes vs. mixture 2 − bornyl acetate	19 37	5 ± 5 23 ± 12	0.1952 NS	23 36	38 ± 10 84 ± 5	0.0007[c]
Mixture 2 − monoterpenes vs. mixture 2	19 19	5 ± 5 38 ± 18	0.0459 NS	23 23	38 ± 10 81 ± 15	0.0035 NS
Mixture 2 − *trans*-pityol vs. mixture 2	9 19	25 ± 25 38 ± 18	0.5259 NS	9 23	20 ± 20 81 ± 15	0.0019[c]
Mixture 2 − *trans*-pinocarveol vs. mixture 2	13 19	30 ± 15 38 ± 18	0.6659 NS	10 23	100 ± 0 81 ± 15	0.2543 NS
Mixture 2 − myrtenol vs. mixture 2	8 19	43 ± 23 38 ± 18	0.7670 NS	10 23	50 ± 10 81 ± 15	0.1060 NS
Mixture 2 − bornyl acetate vs. mixture 2	37 19	23 ± 12 38 ± 18	0.3033 NS	36 23	84 ± 5 81 ± 15	0.8319 NS
Mixture 2 vs. volatiles from females in cones[e]				36 52	81 ± 15 100 ± 0	0.0645 NS

[a] Mixture 1 = *trans*-pityol + *trans*-pinocarveol + myrtenol + cone volatiles.
[b] NS = not significant.
[c] $P \leq 0.0024$ is required for an experiment-wise error rate of $\alpha = 0.05$ (Šidak, 1967; Games, 1977) for each of the 21 comparisons.
[d] Mixture 2 = *trans*-pityol + *trans*-pinocarveol + myrtenol + monoterpenes + bornyl acetate.
[e] Volatiles from aeration of cones infested with females.

TABLE 5. MEAN NUMBERS OF *C. coniperda* CAPTURED IN TRAPS BAITED WITH CONES, BEETLE-INFESTED CONES, PORAPAK Q-TRAPPED HOST- OR BEETLE-PRODUCED VOLATILES OR SPIROACETAL (EXPERIMENT 1 + EXPERIMENT 2) AT USFS BEECH CREEK SEED ORCHARD, NORTH CAROLINA

	Number of beetles captured/trap ($\bar{X}$ ± SE)[a]			
	Experiment 1		Experiment 2	
Single *df* treatment comparisons	Females	Males	Females	Males
Treatments with beetle-produced compounds vs. cones[b]	2.5 ± 0.8[d] 1.4 ± 0.5[NS]	3.0 ± 1.2[d] 0.5 ± 0.2		
Males in cones[c] vs. cones	2.6 ± 0.9[NS] 1.4 ± 0.5[NS]	1.3 ± 0.6[NS] 0.5 ± 0.2[NS]		
Females in cones[c] vs. cones	4.4 ± 1.3[NS] 1.4 ± 0.5[NS]	7.4 ± 2.9[d] 0.5 ± 0.2		
Females in cones vs. males in cones	4.4 ± 1.3[NS] 2.6 ± 0.9[NS]	7.4 ± 2.9[d] 1.3 ± 0.6		
Females + cone volatiles[f] vs. females in cones	1.9 ± 0.6[NS] 4.4 ± 1.3[NS]	3.8 ± 1.5[NS] 7.4 ± 2.9[NS]		
Males + cone volatiles[f] vs. males in cones	2.5 ± 0.9[NS] 2.6 ± 0.9[NS]	1.4 ± 0.5[NS] 1.3 ± 0.6[NS]		
Female + cone volatiles vs. male + cone volatiles	1.9 ± 0.6[NS] 2.5 ± 0.9[NS]	3.8 ± 1.5[NS] 1.4 ± 0.5[NS]	5.9 ± 4.3[d] 0.8 ± 0.5	3.9 ± 2.3[d] 0.3 ± 0.2
(*E*)-(±)-spiroacetal[g] vs. male + cone volatiles	1.0 ± 0.3[NS] 2.5 ± 0.9[NS]	1.1 ± 0.3[NS] 1.4 ± 0.5[NS]	0.5 ± 0.2[NS] 0.8 ± 0.5[NS]	0.4 ± 0.2[NS] 0.3 ± 0.2[NS]
Treatment *F* test	4.92	8.70	50.1	20.01
P	0.0073	0.0005	0.0194	0.0062
df	5, 15	5, 15	2, 5	2, 5

[a] Six Plexiglas barrier traps/treatment in experiment 1, Mar. 24–May 24, 1989; three 12-unit multiple-funnel traps/treatment in experiment 2, Apr. 19–May 24, 1989.
[b] Five mechanically wounded *P. strobus* cones/trap.
[c] NS = not significant.
[d] $P \leq 0.0064$ is required in experiment 1 for an experiment-wise error rate of $\alpha = 0.05$ (Šidák, 1967; Games, 1977) for each of the two comparisons and $P \leq 0.025$ is required in experiment 2 for each of the two comparisons.
[e] Five *P. strobus* cones/trap, each with one male or female beetle.
[f] 20 Male or female beetle equivalents + 20 cone equivalents of volatiles/trap/hr.
[g] 10 ME of (*E*)-(±)-spiroacetal/trap/hr. (Birgersson et al., 1995).

combined with WP cone oil than in those with (±)-*trans*-pityol alone or (+)-*trans*-pityol with WP cones oil (Table 6). Males were attracted to (±)-*trans*-pityol or (+)-*trans*-pityol alone in experiment 4, but only to pityol combined with WP cone oil in experiment 3. Males did not discriminate between (+)-*trans*-pityol and (±)-*trans*-pityol in either experiment. The addition of *E*-(±)-

TABLE 6. MEAN NUMBERS OF *C. coniperda* CAPTURED IN BARRIER TRAPS BAITED WITH (+)-*trans*-PITYOL, (±)-*trans*-PITYOL OR *P. strobus* CONE OIL (WPCO) (EXPERIMENTS 3 + 4) AT BEECH CREEK SEED ORCHARD, NORTH CAROLINA, AND PANCAKE BAY PROVINCIAL PARK, ONTARIO

	Number of beetles captured/trap ($\bar{X}$ + SE)[a]			
	Exp. 3 Beech Creek		Exp. 4 Pancake Bay	
Single *df* treatment comparisons	Females[b]	Males	Females	Males
(±)-*trans*-Pityol[c] vs. unbaited traps	0.0 ± 0.0 0.0 ± 0.0	1.7 ± 0.8 NS[d] 0.0 ± 0.0	0.1 ± 0.1 NS 0.0 ± 0.0	5.9 ± 1.7[e] 0.0 ± 0.0
(+)-*trans*-Pityol vs. unbaited traps	0.0 ± 0.0 0.0 ± 0.0	0.2 ± 0.2 NS 0.0 ± 0.0	0.1 ± 0.1 NS 0.0 ± 0.0	6.1 ± 1.8[e] 0.0 ± 0.0
(+)-*trans*-Pityol vs. (±)-*trans*-pityol	0.0 ± 0.0 0.0 ± 0.0	0.2 ± 0.2 NS 1.7 ± 0.8	0.1 ± 0.1 NS 0.1 ± 0.1	6.1 ± 1.8 NS 5.9 ± 2.3
WP cone oil vs. unbaited traps	0.2 ± 0.2 0.0 ± 0.0	0.0 ± 0.0 NS 0.0 ± 0.0	0.0 ± 0.0 NS 0.0 ± 0.0	0.1 ± 0.1 NS 0.0 ± 0.0
(±)-*trans*-Pityol + WPCO[f] vs. (±)-*trans*-pityol	0.3 ± 0.2 0.0 ± 0.0	4.5 ± 1.7[e] 1.7 ± 0.8	0.4 ± 0.2[e] 0.1 ± 0.1	10.1 ± 2.0 NS 5.9 ± 2.3
(+)-*trans*-Pityol + WPCO vs. (+)-*trans*-pityol	0.3 ± 0.2 0.0 ± 0.0	6.3 ± 2.7[e] 0.2 ± 0.2	0.1 ± 0.1 NS 0.1 ± 0.1	8.4 ± 2.1 NS 6.1 ± 2.2
(+)-*trans*-Pityol + WPCO vs. (±)-*trans*-pityol + WPCO	0.3 ± 0.2 0.3 ± 0.2	6.3 ± 2.7 NS 4.5 ± 1.7	0.1 ± 0.1[e] 0.4 ± 0.2	8.4 ± 2.1 NS 10.1 ± 2.2
(±)-*trans*-Pityol + WPCO vs. unbaited traps	0.3 ± 0.2 0.0 ± 0.0	4.5 ± 1.7[e] 0.0 ± 0.0	0.4 ± 0.2[e] 0.0 ± 0.0	10.1 ± 2.0[e] 0.0 ± 0.0
Treatment *F* test	1.20	11.6	4.05	29.2
P	0.3386	0.0001	0.0040	0.0001
df	5, 25	5, 25	5, 45	5, 45

[a] Six Japanese beetle traps/treatment at Beech Creek Apr. 23–May 10, 1990; 10 traps/treatment at Pancake Bay May 28–Jun. 4, 1990.
[b] *F* test for treatments not significant.
[c] Pityol/trap 4.2 mg week from 1.04 mm ID capillary tube.
[d] NS = not significant.
[e] $P \leq 0.00639$ is required for an experimentwise error rate of $\alpha = 0.05$ (Šidák, 1967; Games, 1977) for each of the eight comparisons.
[f] Volatile oil, 2 ml/week of *P. strobus* cones (Pierce et al., 1995).

spiroacetal to baits containing (±)-*trans*-pityol and α-pinene significantly reduced the trap catch of males but not females in experiment 5 (Table 7).

DISCUSSION

Identification of Pityol and Spiroacetal. The discovery of (+)-*trans*-pityol (1% *cis*) in female WPCB (Figure 7) constitutes the first record of occurrence in this genus. Pityol was first identified in, and named after, the European

TABLE 7. MEAN NUMBERS OF *C. coniperda* CAPTURED IN TRAPS BAITED WITH (±)-*trans*-PITYOL AND α-PINENE, AND (±)-*trans*-PITYOL, α-PINENE, AND (*E*)-(±)-SPIROACETAL AT BEECH CREEK SEED ORCHARD, NORTH CAROLINA

Treatment[a]	Number of beetles captured/trap ($\bar{X}$ ± SE)[b]	
	Females	Males
(±)-*trans*-Pityol and α-pinene	20.2 ± 4.7 a	46.2 ± 10.3 a
(±)-*trans*-Pityol, α-pinene and (*E*)-(±)-spiroacetal	20.0 ± 5.2 a	12.2 ± 3.1 b

[a](±)-*trans*-Pityol released at 100 FE/hr (0.7 mg/week), (±)-α-pinene at 100 mg/week and (*E*)-(±)-spiroacetal at 100 ME/hr (8 mg/week) (Birgersson et al., 1995).
[b]Six Japanese beetle traps/treatment from April 30–June 3, 1990; means within a column followed by the same letter are not significantly different at $P < 0.05$; paired *t* test.

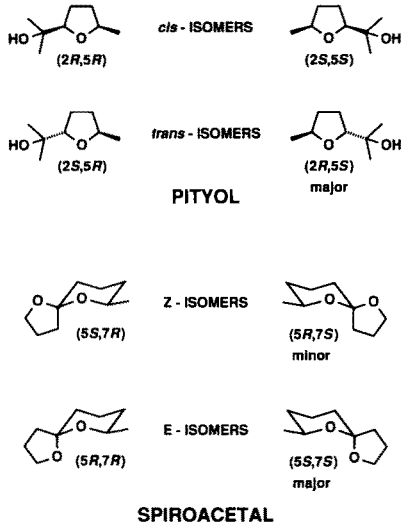

Fig. 7. Stereoisomers of *Conophthorus* pheromones.

species, *Pityophthorus pityographus*, which also contained grandisol (Francke et al., 1987). Klimetzek et al. (1989) isolated *cis*-pityol from the elm bark beetle, *Pteleobius vittatus*, which also produced 3-methyl-3-buten-2-ol and *cis*-vittatol (*cis*-3-hydroxy-2,2,6-trimethyltetrahydropyran). In field tests, the combination of three compounds was far superior to any single compound or two-component mixture. The occurrence of pityol in *Conophthorus* in North America, and

Pityophthorus in Europe supports the hypothesis that these are closely related genera (Wood, 1982).

The spiroacetal produced by WPCB (Figure 7) was previously identified in abdominal glands of social wasps (Francke et al., 1979a). It also occurs in males of *Leperisinus varius* (F.) and in frass of the fir beetle, *Cryphalus piceae*, where it acts as a strong inhibitor of aggregation for these two bark beetle species (Kohnle et al., 1992).

The first spiroacetal identified as a bark beetle-produced compound was isolated from *Pityogenes chalcographus* (Francke et al., 1977). This compound, chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane), is weakly active by itself and is strongly synergized by methyl 2(*E*),4(*Z*)-decadienoate (Byers et al., 1988). Several alkyl-1,6-dioxaspiro[4.5]decanes occur in the social wasps, *Paravespula vulgaris* L., *P. germanica* (F.), and *Dolichovespula saxonica* (F.), and in the ash bark beetle, *L. varius* (F.) (Francke et al., 1979a).

Stereochemistry of Pityol and Spiroacetal. Chiral analyses showed that the *trans*-pityol produced by female WPCB is (2*R*,5*S*)-(+) (Figure 7). Based upon results of our bioassays and field experiments, (+)-*trans*-pityol, synergized by host monoterpene hydrocarbons, is a pheromone for WPCB. Since pityol exists in four different isomers (Figure 7), and only one is produced by the WPCB, it is possible that the stereochemistry is also a key to species separation. In field tests, (+)-*trans*-pityol and racemic grandisol acted synergistically as an aggregation pheromone for *P. pityographus*, whereas a mixture of (−)-*trans*-pityol and grandisol was inactive (Francke et al., 1987). Alone, (+)-*trans*-pityol exhibited only slight attraction for *P. pityographus*. A racemic mixture of *cis*-pityol was active for *P. vittatus*, but *cis*-pityol baits with (−)-*cis*-vittatol attracted more beetles than those with (+)-*cis*-vittatol (Klimetzek et al., 1989).

Isomers of the spiroacetal identified from WPCB were of the 5*S*,7*S* and 5*R*,7*S* configurations in a proportion of ca. 15:1 (Figure 7). However, spiroacetal produced from 1,8-dihydroxynonan-4-one in hydrochloric acid was mostly the more stable *E* isomer ($E/Z \geq 100$). Under acidic conditions the spiroacetal may epimerize at the spirocenter. The 5*S*,7*S* isomer has the methyl group in an equatorial position and, due to the stabilizing anomeric effect, the second oxygen is attached axially at the spirocenter to the tetrahydropyran ring (Francke et al., 1981; Delongchamps et al., 1981). Thus, the natural mixture found in the WPCB is not in a thermodynamically stable equilibrium.

The instability of the bouquet opens the possibility that the "aging" (i.e., equilibration) of the proportion may function as a "built-in clock" providing information about the age of the signal. This effect is even more pronounced in the case of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, which was identified from the cephalic secretion of the solitary bee *Andrena wilkella* (Bergström et al., 1982). The major component, the thermodynamically stable *E*,*E* isomer, has both methyl groups in equatorial positions and a diaxial coupling of the oxygens

at the spirocenter. It is accompanied by an *E,Z* product that also has two methyl groups in equatorial positions, but the oxygens at the spirocenter are in axial and equatorial positions. The ratio between these two compounds in the bees favors the thermodynamically less stable *E,Z* isomer. In *A. wilkella*, the more stable and highly attractive 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane is of 2*S*, 6*R*,8*S* configuration (Tengö et al. 1990); only this enantiomer is recognized by the bees.

Alkyl-1,6-dioxaspiro[4.4]nonanes, such as chalcogran (Francke et al., 1977), are much more sensitive to acidic epimerization at the spirocenter than the two other systems. In *Pityogenes chalcographus*, only (2*S*,5*R*)-2-ethyl-1,6-dioxaspiro[4.4]nonane showed strong activity (Byers et al., 1989). In nature this isomer is accompanied by the behaviorally inactive 2*S*,5*S* diastereomer, in a ratio of about 3:2 (Schurig and Weber, 1984), which almost represents the thermodynamically stable equilibrium.

Beetle Response. The strong male WPCB response to volatiles emanating from cones with females (Figure 6) agrees with the responses reported for male *C. ponderosae* (Kinzer et al., 1972) and *C. resinosae* (de Groot, 1992). However, WPCB males also responded strongly to volatiles from cones infested with pairs of beetles. The strongest response by female WPCB was to volatiles from cones with pairs or male beetles, as was reported for *C. ponderosae* females Kinzer et al., (1972). Few beetles were caught in experiment 1, perhaps because WPCB populations were sparse and the Plexiglas barrier traps were inefficient (Table 5).

Pityol alone elicited only weak WPCB responses in laboratory bioassays (Table 3) and in field experiment 3 (Table 6), but attracted males in field experiment 4. Neither *trans*-pinocarveol nor myrtenol, two other female-produced volatiles, enhanced WPCB responses in the laboratory (Tables 3 and 4). Because male WPCB did not distinguish between (±)-*trans*-pityol and the (+)-*trans*-pityol isomer (Tables 3 and 4), the more easily synthesized, and therefore less expensive, racemic *trans*-pityol may suffice for applied uses in the field.

Host monoterpenes often synergize scolytid pheromones (Borden, 1985). Volatiles collected from cones or a mixture of the four major monoterpene hydrocarbons found in cones, dramatically increased male response to pityol (Tables 3 and 4). Mixtures containing both pityol and monoterpenes produced male responses of about 80%, which were equal to those for volatiles from female-infested cones (Tables 3 and 4). Removing the host volatiles or the monoterpenes greatly decreased male response.

Female WPCB showed weak responses to cone volatiles or monoterpenes (Tables 2–4). Their response was much greater to a mixture of pityol, (+)-*trans*-pinocarveol, and monoterpenes, but was not as strong as to volatiles from female-infested cones (Table 3). Apparently other beetle- or host-produced compounds were emanating from female-infested cones. Bornyl acetate, a host vol-

atile released in prodigious amounts from wounded cones, did not affect beetle response.

The spiroacetal, the major male-produced volatile, did not attract male or female WPCB beetles (Figure 6 and Table 6). However, adding the spiroacetal to the three-compound female-produced mixture reduced male response from 30% to 8%, which was the lowest male response to any bioassay treatment containing pityol (Tables 3 and 4). Had the spiroacetal been added to a mixture of pityol combined with host volatiles, the potential for demonstrating a reduction in male response by the spiroacetal would obviously have been much greater. In the field, the spiroacetal inhibited the attraction of males (Table 7); Pierce et al. (1995) obtained similar field results with *C. resinosae*.

CONCLUSIONS

Independent and parallel chemical analyses undertaken in Georgia and Canada clearly confirmed that pityol and the spiroacetal are present in *C. coniperda*. Furthermore, the compounds do not differ in their chirality between two distinct and widely separated populations. Both pityol and the spiroacetal are potentially useful as behavior-modifying chemicals in an integrated pest management system for WPCB in *P. strobus* seed orchards. Pityol is also an effective attractant for male *C. resinosae* (Pierce et al., 1995), and we propose that it be tested in traps for monitoring WPCB and RPCB populations, or perhaps for mass-trapping populations. The spiroacetal could also have practical application as an inhibitor of normal cone beetle behavior.

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PHEROMONES IN RED PINE CONE BEETLE, *Conophthorus resinosae* HOPKINS, AND ITS SYNONYM, *C. banksianae* McPHERSON (COLEOPTERA: SCOLYTIDAE)

HAROLD D. PIERCE, JR.,[1,*] PETER DE GROOT,[2] JOHN H. BORDEN,[3] S. RAMASWAMY,[1] AND A. C. OEHLSCHLAGER[1]

[1]*Department of Chemistry, Simon Fraser University Burnaby, British Columbia, Canada V5A 1S6*

[2]*Forestry Canada, Forest Pest Management Institute Box 490, Sault Ste. Marie, Ontario, Canada P6A 5M7*

[3]*Centre for Pest Management, Department of Biological Sciences Simon Fraser University Burnaby, British Columbia, Canada V5A 1S6*

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Abstract—Analyses of abdominal extracts and beetle-produced volatiles revealed that female *Conophthorus resinosae* and *C. banksianae* produced optically pure (*E*)-(+)-pityol [(2*R*,5*S*)-(+)-2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran]. In field tests, traps baited with (*E*)-(±)- or (*E*)-(+)-pityol captured only males. Addition of host oils to traps baited with pityol did not significantly enhance the capture of males. Males of these former sibling species produced the spiroacetal (5*S*,7*S*)-(−)-7-methyl-1,6-dioxaspiro[4.5]decane with high optical purity (96%). Addition of the racemate or the optical isomers of the spiroacetal to traps baited with (*E*)-(±)-pityol almost completely inhibited the capture of males. It is hypothesized that to ensure their reproductive success, males produce the spiroacetal to repel rival males. Species specificity in pityol and the spiroacetal was not found between *C. resinosae* and *C. banksianae*, thus supporting their synonymy.

Key Words—*Conophthorus resinosae*, *Conophthorus banksianae*, cone beetle, Scolytidae, sex pheromone, repellent, pityol, 2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran, spiroacetal, 7(*E*)-methyl-1,6-dioxaspiro[4.5]decane.

*To whom correspondence should be addressed.

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INTRODUCTION

Wood (1989) synonymized the morphologically identical sibling species *Conophthorus resinosae* Hopkins, red pine cone beetle, and *C. banksinae* McPherson, the jack pine tip beetle. Taxonomic studies of the karyotypes (de Groot and Ennis, 1990), allozymes (de Groot et al., 1992), cuticular hydrocarbons (Page et al., 1990), life-history traits (de Groot and Borden, 1991), male genitalia (de Groot, 1992), and host acceptance behaviors (de Groot and Borden, 1992) uphold the placement of *C. banksianae* as a junior synonym of *C. resinosae* (Wood, 1989). Another taxonomic character that can be used to distinguish species or host races is pheromone specificity in chemical communication. (Roelofs and Cardé, 1974; Cardé and Baker, 1984).

Evidence that the red pine beetle and the white pine cone beetle, *C. coniperda* (Schwarz), produce sex pheromones has been disclosed by de Groot et al. (1991). This study led us to explore chemical communication in *C. resinosae* and *C. banksianae* as part of a biosystematic study to determine the species validity of the latter (de Groot, 1992). Our objectives were to identify and compare the pheromones of *C. banksianae* and *C. resinosae* and to determine the response of the beetles to synthetic compounds in the field. For clarity, we retain the name *C. banksianae* in this paper to refer to populations of *C. resinosae* found on jack pine, *Pinus banksiana* Lamb.

METHODS AND MATERIALS

Collection and Maintenance of Beetles. Adult *C. banksianae* and *C. resinosae* were collected near Aubrey Falls and Thessalon, Ontario, respectively, during September and October of 1987 and 1988. Infested shoots of jack pine and cones of red pine, *P. resinosa* (Ait.), were placed in paper and burlap bags, respectively, and stored in darkness at 2°C and 70–87% relative humidity. Uninfested jack pine shoots and red pine branches bearing conelets were collected from Thessalon in October of each year. After about two to three days at 20–22°C, paper bags containing host infested with either *C. banksianae* or *C. resinosae* were gently warmed under a 60-W incandescent light bulb to induce beetle emergence. Beetles were sexed by examining the 7th and 8th abdominal tergites (Herdy, 1959) and held at 5°C on moist filter paper in Petri dishes until required. Beetles were assigned to species based on the species of the host tree from which they emerged and their feeding behavior (McPherson et al., 1970).

Collection of Beetle and Host Volatiles. Small branches (33) bearing conelets (1–6) of red pine were stripped of needles, cut 2–3 cm below the terminal bud, and equally distributed between two aeration chambers (Pierce et al., 1984). Male (304) and female (213) *C. resinosae* were introduced into separate chambers and allowed to attack host material. For the control, each conelet was

punctured three to four times with the tang of a small file before placement of branches into the aeration chamber. Humidified and charcoal-filtered air was drawn at 1.65 liters/min through the vertically oriented chambers and traps (ca. 200 mm × 14 mm OD) containing Porapak Q for one to five weeks. Volatiles were recovered by overnight extraction of the Porapak Q with pentane in a Soxhlet extractor. The pentane solution of the volatiles was concentrated (1–5 ml) either by distilling off the pentane through a Dufton column or by evaporation under a stream of helium.

For *C. banksianae*, volatiles were collected for two to seven days from separated males (60) and females (90) feeding on jack pine shoots (10 and 15, respectively) on analytical traps (6 mm OD) packed with a 30-mm length of Porapak Q. Volatiles were recovered by eluting the traps with 1 ml of anhydrous ether. Several collections with *C. resinosae* were also conducted in this manner.

Extraction and Analysis of Beetle Volatiles. After removal from host, beetles were placed in Dry Ice–cold pentane and crushed to a fine powder. After warming to room temperature, the supernatant was analyzed by gas chromatography (GC). Samples that were not immediately analyzed were stored in a freezer. For several samples of *C. banksianae*, the crude extract was placed on glass wool in the upper portion of an analytical Porapak Q trap. The volatiles were transferred from the glass wool to the Porapak Q with nitrogen (ca. 200 ml/min for 0.5 hr) and recovered by eluting the Porapak Q with pentane–ether (1 : 1, v/v). These samples were analyzed by GC for determination of chirality and enantiomeric enhancement (ee).

Isolation of Beetle Volatiles by Steam Distillation. A micro-steam distillation–continuous extraction apparatus (Godefroot et al., 1981) was used for the isolation of volatiles from the pentane extracts of crushed beetles. Pentane was used for extraction. The solutions of the volatiles were stored in the freezer until analyzed by GC.

Isolation of Volatile Oils by Steam Distillation. A simultaneous steam distillation–continuous extraction still head (Flath and Forrey, 1977) was used for the isolation of the volatile oils from macerated (Waring blender) cones, buds, or needles of red pine and shoots of jack pine. The steam distillation was conducted for 4 hr after boil-up, and pentane was used as the extracting solvent. The bulk of the pentane was removed under a steam of argon or nitrogen. Residual solvent was removed by brief vacuum pumping. The oils were stored in a freezer until use.

Instrumental Methods. Hewlett-Packard 5830, 5880, and 5890 gas chromatographs equipped with capillary inlet systems and flame-ionization detectors were used for analyses by GC. Glass columns (30 m × 0.5 mm ID) coated with SP-1000 or Carbowax 20 M (Supelco Canada Ltd., Oakville, Ontario) and a fused silica column (15 m × 0.25 mm ID) coated with DB-1 (J & W Scientific Inc., Folsom, California) were used. A fused silica column (25 m × 0.25 mm

ID) coated with Chirasil-Dex (8) (V. Schurig, University of Tübingen, Germany) was used for determination of chirality and ee. The injection port and detector temperatures were 260° and 270°C, respectively.

A Hewlett-Packard 5895B GC-MS-DS was employed for coupled gas chromatography–mass spectroscopy (GC-MS). Fused silica columns (0.32 mm ID) coated with DB-1 (30 m), DB-WAX (60 m), or SP-1000 (30 m) (J & W Scientific) were coupled directly into the ion source. The injection port, transfer line, and ion source were 260°, 250°, and 200°C, respectively. Helium was the carrier gas for GC and GC-MS.

A Varian 1200 gas chromatography equipped with 10:1 effluent splitter and thermal gradient collector (Brownlee and Silverstein, 1968) was used for micropreparative isolation of pheromones from the Porapak Q-trapped volatiles for chirality determinations. The column was a 3-m × 3.17-mm-OD stainless tube packed with a 10% SP-1000 on Supelcoport (100/120 mesh) (Supelco, Bellafonte, Pennsylvania).

Synthetic Pheromones. (*E*)-(±)-Pityol and (*E*)-(+) pityol were prepared from (±)-sulcatol and (*S*)-(+)-sulcatol (97% ee, Phero Tech, Inc., Delta, British Columbia) by the method of Mori and Paupoomchareon (1987). Distilled (*E*)-(±)-pityol (84%) containing ca. 2.3% of the *Z* isomer and 5% sulcatol was further purified by flash chromatography on silica gel with pentane–ether (4:1) as eluent to give (*E*)-(±)-pityol (98.3%), which contained 0.3% of the *Z* isomer and no sulcatol. The distilled (*E*)-(+)-pityol containing 3.1% of the *Z* isomer and no sulcatol was 86.6% chemically pure and 98.8% ee. The enantiomers and racemate of the spiroacetal 7(*E*)-methyl-1,6-dioxaspiro[4.5]decane were synthesized by procedures recently developed by Ramaswamy and Oehlschlager (1991). The racemic compound was 99.4% pure and contained 0.3% of the *Z* isomer. The (5*R*,7*R*)-(+) and (5*S*,7*S*)-(−) isomers were each ca. 93% chemically pure and contained 1.6 and 1.2% of the *Z* isomer, respectively. Both enantiomers were ca. 97% ee. Samples of 7(*E*)-(±)-methyl-1,6-dioxaspiro[4.5]decane and (±)-pityol (*E*/*Z*, 1:2) were gifts from W. Francke (University of Hamburg, Germany).

Field Experiments. These were conducted to determine the attractiveness of: (1) (±)-pityol and (+)-pityol alone or in combination with host oils (experiments 1 and 4), (2) three (*E*/*Z*) ratios of (±)-pityol with host oils (experiments 2 and 5), (3) (±)-pityol with the racemate of and with the (+) and (−) isomers of the spiroacetal (experiments 3 and 6), and (4) three ratios of (±)-pityol with racemic spiroacetal (experiment 7). Experiments for *C. banksianae* were located in a jack pine plantation in Hurlbert Township ca. 60 km northeast of Sault Ste. Marie, Ontario. A red pine plantation near Thessalon, Ontario, was used for the experiments with *C. resinosae*. Isozyme and karotype studies of *Conophthorus* from these two sites (de Groot et al., 1991; de Groot and Ennis, 1990) showed that *C. coniperda*, which is distinguishable by its morphology, karology,

isozymes, and cuticular hydrocarbons and appears not to use either red or jack pine as hosts (de Groot et al., 1991), was not present. Furthermore, ground surveys indicated that *C. coniperda* was not present within 20 km of these sites. As *C. banksianae* and *C. resinosae* could only be distinguished on the basis of adult size and host, we used these characters to identify the species caught at the sites.

Traps were made from yellow Japanese beetle trap tops (Trécé, Inc., Salinas, California) and Mason jar bottoms containing 50 ml of ethylene glycol. Pityol and the spiroacetal were released from glass capillary tubes (1 mm ID). Release rates from capillary tubes were calculated by determining volume loss at 24°C. Capillary tubes were placed inside 1.5-ml plastic centrifuge tubes in which four 1-mm holes were made to facilitate the release of volatiles. Host oils were released from plastic centrifuge tubes with two 0.5-mm holes. The tubes containing baits were taped to one vane of the trap. Control traps were unbaited. Experiments were laid out as complete randomized blocks, with traps hung in the upper third of the tree crown, one trap per tree, and spaced 15–20 m apart. Captured beetles were preserved in 70% ethanol and later identified and their sex determined. Voucher specimens from each experiment were kept and deposited in the collection of P. de Groot, Natural Resources Canada, Forest Pest Management Institute, Sault Ste. Marie, Ontario. Baits were replaced weekly and treatment locations rerandomized. The dates and number of replicates for each experiment are shown in Tables 1 and 2. Trap catch data were analyzed by the Kruskal-Wallis test with nonparametric multiple comparisons (BMDP program 3s, 1990 version).

RESULTS

Pheromone Identification. Analyses of the Porapak Q-trapped volatiles from female or male *C. banksianae* feeding on jack pine by GC established that beetles of each sex produced at least one unique compound (Figure 1). Sex-specific compounds were also present in pentane extracts of the feeding beetles (Figure 2). The mass spectrum of the female-produced compound exhibited a base peak at *m/z* 59, suggesting a tertiary alcohol, and prominent peaks at *m/z* 85, 102, and 129 but lacked a molecular ion peak. The spectrum of the unknown compound was similar to that of (*E*)-pityol, an aggregation pheromone produced by male *Pityophthorus pityographus* (Franke et al., 1987). Comparison of the mass spectrum and GC retention times of the unknown compound to those of authentic pityol (*E/Z*, 1:2) established that female *C. banksianae* had produced only the *E* isomer of pityol (Figure 3).

The mass spectra of the major and minor unknown compounds in the pentane extract of the males were identical and exhibited a molecular ion at

TABLE 1. CATCHES OF MALE *C. banksianae* IN TRAPS BAITED WITH PITYOL, SPIROACETAL, OR JACK PINE SHOOT OIL (JPSO) ALONE OR IN SELECTED COMBINATIONS IN A JACK PINE PLANTATION, HURLBERT TWP., 60 km NORTHEAST OF SAULT STE. MARIE, ONTARIO

Exp. No.	Treatment[a]	Beetles captured per trap ($\bar{X}$ ± SE)[b]
1	(±)-Pityol	2.6 ± 0.7a
	(+)-Pityol	2.1 ± 0.5a
	(±)-Pityol + JPSO	2.4 ± 0.7a
	(+)-Pityol + JPSO	5.0 ± 1.6a
	JPSO	0.0 ± 0.0b
	Blank	0.0 ± 0.0b
2	(±)-Pityol (*E*/*Z*, 19:1) + JPSO	0.1 ± 0.1a
	(±)-Pityol (*E*/*Z*, 66:1) + JPSO	0.2 ± 0.1a
	(±)-Pityol (*E*/*Z*, 364:1) + JPSO	0.3 ± 0.2a
	Blank	0.0 ± 0.0a
3	(±)-Pityol	1.9 ± 0.5a
	(±)-Pityol + (±)-Spiroacetal	0.3 ± 0.2b
	(±)-Pityol + (+)-Spiroacetal	0.3 ± 0.2b
	(±)-Pityol + (−)-Spiroacetal	0.1 ± 0.1b
	Blank	0.0 ± 0.0b

[a]Experiment 1, May 20–June 7, 1990, 10 traps/treatment; experiment 2, July 4–11, 1990, 10 traps/treatment; experiment 3, June 27–July 4, 1990, 7 traps/treatment. Release rates of (±)-pityol, (+)-pityol, (*E*)-(±)-, (+)-, and (−)-spiroacetal were 0.61, 0.59, 0.95, 1.00, and 0.93 mg/day, respectively, at 24°C in the laboratory.

[b]Means within an experiment followed by the same letter are not significantly different, Kruskal-Wallis test, $P > 0.05$.

m/*z* 156, but were completely different from those of *exo*- and *endo*-brevicomin (MW = 156). The mass spectra matched the partial spectrum reconstructed from data tabulated for 7(*E*)-methyl-1,6-dioxaspiro[4.5]decane (Francke et al., 1979a,b). The mass spectrum and GC retention time of the major male-produced unknown compound were identical to those observed for an authentic sample of this spiroacetal, which contained ca. 1% of the longer-eluting *Z* isomer. The structure of the minor male compound was assigned as the *Z* isomer because its mass spectrum and retention time in GC were, respectively, identical to and considerably longer than that of the *E* isomer (Franke et al., 1979b). The isomer ratio (*E*/*Z*) was 9.4 and 12.4 in two pentane extracts and 10.5 and 27.0 in two samples of Porapak Q-trapped volatiles.

Analyses of the Porapak Q-trapped volatiles (Figure 4) from feeding female and male *C. resinosae* by GC and comparison of the chromatograms to those of Figure 1 indicated that they had emitted the same major sex-specific compounds as had *C. banksianae*. Analysis of the female volatiles by GC-MS not

TABLE 2. CATCHES OF MALE *C. resinosae* IN TRAPS BAITED WITH PITYOL SPIROACETAL OR RED PINE CONE OIL (RPCO) ALONE OR IN SELECTED COMBINATIONS IN A RED PINE PLANTATION, THESSALON, ONTARIO

Exp. No.	Treatment[a]	Beetles captured per trap ($\bar{X}$ ± SE)[b]
4	(±)-Pityol	19.8 ± 2.1a
	(+)-Pityol	25.1 ± 3.0a
	(±)-Pityol + RPCO	18.8 ± 2.4a
	(+)-Pityol + RPCO	28.2 ± 2.2a
	RPCO	0.1 ± 0.1b
	Blank	0.1 ± 0.1b
5	(±)-Pityol (*E*/*Z*, 19:1) + RPCO	11.3 ± 2.2a
	(±)-Pityol (*E*/*Z*, 66:1) + RPCO	16.1 ± 2.6a
	(±)-Pityol (*E*/*Z*, 364:1) + RPCO	15.3 ± 2.7a
	Blank	0.1 ± 0.1b
6	(±)-Pityol	17.9 ± 3.2a
	(±)-Pityol + (±)-Spiroacetal	0.1 ± 0.1b
	(±)-Pityol + (+)-Spiroacetal	2.1 ± 0.8b
	(±)-Pityol + (−)-Spiroacetal	0.4 ± 0.2b
	Blank	0.1 ± 0.1b
7	(±)-Pityol + (±)-Spiroacetal	0.0 ± 0.0a
	(±)-Pityol + (±)-Spiroacetal (20%)[c]	0.1 ± 0.1a
	(±)-Pityol + (±)-Spiroacetal (10%)[c]	0.1 ± 0.1a
	(±)-Pityol	7.9 ± 1.7b
	(±)-Spiroacetal	0.0 ± 0.0a
	Blank	0.1 ± 0.1a

[a]Experiment 4, May 29–June 12, 1990, 10 traps/treatment; experiment 5, July 3–10, 1990, 10 traps/treatment; experiment 6, June 15–26, 1990, 7 traps/treatment; experiment 7, June 4–25, 1991, 7 traps/treatment. Release rates as in Table 1.

[b]Means within an experiment followed by the same letter are not significantly different, Kruskal-Wallis test, $P > 0.05$.

[c]*n*-Decane was used as a diluent.

only confirmed the identity of this compound as (*E*)-pityol but also revealed the presence of a small amount of (*Z*)-pityol. The ratio of the *E* and *Z* isomers was ca. 150:1. The identity of the male compound in a sample of volatiles isolated by steam distillation was confirmed by GC-MS. The ratio of the *E* and *Z* isomers in this sample was 49:1. In the Porapak Q-trapped volatiles the *E*/*Z* isomer ratio was variable and ranged from 19 to 39 ($N = 5$).

(*E*)-Pityol was not detected in pentane extracts of female *C. resinosae* (Figure 5). The chromatogram of the pentane extract of the males (Figure 5) was quite similar to that obtained from male *C. banksianae* (Figure 2), as was the ratio (9.4) of the *E* and *Z* isomers of the spiroacetal. Comparison of the

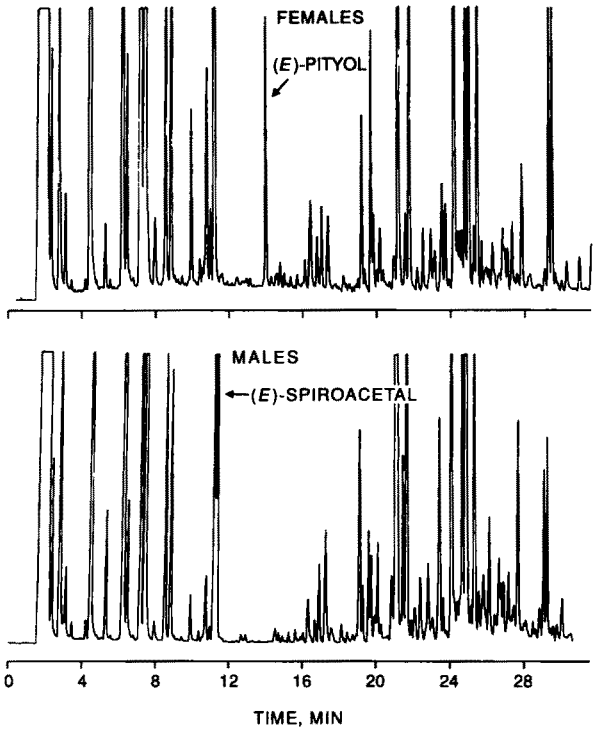

FIG. 1. Gas chromatograms of the Porapak Q-trapped volatiles from female and male *C. banksianae* feeding on jack pine.

chromatograms in Figure 5 indicated that no other sex-specific compound, which might not have been discernible in the complex mixture of Porapak Q-trapped volatiles, was present in these extracts.

Chirality. Analysis of the volatiles in the pentane extract of female *C. banksianae* (Figure 3) on a capillary column coated with Chirasil-Dex(8) revealed that the females had produced optically pure (*E*)-(+)-pityol (Figure 6), as had female *C. resinosae* (Figure 7). These determinations were made difficult by the presence in these samples of several compounds with retention times quite close to that of (*E*)-(−)-pityol. As the lower panels in Figures 6 and 7 show, a small amount of the (*E*)-(−) isomer could have been detected if it had been present in the volatiles. The absolute configuration of (*E*)-(+)-pityol is 2*R*,5*S* (Figure 3).

The *E* isomer of the spiroacetal from the males of the two species had the 5*S*,7*S* configuration and enantiomeric enhancements of ca. 96% as determined

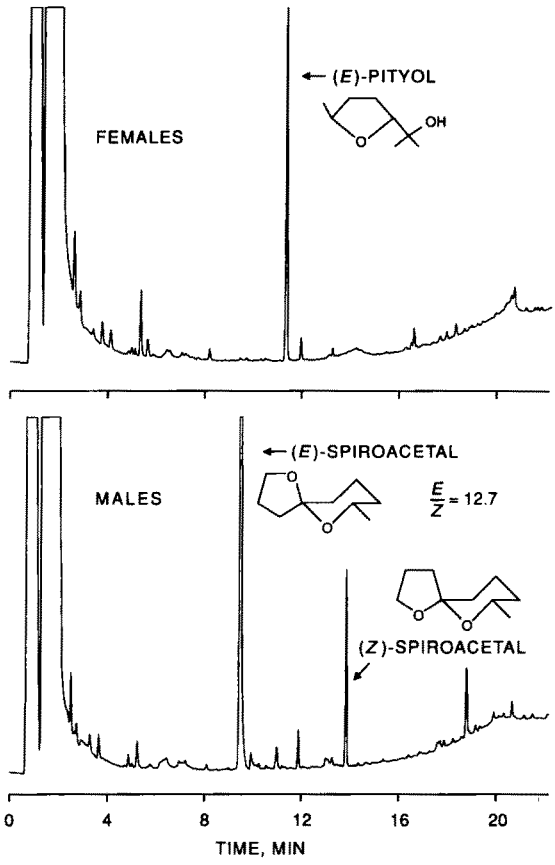

FIG. 2. Gas chromatograms of volatiles in pentane extracts of feeding female and male *C. banksianae* excised from jack pine.

by analyses on the Chirasil-Dex(8) column. Figure 8 shows the chromatogram of the spiroacetal isolated from the Porapak Q-trapped volatiles from feeding *C. resinosae* by micropreparative GC. Since the isomers were not completely resolved, it was fortunate the 5*R*,7*R* isomer eluted first.

Field Experiments. Males of both species responded to (+)- and (±)-pityol (experiments 1–7), except for *C. banksianae* males in experiment 2, where very low trap catches were obtained. No significant differences in trap catch between traps baited with (+)-pityol and (±)-pityol were obtained (experiments 1 and 4). The addition of the host oils to the traps did not appear to enhance male response significantly (experiments 1 and 4), and host oils by themselves were

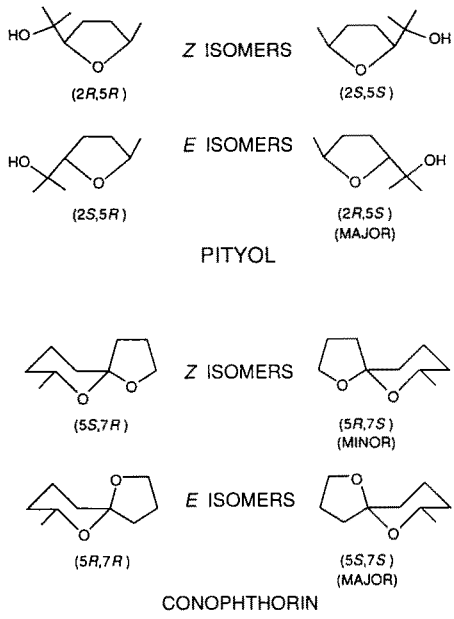

FIG. 3. Stereoisomers of *Conophthorus* pheromones.

unattractive. Male trap catches were not significantly different between the three *E*/*Z* ratios of (±)-pityol for *C. banksianae* and *C. resinosae* (experiments 2 and 5). The addition of (±)-, (+)-, or (−)-spiroacetal to (±)-pityol significantly inhibited the response of males (experiments 3 and 6), and traps baited with the combinations of pityol and the spiroacetal were as attractive as the unbaited traps. When (±)-pityol was released with (±)-spiroacetal (experiment 7) in ratios of 1 : 1, 5 : 1, or 10 : 1, the trap catches of male *C. resinosae* were significantly reduced in comparison to the traps baited with (±)-pityol alone. Neither female *C. banksianae* nor *C. resinosae* responded significantly to any stimulus (trap catches to most treatments were 0 and ranged from 0 to 3).

DISCUSSION

The discovery of (*E*)-(+)-pityol in female *C. resinosae*, *C. banksianae*, and *C. coniperda* (de Groot, 1992; Birgersson et al., 1995) confirms the finding that it is produced by females in this genus. Furthermore, the results clearly demonstrate that (*E*)-(+)-pityol is a sex pheromone that only attracts males. In the closely related genus, *Pityophthorus* (Bright, 1981; Wood 1982), (*E*)-(+)-

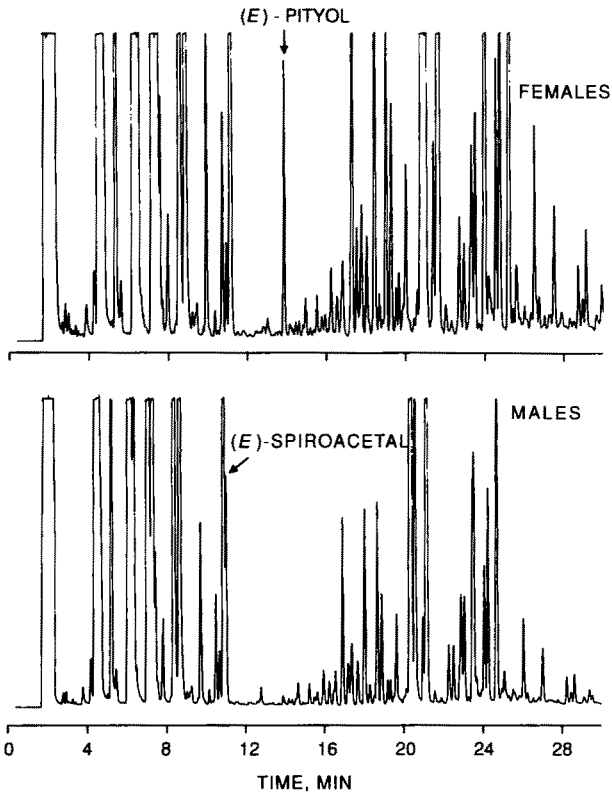

FIG. 4. Gas chromatograms of the Porapak Q-trapped volatiles from female and male *C. resinosae* feeding on red pine.

pityol is produced by male *P. pityographus* Ratz. and with racemic grandisol acts synergistically as an aggregation pheromone (Francke et al., 1987). Although the (+) isomer of (*E*)-pityol generally caught more male *Conophthorus* than the racemate, the differences were not significant, thus indicating that the antipode is benign and the racemate would be suitable in operational use.

The repellency of the spiroacetal to *Conophthorus* is consistent with the finding that its release with *exo-* or *endo*-brevicomin greatly reduced trap catches of both male and female ash bark beetles, *Leperisinus varius* (F.). It thus functions as an antiaggregation pheromone (Kohnle, 1985). Kohnle et al. (1992) found that the spiroacetal reduced the response of *L. varius* regardless of chirality, but only the naturally produced (5*S*,7*S*)-(−) enantiomer reduced the response of the fir bark beetle, *Cryphalus piceae* (Ratz.). The spiroacetal also

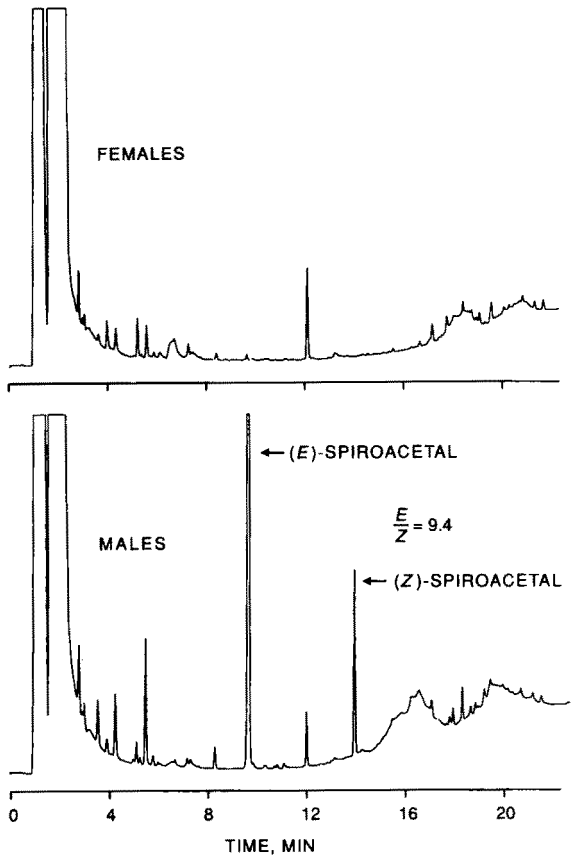

FIG. 5. Gas chromatograms of volatiles in pentane extracts of feeding female and male *C. resinosae* excised from red pine.

repelled worker wasps of several species (Francke et al., 1979b). We suggest that the spiroacetal produced by male *Conophthorus* spp. likely functions as a semiochemical message to warn rival males that they must contest the resident male for the female. Males may also physically protect females from intruders by remaining near the entrance hole between matings and during oviposition (de Groot and Borden, 1991).

Species specificity in (*E*)-pityol and spiroacetal production was not found between *C. banksianae* and *C. resinosae*. Females of both species had produced optically pure (*E*)-(+)-pityol, as had female *C. coniperda* (Birgersson et al., 1995). Similarly, in the males, there was no discernible trend with respect to

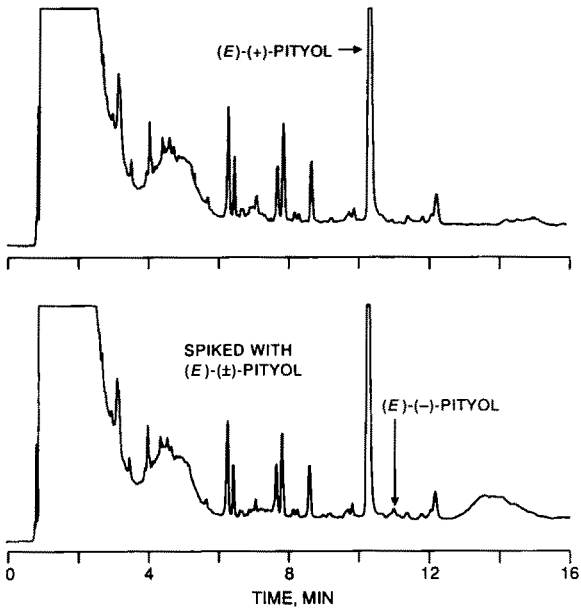

FIG. 6. Gas chromatograms of volatiles in pentane extracts of female *C. banksianae* excised from jack pine (top), and spiked with (*E*)-(±)-pityol (bottom).

species in the structural or optical isomerism of the spiroacetal. The lack of differences in these two pheromones further supports the synonymy of these two sibling species. The release of *E* isomer of the spiroacetal by virgin female *C. coniperda* (Birgersson et al. 1995), but not by female *C. banksianae* and *C. resinosae*, is consistent with other differences that separate the latter two species from *C. coniperda*. After mating, female *C. coniperda* cease production of pityol and continue production of the spiroacetal (Birgersson et al., unpublished results).

Host volatiles do not appear to enhance the attraction to pityol by *C. resinosae* and *C. banksianae*. It appears unlikely that host terpenes confer species specificity to semiochemical-based communication in *Conophthorus* for at least two reasons. First, terpene emissions from pine cones and foliage are highly variable both within and between trees, especially during the growing season (von Rudloff, 1975; Mattson and Strauss, 1986). It is doubtful that *Conophthorus* spp. can rely on such a variable message for highly specific information needed for consistent host finding (Mattson and Strauss 1986) or mate location. Second, *C. resinosae* is not monophagous, suggesting that it does not discriminate among different host "messages" or that differences are detected but are

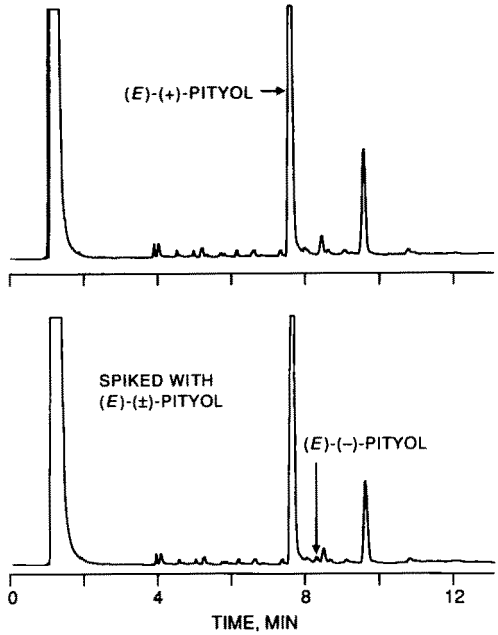

FIG. 7. Gas chromatograms of (*E*)-(+)-pityol isolated from Porapak Q-trapped volatiles from female *C. resinosae* feeding on red pine (top) and spiked with (*E*)-(±)-pityol (bottom).

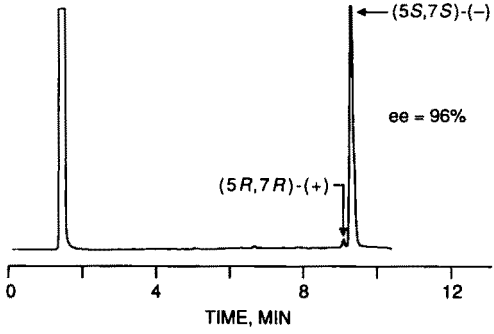

FIG. 8. Gas chromatograms of (*E*)-spiroacetal isolated from the Porapak Q-trapped volatiles from male *C. resinosae* feeding on red pine.

not meaningful. In spite of the apparent vagueness of the chemical message produced by host odors, they may, nevertheless, play a role in host finding, particularly when coupled with gustatory cues and visual stimuli, such as tree silhouettes (Henson 1967) and cone color (Jenkins 1983).

Both pityol and the spiroacetal appear to have great potential in cone beetle pest management programs. As an attractant for males, pityol can be used to detect and monitor beetle populations in seed orchards. Pityol could also be used to disrupt mating or to trap out a sufficient number of males to reduce populations below an economic threshold. As a repellent, the spiroacetal could be used to deter males from finding females and thereby to disrupt mating. A combination of the pheromones could be used in a "push–pull" tactic (Borden, 1993), whereby the spiroacetal is released as a repellent within a seed orchard and pityol-baited traps are placed on the perimeter to trap out repelled males.

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ETHYLENE EMISSION BY A DECIDUOUS TREE, *Tilia americana*, IN RESPONSE TO FEEDING BY INTRODUCED BASSWOOD THRIPS, *Thrips calcaratus*

L.K. RIESKE* and K.F. RAFFA

Department of Entomology
University of Wisconsin
Madison, Wisconsin 53706

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Abstract—Ethylene emission from excised and intact American basswood tissue was increased after seedlings were infested with the bud-feeding herbivore, *Thrips calcaratus*. Gas chromatographic analysis showed that thrips-infested tissue produced significantly more ethylene than mechanically damaged or control tissue. The possibility that thrips feeding enhances ethylene-mediated stress signals is discussed.

Key Words—Ethylene, thrips, *Thrips calcaratus*, *Tilia*, basswood, insects, induction, phytohormones, herbivory, plant-insect interactions.

INTRODUCTION

Ethylene is a volatile gaseous phytohormone that plays important regulatory roles in a number of physiological processes, including leaf senescence, leaf and bud abscission, radial growth, wood formation, leaf epinasty, seed germination, and fruit ripening (Pratt and Goeschl, 1969; Jackson and Osborne, 1970; Lipe and Morgan, 1973; Beyer et al., 1984; Little and Savidge, 1987; Abeles et al., 1992). Endogenous ethylene levels show diurnal, seasonal, and phenological periodicity (Rikin et al., 1984; Eklund, 1990, 1993; Ingemarsson et al., 1991; Abeles et al., 1992).

In addition to maintaining routine metabolic activities, ethylene can also regulate plant secondary chemistry. Both woody and herbaceous plants produce ethylene in response to a variety of biotic and abiotic stresses, including diseases

*To whom correspondence should be addressed.

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(Williamson, 1950; Hislop et al., 1973; Archer and Hislop, 1975), insects (Shain and Hillis, 1972; Wien and Roesingh, 1980; Grisham et al., 1987; Kappel et al., 1987; Martin et al., 1988; Burden et al., 1989; Kendall and Bjostad, 1990), drought (Apelbaum and Yang, 1981; but see Morgan et al., 1990; Narayana et al., 1991), flooding (Tang and Kozlowski, 1984; Yamamoto and Kozlowski, 1987), temperature extremes (Autio and Bramlage, 1986, Kondo and Takahashi, 1989), mechanical wounding (Yu and Yang, 1980), perturbations (Telewski, 1990), and synthetic chemicals (Kimmerer and Kozlowski, 1982; Corey et al., 1987).

American basswood, *Tilia americana* L. (Tiliaceae), is widely distributed across the northeastern United States and is an important component of subclimax forests in the Great Lakes region. The phytochemistry of this species has not been thoroughly studied, but related species are known to contain phytoalexic phenolic sesquiterpenes (Burden and Kemp, 1983).

The introduced basswood thrips, *Thrips calcaratus* Uzel (Thysanoptera: Thripidae), is an early-season defoliator of American basswood. Thrips outbreaks occur immediately following budbreak, with characteristic symptoms including bud or premature leaf abcisson, silvering of the leaf epidermal cells, thinned crowns, branch dieback, and reduction of radial growth (Raffa and Hall, 1989; Rieske et al., 1992).

Wien and Roesingh (1980) and Kendall and Bjostad (1990) showed that feeding by thrips can stimulate ethylene production in herbaceous plants. This response could be partially due to chemical interactions between thrips and host plants, rather than simple mechanical injury. Thrips feeding activity often involves injection of salivary secretions, which predigest plant material (Lewis, 1973). Insect salivary injections may trigger imbalances in phytohormones such as ethylene, and alter plant structure (Purtich and Petty, 1971).

Little research has been conducted on insect-induced ethylene emission by intact deciduous plants. Likewise, our understanding of host-plant chemical interactions associated with bud-feeding is particularly limited. The objectives of this study were to determine whether introduced basswood thrips induced ethylene emission by its deciduous host, *T. americana*, and to compare these emission patterns with those produced by mechanically damaged and uninjured hosts.

METHODS AND MATERIALS

Two-year old *T. americana* seedlings (McKay Nurseries, Waterloo, Wisconsin) with pruned root systems were transplanted into 25-cm-diam. plastic pots in soilless media (Fafard 3B, Carlin Sales, Milwaukee, Wisconsin). Seedlings were grown in a lightly shaded glasshouse under natural lighting and

watered as needed. When leaf expansion reached 5–25 mm, seedlings were fitted with gas collection chambers.

Basswood buds were collected in May 1994 from field sites in northern Wisconsin (Forest County) where there was a heavy infestation of *T. calcaratus*. In the laboratory, thrips were aspirated from the foliage for use in assays.

Ethylene Emission from Excised Tissue. Gas collection chambers consisting of inverted 54 ml (15 × 2.5 cm) glass tubes were fitted on to American basswood seedlings, which were then subjected to one of three treatments: thrips infestation, mechanical injury, and control. Insect-treatment seedlings were infested with 100 newly emerged, field-collected, adult *T. calcaratus*, with an additional 50 insects added 8 hr later. Mechanical damage consisted of puncturing each bud or leaf cluster two times with a group of five 000 sterile insect pins (area = 16 mm^2 per group; puncture depth = 1 mm), and an additional group of punctures to each bud or leaf cluster 8 hr later. Control tissue was untreated. There were five plants each of thrips-infested, mechanically damaged, and control plants, for a total of 15 plants.

Since the basswood buds were not fully opened, and *T. calcaratus* feeds within the opening bud, the actual area of thrips-damaged tissue was impossible to measure. However, the mechanical injury resulted in 48 mm^3 (16 mm^2 per group × 3 punctures × 1 mm deep) of damage per seedling. Modeling clay (VanAken International, El Monte, California) was used to seal the area around the stem and keep the gas collection chamber from contacting soil.

Seedlings were clipped at the base 24 hr following the initial damage, the clay was removed, and each collection chamber was fitted with a gas-tight septum. Headspace was analyzed for ethylene emission immediately and at each successive 8-hr interval, for a total of 96 hr.

Techniques for gas chromatographic analysis of head space were adapted from those of Shain and Hillis (1972). A 2-ml gas sample was withdrawn from each collection chanber using a 5-ml Hamilton Gastight syringe. Headspace samples were injected into a Shimadzu GC-9A gas chromatograph with a flame ionization detector fitted with a capillary Alumina column (30 m × 0.53 mm, J & W Scientific, Folsom, California). The gas chromatograph was operated isothermally at 35°C with an injection port temperature of 100°C; compressed air was used as the carrier gas at a flow rate of 30 ml/min. Samples were integrated with a Shimadzu Chromatopak C-R3A. External standards of 9.900 and 1.060 ppm were used for quantitation. Preliminary experiments demonstrated a highly reliable standard curve for ethylene analysis (peak area = 2400.91 * (ppm ethylene) + 45.000; $R^2 = 0.997$).

Following each sampling, the septum was removed and the collection chamber was flushed for 10–15 sec using a steady stream of ethylene-free air. Assays were maintained at 23°C under constant light for the duration of the experiment.

Buds, leaves, and petioles were dried (96 hr at 65°C) and weighed 120 hr following the initial damage. Ethylene emission was analyzed on a dry weight basis. Nondispersed thrips were counted in an attempt to correlate dispersal with feeding activity, so that the number of dispersed thrips present might be related to ethylene emission.

Ethylene Emission from Intact Plants. Glass globes (1160 ml, 16.5 × 8 cm) fitted with gas-tight septa were placed over American basswood seedlings and sealed with modeling clay as described above. Seedlings were infested with 400 adult *T. calcaratus* initially, followed by 200 additional thrips 8 hr later. Mechanical injury was applied as described above, with each bud or leaf cluster receiving two sets of five punctures, followed by one set of five punctures 8 hr later. There were six plants each of thrips-damaged, mechanically damaged, and control plants, for a total of 18 plants. Assays were maintained at 23°C under constant light for the duration of the experiment.

As described above, headspace samples were analyzed for ethylene emission 24 hr following the initial damage. Samples were taken at 8 hr intervals over 96 hr, with the exception of hour 80. Buds, leaves, and petioles were subsequently dried (96 hr at 65°C) and weighed, and ethylene emission was expressed on a dry-weight basis. Nondispersed thrips were counted in an attempt to correlate the number of dispersed thrips with ethylene emission.

Statistical Analyses. A repeated-measures analysis of variance using SuperANOVA (Abacus Concepts, 1989) was used for data analysis; Fisher's protected LSD was used for mean separations where appropriate.

RESULTS

The retention time for ethylene under the chromatographic conditions used was approximately 2.2 min. Figure 1 shows a typical chromatogram from an uninfested (Figure 1a) versus thrips-infested plant (Figure 1b).

Ethylene Emission from Excised Plants. Adult *T. calcaratus* remained very active on thrips-damaged excised tissue for up to 96 hr following infestation; some remained alive for the duration of the experiment. The first visible signs of thrips feeding were observed 24 hr after infestation.

Mean ethylene production from thrips-damaged tissue was 2.18 times higher than from mechanically damaged tissue, and 2.98 times higher than from control tissue (Figure 2). Ethylene emission from excised, mechanically injured leaves did not differ from controls (Figure 2).

The temporal pattern of ethylene emission from excised *T. americana* tissue over a 120-hr period is illustrated in Figure 3. Ethylene was detectable in all treatments 24 hr after initiation. All three treatments showed similar ethylene emission 8 hr following the initiation of measurements (32 hr following dam-

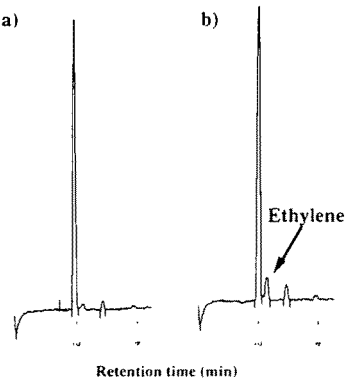

FIG. 1. Gas chromatograms comparing control (a) versus thrips-infested (b) seedlings.

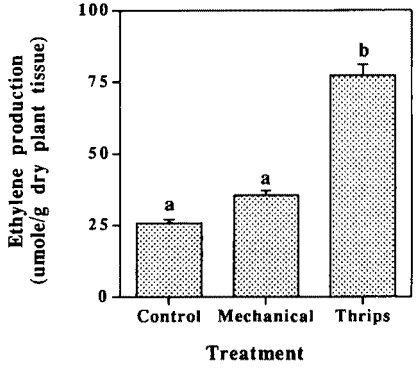

FIG. 2. Mean (± SE) ethylene production by control, mechanically damaged, and thrips-infested excised American basswood tissue. Means followed by the same letter are not significantly different, Fisher's protected LSDD: $F = 2.897$; $df = 2,12$; $P = 0.09$; $N = 5$.

age). However, ethylene production from thrips-infested tissue was greater than control tissue at all subsequent intervals (Figure 3). This increase became statistically significant ($P < 0.1$) at 72 hr, and continued to be significant for the duration of the experiment. Ethylene production from thrips-damaged tissue was also greater than mechanically damaged tissue 40 hr following damage. This difference was statistically significant ($P < 0.1$) at 72, 104, 112, and 120 hr. Although mechanically damaged tissue consistently emitted more ethylene than control tissue, there was no significant difference between mechanically damaged and control tissue at any time interval (Figure 3).

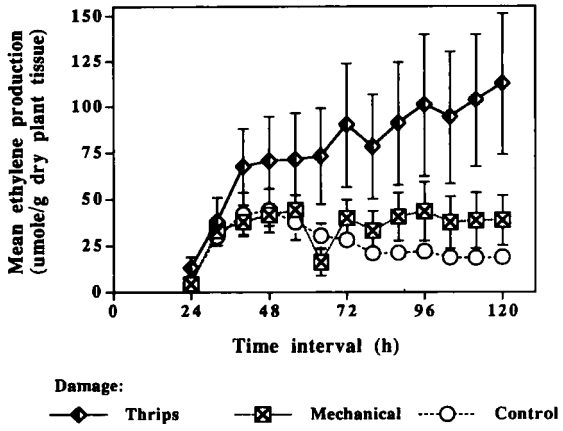

FIG. 3. Mean (± SE) temporal ethylene emission over 120 hr by excised American basswood tissue ($N = 5$). Thrips-infested tissue: $y = 0.801x + 19.587$, $R^2 = 0.833$, $P = 0.0001$; mechanically damaged tissue: $y = 0.170x + 22.272$, $R^2 = 0.211$, $P = 0.1143$; control tissue: $y = -0.122x + 34.652$, $R^2 = 0.124$, $P = 0.2390$.

There was no relationship between the number of thrips present and cumulative ethylene emission from thrips-infested excised plants ($R^2 = 0.021$, $P = 0.8155$).

Ethylene Emission from Intact Plants. Thrips remained active on intact seedlings for the duration of the experiment. The first signs of thrips damage were visible at 24 hr, with symptoms becoming severe by 40 hr.

Figure 4 shows mean ethylene emission from *T. americana* seedlings. Ethylene production from thrips-damaged tissue was 21.60 times higher than from mechanically damaged tissue, and 122.62 times higher than from control tissue (Figure 4). There was no significant difference in ethylene production between mechanically damaged and control plants.

Temporal patterns of mean ethylene production for all treatments are shown in Figure 5. Mean values were higher from thrips-infested than mechanically injured or control seedlings, but ethylene emission varied substantially among plants. Temporal differences between thrips-damaged and control plants appeared statistically significant ($P < 0.1$) beginning at 88 hr and continued to be different for the duration of the experiment (Figure 5). Temporal differences between thrips-damaged and mechanically damaged plants was statistically significant ($P < 0.1$) at 96 hr and remained different throughout. As with the excised tissue, there was no statistical difference between mechanically damaged and control tissue at any time interval (Figure 5).

Also consistent with the excised tissue, there was no relationship between

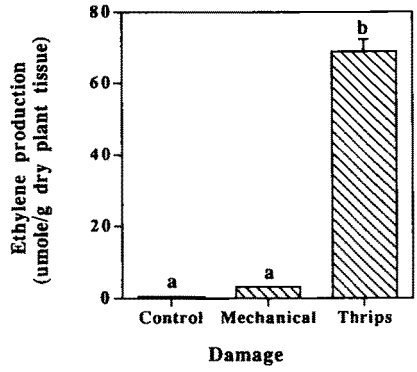

FIG. 4. Mean (± SE) ethylene production by control, mechanically damaged, and thrips-infested American basswood seedlings. Means followed by the same letter are not significantly different, Fisher's protected LSD: $F = 2.761$; $df = 2,15$; $p < 0.1$; $N = 6$.

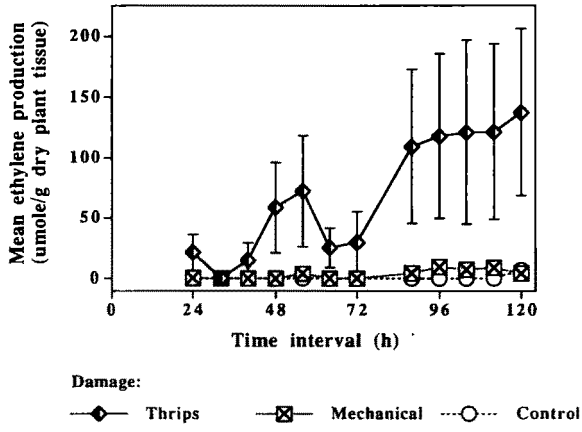

FIG. 5. Mean (± SE) temporal ethylene emission over 120 hr by intact American basswood seedlings ($N = 6$). Thrips-infested tissue: $y = 1.397x - 30.744$, $R^2 = 0.816$, $P = 0.0001$; mechanically damaged tissue: $y = 0.088x - 3.122$, $R^2 = 0.611$, $P = 0.0027$; control tissue: $y = 0.028x - 1.465$, $R^2 = 0.223$, $P = 0.1210$.

the number of thrips present and cumulative ethylene produced ($R^2 = 0.001$, $P = 0.4631$).

Intact infested plants showed a higher rate of increase in ethylene production than did excised, thrips-infested tissue. Moreover, intact plants showed temporal oscillations, whereas excised tissue showed a relatively steady rate of increase (Figures 3 and 5).

DISCUSSION

This is the first report of thrips-induced ethylene production by an intact deciduous perennial host. Feeding by *T. calcaratus* induced ethylene production in both excised and intact basswood plants relative to controls and at levels greater than that of mechanical damage alone. The patterns of ethylene emission among thrips-infested, mechanically damaged and control treatments were comparable between the two experiments (Figures 2–5). The high variability in ethylene emission is consistent with that found by other researchers using both excised tissue (Grisham et al., 1987; Martin et al., 1988) and intact plants (Kendall and Bjostad, 1990).

The duration of increased ethylene emission differed between insect-infested and mechanically injured tissue. Ethylene emission continued to increase in thrips-infested treatments in both experiments up to 120 hr following the initiation of damage (Figures 3 and 5). This contrasts with plant responses to mechanical wounding, which results in a peak rate of ethylene emission within 6 hr (Abeles et al., 1992). Whereas thrips feeding caused continuous injury over a 120-hr period with subsequent increases in ethylene emission, the mechanical injury was not continuous. Therefore, it is not surprising that thrips-induced ethylene emission was far greater than mechanically induced ethylene emission. In working with excised cotton infested with the cotton fleahopper, *Pseudatomoscelis seriatus* (Reuter), Grisham et al. (1987) found ethylene production peaked at 72 hr.

The lack of a correlation between the number of feeding thrips and ethylene emission from both excised and intact tissue was unexpected and suggests that adult thrips may be spending a considerable amount of time in nonfeeding activities. Since *T. calcaratus* is parthenogenetic (Raffa et al., 1992), the primary nonfeeding activity would not be mating, but would most likely be location of suitable oviposition sites.

The pattern of ethylene production by excised tissue shows none of the oscillations seen with intact tissue, suggesting that excision enhanced ethylene production. It is surprising that the overall level of ethylene measured from thrips-infested treatments was greater in the intact tissue, considering the greater than 20-fold increase in chamber volume and considering that wound ethylene caused by excising tissue is itself self-inducing (Abeles et al., 1992). However, the fourfold increase in the number of thrips released seems to have compensated for the increase in chamber volume. In contrast, ethylene emission from mechanically damaged and control treatments were considerably lower in intact than excised plants (Figures 2 and 4).

The low rate of ethylene emission in the mechanically damaged treatments is not inconsistent with that found in other studies (Kendall and Bjostad, 1990). Since ethylene production requires living cells (Abeles et al., 1992), ethylene

emission decreases as leaf necrosis proceeds. Kimmerer and Kozlowski (1982) showed ethylene emission by paper birch decreased when necrosis reached 80%. Although thrips feeding damage was not measured in this study, signs of necrosis were visible in both thrips-damaged and mechanically damaged treatments. The mechanical damage applied is equivalent to or exceeds the estimated 4.93 mm^3 of damage caused by daily feeding of *Thrips tabaci* Lindeman (Quartey, 1982). This suggests that the mechanical damage caused by thrips feeding is not solely responsible for the levels of ethylene emitted. In addition to mechanical wounding, insects can induce ethylene production by the introduction of microorganisms, the injection of hydrolytic salivary secretions, the release of elicitors, and inducing phytohormone imbalances (Abeles et al., 1992). The importance of insect-associated microbes in ethylene production has been shown in other systems (Shain and Hillis, 1972; Grisham et al., 1987). Martin et al. (1988) proposed that the role of the cotton fleahopper in ethylene-induced cotton bud abscission may be due, first, to physical wounding and salivary secretions, and secondly, to providing infection courts for opportunistic microorganisms. The pear thrips, *Taeniothrips inconsequens* (Uzel), which resembles *T. calcaratus* in life history and habits, has been associated with the opportunistic pathogenic fungus, *Discula campestris* (Pass.) von Arx, on sugar maple (Stanosz, 1993). Burden et al. (1989) found that the phytohormone indoleacetic acid and the ethylene precursor ACC (1-aminocycloproprane-1-carboxylic acid) were present in the cotton fleahopper; ethylene emission was enhanced not only by mechanical damage caused by feeding, but also by induced phytohormone changes.

Given the complexity of the roles of ethylene in primary and secondary plant metabolism, it seems reasonable that the mechanisms by which insect feeding increases ethylene evolution would be equally complex. More than likely, insect-induced increases in ethylene are due to a combination of these factors.

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DETERMINING ENANTIOMERIC COMPOSITION OF DISPARLURE

JAMES E. OLIVER[1,*] and ROLLAND M. WATERS[2]

[1]*Insect Chemical Ecology Laboratory, USDA, ARS, B-007*

[2]*Environmental Chemistry Laboratory, B-012*

[1,2]*BARC-West*
Beltsville, Maryland 20705-2350

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Abstract—A method has been developed to convert disparlure (2-methyl-7,8-epoxyoctadecane) to the corresponding *N*-(α-methylbenzyl)aziridine with inversion of configuration at both the 7 and 8 positions. The diastereomeric aziridines can be separated on an efficient gas chromatography column, permitting determination of the enantiomeric constitution of the starting disparlure. As little as ca. 0.1% of the minor enantiomer can be detected.

Key Words—Disparlure, gypsy moth, enantiomer, diastereomer.

INTRODUCTION

Trapping of male gypsy moths (*Lymantria dispar*) with traps baited with synthetic (+)-disparlure [(7*R*, 8*S*)-2-methyl-7,8-epoxyoctadecane] has been used for monitoring populations for a number of years. During this time, differences in effectiveness have been noted among batches of the synthetic pheromone that could not be reconciled with differences in chemical purities as assessed by capillary gas chromatography. It has been reported on several occasions (Plimmer et al., 1977, Cardé et al., 1977, Miller et al., 1977, Odell et al., 1992) that the presence of the (−) enantiomer, even in small amounts, can reduce pheromone effectiveness. Since that time, a number of syntheses of disparlure enantiomers have been reported (Mori, 1992) and, because of its annual use in both control and experimental programs, it has become a commercial item. The optical rotation of pure disparlure is less than 1°, so measurement of rotation

*To whom correspondence should be addressed.

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is of little value for detecting small amounts of the opposite enantiomer. Since there have been no alternative means of assaying samples for the (−) enantiomer, quality control of purchased samples has been impossible except for trapping tests conducted during the short gypsy moth flying season. Accordingly, an analytical method for determining the enantiomeric composition of disparlure is needed.

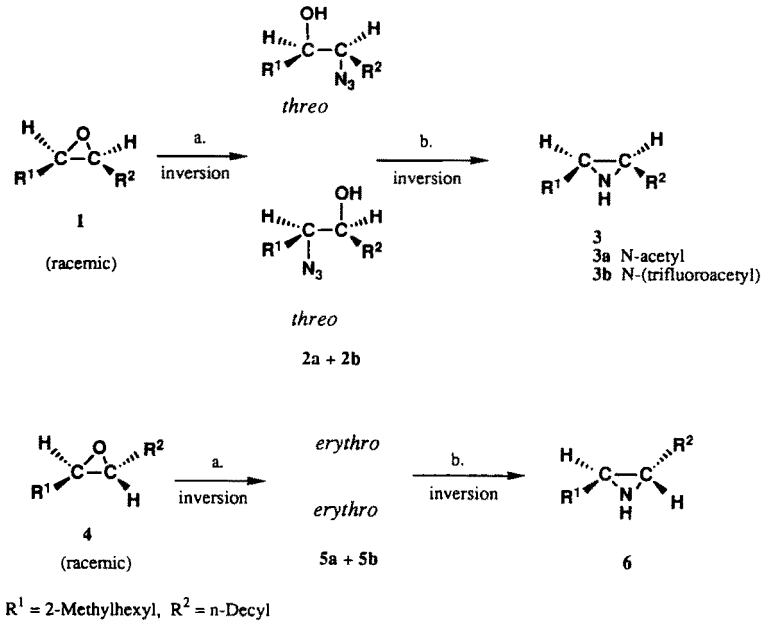
Although considerable progress has been made in HPLC and GLC separation of enantiomers on chiral stationary phases, attempts to apply these methods to disparlure have been discouraging. Undoubtedly, the major problem lies in the similarity of the two alkyl groups on the epoxide ring—each is a generally undistinguished aliphatic alkyl group whose properties in terms of polarity and conformation are very similar. Thus, there is little basis for preferential interaction with a chiral stationary phase. We describe here a stereospecific conversion of disparlure to diastereomeric aziridines and the configurational determination of the disparlure via gas chromatographic analysis of these products.

METHODS AND MATERIALS

Gas chromatography analyses were conducted in a Shimadzu model 14A instrument equipped with a flame ionization detector and a 60-m SPB 608 custom column obtained from Supelco Co. Neutral alumina (Woelm) was oven-dried for about four days at 135°C until constant weight was achieved. ^{1}H NMR spectra were obtained with a General Electric QE-300 NMR spectrometer on deuteriochloroform or deuteriobenzene solutions (significantly better resolution was realized with the α-methylbenzylaziridine spectra recorded with the latter solvent). Mass spectra were recorded on a Finnigan model 4510 GC-MS or a Finnigan Incos 50 GC-MS. Electron ionization spectra were collected at 70 eV and a source block temperature of 150°C. Mention of a proprietary product or company does not imply endorsement by the US Department of Agriculture.

threo-Azidoalcohols **2a** + **2b** *from Disparlure (Scheme 1).* A mixture of racemic disparlure (5.72 g), sodium azide (6.76 g), ammonium chloride (2.14 g), dimethylformamide (100 ml), and water (10 ml) was stirred and heated (bath temperature 122°C) for 4 hr. After cooling, the product was partitioned between water and ether–hexane (1 : 1 v/v), the organic phase was washed well with water, dried, concentrated, and flash chromatographed (5% ethyl acetate in hexanes) to provide 4.72 g of a mixture of **2a** and **2b** as a colorless oil.

Conversion of threo-Azidoalcohols **2a** + **2b** *to Racemic* cis *Aziridine* **3** *(Scheme 1).* A solution of **2a** + **2b** (1.00 g), and triphenylphosphine (0.90 g) in acetonitrile (18 ml) was heated at reflux for 2 hr, then was cooled and concentrated. The residue was extracted with pentane, the extract filtered and

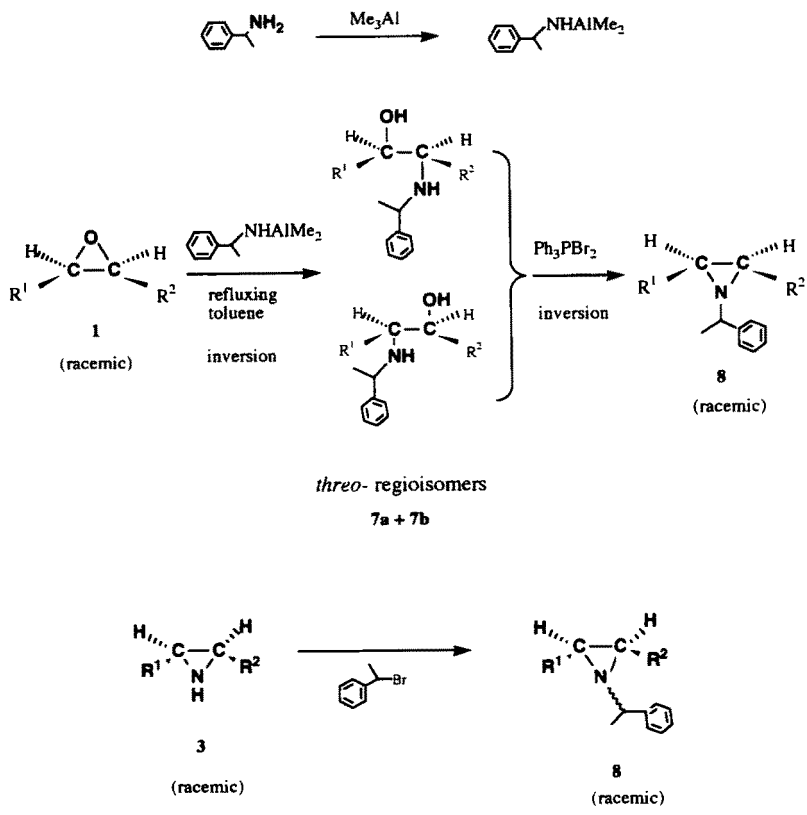


SCHEME 1.

concentrated, and the residue was again extracted with pentane. Flash chromatography (25% ethyl acetate in cyclohexane) gave 590 mg of **3** as a waxy solid. ^{1}H NMR ($CDCl_3$): 0.87 (d, J = 6.6, isopropyl methyl), 0.88 (m, terminal methyl), 1.32 (methylene), 1.52 (m, H-2), 1.98 (m, H-7 and H-8). Small portions of **3** were converted to the acetate **3a** and the trifluoroacetate **3b** with acetic anhydride and trifluoroacetic anhydride, respectively. ^{1}H NMR of **3a** ($CDCl_3$): 0.87 (d, J = 6.6, isopropyl methyl), 0.885 (m, terminal methyl), 1.56 (m, H-2), 2.41 (m, H-7 and H-8), 2.11 (s, acetate methyl). ^{1}H NMR of **3b** ($CDCl_3$): 0.877 (d, J = 6.6, isopropyl methyl), 0.882 (m, terminal methyl), 1.53 (m, H-2), 2.73 (m, H-7 and H-8).

erythro-Azidoalcohols **5a** *and* **5b** *and trans-Aziridine* **6** *(Scheme 1).* The above procedures were repeated starting with racemic *trans*-disparlure **4.** The *trans*-aziridine **6** was surprisingly polar and was eluted from a silica gel column with 75% ethyl acetate in hexanes to give **6** as a colorless oil.

*threo-(α-Methylbenzyl)amino Alcohols (***7a** *and* **7b***) from Disparlure (Scheme 2).* A solution of (−)-α-methylbenzylamine (1.05 g, 85 mmol) in



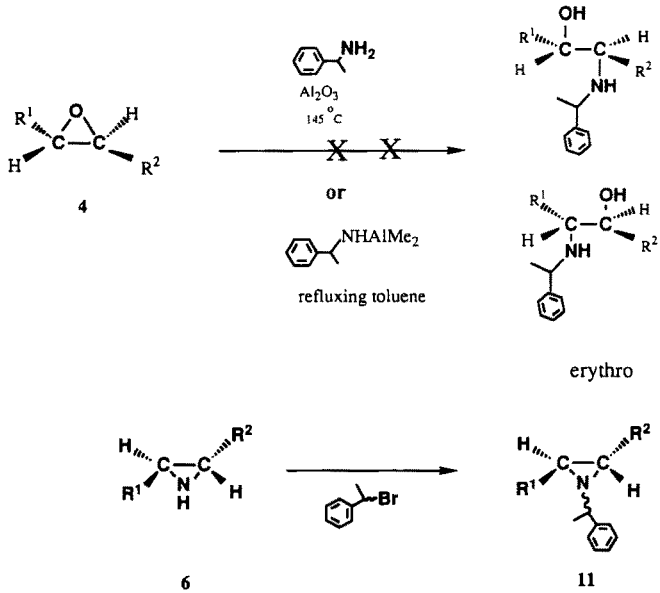
SCHEME 2.

toluene (10 ml) was added dropwise to a cold (ice bath), stirred solution of trimethylaluminum in toluene (5 ml, 2 M, 100 mmol). After the initial reaction had subsided, a solution of racemic disparlure (2.4 g, 85 mmol) in toluene (50 ml) was added and the solution was heated at reflux for 4 hr. After cooling, 6 N NaOH (8 ml) was added, the mixture was extracted with three portions of hexane, and the combined extracts were rinsed well with water, dried, concentrated, and flash chromatographed (20% then 30% ethyl acetate in cyclohexane) to give ca. 2.9 g of a mixture of **7a** and **7b.**

Conversion of threo-(α-Methylbenzyl)amino Alcohols **7a** *and* **7b** *to N-(α-Methylbenzyl)-cis-aziridines* **8** *(Scheme 2).* A solution of **7a** + **7b** (44 mg) in dichloromethane (125 μl) containing triethylamine (50 μl) was treated with meth-

anesulfonyl chloride (10 μl). After stirring overnight, the product was partitioned between aqueous sodium carbonate and pentane, then was flash chromatographed (2%, then 5% ethyl acetate in cyclohexane) to give 32 mg of racemic **8.** The same product was obtained upon treatment of **7a** and **7b** with triphenylphosphine dibromide. Mass spectrum of **8,** *m/e* (relative intensity, %): 385 (M^+, 1), 384, (2), 370 (1), 280 (100), 244 (12), 105 (57), 82 (12), 69 (34), 55 (35). Mass spectrum, chemical ionization, ammonia: *m/z* 386 ($M + H^+$, 100%). ^1H NMR (C_6D_6): **8** from racemic disparlure and (*S*)-(−)-α-methylbenzylamine: (C_{66}) 0.835 (d, $J = 6.6$), isopropyl methyl, 0.92 (m, terminal methyl), 1.30 (methylene), 1.41 (m, H-2), 1.43 (d, $J = 6.6$, α-methyl), 1.52 (m, H-7 and H-8), 2.30 (q, $J = 6.6$), 7.3 (m, phenyl), **8** from (+)-disparlure and (*S*)-(−)-α-methylbenzylamine (C_{66}) 0.84 (d, $J = 6.6$, isopropyl methyl), 0.92 (t, $J = 6.0$, terminal methyl), 1.30 (methylene), 1.38 (m, H-2), 1.43 (d, $J = 6.6$, α-methyl), 1.53 (m, H-7 and H-8), 2.31 (q, $J = 6.6$, α-H), 7.3 (m, phenyl).

N-α-Methylbenzyl-trans-aziridines **11a** *and* **11b** *(Scheme 3).* A solution of the *trans*-aziridine **6** (32 mg), diisopropylethylamine (25 μl), and 1-bromophenylethane (20 μl) in acetonitrile (300 μl) was heated at reflux for 2 hr, then was cooled and partitioned between 5% ethyl acetate in pentane and aqueous sodium carbonate. Flash chromatography of the product afforded a surprising degree of separation of pairs of diastereomers with one pair eluting with 5% ethyl acetate

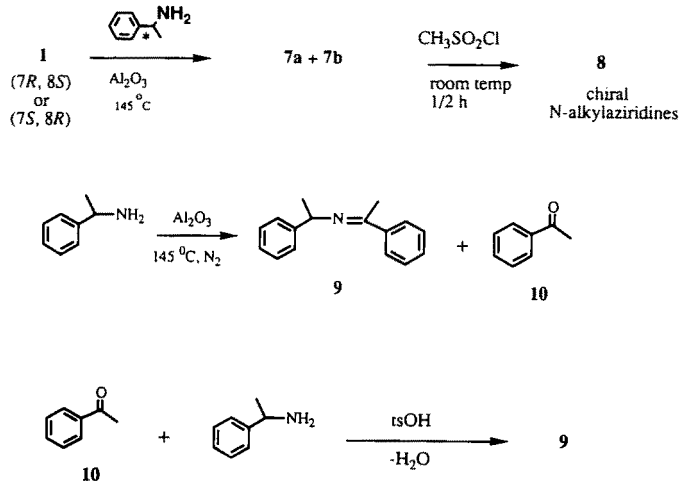


4 6 11

SCHEME 3.

in hexanes and the other with 10% ethyl acetate in hexanes. Gas chromatography of the crude mixture or of a reconstituted mixture showed both pairs of isomers to be later-eluting than the corresponding *cis*-*N*-(α-methylbenzyl)aziridines **8** from *cis*-disparlure. Mass spectrum of **11a** (earlier-eluting isomer from both silica gel and GC): *m/z* 385 (2), 384 (2), 286 (6), 281 (21), 280 (100), 105 (58), 69 (25), 55 (26); ^{1}H NMR (C_6D_6): 0.85 (d, J = 6.6, 3 H, isopropyl methyl), 0.90 (m, terminal methyl), 0.92 (d, J = 6.6, 3 H, isopropyl methyl), 1.29 (methylene), 1.46 (d, J = 6.6, α-methyl), 1.57 (m, H-7 and H-8), 3.10 (q, J = 6.6, α-H), 7.28 (m, phenyl). Mass spectrum of **11b:** 281 (22), 280 (100), 207 (6), 105 (50), 69 (26), 55 (23). ^{1}H NMR (C_6D_6): 0.84 (d, J = 6.6, 3H, isopropyl methyl), 0.92 (d, J = 6.6, 3H, isopropyl methyl), 0.925 (m, terminal methyl), 1.31 (methylene), 1.49 (d, J = 6.6, α-methyl, 3.16 (q, J = 6.6, α-H), 7.37 (m, phenyl).

Isolation and Identification of Imine **9** *(Scheme 4).* (*S*)-(−)-α-Methylbenzylamine (300 μl), anhydrous neutral alumina (3 g), and racemic disparlure (10 mg) were combined in a pressure tube and the tube was heated to 150°C for 16 hr. The tube was cooled and opened, and the contents were extracted with 5% ethyl acetate in pentane to give 330 mg of a pale yellow oil that was recombined with 1.1 g of alumina in an ampoule that was then sealed under nitrogen and heated an additional 2.5 hr at 150°C. The alumina was eluted with 10% ethyl acetate in cyclohexane, and the eluate was flash chromatographed on silica gel with the same solvent to give ca. 20 mg of **9** as a pale yellow oil,



SCHEME 4.

mass spectrum *m/z* 224 (34), 223 (70), 222 (44), 208 (42), 167 (21), 165 (18), 106 (44), 105 (100), 104 (72), 103 (60), 102 (17), 90 (12), 89 (21), 83 (20) 79 (59), 78 (52), 77 (60), 76 (20), 63 (15), 52 (15), 51 (48), 50 (19). ^{1}H NMR ($CDCl_3$) [These chemical shifts vary somewhat from those previously reported (Arjona et al., 1984)]: 1.48 (q, J = 6.6, $\underline{H}CMe$), 1.54 (d, J = 6.6, $C\underline{H}_3CH$), 2.27 (s, $C\underline{H}_3C{=}N$), 7.84 (m, phenyl). The same product could be prepared by refluxing equimolar portions of α-methylbenzylamine and acetophenone in toluene containing toluenesulfonic acid with azeotropic removal of water.

Analytical Procedure for Enantiomeric Evaluation of Disparlure Samples. About 30 mg of alumina (neutral, oven-dried to constant weight) was added to each of two 2-ml ampoules. Then to each is added a solution of disparlure in a volatile solvent (eg. hexane) such that ca. 100 μg disparlure is added. To one ampoule is added 3 μl of pure (−)-α-methylbenzylamine, and to the other is added 3 μl pure (+)-α-methylbenzylamine. A Firestone valve or gentle stream of nitrogen and gentle heat is used to replace simultaneously the air with nitrogen and to evaporate the solvent. The ampoules are sealed and heated at 145–150°C for 2.5 hr. The ampoules are cooled, opened, and to each is added 50 μl of a 10% solution of methanesulfonyl chloride in benzene. After allowing them to stand at room temperature 45–60 min, then 25–50 μl 2N sodium carbonate is added, and they are allowed to stand for 15 or more min. Then 1 ml of 5% ethyl acetate in pentane (in several successive portions) is used to transfer soluble products to (and through) a small column containing ca. 150–160 mg of silica gel. The eluate is concentrated with a stream of nitrogen just to dryness, and an appropriate solvent for gas chromatography is added (e.g., 25–50 μl cyclohexane). The results in Table 1 were obtained using this method with the GC injector operated in the split mode. Satisfactory results have been obtained on less than one tenth this scale using splitless GC injections.

RESULTS AND DISCUSSION

Conversion of enantiomers to diasteromers by the introduction of a second, defined chiral center is a common strategy for assessing the stereochemistry of an analyte (Skidmore, 1993). Diastereomers have different physical properties and are often separable on archiral GLC or HPLC columns. [For example, *cis*-disparlure (the 7*R*, 8*S* and 7*S*, 8*R* pair) is easily separated by GLC or even column chromatography from *trans*-disparlure (7*R*, 8*R* and 7*S*, 8*S*).] Alcohols, amines, acids, etc., with one asymmetric center are easily converted to diastereomeric esters, amides, carbamates, etc., with reagents that introduce a second chiral element. Disparlure, with two chemically similar alkyl groups and the epoxide as its only functionality, presents some limitations: although there are a number of stereospecific epoxide opening reactions, none of these can be

expected to be regiospecific in the disparlure case. Nonregioselective ring opening would lead to multiple products, complicating any subsequent separations and interpretations. Prestwich et al. (1989) described enantioselective enzymatic hydrolysis of disparlure and separation of the resulting diols on a chiral GLC column. In contrast, chemical hydrolysis, although presumably stereospecific, would result in virtual racemization because of nonselective attack of water at either of the two epoxide carbons.

An ideal derivative would be a single product that introduced the required additional chiral center while preserving the stereochemical information of each of the original chiral centers. The original configurations of these two carbons need not necessarily be retained, however; so processes that involved retention of configuration at each center, or inversion at each center, ought to serve equally well.

Conversion of epoxides to unsubstituted aziridines with two inversions of configuration are known, and a procedure based on that of Ittah et al. (1978) was applied to disparlure (Scheme 1). Reaction of disparlure (**1**) with sodium azide plus ammonium chloride in dimethylformamide at >90°C gave a pair of *threo*-azido alcohols **2a** + **2b,** which in turn were reacted with triphenylphosphine to provide the *cis*-aziridine **3** (inversion at each carbon). The *N*-acetyl (**3a**) and *N*-(trifluoroacetyl) (**3b**) derivatives were prepared, but neither pair of enantiomers could be separated on a Cyclodex B gas chromatography column. The unsubstituted aziridine could also be derivatized with Mosher's acid or with 1-(1-naphthyl)ethyl isocyanate; these diastereomeric derivatives ought to be amenable to analyses by HPLC, but the approach was not pursued because we simultaneously developed the procedure for gas chromatographic analysis of *N*-(α-methylbenzyl)aziridines described below.

Martinelli et al. (1990) described the reaction of a 1,2-disubstituted epoxide with α-methylbenzylamine and subsequent conversion of the resulting amino alcohol to the *N*-alkylaziridine upon treatment with triphenylphosphine dibromide or with diethyl azodicarboxylate. Disparlure, in the absence of acids, is remarkably resistant to nucleophilic ring opening and was recovered unchanged after attempting to react it with (+)-α-methylbenzylamine under the conditions (refluxing 1-butanol) described by Martinelli et al., as well as from a variety of more vigorous conditions. Slow ring opening occurred with α-methylbenzylamine and lithium perchlorate in acetonitrile [at 100°C in a sealed tube, less than 50% conversion was realized in 6 hr, in contrast to the room-temperature openings described by Chini et al. (1990)]. We eventually achieved the desired conversion by reacting the amine first with trimethylaluminum (Overman and Flippin, 1981) and then reacting the resulting reagent with disparlure in refluxing toluene (in contrast to the considerably milder conditions found satisfactory for the epoxides studied by the original authors). The resulting mixture (Scheme 2) of positionally isomeric *threo*-amino alcohols **7a** + **7b** could be converted to

the desired *N*-alkylaziridines **8** with either triphenylphosphine dibromide or with methanesulfonyl chloride in the presence of triethylamine. (The same products resulted from alkylation of the unsubstituted *cis*-aziridine **3** with 1-bromo-1-phenylethane).

The racemic *N*-α-methylbenzylaziridine thus prepared was examined on several GLC columns. Varying degrees of separation were observed, and a 60-m proprietary column was found that achieved essentially baseline separation in slightly over 35 min.

Seeking a more convenient alternative to the organometallic reagent, we found that disparlure could also be opened by α-methylbenzylamine on anhydrous neutral alumina (Posner and Rogers, 1977) (Scheme 4). Again the reaction was sluggish (excess amine, 2.5–3 hr at ca. 145°C in the absence of solvent in contrast to 1 hr at room temperature in ether for some examples cited in the original work). Furthermore, some amine oxidation invariably occurred, whether or not an inert atmosphere was used. The odor of acetophenone was always evident, and small amounts of acetophenone **10** could be detected by GLC as could larger amounts of the imine (**9**, Scheme 3) that would formally result from its condensation with α-methylbenzylamine (Arjona et al., 1984). However, these by-products did not interfere with the analyses, and furthermore, ring closure to the desired aziridine could be effected without isolation by simply treating the alumina-adsorbed products with methanesulfonyl chloride. Yields were variable, although not in a recognizably systematic way; from 100 to 200-μg reactions, after passing products through small silical gel columns, they tended to range from 10 to 25%.

As stated, stereospecific conversion of a *cis*-epoxide to a *cis*-aziridine requires an even number of (or zero) inversions of configuration. If one of the two reactions was imperfectly stereoselective, the result would not necessarily be analytically fatal, since the product from an odd number of inversions would be a *trans*-aziridine, which should have a different GLC retention time from either of the *cis*-diastereomers. To determine whether the corresponding *trans*-*N*-(α-methylbenzyl)aziridines would be distinguishable from the *cis*-isomers, we attempted to carry out the epoxide–aziridine conversion starting with *trans*-disparlure (**4**).

Whereas ring opening reactions of *cis*-disparlure with the amine had been difficult, useful ring openings of *trans*-disparlure with α-methylbenzylamine eluded us altogether. Neither the aminodialkylaluminum approach nor the alumina-catalyzed reaction was successful, and the *trans* epoxide was recovered unchanged from conditions that had satisfactorily opened the *cis* isomer as well as from increasingly vigorous conditions, to the point where extensive and undefined decomposition resulted. The difficulty was eventually circumvented, however, by opening *trans*-disparlure with azide as described above for *cis*-disparlure (Scheme 1). In contrast to the unsuccessful attempts with α-methylbenzylamine,

the reaction of *trans*-disparlure with NaN_3/NH_4Cl occurred much as it had for *cis*-disparlure, and the resulting *erythro* pair (positional isomers) of hydroxy azides **5a** and **5b** were converted to the *N*-unsubstituted *trans*-aziridine (**6**) with triphenylphosphine (Scheme 1). Aziridine **6** could be alkylated with 1-bromo-1-phenylethane to give the *trans*-*N*-alkylaziridines **11** (Scheme 4), giving a mixture of diastereomers. Both had GLC retention times longer than those of the corresponding *cis*-aziridines, so that their occurrence in a mixture would not interfere with the analyses. This proved to be a somewhat moot point inasmuch as they were essentially unobservable, their absence from reaction products from *cis*-disparlure testifying to the stereospecificity of each of the two steps in the epoxide–aziridine conversion.

Reliable stereospecificity was also indicated by the fact that one or more samples of each the (+)- and (−)-disparlure samples on hand consistently tested negative for detectable amounts of the opposite enantiomer, whereas many samples reproducibly tested positive for varying amounts of the unwanted enantiomer.

Enantiomeric purity of the starting α-methylbenzylamine was a necessity since any of the wrong enantiomer in the starting amine would give an aziridine product from (+)-disparlure indistinguishable from that formed from the correct amine and (−)-disparlure, i.e., a false positive. Accordingly, each amine (>99% as purchased) was rigorously purified by converting to the appropriate tartrate salt [(+)-amine and (−)-tartaric acid, (−)-amine and (+)-tartaric acid], recrystallizing at least two times from methanol, and regenerating and distilling the amine (Ault, 1973). None of the opposite enantiomer could be detected by conversion to amides with Mosher's acid and examination by gas chromatography.

All samples tested were reacted with both the (+)- and (−)-amines; thus in every case the product from the (−)-disparlure was observed as both the earlier- and later-eluting diastereomer. Consistent results between the two measurements provided confidence against random peaks inadvertently producing false positives, and, importantly, argue against the possibility of conclusions biased by diastereoselectivity. Inconsistent results from the two amines (in reality, very rarely experienced) would be a clear indication that the analyses required repetition. Although near-baseline separation had been achieved on dilute samples of racemic α-methylbenzylaziridines, somewhat larger samples were required when the minor enantiomer constituted only a fraction of a percent of the major enantiomer, and in these cases the minor GLC peak appeared as an incompletely resolved shoulder. It can be seen from Table 1 that in a majority of cases, the estimated value for (−)-disparlure obtained from the use of the (*R*)-(+)-α-methylbenzylamine (minor GLC peak later eluting) was slightly higher than that from the (*S*)-(−)-enantiomer (minor peak earlier eluting). This

TABLE 1. ENANTIOMERIC COMPOSITION OF SYNTHETIC DISPARLURE SAMPLES[a]

Sample	Designation	% (+)-Disparlure [(+)-amine][b]	% (+)-Disparlure [(−)-amine][c]
01	Racemic	50.1	49.9
02	(+)-Disparlure	55.0	55.1
03	(+)-Disparlure	53.9	54.1
04	(+)-Disparlure	51.4	51.5
05	(+)-Disparlure	45.7	43.8
06	(+)-Disparlure	82.3	82.8
		82.4	83.0
07	(+)-Disparlure	100	100
08	(+)-Disparlure	52.1	52.6
09	(+)-Disparlure	86.8	87.3
10	(+)-Disparlure	100	100
11	(+)-Disparlure	92.0	92.4
12	(+)-Disparlure	98.2	98.9
13	(+)-Disparlure	100	100
		100	100
14	(+)-Disparlure	100	100
15	(+)-Disparlure	98.8	99.0
		98.8	99.2
16	(+)-Disparlure	100	100
		99.6	100
17	(+)-Disparlure	100	100
		99.6	100
18	(+)-Disparlure	100	100
		99.6	99.8
19	(+)-Disparlure	100	100
20	(+)-Disparlure	98.3	98.6
		98.1	98.6
21	(+)-Disparlure	98.1	98.3
		98.1	98.3
22	(+)-Disparlure	99.3	99.6
23	(+)-Disparlure	100	100
		99.6	100
24	(+)-Disparlure	100	99.7
25	(+)-Disparlure	97.9	98.2
		97.8	98.3
26	(+)-Disparlure	99.2	99.7
27	(+)-Disparlure	95.4	95.8
28	(+)-Disparlure	97.8	98.2
29	(+)-Disparlure (see Figure)	96.7	97.1
30	(+)-Disparlure from dispenser	99.2	99.6
31	(+)-Disparlure from dispenser	98.5	98.9
32	2% (−)-Disparlure (Std.)	97.4	98.1
33	0.5% (−)-Disparlure (Std.)	99.2	99.5

[a] Many of these samples are proprietary and accordingly are identified only by number for this publication.
[b] Measured value from (*R*)(+)-α-methylbenzylamine.
[c] Measured value from (*S*)(+)-α-methylbenzylamine.

is believed to be a reflection of the integration of incompletely resolved peaks as opposed to any diastereoselectivity in the chemical reactions.

A large number of samples, submitted over several years from a variety of sources as (+)-disparlure, were evaluated by the procedure described (Table 1). It is evident that the enantiomeric composition has varied widely, with some samples being nearly racemic and one actually containing more than 50% of the (−) enantiomer. Several of the samples, in contrast, contain virtually no detectable (−) enantiomer.

Some field tests, designed to examine more systematically the effects of known amounts of the (−) enantiomer, are being conducted by coworkers (B.A. Leonhardt, V. Mastro, among others), and for this purpose, along with that of testing our analytical method, some standard solutions were prepared by careful dilution (with racemic disparlure) of one of the (+)-disparlure samples in which none of the opposite enantiomer was detectable. Two of these dilutions [2.0% and 0.5% (−)-disparlure] were selected for analysis, and the results constitute entries 31 and 32, respectively, of Table 1. Acceptable results were obtained, and as discussed above, the values obtained from the product of (+)-α-methylbenzylamine were slightly higher than those from the (−)-amine. Accordingly, we conclude that the values recorded in column 2 of Table 1 represent reasonably accurate and reliable estimates of the enantiomeric compositions of the disparlure samples examined. Examinations of standard solutions also confirmed the detectability of concentrations of (−)-disparlure as low as 0.1%.

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VOLATILE COMPONENTS IN SCENT GLAND SECRETIONS OF GARTER SNAKES (*Thamnophis* spp.)

WILLIAM F. WOOD,[1]* JOSHUA M. PARKER,[1] and PAUL J. WELDON[2]

[1]*Department of Chemistry*
Humboldt State University, Arcata, California 95521

[2]*Department of Biology*
Texas A & M University, College Station, Texas 77843
and Conservation and Research Center, Smithsonian Institution, 1500 Remount Rd., Front Royal, Virginia 22630

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Abstract—Previous analyses of the scent gland secretions of snakes have focused on the nonvolatile components. Gas chromatography–mass spectrometry of secretions from North American garter snakes (*Thamnophis butleri*, *T. couchi*, *T. elegans*, *T. melanogaster*, and *T. sirtalis*) indicated the following seven major volatile components: acetic, propanoic, 2-methylpropanoic, butanoic, and 3-methylbutanoic acids, trimethylamine, and 2-piperidone. Five or more of these compounds were observed in secretions of select boid, colubrid, pythonid, and viperid snakes, suggesting that they are widespread scent gland products. 3-Methylbutanal also was detected in some snake species.

Key Words—Garter snakes, *Thamnophis*, 2-piperidone, trimethylamine, short-chain carboxylic acids.

INTRODUCTION

The foul-smelling fluids discharged from the cloacal region of snakes are familiar to anyone who has handled an agitated specimen. Contributing to these odoriferous materials are the scent glands, two organs opening through separate ducts at the margin of the cloacal orifice (Whiting, 1969). Previous analyses of scent gland secretions have focused on the nonvolatile components. Both lipids (Oldak, 1976; Weldon et al., 1990, 1991, 1992; Simpson et al., 1993) and

*To whom correspondence should be addressed.

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proteins (Weldon and Leto, 1995) have been reported. Studies on the volatile components of scent gland secretions are confined to an analysis of an ether extract of Drumeril's ground boa, *Acrantophis dumerili*, Boidae (Simpson et al., 1993). Short-chain carboxylic acids (C_3–C_5) and phenol, in addition to higher molecular weight compounds, were identified in this species' secretions.

We report here an analysis by gas chromatography–mass spectrometry (GC-MS) of the volatile components in the scent gland secretions of North American garter snakes (*Thamnophis* spp.). This is the first detailed identification of scent gland compounds from the Colubridae, the most speciose family of snakes. In addition, we provide preliminary results on the volatile secretions of select boid, colubrid, pythonid, and viperid snakes. Our results point to some compounds that likely are widespread scent gland products.

METHODS AND MATERIALS

Scent gland secretions were obtained from adult free-ranging and captive snakes. Free-ranging specimens of the western aquatic garter snake, *T. couchi* (one female), wandering garter snake, *T. elegans* (one female), and eastern garter snake, *T. sirtalis* (three females), were captured in Humboldt County, California; all individuals were released after samples were obtained. Captive secretion donors maintained in the Reptile Ethology Laboratory of the University of Tennessee, Knoxville, were *T. sirtalis* (four females, five males), captured in or born to females from Ottawa County, Ohio; the black-bellied garter snake, *T. melanogaster* (eight females, four males), captured in or born to females from Jalisco, Mexico; and Butler's garter snake, *T. butleri* (five females, three males), born to females captured in Wayne County, Michigan. Captive secretion donors from the Dallas Zoo, Texas, and the Houston Zoological Gardens, Texas, were *T. sirtalis* (one female, one male); *T. elegans* (one male); Dumeril's ground boa, *Acrantophis dumerili*, Boidae (one male); green anaconda, *Eunectes murinus*, Boidae (one female); common kingsnake, *Lampropeltis getula*, Colubridae (one male); ringed python, *Bothrochilus boa*, Pythonidae (one female); copperhead, *Agkistrodon contortrix*, Viperidae (one female); and speckled rattlesnake, *Crotalus mitchelli*, Viperidae (one female).

Scent gland secretions, expressed by gently pressing on the base of the tail, were collected in glass vials with Teflon-lined stoppers. Samples from free-ranging snakes from California were analyzed individually by GC-MS within 30 min. of collection. The pH of the secretions from these snakes was determined with pHydrion Paper (Micro Essential Laboratory, Brooklyn, New York) within a minute of collection. Samples from captive snakes were pooled by species and sex, placed on Dry Ice, shipped to California, and analyzed within a few hours of receipt. Approximately 1 μl of neat secretion was used for each GC-MS analysis.

GC-MS was done on a Hewlett-Packard gas chromatograph (model 5890) fitted with a mass selective detector (model 5970) using a 12-m cross-linked methyl silicone capillary column (HP-1). The gas chromatograph was programmed so the oven temperature was maintained at 40°C for 4 min., then increased to a final temperature of 250°C at a rate of 30°C/min and kept at this temperature for 4 min. For native secretions, mass spectral fragments below $m/z = 20$ were not recorded, and the mass spectrometer was turned on 0.1 min after injection of the sample so that all volatile components could be analyzed. For ether extracts, fragments below $m/z = 35$ were not recorded, and the mass spectrometer was turned on 2 min after injection of the sample. Compounds were identified by comparing their mass spectra and relative retention times of those of authentic standards (Aldrich Chemical Co., Milwaukee, Wisconsin).

To examine the possibility that trimethylamine was an artifact from the decomposition of nonvolatile trimethylammonium salts on the hot GC injector, 0.1 μl of 10% NaOH was added to the vial containing the secretions from *E. murinus*. The vial was immediately sealed with a rubber stopper. After 5 min, a 5-μl headspace sample was analyzed by GC-MS analysis using the same conditions as for native secretions.

To see if trimethylammonium salts of the short-chain carboxylic acids generate trimethylamine and the free carboxylic acids on injection into the GC-MS, a solution was prepared from 50 ml of water and 3 drops of each carboxylic acid. This was adjusted to pH 7 with an aqueous solution of trimethylamine and then analyzed by GC-MS using the same conditions as for the native secretions.

The presence of ammonia or low-molecular-weight amines was investigated by treating the secretions of *T. sirtalis* with 1 ml of 1.0 M NaOH and 1 drop of benzoyl chloride in 1 ml of ether. The ether solution was analyzed by GC-MS for benzamide and *N*-substituted benzamides.

To examine the possibility that 2-piperidone was an artifact from the dehydration of 5-aminopentanoic acid, 1.0 μl of a solution of 100 mg of 5-aminopentanoic acid (Aldrich Chemical Co.) in 50 ml was analyzed by GC-MS using the same conditions as for native secretions.

RESULTS AND DISCUSSION

GC-MS analysis of the neat scent gland secretions from samples of all *Thamnophis* species showed the same seven volatile compounds (in order of elution): trimethylamine, acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, and 2-piperidone (Figure 1).The relative amounts of these compounds varied between samples, and no consistent variation was seen between sex or species. The electron impact-mass spectra of these compounds are as follows: trimethylamine—$m/z = 59(M^+, 42), 58(100), 44(11),$

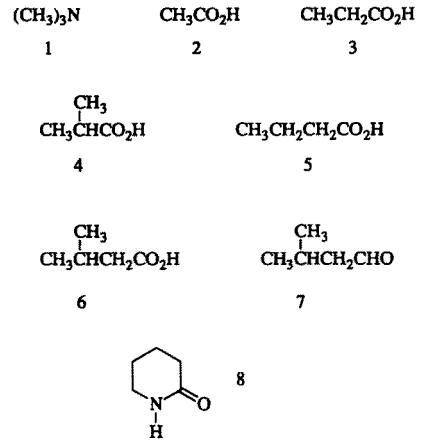

FIG. 1. Volatile components from the scent gland secretions of snakes: trimethylamine (1), acetic acid (2), propanoic acid (3), 2-methylpropanoic acid (4), butanoic acid (5), 3-methylbutanoic acid (6), 3-methylbutanal (7), and 2-piperidone (8).

43(18), 42(92), 41(25), 40(21), 30(50), 28(42), and 27(16), acetic acid—m/z = 61(1), 60(M^+, 48), 45(96), 43(100), 42(16), 41(6), 40(2), 31(7), 29(25), and 28(13), propanoic acid—m/z = 74(M^+, 16), 73(16), 57(9), 45(42), 44(11), 30(14), 29(65), 28(100), 27(68), and 26(35), 2-methylpropanoic acid—m/z = 73(20), 45(68), 44(28), 43(100), 42(21), 41(65), 39(44), 29(28), 28(81), and 27(100), butanoic acid—m/z = 73(39), 60(100), 45(38), 43(24), 42(50), 41(39), 39(32), 29(28), 28(62), and 27(56), 3-methylbutanoic acid—m/z = 87(20), 60(100), 45(68), 43(62), 42(42), 41(68), 39(68), 29(37), 28(24), and 27(62), and 2-piperidone—m/z = 99(M^+, 42), 71(30), 55(26), 44(24), 43(56), 42(74), 41(62), 39(32), 30(65), and 28(100).

The retention time of the amine and carboxylic acids varied between samples, a likely result of water in the secretions binding to the column and altering its polarity. To examine this possibility, we compared the retention times of pure acetic acid and an aqueous solution of it. Glacial acetic acid exhibited a retention time of 1.71 min. When 1 μl of a solution prepared from three drops of glacial acetic acid and 50 ml of distilled water was injected, acetic acid had a retention time of 5.16 min. The retention time of the other short-chain carboxylic acids and trimethylamine also increased when injected as aqueous solutions, but the relative order of elution remained constant. The retention time of 2-piperidone was 8.17 min for all runs.

The possibility that trimethylamine is an artifact from decomposition on the hot GC injector of trimethylammonium salts, a type of Hofmann degradation, was investigated. Trimethylamine was detected in the headspace over a

sample of secretions that was treated with a NaOH solution in a sealed container, indicating the compound is not an artifact.

The pH of the secretions from free-ranging garter snakes from Humboldt County was 7, indicating the trimethylamine and short-chain carboxylic acids exist in the ionized form. An aqueous solution of the short-chain carboxylic acids adjusted to pH 7 with trimethylamine was subjected to the same GC-MS analysis as native scent secretions. Free trimethylamine and carboxylic acids were observed; therefore, we surmise the heat of the injector or separation on the column lead to the generation of these species from trimethylammonium salts of the short-chain acids.

Low-molecular-weight amines or ammonia present with the trimethylamine might not be detectable on GC-MS analysis, so a sample of the secretions from *T. sirtalis* was treated with an ethereal solution of benzoyl chloride and an aqueous solution of sodium hydroxide (Shotten-Baumann reaction). GC-MS analysis of the resulting ether solution failed to show any benzamide or *N*-substituted benzamide derivatives; thus, these amines did not escape detection.

To examine the possibility that 2-piperidone was an artifact produced by the dehydration of 5-aminopentanoic acid, which is known to occur in some mammalian skin gland secretions (Albone *et al.*, 1976), an aqueous solution was analyzed by GC-MS, as was done with the native scent secretions. No cyclic lactam was observed; thus, 2-piperidone appears to be an actual component of the secretions.

A comparison of the scent gland secretions from other snakes demonstrates

TABLE 1. VOLATILE COMPONENTS FROM SCENT GLAND SECRETIONS OF SNAKES

	Compound[a]							
Family and species	1	2	3	4	5	6	7	8
Boidae								
Acrantophis dumerili	X	X	X	X	X	X	X	X
Eunectes murinus	X	X	X	X	X	X	ND[b]	ND
Colubridae								
Lampropeltis getula	X	X	X	X	X	X	ND	ND
Pythonidae								
Bothrochilus boa	ND	X	X	X	X	X	ND	ND
Viperidae								
Agkistrodon contortrix	ND	X	X	X	X	X	ND	X
Crotalus mitchelli	ND	X	X	X	X	X	X	X

[a]Trimethylamine (1), acetic acid (2), propanoic acid (3), 2-methylpropanoic acid (4), butanoic acid (5), 3-methylbutanoic acid (6), 3-methylbutanal (7), and 2-piperidone (8).
[b]ND = none detected.

some of the components found in *Thamnophis* (Table 1). All species were found to contain the same short-chain carboxylic acids. Trimethylamine was detected in *A. dumerili, E. murinus*, and *L. getula*, but not in the other snakes examined. 2-Piperidone was identified in the secretions from *A. dumerili, A. contortrix*, and *C. mitchelli*. *A. dumerili* and *C. mitchelli* secretions contained 3-methylbutanal, a compound not observed in the analysis of *Thamnophis* secretions. This compound had a retention time of 1.34 min and the mass spectrum m/z = 71(13), 58(25), 44(100), 43(50), 42(18), 41(71), 39(31), 29(50), 28(24), and 27(46).

Phenol and 2-methylbutanoic acid detected in a previous study of *A. dumerili* (Simpson *et al.*, 1993) were not seen on analysis of native secretions. Furthermore, the three suspected amines isolated by extraction and thin-layer chromatography of *A. dumerili* secretions were not seen in this study. Injection of neat samples of these secretions likely masks these minor components because of the high concentrations of the short-chain carboxylic acids.

The antipredator function generally attributed to scent gland secretions (e.g., Greene, 1987) needs to be examined. Trimethylammonium salts of short-chain carboxylic acids may themselves be effective defensive compounds. Furthermore, if the secretions are introduced into an alkaline environment, trimethylamine, a pungent gas with an ammonia-like odor, is released. If the secretions encounter an acidic environment, the short-chain carboxylic acid become volatile and more offensive. 2-Piperidone, the least volatile component we observed, while not itself strongly odoriferous, is bitter (tasted by W.F.W.).

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BIORATIONALS FROM *Nicotiana* PROTECT CUCUMBERS AGAINST *Colletotrichum lagenarium* (Pass.) Ell. & Halst DISEASE DEVELOPMENT

B.S. KENNEDY,[1] M.T. NIELSEN,[1,*] and R.F. SEVERSON[2]

[1]*Department of Agronomy*
University of Kentucky
Lexington, Kentucky 40546

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Abstract—Trichome exudate compounds isolated from leaves of *Nicotiana tabacum, N. glutinosa* (accessions 24 and 24a), and 21 other *Nicotiana* species were evaluated for biorational activity against *C. lagenarium*, the anthracnose pathogen of cucumber. Exudate compounds tested were the cembrane diterpenes α- and β-4,8,13-duvatriene-1,3-diols (DVT diols); the labdane diterpenes (13-*E*)-labda-13-ene-8α,15-diol (labdenediol) and (13-*R*)-labda-14-ene-8α,13-diol (sclareol); extracts from *N. gossei*; and various sugar ester isolates. In dose–response experiments test compounds were applied to a water agar surface that was then inoculated with a conidial suspension. Low levels of most test compounds reduced or completely inhibited germination of *C. lagenarium* conidia. IC_{50} values, concentrations (micrograms per square centimeter) at which conidium germination was reduced 50%, were 6.3 for DVT diols, 19.3 for sclareol, 1.0 for labdenediol, 2.8 for a mixture of sclareol and labdenediol, 1.2 for an *N. gossei* sucrose ester and 4.1 for the *N. gossei* crude extract. Higher levels of DVT diols and the sclareol–labdenediol mixture were required to reduce lesion size and number on inoculated cucumber leaves. At the highest concentration tested, 100 $\mu g/cm^2$, the DVT diols and sclareol–labdenediol mixtures protected cucumbers against lesion development by 93 and 98%, respectively. Sugar ester mixtures from 20 *Nicotiana* species and three different sugar ester fractions from *N. bideglovii* had in vitro antifungal activity at a concentration of 48 $\mu g/cm^2$. Sugar esters from nine of the species including *N. acuminata, N. attenuata, N. clevelandii, N. maritima, N. miersii, N. noctiflora, N. occidentalis, N. rustica*, and fractions 10, 12, and 13 from *N. bideglovii* completely inhibited *C. lagenarium* conidium from germinating. Sugar ester mixtures from only four species, *N. plumbaginifolia, N. bonar-*

*To whom correspondence should be addressed.
[2]Deceased, formerly ARS-USDA, Richard B. Russell Agricultural Research Center, Athens, Georgia 30613.

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iensis, *N. simulans*, and *N. palmerii*, had no significant effect on *C. lagenarium* conidium germination. These results suggest the potential of *Nicotiana* exudates as biorationals in reducing disease development.

Key Words—*Nicotiana*, *Colletotrichum lagenarium*, biorationals, trichome exudates, leaf surface chemistry, diterpenes, sugar esters.

INTRODUCTION

Secondary plant products derived from glandular trichome exudates have been implicated in host-plant resistance in certain genera of the Solanaceaous family including *Lycopersicon* and *Solanum* (Gentile and Stoner, 1968; Gibson, 1971). Trichome exudate compounds or crude leaf surface extracts from different *Nicotiana* species have also been shown to have activity against certain phytophagous insects and plant pathogens (Severson et al., 1985, 1991). Earlier, we reported that sporangium germination of *Peronospora tabacina* Adam, the blue mold pathogen of tobacco, was greatly inhibited following exposure to diterpenes and sucrose esters derived from the leaf surface of several *Nicotiana* species (Kennedy et al., 1992). The importance of these compounds as biorationals, natural products with pesticide activity, could increase greatly if they are found to be active against disease and insect pests of other plant species. Recently, foliar applications of sucrose esters, derived from the leaf surface of *N. gossei*, on field grown cotton showed similar or greater control of certain whitefly species than approved pesticides (R.F. Severson, unpublished observations, 1993). The objective of our research was to determine if compounds derived from *Nicotiana* have biorational activity against a fungal pathogen of an unrelated plant species. The following trichome exudate compounds were evaluated for activity against the cucumber pathogen *Colletotrichum lagenarium* (Pass) Ell & Halst.: a mixture of the cembrane diterpenes α- and β-duvatrienediols; two labdane diterpenes, sclareol and labdenediol; extracts from *N. gossei*; and sugar ester mixtures from 20 different *Nicotiana* species.

METHODS AND MATERIALS

Isolation and Purification of Leaf Surface Compounds. Cuticular components were extracted from *N. tabacum* cultivars NC 2326 and NFT, *N. glutinosa* (accessions 24 and 24a), and 24 other species grown in field plots at the Georgia Coastal Plain Experiment Station, Tifton, Georgia, and the Crops Research Laboratory, Oxford, North Carolina. Six to eight weeks after transplanting, tops cut from plants were extracted with methylene chloride (Baker Resianalyzed reagent grade) as previously described (Jackson et al., 1986; Severson et al., 1988). The α- and β-4,8,13,-duvatriene-1,3-diols (from NC 2326) were isolated

in 98 +% purity as described by Severson et al. (1988). *Nicotiana glutinosa* was the source of labdenediol, (13-*E*)-labda-13-ene-8α,15-diol and the sclareols, a mixture of (13-*R*)- and (13-*S*)-labda-14-ene-8α,13-diol (sclareol and episclareol). Labdenediol and sclareols were obtained in 98+% purity as described by Jackson et al. (1986).

Cuticular extracts from the *Nicotiana* species in Table 1 were taken to dryness on a rotoevaporator, and the residue was partitioned between hexane and 80% methanol–water (Severson et al., 1988). The methanol–water-soluble fraction (100–200 μg) containing the sugar esters was dissolved in 0.5 ml of 90% methanol–water, loaded into an injection loop, and chromatographed on a Bio-Sil C-18 (HL Silica, fully encapped; 40–63 μm; Bio-Rad Laboratories) preparative reverse-phase column (1.25 cm ID × 15 cm). The column was eluted with 225 ml 60% methanol–water, 90 ml 90% methanol–water, and 125 ml 100% methanol, and 5-ml fractions were collected. The solvent was removed on a Savant Speed Vac, and fractions with observable residue were derivatized and analyzed by capillary gas chromatography (Severson et al., 1984; Jackson et al., 1986). Fractions containing glucose and/or sucrose esters were combined to yield the sugar ester tested. The preparative chromatography cleanly separated the glucose and sucrose ester fractions in the *N. attenuata* sample, and they were combined separately.

Inoculum Preparation. Colletotrichum lagenarium (Pass) Ell. & Halst. race 1 was obtained from J. Kuć, Department of Plant Pathology, University of Kentucky. It was maintained on green bean agar at ±20–24°C in the dark (Goode, 1958). Conidial suspensions were prepared in sterile distilled H_2O from 7-day-old cultures and conidium concentrations were determined with a hemacytometer.

In vitro Bioassay. Efficacy of the test compounds and extracts was determined by assaying germination of *C. lagenarium* conidia on water agar covered uniformly with the test compounds. Sugar ester mixtures from 20 different *Nicotiana* species were tested at a concentration of 48 $\mu g/cm^2$. In separate experiments, DVTs, sclareol, labdenediol, a mixture of sclareol and labdenediol, a sucrose ester mixture from *N. gossei*, and a crude exudate mixture from *N. gossei* were tested at six concentrations ranging from 0.75 to 48 $\mu g/cm^2$. Desired concentrations were prepared by diluting portions of the stock solution with 100% acetone. Treatments were applied in 50-μl aliquots to the surface of 1.5% water agar in 35- × 10-mm Petri plates. A bent Pasteur glass pipet was used to evenly distribute the compound over the agar surface. Plates were set aside for the carrier solvent to evaporate before a 200-μl conidial suspension of *C. lagenarium* (1×10^5 sp/ml) was added to each plate. Plates were incubated overnight at 24°C in the dark. All treatments were replicated four times and contained two control treatments: (1) no compound or acetone was applied prior to the conidial suspension, and (2) application of 100% acetone, the carrier

solvent. Conidium germination was determined microscopically, and activity of each test compound was expressed as percent inhibition: [(% germination of control − % germination for each treatment)/% germination of control] × 100. Where appropriate, the log dose (concentration)–response relationship was linearized using probits, and inhibitory concentrations (IC_{50}) were estimated (SAS Institute Inc., 1985).

Plant Assay. Seeds of the anthracnose-susceptible cucumber cultivar SMR-58 were sown in 3 1/2-in. plastic pots containing a commercial medium. They were watered daily over the top until emergence of the first cotyledon and then were watered from the bottom of the pot for the duration of each experiment. A water soluble fertilizer, Peters 20:10:20 (N/P/K) was applied weekly. Plants were maintained in a greenhouse at 24–30°C under high-pressure sodium lights for a 14-hr photoperiod. Exudate compounds, α- + β-DVT-diols and mixture of sclareol and labdenediol, were evaluated in the greenhouse for controlling anthracnose disease development on the leaf surface of cucumbers. The DVT diols and the sclareol–labdenediol mixture were assayed in separate experiments. The first true leaves of 13-day-old cucumber plants were uniformly sprayed with different concentrations of the test compounds contained in 35% acetone (v/v) using an airbrush sprayer (Paashe Airbrush Co., Harwood Heights, Illinois) at 50 psi. Plants were returned to the greenhouse bench to dry overnight in absence of artificial light. Approximately 18 hr later, treated plants were inoculated following a procedure adapted by Kuć et al. (1975). Each treated leaf was inoculated with 30 10-μl drops of a conidial suspension of *C. lagenarium* (1×10^5 sp/ml). Thirty 10-μl drops of sterile water were placed on control plants. Plants were incubated in closed moist chambers for 24 hr in the greenhouse and then partially opened within the next 12 hr to establish air temperature equilibrium. Plants were returned to the greenhouse bench under high-pressure sodium lights and rated for symptom development one week after inoculation. Lesion number and necrotic area were determined for each leaf treatment. Treatments were replicated four times in each experiment and both experiments were done twice. Percent protection was calculated in a similar manner for each *in vivo* experiment: % protection = $[(C - T)/C] \times 100$ where C is the percent necrotic area for the acetone control treatments and T is the percent necrotic area for the various treatments.

RESULTS

Conidia germination rates for the control treatments were between 80 and 100% in all *in vitro* experiments (Table 1 and Figure 1). Acetone applied to the agar had no significant effect on conidium germination compared to the untreated agar plates. The acetone apparently evaporated completely and had no residual

TABLE 1. EFFECT OF SUCROSE AND/OR GLUCOSE ESTERS ISOLATED FROM *Nicotiana* SPECIES ON GERMINATION OF *C. lagenarium* CONIDIA IN AN *In Vitro* EXPERIMENT[a]

Treatment (48 $\mu g/cm^2$)	Inhibition (%)
N. plumbaginifolia (43a)	1.1
N. bonariensis (11a)	9.4
N. simulans (66)	13.6
N. palmerii (39)	14.2
N. nudicaulis (36)	26.5[b]
N. debneyi (17)	36.9[b]
N. langsdorffii (28b)	61.8[b]
N. velutina (62)	77.5[b]
N. cavicola (68)	78.6[b]
N. pauciflora (41)	94.9[b]
N. hesperis (67)	98.4[b]
N. acuminata (2)	100.0[b]
N. attenuata (7a)	100.0[b]
N. bigelovii (10)	100.0[b]
N. bigelovii (12)	100.0[b]
N. bigelovii (13)	100.0[b]
N. clevelandii (14)	100.0[b]
N. martima (31)	100.0[b]
N. miersii (33)	100.0[b]
N. noctiflora (35)	100.0[b]
N. occidentalis (37b)	100.0[b]
N. rustica (48)	100.0[b]

[a]The germination rates for the conidia in the control treatments of water and acetone (the carrier solvent) were 94.8% and 93.5%, respectively.
[b]Denotes significant differences ($P < 0.05$) from the acetone control treatment according to a paired t test.

effect on the conidia. Thus, activities of the various chemicals were based on comparisons to the acetone control.

Sugar ester mixtures from 20 *Nicotiana* species and three different sugar ester fractions from *N. bideglovii* had *in vitro* antifungal activity at a concentration of 48 $\mu g/cm^2$. *C. lagenarium* conidia were completely inhibited from germinating when exposed to sugar esters from nine of the species, *N. acuminata*, *N. attenuata*, *N. clevelandii*, *N. maritima*, *N. miersii*, *N. noctiflora*, *N. occidentalis*, *N. rustica*, and fractions 10, 12, and 13 from *N. bideglovii*, when compared to the control treatments (Table 1). Conidia germination was reduced more than 25% when exposed to sugar esters derived from *N. nudicaulis*, *N. debneyi*, *N. langsdorffii*, *N. velutina*, *N. cavicola*, *N. pauciflora*, and *N. hesperis*. Sugar ester mixtures from only four species, *N. plumbaginifolia*, *N. bon-*

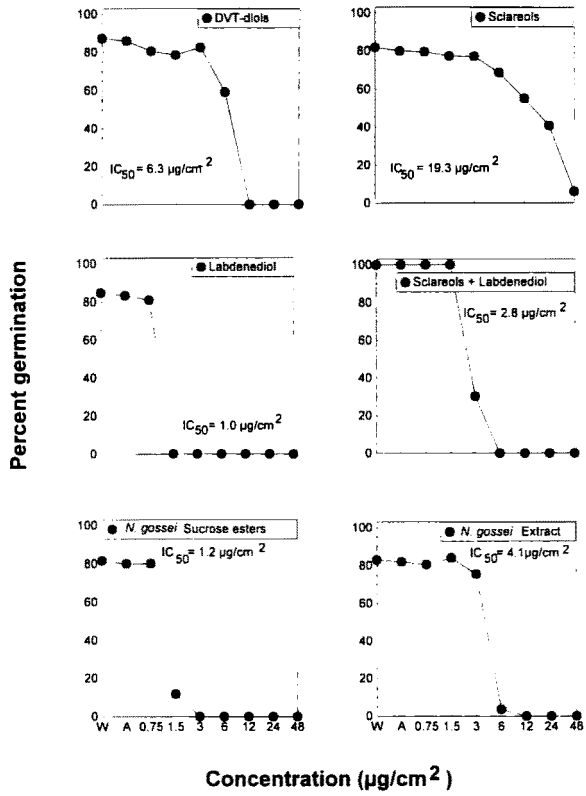

FIG. 1. Percent germination of *C. lagenarium* conidia in vitro after exposure to trichome exudates. Control treatments are represented by: W, no chemical or acetone; and A, acetone only.

ariensis, *N. simulans*, and *N. palmerii*, had no significant effect on *C. lagenarium* conidium germination.

Microscopic examination of conidia in all of the *in vitro* experiments revealed that for those conidia that did germinate, germ tube length and morphology was similar to that observed for conidia in the control treatments.

Inhibition of conidium germination was nearly complete when exposed to the highest concentrations of the six different test compounds for which dose responses were assessed (Figure 1). IC_{50} values, concentrations at which conidium germination was reduced 50%, were well under 5 $\mu g/cm^2$ for labdenediol, the sclareol–labdenediol mixture, *N. gossei* sucrose esters, and the *N. gossei* extract. IC_{50} values were 6.3 $\mu g/cm^2$ for the α- and β-DVT-diol mixture. The

most active compound tested was labdenediol with an IC_{50} value of 1 $\mu g/cm^2$ and the least active was sclareol with an IC_{50} value of 19.3 $\mu g/cm^2$. Figure 1 also reveals that for most of the tested compounds, germination inhibition was not linearly related to concentration. In fact, for labdenediol, DVTs, and the *N. gossei* compounds, a critical response to concentration was observed. This is best illustrated by the experiments with labdenediol in which conidium germination was little affected at 0.75 $\mu g/cm^2$ of labdenediol, but was greatly inhibited at the next higher chemical concentration of 1.5 $\mu g/cm^2$.

Mixtures of: (1) α- and β-DVT-diols and (2) sclareol and labdenediol were evaluated at different concentrations for control of anthracnose disease development on cucumber leaves. These two mixtures were chosen for the disease control experiments because both mixtures are abundant on *Nicotiana* leaf surfaces, DVT diols on most *N. tabacum* genotypes and on *N. glutinosa* accession 24a. Furthermore, abundant quantities of these mixtures were available for testing. Because cucumber leaves are very sensitive to chemical applications, we were initially concerned about using acetone as a carrier solvent for our exudates. However, when the concentration of acetone was reduced to 35%, no phytotoxicity was observed among the sprayed leaves in any of the treatments including the controls.

Preliminary experiments indicated that low concentrations of both mixtures were less effective in controlling anthracnose than they were in preventing *C. lagenarium* conidium germination in vitro. Therefore, higher concentration ranges were chosen for the disease control experiments.

There was significantly less disease as measured by lesion number and total necrotic area at the lowest concentration (40 $\mu g/cm^2$) of DVT diols. At a treatment concentration of 15 $\mu g/cm^2$, the sclareol–labdenediol mixture had significantly lower disease scores than the acetone control. At a concentration of 100 $\mu g/cm^2$, DVT diols and the sclareol–labdenediol mixture protected cucumbers against anthracnose disease development by 93 and 98%, respectively (Tables 2 and 3).

DISCUSSION

Controlling plant diseases in agricultural crops has traditionally been accomplished by using multidisciplinary approaches consisting of host-plant resistance, use of appropriate cultural practices including crop rotation and good sanitation, and the application of pesticides. However, some producer's options for disease control are becoming increasingly limited. This is due primarily to the lack of land available for crop rotation and the growing concern over the use of synthetic chemicals to control plant disease pests. The use of natural plant products, acting as applied pesticides or as factors in host-plant resistance,

TABLE 2. PERCENT PROTECTION OF SMR58 CUCUMBER LEAVES AGAINST DISEASE DEVELOPMENT OF *C. lagenarium* BY α- + β-DVT DIOL FOLIAR SPRAYS IN GREENHOUSE EXPERIMENTS.

Spray treatments	Average No. of lesions per leaf[a]	Average lesion size (mm^2)	Total necrotic area (mm^2)[b]	Protection (%)[c]
Untreated	29.9 a[d]	9.3	277.5 a	
35% acetone	29.3 a	10.5	308.9 a	
40 $\mu g/cm^2$ DVT diols	18.1 b	5.7	107.3 b	65
50	15.1 bc	6.0	78.6 bc	75
60	13.0 bcd	8.6	109.1 b	65
70	9.8 cde	5.4	52.7 bc	83
80	8.6 de	4.9	36.8 bc	88
90	6.5 e	4.5	32.4 bc	90
100	5.0 e	5.5	22.5 c	93
Untreated/water	0.0 e		0.0 c	
35% acetone/water	0.0 e		0.0 c	

[a] Average number of lesions resulting from 30 inoculations per leaf.
[b] Average total necrotic area from each leaf.
[c] Percent protection was calculated for each treatment by using the following formula: % protection = [(35% acetone X TNA–treatment X TNA)/35% acetone X TNA] × 100, where TNA is the total necrotic area.
[d] Means followed by the same letter are not significantly different at $P < 0.05$ according to the least significant difference.

is becoming increasingly desirable. One approach is to identify biorationals—naturally occurring products that have pesticide activity. Our hypothesis was that plant compounds that have already been classified as determinants of resistance in one host plant–pathogen relationship could possibly serve as biorationals against pathogens of other plant species.

Earlier investigations by our group and others (Kennedy et al., 1992, Menetrez et al., 1990) reported that trichome exudates produced on the leaf surface of some *N. tabacum* and *Nicotiana* species significantly reduced germination of *Peronospora tabacina* Adam, the obligate fungal pathogen responsible for the blue mold disease in tobacco. In our present study, we determined that many of these same compounds could be classified as biorationals effective against disease development of anthracnose in cucumbers.

The compounds were selected for our biological assays primarily because of their relative abundance on the leaf surface and their ease in isolation. The duvatriene diols (DVT) were relatively effective in inhibiting germination of *C. lagenarium* conidia in vitro and somewhat effective at higher concentrations in preventing disease development on the inoculated cucumber leaves. These compounds are of particular interest because they are present at high concentrations in the trichome exudate of many *Nicotiana* genotypes. Indeed, we estimate that

TABLE 3. EFFECT OF SCLAREOL AND LABDENEDIOL FOLIAR SPRAYS ON DISEASE DEVELOPMENT OF SMR58 CUCUMBERS BY *C. lagenarium* IN GREENHOUSE EXPERIMENTS

Spray treatments	Average no. of lesions per leaf[a]	Average lesion size (mm^2)	Total necrotic area (mm^2)[b]	Protection (%)[c]
Untreated	27.3 a[d]	5.8	159.5 ab	
35% acetone	29.5 a	6.4	192.8 a	
5 $\mu g/cm^2$ DVT diols	26.8 a	5.1	139.3 ab	23
10	26.0 a	5.5	149.3 ab	28
15	21.3 b	5.3	126.0 b	35
20	13.8 c	3.8	54.8 c	72
40	3.9 d	2.8	12.1 c	94
80	2.5 d	2.4	6.1 c	97
100	1.1 d	3.5	3.1 c	98
Untreated/water	0.0 d		0.0 c	
35% acetone/water	0.0 d		0.0 c	

[a] Average number of lesions resulting from 30 inoculations per leaf.
[b] Average total necrotic area from each leaf.
[c] Percent protection was calculated for each treatment by using the following formula: % protection = [(35% acetone X TNA—treatment X TNA)/35% acetone X TNA] × 100, where the TNA is the total necrotic area.
[d] Means followed by the same letter are not significantly different from $P < 0.05$ according to the least significant difference.

in certain genotypes, the DVTs could represent as much as 10% of the total leaf dry weight.

Sclareol and labdenediol are labdene diterpenes found primarily on the leaf surface of the *Nicotiana* species *glutinosa*. In *in vitro* experiments, we found sclareol to be the least active (IC_{50} value 19.3 $\mu g/cm^2$) and labdenediol to be the most active compound (IC_{50} value 1.0 $\mu g/cm^2$). The two compounds were mixed and concentrated as they naturally occur on the leaf surface in a 65:35 (sclareol–labdenediol) ratio. *C. lagenarium* conidium germination was completely inhibited when exposed to 6 $\mu g/cm^2$ of the mixture in vitro. Foliar applications of this mixture at 40 $\mu g/cm^2$ or greater protected the leaves of cucumbers from disease development by over 90% when compared to the control treatments.

Conidium germination was completely inhibited when exposed to concentrations of 3 $\mu g/cm^2$ and 12 $\mu g/cm^2$ of the *N. gossei* sucrose ester and extract, respectively. These compounds were included in our screen because they have been implicated as biorationals for control of some insects including whiteflies.

Only four of the sugar ester mixtures from the 20 different *Nicotiana* species did not significantly effect germination of *C. lagenarium* conidia. Sugar esters from nine of the species inhibited conidium germination completely. These sugar

esters vary with the type of acid substitution on the glucose or sucrose moieties (Severson et al., 1991) and are considered to be a very diverse class of compounds, having high potential for genetic manipulation. We screened a large number of sugar esters for biorational activity in hopes of correlating this activity with a particular acid substitution. Although we were unable to establish any relationship between biorational activity and any particular type of sugar ester, the positive results from the assays warrant further investigation.

Results of this study further demonstrate the potential of *Nicotiana* exudates as biorationals in reducing disease development. The effectiveness of these exudates in preventing or retarding disease progress under natural disease pressure needs to be investigated. Additional research is also necessary to estimate the overall value of these compounds as antifungal agents at the whole-plant level, including efforts to characterize these exudates for their biorational activity against a wide range of different plant pathogens and to determine appropriate application methodology. *Nicotiana* exudates are quantitatively and qualitatively different among genotypes and can be manipulated through breeding by the type and density of the trichome and by selection for specific compounds (Nielsen and Severson, 1990). New research should be initiated so that biorationals can efficiently and effectively be utilized as sources for developing additional alternatives to pest control that broaden opportunities for reducing fungicide use.

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SEQUESTRATION OF LICHEN COMPOUNDS BY THREE SPECIES OF TERRESTRIAL SNAILS[1]

SONJA HESBACHER,[2] BRUNO BAUR,[3] ANETTE BAUR[3] and PETER PROKSCH[2,*]

[2]*Julius-von-Sachs-Institut für Biowissenschaften*
Universität Würzburg
Mittlerer Dallenbergweg 64
D-97082 Würzburg, Germany

[3]*Zoologisches Institut*
Universität Basel
Rheinsprung 9
CH-4051 Basel, Switzerland

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Abstract—Three species of lichen-grazing snails, *Balea perversa*, *Chondrina clienta*, and *Helicigona lapicida*, all from the Swedish island of Öland, were found to sequester lichen compounds when feeding on the crustous lichen species *Aspicila calcarea*, *Caloplaca flavovirescens*, *Lecanora muralis*, *Physcia adscendens*, *Tephromela atra*, and *Xanthoria parietina*. The lichen compounds detected in the soft bodies of the snail species analyzed included the anthraquinone parietin, the depside atranorin, as well as a presumable degradation product of the latter. Other lichen compounds such as (+)-usnic acid or α-collatolic acid were not found in the soft bodies but were only detected in the feces, suggesting selective uptake of lichen compounds by the snails. In individuals of *C. clienta* initially fed on the lichen *X. parietina*, the amount of sequestered parietin decreased over time on a parietin-free diet but was still detectable in the soft bodies after 28 days. In the ovoviviparous land snail, *B. perversa*, sequestered parietin was transferred from the mother to the eggs in the reproductive tract.

Key Words—*Balea perversa*, *Chondrina clienta*, *Helicigona lapicida*, Gastropoda, Pulmonata, crustous lichens, lichen compounds, sequestration.

*To whom correspondence should be addressed.
[1]Dedicated to Prof. Dr. F.-C. Czygan on the occasion of his 60th birthday.

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INTRODUCTION

Lichens comprise some 20,000 different taxa and show a worldwide distribution (Crittenden and Porter, 1991). In habitats with harsh environmental conditions (e.g., hot or cold deserts) that tend to suppress the growth of most higher plants, lichens can constitute dominant floral elements and may provide important nutritional resources for herbivores.

It was speculated some 100 years ago that the observed unpalatability of lichens to most potential herbivores is largely due to chemical defense relying on the accumulation of copious amounts of lichen compounds (Zukal, 1895; Stahl, 1904), whereas other authors, including for example Zopf (1896), denied a defensive function of lichen compounds. Recent years, however, have witnessed growing experimental evidence in favor of the chemical defense hypothesis (Slansky, 1979; Lawrey, 1986, 1989, 1991). For example, experiments with larvae of the vigorous polyphagous herbivore *Spodoptera littoralis* (Noctuidae) demonstrated pronounced acute toxicity and feeding deterrency for (−)- and (+)-usnic acid and for vulpinic acid at concentrations well below those found in lichen thalli (Emmerich et al., 1993). Other lichen compounds such as oxyphysodic acid, fumarprotocetraric acid, or calycin, which had no acute effects on survival, were found to retard larval growth and to cause developmental malformations of adults of *S. littoralis* originating from larvae that were reared on an artificial diet spiked with the respective lichen compounds at concentrations comparable to those found in lichen thalli (Giez et al., 1994).

The recent studies suggest that lichen compounds act as defense agents against generalist herbivores. However, there are numerous examples of specialized herbivores that feed solely on lichens. Important groups of oligophagous invertebrate lichen feeders include oribatid mites (Seyd and Seaward, 1984; Reutimann and Scheidegger, 1987), insects (especially Lepidoptera of the family Arctiidae) (Rambold, 1985), and terrestrial gastropods (Lawrey, 1986; Baur et al., 1992). How do these specialized lichen feeders cope with the toxic or unpalatable secondary compounds of their food source that effectively deter generalist herbivores (Emmerich et al., 1993; Giez et al., 1994)? Several strategies of dealing with potentially harmful compounds present in the food source have been elucidated in interactions of herbivores with higher plants. Specialized herbivores may, for example, overcome the defensive chemistry of their hosts by rapid excretion of harmful metabolites or by metabolic detoxification (Brattsten, 1986). Another strategy of dealing with harmful plant compounds frequently observed in specialized herbivores is sequestration of the respective compounds, as shown, for example, for arctiid moths that sequester pyrrolizidine alkaloids from host plants (Ehmke et al., 1990), for cardenolides that are sequestered by monarch butterflies (Malcolm, 1990), or for flavonoids sequestered by lycaenid butterflies (Wiesen et al., 1994). The sequestered plant compounds are often

used for the chemical defense of the herbivores (Ehmke et al., 1990; Malcolm, 1990).

As shown for the first time in the present study, specialized lichen-feeding snails (*Balea perversa*, *Chondrina clienta*, and *Helicigona lapicida*) are also capable of storing lichen compounds derived from their hosts. The ecological role of the sequestered lichen compounds in the snails, however, remains open. In addition to the comparative analysis of the sequestered lichen compounds, we present experimental evidence for the transfer of sequestered parietin from the mother to the offspring in the reproductive tract of the ovoviviparous snail *B. perversa*.

METHODS AND MATERIALS

Snail Species. Chondrina clienta (Westerlund) occurs in open limestone areas of central and southeastern Europe and in three isolated areas of Sweden (Kerney and Cameron, 1979). Its cylindroconical shell is dextral and in adults is 5.5–7 mm high (Baur, 1988). *Balea perversa* (L.) is characteristic of dry places among rocks and old stone walls, occurring occasionally on stems of trees. It is widespread in western Europe, and in Scandinavia it occurs mainly along the coast (Kerney and Cameron, 1979). Its narrowly conical shell is sinistral and in adults is 7–10 mm high. *Helicigona lapicida* (L.) is common in holes and crevices in rocky ground and in old woodland and hedgerows in western and central Europe. Fully grown snails have a flattened, sharply keeled shell measuring 12–20 mm in diameter. All three species are particularly well adapted to rocky habitats; they are very resistant to drought, with activity confined to periods of high air humidity, and their specialized radulae enable them to graze epi- and endolithic lichens from rock faces (Schmid 1929; Breure and Gittenberger, 1982).

In the limestone grassland Great Alvar on the Baltic island of Öland (Sweden), 108 species of calcicolous lichens have been recorded (Fröberg, 1989). On a limestone pavement in this grassland, 17 species of terrestrial gastropods have been found, four of them being potential lichen feeders (*Chondrina clienta*, *Balea perversa*, *Clausilia bidentata*, *Pupilla muscorum*) (Baur, 1987). Laboratory experiments demonstrated that individuals of *C. clienta* grazed on 18 of 27 lichen species offered, whereas *B. perversa* fed on all eight lichen species offered (Fröberg et al., 1993). Experiments also showed that these two snail species differed in their lichen preferences and that juveniles of both snail species exhibited differences in growth rate when fed on different lichen diets (Bauer et al., 1994). There is some experimental evidence that both intra- and interspecific competition for lichens occur between these snail species (Baur, 1988, 1990; Baur and Baur, 1990).

Lichen Compounds in Wild-Caught Snails. Specimens of the land snails *C. clienta*, *B. perversa*, and *H. lapicida* were collected at seven sites in the limestone grassland Great Alvar on Öland in August 1993: (1) in fissures of a vertical quarry wall, 1.8 m high, 1.5 km SSW of Vickleby church (*H. lapicida*), (2) a stone wall, 0.9 m high, made of flat pieces of limestone arranged in horizontal layers, 40 m S of site 1 (*C. clienta*, B. perversa); (3) in cracks of a limestone pavement (50 m × 40 m wide), 10 km E of Mörbylanga (*C. clienta*); (4) a stone wall adjacent to site 3, 1 m high, made of flat pieces of limestone (*B. perversa*); (5) in cracks of a limestone pavement (100 m × 120 m wide), 2.5 km N of Frösslunda (*C. clienta*); (6) on a pile of stones (4 m × 25 m wide, 1 m high), 1.5 km N of Frösslunda (*C. clienta*); and (7) a stone wall, 1 m high, separated by a 4-m-wide grass strip from the stone pile of site 6 (*B. perversa*).

To exclude any contamination by ingested lichen tissue, the snails collected were starved by keeping them active for four days on moist paper toweling. This time was found to be sufficient for the snails to empty their digestive tract of ingested lichen material as shown by light-microscopic inspection of dissected snails. HPLC was used to analyze sequestered lichen compounds in groups of four snails each.

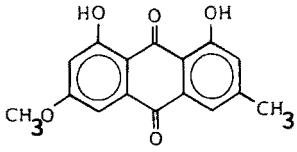
Feeding Experiment. A controlling feeding experiment was designed to examine whether snails sequester particular lichen compounds. Six species of crustous lichens—*Aspicila calcarea* (L.) Mudd, *Caloplaca flavovirescens* (Wulfen) Dalla Torre & Sarnth., *Lecanora muralis* (Schreber) Rabenh., *Physcia adscendens* (Fr.) H. Olivier, *Tephromela atra* (Hudson) Hafellner, and *Xanthoria parietina* (L.) Th. Fr.—that occur in the snails natural habitats were collected in the surroundings of the Ecological Research Station of Uppsala University in Skogsby approximately 8 km N of site 1. Specimens of snails were collected at site 1 (*H. lapicida*) and site 2 (*C. clienta* and *B. perversa*; see above). Snails of all three species were fed a cyanobacteria diet during a conditioning period of six days. During this period, as well as in succeeding experiments, the snails were kept at room temperature (20–22°C) and under natural light conditions. Following a starvation period of eight days (as described above), four individuals of either *C. clienta*, *B. perversa*, or two individuals of *H. lapicida* were placed in a transparent plastic dish (6.5 cm diameter, 2 cm height) lined with paper toweling. A piece of lichen (approximately 3 cm in diameter) was placed on top. This set-up was repeated for each of the six lichen species. Snails of all three species are active only during periods of high air humidity, during which they graze lichens (Schmid, 1929; Neuckel, 1981). To stimulate periodic snail activity, the lichens and paper toweling were moistened and the dishes covered with an acetate film; after 12 h, the waterproof cover was exchanged for a paper towel to dry out the dishes. This procedure was carried out repeatedly at 48-hr intervals, resulting in a rhythm of alternating 12 hr activity and 36 hr resting.

After a feeding period of eight days, the snails were starved for four days (as described above), and the feces collected. Lichen samples, feces, and soft bodies of snails were analyzed for lichen compounds using HPLC.

HPLC Analysis. Lichens as well as feces were ground, extracted in a known aliquot of acetone, and directly subjected to HPLC analysis. Soft bodies of the snails were usually freeze-dried before extraction with known aliquots of acetone. The HPLC system was from Pharmacia (Sweden) and was equipped with a photodiode array detector (Waters). Samples were injected on a Nova-Pak C_{18} column (Waters) (150 × 3.9 mm, 4-μm pore size) and separated using a linear gradient from 100% A (10% MeOH, 90% H_2O adjusted to pH 2 with orthophosphoric acid) to 100% B (MeOH) in 40 min. Identification of lichen compounds was by comparison of retention times and by comparison of the on-line-recorded UV absorption spectra with those of commercially available lichen compounds (Roth) or with lichen compounds isolated and identified previously. Lichen compounds were quantified by the external standard method using commercially available lichen products (Roth).

Retention of Sequestered Parietin. Another laboratory experiment was conducted to examine whether sequestered parietin (**1** in Figure 1) is retained over a long period in snails feeding on a parietin-free diet. Specimens of *C. clienta* were collected from a vertical rock wall close to site 2 on August 8, 1993, and samples of *X. parietina* were collected in the surroundings of the Ecological Research Station. Twelve groups of snails, each consisting of 14 individuals, were kept on a *X. parietina* diet for 10 days. The snails were maintained as described in the feeding experiment (see above). After this period, the snails were randomly assigned to two treatments. Half of the snails were continuously kept on a *X. parietina* diet and the remaining snails were fed a parietin-free diet (free-living cyanobacteria growing on small pieces of limestone). Samples of snails were obtained from both treatments at the beginning of the experiment and after each of four successive periods of eight days. These snails were starved for four days to empty their digestive tract before they were analyzed for sequestered parietin using HPLC.

Sequestered Parietin in Newborn Snails. B. perversa is ovoviviparous, i.e., the young emerge from the egg in the reproductive tract of the mother (Baur, 1990). To examine whether sequestered parietin is passed to embryos in the reproductive tract of the mother, we analyzed neonate *B. perversa* with no feeding experience. Fifty adult *B. perversa* were collected at site 4 on August 8, 1993. The snails were kept in 10 groups each consisting of five individuals on a *X. parietina* diet for 10 days. Maintenance of snails was as described in the feeding experiment (see above). Following this conditioning period, the snails were repeatedly kept on moist paper toweling (no food available) for two days followed by a *X. parietina* diet for two days during a total period of 56 days. Snails born in the parietin-free environment ($N = 51$) were collected for



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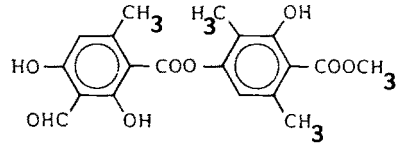

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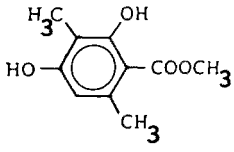

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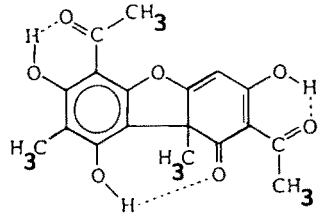

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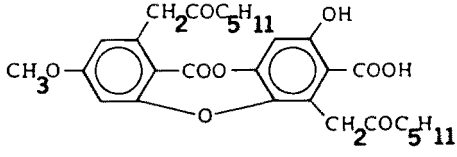

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FIG. 1. Structures of lichen compounds identified: **1**, parietin; **2**, atranorin; **3**, presumed degradation product of **2**; **4**, usnic acid, (+)-enantiomer; **5**, α-collatolic acid.

HPLC analysis of sequestered parietin. For each analysis, 8–12 neonate snails were used.

RESULTS

Lichen Compounds in Wild-Caught Snails. Table 1 summarizes the sequestered lichen compounds found in specimens of three snail species. HPLC analysis of the snails revealed the presence of the lichen compounds parietin (**1**, Figure 1), atranorin (**2**, Figure 1) and of the monomer (**3**, Figure 1)—the latter presumably originating by hydrolytic cleavage of atranorin—in *B. perversa* from two localities. Compounds **2** and **3** were also detected in specimens of *C. clienta* from two localities. Snails of the species *H. lapicida* were devoid of lichen compounds. However, specimens of the latter were only available from one natural habitat, whereas *B. perversa* and *C. clienta* could be collected at three localities each. Since the lichen compounds or their metabolites were detected in snails that were starved following collection to exclude contamination by ingested lichen tissue, a sequestration of lichen compounds must be assumed at least for *B. perversa* and *C. clienta*.

Feeding Experiment. Table 2 summarizes the lichen compounds found in six lichen species and in the soft bodies of *C. clienta*, *B. perversa*, and *H. lapicida* that had fed on the respective lichens. The feces of the three snail species usually resembled those of the corresponding lichen species with regard to the profiles of lichen compounds (e.g., Figures 2 and 3). Lichen products were also present in the soft bodies of the snails analyzed even though the total amounts (in micrograms per snail) of individual compounds showed considerable

TABLE 1. LICHEN COMPOUNDS IN SOFT BODIES AND FECES OF WILD-CAUGHT SNAILS

Snails	Collection sites[a]	Lichen compounds (numbers as in Figure 1)	
		In soft bodies	In feces
B. perversa	4	1, 2, 3	1, 3
	6	1, 2	1, 3
	7	—	1
C. clienta	2	—	—
	3	2	—
	6	2, 3	—
H. lapicida	1	—	—

[a]For description of collections sites see Methods and Materials.

TABLE 2. DISTRIBUTION AMOUNTS, AND CONCENTRATIONS OF LICHEN COMPOUNDS IN SOFT BODIES OF SNAILS FOLLOWING FEEDING ON SIX DIFFERENT SPECIES OF LICHENS[a]

Lichens used for feeding experiments; identified lichen compounds are given in parentheses	Snails	Identified lichen compounds in soft bodies of snails					
		1		**2**		**3**	
		Amount (μg/ind.)	Conc (μg/g) fresh wt	Amount (μg/ind.)	Conc (μg/g) fresh wt	Amount (μg/ind.)	Conc (μg/g) fresh wt
A. calcarea (**1, 2**)	*B. perversa*			+	+	0.40	33.80
	C. clienta	0.06	12.3			0.02	4.10
	H. lapicida					+	+
C. flavovirescens (**1, 2**)	*B. perversa*	0.02	1.7			0.03	2.53
	C. clienta	+	+			0.09	18.45
	H. lapicida	0.06	0.03			0.22	0.11
L. muralis (**2, 4**)	*B. perversa*					+	+
	C. clienta			+	+		
	H. lapicida					+	+
P. adscendens (**2, 3**)	*B. perversa*					0.04	3.38
	C. clienta			0.08	16.40	0.45	92.25
	H. lapicida					0.37	0.18
T. atra (**2, 3, 5**)	*B. perversa*					0.04	3.38
	C. clienta					+	+
	H. lapicida					+	+
X. parietina (**1, 2**)	*B. perversa*	0.13	10.98			0.23	19.43
	C. clienta	0.02	3.57			+	+
	H. lapicida	0.15	0.07			0.23	0.11

[a] Numbers of lichen compounds are as in Figure 1. Compounds **4** and **5** were not detected in soft bodies of snails. (+) = trace amount.

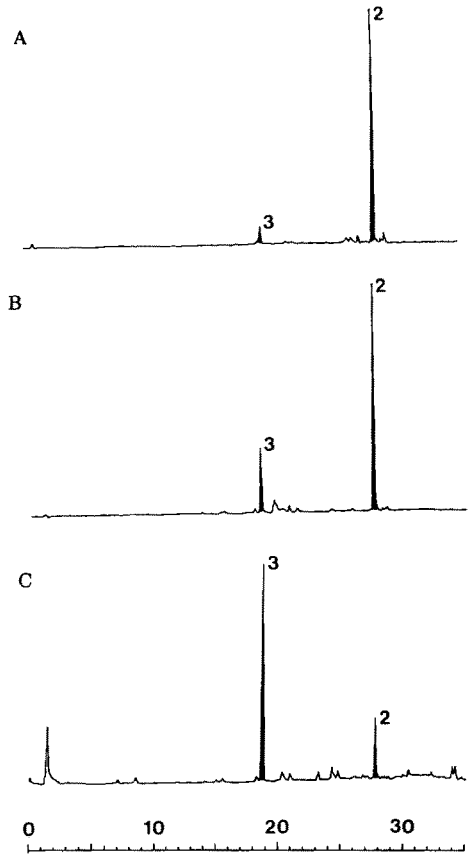

FIG. 2. Comparative HPLC analysis of soft bodies (C) and feces (B) of specimens of *Chondrina clienta* after feeding on thalli of *Physcia adscendens* (A). Specimens of *C. clienta* were treated as described in the feeding experiment. Numbers of compounds are as in Figure 1.

intra- and interspecific variation for the snail species studied. Relatively large amounts of lichen products were detected in specimens of *C. clienta* that fed on *P. adscendens* (Table 2). The major lichen compound detected in the snails was the monomer **3** (0.45 μg/snail), whereas its presumable precursor atranorin (**2**) (0.08 μg/snail) was dominant in both the thallus and feces (Figure 2), suggesting preferential sequestration of the monomer **3** by the snails vs. the depside atranorin (**2**). Similar results that suggest a preferential sequestration of the monomer (**3**) compared to its presumable precursor atranorin (**2**) were found in

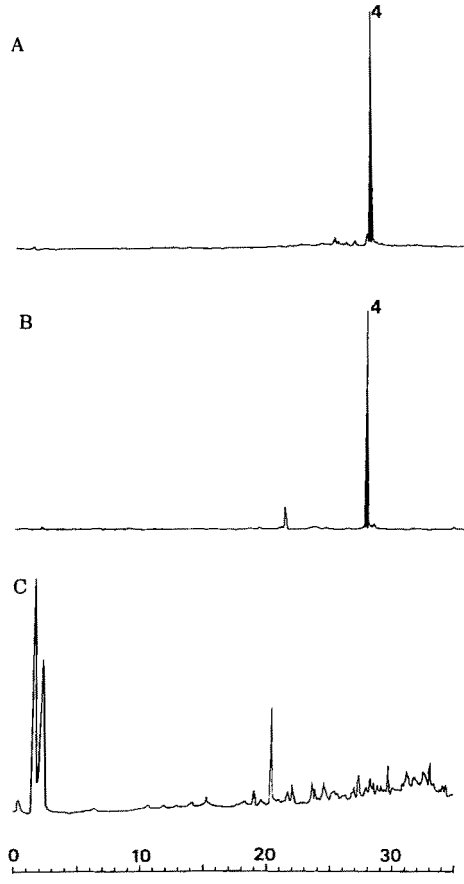

FIG. 3. Comparative HPLC analysis of soft bodies (C) and feces (B) of specimens of *Balea perversa* after feeding on thalli of *Lecanora muralis* (A). Specimens of *B. perversa* were treated as described in the feeding experiment. Numbers of compounds are as in Figure 1.

individuals of *B. perversa* and *H. lapicida* that fed on *P. adscendens*. The monomer (**3**) was the only detectable lichen compound in snails of both species (0.37 μg/snail for *H. lapicida* and 0.04 μg/snail for *B. perversa*), whereas the feces contained both atranorin (**2**) and the monomer (**3**) (data not shown). The anthraquinone parietin (**1**) was another lichen compound detected in specimens of *B. perversa* (0.02–0.13 μg/snail), *C. clienta* (trace–0.06 μg/snail), and *H. lapicida* (0.06–0.15 μg/snail) that fed on thalli of *C. flavovirescens* or *X. parietina*, which both contain parietin (Table 2). Other lichen compounds, however,

such as α-collatolic acid (**5**) (present in *T. atra*) or (+)-usnic acid (**4**) (present in *L. muralis*) were only found in the feces but were not detected in any soft body of the snails analyzed, as exemplified for *B. perversa* in Figure 3. These findings suggest a preferential sequestration of parietin (**1**), atranorin (**2**), and monomer (**3**) by the snails when compared to α-collatolic acid (**5**) or (+)-usnic acid (**4**).

Retention of Sequestered Parietin (1). Following an initial feeding period of 10 days on *X. parietina*, individuals of *C. clienta* were kept either on a parietin-free diet (cyanobacteria) or on a *X. parietina* diet for another 28 days. Subsamples of six snails were removed from both groups at intervals of eight days and analyzed for sequestered parietin (**1**) (Figure 1). The total amount of sequestered parietin (approximately 0.10–0.14 μg/snail) remained almost constant in the group of snails kept on a *X. parietina* diet during the whole experiment, whereas in the group that fed on cyanobacteria the amount of sequestered parietin decreased with time. However, even after being kept on a parietin-free diet for 28 days, the snails still contained traces of the sequestered anthraquinone (0.01 μg/snail compared to 0.10 μg/snail at the beginning of the experiment) (Figure 4).

Sequestered Parietin in Newborn Snails. Sequestered parietin (**1**) was passed on to newborn *B. perversa* that had no feeding experience on lichens. Adult *B. perversa* that were kept on a *X. parietina* diet for 10–66 days gave birth to young that contained parietin at amounts ranging from 2.8 to 3.9 ng/snail (equivalent to concentrations of 3.8–5.3 μg/g fresh wt).

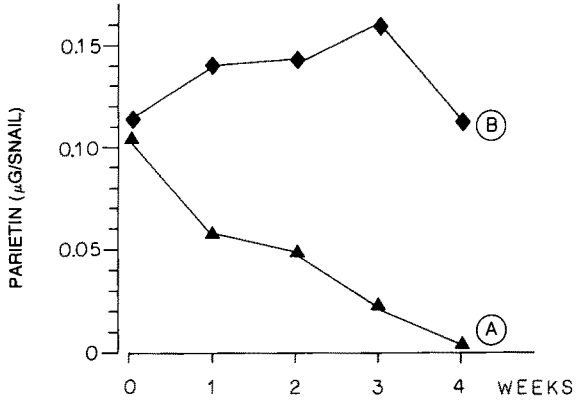

FIG. 4. Time-dependent decrease in the amount of sequestered parietin (**1**) in individuals of *Balea perversa* when fed a parietin-free diet (A). Snails of group B were constantly fed on *Xanthoria parietina*.

DISCUSSION

The present study provides, to our knowledge for the first time, evidence for a sequestration of lichen compounds by lichen-feeding terrestrial gastropods. The lichen compounds parietin (**1**), atranorin (**2**), or the presumable degradation product (**3**) were found (at least in trace amounts) in most of the soft bodies analyzed, whereas both (+)-usnic acid (**4**) as well as α-collatolic acid (**5**) were apparently discriminated by the snails (Table 2 and Figure 3).

Light-microscopic analysis of dissected snails indicated that a starvation period of four days (as used in the feeding experiments) was sufficient to empty the digestive tract of lichen material. The preferential uptake of parietin (**1**) or atranorin (**2**) vs. (+)-usnic acid (**4**), for example, seems not to be due to differences in lipophilicity, which may influence diffusion across membranes. When subjected to partitioning between H_2O and CH_2Cl_2 (the latter representing a lipophilic compartment) almost 100% of compounds **1–5** are recovered from the lipophilic phase (as measured by HPLC; data not shown), indicating that there are no significant differences between the compounds with regard to lipophilicity. Hence, there must be other, so far unknown reasons for the observed differences in sequestration of the various lichen compounds. The discrimination of (+)-usnic acid (**4**) by all of the snails studied (Table 2, Figure 3) is of special interest with respect to the established toxicity of this compound towards invertebrate herbivores. For example, when injected into last-instar larvae of the vigorous insect herbivore *Spodoptera littoralis* (Noctuidae), (+)-usnic acid (**4**) caused pronounced larval mortality (LD_{50} 70 mg/kg body wt), whereas both parietin (**1**) and atranorin (**2**) proved to be nontoxic to the larvae (Emmerich et al., 1993; Giez et al., 1994); α-Collatolic acid (**5**) has not been studied for toxicity towards invertebrates so far. It is possible that the toxicity of (+)-usnic acid (**4**) is not restricted to insects such as *S. littoralis* but extends to other invertebrate grazers including snails. Effective discrimination of usnic acid compared to the demonstrated sequestration of both parietin and atranorin may possibly enable snails to employ even potentially harmful lichens such as *L. muralis* as a food source without a risk of intoxication.

It is so far unknown whether the sequestered lichen substances are of ecological importance to the snails. However, it has been shown that anthraquinone derivatives such as chrysazin and chrysophanol, which are structurally closely related to parietin (**1**), are deterrent towards ants of the species *Myrmica sabuleti* (Hilker et al., 1992). Chrysazin and chrysophanol are used by various chrysomelid beetles for their chemical defense and have also been suggested to protect the eggs of the beetles (Hilker et al., 1992). Marine gastropods of the molluscan subclass Opisthobranchia (the so-called nudibranchs) are notorious for the sequestration of secondary products from dietary sources such as sponges, which in turn are utilized by the nudibranchs for their own chemical defense against

predators such as carnivorous fishes (Hay and Fenical, 1988; Rodgers and Paul 1991; Faulkner, 1992; Proksch, 1994). It is therefore possible that storage of lichen compounds may result in chemical protection of terrestrial snails from predators. In this context it is of interest to note that several species of birds on the island of Öland feed on empty shells of the snails under study but seem to avoid live specimens, suggesting deterrency that might be caused by sequestered lichen compounds (Baur et al., unpublished). Further studies on the ecological role of sequestered lichen compounds in terrestrial snails are underway.

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MORPHOLOGY AND CHEMISTRY OF BROWN RAT, *Rattus norvegicus*, PREPUTIAL AND CLITORAL GLANDS

STEPHAN E. NATYNCZUK,[1] DAVID W. MACDONALD,* and FRANCOISE H. TATTERSALL

Wildlife Conservation Research Unit
Department of Zoology
South Parks Road, Oxford, OX1 3PS, UK

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Abstract—In contrast to previous results from laboratory rats, when sexual dimorphism occurred, clitoral glands from wild female rats were heavier and accounted for a greater proportion of body mass than preputial glands from wild males. Gland length and weight increased with body weight, but gland weight as a proportion of body weight did not. Preputial and clitoral glands showed seasonal differences in size: at one site glands were smaller in December than in April. Maturity, estrus, and pregnancy had no significant effect on clitoral gland size, but immature males had proportionally smaller preputial glands than mature males, and the absolute gland weight, but not gland weight as a proportion of body weight, increased with testes weight. There were no differences between the sexes in lipid production, which increased with preputial or clitoral gland weight. An homologous series of aliphatic acids and their methyl and ethyl esters, plus squalene, cholesterol and lanosterol, was revealed by GC-MS study of the trimethyl silyl derivatized lipid extract. Males and females could be distinguished by principal component analysis of chromatogram peaks.

Key Words—*Rattus norvegicus*, preputial gland, clitoral gland, lipid production.

INTRODUCTION

In rats the specialized sebaceous preputial gland in the male, and the homologous clitoral gland in the female, are situated between the pubic skin and the abdom-

*To whom correspondence should be addressed.
[1]Current address: REBS, Rookery Cottage, Droitwich Road, Bradley Green, Nr Redditch, Worcs. B96 6RT, UK.

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inal muscle. Essentially the same in males and females (Clevedon Brown and Williams, 1972), rat clitoral and preputial glands consist of two pear-shaped lobes connected via short ducts to the free margin of the male prepuce or female clitoris, where they exude their contents.

Albone (1984) stated that in laboratory rats preputial glands measure up to 20 mm in length and contribute 10–20 mg/100 g of body weight. Clevedon Brown and Williams (1972) described preputial glands as being better developed than clitoral glands, but it is not clear whether they referred to the relative sizes or to the chemical complexities of the two glands. Furthermore, there are no data for wild rats, to which laboratory studies (e.g., Clevedon Brown and Williams, 1972; Gawienowski et al., 1975, 1976, Orslak and Gawienowski, 1972; Stacewicz-Sapuntzakis and Gawienowski, 1977; Thody and Dijkstra, 1978) might not apply.

The preputial and clitoral glands are a rich source of lipids of varying complexity (Clevedon Brown and Williams, 1972; Albone, 1984) and are filled with sebaceous material attractive to the opposite sex (Clevedon Brown and Williams, 1972; Brown, 1985). Gawienowski et al. (1976) found that male Sprague-Dawley rats preferred the clitoral gland odors of estrus and sexually receptive females over the odors of anestrus females. Similarly, Thody and Dijkstra (1978) found that males were attracted to the odors of clitoral glands from estrus and proestrus females, but could not differentiate between the two. Males preferred whole-body odors of intact females over those of clitorectomized females. Preputial and clitoral glands also play an essential role in pup survival: dodecyl propionate, an active pheromonelike compound isolated from the preputial and clitoral glands of newborn pups, regulates anogenital licking, a vital maternal behavior (Brouette-Lahlou and Vernet-Maury, 1990). In addition, rats may be able to differentiate between pre- and postpubertal animals by using clitoral or preputial gland odors (Brown, 1985).

We aim to examine the morphology and chemistry of the clitoral and preputial glands in wild rats (*Rattus norvegicus*) by investigating sexual dimorphism in the production and chemical composition of sebaceous material and in gland size, measured as length, weight, and proportion of body weight. We also test for the effects of site, season, maturity, and sexual condition on gland size. Fatty acid metabolism is controlled by sex hormones (Smith et al., 1983), and therefore we would expect differences in the chemical composition of preputial and clitoral gland lipids. Thody and Dijkstra (1978) found no relationship between the size of clitoral glands and their ability to attract males, and so we predict that if female glands function only to attract males, clitoral gland size should not vary with reproductive state. Alternatively, if clitoral and preputial gland secretions have the potential to signal sex and reproductive condition, we might expect the glands to vary in size and/or chemical composition with both sex and hormone status. Testosterone has marked effects on preputial gland

weights (Ebling, 1977; Ebling et al., 1975), and we predict that rat preputial gland size will increase with testis size.

METHODS AND MATERIALS

Anatomical Studies. Using live- and kill-trapping and shooting, 100 male and 150 female wild brown rats, *Rattus norvegicus*, were collected from two Oxfordshire sites: Wytham Farm midden (O.S. grid reference SP 475098) and Tubney Manor Farm, (O.S. grid reference SP 447010). Rats from Wytham were collected during December and April, while rats from Tubney were collected during the end of July, throughout August and at the beginning of September. During April at Wytham, vaginal smears were taken from live-trapped females before they were culled, and estrus was determined by vaginal epithelial cell counts (Zarrow et al., 1964). Scores (1–5) for the abundance of leukocytes (L) and nucleated (N) and cornified (C) cells were used to determine an index of estrus (E) calculated using the formula $E = C/(C + N + L)$. Females were categorized as estrus if $E > 0.7$ or diestrus if $E < 0.3$; intermediate females were not used. Rat carcasses were kept at −20°C until postmortem examination. Culled rats were weighed with the gut removed; left and right preputial or clitoral glands were dissected out, and the mean gland length and weight was recorded for each individual rat. Left and right testes were also excised and a mean testes weight obtained for each male. Sexual maturity was determined by the presence of corpora lutea in females and cauda and caput epididymida in males. Pregnancy was determined by dissection.

Chemistry. Three female and seven male rats, culled from a laboratory colony, were used for chemical analyses. Lipids were extracted from preputial and clitoral glands using a Folch wash (Christie, 1982) prior to being analyzed by gas chromatography with either a FID or mass spectrometer. Preputial or clitoral glands were excised immediately postmortem and were placed in chloroform in clean glass bottles and stored at −20°C. Gland contents were squeezed into chloroform using a clean microspatula. The extract was then pipetted into a clean derivatizing tube and stored under nitrogen at −20°C until saponification and derivatization.

Lipid production was measured by weight of extracted lipid. Each sample was cleared of solvent under a stream of purified nitrogen, and the amount of lipid extracted was weighed before being resuspended in purified hexane. Saponification was accomplished by adding potassium hydroxide (100 μl, 1 M in 95% ethanol) and refluxing the mixture for 2 hr at 100°C. After cooling, hydrochloric acid (60 μl, 3 M) was added. Excess ethanol was removed under a stream of nitrogen, and water was removed by freeze drying. Trimethylsilyl (TMS) ethers were prepared when pyridine was added (50 μml) with *N*-methyl-*N*-trimethylsilyl-trifloroacetamide (100 μl) and the mixture heated for 45 min at 100°C.

A Carlo Erba chromatograph fitted with an OV-1 column (25 m × 0.22 mm ID bonded film) and an FID (air at 300 ml/min, H_2 at 30 ml/min) was used for gas liquid chromatography. The carrier gas was nitrogen at 3 ml/min. A split injection was used with a split ratio of 1 : 100. Chromatograms were recorded and integrated using a Hewlett Packard model 3390A integrator. Naphthalene (20 μg/ml) was used as an internal standard.

Peak identities were confirmed using library references and comparison with authentic compounds. Chromatograms for individual rats were matched by eye. Retention times of peaks from rat lipids were compared to those of a mixture of TMS and ethyl and methyl esters of saturated nC_6 to nC_{24} fatty acids.

Statistical Analysis. Three measures of preputial and clitoral gland size were used: weight (milligrams), proportion of body weight (milligrams per 100 g body weight), and length (millimeters). Gland sizes of rats were analyzed by three-factor analysis of variance, with three two-way interactions, for the effects of body weight, site, and either sex, maturity, pregnancy, estrus, or testes weight. Except when testing for effects of sex, data for males and females were analyzed separately, and except when testing for effects of maturity, only mature rats were used. Within sites, the effects of month, sex, and body weight were tested using three-factor analysis of variance with interactions. One way analysis of variance was used to test within months for sexual dimorphism, and within sexes for pairwise differences between months. Two-way analysis of variance with one interaction was used to test the effects of sex and gland weight on lipid production. Except when modeling factors affecting gland size as a proportion of body weight, models used log-transformed weights and lengths.

Chemical data were summarized using a principal component analysis (PCA) of peak proportions of total peak area for each specimen; glycerol esters were not included. Correlation of chemical peaks was obtained by least-squares regression. Statistical analysis was by SAS (1985).

RESULTS

Preputial and Clitoral Gland Size. Body weight had a significant effect on preputial and clitoral gland lengths (Table 1), with heavier animals having longer glands (Figure 1). Taking into account the effects of body weight, there was a significant difference between preputial and clitoral gland lengths (Table 1): overall females had a shorter mean gland length (143.73 ± 2.26 mm) than males (185.55 ± 3.81 mm). Gland length was also significantly affected by site (Table 1): mean gland length at Tubney was 146.5 ± 3.95 mm, while at Wytham it was 161.07 ± 3 mm. Within sites (Table 2), there remained a significant difference beween sexes ($F_{(1,114)} = 27.13$, $P < 0.0001$ for Wytham; $F_{(1,68)} = 41.7$, $P < 0.0001$ for Tubney). Once sex and body weight effects

TABLE 1. SUMMARY OF ANALYSIS OF VARIANCE FOR EFFECTS OF SEX, BODY, WEIGHT, AND SITE ON THREE MEASURES OF GLAND SIZE

Source of variation	df	MS	*F* value	*P* value
Log gland length				
Log body weight	1	4.512	238.12	<0.0001
Site	1	0.344	18.15	<0.0001
Sex	1	1.063	56.11	<0.0001
Log body wt × sex	1	0.007	0.37	0.5422
Site × sex	1	0.022	1.19	0.2776
Log body wt × site	1	0.000	0.00	0.9509
Residual	190	0.019		
Log gland weight				
Log body weight	1	26.913	98.36	<0.0001
Site	1	4.732	17.29	<0.0001
Sex	1	6.215	22.71	<0.0001
Log body wt × sex	1	0.714	2.61	0.1078
Site × sex	1	2.475	9.05	0.0030
Log body wt × site	1	0.217	0.79	0.3742
Residual	194	0.274		
Gland weight as a proportion of body weight				
Body weight	1	91.679	0.10	0.7480
Site	1	12260.127	13.84	0.0003
Sex	1	10859.764	12.26	0.0006
Body wt × sex	1	4130.547	4.66	0.0320
Site × sex	1	14005.805	15.82	<0.0001
Body wt × site	1	17.785	0.02	0.8875
Residual	194	885.547		

were taken into account, there was no significant difference between July, August, and September samples at Tubney, but at Wytham mean gland lengths were significantly longer ($F_{(1,114)} = 15.73$, $P < 0.0001$) during April than during December (Table 3).

Glands were a constant proportion of body weight (Table 1), with heavier rats having heavier glands (Figure 1). Males had a heavier mean body weight (304.2 ± 9.7 g) than females (238.2 ± 7.2 g), and there was a significant effect of interaction between body weight and sex on gland weight as a proportion of body weight (Table 1). Both sex and site, and the interaction between sex and site, had a significant effect on proportional and absolute gland weight (Table 1). Taking account of body weight, at Wytham females had heavier glands ($F_{(1,119)} = 32.8$, $P < 0.0001$) than males, but at Tubney there was no difference between the sexes (Table 2). Similarly, at Tubney there were no differences between the sexes in gland weight as a proportion of body weight, but at Wytham

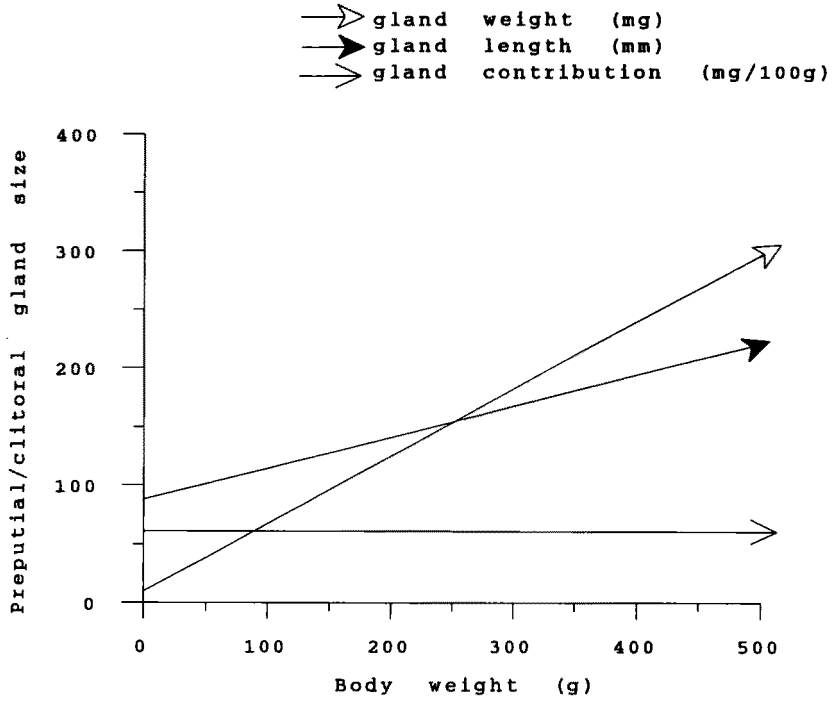

FIG. 1. Lines of best fit showing the relationship between adult rat clean body weight and preputial and clitoral gland size, measured as either gland weight or gland contribution to body weight, and gland length. Gland length and gland weight, but not gland contribution, were significantly affected by body weight (Table 1).

TABLE 2. VARIATION IN ADULT MALE PREPUTIAL AND FEMALE CLITORAL GLAND SIZE WITH SEX AND SITE[a]

			Mean (± SE) gland size		
Site	Sex	*N*	Weight (mg)	Weight (mg)/100 g, clean body weight	Length (mm)
Wytham	F	77	181.6 ± 11.3	66.0 ± 3.5	147.2 ± 2.9
	M	46	127.3 ± 12	38.5 ± 2.9	183.9 ± 4.6
Tubney	F	59	138.1 ± 11	66.9 ± 3.6	139.4 ± 3.5
	M	19	214.0 ± 30	82.8 ± 11	189.3 ± 7.1

[a] Differences between males and females and between Wytham and Tubney were significant for all three measures of gland size.

TABLE 3. THREE MEASURES OF ADULT MALE PREPUTIAL AND FEMALE CLITORAL GLAND SIZES FOR DECEMBER AND APRIL SAMPLES AT WYTHAM

Month	Sex	*N*	Mean (± SE) gland size		
			Weight (mg)	Weight (mg)/100 g clean body weight	Length (mm)
December	F	15	84.7 ± 19	32.6 ± 4.5	122.9 ± 9.6
	M	8	72.9 ± 11	22.0 ± 3.5	173.6 ± 15.2
April	F	62	205.1 ± 11	74.1 ± 3.6	151.9 ± 2.6
	M	38	138.8 ± 14	42.0 ± 3.2	185.9 ± 4.7

female glands were proportionally larger than male glands ($F_{(1,119)} = 34.1$, $P < 0.0001$). At Wytham there was a significant difference between male and female gland size as a proportion of body weight in April ($F_{(1,96)} = 37.9$, $P < 0.0001$), but not during December (Table 3).

Considering seasonal differences in gland weight as a proportion of body weight, month had no effect on gland size at Tubney, which is henceforth considered as a single August sample. For adult females, there was no significant difference in proportional gland weight between August and April, but in December glands were significantly lighter in proportion to body weight than in either April ($F_{(1,75)} = 30.1$, $P < 0.0001$) or August ($F_{(1,73)} = 20.7$, $P < 0.0001$). Adult male preputial gland weight, as a proportion of body weight, was significantly larger in August than in December ($F_{(1,25)} = 12.35$, $P = 0.002$) or April ($F_{(1,55)} = 20.55$, $P < 0.0001$), and again proportional gland weight in December was smaller than in April ($F_{1,44)} = 7.8$, $P = 0.008$).

Immature male rats had, on average, significantly shorter ($F_{(1,88)} = 5.91$, $P = 0.0171$) and lighter ($F_{(1,93)} = 6.09$, $P = 0.0154$) preputial glands than adults, and immature preputial glands were proportionally lighter than glands from mature males ($F_{(1,93)} = 6.28$, $P = 0.014$) (Table 4). For adult males, there was a significant effect of testes weight on preputial gland weight ($F_{(1,58)} = 4.42$, $P = 0.04$). However, testes weight had no significant effect on preputial gland length or preputial gland weight as a proportion of body weight. There was no significant relationship between testes weight and body weight.

When effects of site and body weight were taken into account, there was no significant effect of pregnancy, estrus, or maturity in adult female rats, for any measure of clitoral gland size.

Chemistry. Heavier preputial and clitoral glands produced significantly more lipid than lighter glands ($F_{(1,6)} = 20.6$, $P = 0.004$), but lipid production was not affected by sex or by an interaction between gland weight and sex.

TABLE 4. VARIATION IN THREE MEASURES OF MALE RAT PREPUCE GLAND SIZE WITH SITE AND MATURITY[a]

Month	Maturity	N	Mean (± SE) gland size		
			Weight (mg)	Weight (mg)/100 g clean body weight	Length (mm)
Wytham	Immature	19	45.3 ± 6.3	27.5 ± 2.7	130.2 ± 8.4
	Mature	46	127.3 ± 12	38.5 ± 2.9	183.9 ± 4.6
Tubney	Immature	16	55.7 ± 7.8	42.4 ± 4.5	130.5 ± 5.2
	Mature	19	214.0 ± 30	82.8 ± 11.1	189.3 ± 7.1

[a]Glands from mature males were significantly larger than glands from immature males.

A GC-MS study of the TMS derivatized lipid extract revealed an homologous series of aliphatic acids and their methyl and ethyl esters, plus squalene, cholesterol, and lanosterol. Chromatogram peaks were assigned as shown in Table 5, and chromatograms of TMS-derivatized samples are summarized in Table 6. Correlations between adjacent peaks (Table 7) suggest an even rate of lipid synthesis by the preputial and clitoral glands. Principal components analysis based on the correlation matrix revealed that the first four principal components account for 83% of the variation in the samples and provide a good summary of the data (Table 8). Between-sample variation was explained by a single factor in the first principal component. Peaks A–K share a similar positive loading on the first eigenvector and contrast with the negative loadings for peaks O, P, Q, and R. The interpretation of eigenvectors 2, 3, and 4 is not obvious.

A plot of the first and second principal components (Figure 2) grouped males and females separately along the first principal component axis. The single pregnant female separated from the two nonpregnant females along the first principal component axis, and the juvenile males separated from the adults along both the first and second principle component axes.

DISCUSSION

The compounds identified in our study support previous work with laboratory rats by a variety of authors (reviewed by Clevedon Brown and Williams, 1972; Albone, 1984) with the exception of Stacewicz-Sapuntzakis and Gawienowski (1977). Generally the compounds present in the greatest quantity were the $C_{18:1}$ methyl ester, $C_{18:1}$ ethyl ester, $C_{18:1}$ acid, $C_{18:0}$ acid, squalene, and cholesterol. In addition to the list presented in Table 5, GC-MS revealed a mixture of polyunsaturated methyl esters and glyceryl esters. The presence of

TABLE 5. QUALITATIVE ASSIGNMENT OF SOME CHROMATOGRAM PEAKS BY GC-MS BASED ON PRESENCE OF TMS ESTERS

Peak	Peak assignment
A	phosphate
B	glycerol
C	C_{10} ethyl ester
D	C_{12} acid
E	C_{14} methyl ester
F	C_{14} ethyl ester
G	$C_{16:1}$ methyl ester
H	C_{16} methyl ester
I	$C_{16:1}$ ethyl ester
J	C_{16} ethyl ester
K	$C_{16:1}$ acid
L	O_{16} acid
M	$C_{18:2}$ methyl ester
N	$C_{18:1}$ methyl ester
O	C_{18} methyl ester
P	$C_{18:1}$ ethyl ester
Q	$C_{18:1}$ acid
R	C_{18} acid
S	mixture of polyunsaturated methyl esters
T	glyceryl esters
U	squalene
V	cholesterol
W	lanosterol

glyceryl esters indicates their incomplete hydrolysis at the saponification stage of sample preparation in the particular sample used for GC-MS. To what extent this affected the final analysis (glyceryl esters were not used in the statistical appraisal of the chromatograms) is difficult to determine because it is not known which acids occurred as glyceryl esters, in what proportions, or whether the glyceryl esters were mono-, di-, or triglycerides of mixed or homogeneous acids. However, it seems unlikely that the saponification failed unpredictably because PCA analysis did not reveal a random distribution, but distinguished between males and females.

In contrast to the established view that the preputial gland is better developed than the clitoral gland (Albone, 1984; Clevedon Brown and Williams, 1972; Brown, 1985), we found that in April at Wytham, where there was a significant difference between the sexes, female clitoral glands were heavier and accounted for a higher proportion of body weight than male preputial glands. Preputial glands, however, were consistently longer than clitoral glands. The

TABLE 6. SUMMARY OF CHROMATOGRAMS OF TMS-DERIVATIZED SAMPLES OF RAT PREPUTIAL AND CLITORIAL GLAND LIPID EXTRACTS SHOWING PROPORTIONAL PEAK AREAS FOR FEMALES F1–F3, AND MALES M1–M7, AS A PERCENTAGE OF TOTAL ASSIGNED PEAK AREAS

Peak	F1	F2	F3	M1	M2	M3	M4	M5	M6	M7
A	1.0	0.0	0.6	1.4	1.4	1.5	1.4	1.9	1.7	0.7
B	0.0	0.0	15.9	16.9	13.8	16.5	17.0	14.7	16.3	7.9
C	0.0	0.0	0.0	0.2	0.3	0.0	0.5	0.4	0.4	0.3
D	0.5	0.0	0.0	0.0	0.4	0.8	0.0	0.7	0.6	0.9
E	0.5	0.0	0.2	1.2	1.4	1.3	0.0	1.4	1.2	0.8
F	0.0	0.0	0.0	1.3	1.4	0.2	1.8	1.0	1.6	1.3
G	0.0	0.3	0.4	1.6	1.6	1.9	0.2	2.1	0.8	2.1
H	2.5	0.6	0.3	3.7	1.6	1.6	0.8	1.6	2.2	2.7
I	0.0	1.8	0.7	2.3	2.3	2.0	0.2	2.6	0.8	2.5
J	1.8	1.9	1.9	3.2	4.1	2.7	2.7	2.9	3.3	2.8
K	0.0	2.1	0.7	1.3	3.7	0.6	2.5	2.5	3.5	3.3
L	0.0	11.8	2.9	4.1	9.3	2.5	5.4	7.5	9.7	8.3
M	5.3	2.2	1.4	3.8	2.5	2.3	0.7	3.1	4.5	6.2
N	18.0	6.4	3.5	13.8	7.1	10.4	0.6	6.2	6.3	12.7
O	4.5	9.4	6.7	2.6	6.1	2.3	2.4	4.6	2.7	4.5
P	17.5	10.8	23.6	17.1	16.4	16.3	21.5	9.2	7.8	14.1
Q	15.6	48.7	4.2	4.4	6.0	8.9	3.7	9.9	11.4	1.3
R	10.2	4.0	20.9	8.6	11.5	12.5	17.9	8.8	8.8	9.6
U	17.7	0.0	8.4	9.7	7.5	5.8	8.0	7.2	11.8	9.0
V	3.3	0.0	7.7	0.6	0.0	9.8	13.0	9.1	4.7	5.7
W	1.5	0.0	0.0	2.2	1.8	0.0	0.0	2.7	0.0	3.3

most obvious reason for our unusual results is that we worked with wild rats, whereas former results have been based on laboratory rats. Berdoy and Macdonald (1991) have reviewed instances where extrapolation from the laboratory to natural rat populations proved unjustified.

Seasonal effects on proportional gland weight were evident at Wytham: for both male and female rats, gland sizes were smaller during December than during April. However, it is impossible to say whether differences between the Tubney and Wytham samples were the results of seasonal or site effects. Although the only sample for which there was a significant difference between the sexes was April at Wytham, a reversal in the relative sizes of clitoral and preputial glands was the most marked difference between sites. Effects of site may be at least as important as effects of season: Stockley et al. (1994) found that seminal vesicle weights for male shrews (*Sorex araneus*), measured at the same time of year, differed significantly between sites. Differences between Wytham and Tubney rats might have arisen because of habitat differences. At

TABLE 7. CORRELATION COEFFICIENTS (×100) BETWEEN ASSIGNED CHROMATOGRAPH PEAKS[a]

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	U	V
B	65*																			
C	57	48																		
D	39	−01	15																	
E	74*	43	22	60																
F	56	55	92***	10	33															
G	46	38	20	62	81**	32														
H	37	02	10	36	57	33	44													
I	15	14	06	36	62	18	89***	29												
J	64*	57	57	26	75*	76**	61	39	50											
K	18	19	74*	23	26	75*	33	02	37	67*										
L	−17	−09	40	05	06	37	20	−17	45	37	84**									
M	004	−39	−00	61	36	07	29	77**	18	09	13	01								
N	−01	−47	−45	36	31	−29	20	77**	13	−09	−40	77**								
O	−76**	−60	−45	−34	−42	−54	−27	−53	11	−37	03	42	−17	−16						
P	−20	20	−26	−50	−44	−17	−35	−24	−44	−25	−49	−65*	−45	−11	−07					
Q	−57	−69*	−41	−27	−41	−51	−40	−37	−16	−46	−09	42	−11	−02	68*	−42				
R	06	51	05	−26	−31	02	−26	−44	−48	−13	−25	−52	−53	−45	−21	82**	−59			
U	34	−04	08	23	17	10	−20	53	−50	01	−24	−59	55	53	−48	17	−50	18		
V	34	47	36	20	−16	15	00	−36	−28	−19	−11	−33	−37	−45	−49	28	−43	62	02	
W	19	−12	21	42	47	28	64*	63*	59	32	25	07	65*	51	−07	−21	−35	−33	24	−23

[a] $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

TABLE 8. EIGENVALUES OF CORRELATION MATRIX[a]

Principal component	Eigenvalue	Difference	Proportion	Cumulative
1	6.789	2.014	0.323	0.323
2	4.775	0.738	0.227	0.551
3	4.037	2.209	0.192	0.743
4	1.829	0.517	0.087	0.830
5	1.312	0.223	0.062	0.892
6	1.089	0.500	0.052	0.944
7	0.588	0.204	0.028	0.972
8	0.384	0.186	0.018	0.991
9	0.198	0.198	0.009	1.000

[a]Eigenvalues 10 to 20 make no contribution.

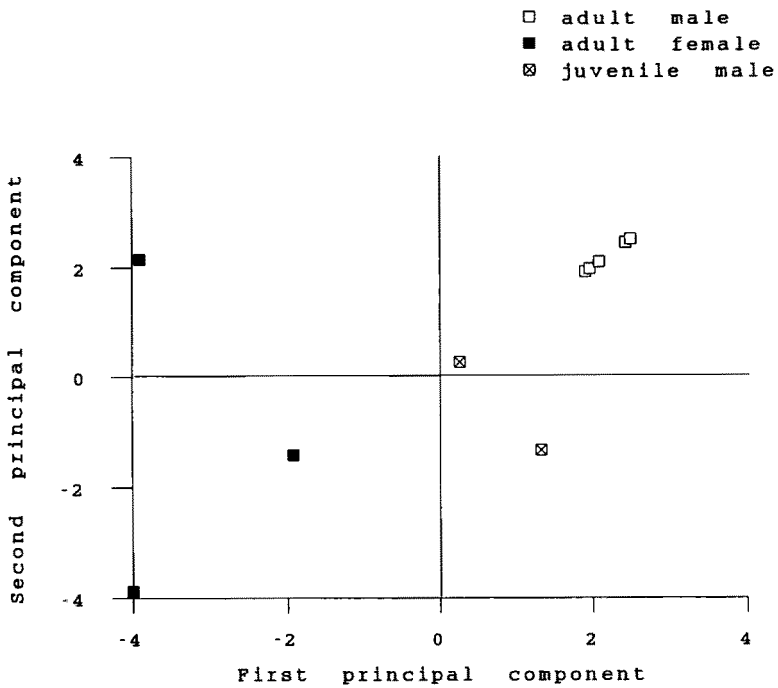

FIG. 2. Plot of first and second principal components, obtained by principal component analysis of a chromatograph peak correlation matrix using lipid produced from the clitoral glands of three females and the preputial glands of seven males. The female in the center of the plot was pregnant.

Wytham rats lived on an agricultural rubbish tip, while at Tubney rats lived in farm buildings and among grain silos (Fenn, 1989).

During December, when gland weight as a proportion of adult body weight was smallest, rats were not breeding (Fenn, 1989), suggesting that seasonal variation in gland size might be the result of seasonal fluctuations in hormones associated with breeding. Ebling (1977) noted that estrogen reduced the output of skin sebaceous glands and had a similar effect on the clitoral glands, as well as on lipogenesis in general. In our wild female rats, however, clitoral gland size did not fluctuate with the reproductive cycle, nor with pregnancy or maturity, although principal component analysis of chromatogram peaks separated a single pregnant female from two nonpregnant females. Females also showed less variation between samples than did males.

In contrast to estrogen, testosterone markedly increases preputial gland weights (Ebling, 1977; Ebling et al., 1975). Sexually mature male rats had larger preputial glands than immature male rats, and gland weight increased with testes weight, although proportional gland weight and gland length did not. The interpretation of these results remains obscure because there was no correlation between testes size and body weight. If the relationship between testes weight and preputial weight was not an artifact of correlation with body weight, testes weight appears to act on preputial glands by increasing lipid production rather than gland capacity.

Changes in gland size reflect changes in the amount of lipid available for secretion. Thus, a decline in gland size during the winter might be indicative of a reduced frequency of scent-marking, associated perhaps with a reduction in territoriality and breeding. Male preputial glands were affected by maturity and possibly by testes size and showed greater variation between seasons than female clitoral glands. Such variability might indicate that the male preputial gland has a greater range of functions than the clitoral gland and that males vary their use of preputial secretion in scent-marking depending on their status. The relatively consistent size of clitoral glands during the breeding season, and their reduction in size during the winter, is consistent with the hypothesis that the glands function only to attract males by signaling their sex, but do not rule out the possibility of chemical changes in gland secretions that might signal hormonal status. Indeed, chemical analysis of clitoral gland secretions suggested that this might be the case.

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Book Review

Chemical Ecology in Thrips–Host Plant Interactions. T.N. Ananthakrishnan and R. Gopichandran. New York: International Science Publisher, 1993. Hardback US$59.00, 125 pp. ISBN1-881570-23-1. (Originally published in India by Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi.)

About 5000 species of thrips are known worldwide. Many species are flower dwellers or live on leaves, a few are predatory, and most feed on fungi. Some are serious economic pests of crops either by feeding on plants or by serving as vectors of plant disease. Considerable progress has been made in understanding the taxonomy and ecology of thrips. This book explores the lesser known biochemical aspects involved in thrips–host plant interactions. The premise is that knowledge of the chemical ecology of thrips–host plant interactions is vital to develop management strategies such as resistant plants for agriculturally important thrips pests.

The book is divided into eight chapters. The first chapter introduces the dimensions of chemical ecology of thrips–host plant interactions. An overview of the biochemical basis of host selection by thrips is presented, and the need to understand resistance mechanisms when developing resistant crops is emphasized. Chapter two generally explores the factors influencing utilization and survival of insects on plant resources, with specific emphasis and review of information on thrips. The chapter is divided into sections on behavioral and physiological aspects, the environment of plants, sensillar diversity in thrips, the role of primary and secondary compounds, and environmental stresses. Chapter three provides a discussion of thrips feeding mechanics and the responses of plant hosts to thrips injury. The biochemical profiles of host plants in relation to thrips infestation along with detailed examples are discussed in the fourth chapter. Chapter five describes the chemical ecology of anthophilous thrips, including the chemical basis of flower color and the chemical components of nectar and pollen. Other aspects of the coadaptation of thrips and flowering plants are discussed. The resistance mechanisms in thrips–host plant interactions, including induced resistance, static plant defense, and tolerance, are given in chapter six. Chapter seven describes the biochemical relationships between thrips galls and plant hosts. The last chapter outlines some commonly employed techniques used in quantitative assays in chemical ecology research.

The authors are experienced researchers in the chemical ecology of thrips. The senior author currently is the Director of the Entomology Research Institute,

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Loyola College, Madris. This book on chemical ecology follows previous books by him on the bioecology and the reproductive biology of thrips. The coauthor is a senior research associate at the Entomology Research Institute who has worked in collaboration with the senior author on research projects.

Thrips are a diverse and biologically interesting group of insects. This book provides a much-needed review and discussion of published information relevant to thrips–host plant interactions. Entomologists interested in chemical ecology of insects in general or thrips specifically will find the book an information resource. Entomologists interested in determining economic injury levels for use in integrated pest management programs will find a good review of literature and discussion of plant response to thrips feeding. Another practical application of knowledge of chemical ecology of thrips–host plant interactions is development of resistant crops. The discussion in this book about expected sustainability of resistant crops developed utilizing different resistance mechanisms is especially relevant. The book is attractive, with numerous tables and figures of varying quality. In some cases it is difficult to determine the appropriate cited source for discussed information. Considering the purchase price, an entomologist should choose to purchase this book for their personal library based on interest in chemical ecology or management of thrips.

Joe E. Funderburk
North Florida search & Education Center
University of Florida
Quincy, Florida 32351

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ANTIGNAWING FACTOR OF CRUDE OIL DERIVED FROM *Thujopsis dolabrata* S. et Z. var. *hondai* SAWDUST AGAINST MICE

YOUNG-JOON AHN,[1,*] SUNG-BAEK LEE,[1] TSUTOMU OKUBO,[2] and MUJO KIM[2]

[1]*Department of Agricultural Biology, College of Agriculture and Life Sciences, and Research Center for New Bio-Materials in Agriculture, Seoul National University, Suwon 441-744, Republic of Korea*

[2]*Central Research Institute, Taiyo Kagaku Co., Ltd., Yokkaichi, Mie 510, Japan*

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Abstract—The antignawing activity of the steam distillate of *Thujopsis dolabrata* S. et. Z. var. *hondai* sawdust against mice was evaluated using a wire-dipping method. This method is suitable for measuring repellent activities of chemicals and plant-derived materials. The steam distillate of the *Thujopsis* sawdust was shown to have potent antignawing activity. Three terpenoid compounds with antignawing activity were isolated from the steam distillate of the *Thujopsis* sawdust: thujopsene, carvacrol, and β-thujaplicine. Carvacrol exhibited the most potent repellent activity. As a naturally occurring repellent, carvacrol could be useful as new preventive agent against various kinds of damage caused by rodents.

Key Words—Rodent, mouse, repellent, antignawing activity, wire-dipping method, *Thujopsis dolabrata* S. et. Z. var. *hondai*, carvacrol, terpenoid.

INTRODUCTION

Rodents are cosmopolitan in their distribution and approximately 1800 species have been described. They cause great damage not only in agriculture, but also contribute significantly to the generation of serious problems for communication

*To whom correspondence should be addressed.

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systems and structures. Control is primarily dependent upon continued or repeated applications of rodenticides. Although they have effectively controlled rodents, their extensive use for the past decades has led to the development of resistance to rodenticides (MacNicoll, 1986), persistence of residues, adverse effects on nontarget organisms, environmental problems, and human health hazards (Lund, 1983; Dubock, 1986). Increasing concern over adverse effects of the earlier types of rodenticides have brought about the need for the development of new types of selective alternatives for controlling rodents. Recently, much concern has been focused on plants as an alternative to the rodent control agents currently used, because plants are the richest source of bioactive organic chemicals (Harborne, 1988).

The most suitable alternative may be a rodent repellent, because for each rat killed with poison there are others in the area who survive. However, relatively little recent work has been carried out on repellents produced by plants compared to other aspects of rodent control. Although the rodent repellent cycloheximide from *Streptomyces griseus* is very effective, this compound irritates human skin very strongly (Budavari et al., 1989). During initial experiments for screening rodent repellents from plants, the tree *Thujopsis dolabrata* S. et. Z. var. *hondai*, the only Japanese *Thujopsis* species, was found to be resistant to rodent attacks. For example, a small pagoda (Nara, Japan) made from *T. dolabrata* is in a well-preserved state with no evidence of damage by various detrimental organisms such as rodents, insects, and microorganisms although it is almost 1200 years old (Kawaguchi et al., 1989). We have therefore examined the rodent repellent components of this tree as potential natural alternatives to toxic chemicals.

METHODS AND MATERIALS

Animals and Housing Conditions. Female SPF mice (Slc: ICR strain, 24–26 g) were purchased from SLC Co., Japan. Animals were allowed to acclimate to their new housing for two weeks prior to the test. They were approximately six weeks of age at the initiation of the study. They were randomly assigned to groups and housed, three per plastic cage, on aspen chip bedding (Soft Chip, Japan SLC Co., Ltd.) under conditions of controlled temperature (25 ± 1°C), 50–60% relative humidity, and a photoperiod of 12L:12D. Cages and food containers were changed every three days. Rodent Chow (MF, Oriental Yeast Industry, Co., Ltd.) and water were provided *ad libitum*.

Sample Preparation. The sawdust of *T. dolabrata* (Family Cupressaceae) was obtained from Aomori Forest Experimental Station, Aomori, Japan.

Isolation and Identification. The sawdust (42 kg) of *T. dolabrata* was subjected to steam distillation (Figure 1). Extraction of the distillate with ether

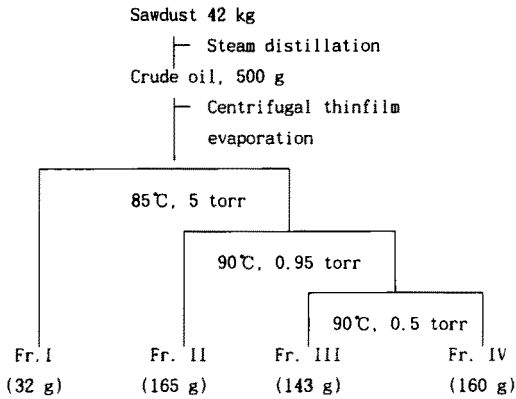

FIG. 1. Procedure of steam distillation of *Thujopsis dolabrata* S. et. Z. var. *hondai* sawdust and partition by centrifugal thin-film evaporation.

gave 500 g of a dark brown crude oil (1.2% yield). The crude oil was separated into fractions I (32 g), II (165 g), III (143 g), and IV (160 g), using a centrifugal thin-film evaporator for subsequent bioassay (Figure 1). Fraction I (1.18 g) was chromatographed on a silica gel column (Fuji-Davidson BW-350, 5 × 100 cm), and eluted with hexane–ethyl acetate (10:1). Column fractions were analyzed by TLC (chloroform–MeOH, 10:3), and the active fractions were combined.

Further separation of the biologically active components was carried out with HPLC (Hitachi 655A-11 LC). Develosil 60-5 (Nomura Chemical Co., Ltd) and Cosmosil $_5C_{18}$ column (Nacalai Tesque Co., Ltd.) were used with hexane–ethyl acetate (9:1) and acetonitrile–water (10:1), respectively. The detector wave length was 254 nm.

Structural determination of the active isolates was made by spectral analysis. ^1H and ^{13}C NMR spectra were recorded with a JEOL GSX-400 spectrometer (TMS as an internal standard) and chemical shifts were given in δ (ppm). UV spectra were obtained on a Shimadzu MPS-2000 spectrometer, IR spectra on a Shimadzu IR-460 spectrophotometer, and mass spectra on a JEOL JMS-DX 30 spectrometer.

Bioassay. Plastic-coated flexible electric cable was cut into 10-cm segments, and dipped in the 10% ethanol solution of the *Thujopsis*-derived materials for 3 min and allowed to dry. Control segments were prepared by dipping the cable in ethanol for 3 min. Ten segments treated with the test material, ten control segments and three female mice were placed in the same cage and the temperature was kept at 25 ± 1°C. All tests were conducted in triplicate. The mouse-induced damage state of the segments was observed two days after treat-

ment (DAT) for a rapid screening of active components. The responses to the *Thujopsis*-derived materials were compared with those to the controls.

The antignawing activities of the most active isolate during the 15-day test period were examined and compared with those of cycloheximide and two commercial terpenoids, thujone and thymol, which are known to have rodent repellent activity (Harding, 1985; Hansson, 1988). Tests were made in triplicate. The antignawing value (AV) of the test samples was calculated from the following formula. $AV = (0 \times n_1 + 1 \times n_2 + 2 \times n_3 + 3 \times n_4 + 4 \times n_5)/(n_1 + n_2 + n_3 + n_4 + n_5)$, where 0 = no damage in the wire segment; 1 = one damage spot in the segment; 2 = two damage spots in the segment; 3 = three to five damage spots in the segment; 4 = heavily damaged; and where n_1 to n_5 are the numbers of individuals showing grade 0 to 4 in each treatment.

Statistical Analysis. Data were subjected to ANOVA. Means were compared and separated by Tukey's studentized range test (SAS Institute, 1985).

RESULTS

During the initial experiments, we observed that the crude oil obtained by steam distillation of *T. dolabrata* sawdust possessed repellent activity on mice in the treated wire assay. The mice did not gnaw the treated wire segments, but gnawed heavily the control ones. Thus, this method is suitable for the measurement of repellent activity of compounds of synthetic or natural origin. Figure

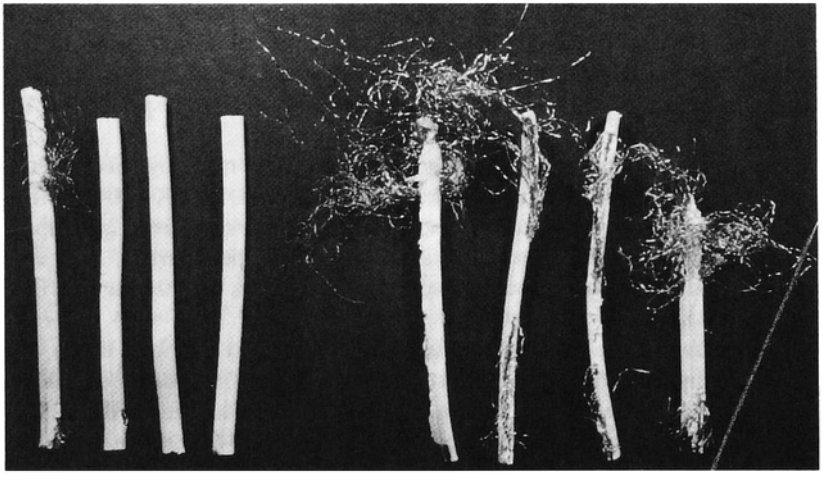

FIG. 2. A typical example of damage to wire after application of steam distillate derived from *T. dolabrata* sawdust (left) compared with control (right).

2 shows a typical example of the protective effect of the crude oil derived from the plant.

The fractions obtained from the crude steam distillate from the *Thujopsis* sawdust were assayed in the same manner. Ten percent ethanol solutions of fractions I and II showed strong (++) and weak (+) antignawing activity against mice. Purification of the biologically active compounds from fraction I (1.18 g) was done by silica gel column chromatography and HPLC, and 10% ethanol solutions of the isolates were bioassayed. Three isolates showed antignawing activity. Structural determination of the isolates was made by spectral techniques, and they were characterized as terpenoids: thujopsene (1530 mg), carvacrol (4 mg), and β-thujaplicine (10 mg) (Figure 3). For example, carvacrol was identified based on the following evidence: the molecular ion peak at *m/z* 150 (M^+) on the EI-MS (70 eV); IR (neat) ν_{max} cm^{-1}, 3375, 1616, 995; ^1H NMR ($CDCl_3$): δ 1.24 (6H, *d*, *J* = 8 Hz), 2.22 (3H, *s*), 2.83 (1H, *d*, *J* = 8 Hz), 6.66 (1H, *s*), 6.73 (1H, *d*, *J* = 8 Hz), 7.03 (1H, *d*, *J* = 8 Hz); ^{13}C NMR ($CDCl_3$); δ 15.3, 24.0, 33.7, 113.0, 118.8 *s*, 130.8, 148.4, 153.6. The structures of thujopsene and β-thujaplicine were identical with authentic samples by ^1H and ^{13}C NMR, MS, and IR.

The antignawing activities of 5% ethanol solutions of the three isolates were investigated. Carvacrol showed potent antignawing activity. Moderate activity was observed for thujopsene and β-thujaplicine.

The persistence of the repellent effects on mice, compared with that of the commonly used cycloheximide, was studied by assaying the damage to wire segments dipped in 5% ethanol solutions of the test materials over a 15-day period (Table 1). Untreated wire segments were heavily damaged during the test period. The antignawing value was 4.0 at six days after treatment. Carvacrol showed antignawing activity for a relatively short period (approximately six days), while cycloheximide provided protection for the full duration of the test.

DISCUSSION

Rodent control is most commonly dependent upon repeated application of rodenticides. However, these compounds have attendant problems (Meeham, 1984). In addition, rodents exhibit shyness behavior after exposure to toxic

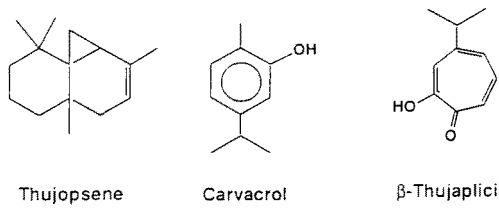

FIG. 3. Structures of terpenoids isolated from *T. dolabrata* sawdust.

TABLE 1. ANTIGNAWING ACTIVITIES OF TEST MATERIALS DURING 15-DAY TEST DETERMINED BY WIRE-DIPPING METHOD

Test material	Antignawing value[a]						
	Day 1	Day 2	Day 3	Day 6	Day 9	Day 12	Day 15
Untreated	2.3 ± 0.2a	2.7 ± 0.2a	2.8 ± 0.2a	4.0a	4.0a	4.0a	4.0a
Thymol	0.8 ± 0.1b	1.1 ± 0.1b	1.5 ± 0.2b	2.3 ± 0.3b	2.7 ± 0.4b	2.9 ± 0.5ab	3.0 ± 0.4ab
Thujone	0.4 ± 0.1bc	0.9 ± 0.2b	0.9 ± 0.3bc	1.8 ± 0.3b	3.0 ± 0.6ab	3.3 ± 0.6a	3.5 ± 0.5ab
Carvacrol	0c	0c	0.5 ± 0.2cd	0.9 ± 0.1c	1.1 ± 0.1c	2.0 ± 0.2b	2.5 ± 0.5b
Cycloheximide	0c	0c	0d	0d	0d	0c	0c

[a]Means followed by the same letter in each column are not significantly different [$P = 0.05$; Tukey's studentized range test (SAS Institute, 1985)].

chemicals (rat: Barnett et al., 1975; Prakash et al., 1975; and mouse: Rao and Prakash, 1980). Prakash (1976) reported that the shyness among rodents persists for 35 to 150 days. The adverse effects and shyness to poisons call for alternative control agents such as repellents rather than attempts to kill the rodents.

Certain plant-derived extracts have repellent properties against rodents (Harding, 1985; Hansson, 1988). In the present study, the steam distillate from *T. dolabrata* sawdust showed potent antignawing activity. Additionally, pharmacological investigations have shown that this plant was antimicrobial (Ito et al., 1980), hypocholesterolemic (Enomoto et al., 1977), and antitermitic effects (Nakashima and Shimizu, 1972; Ikeda et al., 1978).

Various compounds including phenolics, terpenoids, and alkaloids exist in plants and jointly or independently contribute to repellent activities (Hansson, 1988; Harborne, 1988). Many *Thujopsis* species are rich in terpenoids (Hasegawa and Hirose, 1982; Ohgaku et al., 1984; Yatagai et al., 1985). In our study, three terpenoids (carvacrol, thujopsene, and β-thujaplicine) with antignawing activity were isolated from the *Thujopsis* sawdust, and carvacrol was most potent. Harding (1985) also reported that thujone oil of *Artemsia absinthium* strongly repelled rodents.

The *Thujopsis* terpenoids were effective against mice only for a relatively short period of time, typically a few days. Carvacrol was the most effective, probably due to its high volatility in air. It has been also reported that the steam distillate of *Thujopsis* sawdust and its terpenoids carvacrol and β-thujaplicine have potent insecticidal and repellent activities against some stored-product and household insect pests (Lee, 1993) and termites (Ikeda et al., 1978; Chaboussou, 1978; Lee, 1993). These terpenoid compounds might be used as pest controls agent in limited spaces such as storage bins, greenhouses, or buildings, because of their high volatility, although the effects of these terpenoids on humans remain unknown. These results suggest that an appropriate carrier will be needed for slow release into the air. Harding (1985) recommended a suitable liquid or solid carrier such as lacquer, sodium silicate, or paraffin. A combination of thujone oil and lacquer (12:88), when used like a paint, has been reported to keep rodents away from the painted area for between three and five years (Harding, 1985).

Based upon our data and these earlier findings, carvacrol would be expected to protect against damage by rodents for a relatively long time if a carrier with slow-release properties could be selected or developed. Carvacrol has low toxicity (Worthing and Walker, 1987; Budavari et al., 1989) and causes no mutagenicity, when tested against four strains of *Salmonella typhimurium* (Lee, 1993). An average of 4000 tonnes of sawdust per year are produced as a by-product of the timber industry, because *T. dolabrata* is an important timber species in Japan (Kaburagi et al., 1969). Thus there is an adequate source of the crude oil available.

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DEVELOPMENTAL STAGE OF HERBIVORE *Pseudaletia separata* AFFECTS PRODUCTION OF HERBIVORE-INDUCED SYNOMONE BY CORN PLANTS

J. TAKABAYASHI,[1,*] S. TAKAHASHI,[1] M. DICKE,[2] and M.A. POSTHUMUS[3]

[1]*Pesticide Research Institute*
Faculty of Agriculture
Kyoto University
Kyoto 606-01, Japan

[2]*Department of Entomology*
Wageningen Agricultural University
P.O. Box 8031, 6700 EH Wageningen, The Netherlands

[3]*Department of Organic Chemistry*
Wageningen Agricultural University
Dreijenplein 8
6703 HB Wageningen, The Netherlands

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Abstract—The female parasitic wasp *Cotesia kariyai* discriminated between the volatiles of corn leaves infested by younger host larvae *Pseudaletia separata* (first to fourth instar) and uninfested leaves in a Y-tube olfactometer; the wasps were attracted to the infested leaves. In contrast, when corn plants were infested by the later stages (fifth and sixth instar) of the armyworm, the wasps did not distinguish between infested corn leaves and uninfested corn leaves in the olfactometer. Mechanically damaged leaves were no more attractive than undamaged leaves, and host larvae or their feces were not attractive to the parasitoid. Through chemical analysis, the herbivore-induced plant volatiles were identified in the headspace of infested corn leaves. The herbivore-induced volatiles (HIVs) constituted a larger proportion of the headspace of corn leaves infested by early instar armyworms than of corn leaves infested by late instar armyworms. Application of third-instar larval regurgitant onto artificially damaged sites of leaves resulted in emission of parasitoid attractants from the leaf, whereas leaves treated with sixth-instar regurgitant did not. The function of this herbivore-stage related specificity of herbivore-induced synomones is discussed in a tritrophic context.

*To whom correspondence should be addressed.

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Key Words—*Pseudaletia separata*, *Cotesia kariyai*, *Apanteles*, herbivore-induced synomone, Y-tube olfactometer, leaf volatiles, terpenoids, indole, regurgitant, herbivore developmental stage specificity.

INTRODUCTION

Plants defend themselves against herbivores in various ways. These may comprise plant characteristics that affect herbivore performance directly or indirectly through natural enemies of the herbivores (Price et al., 1980; Price, 1981; Rhoades and Cates, 1976; Rhoades, 1985; Dicke and Sabelis, 1988a). These defense mechanisms may function constitutively (Rhoades and Cates, 1976; Price, 1981) or they may be induced by herbivory (Tallamy and Raupp, 1991; Dicke et al., 1990b; Dicke and Sabelis, 1988a; Takabayashi et al., 1994; Turlings et al., 1990).

In this study, we have investigated induced indirect defense of plants, involving the attraction of natural enemies to plants that are infested by herbivores. Plants that are infested by herbivores may emit so-called "herbivore-induced synomones" (Dicke and Sabelis, 1988b; Vet and Dicke, 1992). These are chemicals that are produced by the plant in response to damage done by specific herbivores. The chemicals are not emitted, or are only emitted in trace amounts, upon mechanical damage. Natural enemies of the herbivore use these chemicals in long-/short-range herbivore location (Dicke et al., 1990a,b; Sato, 1979; Turlings et al., 1990). Herbivore-induced synomones may be very specific stimuli: they may be specific for the herbivore species, the plant species, and/or the plant cultivar (Dicke et al., 1990b; Takabayashi and Dicke, 1993; Takabayashi et al., 1994; Turlings et al., 1993; Dicke, 1994). The value of this specificity has been discussed by Vet and Dicke (1992), who hypothesized that natural enemies of herbivores are faced with a reliability–detectability problem during foraging for their herbivore victims. Contact kairomones left by herbivores are the most reliable in indicating herbivore presence and identity, but they are difficult to detect from a distance (high reliability and low detectability). On the other hand, constitutively emitted plant volatile synomones are more detectable at a distance but less reliable in indicating herbivore presence and identity (low reliability and high detectability). Herbivore-induced synomones may combine the high detectability of plant volatiles with a high reliability in indicating herbivore presence. For plants, the specificity of the herbivore-induced synomones may be an important characteristic because it could ensure that the right natural enemy species is attracted.

Moreover, many parasitoid species are more specific and can attack only a limited range of host developmental stages within a species (Waage and Great-

head, 1986). If parasitized herbivores inflict less total damage to a plant than unparasitized herbivores, attraction of the natural enemies during an early phase of herbivore infestation would be beneficial. Yet, if quantities of herbivore-induced synomones are correlated to the extent of herbivory, the natural-enemy attracting stimuli would be expected to be most abundant during the later phase of herbivory. Thus, we were primarily interested in the question of whether the emission of herbivore-induced synomones is specific for the herbivore instars inflicting the damage. In this paper, we describe the effect of herbivore instars that damage plants on the attraction of natural enemies. This is done for a tritrophic system consisting of corn plants, the armyworm *Pseudaletia separata* Walker (Lepidoptera: Noctuidae) and the gregarious parasitic wasp *Cotesia* (= *Apanteles*) *kariyai* Watanabe (Hymenoptera: Braconidae).

Cotesia kariyai is one of the dominant parasitoid species of the common armyworm *P. separata* (Sato, 1988; Hirai, 1984). The wasps search for and attack host larvae actively during the day, while nocturnal host larvae hide in the ground or in the sheath of the host plant (Sato et al., 1983; Kanda, 1988). An intriguing question then is how the diurnal parasitoids find the nocturnal host larvae (Sato et al., 1983). It has been demonstrated that feces, feeding traces, and exuviae of the armyworm on an infested corn plant elicit strong behavioral responses in the parasitoid females upon contact with their antennae: the wasps are arrested and show area-restricted search patterns (Takabayashi et al., 1985; Takabayashi and Takahashi, 1989). This behavior increases the chances of finding a host that hides in the sheath of the infested corn plant or in the ground where host feces are scattered. The kairomones that mediate these behavioral responses were identified to be 2,5-dialkyltetrahydrofuran homologs that were present in host feces, feeding traces, and exuviae (Takabayashi and Takahashi, 1986a,b). Upon antennal contact with a *P. separata* larva, the wasp immediately inserts her ovipositor and lays a clutch of eggs.

The contact kairomones are highly reliable cues of host presence, but the question as to how the parasitoids locate the infested plants from a distance remains unanswered. *Cotesia kariyai* females are known to respond to volatiles from uninfested corn plants (Takabayashi et al., 1991a), but so far it is not known whether they are able to discriminate between uninfested plants and *P. separata*-infested plants. Other *Cotesia* species are known to distinguish uninfested plants from plants infested by their hosts on the basis of volatile infochemicals (Sato, 1979; Turlings et al., 1990; Kaiser and Cardé, 1992; Steinberg et al., 1992, 1993). Here we show that *C. kariyai* also has this ability. In addition we show that in *Cotesia kariyai* this discrimination on the basis of herbivore-induced synomones is affected by the stage of the herbivores infesting the plant.

METHODS AND MATERIALS

Insects

Pseudaletia separata has six larval instars. It takes about 15 days for the newly hatched larva to reach the pupal stage at 25°C, 17L:7D, and 50–70% relative humidity, and about three days at 25°C, 17L:7D for the adult female to initiate oviposition. The newly emerged adult female has a tendency to make a long-distance flight before the first oviposition. Females lay a cluster of ca. 100–300 eggs in one oviposition site.

Pseudaletia separata was introduced to the laboratory from a culture reared at the National Institute of Sericultural and Entomological Science in Tsukuba, Ibaraki, Japan in 1990. The insects were reared on corn leaves or artificial diet (INSECTA-LF: Nihon Nousan Kogyo, Ltd.) under conditions of 25 ± 3°C, 17L:7D, 50–70% relative humidity.

Cotesia kariyai is one of the dominant monophagous parasitoids of the common armyworm *Pseudaletia separata* in Japan (Hirai, 1984). The wasp is a gregarious endoparasitoid of second-instar to second-day sixth-instar larvae at 25°C, 17L:7D (Sato and Tanaka, 1984). The wasp cannot attack first-instar larva, probably because their small size hampers ovipositor insertion. When the wasp oviposits in a late sixth-instar host, the host pupates and the wasp offspring die.

Cotesia kariyai was introduced to the laboratory from a stock culture reared at the National Institute of Sericultural and Entomological Science in 1990. To maintain the wasp culture, fourth- or fifth-instar *P. separata* larvae were offered to female wasps for oviposition. It took about 10 days for the next generation of the wasp to egress at 25°C, 17L:7D. Egressed wasp larvae immediately made cocoons. The cluster of cocoons was collected and placed in a test tube (22 mm diam. × 200 mm long) with a small piece of paper towel moistened with 50% aqueous honey solution as food. Mating generally occurs right after emergence of the wasps. Adult wasps were stored in the dark at 10°C until used (not longer than five days). Naive *C. kariyai* were used in this study.

Corn (*Zea mays* L. vc. Royal Dent) was grown in a greenhouse (25 ± 5°C, 16L:8D). Seedlings of ca. 30–50 cm height were used for the experiments.

Bioassay

A Y-tube olfactometer was used to study the response of the wasps toward leaf volatiles (for details of the olfactometer setup, see Takabayashi et al., 1991b). The number of wasps that walked into the sample cage and the control cage was counted. Sign-tests determined whether the results differed significantly from a 50:50 distribution of the wasps (null hypothesis).

Olfactory Response of C. Kariyai *to Corn Leaf Volatiles*

The attraction of corn leaves infested by *P. separata* larva(e) of different developmental stages was tested. Infested leaves vs. uninfested leaves were compared in the Y-tube olfactometer.

Corn Plants Infested by Young Armyworms. Five intact corn plants (ca. 30 cm height) in a clear plastic cage (50 × 50 × 50 cm) were offered to five second-instar larvae in the climate room (25 ± 3°C, 17:7D, 50–70% relative humidity). Five days later, the armyworms were third instar. The host larvae and feces were then carefully removed from the plants with a brush.

Corn Plants Infested by Old Armyworms. Five intact corn plants (ca. 30 cm height) in a clear plastic cage (50 × 50 × 50 cm) were offered to five fourth-instar larvae in a climate room (25 ± 3°C, 17L:7D, 50–70% relative humidity). Five days later, the armyworms were fifth- to sixth-instar. The larvae and feces were then carefully removed from the infested plant with a brush.

Artificially Damaged Corn Leaves. Ten leaves of an uninfested corn plant (ca. 30 cm) were damaged slightly with sandpaper (ca. 30% of each leaf area) to imitate the young larva's infestation and with scissors (ca. 30% of each leaf area) to imitate the old larva's infestation. One hour later, the leaves were used for the bioassay.

Corn Plants Infested by Armyworms of Each Instar. Infested corn plants were further classified according to the instar of the armyworm that fed on them. Three corn plants were offered to armyworms of each instar during 2–6 hr, at 25 ± 3°C, 50–70% relative humidity. The number of armyworms offered and the infested area of each infested leaf are listed in Table 1.

Imitation of Caterpillar Damage on Corn Leaves. To imitate infestation by armyworm caterpillars, the corn leaf surface (ca. 30%) was damaged artificially with sand paper and then the regurgitant (5 larvae equivalent) of third-instar larvae or sixth-instar larvae was applied to the damaged part of the corn leaf.

TABLE 1. CORN LEAF SAMPLES USED FOR BIOASSAY AND CHEMICAL ANALYSIS

Infested by	Damaged leaf area (%)	Armyworms (*N*)	Bioassay[a]	Chemical analysis[a]
First instar	< 10	150	+	+
Second instar	< 10	100	+	+
Third instar	ca. 10	40	+	+
Fourth instar	ca. 40	10	+	−
Fifth instar	ca. 50	5	+	−
Sixth instar	ca. 50	3	+	+

[a] + = tested, − = not tested.

To collect the one equivalent regurgitant, the host was gently squeezed and the regurgitant was rapidly collected with 5-μl glass capillary tubes. Four leaves were treated. Six hours after treatment, the attraction of the treated leaves vs. undamaged leaves was compared in an olfactometer.

Chemical Analysis of Corn Leaf Volatiles

Infested corn leaves (as listed in Table 1), artificially damaged corn leaves, and uninfested corn leaves were used for chemical analysis. The headspace volatiles of each sample (50–100 g plant material in a 2-liter glass bottle) were collected on Tenax-TA for 30–60 min. The methods used to collect the volatiles of corn leaves were the same as previously reported (Takabayashi et al., 1991b).

The trapped headspace volatiles were subsequently analyzed with a gas chromatograph–mass spectrometer (GC-MS: VG MM7070F) of which the gas chromatograph was equipped with a thermal desorption cold trap injector (TCT, Chrompack). The GC conditions were: a Supelcowax 10 capillary column (60 m, 0.25 mm ID, 0.25-μm film thickness) and the oven temperature programmed from 40°C (4 min hold) to 100°C at a rate of 2°C/min, and then to 270°C at a rate of 6°C/min. The TCT conditions were: thermodesorption at 250°C during 10 min, while at the same time the cold trap was maintained at a temperature of −100°C. The analysis started by fast heating of the cold trap to 220°C. The mass spectrometer was working in the 70 eV electron impact (EI) ionization mode and scanning from 300 to 24 amu at a rate of 1 decade/sec.

The chemical structure of each compound was elucidated by comparison of the mass spectra with those of authentic chemical samples or by comparison with data in the mass spectra library of the Department of Organic Chemistry, Wageningen Agricultural University.

RESULTS

Effect of Armyworm Infestation on Response of Parasitoid to Corn Leaf Volatiles. Naive wasps preferred corn leaves infested by young larvae to uninfested leaves in the olfactometer (Figure 1). However, the wasps did not respond to larvae or their feces, isolated from corn leaves infested by young larvae when compared with clean air in the olfactometer (Figure 1).

The naive wasps did not prefer the odor of corn leaves infested by late-instar armyworms over that of uninfested leaves (Figure 1). The wasps also did not respond to isolated larvae or their feces when compared with clean air in the olfactometer (Figure 1).

The wasps showed no preference for leaves artificially damaged with sandpaper over undamaged leaves, or leaves artificially damaged with scissors over undamaged leaves (Figure 1).

Effect of Developmental Stage of Armyworm on Attractiveness of Infested Plant to Wasps. Corn leaves with ca. 10% (area) infestation by first-, second-, or third-instar host larvae attracted wasps more than uninfested leaves (Figure 2). Leaves with ca. 40% (area) infestation by fourth-instar larvae were also preferred by the wasp (Figure 2). However, when 50% of the area of corn leaves was infested by fifth- or sixth-instar larvae, they were not preferred by the wasp (Figure 2).

The six experiments in Figure 2 were also compared using the *G* test (Sokal and Rohlf, 1981). The attraction of leaves infested by first instars, second instars, third instars, and fourth instars were not significantly different. The attraction

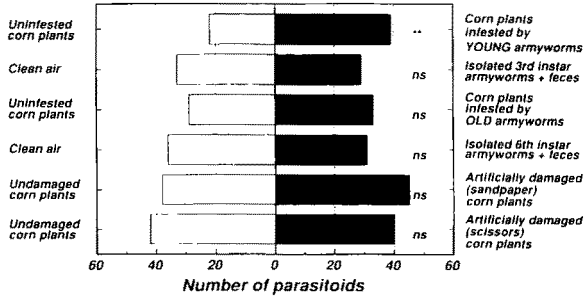

FIG. 1. Olfactory response of *Cotesia kariyai* to infested/uninfested corn plants, and armyworms plus feces. ** $0.01 > P > 0.001$; ns: not significantly different by sign test.

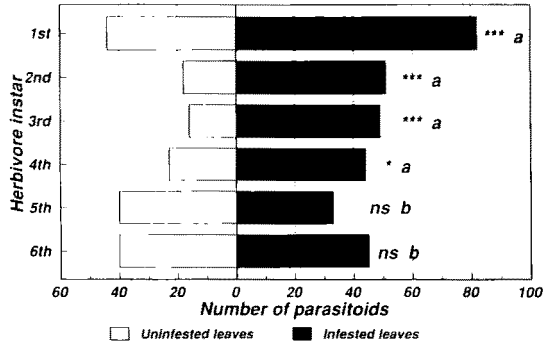

FIG. 2. Olfactory response of *Cotesia kariyai* to corn leaves infested by first- to sixth-instar armyworms separately vs. uninfested leaves. * $0.05 > P > 0.01$; *** $0.001 > P$; ns: not significantly different by sign test. Columns that are followed by the same letter do not differ significantly from each other by *G* test ($P < 0.05$).

TABLE 2. RELATIVE AMOUNTS OF COMPOUNDS FOUND IN HEADSPACE OF UNINFESTED, ARTIFICIALLY DAMAGED, AND INFESTED LEAVES[a]

	Host instar				
Compounds	UD[b]	ADL[c]	1st/ 2nd[d]	3rd	6th
Aldehydes					
Pentanal		0.5			
2-Pentenal		0.7			1.0
Hexanal	8.6	5.0			9.2
2-Hexenals (*E*/*Z*)		4.9	4.4	3.2	16.6
Heptanal	5.6	0.4			
Alcohols					
(*Z*)-3-Hexen-1-ol	8.4	23.5	2.0	1.5	8.4
1-Pentanol	tr[e]	0.2	0.1		
1-Hexanol		0.7			
1-Octanol	3.1		0.4		
Ketones					
4-Methyl-2-pentanone	3.8	1.1			
3-pentanone		1.0	0.5	0.5	4.1
1-Pentene-3-one		1.5	0.2	0.6	5.0
6-Methyl-5-heptene-2-one		0.8			
2-Nonanone	3.0	0.3			
1-Octanone	0.7				
Acetates					
1-Butyl acetate	3.8	0.4	0.1		
(*Z*)-3-Hexen-1-yl acetate	31.8	38.3	5.5	5.4	19.7
1-Hexyl acetate		1.1	0.2	0.1	2.1
Terpenoids					
α-Pinene	5.3	15.7			3.2
β-Pinene	3.3	0.6			
Myrcene	4.2	0.2	0.9	1.2	1.8
Limonene	9.8	1.6	0.1	0.2	4.9
Linalool	3.0	0.6	3.5	16.0	8.3
Others					
Methyl salicylate	3.4	0.4			0.3
Total (%)	97.8	99.5	17.9	28.7	84.6
Total peak area/g leaf	218.3	850.5	184.5	203.9	430.3

[a]Number of replications: 2.
[b]UD: undamaged leaves.
[c]ADL: artificially damaged leaves.
[d]Data of leaves infested by first and second instars were combined.
[e]tr: less than 0.1%

TABLE 3. RELATIVE AMOUNTS OF COMPOUNDS FOUND IN HEADSPACE OF INFESTED LEAVES BUT NOT (OR FOUND IN TRACE AMOUNTS) IN UNINFESTED LEAF HEADSPACE[a]

Compounds	Host instar				
	UD	ADL	1st/ 2nd	3rd	6th
Aldehydes					
2-Methyl-2-propenal				0.1	
3-Methylbutanal				tr	
2-Pentenal				0.1	
Alcohol					
(*E*)-2-Hexen-1-ol				0.1	
2-Methyl-1-propanol			0.4	0.5	1.2
Ketones					
3-Hydroxy-2-butanone			3.5		7.4
Acetates					
1-Pentene-3-ol acetate				0.4	
Isopentyl acetate			0.1	0.2	
2-Methylbutyl acetate			0.6	0.2	
(*E*)-3-Hexen-1-yl acetate			0.4		
2-Hexen-1-yl acetate				0.9	
Benzyl acetate			0.1		
Geranyl acetate			0.3	1.4	
2-Phenylethyl acetate			1.3		
Terpenoids					
(3*E*)-4,8-Dimethyl-1,3,7-nonatriene	2.1	0.4	12.3	22.6	3.4
(3*Z*)-4,8-Dimethyl-1,3,7-nonatriene			1.0	0.9	
α-Copaene			1.1	0.4	
Linalool oxide				0.3	
(*E*)-α-Bergamotene			12.0	7.6	
β-Caryophyllene			1.3		
(*E*)-β-Farnesene			32.3	18.2	
β-Sesquiphellandrene			0.5		
(3*E*, 7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene			1.3	1.0	
unknown terpenoid			0.4	0.9	
Nitriles					
2-Methylpropanenitrile			0.3	0.2	
2-Methylbutanennitrile			0.2	tr	
Oximes					
Butanal *O*-methyloxime			4.4		
2-Methylbutanal *O*-methyloxime				0.2	
2-Methylpropanal *O*-methyloxime			1.2	1.9	
Other					
Indole			7.0	13.0	3.2
Total (%)	2.1	0.4	82.0	71.1	15.2
Total peak area/g leaf	2.6	34.2	846.2	928.8	77.3

[a]See footnotes to Table 2.

of leaves infested by fifth instars and sixth instars was also not significantly different.

Mechanism of Production of Parasitoid Attractants. Leaves treated with third-instar regurgitant on a mechanical wound (five larvae equivalent per leaf) attracted wasps more than untreated leaves (Figure 3). In contrast, leaves treated with sixth-instar regurgitant (five larvae equivalent per leaf) did not attract wasps more than the untreated leaves (Figure 3). A 20-larvae equivalent of regurgitant of third- or sixth-instar larvae impregnated into a filter paper (2 × 2 cm) did not attract wasps (Figure 3).

Chemical Analysis. The main volatile compounds from uninfested corn leaves were hexanal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, and limonene (Table 2). There were many compounds that were found in the volatiles of leaves infested by armyworms that were not detected or detected in only trace amounts in uninfested leaf volatiles (Table 3).

The herbivore-induced plant chemicals in the headspace of corn leaves infested by first- to second- and third-instar larvae were similar, and their combined amounts comprised ca. 70% of the total headspace volatiles emitted (Table 3). The major herbivore-induced compounds were terpenoids, indole, and oximes. (3*E*)-4,8-Dimethyl-1,3,7-nonatriene, (*E*)-α-bergamotene and (*E*)-β-farnesene were found as major compounds in the headspace of leaves infested by first to second instars and leaves infested by third instars. However, when corn leaves were infested by sixth-instar larvae, few compounds were found as herbivore-induced plant chemicals [indole, 3-hydroxy-2-butanone, (3*E*)-4,8-

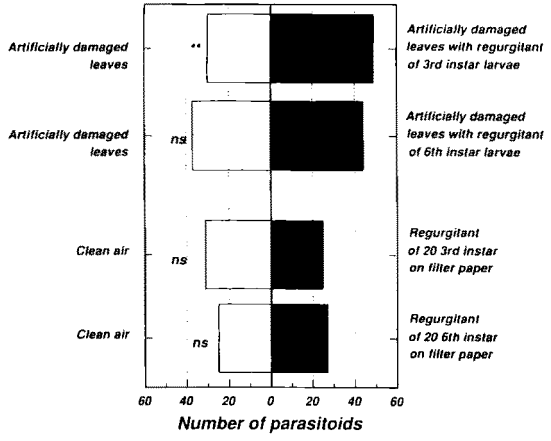

FIG. 3. Olfactory response of *Cotesia kariyai* to artificially damaged corn leaves treated with regurgitant of third-instar and sixth-instar armyworms. ** $0.01 > P > 0.001$; ns: not significantly different by sign test.

dimethyl-1,3,7-nonatriene and 2-methyl-1-propanol] and their total relative amount was only 15.2% (Table 3). The total peak area (arbitrary units) of sixth-instar armyworm-induced plant volatiles per gram leaf (77.3) was smaller than that of first-/second-instar, and third-instar armyworm-induced plant volatiles (846.2 and 928.8, respectively) (Table 3). Thus, there are qualitative and quantitative differences in the production of herbivore-induced plant volatiles depending on the developmental stage of the herbivores.

DISCUSSION

Cotesia kariyai is attracted by uninfested corn leaves, mediated by green leaf volatiles such as (*Z*)-3-hexenol (Takabayashi et al., 1991a). Artificially damaged leaves are not more attractive than undamaged corn leaves, while infochemicals from corn leaves infested by young armyworms were highly preferred compared to uninfested corn leaves. Neither the caterpillars themselves nor their feces are attractive to the parasitoids. The infochemicals that are emitted from host-infested leaves are not just the result of mechanical damage, but are specific for herbivore infestation. Similar phenomena are observed in other tritrophic systems (Turlings et al., 1990, 1991; Steinberg et al., 1992, 1993; Mattiacci et al., 1994). Moreover, the composition of the infochemicals emitted from armyworm-infested corn leaves is dependent on the herbivore developmental stage that feeds on the plant.

Damage by younger instars (first through third) attracts the parasitoids, while damage by older instars (fourth through sixth) does not. This difference was studied more precisely with corn leaves infested by each instar larvae separately. This revealed that corn plants infested by first-, second-, third-, or fourth-instar armyworms attract the wasp, whereas corn plants infested by fifth or sixth instars do not.

Chemical analysis supports our behavioral data. Corn leaves infested by younger instar larvae produce large amounts of herbivore-induced volatiles (70–80% of total volatile emission). However, corn leaves infested by sixth-instar larvae produce few herbivore-induced volatiles and their combined amount comprised only ca. 15% of the total volatile emission (Table 3). This may explain the attraction of leaves infested by young armyworms and the nonattraction of leaves infested by old armyworms.

Application of regurgitant of third-instar larvae onto artificially damaged leaves resulted in the attraction of parasitoids, whereas application of regurgitant of sixth instar leaves did not. It is likely that this effect was caused by buccal secretion or saliva of the host larvae, as has been found for other tritrophic systems (Sato, 1979; Turlings et al., 1990, 1991; Mattiacci et al., 1994). Furthermore, our data suggest that the buccal secretion or saliva of third instars and

those of sixth instar differ in composition and, thus, affect the plant differently with regard to the production of the wasp attractants.

Herbivore-induced synomone (HIS) production by plants for specific natural enemies of herbivores can be of two classes:

1. Unconditional HIS production. This occurs when the interaction mediated by HIS is unconditionally mutualistic, i.e., adaptive to both the plant and the natural enemy.

2. Conditional HIS production. This occurs when the interaction mediated by HIS is conditionally mutualistic in the following three ways. Type 1: Adaptive to the plant and conditionally adaptive to the natural enemy. In this case, the plant should be selected to produce HIS only when it is beneficial for the natural enemy to respond to HIS. Type 2: Adaptive to the natural enemy and conditionally adaptive to the plant. In this case, the plant should be selected to produce HIS only when it is beneficial to the plant. Type 3: Conditionally adaptive to both plant and natural enemy. In this case, plant should be selected to produce HIS only when it is beneficial to both the plant and the natural enemy.

In the case of the *C. kariyai*–armyworm–corn plant interaction, the HIS production is found to be conditional. Thus, the interaction between corn plants and *C. kariyai* is conditionally mutualistic. We examine this mutualistic interaction from the viewpoint of the plant and the wasp:

From the viewpoint for the plant, the wasps can successfully parasitize the larvae during the second to early sixth instar (Sato and Tanaka, 1984). It takes 10 days at 25°C for the parasitizing wasp larvae to develop and egress from the host. *Pseudaletia separata* larvae that are parasitized by *C. kariyai* at these stages do less damage to a plant than unparasitized larvae in this period, and this effect is more pronounced when parasitism takes place in younger host instars (Tanaka et al., 1992).

When a third-instar larva of *P. separata* is parasitized by *C. kariyai*, the total amount of food consumed by this larva until pupation is ca. 36% of the total food consumed by an unparasitized larva of the same developmental stage (Tanaka et al., 1992). Thus, attracting *C. kariyai* is adaptive for the infested corn plant.

When a fifth-instar larva is parasitized by *C. kariyai*, the total food consumed by this larva until pupation is reduced to ca. 63% of the food consumed by an unparasitized larvae of the same developmental stage. The parasitized fifth-instar larva, however, has already consumed food from the first up to the fifth instar developmental stage. Therefore, the total food consumed by a larva that is parasitized at the fifth instar is 76% of the total food consumed by an unparasitized larva of the same developmental stage until pupation (data from Tanaka et al., 1992). Thus, the reduction in feeding damage is much more pronounced when the larva is parasitized in the third instar. When early sixth-

instar larvae were parasitized by *C. kariyai*, the total food consumed by the larva is almost the same as that consumed by the unparasitized host (J. Takabayashi, unpublished data, 1992). Thus, for plants, it is advantageous to attract the parasitoids when the larvae are still small, as the benefit of attracting parasitoids decreases with time. The production of the wasp attractants is likely to have costs, in terms of energy or in terms of being more detectable to other herbivores, that are not affected by the wasps (Dicke and Sabelis, 1989). Therefore, it is adaptive for an infested plant to reduce the production of attractants with decreasing benefit due to herbivore development. This is probably one of the reasons why plants stop attracting wasps when the host is older than fifth instar.

From the viewpoint of *C. kariyai*, it is clearly adaptive for the wasps to find unparasitized hosts. There are three other dominant parasitoid species for *P. separata* larva in Japan: *Cotesia ruficrus*, *Microplitis mediator*, and *Campoletis chloridae* (Sato, 1988). *Cotesia kariyai* can successfully parasitize larvae during the second through the early sixth instar (Sato and Tanaka, 1984), but later-stage armyworms are more likely to be already parasitized by conspecific or heterospecific parasitoids. *Cotesia kariyai* cannot successfully produce offspring in an armyworm that has already been parasitized more than three days earlier by a conspecific wasp (J. Takabayashi, unpublished data). Under such a competitive situation, searching for young armyworms should be advantageous for the wasps. This may also select corn plants to produce HIS when inflicting armyworms are of a younger stage.

The above findings indicate that the interaction between corn plant and *C. kariyai* is type 3—conditionally adaptive to both the plant and the natural enemy. This study provides an example for illustrating a new scheme to evaluate plant–natural enemy interactions on the basis of HIS production. The experimental design developed in this paper is applicable to other tritrophic systems in evaluating the mutualistic interactions between plants and natural enemies of herbivores through HIS.

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EFFECT OF PHYTOTOXIC RESIN GLYCOSIDE ON ACTIVITY OF H^+-ATPase FROM PLASMA MEMBRANE

M.R. CALERA,[1,2] A.L. ANAYA,[1,*] and M. GAVILANES-RUIZ[2]

[1]*Instituto de Fisiología Celular*
[2]*Facultad de Química*
Universidad Nacional Autónoma de México
Apdo. Postal 70-243, México, D.F. 04510, México

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Abstract—A resin glycoside mixture isolated from *Ipomoea tricolor* inhibited radicle growth of *Echinochloa crusgalli*. The effect of the resin was tested on the activity of the plasma membrane H^+-ATPase from *E. crusgalli*. For this purpose, plasma membrane vesicles were purified by the method of aqueous two-phase partitioning. The resin glycoside inhibited by 30% the activity of the plasma membrane ATPase. The same result was obtained with the purified main component of the resin. This indicates that the plasma membrane ATPase can be one of the cellular targets of the resin. Hence it is possible that the mechanism of action of the resin involves an inhibition of the plasma membrane ATPase.

Key Words—*Ipomoea tricolor*, *Echinochloa crusgalli*, allelopathy, allelochemicals, resin glycoside, plasma membrane, plasma membrane H^+-ATPase.

INTRODUCTION

Plants produce and release to the environment secondary compounds that interact in different ways with plants, microorganisms, and animals. These interactions may result in the inhibition or stimulation of root or stem growth. These allelochemicals affect many cellular processes: ion uptake, protein synthesis, photosynthesis, activation or breakdown of hormones, respiration, cell division, energy metabolism, and enzymatic activity (Cameron and Julian, 1980; Mandava, 1985; Alam et al., 1987; Cruz et al., 1990; Wacker et al., 1990).

*To whom correspondence should be addressed at: Depto. de Bioenergética, Instituto de Fisiología Celular, UNAM, Cd. Universitaria 04510, Apdo. Postal 70-243 México, D.F. 04510, México.

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In a previous study, we isolated a resin glycoside from *Ipomoea tricolor* Cav. (Convolvulaceae) that inhibited radicle growth of *Amaranthus leucocarpus* (Amaranthaceae) and *Echinocloa crusgalli* (Gramineae) (Anaya et al., 1990). *Ipomoea tricolor* is used in Mexican traditional agriculture by peasants for weed control. Hence, we explored if the mechanism of action of the resin was related to inhibition of the H^+-ATPase of the plasma membrane. This possibility was considered since the resin has to interact first with the plasma membrane, and one of the most important proteins in this membrane is H^+-ATPase. This enzyme plays a central role in plant cell physiology, i.e., it gives rise to proton gradients that drive the transport of different molecules in and out of the cells. Furthermore, according to the "acid growth theory," the H^+-ATPase produces acidification of the apoplastic space; this increases the plasticity of the cell wall, allowing the cell enlargement. Such actions of ATPase may affect radicle growth (Altabella et al., 1990; Hager et al., 1991). The present experiments show that a resin glycoside from *Ipomoea tricolor* inhibits the activity of plasma membrane H^+-ATPase from *E. crusgalli*. This effect could be related to its overall action on radicle growth.

METHODS AND MATERIALS

Plant Material. Echinochloa crusgalli seeds were germinated at 30°C in the dark on wet filter paper. After three days of growth, the roots were dissected.

Resin Glycoside Mixture. The resin glycoside was isolated from *Ipomoea tricolor* Cav. (Convolvulaceae) by bioactivity-guided fractionation of an ethyl acetate extract (Anaya et al., 1990). Resin (20–200 μg) was dissolved in dimethylformamide; this yielded an approximate concentration of 130–1305 μM using the mol wt of tricolorin A.

Obtaining Tricolorin A. Tricolorin A is the major component from the resin glycoside (it was a kind gift from Dr. Pereda-Miranda). The structure of this compound is shown in Figure 1 (Pereda-Miranda et al., 1993). Tricolorin A dissolved in dimethylformamide was added to the ATP hydrolysis assay in a concentration range of 130–1305 μM.

Microsomal Fraction. The roots (5 g) were ground with a mortar and pestle with the addition of 30 ml of buffer I containing: 250 mM sucrose, 70 mM Tris HCl (pH 8.0), 10% glycerol, 1 mM Na_2ATP, 15 mM 2-mercaptoethanol, and 4 mM DTT (dithiothreitol). EDTA (ethylene diamine tetraacetic acid) 2 mM, 7 μg/ml chymostatin, 10 μg/ml TLCK [L-1-chloro-3-(4-tosylamido)7-amino-2-heptanone], 10 μg/ml TPCK [L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone], and 1 mM PMSF (phenylmethylsulfonyl fluoride) were added as protease inhibitors. All steps were carried out at 4°C. The homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 500*g*. The

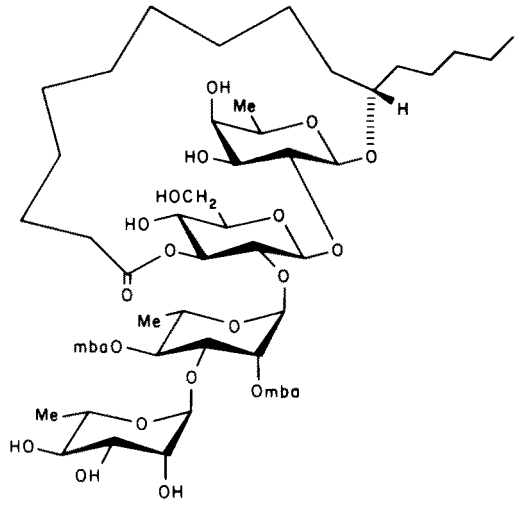

FIG. 1. Structure of tricolorin A, the main compound (85%) of the resin glycoside mixture (Pereda-Miranda et al., 1993).

supernatant was centrifuged at $13 \times 10^3 g$ for 10 min. The resulting supernatant was centrifuged for 1 hr at $80 \times 10^3 g$ to obtain the microsomal pellet.

Aqueous Two-Phase Partitioning. Purified plasma membranes were obtained by the method of aqueous dextran–polyethylene glycol two-phase partition (Larsson et al., 1987) with some modifications. The microsomal pellet from roots was resuspended in buffer II (330 mM sucrose, 5 mM potassium phosphate, pH 7.8, and 4 mM KCl) and brought to a final volume of 1 ml. This suspension was applied to an aqueous-phase partition system. Three tubes containing the aqueous-phase partition system were made as follows: tube 1 contained 2.7 g of a phase mixture consisting of 7% (w/w) dextran (50×10^4 mol wt), 7% (w/w) polyethylene glycol (3350 mol wt), 330 mM sucrose, 5 mM potassium phosphate, pH 7.8, and 5 mM KCl. Tubes 2 and 3 contained 15 g of the phase system identical in composition to tube 1. The microsomal suspension was layered onto the phase mixture in tube 1 (final weight of 3.6 g) and was mixed by inversion 20 times and centrifuged at $1500g$ for 5 min. The resulting upper phase (U_1) was transferred to tube 2, and the mixing and centrifugation were repeated to obtain the U_2 fraction. The procedure was repeated again for tube 3 (to obtain fraction U_3). To remove the polymers, the resulting upper layer (U_2 or U_3) was diluted at least twofold with buffer III (250 mM sucrose and 10 mM Tris HCl, pH 7.5) and centrifuged for 2 hr at $10 \times 10^4 g$. The resulting pellet was resuspended in buffer IV (250 mM sucrose, 5 mM Tris

HCl, pH 7.0, 10% glycerol, 1 mM DTT, and protease inhibitors) to give a final protein concentration of 1.9–2.6 mg/ml; this was stored at −70°C.

ATPase Assay. One microgram of protein from the microsomal fraction or the resuspended U_3 plasma membrane fraction was added to 150 μl of reaction medium consisting of 250 mM sucrose, 20 mM Tris HCl, pH 7.0, 10 mM ATP Tris, pH 7.0, 10 mM $MgCl_2$, and 7 μM CCCP (carbonyl cyanide *m*-chlorphenylhydrazone). When required, the following inhibitors were added as supplements: 2 mM NaN_3 to inhibit mitochondrial ATPase, 2 mM Na_2MoO_4 to inhibit acid phosphate, 150–250 μM Na_2VO_4 to inhibit plasma membrane ATPase, and 100 mM KNO_3 to inhibit tonoplast ATPase. The membranes were preincubated with the resin or the tricolorin A for 15 min when indicated. In preliminary experiments, the inhibitory effect of the resin was the same in the presence or absence of 0.05% Triton X-100; therefore all subsequent experiments were performed without the detergent. ATP hydrolysis was allowed to proceed at 37°C for 30 min; the reaction was stopped by addition of 24% SDS (sodium dodecyl sulfate). Phosphate release was determined by the method described in González-Romo et al. (1992). In each case four repetitions were tested. The results were processed by ANOVA Duncan's multiple-range test.

Protein Determination. Protein concentration was determined by the method of Peterson (1977) using BSA (bovine serum albumin) as standard.

RESULTS

Effect of Resin Glycoside on Radicle Growth of E. crusgalli. The effect of the resin glycoside on radicle growth of *Echinochloa crusgalli* at different times of growth is shown in Figure 2. The resin inhibited radicle growth, evaluated in terms of radicle length, by 82–89%. Fresh weight was inhibited by 78–97%.

Purification of Plasma Membranes. In order to explore the effect of the resin on the H^+-ATPase from the plasma membrane, we first measured the characteristics of the ATPase activity of the microsomal fraction. In this preparation there are four different components, as inferred from the inhibition of ATP hydrolysis by specific reagents (Table 1). The activity was inhibited 18% by vanadate, an inhibitor of plasma membrane ATPase; 33% by nitrate, an inhibitor of tonoplast ATPase; and 52% by a mixture of azide and molybdate, inhibitors of mitochondrial ATPase and acid phosphatase, respectively. Accordingly, plasma membrane vesicles from *E. crusgalli* radicles were purified by the method of aqueous-phase partitioning of Larsson et al. (1987). A polymer concentration of 7% dextran T-500 and 7% PEG-3350 yielded the purest preparation in the U_2 fraction: 76% of the activity was due to plasma membrane ATPase (sensitive to vanadate). Of the overall ATPase activity, 11% corresponded to mitochondrial ATPase plus acid phosphatase and 4% to tonoplast ATPase.

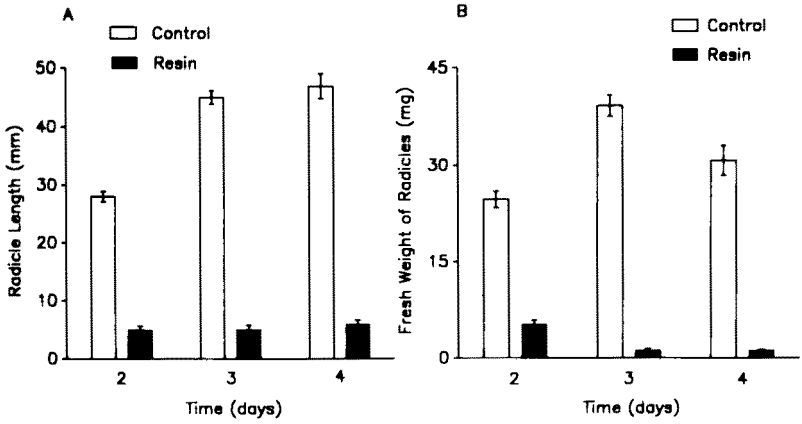

FIG. 2. Effect of the resin glycoside [65 μM (considering the mol wt of tricolorin A)] on the radicle growth of *E. crusgalli*. Resin was dissolved in methylene chloride. The radicle growth in the control was the same in the presence of methylene chloride or water. Resin was added at zero time and the measurements were done at the indicated times. (A) radicle length and (B) fresh weight of the radicles from *E. crusgalli*.

TABLE 1. EFFECT OF RESIN GLYCOSIDE ON ATP HYDROLYSIS OF VESICLES AT DIFFERENT LEVELS OF PURIFICATION: MICROSOMAL FRACTION (MF) AND FRACTIONS OF PLASMA MEMBRANE VESICLES BY SUCCESSIVE PHASE PARTITIONING (U_2 AND U_3)

	ATP hydrolysis (nmol Pi/min/mg protein)		
	MF	U_2	U_3
Control	566.0 ± 0.5 (100.0%)	432.0 ± 1.9 (100.0%)	843.5 ± 1.3 (100.0%)
Plasma membrane ATPase	462.0 ± 0.5 (82.0%)[a]	106.0 ± 0.9 (24.0%)[a]	101.4 ± 2.3 (12.0%)[a]
Tonoplast ATPase	380.7 ± 27.1 (67.0%)[a]	413.9 ± 0.5 (96.0%)	844.4 ± 0.9 (100.1%)
Mitochondrial ATPase, acid phosphatase	270.8 ± 0.5 (48.0%)[a]	382.8 ± 0.5 (89.0%)	758.8 ± 1.0 (90.0%)
Resin (261 μM)	506.5 ± 0.5 (90.0%)	358.8 ± 8.7 (83.0%)[a]	576.8 ± 0.6 (68.0)[a]

[a] $P < 0.05$, ANOVA.

In order to increase the degree of purity of plasma membrane vesicles, the U_2 fraction was repartitioned in a polymer concentration of 7% dextran and 7% PEG-3350 (Table 1). Of the total ATPase activity of the U_3 preparation, 88% correspond to that of the plasma membrane. The rest was due to ATP hydrolysis by mitochondria (10%) and an acid phosphatase. It is noted that no tonoplast ATPase activity was detected.

Effect of Resin on Purified Plasma Membrane Vesicles (U_3 Fraction). Table 1 shows the effect of the resin glycoside on the ATPase activity of vesicles at different levels of purification. In the U_3 fraction, which constitutes a highly purified membrane fraction of plasma membrane vesicles (about 90%), the resin glycoside inhibited ATP hydrolysis by about 30% as compared to inhibition of 10% in the microsomal fraction or 17% in the U_2. In consequence, inhibition of ATP hydrolysis in the U_3 fraction was due to an effect of the resin on the plasma membrane ATPase, since this is its major ATP hydrolytic component.

To determine whether the plasma membrane ATPase was the component affected by the resin glycoside, ATPase activity was measured in the U_3 fraction in a condition where ATP hydrolysis was solely due to the plasma membrane ATPase activity. This condition was attained by adding inhibitors of the mitochondrial ATPase and acid phosphatase. In this fraction, ATP hydrolysis was inhibited 30% by the resin glycoside (Table 2). This indicated that the resin inhibited the activity of the plasma membrane ATPase.

Effect of Resin Concentration on ATP Hydrolysis from Purified Plasma Membranes. The resin glycoside inhibited ATP hydrolysis in the U_3 fraction in a dose-dependent manner (Figure 3). Plasma membrane ATPase activity was reduced to the same extent by equivalent concentrations of tricolorin A, the

TABLE 2. EFFECT OF RESIN GLYCOSIDE ON ATP HYDROLYSIS OF FRACTION MEMBRANES FROM *E. crusgalli* RADICLES[a] IN PRESENCE OF SPECIFIC INHIBITORS

ATPase activity origin	ATPase activity (nmol Pi/min/mg protein)
Plasma membrane ATPase	965.9 ± 0.4
Plasma membrane ATPase + resin (261 μM)	673.7 ± 0.3[b]
Mitochondrial ATPase, acid phosphatase	132.6 ± 0.4
Mitochondrial ATPase, acid phosphatase + resin (261 μM)	154.0 ± 0.4[b]

[a] Total ATPase activity in plasma membrane vesicles was determined in the absence of any inhibitor and was 1077.3 nmol Pi/min/mg protein. Plasma membrane ATPase activity was measured as Pi released in a medium including NaN_3/Na_2MoO_4 (see Methods and Materials) with dimethylformamide (1.3%). Mitochondrial ATPase and acid phosphatase activity were determined in a similar way but in the presence of Na_3VO_4 (see Methods and Materials), with dimethylformamide (1.3%).
[b] $P < 0.05$, ANOVA.

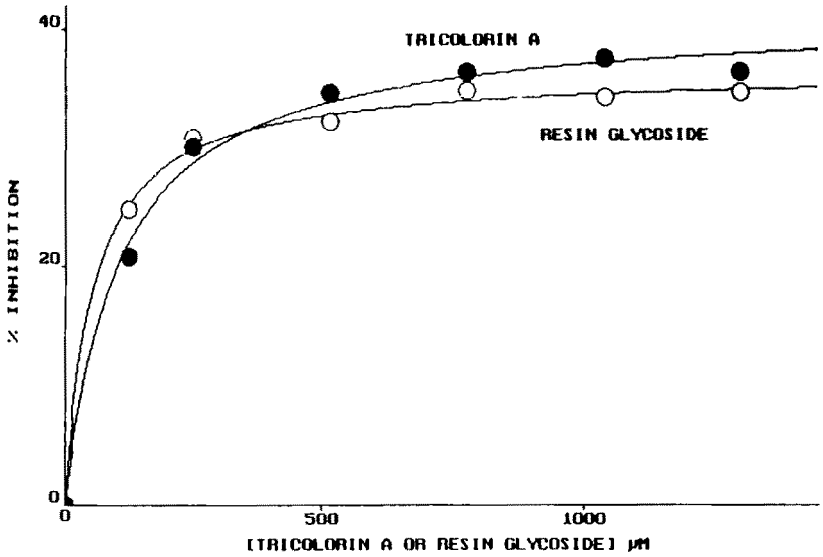

FIG. 3. Effect of the resin glycoside and tricolorin A on the ATPase activity of isolated plasma membranes vesicles of roots from *E. crusgalli*. The U_3 fraction (1 µg of protein) was preincubated with the resin glycoside (○) and tricolorin A (●) at the indicated concentrations during 15 min as described under Methods and Materials and ATP hydrolysis was measured as Pi release.

main component of the resin glycoside. These results indicate that the inhibitory effect displayed by the whole resin glycoside was caused by tricolorin A.

DISCUSSION

The plasma membrane vesicles from *Echinochloa crusgalli* roots prepared in this study by aqueous two-phase partitioning possess a high purity, similar to that obtained in other systems with this method (Buckhout and Hrubec, 1986; Larsson et al., 1987; Sandstrom et al., 1987). The activity of the ATPase of plasma membrane vesicles isolated by this method is similar to that reported by Sandstrom and Cleland (1989) on oat vesicles. The activity of these preparations is higher than those obtained with other methods of purification (DuPont et al., 1981; DeMichelis and Spanswick, 1986).

We have previously reported the effect of another phytotoxic compound, the monoterpene diacetyl-piquerol, on the plasma membrane ATPase activity (Cruz et al., 1990). This monoterpene inhibited radicle growth of *Ipomoea purpurea* (10–100%) in a concentration range of 10–1000 µM. This compound

also inhibited the H^+-ATPase activity in plasma membrane (67.2%) and tonoplast membrane (31.4%) of the microsomal fraction from the radicles of *Ipomoea purpurea* (Cruz et al., 1990). In this study, we obtained results that point out the importance of using membranes with a high degree of purity when effects of allelochemicals are characterized.

The inhibiting action of the resin could result from either a direct interaction of the resin with the enzyme or through its interaction with the immediate lipid environment of the protein, which has been reported to be important for its activity (Serrano et al., 1988; Brauer and Tu, 1989).

The resin glycoside at a concentration of 65 μM inhibits radicle growth by 87%. However, the inhibitory effect of the resin on the plasma membrane H^+-ATPase activity was 32% at 261 μM. This inhibition cannot be underestimated, since the expression of the activity of this enzyme is critical for cell elongation and nutrition, processes that are essential for root growth. The work of Hager et al. (1991) has shown that a decrease in the amount of the H^+-ATPase from plasma membrane is equivalent to a decrease in the elongation rate of maize coleoptiles. The difference in inhibition of radicle growth and H^+-ATPase suggests that besides the effect on ATPase activity, the resin could interfere with other cellular processes, producing an ultimate reduction in the radicle growth. This possibility is supported by the fact that within the cell, allelochemical compounds interfere with a vast number of processes (Cameron and Julian, 1980; Mandava, 1985; Alam et al., 1987, Cruz et al., 1990; Wacker et al., 1990). For instance, two allelochemicals, ferulic acid and vanillic acid, inhibit Ca^{2+} uptake, malate oxidation in mitochondria, and O_2 evolution in chloroplasts (Demos et al., 1975; Moreland and Novitzky, 1987). In the same case, coumarin inhibits Ca^{2+} uptake and respiratory activity in mitochondria and increases indoleacetic acid degradation (Zenk and Muller, 1963; Demos et al., 1975; Moreland and Novitzky, 1987). These data indicate that the toxicity of these kinds of compounds may involve not one, but several molecules present in the cell. However, there is not much information about the specific biological molecules that are affected in these processes.

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SEX ATTRACTANTS OF GEOMETRID AND NOCTUID MOTHS: CHEMICAL CHARACTERIZATION AND FIELD TEST OF MONOEPOXIDES OF 6,9-DIENES AND RELATED COMPOUNDS

TETSU ANDO,[1,*] HIDEKI KISHI,[1,3] NAOKO AKASHIO,[1] XU-RONG QIN,[1] NAMIKO SAITO,[1] HIROSHI ABE,[1] and SATOSHI HASHIMOTO[2]

[1]*Department of Applied Biological Science, Faculty of Agriculture Tokyo University of Agriculture and Technology Fuchu, Tokyo 183, Japan*

[2]*Zoological Laboratory, Faculty of Agriculture Meijo University Tempaku-ku, Nagoya, 468, Japan*

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Abstract—(*Z,Z*)-6,9-Dienes with straight C_{18}–C_{23} chains were synthesized from linoleic acid, and a C_{17} chain was synthesized by hydrogenation of the corresponding 6,9-diyne prepared from propargyl alcohol. Oxidation of the homoconjugated dienes with *m*-chloroperoxybenzoic acid yielded a 1:1 mixture of two monoepoxides that could be separated by repeated medium-pressure liquid chromatography with a Lobar column. The chemical structure of each positional isomer was confirmed by analyses of the ozonolysis products, and the isomers showed characteristic ^{13}C signals in their NMR spectra and fragment ions in their EI mass spectra. In addition to the (*Z,Z,Z*)-3,6,9-trienes with straight C_{18}–C_{23} chains and their monoepoxides, field tests using single source lures incorporating one of the above seven dienes and 14 monoepoxymonoenes were carried out in a forest in Tokyo from 1992 to 1994. Consequently, attraction of six geometrid species and five noctuid species was observed for the first time.

Key Words—Sex pheromone, lepidopterous attractant, field test, monoepoxymonoene, monoepoxydiene, mass spectrum, Geometridae, Noctuidae.

*To whom correspondence should be addressed.

[3]Present address: SDS Biotech K.K., Tsukuba Technology Center, Midorigahara 2-1, Tsukuba-shi 300-26, Japan.

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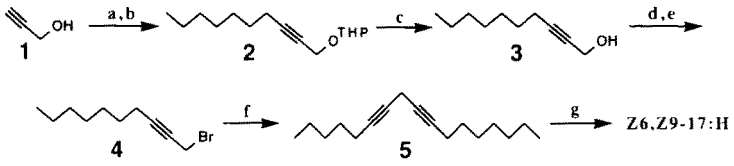
INTRODUCTION

(*Z*,*Z*,*Z*)-3,6,9-Triunsaturated hydrocarbons and their monoepoxides have been identified as sex pheromone components of female moths, mainly in the families Geometridae and Arctiidae (Arn et al., 1992). Furthermore, field screening tests in Canada, Europe, and Japan using synthetic compounds revealed that the trienes and monoepoxydienes attracted not only geometrid species but many noctuid species belonging to the subfamilies Catocalinae, Ophiderinae, Hyperminae, and Herminiinae (Arn et al., 1992; Ando et al., 1993). Recently Ohmasa et al. (1991) reported the major pheromone component of *Oraesia excavata* Butler (Noctuidae: Ophiderinae) to be (*Z*)-6-*cis*-9,10-epoxyheneicosene. Furthermore, the nonadecene analog was tentatively identified from the female gland of *Anacamptodes humaria* Guenée (Geometridae: Ennominae) by Millar et al. (1991). These results indicate that the monoepoxides of (*Z*,*Z*)-6,9-diunsaturated hydrocarbons constitute one group of the lepidopterous sex pheromones. This paper deals with the syntheses and chemical characterizations of monoepoxymonoenes and with their field attractancy as evaluated in a forest in Tokyo, in addition to the parent dienes, (*Z*,*Z*,*Z*)-3,6,9-trienes, and their monoepoxides.

METHODS AND MATERIALS

Synthesis of 6,9-Dienes. Homoconjugated dienes with straight C_{18}–C_{23} chains (Z6,Z9-18:H, Z6,Z9-19:H, and so on)[4] were synthesized starting from linoleic acid by the same method described for 3,6,9-trienes starting from linolenic acid (Ando et al., 1993). Z6,Z9-17:H was synthesized as shown in Scheme 1. After protecting the hydroxyl group, propargyl alcohol (**1**) was alkylated with 1-bromoheptane to yield THP ether of 2-decyn-1-ol (**2**). The THP

[4]Compounds are abbreviated as described in our previous paper (Ando et al., 1993) as follows: Z6,Z9-18:H is (6Z,9Z)-6,9-octadecadiene, epo6,Z9-17:H is (*Z*)-9-*cis*-6,7-epoxyheptadecene.



SCHEME 1. Synthetic route of (6Z,9Z)-6,9-heptadecadiene (Z6,Z9-17:H). a: 3,4-dihydro-2*H*-pyran/*p*-TsOH, b: *n*-BuLi/dry THF, and *n*-$C_7H_{15}Br$/HMPA, c: *p*-TsOH/EtOH, d: MsCl/Et_3N/CH_2Cl_2, e: LiBr/THF, f: *n*-$C_5H_{11}C{\equiv}CMgBr$/CuCl/dry THF, g: H_2/Pd-$BaSO_4$-quinoline/*n*-hexane.

protectional group was removed by acid, and the alcohol (**3**) was converted to 1-bromo-2-decyne (**4**) (37% yield from **1**) via the mesylate. The bromide (**4**) was coupled with hept-1-ynylmagnesium bromide, which was prepared with 1-heptyne and ethylmagnesium bromide, in the presence of copper chloride yielding 6,9-heptadecadiyne (**5**) (80% yield from **4**). Partial hydrogenation of the diyne (**5**) to Z6,Z9-17:H was achieved using Pd-$BaSO_4$ catalyst poisoned with quinoline (86% yield).

Synthesis and Separation of Monoepoxides of 6,9-Dienes. A mixture of epo6,Z9-17:H and Z6,epo9-17:H in a ratio of ca. 1:1 was obtained by oxidation of Z6,Z9-17:H with *m*-chloroperoxybenzoic acid (MCPBA). The two positional isomers were separated by a medium pressure liquid chromatographic system (MPLC), the components of which were a solvent pump (Yanaco L4000W liquid chromatograph), a Lobar column (Merck LiChroprep Si 60, 10 mm ID × 240 mm, 40–63 μm), and an RI detector (Shimadzu RID-2A). Although the mixture (50 mg) applied to the MPLC using 1.0% tetrahydrofuran (THF) in *n*-hexane as a solvent (flow rate: 2.0 ml/min) showed only one peak (R_t = 77.5 min), even in a recyclic mode (two times), the first half (fraction 1, 70.0–77.5 min) and the latter half (fraction 2, 77.5–83.0 min) of the eluent were recovered separately. By repeating this MPLC separation two more times, we obtained two fractionated compounds [8 mg of each with purity >95% by GC (i.e., TIC trace from GC-MS)], and their chemical structures were determined by ozonolysis experiments. Ozone was passed into the *n*-hexane (1 ml) solution of each compound (ca. 0.1 mg), cooled in a Dry Ice–acetone bath for 1 min, and an aliquot (1 μl) of the solution was injected into a capillary column of the GC-MS to analyze the aldehydes, which were produced from the ozonides by heat. Mixtures of two monoepoxides of other diunsaturated hydrocarbons (C_{18}–C_{23}) were prepared and separated into two fractions in the same manner. Their structures were determined also by the GC-MS analyses of ozonolysis products.

Synthesis of 3,6,9-Trienes and Their Monoepoxides. Trienes and their monoepoxides with C_{19}–C_{21} chains had been synthesized (Ando et al., 1993). Compounds with C_{22} and C_{23} chains were prepared by the same method, except for utilizing the bromo derivative of linolenic alcohol in a Grignard-type coupling reaction instead of a tosyl derivative (Fukumoto and Yamamoto, 1992). To yield Z3,Z6,Z9-18:H, the tosyl derivative was mixed with $LiAlH_4$ in dry THF and stirred for one night. The triene was also converted to the 1:1:1 mixture of monoepoxydienes by MCPBA oxidation. These positional isomers were separated more easily by the MPLC with the Lobar column than by the preparative TLC. With a solvent of 1.0% THF in *n*-hexane (flow rate: 2 ml/min), they were well separated from each other after one recycle (R_t = 62.3 min for epo3,Z6,Z9-18:H; 48.5 min for Z3,epo6,Z9-18:H; and 55.9 min for Z3,Z6,epo9-18:H). Monoepoxydienes with C_{22} and C_{23} chains were also separated by the MPLC purification.

Spectroscopy. The NMR spectrum of each compound in $CDCl_3$ was analyzed with a JEOL EX 270 Fourier transform spectrometer (270.2 MHz for ^1H and 67.9 MHz for ^{13}C) using TMS as an internal standard. Electron impact (EI) GC-MS was performed with a JEOL JMS-SX102A mass spectrometer containing a DB-1 capillary column (0.25 mm ID × 30 m, J&W Scientific). The ionization voltage of every measurement was 70 eV. The ion source temperature was 240°C, and the column temperature program was 40°C for 1 min, 50°C/min to 150°C, and finally 8°C/min to 300°C.

Field Evaluation. In the same manner reported previously (Ando et al., 1993), screening tests with seven 6,9-dienes (C_{17}–C_{23}), six 3,6,9-trienes (C_{18}–C_{23}), and their monoepoxides were carried out from August 1992 to July 1994 in a mixed forest area in the suburbs of Tokyo (Rolling Land Laboratory, Tokyo University of Agriculture and Technology, Hachioji-shi, Tokyo). Each chemical at 1 mg with purity >95% by GC was applied to a rubber septum, which was placed in a sticky-type trap (30 × 27-cm bottom plate with a roof, Takeda Chemical Ind., Ltd., Osaka, Japan). Two traps were used for each chemical.

RESULTS AND DISCUSSION

Characterization of Synthetic Compounds

Identification of Monoepoxides of 6,9-Dienes. Ozonolysis of the compound, which was collected as fraction 1 from the monoepoxides of Z6,Z9–17:H by a Lobar MPLC column, produced 3,4-epoxyundecanal with M^+ at *m/z* 184 (4%) and characteristic fragment ions at *m/z* 85 (95%), 127 (63%), 155 (12%), and 166 (19%), while *n*-hexanal, the counterpart of 3,4-epoxyundecanal, was not detected. This small aldehyde eluted from the GC column before starting the measurement of mass spectra. In contrast, ozonolysis of fraction 2 generated two aldehydes, 3,4-epoxynonanal with M^+ at *m/z* 156 (8%) and fragment ions at *m/z* 85 (84%), 99 (61%), 127 (22%), and 138 (18%); and *n*-octanal with M^+ at *m/z* 128 (1%) and fragment ions at *m/z* 99 (40%) and 110 (29%). These results showed that the compound in fraction 1 is Z6,epo9–17:H and that in fraction 2 is epo6,Z9–17:H. The chemical structures of monoepoxides with C_{18}–C_{23} chains were also determined by the same ozonolysis experiments, and it was revealed that 9,10-epoxides were recovered in fraction 1 and 6,7-epoxides in fraction 2.

It is difficult to distinguish between 6,7-epoxides and 9,10-epoxides by ^1H NMR measurements, since these compounds include a common functional group, an epoxy ring homoconjugated with C=C bond. In ^{13}C NMR, however, chemical shifts of some signals of 6,7-epoxides are different from those of the corresponding carbons of 9,10-epoxides, and the ^{13}C NMR spectrum of the crude

products of MCPBA oxidation represented some paired signals. Table 1 shows the ^{13}C NMR assignments (C-1 to C-12) for monoepoxymonoenes of the two types, which were accomplished by two-dimensional experiments and by referring to the ^{13}C NMR data of monoepoxydienes. In this table, carbons from position 4 to position 12 of the 6,7- and 9,10-epoxides are arranged conversely in order to compare the shift values of each carbon in the common structure represented with C-a to C-h (see the footnote of Table 1). Although the epoxy ring and olefinic carbons (C-c, C-d, C-f, and C-g) of the two positional isomers were at the same chemical shift values, each signal of the C-1, C-a, and C-b of the 6,7-epoxides appeared at a higher field than the corresponding signal of the 9,10-epoxides, and each signal of C-2, C-3, and C-h at a lower field. Particular differences of the shift values of C-3 and C-a are about 0.3 ppm; therefore the synthetic monoepoxide of 6,9-dienes can be identified based on the values of these ^{13}C signals.

MS Analyses of Monoepoxides of 6,9-Dienes. In EI-MS measurements, each monoepoxide of 6,9-dienes showed M^+ and $[M-18]^+$ ions with the relative intensities of ca. 5 and 10%, respectively. Reflecting the chemical structures, several abundant fragment ions are expected to be produced after the EI ionization as shown in Figure 1. While ions at *m/z* 71, 153 M-71, and M-153 are listed for both 6,7- and 9,10-epoxides in common, ions at *m/z* 99, 113, 127, M-127, M-114, and M-100 are nominated as characteristic fragments for the 6,7-epoxides and ions at *m/z* 97, 110, 124, M-125, M-111, and M-97 for the 9,10-epoxides. Their relative intensities, which were measured in this experiment, are listed in Table 2. Among them, ions at *m/z* 99, M-114, and M-100 are actually observed to be stronger in 6,7-epoxides, and ions at *m/z* 124, M-125, and M-111 in 9,10-epoxides than the other epoxides. 6,7-Epoxides showed smaller R_t than the corresponding 9,10-epoxides on a DB-1 capillary column (see Table 2). These two types of monoepoxymonoenes are distinguished from each other by using a capillary GC-MS system according to their chromatographic behavior and the above characteristic fragment ions.

Fragmentation at the epoxy ring produces ions at *m/z* 99 from 6,7-epoxides and at *m/z* 124 from 9,10-epoxides, including a moiety composed of C-1 to C-6 and C-1 to C-9, respectively, and ions at *m/z* M-100 from 6,7-epoxides and at *m/z* M-125 from 9,10-epoxides including a terminal carbon. Cleavage of the bond between the allylic carbon and the epoxy ring carbon produces ions at *m/z* M-114 from 6,7-epoxides and at *m/z* M-111 from 9,10-epoxides. When one pays attention to the three pairs of ions and divides the intensity of the latter ion by that of the former ion, namely [124]/[99], [M-125]/[M-100], and [M-111]/[M-114], the intensity ratios of 6,7-epoxides are less than or equal to 1.0, and those of 9,10-epoxides are larger than 1.0 as shown in Table 3. The main pheromonal component of *O. excavata* is Z6,epo9-21 : H, the chemical structure

TABLE 1. ^{13}C NMR ASSIGNMENTS FOR MONOEPOXIDES OF 6,9-DIENES[a]

	Chemical shift (ppm)										
	C-1	C-2	C-3	C-4(a)	C-5(b)	C-6(c)	C-7(d)	C-8(e)	C-9(f)	C-10(g)	C-11(h)
6,7-Epoxide											
epo6,Z9-17:H[b]	14.01	22.60	31.77	26.30	27.76	57.24	56.58	26.24	123.81	132.72	27.46
epo6,Z9-18:H	14.01	22.60	31.75	26.30	27.75	57.24	56.58	26.22	123.80	132.72	27.46
epo6,Z9-19:H	14.01	22.59	31.76	26.29	27.75	57.25	56.57	26.22	123.82	132.71	27.45
epo6,Z9-20:H	14.01	22.60	31.77	26.30	27.76	57.25	56.58	26.24	123.81	132.74	27.46
epo6,Z9-21:H	14.01	22.61	31.77	26.30	27.75	57.25	56.59	26.24	123.81	132.74	27.47
epo6,Z9-22:H	14.01	22.61	31.77	26.31	27.78	57.25	56.59	26.24	123.81	132.74	27.47
epo6,Z9-23:H	14.02	22.62	31.77	26.31	27.77	57.27	56.59	26.24	123.82	132.74	27.48
	C-1	C-2	C-3	C-12(a)	C-11(b)	C-10(c)	C-9(d)	C-8(e)	C-7(f)	C-6(g)	C-5(h)
9,10-Epoxide											
Z6,epo9-17:H	14.07	22.57	31.52	26.62	27.79	57.24	56.58	26.24	123.83	132.72	27.43
Z6,epo9-18:H	14.07	22.57	31.51	26.62	27.79	57.25	56.58	26.24	123.83	132.72	27.43
Z6,epo9-19:H	14.06	22.57	31.51	26.62	27.81	57.24	56.58	26.25	123.84	132.72	27.43
Z6,epo9-20:H	14.07	22.57	31.51	26.62	27.79	57.25	56.58	26.24	123.83	132.72	27.43
Z6,epo9-21:H	14.06	22.57	31.51	26.62	27.81	57.24	56.58	26.23	123.84	132.72	27.43
Z6,epo9-22:H	14.07	22.57	31.52	26.62	27.81	57.24	56.58	26.24	123.83	132.72	27.43
Z6,epo9-23:H	14.06	22.57	31.51	26.62	27.80	57.24	56.58	26.24	123.83	132.73	27.43

[a]Dienes including the common structure represented with: C-a—C-b—C-c(—O—)C-d—C-e—C-f = C-g—C-h.

[b]C-12 to C-14 ~ 29.5 ppm, C-15 31.86 ppm, C-16 22.68 ppm, C-17 14.12 ppm.

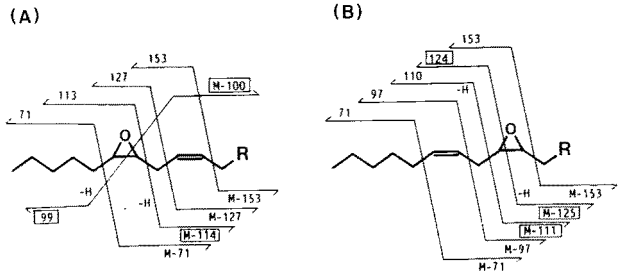


FIG. 1. Fragmentation proposed for 6,7-epoxides (A), and 9,10-epoxides (B) of 6,9-dienes with C_{17}–C_{23} chains after initial EI ionization at the epoxy oxygen and C=C bond. Diagnostic ions shown in squares were used in the calculation for Table 3. Molecular ions: C_{17} compounds (R = n-C_6H_{13}), m/z 252; C_{18} compounds (R = n-C_7H_{15}), m/z 266; C_{19} compounds (R = n-C_8H_{17}), m/z 280; C_{20} compounds (R = n-C_9H_{19}), m/z 294; C_{21} compounds (R = n-$C_{10}H_{21}$), m/z 308; C_{22} compounds (R = n-$C_{11}H_{23}$), m/z 322; C_{23} compounds (R = n-$C_{12}H_{25}$), m/z 336.

of which has been identified by ozonolysis and other experiments (Ohmasa et al., 1991). The values of the ratios calculated from the published mass spectrum of the natural pheromone are larger than 1.0 (see Table 3), indicating that it is a 9,10-epoxide. Because these three ratios reflect the position of the epoxy ring, the structure of monoepoxymonoenes of 6,9-dienes can be reliably estimated by examining the intensity ratios.

Characterization of Monoepoxides of 3,6,9-Trienes. The chromatographic behavior of the monoepoxydienes with C_{18}, C_{22}, and C_{23} chains on a normal-phase MPLC correspond to those of the C_{19}–C_{21} monoepoxydienes on a normal-phase TLC plate (Ando et al., 1993). NMR data of the compounds possessing the epoxy ring at the same position are almost the same except for the methylene signals of the long chains. The intensity of the methylene proton signals at ca. 1.3 ppm and the number of methylene carbon signals at ca. 29.5 ppm depends on the chain length. On EI-MS, fragmentation properties of the compounds previously synthesized (Ando et al., 1993) were also observed in the spectra measured for the new compounds as follows: ions at m/z M-29, M-72, and 79 for 3,4-epoxides; at m/z M-29, M-69, and 111 for 6,7-epoxides, and at m/z M-29, M-69, M-109, 108, and 79 for 9,10-epoxides. These results confirmed that these are diagnostic ions for identifying epoxydienes with C_{18}–C_{23} chains from a natural pheromone extract. By further examination of these spectra, it was revealed that other diagnostic ions, which were produced by fragmentation at the epoxy ring, were additionally characterized as follows: ions at m/z M-58 for 3,4-epoxides, at m/z 97 and M-83 for 6,7-epoxides, and at m/z 122 and M-123 for 9,10-epoxides.

TABLE 2. GC-MS DATA OF MONOEPOXIDES OF 6,9-DIENES[a]

	R_t (min)	Base ion (*m/z*)	M^+ (*m/z*)	Relative intensity of fragment ions, *m/z* (%)															
				71	79	99	110	113	124	127	153	M-153	M-127	M-125	M-114	M-111	M-100	M-97	M-71
6,7-Epoxide																			
epo6,Z9-17:H	7.53	81	252	21	48	22	48	23	20	10	17	21	8	10	27	27	10	2	12
epo6,Z9-18:H	8.46	67	266	28	56	36	33	23	30	10	19	23	9	5	24	16	13	1	11
epo6,Z9-19:H	9.43	81	280	49	63	85	33	58	20	8	12	8	12	4	17	11	7	1	13
epo6,Z9-20:H	10.42	67	294	35	46	44	29	35	15	5	12	1	6	1	9	6	4	1	8
epo6,Z9-21:H	11.48	96	308	35	58	56	46	47	29	8	14	1	4	2	16	13	4	1	11
epo6,Z9-22:H	12.46	81	322	40	57	49	33	34	21	5	13	1	4	1	8	5	4	1	9
epo6,Z9-23:H	13.49	67	336	51	48	96	38	45	23	10	17	2	7	2	11	6	7	1	15
9,10-Epoxide																			
Z6,epo9-17:H	7.54	69	252	17	37	8	56	35	26	10	12	8	11	15	6	12	3	4	13
Z6,epo9-18:H	8.48	81	266	28	45	16	80	23	37	6	22	11	10	21	7	21	2	10	15
Z6,epo9-19:H	9.47	81	280	38	49	9	67	24	26	3	18	3	18	38	3	29	2	6	12
Z6,epo9-20:H	10.47	81	294	28	43	11	44	19	21	2	13	1	4	16	2	16	1	2	10
Z6,epo9-21:H	11.52	81	308	33	50	17	71	32	31	3	20	2	8	24	5	27	2	5	13
Z6,epo9-22:H	12.50	81	322	29	46	7	51	20	24	3	10	0	2	14	1	13	1	3	9
Z6,epo9-23:H	13.54	81	336	48	44	10	55	17	24	3	18	1	0	38	3	21	1	6	8

[a]Each compound was chromatographed on a DB-1 capillary column (0.25 mm i.d. × 30 m) under programed temperature (40°C 1 min, 40°C to 150°C at 50°C/min, 150°C to 300°C at 8°C/min, and 300°C 5 min), and the MS spectrum was measured by EI ionization operated at 70 eV.

TABLE 3. INTENSITY RATIOS OF SOME CHARACTERISTIC FRAGMENT IONS OBSERVED IN MASS SPECTRA OF SYNTHETIC MONOEPOXIDES OF 6,9-DIENES AND A MAIN COMPONENT OF NATURAL PHEROMONE OF *Oraesia excavata*

	Intensity ratio							
	Synthetic monoepoxymonoene[a]							Natural pheromone[b]
Ratio, [ion]/[ion]	C_{17}	C_{18}	C_{19}	C_{20}	C_{21}	C_{22}	C_{23}	C_{21}
6,7-Epoxide								
[124]/[99]	0.9	0.8	0.2	0.3	0.5	0.4	0.2	
[M-125]/[M-100]	1.0	0.4	0.6	0.3	0.5	0.3	0.3	
[M-111]/[M-114]	1.0	0.7	0.6	0.7	0.8	0.6	0.5	
9,10-Epoxide								
[124]/[99]	3	2	3	2	2	3	2	2
[M-125]/[M-100]	5	11	19	16	12	14	38	65
[M-111]/[M-114]	2	3	10	8	5	13	7	10

[a] Spectra were measured with a JEOL JMS-SX102A mass spectrometer operated at 70 eV.
[b] Spectrum was measured with a JEOL JMS-D300 mass spectrometer operated at 70 eV (Ohmasa et al., 1991).

Sex Attractants Discovered by Screening Tests

Field Evaluation from 1992 to 1994. By the field screening tests of 6,9-dienes, 3,6,9-trienes, and monoepoxydienes with C_{19}–C_{21} chains, sex attractants for 14 geometrid and four noctuid species had already been found in Japan (Ando et al., 1993). This time field evaluation mainly with their C_{18}, C_{22}, and C_{23} chain analogs and monoepoxymonoenes with chains from C_{17} to C_{23} showed attraction of male moths of another six geometrid and five noctuid species. Table 4 lists the scientific names of the new attracted insects, the abbreviated names of the attractants, the time of flight, and the total number of moths captured by dual traps. All of the six geometrid species belong to the subfamily Ennominae. Recently sex pheromones were investigated in geometrid moths closely related to three of these species. Two C_{17} components, epo3,*Z*6,*Z*9–17:H and *Z*3,*Z*6,*Z*9–17:H, have been identified as sex pheromone components from the pheromone gland of *Abraxas grossulariata* L., the 10:1 mixture of which attracted the male moths (Tóth et al., 1992), while the corresponding C_{18} epoxide epo3,*Z*6,*Z*9–18:H attracted *A. niphonibia* in our field test. Other sex pheromones, *Z*3,epo6,*Z*9–18:H and *Z*3,*Z*6,*Z*9–18:H, have been identified from the gland extract of *Ectropis obliqua* Prout (Yao et al., 1991) in China. The monoene derivative epo6,*Z*9–18:H more effectively attracted *E. excellens* than the

TABLE 4. SEX ATTRACTANTS DETECTED IN FIELD SCREENING TESTS IN A FOREST IN TOKYO FROM 1992 TO 1994

Family, subfamily, and species	Attractant	Time of flight	Attracted moths (*N*)
Geometridae			
Ennominae			
Abraxas niphonibia Wehrli	epo3,Z6,Z9-18:H	June	17
Colotois pennaria ussuriensis Bang-Haas[a]	Z3,epo6,Z9-20:H	Nov.-Dec	99
Ectropis excellens (Butler)	epo6,Z9-18:H	Apr.	291
	Z3,epo6,Z9-18:H		38
	Z6,epo9-18:H		16
Erannis golda Djakonov	Z3,epo6,Z9-18:H	Dec.	6
Menophra senilis (Butler)	epo6,Z9-19:H	Apr.-May	27
Rhynchobapta cervinaria bilineata (Leech)	Z6,Z9-17:H	Aug.	6
Synegia esther Butler	Z6,Z9-21:H	Sep.	10
Noctuidae			
Ophiderinae			
Hypenomorpha calamina (Butler)	Z6,epo9-20:H	June	74
Neachrostia bipuncta Sugi	Z3,Z6,Z9-18:H	Sep.-Oct.	21
Pangrapta trimantesalis (Walker)	Z3,Z6,epo9-23:H	Aug.-Sep.	13
Rivula leucanioides (Walker)	Z3,epo6,Z9-18:H	Oct.-Nov.	11
Herminiinae			
Adrapsa notigera (Butler)	Z3,epo6,Z9-23:H	Aug.-Sep.	97
	Z3,epo6,Z9-22:H		14

[a] This species was attracted by epo3,Z6,Z9-20:H and Z3,epo6,Z9-19:H in our previous field tests (Ando et al., 1993).

monoepoxydiene in Japan. The mixture of two C_{19} pheromone components, Z3,epo6,Z9-19:H and Z3,Z6,Z9-19:H (10:3), attracted *Erannis defoliaria* Clerck (Hansson et al., 1990). In this test the C_{18} compound Z3,epo6,Z9-18:H caught males of *E. golda*. Sex pheromones and attractants have not been reported so far for any species in the same genera as the other three species, *Menophra senilis* Butler, *Rhynchobapta cervinaria bilineata* Leech, and *Synegia esther* Butler. In addition to these six species, *Colotois pennaria ussuriensis* Bang-Haas is also listed in this table, since Z3,epo6,Z9-20:H attracted this species more strongly in the winters of 1992 and 1993 than epo3,Z6,Z9-20:H and Z3,epo6,Z9-19:H, which had been announced to be its attractant in the field evaluation in 1991 (Ando et al., 1993).

Noctuid species attracted in this test are *Hypenomorpha calamina* Butler, *Neachrostia bipuncta* Sugi, *Pangrapta trimantesalis* Walker, *Rivula leucanioides* Walker, and *Adrapsa notigera* Butler. The first four species belong to

the subfamily Ophiderinae, and the last to Herminiinae. Although sex attractants of the species in the genus *Rivula* (*R. propinqualis*, *R. sericealis*, and *R. sasaphila*) are now known (Millar et al., 1990; Ando et al., 1993), no reports have been published concerning the pheromones and attractants of the moths in the other genera—*Hypenomorpha*, *Neachrostia*, *Pangrapta* and *Adrapsa*.

Structural Peculiarities of Discovered Sex Attractants. By field trials from 1991 to 1994, we finished the evaluation of 3,6,9-trienes with C_{18}–C_{23} chains, 6,9-dienes with C_{17}–C_{23} chains, and all of their monoepoxides. These systematic tests in Japan revealed the sex attractants for a total of 29 species. Although lepidopterous sex pheromones are usually composed of multiple components, not a few species were successfully attracted by single-source lures. The peculiarities of their chemical structures are summarized in Table 5. The number of the species attracted by the trienes and the monoepoxides (monoepoxydienes) is larger than that by the dienes and the monoepoxides (monoepoxymonoenes), and the epoxides attracted more species than the parent hydrocarbons. In particular, a considerable number of noctuid species were trapped by the lures baited with monoepoxydienes. By field evaluation of monoepoxymonoenes of C_{17}–C_{21} in Saskatoon (Canada), Millar et al. (1991) observed attraction of four geometrid species. Our test of the monoepoxymonoenes in Japan showed attrac-

TABLE 5. NUMBER OF LEPIDOPTEROUS SPECIES, SEX ATTRACTANTS FOUND TO BE UNSATURATEI HYDROCARBONS, AND THEIR MONOEPOXIDES BY FIELD SCREENING TESTS IN JAPAN

	Geometridae				Noctuidae		
Attractant	Ennominae	Oenochrominae	Geometrinae	Larentiinae	Ophiderinae	Herminiinae	Tot
Functional group							
Triene	0	1	2	3	1	0	7
Monoepoxy-diene	6	2	0	1	5	2	16
Diene	2	0	1	0	0	0	3
Monoepoxy-monoene	2	0	0	0	1	0	3
Carbon length of chain							
C_{17}	1	0	0	0	0	0	1
C_{18}	3	0	0	0	2	0	5
C_{19}	3	1	0	2	2	0	8
C_{20}	1	0	3	0	1	1	6
C_{21}	2	2	0	2	1	0	7
C_{22}	0	0	0	0	0	0	0
C_{23}	0	0	0	0	1	1	2

tion of two other geometrid species and, interestingly, one noctuid species. While the number of the species attracted by monoepoxymonoenes is much smaller than those attracted by monoepoxydienes, these results indicate that the monoepoxymonoenes are also important pheromonal components of the geometrid and noctuid species.

Carbon lengths of the sex attractants discovered in Japan are mainly C_{18}–C_{21}. Since monoepoxydienes of C_{17} have not been tested, it cannot be concluded that the C_{17} compounds are novel for Japanese Lepidoptera. A biosynthetic pathway has been proposed for the hydrocarbons with a homoconjugated polyene system and their monoepoxides (Rule and Roelofs, 1989). Starting from the fatty acids with the polyene system, linoleic and linolenic acids, which commonly occur in insects, these compounds might be biosynthesized by a combination of chain elongation and reductive decarboxylation. When the chain elongation proceeds only with acetyl CoA, compounds with carbons of odd numbers are selectively produced. However, not a few hydrocarbons and epoxides with an even carbon chain are recognized as pheromone components (Arn et al., 1992). More than one third of the attractants found in our experiments have an even-number carbon chain. It is necessary to understand well the biosynthetic pathways of the geometrid and noctuid pheromones.

Our field tests utilized a racemic mixture of *cis*-epoxides. It is desirable to examine the attractancy of optically pure samples. Another valuable method to obtain further information about the geometrid and noctuid pheromones is the evaluation of multicomponent lures.

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IDENTIFICATION OF SENSILLA INVOLVED IN TASTE MEDIATION IN ADULT WESTERN CORN ROOTWORM (*Diabrotica virgifera virgifera* LeConte)

SYLWESTER CHYB,* HERBERT EICHENSEER, BENEDICT HOLLISTER, CHRISTOPHER A. MULLIN, and JAMES L. FRAZIER

Department of Entomology
The Pennsylvania State University
University Park, Pennsylvania 16802

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Abstract—A group of sensilla present on the maxillary galea of adult western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) beetles has been identified morphologically and physiologically to be involved in taste mediation. There are approximately 15 chemosensory hairs on each galea. Bilateral removal of these structures resulted in a significantly reduced consumption of a strongly phagostimulant triterpenoid, cucurbitacin B, and led to increased ingestion of a phagodeterrent alkaloid, strychnine. Electrophysiological responses obtained via tip-recording of galeal chemosensilla with submillimolar concentrations of host and nonhost plant compounds resulted in dose responses overlapping with the effective behavioral ranges. Cucurbitacin B was found to evoke chemosensory responses at levels as low as 0.1 μM. Since γ-aminobutyric acid (GABA) is an agonist, (−)-β-hydrastine and strychnine are antagonists, and cucurbitacin B has been proposed to act at a separate modulatory site of classical synaptic GABA and glycine receptor-channel complexes, results reported here raise the possibility that there are peripheral chemosensory receptor sites that may resemble, functionally and structurally, synaptic receptor sites in the central nervous system.

Key Words—Insect-plant interactions, chemoreception, feeding behavior, phagostimulant, deterrent, antifeedant, GABA, cucurbitacins.

*To whom correspondence should be addressed.

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INTRODUCTION

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a major pest of corn (*Zea mays* L., Gramineae) in the midwestern United States. Despite intensive insecticide applications, the geographical range of this species has been expanding eastward in recent decades. The annual costs of pesticide treatments and crop losses due to this insect amount to about one billion dollars in the United States. Most damage is done by the monophagous larvae, which attack the root system of young corn, leading to plant lodging. The polyphagous adult beetles feed on corn pollen, silk, tassels, and sometimes leaves, or flowers and foliage of alternative host plants, e.g., squash (Krysan and Miller, 1986; Metcalf and Metcalf, 1992, 1993). Although adults exert less damage on corn, this obligatory feeding leads to the production of about 500–1000 eggs per female that overwinter and constitute the next season's generation of rootworm larvae.

Despite the clear and long-standing agricultural pest status of *D. virgifera virgifera*, little is known about the physiological basis of its feeding, especially the sensory aspects of host-plant recognition. Cucurbitacins, a family of triterpenoids found in Cucurbitaceae, are the most powerful feeding stimulants established for this and closely related leaf beetles (Metcalf et al., 1980). On the other hand, compounds that reduce food intake in adult corn rootworms have only recently been discovered (Mullin et al., 1992, 1994). These findings contribute to other recent developments in antifeedant design and technology that have opened new opportunities for biological and chemical insect control, particularly as pest-specific formulations (Jermy, 1990; Frazier and Chyb, 1995).

We have previously shown that a number of sesquiterpenoid epoxides isolated from cultivated sunflower (*Helianthus annuus* L., Compositae), a marginal host plant for adults (Siegfried and Mullin, 1990), are able to reduce food intake by adult *D. virgifera virgifera* in a squash disk consumption bioassay (Mullin et al., 1992). In turn, isoquinoline alkaloids, commonly found in spring wildflowers of the northeastern United States (Papaveraceae, Fumariaceae, Ranunculaceae, and Berberidaceae) and thus temporally unavailable for consumption by adult rootworms, were potent antifeedants for *Diabrotica*. The antifeedant potency of selected terpenoids and alkaloids was high enough to counteract and overcome the phagostimulatory action of cucurbitacins present in the medium. Some of the deterrents identified to date are well-known antagonists of γ-aminobutyric acid (GABA) and glycine receptor-channel complexes in synaptic sites of the vertebrate central nervous system (CNS) (see for example Hall, 1992). GABA, glycine, and other agonists of these receptors are at least weakly phagostimulatory for *Diabrotica* (Mullin et al., 1994). These observations opened the possibility that a neurotransmitter receptor structurally related to one found in the insect CNS might be localized peripherally, within insect sensilla, and therefore be more susceptible to chemicals designed for insect control.

The aim of the present study is to identify and preliminarily characterize, morphologically and physiologically, sensory structures involved in taste mediation, specifically for phagostimulant and phagodeterrent detection, by adult beetles of *D. virgifera virgifera*. Knowledge of the mode of action of compounds affecting corn rootworm feeding behavior could potentially lead to the design of more effective and specific control agents and perhaps to a structural template for gustatory chemoreception in phytophagous insects.

METHODS AND MATERIALS

Insects. Pupae of a nondiapausing laboratory strain of western corn rootworm were obtained from a commercial colony (French Agric. Res., Lamberton, Minnesota) and kept in moist soil until adult emergence. Newly emerged beetles were placed in a separate container with access to water but were deprived of food prior to the electrophysiological recordings. For behavioral experiments, beetles were maintained on lettuce, winter squash, and an artificial pollen diet (Krysan and Miller, 1986) until their use in feeding bioassays.

Chemicals. GABA, (−)-β-hydrastine, and strychnine HCl were obtained from Sigma Chemical Co. (St. Louis, Missouri). Cucurbitacin B was isolated from squash, *Cucurbita maxima* Duchesne var. Blue Hubbard hypocotyls by a modified method of Metcalf et al. (1982). The residue remaining in the chloroform extract of the hypocotyl was partitioned between petroleum ether and methanol–water (9 : 1 v/v), and the aqueous methanol residue dissolved in ethyl acetate and purified using silica gel TLC. The UV-quenching band cochromatographing with cucurbitacin B was scraped and eluted with 2 : 1 $CHCl_3$–MeOH from a dry packed column.

Scanning Electron Microscopy. Excised heads or isolated maxillae of adult beetles were mounted on a stub by using silver paint, kept in a desiccator for 24 hr, and subsequently sputter-coated with particle gold. No cleaning or fixing was necessary to achieve the desired resolution. Preparations were viewed and photographed with a JEOL JSM 5400 scanning microscope at the Electron Microscope Facility of The Pennsylvania State University.

Galeal Ablation and Feeding Bioassays. Chilled beetles were placed in a plastic pipet tip from which only the head could protrude. Galeae on one (unilateral ablation) or both (bilateral ablation) sides were removed with a fine pair of forceps; control insects had no operation. After a two-day recovery period with food and overnight starvation, ablated beetles were given a choice between two different disks.

In one experiment, disks (177 mm^2) were punched out of squash blossoms and one beetle could choose between a solvent-treated and a 6.0-nmol strychnine-treated disk; consumption levels were evaluated at 5, 24, and 48 hr. In

the second experiment, two beetles could choose between two regenerated cellulose disks (30 mm^2, 0.45-μm pore size; Schleicher and Schuell, Keene, New Hampshire) stuck to insect pins 1–2 mm above the filter paper opposite one another. One disk contained only 10.7 nmol cucurbitacin B and the other disk contained the same amount of cucurbitacin B and 2.2 nmol strychnine. Beetles were removed 24 hr later, disks were air-dried, and the area of each disk measured with an image analyzer to the nearest 0.1 mm^2.

Electrophysiological Recordings. Responses of sensory hairs to phagostimulants and phagodeterrents were obtained by a standard tip-recording technique (Hodgson et al., 1955; Frazier and Hanson, 1986). Newly emerged adults were used in the experiments. This helped decrease the inherent variability of responses, and kept the signal-to-noise (S/N) ratio of electrophysiological recordings high as well, thus greatly facilitating subsequent data analysis. The insect's head was mounted on a glass capillary filled with 0.1 M KCl. Maxillae were gently pulled away from the head with a surgical thread lasso and immobilized by waxing the thread to the ventral surface of the prothoracic legs. Another glass capillary, pulled and sized to approximately 15 μm in external diameter, was filled with a solution of the test compound in 0.01 M KCl (which by itself evoked a low firing activity of 12.4 $\pm$ 2.1 spikes/sec, $N = 122$), and used as a stimulating/recording electrode. Its tip opening was just large enough to allow placing it over a single sensillum. Both microelectrodes were connected to a high-impedance, baseline-restoring preamplifier and a filter amplifier with a bandpass of 50–2000 Hz. Spike trains were viewed on a storage oscilloscope, monitored with a loudspeaker, and stored on an FM magnetic tape together with a voice commentary provided by the experimenter (Frazier and Hanson, 1986). Records were subsequently digitized at 10,000 Hz and analyzed with customized software for Macintosh computers, SpikeAnalysis 3.0 (Roessingh et al., 1992). For dose–response curves, stimuli were presented in the order of increasing concentrations and at least 2-min recovery periods between applications were allowed. At least 30 records were obtained, and the spike counts were averaged across trials within each concentration.

Functional differences among galeal sensilla were evaluated by comparing firing rates of different sensilla in response to a series of six concentrations of GABA or (−)-β-hydrastine. Hairs were numbered according to position from 1 (most medial) to 6 (most lateral). Often, because of the galeal orientation of a particular specimen, it was not possible to record from the most lateral hair (number 6); therefore, most of the data presented here do not include responses from this hair. Results were analyzed statistically using two-way analysis of variance.

RESULTS

Morphology. Adult *D. virgifera virgifera* have well-developed, paired maxillae that under natural conditions are located just below and behind the mandibles (Figure 1) and constitute crucial components of the insect's chewing

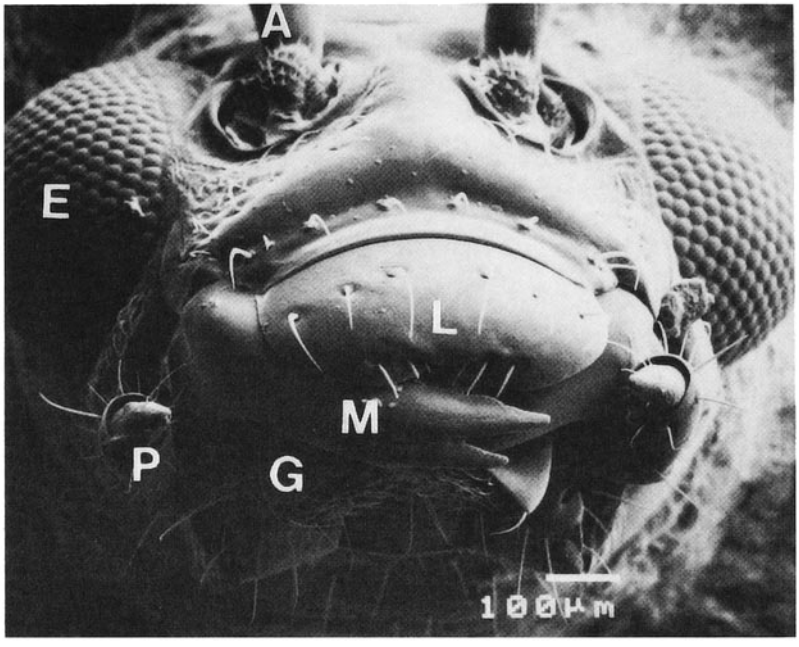

FIG. 1. Scanning electron micrograph showing a frontal view of the head of adult *D. virgifera virgifera*: antennae (A), compound eye (E), maxillary galea (G), labrum (L), mandible (M), maxillary palp (P).

apparatus. Each maxilla is composed of three structural elements: palp, galea, and lacinea (Figure 2).

The maxillary palps are the outermost parts of the maxillae. They are composed of four segments, with each segment having a complement of several long thin setae, variable in length. Based upon their morphology, at least some of these setae may have a mechanosensory function (Figure 2).

Located medially from the maxillary palp is the galea (Figure 2). It is roughly triangular in shape, measuring 50 μm at the base and widening to about 120 μm distally; and is approximately 250 μm long. The sensilla on the galea fall into two morphologically distinct groups: longer ($\sim$120 μm) and thinner ($\sim$3 μm) hairs localized on the perimeter of the galea, and shorter (see below), blunt-tipped hairs in the middle of the flattened surface. The latter are distributed in a relatively regular fashion along the medial–lateral axis of the galea surface, forming two rows of six to seven hairs each (Figure 3). Only these hairs are responsive to chemical stimulation. Neighboring taste sensilla, within and between the two rows, are separated by a distance of approximately 15–20 μm. From the medial to the lateral side of the galea, there is a noticeable decrease

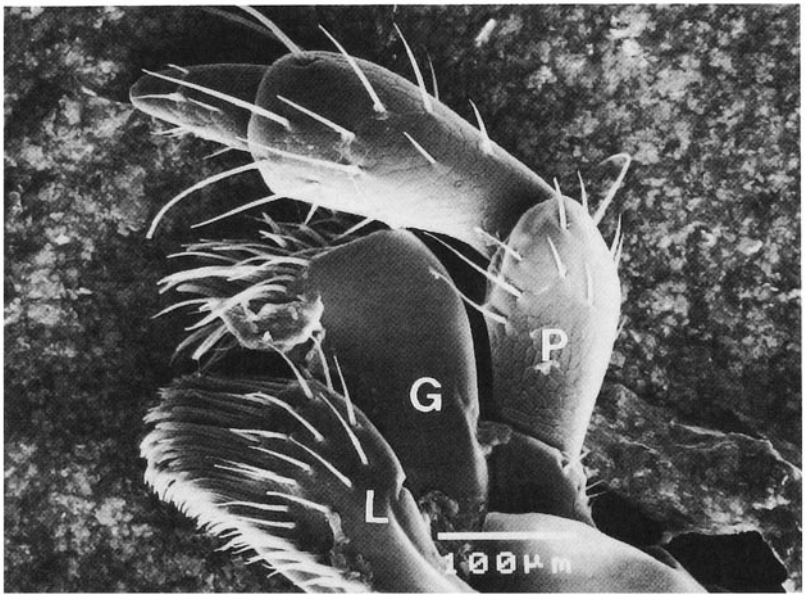

FIG. 2. Scanning electron micrograph of an isolated maxilla of adult *D. virgifera virgifera*, showing morphological details of maxillary palp (P), galea (G), and lacinea (L).

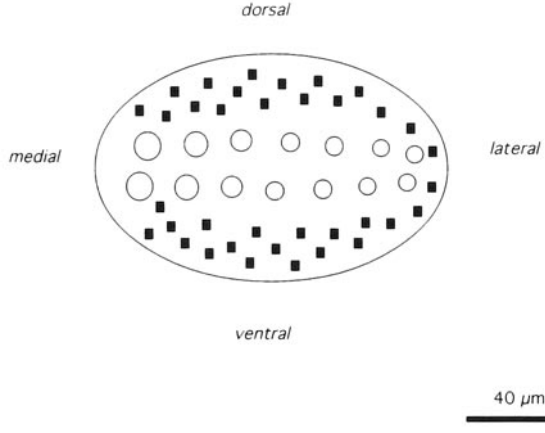

FIG. 3. Diagrammatic distribution of chemosensilla (open circles) and putative mechanosensilla (closed rectangles) on the galea of adult *D. virgifera virgifera* (reconstructed from a digitized video recording).

in length of chemosensilla from 70 μm to 30 μm, accompanied by a corresponding reduction in hair diameter. All chemosensilla are slightly curved along their length, with their tips pointing medially (Figure 4).

The lacinea, the most medial part of maxilla (Figure 2), is similar in shape and size to the galea, but equipped with a very dense cover of long (~100 μm) and relatively straight hairs on its proximal surface. The functional significance of these structures is unknown since individual hairs are not readily accessible for electrophysiological investigations. There are also several setae located along the sides of lacinea that may be involved in mechanoreception.

Although the major group of maxillary chemosensilla has been observed on the maxillary galea, it cannot be ruled out that there are other contact chemoreceptors present on the other parts of maxillae (e.g., lacinea), as well as associated with other body regions, including the other mouthparts. An important question, therefore, is whether the galeal chemosensilla are the ones responsible for food selection and host vs. nonhost discrimination in adult *D. virgifera virgifera*.

Galeal Ablation. In the deterrent-detection experiment (Figure 5A), bilaterally ablated beetles consumed significantly more of the strychnine-treated disks

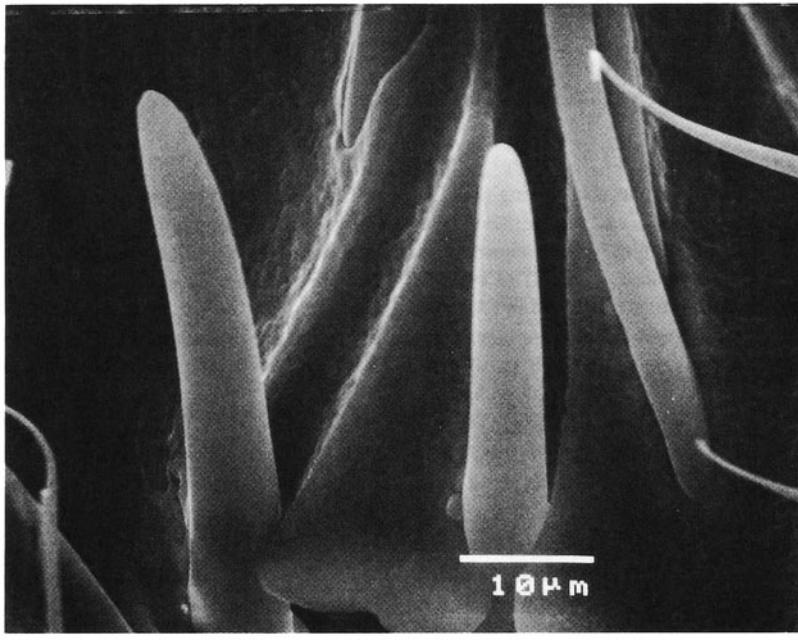

FIG. 4. Scanning electron micrograph of two of the galeal chemosensilla. The tips of two of the putative galeal mechanosensilla are visible to the right of chemosensilla.

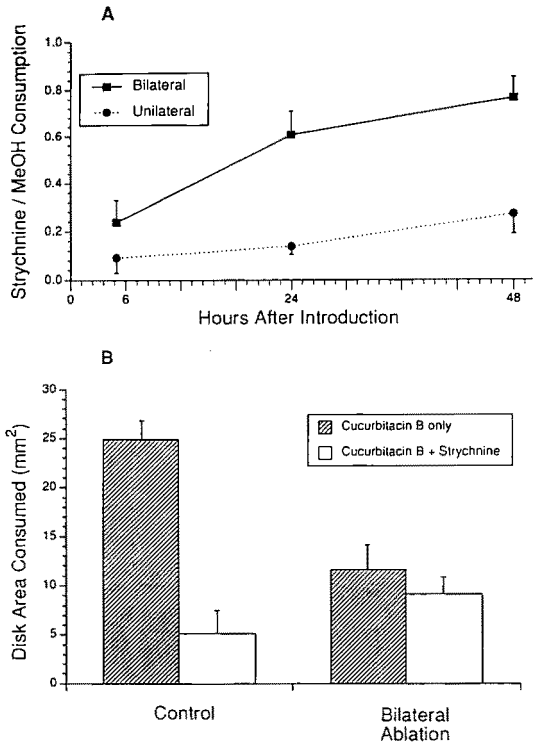

FIG. 5. Feeding by insects lacking maxillary galea: results of two bioassays using squash blossom disks (A), and regenerated cellulose disks (B). Consumption in A indicates beetle consumption of strychnine-treated disks relative to solvent-treated disks after 5, 24, and 48 hr (mean ± SEM) in bilaterally and unilaterally ablated beetles. (B) The amount of disk area consumed in 24 hr by beetles with both galeae intact (control) and both galeae ablated (bilateral ablation) when offered a choice between a cucurbitacin B-treated disk (Cucurbitacin B only) and a disk treated with both cucurbitacin B and strychnine (Cucurbitacin B + Strychnine).

than unilaterally ablated beetles at 24 and 48 hr (two-sample t-test, 24 hr: $P \leq 0.01$; 48 hr: $P < 0.001$) but not at 5 hr ($P > 0.05$). During the entire experiment, both sets of beetles fed more on solvent-treated disks than on strychnine-treated disks, as demonstrated by consumption ratios of less than 1.0. Strychnine was less deterrent to bilaterally ablated beetles than for unilaterally ablated beetles.

In the phagostimulant-detection experiment (Figure 5B), bilaterally ablated beetles consumed significantly less of the cucurbitacin B-treated disks than non-

ablated beetles ($P < 0.001$). Both sets of beetles consumed similar amounts of the cucurbitacin B plus strychnine-treated disks ($P > 0.05$). Galeal ablation affected consumption on both disks because a smaller percentage of total disk area was eaten by ablated beetles than by the controls. Nevertheless, beetles without galea could not discriminate between a disk treated with cucurbitacin B with or without strychnine.

Electrophysiology of Chemosensory Responses. Galeal chemosensilla of adult *D. virgifera virgifera* exhibited clear sensitivity to several compounds shown previously to enhance or reduce feeding. Although gustatory responses were usually unicellular, two-cell responses were also observed.

GABA stimulated a single cell (Figure 6A) over a concentration range of 1 μM to 10 mM. The dose–response curve within the lower limits of this range was relatively flat, and a substantial increase in the firing rate was observed above 100 μM (Figure 7A). It is worth noting here that responses to this compound were often quite variable, with the same concentration evoking different levels of firing activity in different insects. Spike trains were frequently characterized by a low S/N ratio, which made it difficult to separate spikes from background noise levels typical for electrophysiological recordings.

Cucurbitacin B (Figure 7B) evoked a vigorous response with a threshold

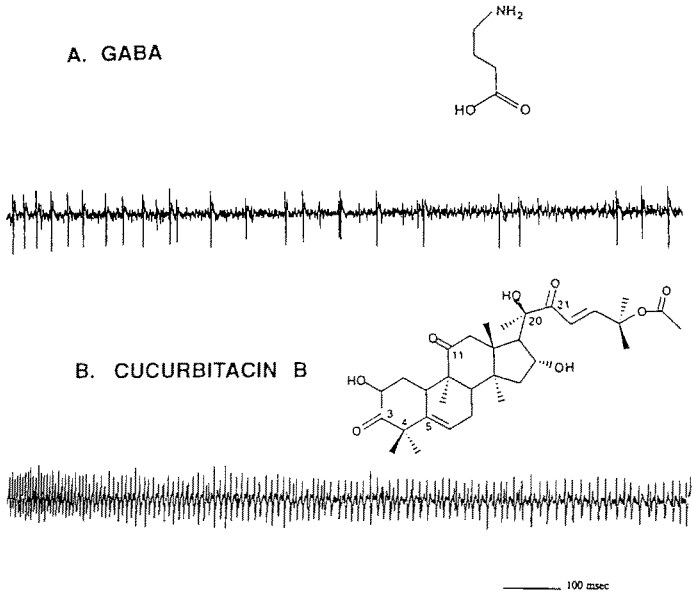

FIG. 6. Representative records of chemosensory responses of galeal sensilla to 10 μM concentrations of phagostimulants: GABA (A) and cucurbitacin B (B).

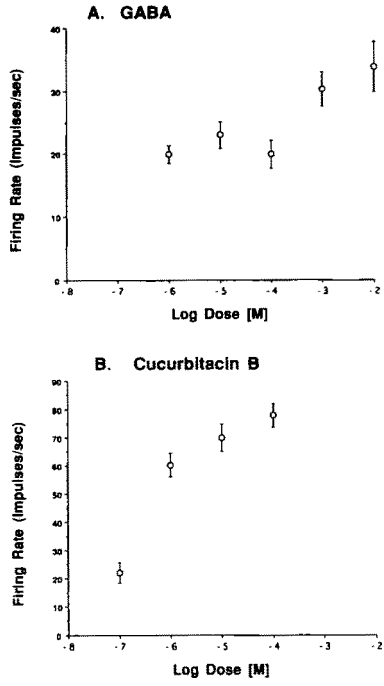

FIG. 7. Dose-response curves for phagostimulants: GABA (A), cucurbitacin B (B). Firing rates are expressed as the number of impulses within the first second of stimulation (means ± SEM, all data points based on ≥30 records).

at approximately 0.1 μM. Increasing the concentration to 100 μM activated a second cell. There was usually a response delay in the range of 10–20 msec followed by a period of rapid firing with very little sensory adaptation. Doses higher than 100 μM were not tested due to the limited solubility of cucurbitacin B in the reference solution (0.01 M KCl).

(−)-β-Hydrastine evokes responses from a single cell (Figure 8A), giving a sigmoid pattern of dose-response (Figure 9A). There were noticeable changes in the spike amplitude within the first 150–200 msec of the response, and little adaptation occurred within the first second of stimulation with this compound.

Strychnine stimulated a single cell with clear patterns of phasic and tonic components of responses (Figure 8B). Records from this compound were usually characterized by a high S/N ratio. A linear dose-response curve with saturation at 100 μM was found (Figure 9B). A further 10-fold increase in concentration did not significantly increase the intensity of response (one-way ANOVA, $P > 0.05$).

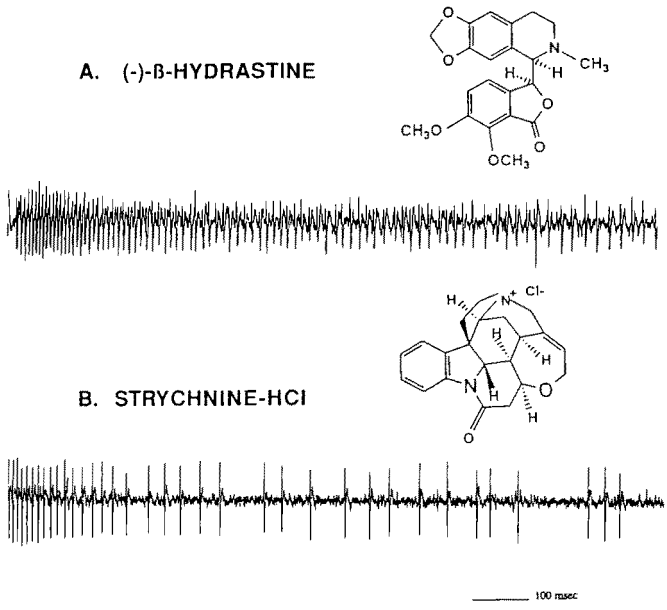

FIG. 8. Representative records of chemosensory responses of galeal sensilla to 10 μM concentrations of phagodeterrents: (−)-β-hydrastine (A) and strychnine HCl (B).

Functional Differences among Sensilla. No statistically significant differences in firing rate during the first second of stimulation were found among sensilla from the same galea when several individual hairs were tested with various doses of GABA or (−)-β-hydrastine (two-way ANOVA: $df = 4$, $F = 1.06$, $P = 0.38$ for GABA; $df = 5$, $F = 0.46$, $P = 0.81$ for (−)-β-hydrastine). Similarly, the interaction between the dose tested and the sensillum position along the medial–lateral axis of the maxillary galea was not statistically significant in either case ($df = 20$, $F = 0.35$, $P = 0.99$, and $df = 25$, $F = 1.02$, $P = 0.46$, respectively). Temporal patterns of observed responses were also similar (but were not quantified) regardless of the hair position.

DISCUSSION

Adults of western corn rootworm have a distinct population of galeal contact chemoreceptors exhibiting sensitivity to a number of compounds shown previously to evoke or deter feeding. Particularly novel is the potent electrophysiological response elicited by cucurbitacin B in the galeal chemosensilla of this *Diabrotica* species.

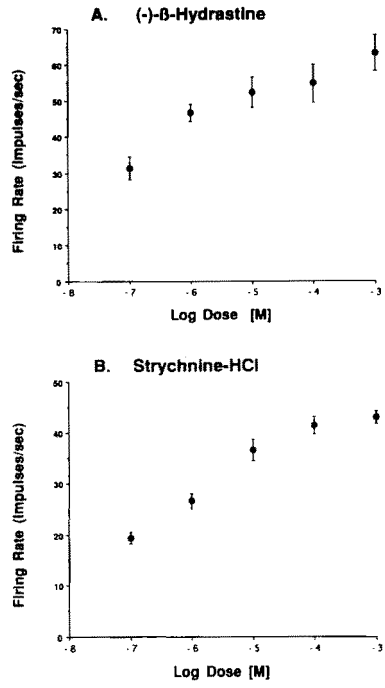

FIG. 9. Dose-response curves for phagodeterrents: (−)-β-hydrastine (A) and strychnine HCl (B). Firing rates are expressed as the number of impulses within the first second of stimulation (means ± SEM, all data points based on ≥30 records).

Mitchell and Harrison (1984) studied galeal chemosensilla of another chrysomelid, Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and found a similar number of chemosensory sensilla they term apical pegs; the spatial distribution of these structures was reported by Sen and Mitchell (1987) as irregular, unlike the two-row formation observed here. Galeal sensilla in *L. decemlineata* were indistinguishable morphologically but, surprisingly, were functionally different. In particular, one of the hairs, dubbed the α-sensillum, was significantly more sensitive to stimulation with GABA (and L-alanine) than all other hairs; ultrastructurally, the α-sensilla had three, rather than four (like other galeal chemosensilla in this beetle) dendrites associated with its lumen. Our careful investigation of this possibility in *D. virgifera virgifera* provided no indication of sensitivity differences among galeal sensilla in this species. It appears, therefore, that the more polyphagous adult *D. virgifera virgifera* may have at its disposal a functionally uniform complement of gustatory hairs on the galea.

Ultrastructural studies of galeal sensilla of adult *D. virgifera virgifera* are underway.

D. virgifera virgifera feeds on nutrient-rich reproductive tissues of plants, including pollen. Although detailed chemical composition of these food sources has not been adequately determined, especially when compared with other plant tissues (e.g., leaves), high levels of amino acids and other nitrogenous compounds have been detected. Since GABA is one of the major components of pollen in some plant species, it raises the possibility that pollinivorous insects could possess gustatory receptors tuned to this and related compounds (Mullin et al., 1993, 1994). Clearly, as shown by our results, *Diabrotica* can perceive the presence of GABA in their diet and, as more recent preliminary data indicate, other short-chain neutral amino acids: glycine, alanine, β-alanine, and proline (Chyb and Hollister, unpublished data).

Cucurbitacin B was a potent stimulant of the chemosensory cells in our studies. It was effective at concentrations as low as 0.1 μM, the lowest of the tested doses, well below the thresholds for phagostimulation seen in any other insect species and for any other feeding stimulant where millimolar doses are usually required to evoke a chemosensory response. Clearly, the phagostimulant action of this compound was mediated by the galeal chemosensilla, as shown by the ablation study. A QSAR study correlating potential differences in efficacy of phagostimulation with discreet modifications in chemical structures of different cucurbitacins could provide much needed information on the binding site of the receptor involved in sensory transduction.

Bilateral ablation of galeae reduced but did not completely abolish the beetle's sensitivity to cucurbitacin B or to strychnine. That bilaterally ablated beetles fed on cucurbitacin B-treated disks and less on strychnine-treated disks in squash disk bioassay suggests that other mouthpart sensilla are involved in taste mediation. Metcalf et al. (1980) suggested that only maxillary palps are involved in cucurbitacin detection since adult *D. undecimpunctata* could not find cucurbitacin B spots on thin-layer plates after ablation of these structures. Branson and Ortman (1969) have shown that maxillectomy in *D. virgifera virgifera* larvae reduced sucrose sensitivity but did not affect deterrence to oat roots. This suggests that larval maxillae may mediate phagostimulant chemoreception, while other mouthparts are also probably involved in chemosensory response to deterrents.

D. virgifera virgifera responded to the phagostimulant cucurbitacin B and the phagodeterrent strychnine in a dose-dependent manner over a range of concentrations from 0.1 μM to 1 mM. This work presents the first electrophysiological records obtained with cucurbitacin and also the first description of chemosensory responses of any diabroticite. Mitchell and Harrison (1985) investigated the effects of some of the *Solanum* antifeedant glycoalkaloids, including solanine and tomatine, on galeal chemosensilla of the adult Colorado potato

beetle. None of these compounds stimulated a direct chemosensory response, but rather caused bursting in taste cells after a relatively long (on average 6–12 sec) period of latency. These changes in cell activity were attributed to membrane disruption resulting from chemical stress posed by applied chemicals and are qualitatively different from the results presented in this study. Several other alkaloids (e.g., bicuculline, quinine) and terpenoids (e.g., azadirachtin, picrotoxinin) have been observed to stimulate galeal chemosensilla in *D. virgifera virgifera* (Chyb, unpublished data).

Signaling the presence of nonhost chemicals could be accomplished through one or a combination of four basic mechanisms: stimulating a specialized deterrent cell, stimulating a general sensitivity cell, inhibiting a cell responding to phagostimulant, or evoking irregular activity of chemoreceptor cells, e.g., bursting (Schoonhoven, 1982). At present, we do not have a definitive answer as to which of these modes of deterrency coding is employed by adult *D. virgifera virgifera*. It is possible that one or more deterrent cells are present since strychnine and (−)-β-hydrastine seem to stimulate separate cells (e.g., they had different time courses of response, especially within the first 200 msec; there were changes in spike amplitude and shape visible in "hydrastine" but not "strychnine" spike trains). It is also possible that these and other deterrent compounds inhibit the activity of phagostimulant-detecting cells. We need to establish whether there is any overlap of sensitivity spectra for deterrent and stimulant-activated cells. It is also unclear how many functional chemoreceptor neurons are contained within each of the galeal sensilla. Spike extraction procedures combined with analysis of spike shapes have not, as yet, provided conclusive evidence. Results of such analysis also need to be correlated with histological and ultrastructural investigations.

There is a strong correlation (Figure 10) between the amount of feeding (Mullin et al., 1993, 1994; Mullin et al., unpublished data) and the sensory input from galeal chemosensilla presented here for cucurbitacin B, strychnine HCl, and (−)-β-hydrastine (correlation coefficient, $R = 0.92$, 0.88, and 0.95 for cucurbitacin B, strychnine, and (−)-β-hydrastine, respectively). The increase in firing rate due to the increase in phagostimulant (cucurbitacin B) concentration corresponds to the increase in feeding consumption, while the reverse is true for the two deterrent compounds (increased firing corresponds to reduction in feeding consumption). It is also worth noting that while the feeding response to cucurbitacin B is proportional to the chemosensory input over almost the whole range of firing rates, the deterrent activity of (−)-β-hydrastine and strychnine seems to involve a more elevated chemosensory threshold for behavioral response, resulting in decreased consumption (above 30 spikes/sec for strychnine and almost 50 spikes/sec for hydrastine). It is, however, important to note that, due to the bioassay used to assess antifeedancy (squash disk bioassay), there was a residual dose of phagostimulant cucurbitacin B in the disks that needed

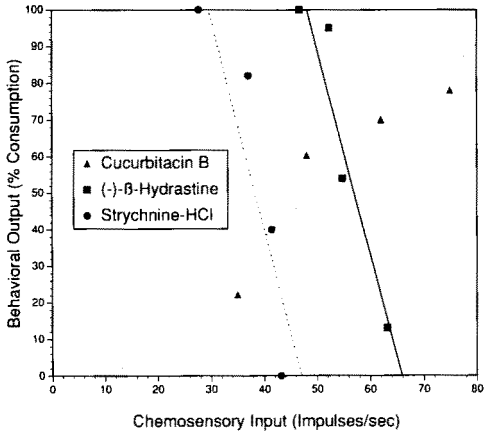

FIG. 10. Correlation between chemosensory input (firing rate of galeal chemosensory cell within the first second of stimulation) and behavioral output (percentage of disk consumed by adult beetle over 24 hr) for cucurbitacin B (triangles), (−)-β-hydrastine (squares), and strychnine HCl (circles). Points in each data series represent mean responses to (from left to right) 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M for cucurbitacin B, and 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M for the two deterrents: (−)-β-hydrastine and strychnine HCl.

to be overcome by the presence of deterrent. This may explain the threshold seen in the correlation plots for strychnine and (−)-β-hydrastine (Figure 10). Antifeedant potency was similar at the behavioral and chemosensory level; strychnine and (−)-β-hydrastine were the strongest antifeedants among the deterrent compounds tested so far (ED_{50} for behavioral response of 2 and 5 nmol/disk, respectively) and also evoked the most potent chemosensory responses (ED_{50} = 0.1 μM, both compounds). The large difference in slope between the chemosensory input–behavioral output lines for the phagodeterrent alkaloids and the phagostimulant cucurbitacin indicates a fundamental divergence in receptor cell binding of these compounds. The increase in firing frequency in response to a 10-fold increase in the deterrent concentration causes ~60% change in consumption for both strychnine and (−)-β-hydrastine, which is about four times as much as cucurbitacin B. This slope differential may represent significantly different sensory weights associated with inputs from phagostimulant and deterrent cells, as postulated by Schoonhoven (1982).

Currently very little is known about sensory processing of taste information in insect CNS, and we can only hypothesize as to how the input from contact chemosensilla is interpreted by the insect's brain. The data presented here show that *D. virgifera virgifera* beetles are able to detect alkaloids at concentrations

of 10^{-6} M, far below those naturally occurring in plants (Levinson, 1976; Wink, 1992). A 1000-fold increase in alkaloid concentration results in complete rejection of previously acceptable diet. At the same time, the taste system is finely tuned to detect the presence of phagostimulants such as cucurbitacins or amino acids. Adults of western corn rootworm feed selectively on pollen, a plant tissue low in deterrent content (Chou and Mullin, 1993, and references therein) and high in phagostimulatory amino acids (Mullin et al., 1993), but readily reject other food sources such as leaves and disk flowers where antifeedant content exceeds the levels expected to evoke a maximal firing rate from the galeal taste cell.

Our results support the hypothesis that a protein complex related to a synaptic GABA/glycine receptor may mediate host and nonhost plant discrimination in *D. virgifera virgifera*. GABA receptors are involved in sensory processing, e.g., in vertebrate vision. A potential role for this GABA receptor in insect taste has been presented elsewhere (Mullin et al., 1994). That net sensory processing of feeding stimulants and deterrents may be determined peripherally, and not exclusively in the CNS, by chemical interactions at amino acid taste receptor proteins is an intriguing hypothesis that needs experimental verification.

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OUTDOOR ATTRACTANCY OF MALES OF *Matsucoccus josephi* (HOMOPTERA: MATSUCOCCIDAE) AND *Elatophilus hebraicus* (HEMIPTERA: ANTHOCORIDAE) TO SYNTHETIC FEMALE SEX PHEROMONE OF *Matsucoccus josephi*

ZVI MENDEL,[1,*] LEV ZEGELMAN,[2] ALFRED HASSNER,[2] FABIENNE ASSAEL,[1] MIRIAM HAREL,[1] SHLOMO TAM,[1] and EZRA DUNKELBLUM[1]

[1]*Institute of Plant Protection, ARO*
The Volcani Center
Bet Dagan 50250, Israel

[2]*Department of Chemistry*
Bar-Ilan University
Ramat Gan 52100, Israel

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Abstract—The active component of the sex pheromone[3] of *Matsucoccus josephi* is (2*E*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one; the chemical is also a powerful kairomone of adult males and females of the bug *Elatophilus hebraicus* the principal predator of *M. josephi*. The presence of the *Z* isomer (2*E*,6*Z*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one does not interfere with the attractancy of the active *E* component for *M. josephi* males or the bug. Our results show a clear dose–response between trap catch of *M. josephi* males and *E. hebraicus*. Conversely, increasing amounts of the *Z* isomer in the mixture did not affect the attraction of the scale insect males or the bug. The catch of *M. josephi* males did not differ significantly among traps of different color, and was significantly higher with traps attached to the tree trunk than those suspended between trees. Comparison of the catch of *M. josephi* among the three forests and between pine species suggests that the level of infestation of *Pinus halepepsis* and *Pinus brutia* ssp. *brutia* is similar, despite the fact that the latter pine is resistant to the scale insect. Both sexes of *E. hebraicus* were trapped in much lower numbers at the more infested sites. This may be related to interference with the activity of *E. hebraicus* due to deterioration

*To whom correspondence should be addressed.

[3]Initially, this compound was incorrectly named as (2*E*,4*E*,8*E*)- and (2*E*,4*Z*,8*E*)-4,6-dimethyl-2,4,8-decatrien-7-one.

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and drying of parts of the tree crowns and heavy colonization by generalist predators in injured trees.

Key Words—*Matsucoccus josephi*, *Elatophilus hebraicus*, Homoptera, Heteroptera, Matsucoccidae, Anthocoridae, scale insect, pine bast scale, predator, *Pinus halepensis*, *Pinus brutia*, sex pheromone (2*E*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one, trap color, trap position.

INTRODUCTION

The Israeli pine bast scale, *Matsucoccus josephi* Bodenheimer et Harpaz, (Homoptera, Matsucoccidae) is the most serious pest of pine stands in Israel, killing large areas planted with Aleppo pine, *Pinus halepensis* Mill. (Mendel et al., 1994). The natural distribution of *M. josephi* is in areas in the Near East where its principal host, the brutia pine (*Pinus brutia* Ten. spp. *brutia* Nahal) occurs.

The larvae of *M. josephi* are hidden under bark crevices and at the base of needles. The cryptic occurrence of the scale on adult trees makes visual estimation of the pest densities impractical. At present, sampling of stem sections and crowns for counting the larvae is necessary in order to study infestation levels and population trends of the scale insect. This makes monitoring of *M. josephi* very tedious and expensive.

As in other members of the superfamily Coccoidea, the females display neoteny and the alate males locate their mates over long distances by being attracted to the female sex pheromone. First indications of the presence of a female sex attractant in the Matsucoccidae were observed by Doane (1966). The female sex pheromone has been identified for *Matsucoccus matsumurae* (Kuwana) (=*resinosae* Bean et Godwin) (Lanier et al., 1989), *Matsucoccus feytaudi* Ducasse (Einhorn et al., 1990), and *M. josephi* (Dunkelblum et al., 1993). The identification and the synthesis of the sex pheromone of *M. josephi* (Zegelman et al., 1993) have been a turning point in the possible development of monitoring techniques for this pernicious forest pest.

Unlike other Matsucoccidae, which display one (e.g., *M. feytaudi*) or two (*M. matsumurae* or *Matsucoccus pini* Green) separate generations per year, *M. josephi* produces four to five overlapping generations (Bodenheimer and Neumark, 1955; Mendel and Saphir, in preparation). Hence, year-round male flight in the forest and availability of an efficient mass rearing program (Mendel et al., 1990) allow continuous field and laboratory experiments.

Most insect predators associated with the Matsucoccidae are polyphagous; one particular group, members of the genus *Elatophilus* Reuter (Hemiptera: Anthocoridae) displays specific affinity for the genera *Pinus* and *Matsucoccus* Cockerell (Mendel et al., 1991). *Elatophilus hebraicus* Pericart is a common monophagous predator of *M. josephi* and feeds on all developmental stages. The bug occurs in the natural brutia pine forests in Asia Minor, Cyprus and

FIG. 1. Two geometrical isomer components.

Lebanon (Mendel, 1992; Mendel and Schiller, 1993), as well as in the stands of brutia pine and Aleppo pine in Israel and Jordan (Mendel et al., 1994). Preliminary observations indicated that *E. hebraicus* is attracted to the racemic female sex pheromone of *M. josephi* (Zegelman et al., 1993). The structures of the identified Matsucoccidae sex pheromones are similar. All three known pheromones are unsaturated aliphatic ketones, sharing a common C_8H_{13} diene moiety consisting of one chiral center. The second subunit is different; in *M. feytaudi* and *M. matsumurae*, the side chains are saturated and have a second chiral center (Einhorn et al., 1990; Lanier et al., 1989), whereas in *M. josephi* the chiral center is replaced by a double bond (Dunkelblum et al., 1993). Two geometrical isomer components (Figure 1) in the ratio of 75E:25Z were identified in the natural sex pheromone of *M. josephi* (Dunkelblum et al., 1993). The same isomers were produced in the synthesis of the pheromone at a ratio of 56*E*:44*Z* (Zegelman et al., 1993).

The objectives of the present study were to: (1) study the role of the *E* and *Z* isomers combined and separately in the response of the males of *M. josephi* and *E. hebraicus*; (2) study the dose–response of both insects to the sex pheromone; (3) examine the effect of layout of traps, viz., traps attached to the stem vs. traps suspended between trees; (4) evaluate the effect of trap color on catch efficacy; and (5) conduct a preliminary comparison between populations of the scale insect and its predator in stands planted with brutia pine with (a) high resistance to the scale insect and damage practically nil, and (b) the highly susceptible plantations of Aleppo pine.

METHODS AND MATERIALS

Chemicals. The racemic pheromone mixture of (2*E*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one (56% *E* isomer) and (2*E*,6*Z*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one (44% *Z* isomer) was prepared using the Reformatzky and Wittig reaction as key steps (Zegelman et al., 1993). The isolated *E* and *Z* isomers were obtained by GC separation of the mixture on a packed OV-1 5% column (2 m × 4 mm on CWHP) at 120°C, using a nondestructive thermal conductivity detector.

Study Areas, Trap Activation, and Data Collection. The experiments were conducted in adult plantations of Aleppo pine and brutia pine in the Judean

foothills, Samaria, and Galilee in Israel. Triangular sticky traps made of white plastic, containing a rubber septum as dispenser (Dunkelblum et al., 1987), were used. Traps were attached to the north side of the trunk of living trees, 1.8 m above the ground. The experiments were arranged in a randomized block design. The scale insect males and the anthocorids were counted under a magnification of ×20.

Experiment 1 was conducted during two time periods in the Harel Forest (Judean foothills). Trapping period 1 was between March and April 28, 1993. Ten isomer mixtures were tested; the ratios of *E*:*Z* isomers and concentrations (in micrograms in parentheses) were: 5:95 (100), 5:95 (250), 55:45 (100), 55:45 (200), 55:45 (250), 55:45 (500), 75:25 (100), 90:10 (100), 95:5 (100), and 95:5 (250). Each treatment was replicated five times. The sticky plates were replaced twice during this period. Period 2 was between June 8 and 21, 1994, the ratios and concentrations (in micrograms) of *E*:*Z* isomers being: 3:97 (5) 3:79 (50) 98:2 (5), and 98:2 (50). Each treatment was replicated four times. The sticky plates were replaced once during this period. Traps with an unloaded dispenser served as the control in both periods. The total number of males of *M. josephi* and adults of *E. hebraicus* trapped throughout each period was combined.

Experiment 2 was conducted between February 18, and March 22, 1993 in the Harel Forest to test the effect of trap color and position on the catch of *M. josephi* males. Traps made from plastic board of colors white, black, yellow (yellow-orange group 15A), blue (blue group 101A) and red (red group 52A) (Anon., 1966) were baited with dispensers containing 500 μg of the racemic *E*:*Z* pheromone mixture and attached to the tree or suspended 1.8 m above the ground between trees by a wire, at a minimum distance of 3.5 m from the nearest trunk. Each color and position was replicated four times in a completely randomized block design.

Experiment 3 was conducted in March and April 1993 in three types of pine plantations located in different areas: (1) Midrakh 'Oz forest (Samaria), where Aleppo pine is severely injured by the scale insect, (2) Hanita Forest (Western Galilee), and (3) Biriyya Forest (Upper Galilee), where Aleppo pine was only slightly affected by *M. josephi*. Within each forest two adult stands, one of Aleppo pine and the other of brutia pine, were selected. In each stand four traps, baited with 500 μg of the racemic mixture of the synthetic pheromone, were used.

RESULTS

Results of experiment 1 (Table 1) show a clear dose–response between trap catch of *M. josephi* males and the increasing amounts of the *E* isomer ($R^2 = 0.87$, $P < 0.01$). Conversely, increasing amounts of the *Z* isomer in the mixture

TABLE 1. MEAN CATCH OF ADULT MALE *Matsucoccus josephi* AND ADULTS MALE AND FEMALE *Elatophilus hebraicus* IN STICKY TRAPS BAITED WITH DIFFERENT AMOUNTS OF SYNTHETIC SEX PHEROMONE MIXTURE OF FEMALE OF *M. josephi*[a]

Total amount (μg) of the pheromone mixture (% of *E*)	Amount (μg) of component		Catch/trap/day[b]	
	E	*Z*	*M. josephi*	*E. hebraicus*
Period 1				
0	0	0	0.0 f	0.0 f
100 (5%)	5	95	6.1 e	0.4 e
250 (5%)	12	238	12.6 d	0.7 de
100 (55%)	55	45	14.0 cd	0.9 cde
100 (75%)	75	25	21.1 bc	0.9 cde
100 (90%)	90	10	31.8 b	1.4 cd
100 (95%)	95	5	29.4 b	1.2 cde
200 (55%)	110	90	30.7 b	1.9 c
250 (55%)	138	112	25.9 b	1.9 c
250 (95%)	238	12	54.5 a	3.0 b
500 (55%)	276	224	71.7 a	5.0 a
Period 2				
5 (3%)	0.15	4.85	0.1 d	0 c
50 (3%)	1.5	48.5	1.5 c	0 c
5 (98%)	4.9	0.1	3.1 b	0.1 b
50 (98%)	49.0	1.0	4.6 a	1.5 a

[a]In both periods the tests were conducted in the Harel Forest. The traps were activated between March 22 and April 28, 1993, and between June 8 and 21, 1994, in period 1 and period 2, respectively.
[b]Within columns, in each period, means followed by the same letter are not significantly different. Duncan's (1955) multiple-range test, $\alpha = 0.05$.

did not affect the attraction of the males. The correlation coefficient of the amount of the *Z* isomer to male catch was close to zero ($R^2 = 0.07$). A similar pattern was observed in the catch of the predatory bug *E. hebraicus*. The correlation coefficients between the dosages of *E* and *Z* isomers and the trapped bugs were $R^2 = 0.81$ ($P < 0.01$) and $R^2 = 0.15$, respectively. Both sexes of the bugs were trapped, but sex ratios were not determined in this experiment due to technical difficulties.

The effect of the trap color and position (experiment 2) on the catches of *M. josephi* is shown in Table 2. The catch with white traps was somewhat less than with other colors, but did not differ significantly. For all colors tested, catches of *M. josephi* males were significantly ($P < 0.01$) higher with traps attached to the tree trunk than those suspended between trees, particularly in the case of red, yellow, and blue traps. The catches of the predatory bugs were

too low in this experiment for proper analysis of the level of attraction to different trap colors or position.

The catches of both *M. josephi* and *E. hebraicus* among three planting areas and between stands of Aleppo pine and brutia pine are given in Table 3. The number of *M. josephi* males and adult bugs caught in the sticky traps differ

TABLE 2. EFFECT OF TRAP COLOR AND POSITION ON MALE CATCH OF *Matsucoccus josephi* IN TRAPS BAITED WITH 500 μg OF SYNTHETIC FEMALE SEX PHEROMONE (MIXTURE OF 56*E*:44*Z*)

Trap color	Catch/trap/day[a]	
	On tree trunk	Between trees[b]
Red	34.9 a	12.5 b
Yellow	37.3 a	12.6 b
Blue	34.5 a	11.0 b
Black	34.2 a	17.3 a
White	23.8 a	13.0 a
Overall	32.9 a	13.2 b

[a]Within rows, means followed by the same letter are not significantly different. No statistical differences were found within columns. Duncan's (1955) multiple-range test, $\alpha = 0.05$.
[b]Two to 3 m from the nearest stem.

TABLE 3. MEAN ± SD OF *Matsucoccus josephi* MALES AND NUMBER AND FEMALE-TO-MALE RATIO OF *Elatophilus hebraicus* DETERMINED BY STICKY TRAPS IN THREE FORESTS DURING 44 DAYS IN FEBRUARY–MARCH 1993

Forest	Pine species	Catch/trap/day[a]		
			E. hebraicus	
		M. josephi	Males + females	Sex ratio
Midrakh Oz	*P. halepensis*	28.7 ± 7.5a	0.2 ± 0.1c	1:2.3
	P. brutia	23.6 ± 4.1a	0.1 ± 0.0c	1:3.1
Hanita	*P. halepensis*	4.2 ± 2.0bc	1.3 ± 0.6b	1:1.3
	P. brutia	4.3 ± 0.5b	5.7 ± 1.1a	1:1.4
Biriyya	*P. halepensis*	2.1 ± 0.9c	0.3 ± 0.2c	1:1.7
	P. brutia	1.6 ± 0.3c	0.5 ± 0.7bc	1:1.3

[a]Within columns, means followed by the same letter are not significantly different. Duncan's (1955) multiple-range test, $\alpha = 0.05$.

significantly between the study areas. The highest male capture was in Midrakh 'Oz and the lowest in Biriyyia. For each forest, the catches of *M. josephi* males did not differ significantly between Aleppo pine and brutia pine. The catch of *E. hebraicus* was the highest in brutia pine at Hanita, and the lowest in Aleppo pine at Midrakh 'Oz (5.7 and 0.1/trap/day, respectively). The overall male–female ratio among the trapped bugs was 1 : 1.4 (range 1 : 1.3–1 : 3.1).

DISCUSSION

The results indicate that the active component of the pheromone of *M. josephi* is (2*E*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one. Our findings also suggest that the presence of the *Z* isomer, (2*E*,6*Z*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one, does not interfere with the attractancy of the active *E* component. This was demonstrated when dispensers loaded with 5 μg of a ratio of 3*E*:97*Z* were significantly more active than the control. Therefore, traps baited with the *E*:*Z* mixture, which is produced by a relatively simple and cheap procedure, can be used for practical purposes.

Despite the fact that the *Z* isomer was found in the volatile natural pheromone of *M. josephi* in a relatively high percentage (25%), it appears that it is not a pheromone component, i.e., neither a synergist nor an inhibitor. In *M. feytaudi*, the corresponding *Z* isomer also was identified in the volatile natural pheromone, but in a low amount (3%), and its possible biological activity is questionable (Einhorn et al., 1990). In the case of *M. matsumurae*, the corresponding *E* isomer was identified as the sole pheromone component; the attractancy of the corresponding synthetic *Z* isomer to the male of *M. matsumurae* was quite similar to the control (analog 2, page 95, Hibbard et al., 1991). Hence, it is suggested that the corresponding *Z* isomers of the three *Matsucoccus* spp. lack biological activity.

In this study we utilized the racemic pheromone of the scale insect. Since the pheromone of *M. josephi* contains one chiral center, the important question relates to the absolute configuration of the natural pheromone and whether one of the enantiomers is an inhibitor. Chemical identification of the pheromone chirality and the activity of the enantiomers is in progress. Preliminary field data indicate that none of the enantiomers displays inhibitive activity. A similar finding was reported by Jactel et al. (1994) regarding the activity of the stereoisomers of the *M. feytaudi* pheromone.

Our findings demonstrate that the sex pheromone of *M. josephi* is also a strong kairomone for *E. hebraicus*, the principal predator of the scale insect. This is the first evidence for the use of a sex pheromone by a predator of homopterous species for prey location. According to Tumlinson et al. (1992), all of the long-range kairomones so far identified are sex pheromones of the

hosts. The use of sex pheromones as olfactory cues for host detection was recorded in several cases among egg parasitoids of lepidopteran pests (e.g., Batisti, 1989; Noldus et al., 1991). Thus, the higher rates of parasitism by *Trichogramma* spp. of moth eggs in previously pheromone-treated plots are explained by the fact that the host sex pheromone caused the parasitoids to land shortly after takeoff (Noldus et al., 1991). Niwa and Daterman (1989) showed that sex pheromone treatment in pine plantations affected either the species distribution or the abundance of the parasitoid complex; abundance of a pupal parasitoid was higher in pheromone-disrupted areas than in the controls. Among the Homoptera, honeydew serves as a kairomone for parasitoids and predators of aphids, whiteflies, and mealybugs (e.g., Budenberg, 1990; Meiracker et al., 1988). Among armoured scale insects, there is ample evidence that aphelinid parasitoids are attracted to the sex pheromone of the scale hosts (e.g., Abdel-Kareim and Kozar, 1988; Kyparissoudas, 1987; McClain et al., 1990; Samways, 1988). The feeding behavior of the Matsucoccidae is very similar to that of armored scale insects; they do not produce honeydew as do members of other closely related margarodids. Hence, it is not unexpected that the specific predator of *M. josephi* uses the sex pheromone as a kairomone (the pine bast scales are not attacked by parasitoids).

Many Anthocoridae of coniferous forests oviposit in the tree needles. *E. hebraicus* readily uses needles of any pine species for oviposition (Carmi, 1989), but not of other tree genera (Mendel, Assael and Carmi, in preparation). Therefore, it is suggested that the sex pheromone of *M. josephi* also serves for host-plant finding. It is important to note that in natural brutia pine forests, *M. josephi* occurs at low levels (Mendel, 1992). Hence, the efficient use of sex pheromones as an olfactory cue to find infested trees hosts is probably the result of the high sensitivity to the pheromone of *M. josephi* of both sexes of *E. hebraicus*. This may explain the latest findings (Mendel, Dunkelblum, Assael, in preparation) that in several cases hundreds of *E. hebraicus* were seen swarming and congregating around traps baited with the racemic mixture of the sex pheromone of *M. josephi*.

Our results indicate that the males of *M. josephi* do not respond better to any of the five colors tested in the present study. Many insect species are highly attracted to yellow. However, small flying insects, especially males of scale insects, are not associated with the crown of the host plant and do not display attraction to yellow (Kirk, 1984; Prokopy and Owen, 1987). Many insects that colonize the woody part of the tree, such as bark beetles, cerambycids, or curculionids, respond to dark objects or dark traps exposed against a bright background (e.g., Tedder and Wood, 1994). McClain et al. (1990) suggested that the reason parasitoids of the San Jose scale, *Quadraspidiotus perniciosus* (Comstock), respond better to black sticky traps baited with the sex pheromone of their host than to traps of other colors is due to the contrast of the trap color

against a given background. Males of *M. josephi*, similar to other *Matsucoccus* species, pupate on the forest floor, usually in the litter, while males of other scale insects that colonize woody plants pupate near the feeding plant tissue. This may explain the higher attraction of the males to traps attached to the tree trunk than to those that were suspended between trees. Naturally, it would be interesting to examine whether the strong attraction to traps attached to the trunks is related to the trunk shape or tree kairomones, or both.

The rate of increase of the *M. josephi* population in *P. halepensis* forests is related to environmental factors, tree age, and time until infestation after natural seeding or planting (Golan et al., 1983; Mendel et al., 1994). Comparison of the catch of *M. josephi* among the three studied forests and between pine species suggests that the level of infestation of *P. halepensis* and *P. brutia* is similar, despite the fact that the latter pine is tolerant to the scale insect (Liphschitz and Mendel, 1989). At present we may not exclude the possibility that equal catches recorded in stands of both host-plant species are due to male flight between plots. The different numbers of searching males among the studied forests are probably related to the effect of diverse climatic conditions. Damage in the Judean foothills and Samaria is higher than in Upper Galilee (Mendel et al., 1988, Mendel et al., 1994).

Elatophilus hebraicus of both sexes were trapped in much lower numbers in the more infested sites. This may be related at least partly to the fact that (1) a high level of injury, as observed in Midrakh 'Oz forest, is associated with deterioration and drying of the low and middle parts of the tree crowns (Mendel and Liphschitz, 1988), which increase the distance between oviposition and feeding sites of the bugs; and that (2) trees severely injured by *M. josephi* are heavily colonized by generalist predators (Halevy, Rosen, and Mendel, in preparation) which feed on, or interfere with, the activity of, *E. hebraicus*.

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LEAF PROFILE OF MAIZE RESISTANCE FACTORS TO EUROPEAN CORN BORER, *Ostrinia nubilalis*[1]

DAVID J. BERGVINSON,[2,*] ROBERT I. HAMILTON,[2] and JOHN T. ARNASON[3]

[2]*Plant Research Centre, Agriculture Canada*
Central Experimental Farm, Bldg. 121
Ottawa, Ontario, Canada K1A 0C6

[3]*Department of Biology*
University of Ottawa, Ottawa
Ontario, Canada K1N 6N5

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Abstract—The feeding preference of European corn borer larvae for immature whorl tissue of maize was examined by conducting leaf bioassays and quantifying resistance factors along the length of mid-whorl leaves from the maize synthetic BS9(C4) developed by recurrent selection for resistance. Potential resistance factors that were quantified included percent foliar nitrogen, gravimetric determination of soluble metabolites and fiber, soluble phenolics and hydroxamic acids, cell-wall-bound phenolics, leaf toughness, and UV absorbance of the epidermal cell wall determined by microspectrophotometry. Larvae consumed immature tissue at a higher rate than more mature tissue outside of the whorl, despite higher levels of DIMBOA in immature tissue. Consumption rate was highly negatively correlated with epidermal cell wall absorbance and leaf toughness. Fiber content and phenolic fortification of cell walls are proposed as the major resistance components that influence European corn borer feeding preference within the resistant synthetic BS9(C4).

Key Words—*Ostrinia nubilalis*, Lepidoptera, Pyralidae, European corn borer, maize, resistance, phenolics, fiber, diferulic acid, truxillic acid, DIMBOA, toughness.

INTRODUCTION

Maize resistance to the European corn borer (ECB), *Ostrinia nubilalis* (Hbn), (Lepidoptera: Pyralidae) has been extensively studied. For over two decades, the hydroxamic acid 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3-(4H)-one

*To whom correspondence should be addressed. Present address: CIMMYT, Apdo Postal 6-64, 06600 Mexico DF, Mexico.
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(DIMBOA) has been recognized as the major component in leaf-feeding resistance (Klun and Robinson, 1969) and is a feeding deterrent in both field and laboratory studies (Robinson et al., 1978). DIMBOA acts on growth and development of corn borer larvae by noncompetitive inhibition of digestive proteases (Campos et al., 1989; Houseman et al., 1992). Other resistance factors that have been less extensively studied include silica, lignin, and fiber content, which may act by reducing the nutritional quality of the leaf or increasing tissue toughness and thereby rendering nutrients less accessible (Rojanaridpiched et al., 1984; Coors, 1987; Peterson et al., 1988; Buendgen et al., 1990).

Breeding for resistance to ECB has resulted in the release of several resistant inbred lines, cultivars, and synthetics. BS9 is a synthetic developed by open pollination of 10 inbreds that had good agronomic traits as well as moderate resistance to ECB damage (Russell and Guthrie, 1982). Using an artificial infestation technique for ECB, four consecutive cycles of selection were conducted to select plants with reduced leaf feeding damage by ECB larvae. Since this germplasm was developed specifically for ECB resistance, changes in its phytochemical composition over cycles of selection have been investigated to better understand maize resistance mechanisms (Buendgen et al., 1990).

Intraplant variation in secondary compounds and nutrients has been studied in maize and would be of value to further our understanding of insect feeding behavior and feeding site preference. The objective of this study was to monitor changes in various secondary compounds and nutritional parameters along the length of mid-whorl leaves of ECB-resistant maize and to relate these changes to ECB feeding performance in both laboratory bioassays and the field. Resistance factors investigated included foliar nitrogen content, leaf toughness, fiber content, soluble and cell-wall-bound hydroxycinnamic acids, hydroxamic acids, and cell wall absorbance using a microspectrophotometer to localize biochemical resistance factors along the entire length of the leaf.

METHODS AND MATERIALS

Plant Samples. The fourth cycle of selection from the maize population BS9 was used with the method of selection outlined by Russell and Guthrie (1982). BS9 is a synthetic that has the following inbreds in its genetic background: B49, B50, B52, B54, B55, B57, B68, CI31A, Mo17, and SD10. Planting occurred in mid-May of 1992 at the Central Experimental Farm, Agriculture Canada, Ottawa, Ontario, Canada. The rows (4.5 m) were spaced 0.9 m apart with 30 plants per row in a sandy loam soil. Three blocks of four rows were planted with one row from each block being infested at the mid-whorl stage with four egg masses (ca. 200 eggs) for field observations. Univoltine ECB normally oviposit at this time on the undersurface of the later-emerging leaves

(leaves 10 through 13). At the mid-whorl stage (approx. 40 days from sowing), the 13th leaf was harvested by pulling the 11th leaf and above whorl out of the plant. Leaves were unwrapped from the whorl to expose the 13th leaf within the whorl. This leaf was green along one quarter, green–yellow along one half, and yellow along one quarter of its length and would emerge from the whorl within four days. Fifteen leaves from each block were harvested for phytochemical analysis. The midrib was removed and the leaves were then cut into eight sections of equal length along the leaf length with four sections being predominantly green and four sections being predominantly yellow. Section 1 was located at the leaf tip and section 8 was located at the auricles. Tissue was frozen at −20°C, thawed for 1 hr to release DIMBOA from its glucoside, refrozen, freeze-dried, and milled on a UD cyclone mill (UD Corp., Boulder, Colorado) with a 1-mm screen. Milled samples were stored at −20°C until analyzed.

Bioassays. Thirty plants were dissected and sectioned as above. Tissue was stored in water for up to 6 hr prior to incorporation into bioassays. A modified Ascher et al. (1981) apparatus was used to determine consumption of a 1.25-cm^2 disk of the leaf tissue exposed to two third-instars. The bioassay apparatus consisted of two top and one bottom halves of plastic Petri plates (5 cm diam.) with the bottom plate having a 1.25-cm-diam. hole. The top plate was inverted and wet cotton was placed inside and covered with filter paper. Leaf tissue was placed onto the filter paper with the top surface facing down. The bottom plate with the hole was taped on top to expose a defined feeding surface. Two early third-instars were placed into the apparatus, a small plastic covering was placed inside to shade the larvae, and the third plate was taped over the top to seal the larvae into the apparatus. The larvae were allowed to feed for 48 hr under controlled conditions of 25:18°C light–dark, 85% relative humidity, and light regime of 16:8 hr light–dark. Mean area consumed was determined from 30 leaf disks per section. Area consumed was measured by placing the consumed disk on top of 1-mm-grid graph paper.

Leaf Toughness. Using the method reported in Bergvinson et al. (1994a), force measurements were taken from the abaxial leaf surface between veins using a 1-mm-diam. probe mounted to an instron (model TM-M, Instron Corp., Canton, Massachusetts). Leaf tissue was held in place using a stainless steel platform fitted with a Plexiglas plate with a 2-cm-diam. hole for probe penetration. Ten samples for each of the eight sections were measured and the peak force recorded. Leaf samples were stored in distilled water at 4°C for up to 2 hr before force measurements were taken.

Protein Determinations. Protein content was estimated with an automatic micro-Kjeldahl nitrogen analyzer (Tecator model 1030, Höganäs, Sweden) using the conversion factor 6.25 to estimate percent protein from percent nitrogen.

Cell Wall UV Absorbance. Five samples from leaf sections 2, 4, 6, and 8

were collected and kept at −20°C. Samples were embedded in water containing glycerol (5 drops/10 ml water). Sections 6 μm were prepared and loaded onto Zeiss quartz slides and cover slips using glycerol. A computer-controlled Zeiss UMSP 80 microspectrophotometer equipped with a high-pressure xenon lamp (XBO 75W/2) was used to determine the absorbance at 326 nm of the adaxial epidermal cell wall (see Bergvinson et al., 1994b for more details).

Phytochemical Analysis. Phenolic conjugates and hydroxamic acids were extracted from a 0.5-g sample of dry tissue. Samples were extracted for 20 sec at ambient temperature in 70% methanol (4 × 20 ml) and mixed with a polytron mixer (model PT-1200, Brinkmann, Westbury, New York). After centrifugation at 500*g* for 10 min, the supernatants were pooled, methanol was removed by rotary evaporator (35°C), and the pH lowered to 2.0 using 1 M HCl. The aqueous fraction was extracted at ambient temperature by solvent partitioning with ethyl acetate (4 × 50 ml) (BDH, Omni-Solv grade). Ethyl acetate fractions were pooled, dried by a rotary evaporator, and stored at −20°C until HPLC analysis.

After extraction, the pellet that remained was washed in a Büchner funnel with 30 ml each of water, methanol, and ethyl acetate to remove chlorophyll and provide a crude cell wall preparation. Cell wall samples were dried in a desiccator for four days. This preparation was weighed, and the weight loss was used as the gravimetric measure of soluble metabolites. Cell wall preparations were shaken in 20 ml of 2 M NaOH for 4 hr under N_2 and wrapped in foil to hydrolyze cell-wall-bound hydroxycinnamic acids. Samples were neutralized with 6 M HCl and the pH lowered to 2.0. After centrifugation, the supernatant was extracted with ethyl acetate (3 × 50 ml). The pellet was resuspended in water and centrifuged twice, with both fractions pooled, and extracted with ethyl acetate (3 × 50 ml). Ethyl acetate fractions were pooled and dried by a rotary evaporator under darkness and stored at −20°C until HPLC analysis. The pellet that remained after extraction was dried and weighed to provide an estimate of fiber content.

HPLC Analysis. All analyses were performed with a Perkin-Elmer (Beaconsfield, England) system consisting of an LC 480 diode scan array detector and a Perkin-Elmer LC250 binary pump fitted with a 10-μl injection loop. Separations were achieved using a C18 ODS reverse-phase column (250 × 4.6 mm, 5-μm particle size, Beckman, Fullerton, California).

Soluble extracts were suspended in 4 ml of 50% methanol and centrifuged at 500*g* for 5 min to remove excess chlorophyll. The supernatant was filtered and injected onto the column. The solvent system consisted of methanol (A) and 10 mM H_2PO_4, pH 2.4 (B), at a flow rate of 1.5 ml/min using the following gradient: 25 to 55% A in 15 min, 55 to 80% A in 5 min, 80 to 100% A in 2 min, 100% A for 8 min, 100 to 25% A in 2 min, and 25% A for 3 min. DIMBOA (R_t = 10.5 min) and MBOA (R_t = 13.6 min) standards were syn-

thetically prepared according to Atkinson et al. (1991). Peak identity was confirmed by on-line UV spectra and spiking of extracts with authentic standards.

Cell-wall-bound phenolic acids were suspended in 1 ml of methanol, diluted 10-fold in methanol, filtered, and injected onto the column. The solvent system was the same as above except the starting mixture was 15% methanol. Standards of (*E*)-*p*-coumaric (R_t = 15.2 min) and (*E*)-ferulic acid (R_t = 15.6 min) were purchased from Sigma (St. Louis, Missouri). Truxillic acid and 5,5′-diferulic acid were obtained from GHN Towers (Vancouver, Britich Columbia).

Statistical Analysis. All statistical analyses were performed on SAS V.6.03 (SAS Institute, 1988). Data satisfied the assumptions of the general linear model and were not transformed. Statistical significance of data was assessed by analysis of variance (ANOVA). When ANOVA indicated there were significant effects, the Student-Neuman-Keuls (SNK) test was used to compare means. Regression analyses between larval consumption and phytochemical parameters were done using the mean values for each of the eight leaf sections.

RESULTS AND DISCUSSION

Field observations of recently hatched European corn borer larvae showed that approximately 80% of the larvae move towards the center of the whorl during daylight hours (unpublished data). Similar observations have been reported for other stem borers such as *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) in which 95–100% of live larvae are within the whorl (Ampofo and Kidiavai, 1987). A possible explanation for this behavior is avoidance of the hot, dry microenvironment on the exposed whorl leaves, which can result in desiccation of neonate larvae. This explanation is supported by the fact that egg mortality is higher at lower relative humidities (Lee, 1988) and larval mortality often exceeds 80% during the first 48 hr after hatching (Ross and Ostlie, 1990).

A profile of leaf consumption by ECB on BS9(C4) is depicted in Figure 1. ECB larvae show the highest consumption rate on immature tissue (sections 6 to 8). The leaf section with the lowest consumption was at the point where the leaf subtends from the whorl (section 4). By conducting leaf feeding bioassays in growth chambers, the effect of relative humidity over the leaf length is controlled and the degree of feeding on mature tissue is obviously lower even when relative humidity is not a restricting factor. This suggests that factors other than microenvironment are influencing larval preference for feeding within the whorl of maize.

Other parameters thought to be associated with feeding behavior are shown in Figure 1. Protein was lower for the sections around the green–yellow interface, reaching a level as low as 17%. Given the low protein levels at the green–yellow interface, one would expect consumption to be higher for this tissue so

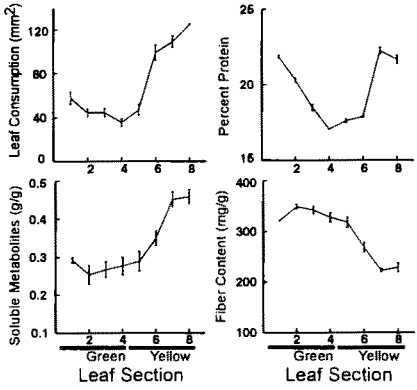

FIG. 1. Maize leaf profile for European corn borer consumption, protein, soluble metabolites, and fiber content for resistant synthetic BS9(C4). Leaf tip is section 1 and leaf attachment is section 8. Standard error of mean shown by vertical lines.

that it is able to fulfill nutritional requirements for development (Scriber and Slansky, 1981), but this was not observed during the 48-hr bioassay. Soluble metabolites washed from milled leaf tissue included sugars, soluble proteins, chlorophyll, phenolic conjugates such as flavonoids and hydroxycinnamic acids, and hydroxamics such as DIMBOA. The trend for soluble metabolites follows that of leaf consumption. Phenolic conjugates from maize have been shown to be phagostimulants (Bergvinson, 1993) and may account for higher consumption, as the level of sugar conjugates of *p*-coumaric acid are higher for immature whorl tissue (Figure 2). Other soluble secondary metabolites such as ferulic acid conjugates or HMBOA fluctuate and show no consistent trend.

DIMBOA was found to be at the highest levels within the yellow whorl tissue, which was also the most preferred by ECB larvae (Figure 2). Based on previous feeding preference studies, the converse would be expected (Robinson et al., 1978). Nutritional studies have shown that DIMBOA incorporated into meridic diet increases larval consumption while reducing the efficiency of nutrient assimilation and various fitness parameters (Houseman et al., 1992). This in part may explain the higher consumption rate of immature, yellow whorl tissue with elevated DIMBOA levels. Elevated levels of DIMBOA do not appear to be a significant deterrent to larval feeding during a 48-hr bioassay but may affect insect performance through reduced fecundity and prolonged development if feeding was restricted to this tissue throughout larval development.

The high feeding preference for tissue with elevated levels of DIMBOA can be rationalized by observing the relative absence of physical defense mechanisms in immature whorl tissue. Fiber content in immature tissue is very low

(Figure 1), and the relative absence of phenolic fortification in epidermal cell walls, as demonstrated by the low microspectrophotometer readings (Table 1), renders nutrients within the leaf more accessible and hence makes the tissue more desirable (Scriber and Slansky, 1981; Bernays and Barbehenn, 1987). The tissue toughness profile found in Figure 3 best illustrates the absence of mechanical resistance factors vis-à-vis fiber and hydroxycinnamic acid fortification of cell walls (Figure 4).

The toughness profile could account for field observations of neonate behavior. Immature whorl tissue would be easier to consume by neonates than tougher, mature leaf tissue. By migrating to the inner whorl, larvae would not

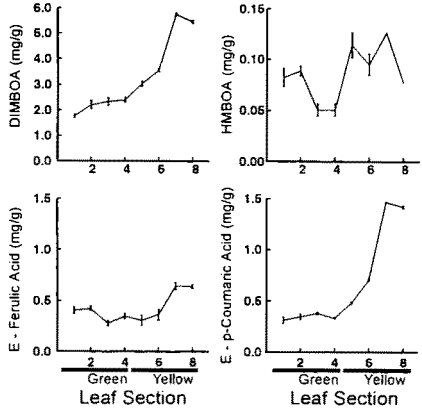

FIG. 2. Maize leaf profile of soluble secondary metabolites for resistant synthetic BS9(C4). Leaf tip is section 1 and leaf attachment is section 8. Standard error of mean shown by vertical lines.

TABLE 1. EPIDERMAL CELL WALL ABSORBANCE OF MAIZE SYNTHETIC BS9(C4) FROM SECTIONS ALONG LEAF LENGTH

Leaf sections[a]	Absorbance at 326 nm (mean ± SEM)[b]
2	1.49 ± 0.11 a
4	1.46 ± 0.10 a
6	0.45 ± 0.06 b
8	0.04 ± 0.01 c

[a]Leaf sections are from the leaf tip (section 1) to the point of attachment to the stalk (section 8).
[b]Means followed by the same letter are not significantly different, Student-Newman-Keuls test, $P < 0.05$.

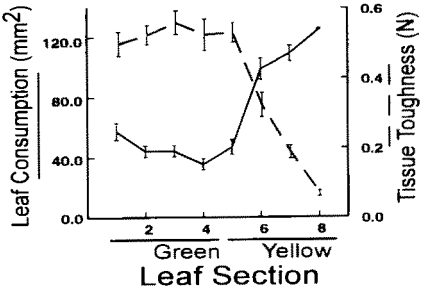

FIG. 3. Maize leaf toughness profile of abaxial epidermis (dashed line) from resistant synthetic BS9(C4) in relation to leaf consumption by European corn borer larvae (solid line). Standard error of mean shown by vertical lines.

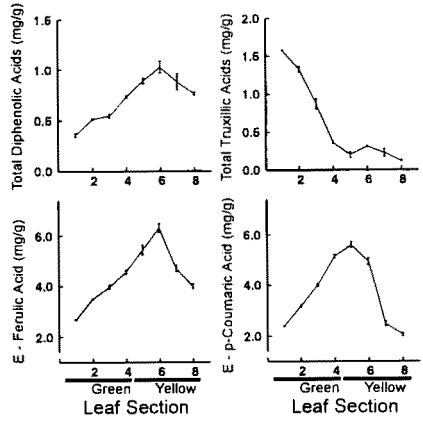

FIG. 4. Maize leaf profile of cell-wall-bound hydroxycinnamic acids for resistant synthetic BS9(C4). Total diphenolic acids include dehydrodiferulic acid and related dimers. Total cyclobutane dimers include both truxillic and truxinic acids. Leaf tip is section 1 and leaf attachment is section 8. Standard error of mean shown by vertical lines.

only be in a higher humidity microenvironment but would also be able to easily ingest water and nutrients to avoid desiccation and starvation during early stages of development. During the field study, another insect, the corn leaf aphid, *Rhopalosiphum maidis*, was observed to be most prominent within the whorl of several maize varieties where higher levels of DIMBOA are found (Bergvinson, 1993). DIMBOA in wheat has been identified as a feeding deterrent for aphids (Niemeyer et al., 1989) as well as accounting for varietal differences in maize resistance to the corn leaf aphid (Beck et al., 1983). However, like the ECB,

there may be physical, environmental, or nutritional factors that are of greater importance to aphid performance that necessitate feeding within the whorl despite high DIMBOA levels.

The major cell-wall-bound phenolic acids are (*E*)-ferulic and (*E*)-*p*-coumaric acids, which are attached to hemicellulose through pentose sugars (Kato and Nevins, 1985). Both phenolic acids reach their highest levels in sections 5 and 6, the yellow tissue that will emerge within two days (Figure 4). Cell-wall-bound ferulic and *p*-coumaric acids can form dimers either enzymatically through peroxidase to form 5,5′-diferulic acid (Hartley and Jones, 1976) or through photochemical reactions to form truxillic and truxinic acids (Hartley et al., 1988; Ford and Hartley, 1989). Formation of such dimers may increase the mechanical strength of the cell wall by cross-linking hemicellulose (Ford and Hartley, 1989). From the profiles in Figure 4 no individual biochemical component provides a suitable explanation for leaf consumption or toughness. However, when taken together, hydroxycinnamic acids provide a biochemical explanation for feeding performance. The leaf tip has been exposed to sunlight the longest and shows the highest levels of the photodimers truxillic and truxinic acids, which could cross-link the hemicellulose of mature tissue to provide structural resistance. For sections 4–6, elevated levels of cell-wall-bound *p*-coumaric, ferulic, and diphenolic acids sustain leaf toughness and thereby maintain a moderate leaf consumption rate. For sections 7 and 8, all cell wall phenolics are at their lowest levels, corresponding to increases in leaf consumption (Figure 1).

It is of interest to note the dramatic reduction in cell-wall-bound *p*-coumaric acid, which has been shown to have a positive correlation with lignin in bromegrass, *Bromus inermus* Leyss. (Jung and Casler, 1990). It may be that cell-wall-bound *p*-coumaric acid is an indicator for lignin by facilitating its attachment to the cell wall, as has been proposed for ferulic acid in wheat (Iiyama et al., 1990). Moreover, Akin et al. (1992) showed increased digestibility of bermudagrass leaf with alkaline release of phenolic monomers and their photoactivated dimers. This increase in digestibility was especially noticed in the epidermis. These observations support the hypothesis that phenolic acid monomers and dimers contribute to the mechanical resistance of maturing tissue.

Simple linear regression analysis of leaf consumption against the 12 parameters studied identified three parameters that could account for over 90% of the variation in leaf consumption (Table 2). Epidermal cell wall absorbance, toughness, and fiber content are all components or indicators of mechanical resistance. Elevated fiber content would not only increase the bulk density of the insect's diet to make nutrient and water requirements less attainable (Bernays, 1986), but would also increase the area for phenolic cross-linking. Acting in concert, fiber and hydroxycinnamic acid fortification in epidermal cell wall tissue could increase leaf toughness of "apparent" (*sensu* Feeny, 1976) mature leaf tissue, making it inaccessible to neonate larvae, which are forced to feed on softer,

TABLE 2. REGRESSIONS BETWEEN *Ostrinia nubilalis* CONSUMPTION OF LEAF TISSUE AND BIOCHEMICAL RESISTANCE FACTORS FOR MAIZE SYNTHETIC BS9(C4)

Parameter	Regression equation[a]	r^2	F value	P
Absorbance	LF = 126(±5) − 58(±4) × Abs	0.98	181.1	0.005
Toughness	LF = 142(±7) − 181(±17) × T	0.94	111.9	0.001
Fiber	LF = 540(±46) − 2688(±312) × Wt	0.93	74.2	0.001
Solubles	LF = −62(±18) + 403(±52) × Ratio	0.89	59.1	0.001
HMBOA	LF = 29(±10) + 58(±12) × HMBOA	0.76	23.1	0.03
DIMBOA	LF = −13(±25) + 198(±58) × DIMBOA	0.61	11.7	0.01

[a]LF = leaf feeding (mm^2/48 hr); T = leaf toughness in newtons (N); Wt = weight in mg/g; Ratio = grams of soluble/total weight; DIMBOA = mg/g dry weight. Regressions based on means from eight sections of leaf tissue from along the length of the leaf (N = 8).

immature whorl tissue that is defended by high levels of DIMBOA. As the insect matures, its mandibles may be better able to cope with tougher, mature tissue (Bernays, 1986) that has lower levels of DIMBOA. Based on within-leaf variation of feeding preference and biochemical factors, leaf toughness and the biochemical factors responsible for leaf toughness appear to be the predominant factors that influence ECB feeding behavior within maize during the mid-whorl stage of plant development.

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CONTROLLED-RELEASE PHEROMONE DISPENSER FOR USE IN TRAPS TO MONITOR FLIGHT ACTIVITY OF FALSE CODLING MOTH

J.H. HOFMEYR[1,*] and B.V. BURGER[2]

[1]*Outspan International*
P.O. Box 212
Citrusdal 7340, South Africa

[2]*Department of Chemistry*
University of Stellenbosch
Stellenbosch 7600, South Africa

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Abstract—Field trials were conducted in the western Cape Province, South Africa, to develop a sex pheromone dispenser suitable for monitoring the flight activity of false codling moth. A controlled-release dispenser capable of releasing sex pheromone at a predetermined and constant release rate without replacement for more than seven months was produced.

Key Words—*Cryptophlebia leucotreta*, controlled-release pheromone dispenser, sex pheromone.

INTRODUCTION

Various methods of releasing sex pheromone for both the monitoring and mating disruption of insect pests are used worldwide. A variety of dispensers, such as sachets made from polymer film, polyethylene (PE) vial caps and tubes, rubber septa, hollow fibers, and other materials, are utilized (Butler and McDonough, 1981; Kauth and Darskus, 1980; Urahama, 1982; Vrkoč et al., 1988; Weatherston et al., 1985; Yamamoto, 1982a,b). Generally, the inherent shortcoming of these dispensers is having to fulfill a dual function in providing a pheromone-releasing surface as well as serving as a reservoir for the pheromone. As the pheromone is partly or totally dissolved in the dispensing material, the rate at

*To whom correspondence should be addressed.

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which it is released decreases as its concentration in the dispensing device, or the surface area wetted by the pheromone, decreases. This means that the dispensers need to be replaced at regular, relatively short intervals to maintain both the integrity of the trapping system and optimum pheromone release levels for the insect concerned. For a trained scientist or technical officer, this requirement does not present a problem, but in practice, recommendations by the supplier to replace dispensers at fixed intervals are often either disregarded as unnecessary or are neglected. This can have serious consequences when the implementation of control procedures depends solely upon data supplied by the monitoring system.

For many years, low-density PE vial caps were used in trapping studies on false codling moth (FCM) *Cryptophlebia leucotreta* (Meyr.). Liquid pheromone was pipetted into the caps, which were then suspended in PVC pipe traps (Hofmeyr and Calitz, 1991). However, the pheromone only partially penetrated into the PE and was released mainly from the liquid phase, necessitating the regular replacement of the dispensers at monthly intervals. Pheromone release rates decreased rapidly to below optimum levels if this interval was exceeded, reducing moth catches. Subsequent dispenser replacement restored the initial pheromone release rate, but also sometimes resulted in the development of artificial population peaks as moth catches increased in response to the higher pheromone levels.

In developing a monitoring system, the following requirements for a pheromone dispenser had to be considered: (1) transport of centrally produced dispensers without fear of pheromone spillage; (2) continual pheromone release for approximately five months, without replacement or replenishment; and (3) pheromone release at a constant, predetermined rate.

In this paper we report the development of a controlled-release pheromone dispenser (CRPD) and the results obtained in preliminary field trials conducted in the western Cape Province, South Africa.

METHODS AND MATERIALS

The prototype CRPD consisted of two main parts: a glass tube acting as a pheromone-impermeable reservoir and, attached to it, a short length of PE tubing, through which the pheromone can diffuse. Although this basic design, as originally suggested by the second author, has been retained, it has evolved through several stages to eliminate various problems encountered during field trials. The design currently used is constructed as described below (Figure 1).

Both ends of a length of Pyrex glass tube (7 mm diameter and 0.9 mm wall thickness) are drawn out in the flame of a glass-blower's torch to form two tapered tips, the resulting glass reservoir having a length of approximately 20

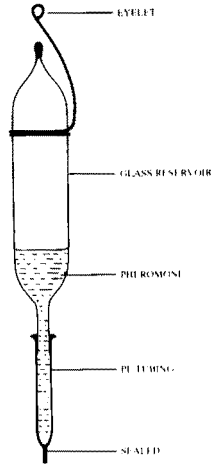

FIG. 1. Schematic presentation of the controlled-release pheromone dispenser.

mm and an overall length, including tapered ends, of 40 mm. The diameter of one of the tapered tips is critical and should be approximately 1.5 mm.

One end of a 15-mm length of low-density PE tubing (1.9 mm outside diameter and 0.25 mm wall thickness; Intramedic Micro Bore, Clay Adams 7400 Series; Becton, Dickinson and Co., Parsippany, New Jersey 07054) is heat-sealed with an impulse heat sealer (TEW Tish-200, Acepak Packaging Systems, P.O. Box 634, Goodwood 7460, South Africa). The other end is heated momentarily in an open flame and cooled down immediately, resulting in a thickened reinforcing collar. The collar end of the PE tubing is slipped over the 1.5-mm-diameter tip of the glass tube. Cyanoacrylate adhesive is used to strengthen this bond, although it is unnecessary if the fit between the glass and PE tubing is tight. A prefabricated eyelet, made from a length of copper wire, is slipped over the glass tube and glued into place with cyanoacrylate adhesive. This eyelet is used to suspend the dispenser from a hook in the trap.

With the aid of a microliter syringe, pheromone is injected into the PE tubing through the open end of the glass tube. Care is taken to prevent any air bubbles from forming during this process. The filled dispenser is placed in a refrigerator at 5°C. Once it has cooled down, the dispenser is removed with a long-nosed forceps, and the open end of the glass tube is immediately heat-sealed with a small glass-blower's torch. The dispenser is held upright momentarily until the glow has faded from the sealed end and is then cooled down with a wet cloth to prevent an excessive increase in temperature and a resulting build-up of air pressure in the dispenser. The object of this procedure is to create a slight positive air pressure in the dispenser at ambient temperature. If this is not

done, an air bubble may eventually be formed in the PE tubing (probably created by a negative pressure caused by pheromone release from the dispenser), which will interfere with the predetermined release rate.

A standard three-component mixture of the following compounds was used in all dispensers: 50% (*E*)-7-dodecenyl acetate plus 50% of a 5:1 mixture of (*E*)-8-dodecenyl acetate and (*Z*)-8-dodecenyl acetate. The (*E*)- and (*Z*)-8-dodecenyl acetate are major components of the sex pheromone of FCM (Persoons et al., 1977; Angelini et al., 1980; Attygalle et al., 1986), while (*E*)-7-dodecenyl acetate was added as a synergist (Hofmeyr and Calitz, 1991). In all experiments, 80 mg of pheromone were used per CRPD. For comparison of CRPD and PE caps, 8 mg of pheromone were pipetted into each PE cap. Although experiments were conducted using up to 40 mg of liquid pheromone per PE cap, this was considered impractical, as the danger of pheromone spillage was already high when only 8 mg of pheromone were used per cap. Furthermore, a fivefold increase in the total amount of pheromone per cap did not result in a corresponding extension in exploitable life, as the release rate was much higher than required (Hofmeyr, unpublished data).

Comparison of CRPDs with Different Sizes of PE Tubing (Experiment 1). To determine the influence of the PE tubing on the release rate of the CRPD, dispensers with tubing of differing lengths and diameters were prepared. One dispenser of each type was suspended from a hook beneath a wooden rack. Five of these racks were, in turn, suspended inside the canopy of as many fruit trees. They were removed once a week and transported to the laboratory, where mass loss of the dispensers was determined with the aid of an analytical balance.

Quantitative Comparison of Pheromone Released from PE Caps and CRPDs (Experiment 2). Ten CRPDs (PE tubing size 15 × 1.9 mm) were compared with 10 low-density PE caps (internal diameter and depth 9 × 9 mm, average mass 0.63 g). All dispensers were field-aged as in experiment 1. Comparisons were terminated after six weeks and the PE caps removed. The CRPDs were left in situ for a further 18 weeks, after which they were taken to the laboratory and maintained at temperatures of 25–27°C. At this stage, 10 fresh CRPDs were added to the trial for comparison, and weekly mass determinations were resumed.

Efficacy of Dispensers in Traps (Experiment 3). A trapping experiment was conducted to compare the efficacy of the CRPDs with that of the standard PE caps. Sticky pipe traps were evenly distributed at a minimum of 50-m intervals across an orchard and suspended from a convenient branch at a height of 1.9 m on the southern side of trees, just inside the foliage canopy. A randomized block design was used for the experiment. Each treatment, consisting of a trap plus dispenser, was randomly allocated to each block. Six blocks per treatment were used. At weekly intervals trapped moths were counted, removed, and each trap moved one position to the adjacent block.

PE caps were replaced with fresh dispensers after six weeks. The original CRPDs were retained for the duration of the experiment. All experiments were conducted between February and August.

Analysis of Trap Data. To test differences between treatments, *t* tests for independent samples were performed.

RESULTS AND DISCUSSION

Pheromone Release Rate of Various Sizes of PE Tubing (Experiment 1). It was found that the release of pheromone from the CRPD could be calibrated for a desired rate simply by adjusting the length and/or diameter of the attached PE tubing (Figure 2). During the sealing process approximately 6 mm of the total length of PE tubing is lost. This loss is constant, regardless of the length of PE tubing used. The effective external surface area of the different dispensers therefore varied from 53.7 mm^2 (15 × 1.9 mm) to 59.7 mm^2 (25 × 1 mm) to 113.4 mm^2 (25 × 1.9 mm). Increasing the tubing length from 15 to 25 mm, while maintaining the same diameter (111% increase in surface area), increased the mean release rate by 135%. The surface area from a 25-mm × 1-mm length of PE tubing, which was 11% greater than that of 15-mm × 1.9-mm tubing, increased the mean release rate by 15%. Theoretically the release rate should have been directly proportional to the surface area of the PE tubing. The reason

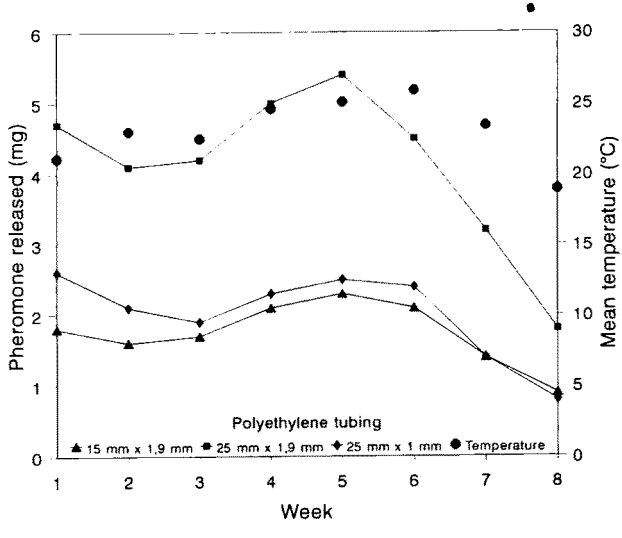

FIG. 2. The influence of polyethylene tubing of different lengths and diameter on the release rate of the controlled-release pheromone dispenser.

for these discrepancies is unknown, but is of little practical importance, since the release rate is easily adjusted.

During the first week of exposure in this experiment, the release rate from all CRPDs did not follow a temperature-related curve, but was higher than expected. This could be a logical consequence of too high a pressure inside the CRPDs, but as the release rate stabilized from the third week onwards, it does not seem to be of practical significance. This phenomenon was, however, also noted in similar experiments with BASF double-receptacle pheromone ampoules used for male disruption. As the internal air pressure probably does not play a significant role in these ampoules, a further unknown factor could be involved.

Pheromone Release Rate of CRPDs and PE caps (Experiment 2). The PE caps maintained an acceptable rate of release for the first four weeks, after which there was a rapid and permanent reduction (Table 1). In contrast, pheromone release from the CRPDs was not reduced. A rise in mean temperatures occurred during the second half of the six-week exposure period, which was responsible for the increase in pheromone release from the CRPDs. However, pheromone had already been depleted to such an extent in the PE caps that temperature had no noticeable effect on the release rate.

After exposure in the orchard for 24 weeks, pheromone was being released from the field-aged CRPDs at a mean rate of approximately 30% less than from

TABLE 1. COMPARISON OF WEEKLY RELEASE RATES OF CONSTANT RELEASE PHEROMONE DISPENSERS (CRPD) AND POLYETHYLENE (PE) VIAL CAPS

Week	Mean release rates (mg) ± SEM	
	CRPD	caps
Field	1.8 ± 0.08	2.2 ± 0.14
1		
2	1.6 ± 0.13	2.3 ± 0.02
3	1.7 ± 0.07	1.2 ± 0.07
4	2.1 ± 0.07	1.1 ± 0.05
5	2.3 ± 0.05	0.4 ± 0.03
6	2.1 ± 0.16	0.3 ± 0.04
	CRPD (field aged)	CRPD (new)
(Laboratory)	1.9 ± 0.11	2.7 ± 0.08
24		
25	2.1 ± 0.11	2.8 ± 0.05
26	1.9 ± 0.11	2.7 ± 0.05
27	1.7 ± 0.12	2.6 ± 0.09
28	1.6 ± 0.07	2.6 ± 0.05

new CRPDs. This is still well within the optimum range for monitoring FCM of 1.0–2.5 mg per trap per week, as found in trapping experiments using PE caps (Hofmeyr, unpublished data).

Efficacy of CRPDs and PE caps in traps (Experiment 3). Moth activity fluctuated inherently from week to week independent of the influence of the two treatments. The results are therefore presented as the size of the moth catch with the PE caps relative to that attracted to the CRPD (Figure 3).

Under late summer conditions (February–March) traps equipped with PE caps remained competitive for only three weeks, before the number of attracted males decreased in comparison to the CRPD-baited traps. After replacement, this period was increased to five weeks as the lower autumn temperatures resulted in a decrease in pheromone evaporation from the PE caps.

During the first three weeks, 23 males were attracted to the six PE cap dispensers, while 28 males were caught in the six traps equipped with the CRPDs. The difference became significant ($P < 0.01$) during the ensuing three weeks when eight and 58 males, respectively, were trapped. Corresponding figures for the period following replacement of the PE caps are as follows: First five weeks, 136 (PE caps) and 160 males (CRPD): ensuing six weeks, 34 and 106 males, respectively.

Experiment 2 did not run concurrently with experiment 3, but was conducted during cooler conditions later in the same season. This explains why

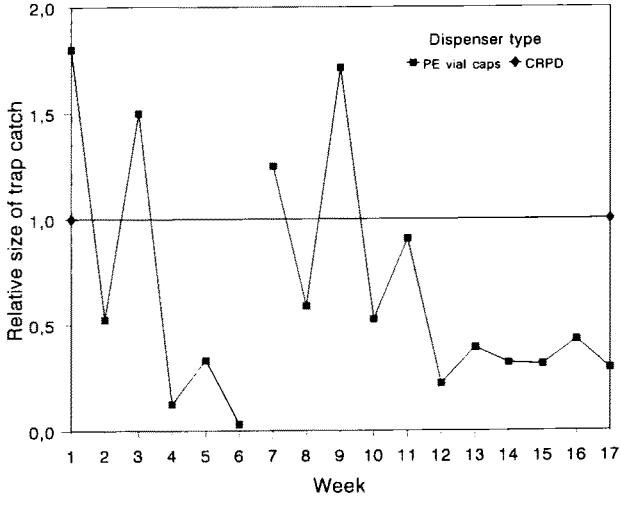

FIG. 3. The relative size of male moth catches in traps baited with sex pheromone released from polyethylene (PE) vial caps and controlled-release pheromone dispensers (CRPD). PE dispensers were replaced at the end of week 6.

pheromone release during week 4 was still at an acceptable level in experiment 2 (Table 1), while male attraction to PE caps had already decreased during week 4 in experiment 3 (Figure 3).

It was demonstrated in the quantitative assessment that the CRPD will release pheromone at predetermined levels for at least 30 weeks under prevailing climatic conditions. The CRPDs retained their activity in traps without replacement for 17 weeks before the experiment was terminated. This is a significant improvement on the performance of the PE caps, bearing in mind that the pheromone in the CRPDs was far from depleted and that the release rate in these dispensers would have been maintained for an even longer period.

In the present study, glass was used for the construction of the pheromone-impermeable reservoir. This allowed detailed observation of the physical behavior of the pheromone in the dispenser. A reservoir constructed from a pheromone-impermeable plastic might, however, be preferred for mass-production purposes.

The influence of oxygen on the integrity of the pheromone composition was not studied in detail. However, in a preliminary investigation of FCM pheromone that had been exposed in CRPDs in traps in orchards for seven months, no evidence of isomerization of any practical importance was found. It remains to be determined whether this CRPD can be used for more labile pheromones having, for example, conjugated or skipped diene systems and whether the use of antioxidants could be effectively used to stabilize such sensitive pheromones.

Using a reservoir made from a pheromone-impermeable material, to keep a short length of PE tubing totally filled with pheromone, was seen as a possible solution to the problem of the continually decreasing rate of pheromone release from other types of dispensers. In such a device, the pheromone-releasing surface, as well as the concentration of the pheromone in the polymeric material acting as releasing agent, remains constant. The rate at which the pheromone is released therefore also remains constant. The relatively simple pheromone dispensers constructed along these guidelines proved to satisfy all the requirements set out in the introduction.

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HYDROCARBON DYNAMICS WITHIN AND BETWEEN NESTMATES IN *Cataglyphis niger* (HYMENOPTERA: FORMICIDAE)

VICTORIA SOROKER,[1,*] CATHERINE VIENNE,[1,2] and ABRAHAM HEFETZ[1]

[1]*Department of Zoology*
Tel Aviv University
Ramat Aviv, Tel Aviv 69978, Israel

[2]*Laboratoire d'Ethologie Expérimentale et Comparée*
Université Paris Nord
93430 Villetaneuse, France

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Abstract—The objective of the present study was to evaluate the interrelationship between the cuticular and postpharyngeal glands' hydrocarbons, both in the individual ant and during its interaction with nestmates. In vivo radiochemical assays were employed to monitor the de novo hydrocarbon biosynthesis from acetate in the ant's body. The newly synthesized hydrocarbons appeared first internally and after 24 hr they accumulated in the postpharyngeal gland and on the cuticular surface. Blocking the possibility of external transfer of hydrocarbons between cuticle and postpharyngeal gland led to a significant decrease of labeled hydrocarbons in the postpharyngeal gland. In addition, during encounters between labeled and unlabeled ants, newly synthesized hydrocarbons were transferred, mainly via trophallaxis, but also by allogrooming and physical contact. In view of these results, we propose as a model for their dynamics that hydrocarbons are synthesized in tissues associated with the integument. Through self-grooming, there is a constant exchange of hydrocarbons between the cuticular surface and the postpharyngeal gland. Furthermore, in encounters between nestmates, hydrocarbons are exchanged among them mostly by trophallaxis, with the mediation of the postpharyngeal gland. Thus, this gland acts as a pool for mixing colonial hydrocarbons and may serve to attain a unified colony odor.

Key Words—Postpharyngeal glands, cuticle, hydrocarbons, nestmates recognition, ants, *Cataglyphis niger*.

*To whom correspondence should be addressed.

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INTRODUCTION

Nestmate recognition, i.e., the ability to discriminate individuals that belong to the same colony from alien individuals, has been reported in a large number of social Hymenoptera species (see reviews in Hölldobler and Michener, 1980; Gadagkar, 1985; Breed and Bennett, 1987). It provides an effective means for maintaining the coherence of the colony and preventing the invasion of conspecific competitors. It is generally believed that nestmate recognition cues reside among the chemicals found on an individual's cuticle (Hölldobler and Michener, 1980; Bradshaw and Howse, 1984). The cuticular lipids of insects vary in composition and may include fatty acids, alcohols, esters, glycerides, sterols, aldehydes, ketones, wax esters, and hydrocarbons (Lockey, 1988). In ants, hydrocarbons are usually the dominant chemical class found on the cuticle, forming complex mixtures. Hydrocarbon patterns have thus been used as species and/or colony markers, e.g., hydrocarbon patterns are species and colony specific in the fire ant complex (Brill et al., 1985; Ross et al., 1987; Vander Meer, 1986).

Although there is no conclusive evidence linking nestmate recognition behavior with cuticular hydrocarbons, multiple experimental data support this possibility. The acquisition of the host ant cuticular hydrocarbon pattern has been correlated with the acceptance of inquilines into the ant's colony (Dettner and Liepert, 1994). Recognition between alien societies of ants is correlated with a similarity in composition of cuticular hydrocarbons (Bonavita-Cougourdan et al., 1987, 1989; Henderson et al., 1990; Nowbahari et al., 1990). Studies with mixed species groups of ants showed that nonaggressive cohabitation between the species may be correlated with a congruency in hydrocarbon profile. Especially intriguing is the occurrence of heterospecific compounds in members of these groups (Bagnères et al., 1971; Franks et al., 1990; Vienne, 1993).

It was recently demonstrated in several ant species that the chemical profile of the cuticular hydrocarbons is congruent with that of the postpharyngeal gland contents (Bagnères and Morgan, 1991; Do Nascimento et al., 1993; Soroker et al., unpublished data). The postpharyngeal gland, which is specific to ants, occupies a large portion of the head overlaying the brain, with a lateral opening in the bottom of the buccal cavity just in front of the esophagus (Barbier and Delage, 1967). Recently, we were able to show that exudates of the postpharyngeal gland modify nestmate recognition in *Cataglyphis niger* (Soroker et al., 1994). This congruency in hydrocarbon profile between the cuticle and the postpharyngeal gland could be the consequence of several processes: synthesis in the tissues underlying the cuticle and sequestration by the postpharyngeal gland; synthesis in the postpharyngeal gland and application on the cuticle by grooming; or an independent synthesis in both tissues. In addition, workers of mixed groups of *Manica rubida* and *Formica selysi* were shown to have similar

hydrocarbon profiles on both the cuticular surface and in the postpharyngeal gland (Hefetz et al., 1992). One possible route for the acquisition of heterospecific substances in these mixed species groups is a continuous flow of hydrocarbons between members of a colony. Since the postpharyngeal gland exudes to the mouth, it could play a role in the distribution of hydrocarbons between nestmates by trophallaxis and/or grooming.

We present here studies that evaluate the interrelationship between the cuticular and postpharyngeal gland hydrocarbons and its impact on hydrocarbon dynamics between nestmates.

METHODS AND MATERIALS

Collection and Maintenance of Ant Colonies. Colonies of *Cataglyphis niger* were collected from the Tel-Aviv area during October 1992–November 1993. In the laboratory they were transferred to artificial nests and reared in an incubator under controlled conditions of 28 ± 2°C and a photoperiod of 8L : 16D. Colonies were provided with an identical diet of sugar water and minced insects three times a week.

Studies on Hydrocarbon Distribution In Vivo Using Labeled Sodium Acetate. In order to follow the biosynthesis and accumulation of the hydrocarbons in the ants, an *in vivo* radiochemical assay was used. Ants were each injected into the hemolymph through the intersegmental membrane of the abdomen with 0.5 μl medium, prepared according to Katase and Chino (1984) and containing 1 μCi (56 mCi/mmol) [1-^{14}C]sodium acetate (NEN, Boston, Massachusetts). Thereafter, at various time intervals for the time-course studies, or after 24 hr for all the other experiments, the ants were frozen and dissected.

The effect of self-grooming on hydrocarbon distribution within the ants was monitored using ants with blocked mouth parts. The sealing of the mouth parts was done prior to injection by using melted beeswax while the ants were immobilized on ice. Twenty-four hours later, the ants were dissected and monitored for the presence of labeled hydrocarbons on their cuticular surface (cuticle), in their postpharyngeal gland (PPG), and internally as described below. Only those ants that were alive and in good condition were used.

Hydrocarbon Transfer between Nestmates. Donor ants were injected with [1-^{14}C]sodium acetate as described previously and after 24 hr of incubation (26°C) they were presented with a nestmate (recipient), prestarved for 24 hr, in a Petri dish (4.8 cm diameter). To test whether hydrocarbon transfer occurs via trophallaxis, the behavior of the ants was recorded. Each encounter was stopped 30 min after trophallaxis was first observed and encounters did not exceed 90 min. Ants that did not engage in trophallaxis within the first hour were not taken into consideration. To test the successive transfer of hydrocar-

bons between nestmates, the first dyadic encounters were followed by another encounter, this time using the recipient as a donor for a third nestmate. The length of each encounter was 24 hr. Food transfer by trophallaxis was verified by feeding the donor ants with colored food and following its occurrence in the recipient. To test whether hydrocarbon transfer occurs otherwise than by trophallaxis and to assess the magnitude of the label transfer, prelabeled ants were presented with nestmates that had their mouth parts sealed with beeswax (blocked ants) to prevent any trophallaxis or licking, and, vice versa, prelabeled blocked ants were presented with normal recipient nestmates. In addition, encounters between donors and recipients that were both blocked were also conducted. All encounters were stopped 24 hr later by freezing the ants, and both donor and recipient were monitored for the presence of labeled hydrocarbons in the postpharyngeal gland and on the cuticular surface. For comparison between the magnitude of labeled hydrocarbon transfer to the various recipients, the relative amounts of labeled hydrocarbons transferred from their donor's to the recipient's postpharyngeal gland or cuticle were calculated individually for each pair using the following equation (example given for PPG):

$$\% \text{ Hydrocarbons transferred to PPG} = \frac{\text{Labeled hydrocarbons in recipient PPG}}{\text{Total labeled hydrocarbons in donor (PPG + cuticle)}}$$

Extraction and Separation of Labeled Hydrocarbons. Postpharyngeal glands were extracted for 24 hr in 100 μl of pentane. To separate cuticular from internal hydrocarbons and to eliminate the possibility of sample contamination by Dufour's gland hydrocarbons, headless and Dufour's glandless carcasses were first washed by immersion in 400 μl of pentane for 5 min and subsequently extracted for longer periods (24 hr or more). The extracts were subjected to silica gel (polygram Sil G) thin-layer chromatography (TLC) using petrol ether–diethyl ether–acetic acid (95 : 5 : 1) as a running solvent. The various lipid classes were identified by comparing their R_f values with those of cochromatographed standards purchased from Sigma (tricosene, triolein, oleic acid, and palmitoleic acid stearyl ester) and visualized by iodine vapor. The area corresponding to hydrocarbons was cut and placed in scintillation fluid (Opti Fluor, Packard) and its radioactivity was monitored by a scintillation counter (Packard Tri-Carb 4530). The presence of hydrocarbons in this TLC faction was ascertained by comparing their GC profile to that obtained by combined GC-MS analysis of PPG extracts.

Statistics. In all experiments individual ants were analyzed. Different groups were compared by Mann-Whitney U test and Kruskal-Wallis test performed using a Statview 4.01 package in a Macintosh computer. The data from the same individuals were compared by Wilcoxon's signed-ranks test according to Sokal and Rohlf (1981).

RESULTS

Hydrocarbon Distribution within the Ant. The results of the radiochemical assay showed that adult workers synthesized hydrocarbons de novo from sodium acetate (Figure 1A). While the amount of labeling increased with time in all the tissues examined, their distribution in the various tissues progressively changed. At first, a fast increase in labeled internal hydrocarbons was observed, reaching significantly higher levels than on the cuticular surface or in the postpharyngeal gland. Thereafter, the amount of labeling in hydrocarbons increased with time, especially in the postpharyngeal gland. However, after 24 hr, the amount of hydrocarbons in the postpharyngeal gland was not significantly higher than on the cuticular surface. Results of a previous study with decapitated ants, i.e.,

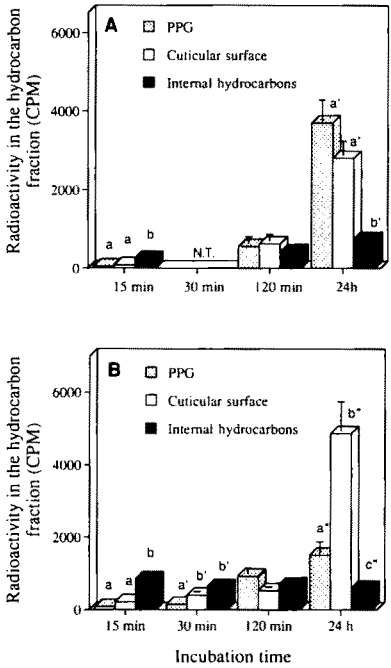

FIG. 1. Time-dependent changes in *in vivo* incorporation of [1-^{14}C]acetate into the hydrocarbon fraction in *C. niger* workers. (A) In normal ants (N.T.: not tested); (B) in ants with blocked mouthparts. Data are means ± SEM of a minimum of seven replicates. For every incubation time, different letters indicate the groups that are statistically different (Wilcoxon's signed-ranks test): (A) a vs. b, $P < 0.05$; a′ vs. b′, $P = 0.0001$; (B) a vs. b, $P = 0.0033$; a′ vs. b′, $P = 0.01$; a″ vs. b″ and b″ vs. c″, $P = 0.0001$; a″ vs. c″, $P = 0.018$.

lacking their postpharyngeal gland, that were injected with [1-^{14}C]sodium acetate, implied that this organ is not the site of hydrocarbon synthesis, but that the gland does sequester them (Soroker et al., 1994). The mode of sequestration of these hydrocarbons was further investigated while preventing their external passage via self-grooming by blocking the ants' mouthparts with melted beeswax (Figure 1B). At first, as for untreated ants, a fast increase in labeled internal hydrocarbons was observed, reaching significantly higher levels than on the cuticular surface or in the postpharyngeal gland. Thereafter, the amount of labeling in hydrocarbons increased with time first on the cuticular surface and subsequently in the postpharyngeal gland, but stayed relatively stable in the long-term extracts for internal hydrocarbons ($P = 0.45$, Kruskal-Wallis). In contrast to the previous experiment conducted with normal ants (Figure 1A), after 24 hr of incubation, ants with blocked mouthparts contained significantly higher amounts of labeled hydrocarbons on their cuticular surface than in their postpharyngeal gland. Nevertheless, the postpharyngeal gland still contained significant amounts of newly synthesized hydrocarbons, suggesting an uptake from an internal pool. The comparison with normal ants suggests that the presence of hydrocarbons in the postpharyngeal gland results from both this uptake and an assimilation via self-grooming.

The possibility that hydrocarbons also accumulated in the phostpharyngeal gland by self-grooming is emphasized when we compare the ratio of labeled hydrocarbons on the cuticle and in the postpharyngeal gland between the normal and blocked ants (Table 1). During the first 2 hr, there was little change in the ratios between blocked and normal ants. In contrast, 24 hr later, the ratio in blocked ants was significantly higher than in normal ones. We can conclude that, by that time, sufficient self-grooming was performed by the normal ants to transfer labeled hydrocarbons from the cuticular surface to the postpharyngeal gland, while blocked ants were deprived of this ability.

TABLE 1. EFFECT OF MOUTH BLOCKING ON DISTRIBUTION OF LABELED HYDROCARBONS BETWEEN POSTPHARYNGEAL GLAND AND CUTICLE IN WORKERS OF *C. niger*[a]

Incubation time (min)	Ratio between labeled hydrocarbons in cuticle/postpharyngeal gland		
	Control	Blocked	Probability[b]
15 min	1.83 ± 0.34 ($N = 14$)	2.66 ± 0.67 ($N = 11$)	NS
120 min	2.65 ± 1.20 ($N = 7$)	1.09 ± 0.23 ($N = 13$)	NS
24 hours	1.38 ± 0.27 ($N = 25$)	7.62 ± 2.32 ($N = 22$)	$P = 0.0001$

[a]Results are presented as means ± SEM.
[b]Significant differences between control and blocked ants were assessed by Mann-Whitney U test.

Hydrocarbons Transfer between Nestmates. The possible transfer of newly synthesized hydrocarbons between nestmates was examined by first evaluating the role of trophallaxis in this process. When a prelabeled donor ant was confronted with a nestmate, trophallaxis occurred rather quickly with a consequent transfer of labeled hydrocarbons from the donor to the recipient within 30 min from the beginning of trophallaxis (Figure 2). The amount of labeled hydrocarbons in the recipient's postpharyngeal gland was significantly higher than on their cuticle ($P < 0.01$; Wilcoxon test). The passage of hydrocarbons between nestmates was further tested in two successive dyadic encounters (Figure 3). After 24 hr, a conspicuous accumulation of radiolabeled hydrocarbons was observed in the postpharyngeal gland of both recipients. The labeling in the first recipient's postpharyngeal gland was equal to the level in the donor corresponding to a transfer of 40 ± 11%. However, the amount of labeled hydrocarbons in the second recipient's postpharyngeal gland was significantly lower ($P = 0.001$, Mann-Whitney), reaching only 8 ± 2% of the labeled hydrocarbons observed in the ant it encountered (the first recipient). The labeling of cuticular hydrocarbons in recipients was much lower than in the postpharyngeal gland but remained the same in the two encounters (1.4 ± 0.3% for the first recipient and 1.1 ± 0.3% for the second, $P = 0.4$, Mann-Whitney).

In addition to trophallaxis, the impact of other modes of label transfer between nestmates, such as allogrooming and body contact, were assessed in a series of encounters in which either the donor ants or the recipients or both had their mouthparts sealed. Figure 4 depicts the relative amounts of labeled hydrocarbons present in the recipient's postpharyngeal gland and on the cuticle,

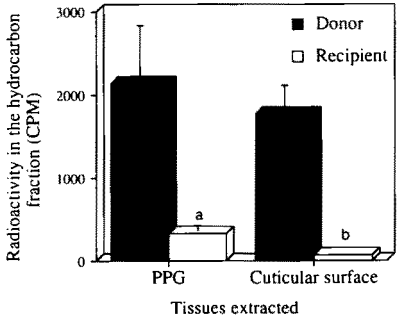

FIG. 2. Hydrocarbons transfer by trophallaxis between *C. niger* workers. Data are expressed as mean labeling in hydrocarbon fraction in the postpharyngeal gland and on the cuticular surface of donors and recipients ± SEM of 15 replicates. Different letters indicate the groups that are statistically different ($P = 0.0019$, Wilcoxon's signed-ranks test).

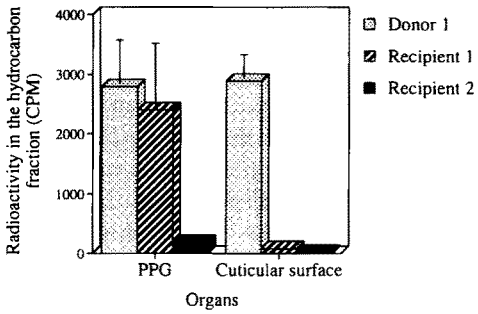

FIG. 3. Hydrocarbons transfer in two successive dyadic encounters between nestmates of *C. niger*. Data are expressed as mean labeling in hydrocarbon fraction in the postpharyngeal gland and on the cuticular surface of donors and recipients ± SEM of nine replicates.

respectively, as a consequence of all the encounters. As expected from the previous experiment, there was a substantial transfer of newly synthesized hydrocarbons to the recipient when free trophallaxis was permitted (RO/DO, Figure 4). On the other hand, transfer to the postpharyngeal gland was abolished completely when the mouthparts of the recipient were blocked (RB/DO or RB/DB). However, normal recipients that encountered blocked donors (RO/DB) acquired radioactive hydrocarbons in their postpharyngeal gland. Since trophal-

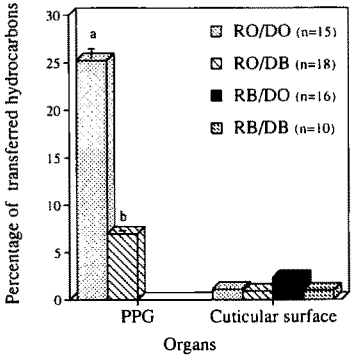

FIG. 4. Hydrocarbons transfer between nestmates of *C. niger*. Data are relative amount of labeling in hydrocarbon fraction found in the recipients, mean percent ± SEM of a minimum of 10 replicates. RO = normal recipient; RB = blocked recipients; DO = normal donor; DB = blocked donor. Statistics: different letters indicate the groups that are significantly different [RO/DO vs. RO/DB, $P = 0.02$, Mann-Whitney U test].

laxis between these two ants was impossible, this acquisition was probably accomplished when the recipient ant groomed the donor, picking up newly synthesized hydrocarbons from its cuticle.

In contrast to the variabilty in hydrocarbon transfer into the postpharyngeal gland in the various treatments, the transfer to the cuticular surface was low (but above the background) and in similar proportions. Nevertheless, the level of labeling that occurred on the cuticle of blocked recipients that encountered nonblocked donors (RB/DO) tended to be the highest. It was also evident that in these ants, the radioactivity on the cuticle was higher than in the postpharyngeal gland (which had only a background level of radioactivity, RB/DO 179 $\pm$ 30.6 cpm vs. 32 $\pm$ 1.8 cpm; $P < 0.01$; Wilcoxon test). These data imply that the donor smeared its postpharyngeal gland secretion on the recipient through licking.

The possibility that the labeled hydrocarbons observed in the recipients were biosynthesized de novo from free [1-^{14}C]acetate that was transferred as such from the donor was examined by comparing the distribution of labeled hydrocarbons in the postpharyngeal gland and on the cuticular surface between donors and recipients (Table 2). Significantly higher ratios of labeled hydrocarbons between cuticle and postpharyngeal gland were found in the donor rather than in the recipient, indicating that there was a transfer of labeled hydrocarbons from donor to recipient's postpharyngeal gland rather than a de novo synthesis from acetate transferred to the recipient.

DISCUSSION

It is currently believed that cuticular hydrocarbons are involved in the process of nestmate recognition in social Hymenoptera, including ants (Blum, 1987). This hypothesis is supported by the findings that the postpharyngeal gland's secretion modifies the aggressive behavior that is generally directed towards alien ants and the congruency between cuticular and postpharyngeal

TABLE 2. DISTRIBUTION OF LABELED HYDROCARBONS IN BODY OF *C. niger* WORKER[a]

	Donor	Recipient
Postpharyngeal gland	3575 $\pm$ 634	1876 $\pm$ 485
Cuticular surface	3674 $\pm$ 421	104 $\pm$ 12
Ratio (Cuticle/PPG)*	1.7 $\pm$ 0.4	0.3 $\pm$ 0.17

[a]Results are presented as means $\pm$ SEM.
[b]The significant difference between donor and recipient was calculated by Mann-Whitney U test ($P = 0.0001$), $N = 15$.

gland hydrocarbons that exists in several ant species (Bagnères and Morgan, 1991; Do Nascimento et al., 1993; for *C. niger*, Soroker et al., unpublished data). The primary aim of this study was to explore the dynamics of hydrocarbon synthesis and their distribution in the ant's body tissues as well as between the ants in a colony.

Workers of *C. niger* synthesized hydrocarbons de novo from sodium acetate, as was described previously in other insects (Howard and Blomquist, 1982). Time-course analyses of radioactive hydrocarbon distribution showed an initial elevation in internal hydrocarbons that was followed by an increase in labeled hydrocarbons on the cuticular surface and in the postpharyngeal gland. This corroborates the current knowledge that cuticular hydrocarbons are synthesized by cells associated with the epidermis or the peripheral fat body, probably oenocytes, and are subsequently transferred to the cuticle and other locations through the hemolymph (De Renobales et al., 1988, 1991; Lockey, 1988). The results of the experiments using ants that had their mouthparts blocked indicate that in *C. niger* hydrocarbons reach the gland both internally through the hemolymph and externally through the mouth, probably by self-grooming. The hypothesis that the postpharyngeal gland sequesters rather than synthesizes hydrocarbons is further corroborated by the fact that in decapitated ants (i.e., lacking a postpharyngeal glands) the magnitude of hydrocarbon synthesis is not hampered, but these hydrocarbons accumulate internally (Soroker et al., 1994). Moreover, preliminary *in vitro* experiments revealed that the gland failed to biosynthesize de novo hydrocarbons from acetate. Lipid passage from hemolymph to postpharyngeal gland cells was previously suggested by Barbier and Delage (1967). Since Philips and Vinson (1980) found no evidence for fatty acid and triglyceride sequestration by the postpharyngeal gland, we suggest a specific sequestration of hydrocarbons. Our results suggest that in *C. niger*, and possibly other ant speices, the postpharyngeal gland might function as a site for hydrocarbon storage, thus explaining the congruency between cuticular and postpharyngeal gland hydrocarbons.

The purpose of hydrocarbon storage in the postpharyngeal gland is still unknown. However, our recent data show the importance of postpharyngeal gland constituents in nestmate recognition (Soroker et al., 1994). Considering discrimination cues, there might be an advantage for localization of such cues in the postpharyngeal gland. In encounters between ants, the first contact is an antennal one. The importance of these contacts for the organization of ant colonies and nestmate recognition was recently discussed by Gordon et al. (1993). During our experiments, antennal contacts were often interrupted by antennal cleaning and licking. Hydrocarbons stored in postpharyngeal glands can be easily smeared over the antennae and other body parts during their cleaning and licking, thus providing the cuticular surface with a fresh supply of individual recognition cues.

In dyadic encounters, we clearly showed a significant transfer of hydrocarbons between nestmates within 24 hr. During the passage of labeled hydrocarbons from one ant to the other in succession, the radioactivity declined, probably due to a dilution of the transferred hydrocarbons with the nonlabeled hydrocarbons of the recipient. The low level of labeled hydrocarbons on the recipient's cuticular surface could be the result of a relatively low level of interaction during these encounters and/or to a short exposure time. In the nest, ants are constantly exposed to a large number of nestmates, whereas our experimental setup was limited to dyadic encounters for 24 hr. Within this time interval, transfer to the recipient's postpharyngeal gland seems to be the major route. It is important to point out that hydrocarbon transfer is not merely a side effect of food transfer. Experiments with starved prelabeled donors presented with satiated recipients showed that the direction of the hydrocarbon transfer occurs independent of the direction of the food flow (Soroker et al., 1994). Nonfeeding trophallaxis was reported earlier for other species of ants (Hölldobler and Wilson, 1990) and in honeybees (Korst and Velthuis, 1982). This implies that it may constitute a method for signal transfer between nestmates in the social insects. When two nestmates meet, interactions such as trophallaxis, licking, body contact, and allogrooming normally take place. The distribution of labeled hydrocarbons in the recipients suggests that all these interactions are involved in hydrocarbon transfer between nestmates. From the results of different sets of encounters, using alternatively blocked or normal donors and recipients, we can conclude that hydrocarbons are mostly transferred from the postpharyngeal gland of one nestmate to the postpharyngeal gland of the other by trophallaxis; other ways of transfer are from the donor cuticle to the recipient postpharyngeal gland and vice versa by licking and from cuticle to cuticle by body contact.

These results fit well with accumulating data for the social distribution of colony odor in ants: from older workers to adopted callows through allogrooming (Morel, 1983; Errard, 1986; Morel and Blum, 1988); from nurses to callow workers (Morel, 1983; Morel et al., 1988; Morel and Vander Meer, 1987); between workers of the same colony by grooming (Crosland, 1989; Franks et al., 1990), and among conspecifics from different colonies during relatively short periods of contact (Breed et al., 1992). Our results also support the evidence that harmonization of the changes in hydrocarbon profile, reported by Provost et al. (1993) and by Vander Meer et al. (1989), can be a result of reciprocal transfer of the hydrocarbons. The "Gestalt model" (Crozier and Dix, 1979; Crozier, 1987; Crosland, 1989), states that the individual specific worker cues are distributed to the other workers via social interactions so that each worker's cuticular chemistry (heritable and environmental components) becomes a mean value representing the colony as a whole. The effect is to level out within-colony variation. The results of the present study provide evidence for the involvement

of the postpharyngeal gland in this process during trophallaxis and licking. The postpharyngeal gland, as a site where the ant's own hydrocarbons are mixed with those of conspecifics, may function as a source for the production of a colony Gestalt odor. The transfer of hydrocarbons between nestmates can partly explain the congruency of hydrocarbon patterns between the two ant species of heterospecific colonies (Errard and Jallon, 1987; Bagnères et al., 1991; Franks et al., 1990; Vienne, 1993) and between the myrmecophile insects and their host as result of the integration process into the ants' nest (Dettner and Liepert, 1994). Indeed, working on heterospecific groups of *Manica rubida* and *Formica selysi*, using the same methodology together with behavioral observations, we recently were able to provide evidence for hydrocarbon transfer between allospecific nestmates (Vienne et al., 1995).

Until the nature of recognition cues is defined, the results of the present research can serve as a model for the transfer of heritable chemical cues between nestmates and shed some light on the function of postpharyngeal glands in ants.

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ON INSECT ATTRACTANTS FROM PITCHER PLANTS OF THE GENUS *Heliamphora* (SARRACENIACEAE)

KLAUS JAFFÉ,[1] MURRAY S. BLUM,[2] HENRY M. FALES,[3] ROBERT T. MASON,[4] and AIVLÉ CABRERA[1]

[1]*Universidad Simón Bolívar*
Dpto. de Biología de Organismos
Apartado 89000, Caracas 1080, Venezuela

[2]*Department of Entomology*
University of Georgia
Athens, Georgia 30602

[3]*Laboratory of Physical Chemistry*
National Heart, Lung and Blood Institute
Bethesda, Maryland 20892

[4]*Department of Zoology*
Oregon State University
Corvallis, Oregon 97331

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Abstract—Examination of *Heliamphora heterodoxa* and *H. tatei* from the Guayana Highlands of Venezuela reveals that the enol diacetal monoterpene, sarracenin, is the major volatile compound present in the spoon-shaped structures of leaves of the pitchers. In addition, erucamide, phenol, cinerone, phenylacetaldehyde, and a series of methyl esters also occur in extracts of the spoon-shaped appendages of pitchers at the time during which they attract insects.

Key Words—*Heliamphora*, carnivorous plants, pitcher plants, sarracenin, insect attractants.

INTRODUCTION

Carnivorous plants are an extreme example of the diversity of insect–plant relationships (Darwin, 1888). The arthropod prey is attracted, caught, and degraded by specially adapted leaves supplementing the limited amount of nutrients available to these plants (Lloyd, 1942; Rymal and Folkerts, 1982; Juniper et al., 1989).

*To whom correspondence should be addressed.

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Plants in the genus *Heliamphora* Benth. (Sarraceniaceae) endemic to the Precambrian sandstone formations ("Tepuy") in Venezuela were not included by Darwin (1888) in the first extensive revision of carnivorous plants, and only recently have they been studied in some detail (Jaffé et al., 1992). These plants possess visual cues (Joel et al., 1985) in addition to special leaf appendages producing chemical secretions in order to attract their prey to the pitcher (Jaffé et al., 1992). Brewer-Carias (1972) was the first to describe the feeding behavior of the plant, reporting that a honeylike fluid produced by nectaries in the spoon-shaped appendage of the leaves attracted a variety of insects.

The macroscopic anatomy of a *Heliamphora* pitcher has been described in considerable detail (Lloyd, 1942). The transformed leaf or pitcher has a nectary or spoon, a bell zone, an intermediary constriction, and a funnel or tank (Jaffé et al., 1992). *Heliamphora* leaves have a reddish coloration on the spoon and the surrounding area, which may further act to attract potential prey items. UV light-reflecting guides also have been reported on *Heliamphora* leaves that probably enhance the attraction of certain insect groups (Joel et al., 1985; Juniper et al., 1989). Sun-exposed *Heliamphora* plants have pitchers of a generally constant size within a given species. They have well-developed spoons that are conspicuously red colored and fragrant. However, in instances where pitcher plants grow in the shade of crevasses, shrubs, or small trees, the appearance of the plants is very different. These pitchers are bright green without the red pigment, the hairs in the upper part of the pitcher are lost, and the size of the spoon is reduced. The bell or upper part of the leaf increases while the tank is reduced, becoming thinner and resulting in the pitcher being larger and more slender (Jaffé et al., 1992).

Pitcher leaves have an optimum age for attracting and capturing prey. For example, we know from field observations that fully developed leaves capture the largest number of prey items (arthropod carcasses) compared to very young or very old leaves (Jaffé et al., 1992).

Heliamphora pitchers attract and trap a large variety of arthropod species. Prey is apparently attracted to the spoon-shaped appendage at the tip of each pitcher, which accumulates a sweet-smelling and highly palatable sticky secretion. The odor of the secretion varies according to the location (Tepuy) or species. This secretion is very attractive to insects in various orders, but ants seem to constitute the main prey items (Gonzales et al., 1991; Jaffé et al., 1992).

The present study identifies several of the chemicals produced by the spoon-like leaf appendage in plants actively attracting insects.

METHODS AND MATERIALS

Nectaries of *Heliamphora heterodoxa* Steyermark were obtained at Auyantepuy, Estado Bolivar, by cutting the spoonlike leaf appendages and collecting

them in 5-ml vials with ethyl acetate or methylene chloride. For laboratory studies, we collected plants of *H. heterodoxa* at Auyantepuy, whereas those of *H. tatei* Gleason were from Aracamuni, Estado Amazonas, Venezuela. Nectaries from plants maintained in the laboratory were collected monthly by placing them in glass vials containing 5 ml of either ethyl acetate or methylene chloride. Each extract was concentrated to approximately 0.5 ml using a gentle nitrogen stream, and 1 μl of each extract was injected into the gas chromatograph (GC) or gas chromatograph–mass spectrometer (GC-MS).

GC and GC-MS analyses were performed in two different laboratories. The first GC-MS data identifying sarracenin were obtained on an LKB-2091 mass spectrometer equipped with a Shimadzu model GC-9A gas chromatograph using a J & W Instrument Co. 20-m × 0.18-mm-ID capillary column coated with their DB-5 methyl silicone. The column was programmed from 50 to 270°C at 10°C/min. The ion chamber was set at 270°C and spectra were run at 70 eV. Other GC-MS data in this laboratory came from a Hewlett-Packard 5890A gas chromatograph programmed from 50 to 275°C at 7°C/min using a 30-m Restec RTX-1 dimethylsiloxane capillary column 0.25 mm ID and 0.25 μm film thickness with data collected on a Finnigan Ion Trap Mass Spectrometer (ITMS) using helium as a buffer gas at 150°C. Sarracenin was also isolated here from the nectary extracts by preparative TLC on silica gel using 50:50 toluene–ethyl acetate, its identity being confirmed by GC-MS on the LKB-2091 GC-MS system.

In the other laboratory, samples from *H. heterodoxa* and *H. tatei* plants grown in the laboratory and *H. heterodoxa* grown in the field were analyzed using a Hewlett Packard model 5971A Mass Selective Detector (GC-MS) fitted with a HP 5908 Series II gas chromatograph and an MS-Chemstation data system. The instrument was operated in scan mode at an ionization energy of 70 eV. The temperature program and column were the same as described above.

In order to evaluate qualitatively the attractiveness of spoons (nectaries) of *H. heterodoxa* and *H. tatei* growing in the laboratory, they were cut from the plants and placed in a closed 1-m^3 chamber. Five house flies were released in the chamber and their landing sites observed for over 2 hr. Spoons were also placed on the foraging table of a *Solenopsis geminata* ant colony, and the number of workers recruited to the spoon were recorded.

RESULTS

Analyses of *H. heterodoxa* spoons from field collections revealed the presence of a number of chemical compounds. Sarracenine (an iridoid) was identified using all of the above systems by comparison with mass spectral data reported by Miles et al. (1976). Figure 1 (top) (from *H. tatei* run on the Hewlett Packard 5971A spectrometer) shows a typical chromatogram. Direct comparison of the retention time and mass spectrum was made using the LKB-2091 GC-MS system

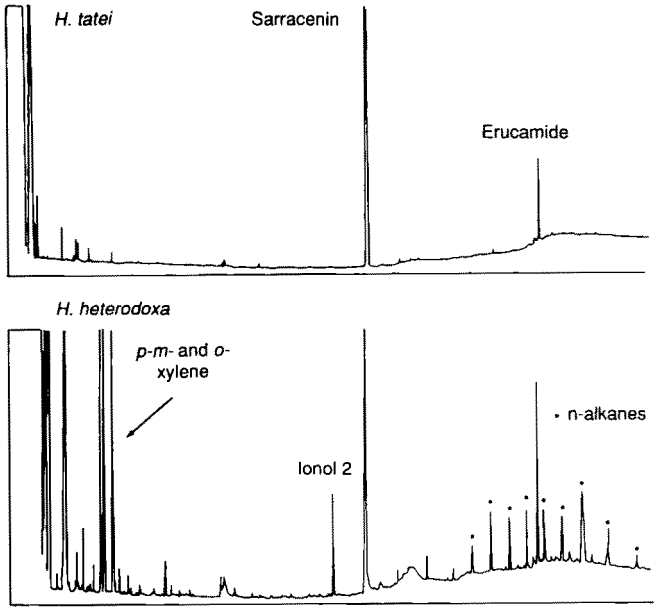

FIG. 1. Gas chromatograms of extracts of attractive spoons from *Heliamphora* pitchers. Samples of *H. tatei* came from CH_2Cl_2 extracts of pitchers growing in the laboratory; the sample of *H. heterodoxa* was an ethyl acetate extract from pitchers collected in the field.

using a sample of sarracenin kindly supplied by Professor J.K. Whitesell, Department of Chemistry, University of Texas. In addition, a sample of sarracenin purified by TLC was examined by gas chromatography–high-resolution mass spectrometry (Edward Larkin, University of Minnesota Mass Spectrometry Service Laboratory) giving a molecular ion at *m/z* 226.0854 (sarracenin $C_{11}H_{14}O_5$, requires 226.0867). Erucamide (13-docosenamide) also seen in Figure 1 as a late-eluting peak, was identified by comparison with its mass spectrum in the Wiley data base. Figure 1 (bottom) also reveals the presence of *o*-, *m*-, and *p*-xylene, 2,6-di-*tert*-butyl 4-ethylphenol (ionol-2), and a series of *n*-alkanes ranging from C_{21} to C_{33}, all identified by comparison with spectra in the Wiley data base. Ionol-2 is a likely contaminant from the solvents since it is employed as a commercial antioxidant.

Further analyses of *H. tatei* on the ion trap mass spectrometer resulted in the identification of phenol and phenylacetaldehyde in approximately equal concentrations. The terpene cinerone, also a significant constituent, was easily identified by its molecular ion at *m/z* 150 and its characteristic mass spectrum with

a base peak at 41 as seen in the NIST-NIH data base (Version 4.5, US Department of Commerce, NIST, Gaithersburg, Maryland). Three esters, methyl palmitate, ethyl palmitate, and methyl linolelaidate were detected in the ethyl acetate extracts.

Using the simple landing-site bioassay (Methods and Materials) the nectaries of the leaves of *H. tatei* and *H. heterodoxa* raised in the laboratory [(as opposed to the field, Jaffé et al. (1992)] did not, in fact, attract flies or ants. GC-MS analyses of three samples of each species of these nonattractive spoons revealed that neither sarracenin nor erucamide were present in these samples. However, on one occasion we did detect nectaries that were attractive to insects. These were found on leaves of a single large *H. tatei* plant grown in the laboratory that produced a sticky secretion on the inside of the spoons similar to that observed in plants in the field (Jaffé et al., 1992). Thus, approximately one third of a spoon was removed from the plant and of 10 observed fly landings, six were on the spoon section. The same section attracted a modest group (> 10) of ant workers during a 15-min period following the placement of the section on the foraging table of the ant colony. Accordingly, the remaining parts of the spoon were extracted with CH_2Cl_2 and is the source of the GC-MS in Figure 1 revealing the presence of sarracenin and erucamide. Figure 1 (bottom) shows a similar chromatogram from field-grown *H. heterodoxa* and these same two compounds are seen to be present. Unfortunately, no samples of wild growing *H. tatei* were available due to difficult and restricted access to the site.

DISCUSSION

The enol diacetal monoterpene sarracenin, first identified in leaf extracts of *Sarracenia flava* (Miles et al., 1976), is seen to be the major volatile compound from the spoon-shaped structure of leaves of *H. heterodoxa* and *H. tatei*. In addition, *o*- and *m*-xylene and phenylacetaldehyde, identified here, were also found in *S. flava* (Miles et al., 1976). Interesting among the other compounds produced by the spoon-shaped appendages of attractive pitchers was erucamide. This compound appears in the literature as an anti-block additive for inks and sticky substances (Nagura et al., 1992; Radosta and Riley, 1991), raising the possibility that plants may produce this compound to endow its nectar secretion with certain advantageous physical properties to entangle or trap insects.

The fact that sarracenin and erucamide were rarely produced in plants growing in the laboratory, and when present, accompanied attraction of insects, suggests they have a biological role. Possible functions could include attraction, trapping, killing, or anesthetizing insects; facilitating feeding on the secretion; maintaining a low viscosity of the secretion; etc. In the case of *S. flava*, Mody et al. (1976) have shown that the insect paralyzing-activity is due to coniine, and it would be interesting to try to detect this base in these plants.

We have previously shown that plants growing in the laboratory lose their attractive properties. This suggests that the morphological changes plants suffer when they lack light in the field (Jaffé et al., 1992) are equivalent to those they suffer when growing in the laboratory. This is in accord with previous proposals (Givinish et al., 1984) suggesting that carnivory is only advantageous to the plant in light-saturated habitats. If plants lose the ability to capture additional nutrients in light-poor environments, they may save energy by not producing attractive chemical secretions or undergoing costly morphological features (Jaffé et al., 1992).

It would be very interesting to know if the ability of the pitchers to attract a large variety of different arthropods (ants, moths, flies, crickets, bees, and scorpions) is due to some or all of the compounds reported in this work.

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ON THE APPARENT PERSISTENCE OF DISPARLURE IN THE HUMAN BODY

E. ALAN CAMERON

Department of Entomology
Penn State University
University Park, Pennsylvania 16802

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Key Words—Gypsy moth; disparlure, *Lymantria dispar*.

Previously I reported on long-term persistence of personal attractiveness to the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) following my exposure to the racemic form of its attractant, disparlure (*cis*-7-8-epoxy-2-methyloctadecane) (Cameron, 1981, 1983). My last known direct contact with disparlure was during the 1977 field season.

For the historical record, I note the following facts. Even though rigorous testing, such as that reported in my 1983 paper, has not been repeated, I have noted during each succeeding field season whether or not attractiveness persisted. Each field season through and including the summer of 1993, male gypsy moths continued to orient to and commonly make contact with me under a variety of conditions; no plausible explanation other than my persisting personal attractiveness to these insects could explain the phenomenon. For example, during casual conversations with colleagues while we were standing in paved parking lots at least tens of meters from the nearest tree, we have observed male moths exhibiting typical response behavior oriented towards my legs and torso but not to other individuals. Subjectively, I suspect a modest diminution of my attractiveness in recent years, but this may only reflect changes in naturally occurring populations.

During the summer of 1994, I participated in trapping tests during which I baited numbers of gypsy moth traps with synthetic lures. Although I took rigorous precautions to avoid direct contact with any of the lures, doubtless at a minimum I inhaled at least small quantities of pheromone. My original "contamination" was through an undetermined route(s), but certainly inhalation would

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not be excluded. I believe that it reasonably can be argued that my personal "experiment" must be concluded. A few early-season observations in 1994, before my first possible exposure to any disparlure, strongly suggested that I retained attractiveness for 17 years following my last previous known exposure. At a minimum, the period of attractiveness was 16 years, or through the 1993 field season.

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FOLIAGE CONSTITUENTS OF DOUGLAS FIR (*Pseudotsuga menziesii* (Mirb.) Franco (Pinaceae)): THEIR SEASONAL VARIATION AND POTENTIAL ROLE IN DOUGLAS FIR RESISTANCE AND SILVICULTURE MANAGEMENT

JIPING ZOU and REX G. CATES*

Chemical Ecology Laboratory
Department of Botany and Range Science
Brigham Young University
Provo, Utah 84602

(Received June 27, 1994; accepted December 9, 1994)

Abstract—Seasonal changes in the production of primary nutrients (soluble carbohydrates) and secondary metabolites (terpenes, monomeric phenolics, and tannins) in the current needle tissue of Douglas fir were investigated. All four classes of compounds showed significant seasonal changes in concentration during foliage development. Most terpenes increased significantly in concentration from June 11 to August 3, and then showed declining concentrations to September 20. The most dramatic and significant seasonal increases occurred in α-pinene, camphene, and bornyl acetate concentrations. The monomeric phenolics chlorogenic acid, taxifolin glucoside, quercetin galactoside, and those unknown phenolics showed an overall trend of declining in concentration from June 11 through September 20. However, considerable variation between sampling dates in the concentration of these phenolics was noted. Tannin concentration decreased significantly from June 11 to July 9, and then increased in concentration to the September 20 sampling date. Fructose, galactose, glucose, and sucrose tended to decrease from June 11 to September 20. However, significant variation between sampling dates was evident in these compounds as well. Galactose was the major compound in the soluble carbohydrate fraction, amounting to almost 80% of the total concentration throughout the growing season. These data suggest that if phenolics and tannins function as defenses, they would only affect second- and possibly third-instar budworm larvae during the time that these instars mine the buds. Camphene, α-pinene, and bornyl acetate increased in concentration throughout the growing season and may be effective deterrents to the budworm. Both bornyl acetate and

*To whom correspondence should be addressed.

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camphene have been shown in field and laboratory studies to increase larval mortality and adversely affect budworm larval growth. Carbohydrates generally act as nutrients that enhance herbivore growth. However, in a previous study, galactose was found to cause reduced budworm larval growth and increased larval mortality.

Key Words—Douglas fir, seasonal variation, carbohydrate, galactose, phenolic, terpene, tannin.

INTRODUCTION

Plant secondary compounds, such as terpenes, monomeric phenolics, and tannins have been reported to confer resistance in plants against phytophagous insects (Cates and Zou, 1990; Lindroth et al., 1988; Rosenthal and Janzen, 1979). Additionally, several types of variation in the production of primary nutrients and secondary metabolites have been suggested as important factors in the interactions between host plants and herbivores (Cates and Zou, 1990; Wagner et al., 1989; Clancy et al., 1988; Denno and McClure, 1983).

The objectives of this study were to investigate the seasonal patterns of variation in individual terpenes, phenolics, soluble carbohydrates, and total tannins in the current year's growth of Douglas fir (*Pseudotsuga menziesii*). As the current year's growth matures, it generally proceeds from a somewhat acceptable food resource to one that adversely affects budworm larval performance. While some terpenes have been found to affect the budworm adversely (Cates and Zou, 1990, Cates and Redak, 1988), little is known about the effect of individual compounds on larval development or of the interaction of carbohydrates and other primary nutrients with secondary metabolites.

METHODS AND MATERIALS

Preparation of Materials. Seven trees of similar age, height, vigor, and crown diameter were selected from a population of about 50 trees at a study site near Island Park, Idaho. These trees were selected because they had been involved since 1985 in all studies at this site dealing with budworm–host tree interactions. Needle samples were collected from four randomly chosen branches in each cardinal direction in the mid-crown of each tree. Two-gram samples of needles were collected from each branch on June 11, June 22, July 9, August 3, and September 20, 1989. After collection, needles were immediately placed in liquid nitrogen, transported to the Chemical Ecology Laboratory, and stored at −80°C.

Isolation and Quantification of Carbohydrate. For each sample, 500 mg frozen tissue were ground with pestle and mortar in liquid nitrogen. The leaf

powder was then extracted with 20 ml methanol and water (80% MeOH–20% water) (2 × 10 ml each). Five milliliters of the MeOH–water extract were placed in a separate vial, 100 μl of the internal standard inositol were added, and then all was taken to dryness. The extract was derivatized by adding 1 ml pyridine, 0.2 ml hexamethyldisilazane (HMDS), and 0.1 ml trimethylchlorosilane (TMCS). Samples were mixed by shaking, kept at 45°C for 10 min, centrifuged (2000 rpm) for 15 min, and then transferred into an autosampler vial using a disposable syringe. Carbohydrate analysis was done on a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector. Sample solutions were injected into a 25-m 100% methyl-polysiloxane stationary phase column (0.17 μm film thickness). Temperature programming was as follows: initial temperature was set at 100°C, after 1 min increased to 145°C at a rate of 15°C/min followed by immediate increases of 2.5°C/min to 200°C. Then the temperature was increased at 15°C/min to 245°C, and the oven was kept at 245°C for 15 min. Both cochromatography and GC–mass spectroscopy (GC-MS Hewlett Packard 5995) were used to identify the carbohydrate compounds. Unknown compounds were assigned numbers. The concentration of each compound was determined by area counts and calculated as milligrams per gram fresh tissue. Compounds with area values below 1000 were excluded.

Isolation and Quantification of Terpenes. Frozen tissue (500 mg) was ground with pestle and mortar in liquid nitrogen, and 20 ml of 100% chloroform were added to extract the terpenes (2 × 10 ml each). Five milliliters of the extract were removed and 100 μl of γ-terpene were added as the internal standard. This was reduced to 1 ml, and 2 μl were transferred to an autosampler vial. Temperature programming was as follows: initial temperature was set at 60°C, after 4 min increased to 100°C at a rate of 5°C/min, then followed by an immediate increase of 15°C/min to 220°C, and then held on 220°C for 1 min. Finally, temperature increased at 5°C/min to 250°C and was held at 250°C for 5 min. Concentration of each compound was determined by area counts and calculated as milligrams per gram fresh tissue.

Isolation and Quantification of Tannins. Tannin content was analyzed by the astringency method of Martin and Martin (1984) with the following modifications. Plant needles were carefully separated from bud scales and stem tissue. Approximately 100 mg of frozen leaf tissue were ground in liquid nitrogen in a mortar, extracted in 70% aqueous acetone in a test tube by sonication for 3 min at room temperature, and then contrifuged (4500 rpm) for 10 min. The supernatant was then ready for testing. Hemoglobin solution (2.2 mg of bovine hemoglobin substrate powder type II obtained from Sigma Chemical Corporation per milliliter of phosphate buffer, pH 6.5) was used as the reaction protein. From the Douglas fir needles tannins were extracted, purified, and used as the reference standard.

Isolation and Quantification of Phenolics. Plant needles (300 mg) were

ground in liquid nitrogen in a mortar, and extracted twice each with 10 ml of MeOH–water (80:20). Four milliliters of this solution were then blown dry under nitrogen. One milliliter of MeOH–water (75:25) was then added and sonicated to redissolve the sample. This solution was filtered and injected into a high-pressure liquid chromatograph (Hewlett Packard 1090). HPLC solvents were: solvent A = water–acetic acid (98:2), solvent B = acetonitrile–acetic acid (98:2). Temperature was 50°C, flow was 1 ml/min, and wavelength detection was at 254 nm. The column was a Phenomenex Ultracarb 20, C_{18} reverse-phase 5-μm column. The column was 25 cm × 4.6 mm and fitted with a matching guard column. Compounds were identified by cochromatography, and concentration was calculated as milligrams per gram tissue fresh weight.

Statistical Analyses. Data from all sampling dates were compared using a repeated measures model with season as the fixed factor. From this analysis a determination was made as to whether significant seasonal changes in individual chemicals occurred across all sampling dates (Tables 1, 3, 5, 7; $P < 0.05$). To determine where significant differences ($P < 0.05$) within the growing season occurred, the repeated measures model was used for all possible comparisons taken two at a time (Tables 2, 4, 6, 8). All statistical procedures were accomplished with SAS programs (SAS Institute, 1985).

RESULTS

Seven monoterpenes (α-pinene, β-pinene, camphene, limonene, myrcene, terpinolene, and tricyclene), two sesquiterpenes (α-humulene and longibornyl), and bornyl acetate were identified in the seasonal growth of Douglas fir. Four unknown compounds also were isolated from Douglas fir needles, and tentative identification suggested these to be sesquiterpenes. The major components of the terpene fraction were camphene, bornyl acetate, and α-pinene (79% of the compounds resolved at the July 9 sampling) (Table 1). Total concentration of the volatiles varied significantly between sampling dates within the growing season ($P = 0.006$). Total volatile concentrations in Douglas fir foliage increased significantly from June to August except between the July 9 and August 3 sampling dates and then decreased after the August sampling (Tables 1 and 2). α-Pinene, camphene, limonene, myrcene, terpinolene, tricyclene, and bornyl acetate changed significantly through the growing season. β-Pinene, α-humulene, longibornyl, terpinolene, and unknowns 3, 4, and 5 showed small, and sometimes significant, changes during needle maturation. Unknown 6 decreased throughout the growing season.

Six phenolics were resolved by HPLC in the current year's needles of Douglas fir. Significant changes in the total monomeric phenolic concentration occurred during the growing season ($P = 0.011$) (Table 3). However, the pattern

of variation was the reverse of the volatiles in that the concentrations of phenolic compounds was highest in the early season samples, decreased to the August sample, and then increased slightly by the September sample. Chlorogenic acid did not show a significant change overall (Table 3), but significant decreases did occur from June 11 through August 3, (Table 4). The concentrations of taxifolin glucoside, unknown 1, and unknown 3, along with the major phenolic components quercetin galactoside and unknown 2 decreased significantly during the growing season (Tables 3 and 4).

Condensed tannin, as measured by astringency or by binding capacity of the extract, was significantly higher in swollen buds (June 11), decreased by the July 9 sample, and then increased by the September 20 sampling to concentrations similar to those found in swollen buds (Tables 5 and 6). Although a significant change in tannin concentration was noted over all sampling dates (Table 5), the most significant decreases in tannin occurred between June 11 and July 9 (Table 6).

Concentration of the total soluble carbohydrate fraction, which consisted of five identified compounds, remained at high levels to August 3 and then decreased significantly by September ($P = 0.001$) (Table 7). Galactose made up over 80% of the total carbohydrate concentration (Table 7), and was significantly higher in concentration throughout the growting season compared to all other carbohydrates (Table 7). Galactose concentrations did not change significantly from June to August, but did decrease significantly from August to September ($P = 0.001$) (Table 8). All other carbohydrates showed only small changes throughout the growing season even though some changes were significant (Table 8).

Figure 1 illustrates the seasonal changes for all classes of compounds. At the July 9 sampling date, carbohydrates were highest in concentration (75%), followed by the condensed tannins (14%), terpenes or volatiles (8%), and finally, the monomeric phenolics (3%).

DISCUSSION

The only compounds showing dramatic and consistent seasonal increases were the terpenes α-pinene, camphene, tricyclene, and bornyl acetate (Tables 1 and 2; Figure 1). However, these compounds decreased significantly after the August 3 sampling date. Alternatively, monomeric phenolics decreased from June 11 to August 3 and then significantly increased in concentration to September 20 (Tables 3 and 4; Figure 1). Tannin concentration decreased significantly from June 11 to July 9 and then increased significantly following the July 9 sampling (Tables 5 and 6; Figure 1). Western spruce budworm vigorously feed on the current year's foliage of Douglas fir from June 1 to about mid-July.

TABLE 1. VARIATION IN TERPENE CONCENTRATION WITHIN GROWING SEASON IN DOUGLAS FIR CURRENT YEAR'S GROWTH[a]

	Sampling date					
Volatiles	June 11 $\bar{X} \pm$ SE	June 22 $\bar{X} \pm$ SE	July 9 $\bar{X} \pm$ SE	August 3 $\bar{X} \pm$ SE	September 20 $\bar{X} \pm$ SE	P
α-Pinene	0.43 ± 0.03	0.98 ± 0.10	2.01 ± 0.24	2.32 ± 0.26	1.42 ± 0.022	0.022
β-Pinene	0.39 ± 0.03	0.98 ± 0.10	2.01 ± 0.24	0.54 ± 0.10	0.38 ± 0.08	0.144
Camphene	0.49 ± 0.05	1.45 ± 0.18	3.31 ± 0.42	3.88 ± 0.45	2.62 ± 0.16	0.006
Limonene	0.19 ± 0.06	0.22 ± 0.06	0.34 ± 0.09	0.38 ± 0.10	0.27 ± 0.07	0.025
Myrcene	0.098 ± 0.02	0.103 ± 0.02	0.149 ± 0.02	0.166 ± 0.02	0.112 ± 0.01	0.012
Terpinolene	0.006 ± 0.00	0.028 ± 0.01	0.008 ± 0.00	0.004 ± 0.00	0.044 ± 0.01	0.030
Tricyclene	0.077 ± 0.01	0.221 ± 0.03	0.495 ± 0.06	0.589 ± 0.07	0.399 ± 0.02	0.006
Bornyl acetate	0.35 ± 0.04	1.14 ± 0.16	2.67 ± 0.41	2.82 ± 0.34	1.92 ± 0.12	0.001
α-Humulene	0.02 ± 0.01	0.01 ± 0.001	0.09 ± 0.03	0.11 ± 0.03	0.07 ± 0.02	0.079
Longibornyl	0.001 ± 0.00	0.008 ± 0.00	0.026 ± 0.01	0.044 ± 0.01	0.024 ± 0.01	0.313
Unknown 3	0.004 ± 0.00	0.004 ± 0.00	0.005 ± 0.00	0.006 ± 0.00	0.004 ± 0.00	0.207
Unknown 4	0.021 ± 0.00	0.023 ± 0.00	0.028 ± 0.00	0.029 ± 0.01	0.02 ± 0.004	0.185
Unknown 5	0.021 ± 0.00	0.042 ± 0.01	0.048 ± 0.02	0.054 ± 0.01	0.037 ± 0.008	0.150
Unknown 6	0.062 ± 0.01	0.033 ± 0.00	0.03 ± 0.00	0.028 ± 0.00	0.019 ± 0.003	0.005
Total	2.367 ± 0.21	4.922 ± 0.54	10.086 ± 1.30	11.372 ± 1.27	7.609 ± 0.49	0.006

[a] Values are milligrams per gram fresh weight. A repeated measures model was used to determine if a significant change occurred in the concentration of each compound across all five sampling dates. Table 2 indicates significant changes for each compound for all possible comparisons between dates.

TABLE 2. PROBABILITY VALUES COMPARING ALL POSSIBLE COMBINATIONS OF VOLATILE CONCENTRATIONS TAKEN TWO AT A TIME FOR EACH VOLATILE COMPOUND[a]

	Sampling date									
	June 11 vs.				June 22 vs.			July 9 vs.		August 3 vs.
Volatiles	June 22	July 9	August 3	September 20	July 9	August 3	September 20	August 3	September 20	September 20
α-Pinene	0.001	0.000	0.000	0.001	0.001	0.000	0.082	0.088	0.058	0.027
β-Pinene	0.011	0.012	0.209	0.853	0.069	0.037	0.251	0.761	0.015	0.015
Camphene	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.066	0.071	0.016
Limonene	0.037	0.001	0.001	0.001	0.004	0.000	0.037	0.066	0.051	0.009
Myrcene	0.514	0.001	0.000	0.077	0.002	0.000	0.338	0.077	0.035	0.004
Terpinolene	0.033	0.418	0.481	0.008	0.119	0.049	0.101	0.117	0.028	0.014
Tricyclene	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.049	0.084	0.016
Bornyl acetate	0.001	0.001	0.000	0.000	0.002	0.000	0.000	0.436	0.045	0.014
α-Humulene	0.567	0.055	0.050	0.106	0.017	0.014	0.006	0.134	0.111	0.034
Longibornyl	0.058	0.010	0.020	0.016	0.013	0.031	0.046	0.147	0.787	0.039
Unknown 3	0.766	0.279	0.219	0.766	0.025	0.017	0.436	0.654	0.418	0.256
Unknown 4	0.549	0.123	0.158	0.905	0.053	0.064	0.456	0.775	0.047	0.009
Unknown 5	0.014	0.034	0.014	0.083	0.254	0.078	0.326	0.217	0.079	0.003
Unknown 6	0.001	0.000	0.000	0.000	0.232	0.121	0.001	0.451	0.013	0.003
Total	0.001	0.001	0.000	0.000	0.001	0.000	0.002	0.120	0.048	0.015

[a]Actual concentrations for each sampling period for this Idaho population are found in Table 1.

TABLE 3. VARIATION IN MONOMERIC PHENOLIC CONCENTRATION WITHIN GROWING SEASON IN DOUGLAS-FIR GROWTH[a]

Phenolics	Sampling date					
	June 11 $\bar{X} \pm$ SE	June 22 $\bar{X} \pm$ SE	July 9 $\bar{X} \pm$ SE	August 3 $\bar{X} \pm$ SE	September 20 $\bar{X} \pm$ SE	*P*
Chlorogenic acid	0.53 ± 0.09	0.45 ± 0.08	0.33 ± 0.07	0.25 ± 0.04	0.23 ± 0.04	0.11
Taxifolin glucoside	0.72 ± 0.40	0.54 ± 0.54	0.78 ± 0.43	0.09 ± 0.08	1.43 ± 0.82	0.05
Quercetin galactoside	3.23 ± 0.44	1.65 ± 0.21	1.09 ± 0.17	0.99 ± 0.14	0.79 ± 0.11	0.05
Unknown 1	0.96 ± 0.11	0.51 ± 0.08	0.09 ± 0.04	0.42 ± 0.06	0.41 ± 0.06	0.01
Unknown 2	2.84 ± 0.45	2.17 ± 0.24	0.58 ± 0.17	0.36 ± 0.06	0.09 ± 0.07	0.009
Unknown 3	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.09 ± 0.00	0.01
Total	8.73 ± 1.09	5.20 ± 0.60	3.09 ± 0.54	2.47 ± 0.28	3.50 ± 0.24	0.011

[a]Values are milligrams per gram fresh weight. A repeated measures model was used to determine if a significant change occurred in the concentration of each compound across all five sampling dates. Table 4 indicates significant changes for each compound for all possible comparisons between dates.

TABLE 4. PROBABILITY VALUES WHEN COMPARING ALL POSSIBLE COMBINATIONS OF MONOMERIC PHENOLIC CONCENTRATIONS TAKEN TWO AT A TIME FOR EACH PHENOLIC COMPOUND[a]

	Sampling date									
	June 11 vs.				June 22 vs.			July 9 vs.		August 3 vs.
Phenolics	June 22	July 9	August 3	September 20	July 9	August 3	September 20	August 3	September 20	September 20
Chlorogenic acid	0.039	0.002	0.002	0.002	0.002	0.004	0.002	0.031	0.013	0.179
Taxifolin glucoside	0.094	0.335	0.029	0.308	0.039	0.632	0.601	0.039	0.691	0.002
Quercetin galactoside	0.002	0.001	0.000	0.001	0.016	0.002	0.001	0.479	0.010	0.104
Unknown 1	0.015	0.001	0.001	0.003	0.003	0.204	0.161	0.006	0.001	0.870
Unknown 2	0.210	0.001	0.001	0.001	0.000	0.000	0.000	0.166	0.396	0.023
Unknown 3	0.068	0.899	0.883	0.020	0.011	0.044	0.000	0.753	0.001	0.014
Total	0.010	0.000	0.000	0.002	0.004	0.002	0.032	0.201	0.409	0.008

[a]Actual concentrations for each sampling period for this Idaho population are found in Table 3.

TABLE 5. VARIATION IN TANNIN CONCENTRATION WITHIN GROWING SEASON IN DOUGLAS-FIR CURRENT YEAR'S GROWTH[a]

	Sampling date					
	June 11 $\bar{X} \pm$ SE	June 22 $\bar{X} \pm$ SE	July 9 $\bar{X} \pm$ SE	August 3 $\bar{X} \pm$ SE	September 20 $\bar{X} \pm$ SE	P
Tannin	31.99 ± 3.05	18.9 ± 1.65	16.70 ± 1.23	24.32 ± 2.24	27.05 ± 2.77	0.005

[a]A repeated-measures model was used to determine if a significant change occurred in the concentration of tannin across all sampling dates. Table 6 indicates significant changes for tannin for all possible comparisons between dates.

Since monomeric phenolics and tannins decreased in concentration in the current year's growth during this time, they probably are not significant in deterring third- through sixth-instar feeding (Wagner et al., 1989). However, the possibility exists that phenolics or tannins may deter second instars that disperse and mine buds high in tannin and phenolic compounds.

The soluble carbohydrates fructose, glucose, mannose, and sucrose remained at low concentrations during the growing season (Tables 7 and 8). Noteworthy, and the first report we are aware of, is the finding that galactose was produced in significantly higher concentrations during all sampling periods as compared to the other soluble carbohydrates. This occurred despite the significant decrease in galactose that began near the August 3 sampling date and continued to the September sampling (Tables 7 and 8; Figure 1). Coinciding with the decrease in galactose in August is a significant increase in tannin concentration (Tables 5 and 7; Figure 1). Perhaps galactose may be involved in the phenolic pathway being utilized as a source of energy or substrate for various reactions in this metabolic pathway. Because of the high concentration of galactose across the growing season when budworms feed on the current year's growth, we tested this compound against the budworm. Concentrations of total soluble carbohydrates containing galactose in concentrations typical of the current year's foliage, and 6% galactose by itself, resulted in 64–96% and 68% larval mortality, respectively (Zou and Cates, 1994).

The patterns of change in volatiles in the current year's foliage of Douglas fir discussed in this study are similar to those found in other investigations, even though the chemical methods, Douglas fir populations, and sampling dates were different. Since somewhere between 14 and 27 volatiles in the current year's growth of Douglas fir have been reported in various studies, several patterns could be discussed. In this section only the patterns that may impact budworm feeding are addressed for both major and minor components.

TABLE 6. PROBABILITY VALUES WHEN COMPARING ALL POSSIBLE COMBINATIONS OF TANNIN CONCENTRATIONS TAKEN TWO AT A TIME[a]

	Sampling date									
	June 11 vs.				June 22 vs.			July 9 vs.		August 3 vs.
	June 22	July 9	August 3	September 20	July 9	August 3	September 20	August 3	September 20	September 20
Tannin	0.0075	0.000	0.000	0.215	0.446	0.108	0.280	0.004	0.020	0.559

[a] Actual concentrations for each sampling period for this Idaho population are found in Table 5.

TABLE 7. VARIATION IN SOLUBLE CARBOHYDRATE CONCENTRATION WITHIN GROWING SEASON IN DOUGLAS-FIR CURRENT YEAR'S GROWTH[a]

	Sampling date					
Carbohydrate	June 11 $\bar{X} \pm$ SE	June 22 $\bar{X} \pm$ SE	July 9 $\bar{X} \pm$ SE	August 3 $\bar{X} \pm$ SE	September 20 $\bar{X} \pm$ SE	P
Fructose	5.47 ± 0.42	6.75 ± 0.56	4.48 ± 0.85	3.70 ± 1.07	4.11 ± 0.43	0.06
Galactose	77.72 ± 3.43	77.99 ± 2.08	80.30 ± 4.31	76.50 ± 6.12	39.83 ± 1.68	0.004
Glucose	5.93 ± 0.30	6.77 ± 0.59	2.92 ± 0.52	4.06 ± 0.46	4.22 ± 0.58	0.02
Mannose	0.82 ± 0.07	1.15 ± 0.08	0.99 ± 0.18	0.91 ± 0.27	0.78 ± 0.07	0.20
Sucrose	4.64 ± 1.12	2.77 ± 0.64	2.19 ± 0.67	1.30 ± 0.26	1.45 ± 0.31	0.05
Total	94.57 ± 4.52	95.42 ± 2.79	90.89 ± 3.74	86.45 ± 6.48	50.39 ± 1.61	0.001

[a] Values are milligrams per gram fresh weight. A repeated-measures model was used to determine if a significant change occurred in the concentration of each compound across all five sampling dates. Table 8 indicates significant changes for each compound for all possible comparisons between dates.

As in this study, Gambliel and Cates (1995) and Wagner et al. (1989) found that total terpene concentration increased from June to early July in populations located in New Mexico and Arizona. Individual terpenes, notably α-pinene, tricyclene, camphene, and bornyl acetate, increased within the growing season in the current year's growth of Douglas fir populations from New Mexico (Gambliel and Cates, 1995), Arizona (Wagner et al., 1989) and Idaho (this study). Concentrations of some compounds were similar throughout the growing season in Idaho, Arizona, and New Mexico populations, but other compounds generally showed decreased concentrations in the Idaho (this study) and Arizona (Wagner et al., 1989) populations when compared to the New Mexico population (Gambliel and Cates, 1995). Populations do show several unique changes in concentrations of individual terpenes and volatiles. β-Pinene and myrcene, for example, increased significantly during the June, July, and August sampling dates in the Idaho population reported here, but Gambliel and Cates reported no significant changes within the growing season in the concentration of these two compounds in the New Mexico population. For the Arizona population, β-pinene increased almost sevenfold between the June 6 and 20 sampling dates, and then declined by July 25 to less than a fourth of the highest concentration. Limonene showed little change during the growing season in the current year's growth of the Arizona population (Wagner et al., 1989), but in the Idaho population significant increases occurred through the July 9 date.

Within-season variation in the production of allelochemicals suggests that several compounds may be toxic to the budworm since they were present in high concentrations during the time that the budworm actively feeds on the

TABLE 8. STATISTICAL RESULTS COMPARING ALL POSSIBLE COMBINATIONS OF SOLUBLE CARBOHYDRATE CONCENTRATIONS TAKEN TWO AT A TIME[a]

	Sampling date									
	June 11 vs.				June 22 vs.			July 9 vs.		August 3 vs.
Carbohydrate	June 22	July 9	August 3	September 20	July 9	August 3	September 20	August 3	September 20	September 20
Fructose	0.078	0.229	0.118	0.006	0.002	0.009	0.006	0.149	0.638	0.674
Galactose	0.956	0.637	0.879	0.000	0.598	0.839	0.000	0.669	0.000	0.001
Glucose	0.358	0.001	0.024	0.015	0.008	0.005	0.021	0.215	0.174	0.827
Mannose	0.014	0.304	0.732	0.242	0.219	0.316	0.008	0.594	0.230	0.619
Sucrose	0.090	0.010	0.030	0.016	0.132	0.034	0.009	0.225	0.120	0.597
Total	0.898	0.526	0.421	0.000	0.335	0.271	0.000	0.648	0.000	0.002

[a]Actual concentrations for each sampling period for this Idaho population are found in Table 7.

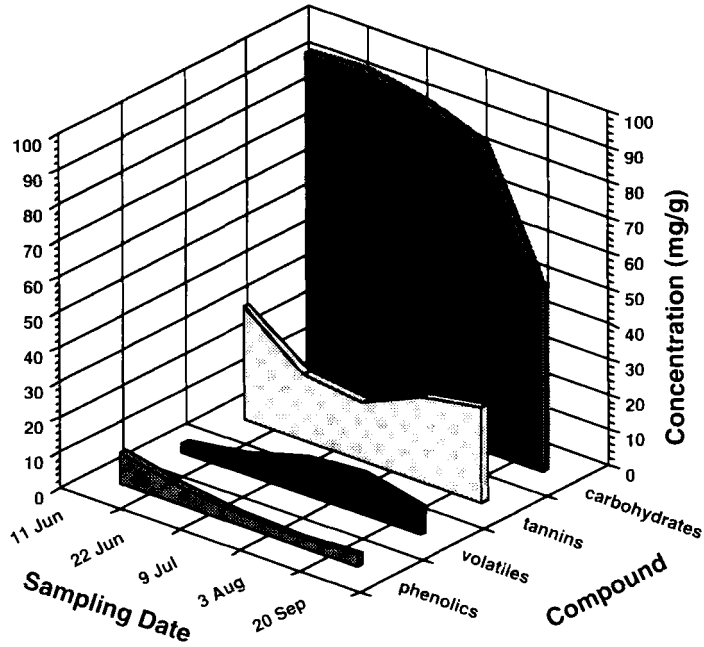

FIG. 1. Seasonal changes in monomeric phenolics, volatiles, tannins, and soluble carbohydrates in Douglas fir from Idaho. Means are plotted. Means and standard errors for the total concentration for each class of compound for each sampling date are found in Tables 1, 3, 5, and 7, respectively.

current year's growth of Douglas fir. For example, seasonal maturation patterns suggest that the volatiles tricyclene, camphene, α-pinene, and bornyl acetate may be associated with resistance in Douglas fir to the budworm. Laboratory and field studies show that camphene and bornyl acetate are highly toxic to several populations of budworm in Idaho, Montana, and New Mexico (Cates et al., 1983; Cates and Redak, 1988; Cates and Zou, 1990; Zou and Cates, unpublished data). Other volatile components enhance this toxicity by forming a mixture of compounds of varying toxicity. In addition, a suite of sesquiterpenes have recently been found to be toxic to the western budworm (Zou and Cates, unpublished data). A new finding is that galactose represents nearly 80% of the total soluble carbohydrate produced by Douglas fir and, either alone or in the presence of a mixture of carbohydrates and terpenes, significantly increased larval mortality (Zou and Cates, 1994).

Significant differences exist among populations both in their qualitative and

quantitative production of individual and total terpenes, as well as different types of terpenes (e.g., sesquiterpenes) (Cates and Zou, 1990, Wagner et al., 1989; Cates and Redak, 1988; Gambliel and Cates, 1995; Zou and Cates, unpublished data). These differences occur during June and July when budworm are feeding heavily on the new foliage. Variation in allelochemics, high concentrations of galactose and other carbohydrates (Zou and Cates, 1994), time of budbreak (Cates et al., 1983), and other foliage parameters (Zou and Cates, 1994, Cates and Redak, 1988), play an important role in creating a diverse, ever-changing mosaic of host resources (among individuals within a population and among populations) that the budworm and other needle-feeding insects regularly encounter. These mosaics are significant to the development of natural stands possessing reduced susceptibility to herbivores and pathogens and must be maintained in current silviculture management schemes (Cates and Zou, 1990; Wulf and Cates, 1987; Carlson et al., 1983).

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SPINED CITRUS BUGS, *Biprorulus bibax* BREDDIN (HEMIPTERA: PENTATOMIDAE), DO NOT DISCRIMINATE BETWEEN ENANTIOMERS IN THEIR AGGREGATION PHEROMONE

DAVID G. JAMES[1,*] and KENJI MORI[2]

[1]*NSW Agriculture, Yanco Agricultural Institute*
PMB Yanco NSW 2703 Australia
[2]*Department of Agricultural Chemistry*
The University of Tokyo
Yayoi 1-1-1 Bunkyo-ku, Tokyo 113, Japan

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Abstract—Female spined citrus bugs, *Biprorulus bibax*, in outdoor flight cage tests did not discriminate between synthetic aggregation pheromone blends containing either the racemate, natural (3*R*, 4*S*)-(−) or unnatural (4*S*, 3*S*)-(+) enantiomers of the major hemiacetal component (3*R*, 4*S*, 1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol. Two to three times as many bugs were attracted to pheromone-baited sites compared to unbaited sites. The racemate of the hemiacetal is easier and less costly to synthesize than the enantiomers and will be used in development of the pheromone as a management tool for this pest.

Key Words—*Biprorulus bibax*, Hemiptera, Pentatomidae, aggregation pheromone.

INTRODUCTION

The spined citrus bug, *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) is an important pest of citrus in inland southeastern Australia (James, 1989). James and Warren (1989) reported the presence of enlarged dorsal abdominal glands (DAGs) in male *B. bibax*, which produce an aggregation pheromone comprised of linalool, nerolidol, two isomers of farnesol, and a novel hemiacetal identified by Oliver et al. (1992) as *cis*-3,4-bis[(*E*)-1′-butenyl]tetrahydro-2-furanol. The

*To whom correspondence should be addressed.

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hemiacetal and linalool are major components and comprise 85–90% of DAG secretions. Absolute configuration of the hemiacetal was reported by Mori et al. (1993) to be (3*R*, 4*S*, 1′*E*)-3,4-bis(1′-butenyl)tetahydro-2-furanol.

Teneral and reproductive female *B. bibax* showed attraction in flight cage tests to synthetic blends of aggregation pheromone incorporating the racemate of the hemiacetal (James et al., 1994). Sites baited with synthetic blend attracted 2.3–4.7 times as many bugs as unbaited sites. This pheromone has potential for use in management of *B. bibax* as an aid to population monitoring, but it needs to be determined whether attractivity of the hemiacetal racemate rivals that of the natural enantiomer (3*R*, 4*S*)-(−). The latter is more difficult and costly to synthesize. This paper presents data on the comparative attractancy of natural and unnatural enantiomers and the racemate of the hemiacetal in pheromone blends to *B. bibax*.

METHODS AND MATERIALS

A large (6.6 × 3 × 1.8-m) shade-cloth-covered, steel-framed structure was used as an outdoor flight cage. The cage was situated in a sunlit, grassy area at the Yanco Agricultural Institute and contained two potted citrus trees (var. Valencia orange) of the same age (3 years) and size (1.5 m) at either end. The attractivity of three blends of *B. bibax* aggregation pheromone containing either the racemate or natural (3*R*, 4*S*)-(−) or unnatural (3*S*, 4*R*)-(+) isomers (both ca. 100% enantiomeric purity) of the major hemiacetal component, (3*R*, 4*S*, 1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol, was evaluated in three experiments, each conducted over four days during February–March 1994. The racemate and two isomers were synthesized in Japan (Mori et al., 1993) and shipped to Australia. In each blend, 17 mg (46%) of the hemiacetal was used with 15 mg (40%) of linalool, 4.5 mg (12%) of farnesol, and 0.5 mg (2%) of nerolidol. This component ratio approximates that found in male DAG extracts and extracts from airborne volatiles of live calling males (James et al., 1994). Linalool, farnesol, and nerolidol (all racemic mixtures) were obtained from Sigma-Aldrich Pty Ltd (Castle Hill, New South Wales). Blends were mixed with 1 ml of heptane in a plastic film-capped glass vial. *B. bibax* shows no response to heptane alone (James et al. 1994). The plastic film was pierced with a pin and a single vial was attached to the trunk of the trees and hidden from direct sunlight to retard evaporation. A vial containing 1 ml of heptane only was placed on the other tree. A new sample of pheromone was used after two days. Baited and unbaited trees were alternated daily. Forty to 50 reproductively active female *B. bibax* collected from district orchards were released in the center of the flight cage in each experiment. An equal number of fruit (oranges and lemons) was placed in each tree to provide food for the bugs. A daily count of bugs on the baited and unbaited trees was conducted at 16:00 hr. Traps were not used

because a type suitable for *B. bibax* has not yet been developed. After counting, bugs were released again in the center of the cage. Records were kept of daily temperature and weather conditions. A limited supply of synthetic pheromone prevented replication of individual experiments, although results for each of the four test dates were treated as replicates.

RESULTS AND DISCUSSION

Weather conditions during the three experiments were always favorable for flight activity of *B. bibax*, with daytime temperatures ranging from 20 to 32°C. Significantly greater numbers of *B. bibax* were attracted to the pheromone-baited tree regardless of the enantiomeric status of the hemiacetal component (Table 1). Two to three times as many bugs were counted on the pheromone-baited tree as on the unbaited tree throughout the experiments. Attraction ratios (baited–unbaited) did not differ significantly between the three experiments ($P < 0.05$, anova). Although linalool appears to occur in *B. bibax* pheromone as a single enantiomer (*S*)-(+) (J. Oliver personal communication), attraction of *B. bibax* in this study to blends using racemic linalool suggests chirality of this compound is also unimportant.

These data indicate that no advantage would be gained in using the natural enantiomer (3*R*, 4*S*)-(−) instead of the racemate of the hemiacetal in *B. bibax*

TABLE 1. ATTRACTIVITY OF FEMALE *B. bibax* IN FLIGHT CAGE TESTS TO AGGREGATION PHEROMONE CONTAINING RACEMATE, NATURAL ENANTIOMER, OR UNNATURAL ENANTIOMER OF MAJOR HEMIACETAL COMPONENT

Enantiomeric status of hemiacetal in pheromone	Female *B. bibax* responding (*N*)	
	Total	Mean ± SE/day
Experiment 1		
Racemate	82.0	20.5 ± 1.1[a]
Heptane	30.0	7.5 ± 0.6
Experiment 2		
Natural enantiomer (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol	44.0	11.0 ± 1.6[a]
Heptane	16.0	4.0 ± 0.3
Experiment 3		
Unnatural enantiomer (4*S*,3*S*,1′*E*)-3,4-bis-(1′butenyl)tetrahydro-2-furanol	61.0	15.2 ± 3.9[a]
Heptane	24.0	6.0 ± 1.5

[a]Significantly greater than heptane treatment, $P < 0.05$ (ANOVA).

pheromone in management of this pest. The natural enantiomer is more difficult and costly to synthesize than the racemate (Mori et al., 1993). Interestingly, the unnatural enantiomer of the hemiacetal was also bioactive, indicating that *B. bibax* does not discriminate between stereoisomers, as is the case for the German cockroach and its sex pheromone (Mori et al., 1981, Mori and Takikawa, 1990).

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OLFACTORY RESPONSES OF THE PARASITOID *Diaeretiella rapae* (HYMENOPTERA: APHIDIIDAE) TO ODOR OF PLANTS, APHIDS, AND PLANT-APHID COMPLEXES

H.C. REED,[1,*] S.H. TAN,[1] K. HAAPANEN,[1] M. KILLMON,[1] D.K. REED,[2] and N.C. ELLIOTT[2]

[1]*Biology Department*
Oral Roberts University
Tulsa, Oklahoma 74171

[2]*USDA/ARS Plant Science Research Laboratory*
Stillwater, Oklahoma 74075

(Received July 11, 1994; accepted December 16, 1994)

Abstract—*Diaeretiella rapae* (M'Intosh) (Hymenoptera: Aphidiidae) is a parasitoid of several aphid species, including the Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), and the cabbage aphid (CA), *Brevicoryne brassicae* (L.). The response of mated *D. rapae* females to odors from wheat, cabbage, and plant-host complexes was investigated using a four-choice olfactometer. Experienced parasitoids, but not inexperienced females, responded positively to odors of the wheat-RWA complex in a no-choice test. In choice tests, experienced parasitoids did not respond to odors of uninfested cabbage and wheat leaves, but did respond positively to aphid-infested plants and to aphids alone. The response of *D. rapae* to the cabbage-CA complex and to CA alone was significantly greater than to the wheat-RWA complex and RWA alone, suggesting an innate odor preference for crucifer-feeding aphids.

Key Words—Kairomone, biological control, cabbage, wheat, *Diuraphis noxia*, *Brevicoryne brassicae*, olfactometer, infochemical, preference, host plants.

INTRODUCTION

Chemical orientation plays an important role in host-habitat location and host location by insect parasitoids (Vinson, 1981; Weseloh, 1981). The olfactory

*To whom correspondence should be addressed.

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cues eliciting the searching process may originate from the host plant, the host, or a combination of plant and host factors (Vet and Dicke, 1992). The utility of olfactory cues depends on their reliability in indicating herbivore presence, identity, accessibility, suitability, and detectability (Steinberg et al., 1993; Vet and Dicke, 1992). Therefore, the information value of odor stimuli from the host and host plant is important to aid the parasitoid in selecting a suitable host.

Olfactometry has been widely used to demonstrate responses of aphid parasitoids to host food plant odors and their aphid hosts (Bouchard and Cloutier, 1985; Powell and Zhang, 1983; Read et al., 1970; Wickremasinghe and van Emden, 1992). In a few studies, flight responses of aphidiids to host plants and hosts have been investigated in wind tunnels (Grasswitz and Paine, 1993; Guerrieri et al., 1993; Sheehan and Shelton, 1989a) or in the field (Sheehan and Shelton, 1989b; Titayavan and Altieri, 1990). Some of these studies have addressed the relative value of volatile infochemicals from the aphid host plant, the aphid host, and the plant-aphid complex (Grasswitz and Paine, 1993; Guerrieri et al., 1993; Wickremasinghe and van Emden, 1992).

Diaeretiella rapae (M'Intosh) (Hymenoptera: Aphidiidae) parasitizes over 30 aphid species on a variety of plants (Mackauer and Stary, 1967), including the cabbage aphid, *Brevicoryne brassicae* (L.), a worldwide pest of cruciferous crops, and cereal aphids such as the Russian wheat aphid (RWA), *Diuraphis noxia* Mordvilko. Various strains of exotic *D. rapae* have been released in the United States for establishment as a natural enemy of the RWA (McKinnon et al., 1992). Some researchers (e.g., Sheehan and Shelton, 1989a) indicate that the cabbage aphid (CA) is the preferred host of this parasitoid, but olfactory or ovipositional preference of this generalist aphid parasitoid has not been directly tested. This study examines the role of experience and odor preferences of mated *D. rapae* females in choice tests to odors from cabbage and wheat, the respective plant-aphid complexes, and the two aphid species alone. An understanding of the olfactory preference of *D. rapae* between the cabbage and wheat systems will aid in our understanding of host-finding behavior, which in turn will be important in the design of programs to manage, manipulate, or select potential parasitoids in biological control.

MATERIALS AND METHODS

The *D. rapae* females used in all tests were a strain (T92008) obtained from China supplied by the USDA/ARS Plant Science Research Laboratory. All parasitoids were reared continuously on the RWA and the TAM-101 or Mit cultivars of winter wheat (*Triticum aestivum* L.). The colony was maintained in a controlled environment chamber at 25°C under a 16L:8D photoperiod. Both the winter wheat and cabbage, *Brassica oleracea* L., were reared in a

greenhouse under a natural light regime. The CA and the RWA were reared on their respective host plants in a cage in a greenhouse under a natural light regime.

The olfactometer used was similar to that designed by Vet et al. (1983) for testing odor responses of small Diptera and Hymenoptera (Bouchard and Cloutier, 1985; De Jong and Kaiser, 1991; Eller et al., 1988; Turlings et al., 1989, 1990). Illumination was provided by a 20-W circular fluorescent lamp (3700 lux) 20 cm above the olfactometer. An airflow meter (Cole-Parmer: P04-N112-02 G) maintained an airflow rate of 300 ml/min through each of the four quadrants. A vacuum pressure pump, placed on the floor, pulled room air through charcoal filters before entering the olfactometer chamber and then was vented into a fume hood. Odor fields were visualized using smokes generated by NH_4OH and HCl. The boundaries of each odor field were marked on the external surface of the olfactometer.

General Experimental Procedures. Parasitized aphids (i.e., mummies) were cut from the wheat plants and isolated in gelatin capsules. Upon emergence, individual parasitoid females were mated with individual males and, in the choice tests, were allowed to oviposit in 7–12 aphids on a plant leaf for 20 min. The parasitoids were then fed honey water and isolated into glass vials prior to testing. All parasitoids were tested within 1 hr of this postemergence treatment.

Test materials were put into vials of two opposite arms, and the two other arms had vials of water to act as humidified air controls. The experimental design of the six choice tests are depicted in Table 1. The odor sources for tests 1 and 2 were two to three small cabbage leaves and 20 tillers of wheat leaves. The odor sources for tests 3 and 4 were two to three cabbage leaves with approximately 250 CA and four to six tillers of wheat with about 250 RWA. Such densities reflect heavy infestations in the field. The cut edge of intact

TABLE 1. EXPERIMENTAL PROTOCOL OF CHOICE TESTS CONDUCTED IN FOUR-ARM OLFACTOMETER

Test	Pretreatment oviposition experience	Odor sources[a]	Number tested
1	RWA and wheat	uninfested wheat and cabbage leaves	60
2	CA and cabbage	uninfested wheat and cabbage leaves	41
3	RWA and wheat	infested wheat and cabbage leaves	85
4	CA and cabbage	infested wheat and cabbage leaves	44
5	RWA and wheat	CA and RWA	30
6	CA and cabbage	CA and RWA	30

[a]Controls were humidified air in all tests.

leaves was submerged in water to minimize the release of volatiles from the cut stem base (Turlings and Tumlinson, 1991). The odor choices for tests 5 and 6 were 200 CA and RWA that had been removed from their host plant 45 min prior to testing.

Experiments were conducted during the mid-morning through mid-afternoon (4–9 hr after lights on) at a room temperature of 22.5°C. Parasitoids to be tested were introduced singly into the olfactometer exposure chamber through the exit port in the floor. Once in the chamber, the parasitoids were given 5 min to make their choice between the four air fields. The amount of time spent in each odor field and the number of final choices were recorded for each parasitoid. A final choice was recorded when a parasitoid entered a treatment arm and remained there for at least 10 sec. Once a final choice was made, the remaining time (up to 5 min) was added to that odor field. A positive response was indicated by a significantly greater percentage of time spent in the test fields over the controls, as indicated by the Kruskal-Wallis test (SAS Institute, 1985).

Prior to testing the odor sources, a system bias check was conducted on the olfactometer system by testing the responses of 60 parasitoids to humidified air in all four quadrants. No differences in mean time spent per quadrant were found, and no final choices were made. Before conducting the choice tests, the role of ovipositional experience on the response of *D. rapae* to the wheat–RWA complex was examined in two tests. The protocol for these tests was identical to that used in the choice tests except for the following differences: (1) experienced females were housed with wheat and RWA for 24 hr and inexperienced females were kept in cages without wheat and RWA; (2) the odor source consisted of four tillers of TAM-101 winter wheat (one tiller was finely chopped) and 100 RWA in each of two quadrants with the remaining two quadrants serving as humidified air (Bouchard and Cloutier, 1985); (3) 20 inexperienced and experienced females (four replicates each) were tested singly for 10 min each in the olfactometer; and (4) once a final choice was made the test was completed, and the remaining time was *not* added to the chosen quadrant. Data from the two identical treatments used in opposite quadrants were combined, resulting in a two-group design. The mean time spent in each quadrant was analyzed using the Wilcoxon test (SAS Institute, 1985).

The olfactometer chamber was washed thoroughly with detergent and warm water and rinsed in 75% alcohol at the end of each day of testing or after a set of replicates (five to seven test females).

RESULTS

Only the experienced females showed a significant response to the wheat–RWA complex in the no-choice 10-min tests (Table 2). Seven of the 20 experienced females and no inexperienced females made a final choice to the wheat–

TABLE 2. RESPONSES OF INEXPERIENCED AND EXPERIENCED *D. rapae* FEMALES TO WHEAT-RWA COMPLEX DURING 10-MIN TESTS

Experimental group (N = 20)	Mean time ± SE in wheat/RWA odor (sec)	Mean time ± SE in humidified air (sec)
Inexperienced	300.3 ± 46.4[a]	273.4 ± 46.9
Experienced	293.2 ± 43.8[b]	156.0 ± 34.0

[a] Mean not significantly different from control by Wilcoxon test (P = 0.870).
[b] Mean significantly different from control by Wilcoxon test (P = 0.025).

RWA odor. In the first choice test, *D. rapae* with ovipositional experience on RWA did not show any significant response to either the uninfested cabbage leaves or wheat leaves (Kruskal-Wallis P = 0.2671) (Figure 1A). The reciprocal test 2, using parasitoids with experience on CA, also did not show a positive response to the two plant species (Kruskal-Wallis, P = 0.09) (Figure 1B). However, in the subsequent tests 3 and 4, experienced parasitoids responded positively to aphid-infested leaves (Figure 2). The RWA-experienced parasitoids demonstrated a significantly greater response to infested cabbage leaves than to infested wheat leaves (Kruskal-Wallis P = 0.0001) (test 3, Figure 2A). An odor preference for infested cabbage leaves was also observed in test 4 using CA-experienced parasitoids (Kruskal-Wallis P = 0.01) (Figure 2B). The type of aphid ovipositional experience also did not affect their response in aphid choice tests 5 and 6, as parasitoids responded preferentially to CA over RWA (Figure 3).

DISCUSSION

Volatiles from host plants are commonly found to be the sources of attractancts for parasitoids (Nordlund et al., 1988; Powell and Zhang, 1983; Vet and Dicke, 1992). Read et al. (1970) suggested that female *D. rapae* locate the habitat of potential host aphids by an olfactory response to volatile chemicals released by aphid host plants. Sheehan and Shelton (1989a) demonstrated that *D. rapae* flew upwind in response to collard odor, but less so to a nonhost plant, the potato. Evidence indicates that *D. rapae* locates aphids on crucifers by responding to mustard oils, especially allylisothiocyanate (Read et al., 1970; Titayavan and Altieri, 1990), although Sheehan and Shelton (1989b) did not obtain significant attraction to ecologically relevant concentrations of this compound in the field.

Wheat volatiles may also be important plant synomones for parasitoids (Buttery et al., 1985). Wickremasinghe and van Emden (1992) found three

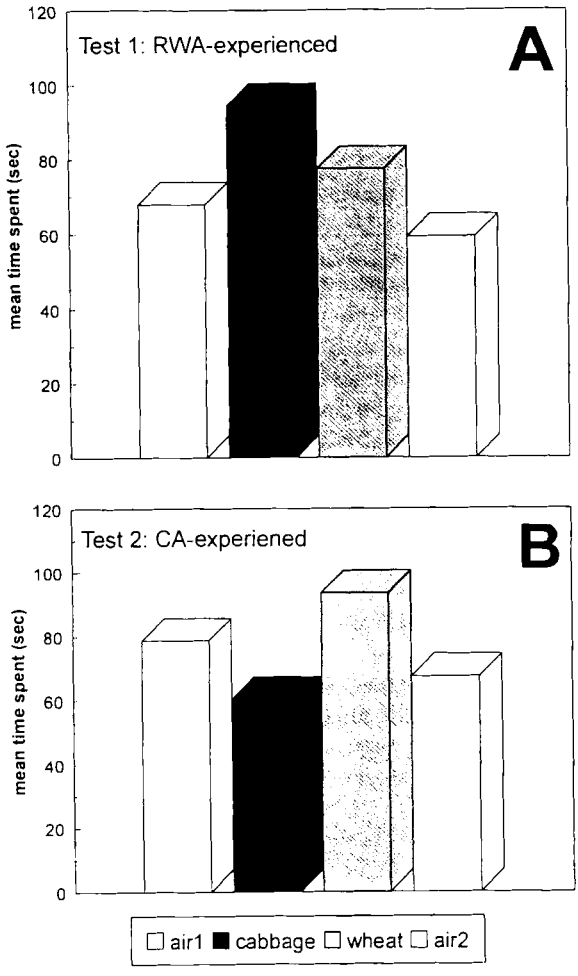

FIG. 1. Mean time spent (seconds) by *D. rapae* females in odor fields of cabbage and wheat leaves and humidified air controls in 5-min tests: (A) test 1 using 60 RWA-experienced females (not significantly different, Kruskal-Wallis, $P = 0.2671$); (B) test 2 using 41 CA-experienced females (not significantly different, Kruskal-Wallis, $P = 0.09$).

volatiles compounds emanating from wheat leaves to be attractive to the cereal aphid parasitoid *Aphidius rhopalosphi*. Females of the generalist aphid parasitoid *Lysiphlebus testaceipes* given postemergence experience with wheat were attracted to uninfested wheat plants in a wind tunnel study (Grasswitz and Paine,

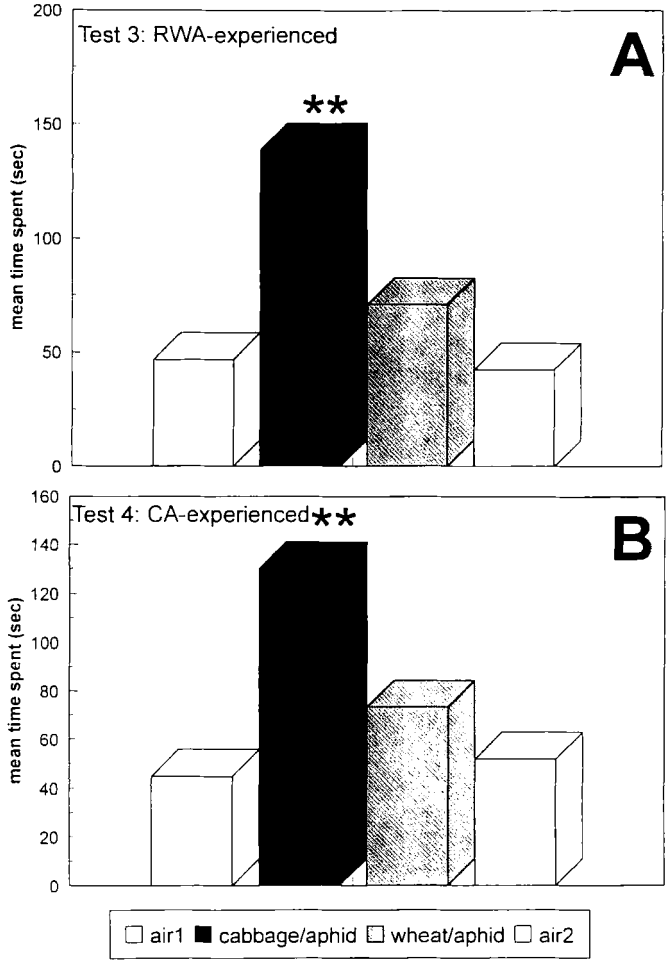

FIG. 2. Mean time spent (seconds) by *D. rapae* females in odor fields of cabbage–CA and wheat–RWA complexes, and humidified air controls in 5-min tests: (A) test 3 using 85 RWA-experienced females (** significantly different, Kruskal-Wallis, $P = 0.0001$); (B) test 4 using 44 CA-experienced females (** significantly different, Kruskal-Wallis, $P = 0.0134$).

1993). Consequently, it is a bit surprising that in our study female *D. rapae* were not significantly attracted to the odor from either uninfested cabbage or Mit winter wheat. However, differing experimental designs may not allow direct comparisons of studies.

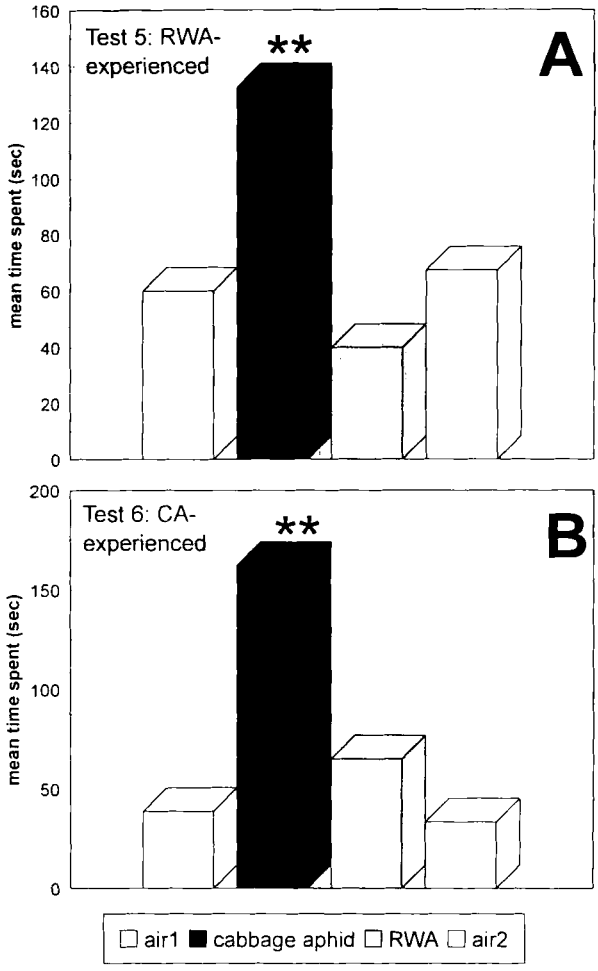

FIG. 3. Mean time spent (seconds) by *D. rapae* females in odor fields of CA and RWA, and humidified air controls in 5-min tests: (A) test 5 using 30 RWA-experienced females (** significantly different, Kruskal-Wallis, $P = 0.0001$); (B) test 6 using 30 CA-experienced females (** significantly different, Kruskal-Wallis, $P = 0.0001$).

The olfactometer used by Read et al. (1970) was a Y-tube design, and tests were limited to one plant odor versus a blank, with no choice tests between two plant materials. Sheehan and Shelton (1989a) also did not offer plant choices to their test insects. Although Wickremasinghe and van Emden (1992) performed

extensive choice tests in their modified Y-tube olfactometer, they introduced 12–40 female parasitoids simultaneously, which may affect their olfactory responses. Perhaps the differential attraction to either plant odor is not strong enough to be discriminated in our olfactometer system. Indeed, the aphid parasitoid *L. testaceipes* responded nearly equally to host plant odor and to host aphid odor in a choice test in a Y-tube olfactometer (Schuster and Starks, 1974).

Another possible reason for the differing results might be that we did not cut the cabbage plants, as did Sheehan and Shelton (1989a), so as to minimize release of plant volatiles. Aphid feeding typically does not cut the tissue. Other studies have shown that aphid parasitoids are not always attracted to plants of their host. Bouchard and Cloutier (1985) found no evidence that female *Aphidius nigripes* responded to host-plant foliage volatiles. Host plants alone did not elicit oriented flight in a related aphid parasitoid, *Aphidius ervi*, in a wind-tunnel study (Guerrieri et al., 1993).

The infested cabbage plants in our study stimulated a very strong response by *D. rapae*, results that are consistent with studies on other parasitoids. More *A. ervi* females exhibited oriented flight to the bean–aphid complex than to the host-damaged plants, aphids alone, or plant alone (Guerrieri et al., 1993). The six species of aphid parasitoids tested by Wickremasinghe and van Emden (1992) each showed an odor preference to the plant–aphid complex over the host plant alone. In a study of *Microplitis croceipes* (Cresson) in a four-choice olfactometer, Eller et al. (1988) found that parasitoids responded in a dose-dependent fashion to volatiles from the plant–host complex and oviposition experience enhanced their response. Furthermore, Steinberg et al. (1993) showed that the plant–host complex was the most attractive odor source for parasitoids in their experiment with *Cotesia glomerata* (L.). Other parasitoids are known to discriminate between volatile chemicals emitted by uninfested plants and plants infested by a particular herbivore species (Vet and Dicke, 1992).

Cabbage aphids alone elicited a response nearly as strong as the cabbage–CA complex. Aphid odor alone has been shown to stimulate olfactory responses (Bouchard and Cloutier 1984, 1985; Powell and Zhang, 1983; Read et al., 1970). The possibility exists that honeydew, a known contact kairomone for aphid parasitoids (Budenberg, 1990; Grasswitz and Paine, 1992; Wickremasinghe and van Emden, 1992), may contain attractive volatile chemicals. However, some experiments have not demonstrated a positive response to aphid odor alone (Guerrieri et al., 1993) or to washed aphids (Wickremasinghe and van Emden, 1992). Read et al. (1970) demonstrated that *D. rapae* females responded to CA freshly removed (15 min prior to test) from collards, but not to CA removed from host plants 24 hr prior to the test, suggesting that the positive response may be due to substances from the plant and not from the aphids. Perhaps our 45-min time was not sufficient to allow the cabbage–CA complex compounds to dissipate. Our results, together with those of Read et al. (1970)

and Sheehan and Shelton (1989a,b), suggest that the source of attractant to *D. rapae* females may originate from the interactions between plants and feeding aphids, i.e., herbivore-induced synomones (Vet and Dicke, 1992).

Although experienced parasitoids did respond positively to the wheat–RWA complex when given no choice of another plant–aphid complex (Table 2), this response greatly decreased in the presence of odor from the cabbage–CA complex. Experienced *D. rapae* preferred infested cabbage over infested Mit winter wheat despite their ovipositional experience. Usually such postemergence exposure tends to enhance parasitoid responses to the experienced host or plant (Vet and Dicke, 1992; Sheehan and Shelton, 1989a, Turlings et al., 1989, 1990). Our data indicate that *D. rapae* has a strong innate odor preference to the CA and the cabbage–CA complex that is not easily overridden by preemergence (i.e., rearing) or postemergence (i.e., oviposition) experience. Perhaps the odor preference for CA is due to the copious amounts of honeydew produced by CA on cabbage in comparison with the relatively minute quantities produced by RWA on wheat. This odor preference may also reflect an innate host preference for CA and other crucifer-feeding aphids, as has been suggested by Sheehan and Shelton (1989a). However, various strains of *D. rapae* may differ in their host preferences. Furthermore, laboratory rearing of the parasitoid on RWA and wheat may decrease the population's sensitivity to cues from the reared host or in some way alter searching efficiency (Geden et al., 1992).

Odor preference is an important factor to consider when attempting to release this generalist aphid parasitoid for control of the RWA and other cereal aphids. Testing of ovipositional preference in laboratory and field experiments would further clarify the host preferences of this generalist aphid parasitoid. Analysis and testing of collected volatiles from plants, aphids, and the plant–aphid complexes are needed to clarify the relative importance of these infochemicals in the host-finding mechanism of *D. rapae*.

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ANTIMICROBIAL ACTIVITY OF DOGWOOD FRUITS (*Cornus drummondii*) FROM WINTER FOOD CACHES OF EASTERN WOODRATS (*Neotoma floridana*)

DIANE M. POST* and JAMES E. URBAN

Division of Biology
Kansas State University
Manhattan, Kansas 66506-4901

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Abstract—Post-harvest loss of resources to microbial competitors affects a number of species of animals, and many of them have evolved behaviors that reduce the likelihood of such loss. We previously described the inhibition of *Bacillus psychrophilus* by dogwood fruits (*Cornus drummondii*), which can comprise a significant portion of the winter food cache of the eastern woodrat (*Neotoma floridana*). The present study is a further investigation of the ability of dogwood fruits to inhibit growth of *B. psychrophilus*. Additionally, we tested the antimicrobial activity of dogwood fruits on *Escherichia coli* and *Staphlococcus aureus*. Results of our study indicate that fresh fruits inhibit the growth of *B. psychrophilus* and *S. aureus* but not *E. coli*. However, fruits taken from woodrat dens after four and six months storage inhibited growth of all three species of bacteria. Further, the strength of inhibition increased over the test period. We suggest the presence of an inhibitor allows woodrats to compete effectively for a valuable and limited food resource.

Key Words—Caching, antimicrobial activity, *Neotoma floridana*, eastern woodrat, *Cornus drummondii*, dogwood fruit, *Escherichia coli*, *Staphlococcus aureus*, *Bacillus psychrophilus*

INTRODUCTION

Many animals, including humans, that larderhoard food, experience post harvest loss of food resources to microbial competitors. Acquisition of a larderhoard requires a significant investment of resources and loss could have an important negative impact on the hoarder. For example, loss of stored fruit and vegetable

*To whom correspondence should be addressed.

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crops has a significant economic impact on human populations, particularly those of less-developed tropical countries (Lund, 1981; 1983). Nonhuman species that larderhoard may also experience loss of resources to microbes (Smith and Reichman, 1984; Vander Wall, 1990), and some have evolved prestorage food-handling behaviors that reduce the likelihood that food stores will be lost to microbes. For example, squirrels (*Tamiasciurus hudsonicus*) (Smith, 1968) and pikas (*Ocotona princeps*) (Connor, 1983) allow foods to dry before they are stored. Clark's nutcracker does not store spoiled food items (Vander Wall and Balda, 1977).

Other species have developed behaviors that act in the poststorage period to reduce loss of stored food to spoilage by microbes. In a laboratory study, kangaroo rats moved seeds between areas with different humidity levels in response to the amount of mold present on the seeds, a behavior that has been suggested to limit loss of resources to microbes (Reichman et al., 1986; but see Frank, 1988).

Our previous investigation of caches from eastern woodrat (*Neotoma floridana*) dens showed most cache foods harbored *Bacillus psychrophilus* and in relatively high amounts as compared to the amount of other bacteria present. However, the bacterium was absent from dogwood (*Cornus drummondii*) fruits (Post and Urban, 1993). The absence of *B. psychrophilus* was intriguing because as a psychrophile, *B. psychrophilus* should have a competitive advantage over other bacteria in woodrat caches. Woodrats begin to store food in early fall and rely on stored resources to supply a portion of the winter diet. Thus, a bacteria that is cold-tolerant should have a competitive advantage over other microbes for access to food stored by eastern woodrats.

In our initial screening of woodrat cache contents for microbes, our storage procedures exposed dogwood fruits to the den environment, but prevented loss of sample material to the resident woodrat (Post and Urban, 1993). However, we were unable to determine whether the absence of *B. psychrophilus* on dogwood fruit was a consequence of the effects of the fruits or a consequence of passive treatment of the fruits by air, liquid, dust, or soil particles within the den. In the present study, we further examined the relationship of cached dogwood fruits and microorganisms and have demonstrated an antimicrobial activity of dogwood fruits on *B. psychrophilus*, *Staphlococcus aureus*, and *Escherichia coli*.

METHODS AND MATERIALS

Samples of dogwood fruits (and attached stem material) were collected on September 8, 1993, and air dried in the laboratory until October 4, 1993. After drying, fruits were packaged in tubes constructed of 11.4 × 3.8-cm PVC pipe

or 8.8 × 5-cm plastic bottles. Pipes and bottles were covered on each end with 2-mm aluminum wire screen. Groups of two tubes and one bottle were then placed in 1.5 × 1.5-cm wire mesh containers, which resulted in six packages of samples. Containers of sample tubes were inserted into woodrat dens (one sample container per den) in the space within the den where the cache material was located. Dens were located at a site approximately 20 km south of Manhattan, Kansas, which consisted of hedgerow habitat surrounded by agricultural land. Sets of sample tubes were removed from each of three woodrat dens on January 10, 1994. The three remaining sets of tubes were removed on March 10, 1994. Fruits retrieved from woodrat dens in January and March were held at 5°C for 24 hr or less prior to being tested for antimicrobial activity. Samples of fresh dogwood fruit and stem material were collected into new plastic food bags (a relatively sterile environment) from trees within a 10-km radius of Manhattan, Kansas on October 4, 1993. Fruits were held at 5°C for 24 hr or less after harvesting and were then tested for antimicrobial activity. Samples that were collected to assess antimicrobial activity, and samples that were collected and stored in dens were gathered after fruits had ripened.

Inhibition assays were modeled after the Bauer-Kirby qualitative antibiotic susceptibility assay (Bauer et al., 1966) routinely used in clinical laboratories. Actual assays were begun by spreading 0.2-ml suspensions of individual bacterial species (which had been adjusted to a density of 5.0×10^7 cells/ml) onto an agar surface in 150 × 15-mm Petri dishes (Falcon 1058, Becton Dickinson Labware, Lincoln Park, New Jersey). The bacteria tested were *Bacillus psychrophilus* [a natural isolate as described in Post and Urban (1993)] and *Staphlococcus aureus* (a gram-positive bacteria; strain S_6 obtained from J. Iandolo, College of Veterinary Medicine, Kansas State University, Manhattan) and *Escherichia coli* (a gram-negative bacteria; American Type Culture collection 25922). *B. psychrophilus* and *E. coli*, were grown on R2A agar and *S. aureus* was grown in TSA agar. R2A agar (Difco, Detroit, Michigan) is described in Post and Urban (1993) and TSA agar consists of 15 g Tripticase, (Difco, Detroit, Michigan) 5 g Phytone, (Difco, Detroit, Michigan), and 5 g NaCl per liter (pH 7.3). Cultures on R2A agar were incubated at 23°C and *S. aureus* was incubated at 30°C.

While incubated plates dried and equilibrated, fruits and receptacles obtained from stem material were prepared for assay. A preliminary study showed that inhibitory activity of dogwood fruit occurred in the area of the receptacle (the site on the stem where the fruit is attached). Therefore, fruits were placed on inoculated plates such that the part of the fruit previously attached to the receptacle was in direct contact with the agar surface of the inoculated medium. The receptacle was severed from the stem and placed in contact with the agar surface. Forceps and scissors that had been rinsed with 95% ethanol and flamed were used to separate and manipulate the fruit and receptacle.

To serve as both standard and control, antibiotic sensitivity disks were placed on prepared and inoculated dishes identical to those used for the inhibition study. Antibiotic sensitivity disks containing ampicillin (10 μg/7-mm-diameter paper disk), chloramphenicol (30 μg/7-mm-diameter paper disk), novobiocin (30 μg/7-mm-diameter paper disk), streptomycin (10 μg/7-mm-diameter paper disk), and tetracycline (30 μg/7-mm-diameter paper disk) were used.

Plates containing fruit bodies, stem nodes, or antibiotic disks were incubated for 48 hr, after which they were examined for zones of growth inhibition. Use of zones of growth inhibition is a typical method used to assess antibiotic activity (Bauer et al., 1966; Ziaka, 1988). When inhibition was observed, the diameter of the zone was measured with a clear plastic ruler with metric markings. The ruler was always held such that the diameter measure passed through the center of the disk, node, or fruit body. Data were analyzed by use of the General Linear Model procedure on SAS (SAS Institute, Cary, North Carolina) computer software. The analysis tested the null hypothesis of no difference in the diameter of the zone of inhibition between testing periods.

RESULTS

Results of our experiments showed no inhibition of bacterial growth by dogwood fruit in any period for any of the bacteria tested. Receptacles from fresh dogwood stem material showed inhibition of *B. psychrophilus* and *S. aureus*. However, no inhibition of *E. coli* by fresh material (Figure 1) was found. Receptacles from dogwood stems taken from woodrat dens in January and March 1994 showed inhibition of growth of all three bacteria.

For *Bacillus psychrophilus* inhibition of growth increased over the test period ($F = 4.03$, $df = 3$, $P = 0.01$). The strength of the inhibition was greater in January than October ($P = 0.001$) and greater in March than October ($P = 0.001$). There was no difference in inhibition noted between January and March ($P = 0.30$). An increase in inhibitory activity was noted for *Staphylococcus aureus* between all three test periods ($F = 73.87$, $df = 3$, $P = 0.0001$; October < January, $P = 0.0001$; October < March, $P = 0.0001$; January < March, $P = 0.0001$). As indicated above, no inhibition of *Escherichia coli* was observed by fresh material tested in October. However, inhibition of growth by *E. coli* was seen in January and March, and there was no difference in the amount of inhibition between these two periods ($F = 5.44$; $df = 3$, $P = 0.002$; October < January, $P = 0.0006$; October < March, $P = 0.002$; January = March, $P = 0.31$).

The efficacy and comparability of individual fruit and receptacle assays were confirmed by the control antibiotic sensitivity tests run with each assay. Each of the control antibiotic sensitivity tests showed levels of inhibition within

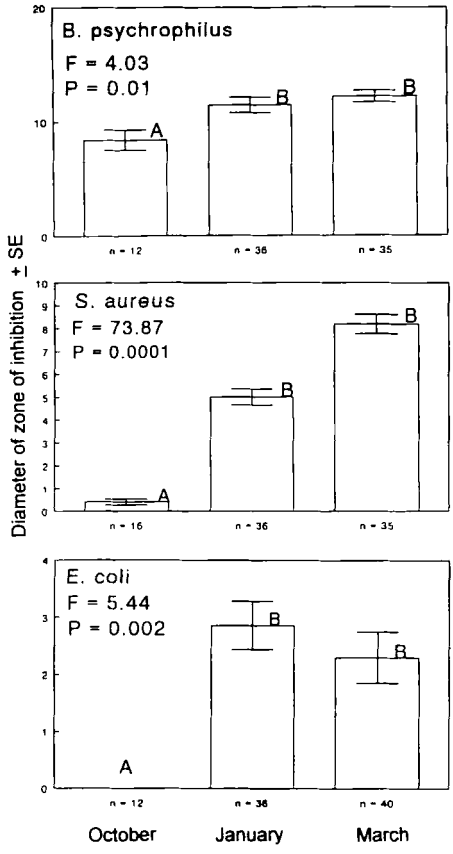

FIG. 1. Inhibition of *Bacillus psychrophilus*, *Staphylococcus aureus*, and *Escherichia coli* by receptacles of stems connected to fresh dogwood fruits (*Cornus drummondii*) and fruits stored in woodrat dens for four and six months. Data shown are means ±SE. Bars with different letters indicate significant differences in the strength of inhibition between test periods. Note the difference in scale on the *y* axis of the three graphs.

the expected range for each of the tested bacteria–antibiotic combinations (Table 1).

DISCUSSION

Results of our experiments suggest the substance responsible for the antimicrobial properties of dogwood fruit is produced within the area of the recep-

TABLE 1. RESULTS OF ANTIBIOTIC/BACTERIA SENSITIVITY ASSAYS[a]

Bacteria and date	Ampicillin	Chloramphenicol	Novobiocin	Streptomycin	Tetracycline
E. coli					
October	21 ± 1.4	26.5 ± 2.1	0	12 ± 0	27.5 ± 0.7
January	24 ± 2.8	23 ± 1.4	0	10.5 ± 0.7	17 ± 0
March	24 ± 0	22 ± 0	0	13 ± 0	19 ± 0
B. psychrophilus					
October	54.6 ± 5	36.6 ± 4.9	45 ± 4.4	20.6 ± 1.2	31.3 ± 1.5
January	45 ± 4.2	31 ± 1.4	40.5 ± 2	18.5 ± 0.7	26 ± 2.8
March	48 ± 0	33 ± 1.4	52 ± 2.1	20 ± 2.8	34 ± 0
S. aureus					
October	37.6 ± 0.7	27 ± 1.7	26.6 ± 1.5	22.3 ± 0.6	27 ± 1
January	33 ± 1.4	20 ± 2.8	23 ± 1.4	15 ± 5.6	25 ± 3.5
March	27 ± 0.7	17.5 ± 0.7	28 ± 1.4	13 ± 0	24 ± 0

[a] Numbers indicate the diameter of the zone of inhibition (mm) ± SD. Data with an SD of zero are the result of three equal measurements. The strength of inhibition is within the expected range for each antibiotic/bacteria combination.

tacle. Furthermore, the strength of the inhibitor appears to increase over time. There are several possible explanations for the observed increase in the strength of the inhibitor. Plant material is biologically active and may undergo a variety of biochemical changes in the postharvest period. Thus, the observed increase in inhibitor strength may be the result of an increased production of the inhibitor or the breakdown of some inhibition repressor. Alternatively, desiccation of stored material may play a part in the observed increase of the strength of the inhibitor. Dogwood fruits loose water rapidly after harvest and continue to decline in water content during storage (Post, 1992). Thus, the relative amount of the inhibitor may remain constant, but its specific activity may increase with a concomitant reduction in the water content of the receptacle.

Regardless of the mechanics by which dogwood fruits inhibit microbial growth, the presence of the inhibitor effectively allows woodrats to store a large supply of a valuable resource. Dogwood fruits are an energy-dense and lipid-rich resource used by a number of species, notably fall migrating birds and, once ripe, the supply of fruits declines rapidly. Woodrats have been observed to store large amounts of dogwood fruit and include it in their diet from fall until early spring (Post, 1991). If dogwood fruits were not resistant to microbially mediated spoilage, woodrats would not be expected to store large amounts of the fruit because to do so would result in a loss of investment in the cache. Therefore, we suggest that the presence of an inhibitor allows woodrats to compete effectively for a valuable and limited winter food resource.

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A CRITICAL BODY SIZE FOR USE OF PHEROMONES IN MATE LOCATION

DAVID B. DUSENBERY* and TERRY W. SNELL

School of Biology
Georgia Institute of Technology
Atlanta, Georgia 30332-0230

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Abstract—Pheromones have demonstrated importance in mate location in many insect species. Because chemoreception is the most universal sense, it has been assumed that pheromones also are important in aquatic organisms, including bacteria, but few have been found. The physical limits on effective strategies for organisms to come into contact for mating were modeled with assumptions appropriate for organisms less than a millimeter in size in an open aquatic environment. One sex was assumed to be motile, while the other sex was passive or devoted energy to locomotion or to diffusible pheromone production. Assuming spherical organisms, random locomotion by the second sex at the same velocity as the first sex increases the chances of contact by a factor of 4/3 over being passive; this ratio is independent of size. For detection by contact, the effectiveness of searching increases with the third power of the radius of the organisms; for detection by pheromones, search effectiveness increases with the seventh power of the radius above a critical size. Diverting energy from motility to pheromone production is not productive for organisms smaller than the critical size, which corresponds to a radius of 1.8 times the square root of the diffusion coefficient of the pheromone times the threshold concentration for detection divided by the rate of pheromone production per unit volume of organism. Thus, pheromone production is very favorable for organisms much above the critical size, which appears to be between 0.2 and 5 mm in water. On the other hand, bacteria are probably too small to use diffusable pheromones for mate location; most protozoans and rotifers may also be too small.

Key Words—Attraction, bacteria, chemoreception, crustacean, efficiency, mate location, pheromone, plankton, protozoan, rotifer, sex, size.

*To whom correspondence should be addressed.

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INTRODUCTION

A common problem for sexually reproducing species is for the two sexes to come together. In species with motile adults, the first step is for one or both adults to direct their locomotion so that two adults of opposite sex come together. The ability of a species to accomplish this affects the minimum population density required for its survival and has implications for its life history strategies (Gerritsen, 1980). Among vertebrates, a variety of sensory systems are employed. In simpler animals, including many crustaceans, released chemical pheromones are believed to be important (Dunham, 1988), and researchers often assume microorganisms and plankton likewise use released pheromones (Kochert, 1978; Crease and Hebert, 1983; Stephens, 1986). However, observations on rotifer zooplankters suggest that mates are not recognized at a distance; rather they rely on random locomotion and contact chemoreception (Gilbert, 1963; Aloia and Moretti, 1973; Snell and Hawkinson, 1983; Snell and Nacionales, 1990). This raises the question of whether diffusible pheromones are generally useful to small free-swimming organisms.

In an open aquatic environment, the utility of diffusible pheromones in mate location can be addressed rigorously for rotifers and other small organisms that are less than a millimeter in size, because the spread of a pheromone in this size range is dominated by diffusion, and turbulence has little effect (Atema et al., 1988). In addition, organisms in this size range swim at Reynolds numbers less than 1 and the minimum energetic cost of locomotion can be reliably estimated from Stoke's law (Rouse, 1961, p. 215; Vogel, 1981, p. 245).

We assume that one sex is always motile, recognition occurs on contact, and contact is efficiently achieved once a pheromone is detected. We then ask what the relative chances of contact are if the other sex: (1) does nothing, (2) moves in random directions, or (3) produces a diffusible pheromone that attracts the other sex; and whether the relative effectiveness of each behavior varies with the size of the organism.

METHODS AND MATERIALS

For simplicity, we assume that both sexes can be represented as spheres of the same size (radius = r_0).

Power. We assume that both sexes have the same amount of metabolic energy available for mate location. Furthermore, the available energy is proportional to the volume of the organism, and the power (rate of energy use) per unit volume of organism (power density) available for mate location is a constant P_V. This is the power available at the metabolic level. To determine the power effectively applied to propulsion, the metabolic power must be multiplied by the

locomotor efficiency, E. To determine the power effectively applied to pheromone release, the metabolic power must be multiplied by a conversion factor, K.

The magnitude of the metabolic power potentially available for propulsion or synthesis of pheromone can be estimated from the rate of oxygen consumption. Consulting Altman and Dittmer (1974, pp. 1613–1659), one finds values for oxygen consumption (milliliter if O_2 per gram wet weight of tissue per hour) of 0.01–0.1 for algae, reptiles, and amphibians; 0.1–1 for fungi and most animals; and 1–10 for bacteria and birds. Measurements for a rotifer gave 1.0 and 1.5 for females and males, respectively (Epp and Lewis, 1979), and we assume a value of 1. These numbers can be multiplied by 5.6×10^4 to convert to ergs per cubic centimeter per second. This is the total metabolic power density available to the organism for all functions including inefficiencies. If 10% of the energy budget is devoted to mate location, $P_V = 6 \times 10^3$ erg/cm^3/sec.

Propulsion. The organisms are assumed to be sufficiently small and slow-moving that Stoke's law describes the power required for propulsion; this means a Reynolds number less than 1. Stokes' law says that the force necessary to propel a sphere through a viscous fluid is equal to $6\pi\eta r_0 v$, where η is the viscosity of the medium, r_0 is the radius of the sphere (organism), and v is the velocity (of locomotion). Since work is force times distance or force times velocity and time, and power is work per unit time, the power required for propulsion is $6\pi\eta r_0 v^2$. If the power devoted to propulsion per unit volume of the organism is P_V, then

$$\tfrac{4}{3}\,\pi r_0^3 P_V E = 6\,\pi\eta r_0 v^2$$

Solving for v, gives

$$v = \frac{\sqrt{2}}{3}\sqrt{\frac{EP_V}{\eta}}\,r_0 \tag{1}$$

The first fraction is a pure number ($\cong 0.47$), and the second has units of inverse time. This relation illustrates why one sees a proportionality between velocity and size across a wide range of organisms.

Reasonable values for the overall efficiency of propulsion are based on the following considerations. The mechanical efficiency of propulsion of bacteria is thought to be not more than 1% (Purcell, 1977; Berg, 1983) and the efficiency of conversion of electrochemical energy of the proton gradient to torque in bacteria is estimated as 5% (Meister et al., 1987). The overall efficiency is the product of these or $E \leq 0.05\%$ for bacteria. Measurements indicate that a rotifer must be at least $10^{-4}\%$ efficient (Epp and Lewis, 1979), and a copepod is at least 0.1% efficient (Vlymen, 1970). We assume an efficiency of 0.1% for Figure 1. Others have assumed an efficiency of 1% (Fenchel and Finlay, 1983;

Crawford, 1992). Note below (equation 8) that the efficiency of propulsion does not enter into determining the critical size.

Search. We assume a clean-sweep search (Dusenbery, 1992, p. 386) with the searching organisms moving in straight paths. Search rate (S) is the rate at which a volume is swept for detection of a target. It is equal to the velocity of swimming (v) times the cross section for detection. The latter is equal to the square of the range of detection (r_d) times pi. (All distances are measured from the center of the spherical organisms.) In symbols:

$$S = \pi r_d^2 v \tag{2}$$

Pheromones. The energy required for synthesis and release of a pheromone is difficult to estimate. Taking data from the heat of combustion of organic molecules, 6- to 18-carbon molecules have heats of combustion ranging from about 1000 to 2000 kcal/mol (Weast, 1985, pp. D272–D278). This equals the minimum energy necessary to synthesize these compounds from carbon dioxide and water. However, animals would make them from other molecules, which might reduce their costs. On the other hand, there will be inefficiencies in synthesis that will increase the energetic costs. We assume the efficiency of conversion, K, is 10^{-3} mol/kcal or about 10^{-14} mol/erg. Thus, the best estimate of pheromone release is 10^{-14} mol/erg times 6000 erg/cm^3/sec $= 6 \times 10^{-11}$ mol/cm^3/sec.

The diffusion coefficient, D, varies relatively little around 1×10^{-5} cm^2/sec for small molecules in water [For example, glycine is 1.06×10^{-5} and sucrose is 0.52×10^{-5} (Weast, 1970, p. F-47) and even small globular proteins are only 0.1×10^{-5} (Tanford, 1961, p. 358).] We assume a value of 1×10^{-5} cm^2/sec.

Threshold concentration, C_{Th}, can vary over a wide range. Theoretical analysis suggests that the lowest practical thresholds for small organisms are about 10^{-12} molar (Dusenbery, 1992, p. 129) or 10^{-15} mol/cm^3. Actually measured thresholds among microorganisms are generally in the range 10^{-9} to 10^{-12} molar (Maier and Müller, 1986; Dusenbery, 1992, p. 137). This range also applies to many fish responses, although there are a few examples of greater sensitivity (Hara, 1993). However, during the initial evolution of pheromone use, detection sensitivities would not generally be optimized, and the pheromone may have to function with less sensitive receptors. The lowest thresholds for chemotaxis of bacteria are 10^{-7} molar (Adler, 1975), and we consider this a more appropriate value.

RESULTS

Detection by Contact. If no pheromones are involved, mates can detect one another only upon contact. In this case, the range of detection is equal to the

sum of the radii of the two organisms ($r_d = 2\ r_0$). Consequently, when one organism is stationary and one moves at velocity v, we can use equation 2, and

$$S = 4\pi r_0^2 v \tag{3}$$

If both organisms move at velocity v, the search rate is increased by a factor of 4/3 (Kauzmann, 1966; pp. 174–175; Gerritsen and Strickler, 1977). Substituting in equation 1 for v,

$$S = 4\pi r_0^2 \frac{\sqrt{2}}{3} \sqrt{\frac{EP_V}{\eta}}\, r_0$$

which we rearrange to get

$$S = \frac{4\pi\sqrt{2}}{3} \sqrt{\frac{EP_V}{\eta}}\, r_0^3 \tag{4}$$

This indicates that the search rate increases with the cube of the size of the organism and in direct proportion to its volume, assuming a constant amount of energy per unit volume is devoted to propulsion.

Detection by Pheromone. In order to improve the chances of a successful search, the target organism can release a chemical, which may extend the range of detection. We assume that the organism devotes a certain amount of metabolic energy per unit volume to producing the chemical and that there is a conversion factor of K relating the amount of energy to the amount of chemical released. We assume that the chemical is produced and released at a constant rate, so energy is used at a constant rate, and the power consumed is the constant P_V. The total rate of chemical release (J) is then K times P_V times the volume of the organism:

$$J = KP_V \frac{4\pi}{3} r_0^3$$

On a scale of less than a millimeter, transport of chemicals is dominated by diffusion, and we can generally ignore convection. In this circumstance, the steady-state concentration (C) of chemical around a small source releasing a chemical at a constant rate (J) is described by (Dusenbery, 1992, p. 66):

$$C(r) = \frac{J}{4\pi Dr}$$

where D is the diffusion coefficient of the chemical in water, and r is the distance from the source. If detection occurs at a concentration C_{Th}, the range of detection will be

$$r_d = \frac{J}{4\pi DC_{\mathrm{Th}}}$$

Substituting for the rate of chemical production,

$$r_d = \frac{1}{4\pi DC_{\mathrm{Th}}} KP_V \frac{4\pi}{3} r_0^3$$

which we rearrange to

$$r_d = \tfrac{1}{3} \frac{KP_V}{DC_{\mathrm{Th}}} r_0^3 \tag{5}$$

The range of detection now increases as the cube of the size of the organism rather than being equal to $2r_0$ as for contact detection. If the chemical is to be at all effective, it must be present at a concentration above threshold outside the organism. This requires that $r_d > 2r_0$ or, from equation 5,

$$\frac{1}{3} \frac{KP_V}{DC_{\mathrm{Th}}} r_0^3 > 2r_0$$

which is rearranged to

$$r_0 > r_0(\min) = \sqrt{\frac{6DC_{\mathrm{Th}}}{KP_V}} \cong 2.45 \sqrt{\frac{DC_{\mathrm{Th}}}{KP_V}} \tag{6}$$

Below this minimum size, pheromone diffuses away too fast relative to its rate of production for a detectable concentration to build up around the organism.

Substituting for r_d (equation 5) and v (equation 1) into equation 2,

$$S = \pi \left(\frac{1}{3} \frac{KP_V}{DC_{\mathrm{Th}}} r_0^3 \right)^2 \frac{\sqrt{2}}{3} \sqrt{\frac{EP_V}{\eta}}\, r_0$$

Rearranging gives

$$S = \frac{\pi\sqrt{2}}{27} \sqrt{\frac{EP_V}{\eta}} \left(\frac{KP_V}{DC_{\mathrm{Th}}} \right)^2 r_0^7 \tag{7}$$

The first term is a pure number ($\cong 0.165$), the second has units of inverse time, and the third term has units of length to the inverse fourth power. Note that the search rate varies with an exponent of 2.5 on the metabolic power density devoted to mate location. However, the most important feature is that the search rate depends on the seventh power of the size of the organism, indicating that diffusing pheromones are much more useful to large organisms.

Figure 1 shows a plot of the search rates for the three types of alternative behaviors of the target organism (passive, random locomotion, and pheromone production), assuming reasonable values for the parameters, as discussed in Methods and Materials. The important thing to note about the plot is that there is a critical size of organism below which pheromone production is useless. In

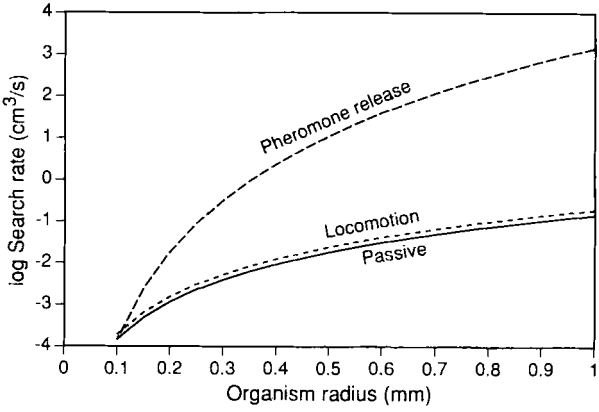

FIG. 1. Search rate dependence on size and target behavior. The target organism can remain passive (equation 3) or devote energy to random locomotion (equation 3 times 4/3) or to pheromone release (equation 7). Calculations assumed spherical organisms of the indicated radius; power applied to mate location, $P_V = 6000$ erg/sec/cm^3 of organism; conversion efficiency for pheromone release, $K = 1 \times 10^{-14}$ mol/erg; threshold sensitivity to pheromone, $C_{Th} = 1 \times 10^{-7}$ molar; efficiency of propulsion of 0.1%, $E = 0.001$, which gives a swimming speed of about 10 diameters per second. The justification for these assumptions is given in Methods and Materials. With these assumptions, the Reynolds number is 0.1 at the critical size and increases to 23 at 1 mm diameter; consequently, at the larger sizes, there is more drag than we have calculated and the advantages of pheromone release are underestimated. The critical size in this case is defined by $r_0(\text{min}) = 100$ μm and $r_0(\text{equiv}) = 108$ μm, and the critical diameter is 215 μm.

contrast, pheromone production by larger organisms can increase the search rate thousands of times above what could be obtained by devoting the energy to locomotion.

Size Limit. What is the critical size where pheromone production becomes useful, and what parameters influence it? In order to answer these questions, we determined the size at which the equal search rates are obtained when one organism (the target) devotes its energy to propulsion (equation 4 times 4/3) or alternatively to pheromone production (equation 7):

$$\frac{4}{3}\frac{4\pi\sqrt{2}}{3}\sqrt{\frac{EP_V}{\eta}}\,r_0^3 = S = \frac{\pi\sqrt{2}}{27}\left(\frac{KP_V}{DC_{Th}}\right)^2\sqrt{\frac{EP_V}{\eta}}\,r_0^7$$

Solving for r_0, we get

$$r_0(\text{equiv.}) = \sqrt[4]{48}\sqrt{\frac{DC_{Th}}{KP_V}} \cong 2.63\sqrt{\frac{DC_{Th}}{KP_V}} \qquad (8)$$

TABLE 1. BREAK-EVEN RADIUS IN MICROMETERS

Pheromone release, KP (mol/cm^3 sec)	Threshold concentration, C_{Th} (mol/liter)					
	1×10^{-12}	1×10^{-11}	1×10^{-10}	1×10^{-9}	1×10^{-8}	1×10^{-7}
1×10^{-10}	0.3	0.8	3	8	30	80
1×10^{-11}	0.8	3	8	30	80	300
1×10^{-12}	3	8	30	80	300	800
1×10^{-13}	8	30	80	300	800	3000
1×10^{-14}	30	80	300	800	3000	8000

It can be seen that the break-even size for pheromone production varies with the square root of the ratio of threshold sensitivity over rate of production (KP_V). In fact, the break-even size (equation 8) is a constant factor (fourth root of $4/3 \cong 1.075$) times the minimum size for pheromone production to be at all effective (equation 6). Thus once the minimum size is reached, only a trivial increase in size makes it more efficient for one sex to devote energy to pheromone release rather than locomotion.

What is the magnitude of this size limit above which pheromone production is effective? We inserted some specific estimates for the parameters into the two equations and made quantitative estimates. Using a range of plausible values discussed in Methods and Materials, the results shown in Table 1 are obtained. With this wide range of possible values, the threshold size could lie anywhere between 1 μm and a few millimeters. This wide range of possible values is a consequence of the lack of quantitative data on chemical communication.

DISCUSSION

Relatively few observations have been made on the mechanisms of mate location among aquatic organisms (Carr, 1988). The data we could find is presented in Table 2. All crustaceans larger than 5 mm seem to employ pheromones, while cladocerans, rotifers, and bacteria do not. In well-studied rotifers, males contact females entirely by random locomotion (Gilbert, 1963; Snell and Garman, 1986). Among ciliates, there is strong evidence for such a pheromone in one genus that is 200 μm in size, but not in many other ciliates examined. In short, there is a strong correlation between the use of mate location pheromones and size, as predicted by the model. The critical size appears to be between 0.2 and 5 mm.

In air, the same basic argument applies, except that the energy of locomotion is more difficult to estimate. It might be thought that the 10^4 increase in

TABLE 2. OBSERVATIONS OF DIFFUSIBLE PHEROMONES FOR MATE LOCATION

Organisms	Adult size (mm)	Mate location pheromones?	References
Bacteria	0.001–0.01	No	Stephens (1986), Dworkin (1992)
Flagellates			
Chlamydomonas	0.008	No	Raper (1970), Goldstein (1992)
Ciliates			
Blepharisma	0.2	Yes	Honda and Miyake (1975), Miyake (1981)
Other ciliates	0.01–3	No	Miyake (1981)[a]
Rotifers (3 species)	0.2–1.2	No	[b]
Crustaceans			
Cladocerans (1 species)	2–4	No	Crease and Hebert (1983)
Copepods (9 genera)	1–5	Yes	[c]
Amphipods (2 species)	5–20	Yes	Borowsky et al. (1987), Stanhope et al. (1992)
Crayfish (1 species)	40–60	Yes	Ameyaw-Akumfi and Hazlett (1975)
Crabs (2 species)	100	Yes	Ryan (1966), Gleeson et al. (1984)
Lobster (1 species)	100	Yes	McLeese (1970), Atema and Engstrom (1971)

[a]Although there is evidence for pheromones in ciliates other than *Blepharisma* (Luporini and Miceli, 1986), there is no evidence that they are involved in attracting one mating type to another.
[b]Rotifers: Gilbert (1963), Aloia and Moretti (1973), Snell and Hawkinson (1983).
[c]Copepods: Jacobs (1961), Haq (1972), Katona (1973), Griffiths and Frost (1976), Blades (1977); Blades and Youngbluth (1979, 1980); Jacoby and Youngbluth (1983), Watras (1983), Burton (1985), Chow-Fraser and Maly (1988), Uchima and Murano (1988), Lazzaretto et al. (1990).

diffusion rate in air would increase the critical size by 100-fold, but there is reason to expect that the increased diffusion rate will decrease the potential threshold concentration proportionally (Dusenbery, 1992, pp. 128–129). Consequently, the critical size may be similar in air.

There is evidence that gametes of water molds (Allomyces) (Pommerville, 1981) and brown algae (Boland et al., 1984), which are only about 10 μm long, make use of diffusible pheromones. This is at the low end of our estimates of the critical size. However, gametes are a special case because they can utilize energy provided by a much larger parent. Consequently, it may be expected that the critical size for gametes may be smaller than for independent organisms.

Although ameboid cells of the cellular slime mold *Dictyostelium discoideum* are only about 10 μm in diameter (Raper, 1935; Soll, 1988), they employ a pheromone (cyclic AMP) in a complicated pulsed system of communication to facilitate aggregation of the cells for the formation of fruiting stalks (Devreotes, 1989). Two factors may contribute to making it feasible for these small cells to employ a diffusible pheromone. Most obviously, many cells work in concert to build up the concentration of pheromone, less obviously, these organisms normally live in the two-dimensional world of a thin film of water. Consequently,

the pheromone is not diluted nearly as rapidly as in the three-dimensional environment we have considered.

The realization that the benefit of pheromones varies with size offers a potential explanation for why the two gametes of most species are very different in size. Having a large immobile gamete (egg), which can efficiently release an attractant pheromone, and many small mobile gametes (sperm) improves the probability of appropriate gametes coming together compared to the situation of equal-size gametes.

This model makes very specific predictions relating the amount of pheromone release, detection threshold, and range of detection. Studies of chemical communication among small organisms is particularly attractive, because the spread of the signal is by diffusion, which can be analyzed rigorously. This situation contrasts strongly with that of insects, where air turbulence makes the spread of the signal very difficult to analyze. We hope this analysis will stimulate more quantitative studies of chemical communication among small organisms.

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METABOLISM OF 1,8-CINEOLE IN TEA TREE (*Melaleuca alternifolia* and *M. linariifolia*) BY PYRGO BEETLE (*Paropsisterna tigrina*)

IAN A. SOUTHWELL,[1,*] CRAIG D.A. MADDOX,[1] and MYRON P. ZALUCKI[2]

[1]*Wollongbar Agricultural Institute*
Wollongbar, NSW, 2477, Australia
[2]*Department of Entomology*
University of Queensland
St. Lucia, Qld, 4072, Australia

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Abstract—The frass of the pyrgo beetle (*Paropsisterna tigrina*) feeding on commercial plantations of the terpinen-4-ol chemical variety of the Australian tea tree, *Melaleuca alternifolia*, was found to contain a volatile oil almost identical to the essential oil of the ingested leaf. When beetles were fed leaf containing substantial quantities of 1,8-cineole, the predominant frass metabolite as determined by MS, IR, ^{13}C and ^{1}H NMR, GC, and CoGC was (+)-2β-hydroxycineole. Both male and female adults and larvae metabolized *Melaleuca* oils in similar ways.

Key Words—Coleoptera, Chrysomelidae, *Paropsisterna tigrina*, pyrgo beetle, *Melaleuca alternifolia*, *M. linariifolia*, Myrtaceae, volatile oil, metabolism, 1,8-cineole, hydroxycineole, (+)-2β-hydroxycineole.

INTRODUCTION

Extensive plantings of *Melaleuca alternifolia* (Maiden and Betche) Cheel (Myrtaceae) over the last 10 years have been responsible for the production of Australian tea tree oil increasing beyond one hundred tonnes per annum (Southwell, 1988; Brophy et al., 1989; Merry, 1991). To distinguish the commercial product from other less efficacious *Melaleuca* oils, this oil is designated as Oil of *Melaleuca*, Terpinen-4-ol type (Standards Association of Australia, 1985; Interna-

*To whom correspondence should be addressed.

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tional Standards Organisation, 1992) derived from *M. alternifolia*, *M. linariifolia*, *M. dissitiflora*, and any other *Melaleuca* species giving similar (+)-terpinen-4-ol (**1**) -rich oils. The abundant intermediate and high 1,8-cineole (**2**) chemical varieties are considered unsuitable sources of oils for antimicrobial application (Brophy et al., 1989).

Paropsisterna tigrina Chapuis (Coleoptera: Chrysomelidae), commonly known as the pyrgo beetle (Colton and Murtagh, 1990), is a major defoliator of plantations of *M. alternifolia* in the Northern Rivers district of New South Wales. Both larvae and adults show a strong feeding preference for the young flush growth (Maddox, 1994), which contains substantial quantities of terpenoids (Brophy et al., 1989; Southwell and Stiff, 1989, 1990; Southwell et al., 1992).

The use of dietary plant volatiles by insects both with and without structural modification as pheromones is well known (Harborne, 1988), especially in the case of *Dendroctonus* and *Ips* pine bark beetles (Wood, 1982; Byers et al., 1979, 1984; Birgersson et al., 1984). Dietary monoterpenes also play a role in the stimulation of the mixed function oxidase (MFO) activity in the mid-gut of some insects, and the ingestion of monoterpenes can enhance the tolerance of the insect to host plant allelochemicals (Brattsen et al., 1977; Yu et al., 1979; Yu 1987).

This investigation identifies the volatile frass constituents of adult and larval male and female *P. tigrina* beetles feeding on terpinen-4-ol (**1**), cineole (**2**), and terpinolene (**3**) chemical varieties of *M. alternifolia* and *M. linariifolia* (Figure 1). The frass volatiles were compared with the leaf volatiles ingested and the structure of the major metabolite of 1,8-cineole determined as the first stage of our investigations into the chemical ecology of *Paropsisterna–Melaleuca* relationships.

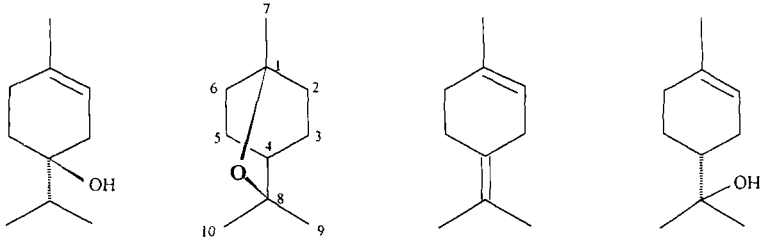


FIG. 1. Chemical structures of compounds in leaves fed to *P. tigrina* beetles. (**1**) terpinen-4-ol, (**2**) 1,8-cineole, (**3**) terpinolene, (**4**) (+)-α-terpineol.

METHODS AND MATERIALS

Melaleuca Food Source. Trees for metabolite screening were 2 to 3-year-old garden specimens of known oil type growing in the Lismore district, New South Wales, Australia. The *M. alternifolia* specimens of commercial oil type (MA1, MA2) were obtained from Australian Plantations, Wyrallah, and cultivated at the Wollongbar Agricultural Institute (28°49'S, 153°24'E). The low cineole *M. linariifolia* (ML1) specimen came from Bryants Nursery, Corndale; the intermediate cineole *M. alternifolia* (MA3) from South Lismore; and the intermediate cineole *M. linariifolia* (ML2) from Wollongbar. The high terpinolene (MA4) and high cineole (ML3) samples came from plants previously identified during surveys and feeding studies growing at South Lismore and Richmond Hill, respectively (Maddox, 1994). Freshly growing tips were fed to the beetles and typical samples retained for GC analysis.

Beetles and Frass Collection. Adult and third– fourth-instar *P. tigrina* larvae were collected from the Wollongbar Agricultural Institute commercial grade (terpinen-4-ol type) plantings. Experimental groupings consisted of mixed sex adults, male adults, female adults, or larvae. Adults were sexed using fore/mid-tarsal structure and body size/shape (Reid and Ohmart, 1989; Maddox, 1994). Each group was kept separate in 70-ml sterile plastic containers in groups of 7–10 and maintained at room temperature with normal daylight. Each group was prefed for two days on new foliage before being rehoused, and frass collected at 8 hr intervals using stainless steel probes to lift the pellet from the leaf or container surface into the receptacle. Frass was stored at −10°C until extraction. Fresh leaf was provided daily. Flush (leaves 1–3 from tip) and mature leaves (> 15 from tip) were retained at the beginning and end of frass collection, and frozen until GC analysis.

For larger-scale frass collections for NMR analysis, *M. linariifolia* twigs with flush growth were placed in water and suspended over filter paper with up to 100 insects. A lag time of 24 hr after a change of diet was more than sufficient to clear the gut of the previous diet. Frass was collected with small spatulas and brushes, even though the chance of contamination by leaf fragments was greater. Samples were collected directly into ethanol and stored at −10°C to prevent the excessive loss of volatiles.

Instrumentation. Analytical chromatography was performed on a Hewlett Packard 5890 gas chromatograph with HP7673A controller, HP3393 integrator, a 60-m × 0.25-mm-ID FSOT AT35 column, H_2 as carrier gas, FID detector, and a 10°/min program rate from 50° (1 min hold) to 250°C. Retention indices were measured against *n*-alkanes. Tridecane was used as a quantitation internal standard. GC-MS was carried out on a Shimadzu QP 1000 with a 30-m × 0.22-mm-ID FSOT DB-17 column for both 70 and 20 eV determina-

tions. Both ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were determined in $CDCl_3$ on a Bruker AC-3 instrument.

Comparison of Ingested and Excreted Volatile Constituents. Leaf volatile constituents of all food sources were determined by leaf extract analysis in ethanol by a modified microanalytical method (Brophy et al., 1989) in which 30-hr extraction times (20°C) were reduced to 1 hr following 10 sec of microwave (700 W) irradiation. GC peak identification was by retention time, comparison with authentic samples, and correlation with published data (Brophy et al., 1989; Southwell and Stiff, 1989, 1990; Southwell et al., 1992). Frass constituents were identified similarly, with the addition of GC-MS data for the metabolites present in the frass extracts and absent from the leaf extract.

Isolation and Identification of (+)-2β-Hydroxycineole (8). Larval frass (0.96 g, contaminated with minute leaf fragments) in ethanol was filtered and the filtrates made up to 10 ml with absolute alcohol. Water (5 ml) was added and nonpolar constituents were partitioned into hexane (25 ml). The ethanol–water layer was concentrated under vacuum, extracted into $CDCl_3$, and dried ($MgSO_4$) to give (+)-2β-hydroxycineole (57.4 mg) (**8**). IR ν_{max} cm^{-1} (film): 3261, 1259, 1212, 1072, 1036, 729, 703; ^{1}H NMR, δ ($CDCl_3$) 1.10 (3 H s, 7-Me), 1.26, 1.28 (2 × 3 H s, 9,10-Me), 0.80–2.10 (numerous multiplets; 3,4,5,6-Hs), 3.50 (1 H dd, $J_{3\beta}$ 3.4 Hz, $J_{3\alpha}$ 9.1 Hz, 2-H); ^{13}C NMR 22.1 (5-C), 23.1 (7-C), 28.2 (9 or 10-C), 29.0 (6 and 9 or 10-C), 33.4 (4-C), 35.4 (3-C), 70.4 (2-C), 72.6 (1-C), 74.0 (8-C). GC-MS (20 eV) *m/z* (%): 170(5), 137(2), 126(50), 111(38), 108(75), 93(40), 83(25), 71(75), 69(45), 43(100). GC-MS (70 eV) *m/z* (%): 170(3), 126(24), 111(18), 108(36), 83(18), 71(36), 69(21), 43(100)·$[\alpha]_D$+21.8° (c 0.03 in $CHCl_3$), lit. + 28.0° (Carman et al., 1986).

RESULTS AND DISCUSSION

A GC comparison of the volatile constituents of leaf ingesta and frass excreta was made for *P. tigrina* male adults, female adults, and stage three and four instars on seven different diets (Table 1). The four *M. alternifolia* diets represented very low cineole (MA1), low cineole (MA2), intermediate cineole (MA3), and terpinolene (MA4) chemical varieties. The three *M. linariifolia* diets included low cineole (ML1), high cineole (ML2), and very high cineole (ML3) varieties. These diets were typical of the varieties likely to be encountered in nature with only MA1, MA2, and ML1 being sources of commercially acceptable oils (International Standards Organisation, 1992). The predominance of sabinene and sabinene hydrates in the flush growth, and of γ-terpinene and terpinen-4-ol in the mature leaf, of *M. alternifolia* and *M. linariifolia* has been described (Southwell and Stiff, 1989; 1990). *Paropsisterna* feed almost entirely on flush growth for reasons (physical or chemical) currently under investigation (Maddox, 1994).

TABLE 1. PERCENTAGE COMPOSITION OF VOLATILE MONOTERPENOID CONSTITUENTS IN FLUSH GROWTH OF *M. alternifolia* (MA) AND *M. linariifolia* (ML) DIETS FOR *P. tigrina*

Peak No.[a]	Constituent	MA1	MA2	MA3	MA4	ML1	ML2	ML3
1	α-Thujene	0.3	0.7	1.4	1.0	4.1	0.8	0.1
2	α-Pinene	0.8	1.7	2.5	2.1	2.2	3.0	4.2
3	Sabinene	7.1	6.6	6.0	4.8	27.1	17.1	1.5
4	β-Pinene	0.2	0.5	0.7	0.9	0.4	1.2	1.7
5	Myrcene	0.3	0.8	1.9	2.1	1.1	3.0	4.0
6	α-Terpinene	1.8	2.1	3.4	2.7	2.7	1.0	0.2
7	Limonene	0.4	0.6	2.8	3.5	0.9	6.3	9.2
8	1,8-Cineole	**0.4**	**2.0**	**16.1**	**22.9**	**4.3**	**46.4**	**62.1**
9	γ-Terpinene	3.8	3.7	5.5	4.4	4.6	1.5	0.3
10	*trans*-Sabinene hydrate	5.5	6.3	4.9	2.2	16.2	1.2	0.1
11	Terpinolene	0.8	0.7	1.2	21.1	0.9	0.4	0.1
12	*cis*-Sabinene hydrate	34.5	38.6	25.3	12.6	10.6	1.3	0.1
13	Terpinen-4-ol	4.0	5.1	4.9	2.9	3.8	1.0	0.5
14	α-Terpineol	0.8	1.6	2.1	3.2	0.5	3.2	9.5

[a] Peak No. refers to peaks in chromatogram of figure 3.

The ability of adults and larvae of *P. tigrina* to excrete low-cineole tea tree leaf volatiles virtually unchanged was shown by the almost identical chromatograms for volatiles from leaf ingesta and frass excreta for MA1, MA2, and ML1 diets. The GC-determined constituent percentages for the volatile components of host *M. alternifolia* MA1 (flush growth and mature leaf) and frass extracted by adult males, adult females, and larvae are shown in Table 2. The *M. linariifolia* ML1 diet gave similar results. Diets containing significant concentrations of cineole (MA3, MA4, ML2, and ML3) gave frass volatiles containing a constituent not present in the ingesta with a retention index (1316) higher than (+)-terpinen-4-ol (**1**) (1258) and (+)-α-terpineol (**4**) (1277) (Figure 1). The percentage of this frass constituent was proportional ($R^2 = 0.99$) to the percentage of cineole ingested (Figure 2). An increase in percentage of dietary cineole corresponded with an increase in the concentration of this constituent, suggesting that it was a metabolite of cineole. Comparative gas chromatograms of high-cineole (ML3) ingesta and excreta are shown in Figure 3.

GC-MS analysis of the laval frass volatiles from a *M. linariifolia* high-cineole diet (ML3) indicated a molecular ion at *m/z* 170 for the major constituent, consistent with one of the 12 isomers possible for hydroxy-1,8-cineole (**5–16**) (Figure 4). Retention time comparison with authentic samples of (−)-2α-

TABLE 2. LEAF AND EXCRETA ETHANOLIC EXTRACT COMPOSITION FOR *P. tigrina* ON LOW-CINEOLE, FLUSH GROWTH *M. alternifolia* DIET (MA2) WITH COMPOSITION OF UNBROWSED MATURE LEAF FOR COMPARISON

Peak No.[a]	Constituent	MA2 host tree		FRASS excreta		
		Flush growth	Mature leaf	Male adults	Female adults	Larvae
1	α-Thujene	0.7	0.9	0.3	0.1	0.6
2	α-Pinene	1.7	3.0	0.9	0.7	1.1
3	Sabinene	6.6	0.0	2.1	1.1	3.2
4	β-Pinene	0.5	0.6	0.1	0.0	0.0
5	Myrcene	0.8	1.0	0.3	0.1	0.5
6	α-Terpinene	2.1	6.0	2.0	1.2	2.2
7	Limonene	0.6	0.7	0.2	0.1	0.4
8	1,8-Cineole	2.0	2.2	0.1	0.1	0.3
9	γ-Terpinene	3.7	24.0	3.9	3.2	5.2
10	*trans*-Sabinene hydrate	6.3	0.0	0.9	0.8	2.5
11	Terpinolene	0.7	2.7	0.5	0.6	0.6
12	*cis*-Sabinene hydrate	38.6	0.0	8.7	6.4	14.8
13	Terpinen-4-ol	5.1	38.8	11.9	11.4	19.7
14	α-Terpineol	1.6	3.2	0.5	0.5	3.3
15	(+)-2β-Hydroxycineole	0.0	0.0	0.4	0.1	1.6

[a]Peak No. refers to peaks in chromatogram of Figure 3.

hydroxycineole (**5**),[3] (+)-2α-hydroxycineole (**6**), (−)-2β-hydroxycineole (**7**), (+)-2β-hydroxycineole (**8**), (±)-3β-hydroxycineole (**11, 12**), 4-hydroxycineole (**13**), 7-hydroxycineole (**14**), (+)-(9S)-hydroxycineole (**15**), and (−)-(9*R*)-hydroxycineole (**16**) showed that only (−)- and (+)-2β-hydroxycineole had identical retention times to the *P. tigrinis* metabolite. The only isomers unavailable for comparison were (−)- and (+)-3α-hydroxycineole (**9**, **10**). Coinjection with both (−)- and (+)-2β-hydroxycineole (**7** and **8**) showed peak enhancement. The high-cineole *M. linariifolia* diet (ML3) was used for larger-scale feeding with approximately 100 larvae to obtain enough hydroxycineole for NMR spectroscopy. Measurement of absolute quantities ingested and excreted was not possible because of the small size of the larvae and the difficulty in separating frass from leaf fragments. Ingesta containing 1.5 mg of 1,8-cineole per 1 g leaf

[3]The trivial stereochemical α and β nomenclature is used here in preference to *exo* and *endo* terminology because of the ambiguity arising from some authors referencing substituent groups with respect to the cyclohexane ring (Asakawa et al., 1988a; Gandini et al., 1972; Liu et al., 1988; Orihara and Furuya, 1994; Miyazawa et al., 1991a, b; 1992a–c) and others, with respect to the pyran ring (Bitteur et al., 1990; Liu and Rosazza, 1990; MacRae et al., 1979; Nishimura et al., 1982; Williams et al., 1989).

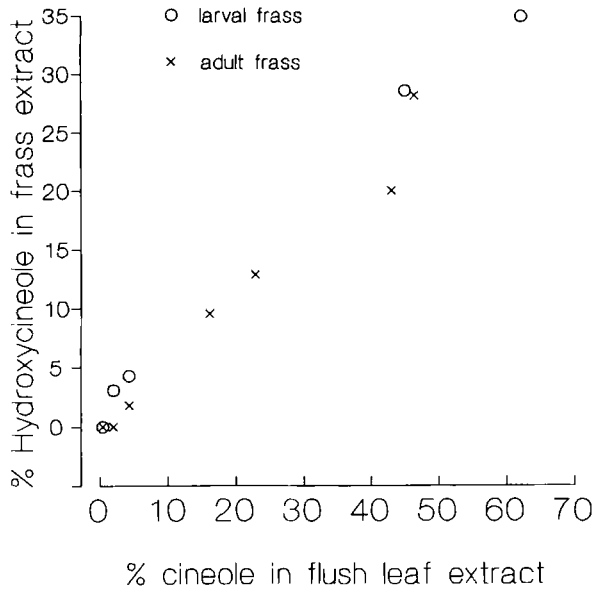


FIG. 2. Percent of (+)-2β-hydroxycineole (**8**) in *P. tigrina* frass extracts with respect to the percentage of 1,8-cineole in the diet.

(62.1% of the volatiles) gave 2.0 mg of hydroxycineole per gram of frass (39.8% of the volatiles). Both ^{1}H and ^{13}C NMR shifts were identical with published values for the enantiomers of 2β-hydroxycineole (Gandini et al., 1972; Carman and Fletcher, 1984; de Boggiatto et al., 1987; Asakawa et al., 1988a; Miyazawa et al., 1989; Carman et al., 1994). Assignment as (+)-2β-hydroxycineole[(1S, 4*R*, 6*R*)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ol] (**8**) followed measurement of optical rotation ($[\alpha_D]+21.8°$).

Comparison of the gas chromatographic percentages of cineole and hydroxycineole in ingesta and excreta suggests that the metabolism of the former to the latter proceeds in approximately 84% yield. The meticulous separation of all visible leaf fragments from the frass did not increase this percentage. Much of the remaining 16% of seemingly unmetabolized cineole may arise from undigested leaf within the frass. Very few other metabolites were detected on GC that could not be accounted for as unmetabolized ingesta. The most significant of these was a 1% constituent with an identical retention time and mass spectrum to 2α-hydroxycineole (**5** or **6**).

There have been numerous attempts to relate levels of herbivory by insects to plant chemistry in Australian Myrtaceae, mainly *Eucalyptus* spp. (Fox and

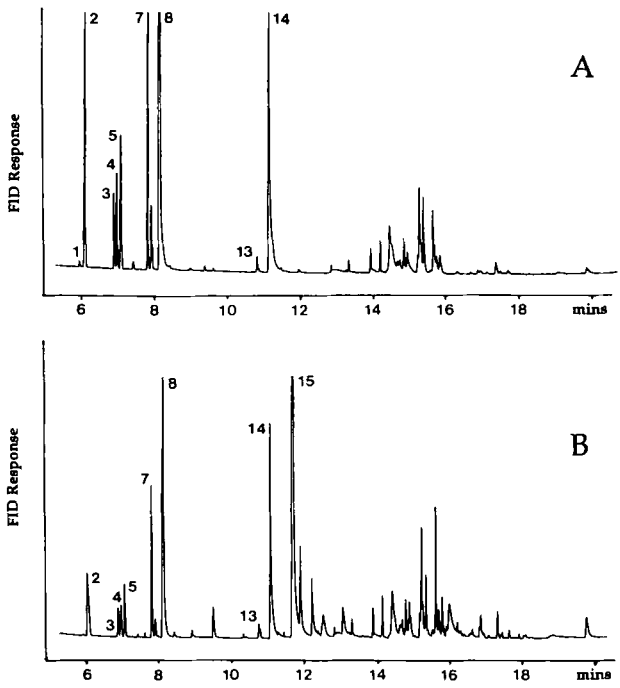
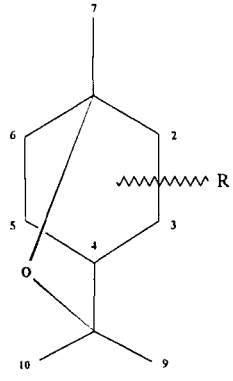


FIG. 3. Chromatograms of high-cineole *M. linariifolia* leaf ingesta (ML3) (A) and subsequent *P. tigrina* frass excreta (B). Peak numbers correspond to constituents listed in Table 2.



(5) $R_{2\alpha}$ = OH

(6) $R_{4\alpha}$ = OH

(7) $R_{2\beta}$ = OH

(8) $R_{6\beta}$ = OH

(9) $R_{3\alpha}$ = OH

(10) $R_{5\alpha}$ = OH

(11) $R_{3\beta}$ = OH

(12) $R_{5\beta}$ = OH

(13) $R_{4\alpha}$ = OH

(14) R_7 = CH_2OH

(15) R_9 = CH_2OH

(16) R_{10} = CH_2OH

FIG. 4. Chemical structures of the 12 possible isomers for hydroxy-1,8-cineole isolated from *P. tigrina* frass. Compound names are given in the text, identified by number.

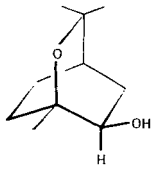
Macauley, 1977; Fox and Morrow, 1983; Morrow and Fox, 1980; see Ohmart and Edwards, 1991, for a review). Most recent studies have concentrated on terpenoid composition, with levels of cineole in particular implicated in tree variation in herbivore damage, both within and among tree species (Edwards et al., 1990, 1993; Stone and Bacon, 1994). There is much conjecture on how insects that feed on Myrtaceae process terpenoids, mainly due to the lack of detailed studies. Thus, Morrow and Fox (1980) concluded that the eucalypt herbivores they tested tolerated these compounds. This conclusion was based on the "close similarity" of terpenoid profiles of leaf ingesta and frass. Edwards et al. (1993) concluded that eucalypt herbivores (specifically *Anoplognathus* spp.) could not detoxify or metabolize terpenoids. This conclusion was based on a negative correlation between levels of tree damage and the percentage of cineole in the terpenoid composition of the trees. More detailed studies showed sequestrian in sawfly larvae (Morrow et al., 1976) and possible absorption of some terpenoids in a chrysomelid (Ohmart and Larsson, 1989). The latter authors reported a terpenoid budget for *Paropsis atomaria* (Olivier) feeding on *E. blakelyi* and concluded that larvae of this beetle absorbed and/or somehow metabolized to other compounds 86% of ingested cineole. Other terpenoids present in the diet were also greatly reduced in the egesta. Although they did not identify the metabolites, their efficiency of conversion figure, based on a nutrition budget approach, is very similar to our own estimates for another chrysomelid. Pierce et al. (1987) found that pine bark beetles could also detoxify terpenoids.

An obvious question that stems from our results is: Are the terpenoids simply being detoxified or are they being utilized as an essential component of the diet? If the latter, then high levels of herbivore damage on low-cineole trees or species may simply reflect higher feeding rates to increase the rate of intake of cineole. Studies based on levels of damage alone will not answer this question. In fact, studies that concentrate solely on levels of herbivore damage are likely to be misleading on a number of counts. Damage is the end product of the number of adults and immatures feeding on a tree, reflecting the various recruitment and attritional processes affecting the population as well as the physiology of feeding. Correlation of feeding damage with plant chemical attributes without independent measures of the contributing processes would not be a sensible research paradigm to follow.

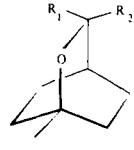
CONCLUSION

With the exception of 1,8-cineole (**2**), *Paropsisterna tigrina* excretes all volatile *Melaleuca* ingesta constituents unchanged in the frass. Some of the less chemically stable constituents (e.g., the thujenes: sabinene, *cis*-sabinene hydrate and *trans*-sabinene hydrate) are present in reduced concentrations. Adult male,

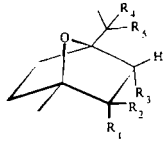
adult female, and larvae metabolized *Melaleuca* volatiles similarly; 1,8-cineole is largely metabolized to (+)-2β-hydroxycineole or (1*S*, 4*R*, 8*R*)-1,3,3-trimethyl-2-oxabicyclo[2,2,2]octan-6-ol (**8**), with a trace of the 2α-hydroxycineole (**5** or **6**). This oxidation is in contrast to the metabolism of 1,8-cineole in the brushtail possum, *Trichosurus vulpecula* (Flynn and Southwell, 1979; Southwell et al., 1980; Carman and Klika, 1992; Bull et al., 1993) where oxidation of the most sterically available positions gives as major urinary metabolites the 9-hydroxycineoles (**15** and **16**) and cineole-9-oic acids (**17** and **18**). Trace amounts of the 2α, 3α, 3β, 2α-(8*R*), and 2α-(8*S*) alcohols and diols have also been isolated from the possum (Carman et al., 1994). The rabbit metabolizes cineole similarly to *Paropsisterna* to give urinary (+)-2β-hydroxycineole (**8**) as the major component (Miyazawa et al., 1989). The fungus *Glomerella cingulata* transforms 1,8-cineole into (+)-2α-hydroxy-1,8-cineole (**6**) (Miyazawa et al., 1991a). The less common 1,4-cineole (**19**) is metabolized by *Bacillus cereus*, *Streptomyces griseus*, and *Aspergillus niger* to 2α(**20**), 2β(**21**), 3α(**22**), 8(**23**), and 9(**24**) -hydroxy-1,4-cineole (Liu et al., 1988; Miyazawa et al., 1991b; 1992a–c). The rabbit metabolizes 1,4-cineole to 9-hydroxy-1,4-cineole (**24**) 1,4-



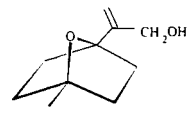
(8)



(17) R_1 = COOH R_2 = CH_3
(18) R_1 = CH_3 R_2 = COOH



(19) $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = CH_3$
(20) $R_1 = OH$, $R_2 = R_3 = R_4 = H$, $R_5 = CH_3$
(21) $R_2 = OH$, $R_1 = R_3 = R_4 = H$, $R_5 = CH_3$
(22) $R_3 = OH$, $R_1 = R_2 = R_4 = H$, $R_5 = CH_3$
(23) $R_4 = OH$, $R_1 = R_2 = R_3 = H$, $R_5 = CH_3$
(24) $R_5 = CH_2OH$, $R_1 = R_2 = R_3 = R_4 = H$
(25) $R_5 = COOH$, $R_1 = R_2 = R_3 = R_4 = H$
(26) $R_3 = R_4 = OH$, $R_1 = R_2 = H$, $R_5 = CH_3$
(27) $R_4 = OH$, $R_5 = CH_2OH$, $R_1 = R_2 = R_3 = H$



(28)

FIG. 5. Structures for metabolites of 1,8-cineole and 1,4-cineole. Compound **8** is (+)-2β-hydroxycineole, the major metabolite in frass of *P. tigrina* after ingestion of 1,8-cineole. Compounds **17** and **18** are cineole-9-oic acids, metabolites of 1,8-cineole by brushtail possum. Compounds **19–24** are major metabolites produced by various microorganisms from 1,4-cineole, and compounds **24–28** are metabolites of 1,4-cineole by rabbits.

TABLE 3. METABOLITES OF 1,8- AND 1,4-CINEOLE

Substrate	Species	Metabolite	Ratio[a]	References
1,8-Cineole (**2**)	*Aspergillus niger*	(±)-2α-OH-1,8-cineole	100	Nishimura et al. (1982)
		(±)-3α-OH-1,8-cineole	92	Nishimura et al. (1982)
		(±)-3-oxo-1,8-cineole	9	Nishimura et al. (1982)
		(±)-2-oxo-1,8-cineole	2	Nishimura et al. (1982)
		(±)-3β-OH-1,8-cineole	2	Nishimura et al. (1982)
	Bacillus cereus	(−)-2α-OH-1,8-cineole	100	Liu and Rosazza (1990)
	Eucalyptus perriniana[b]	(+)-2α-OH-1,8-cineole[c]	100	Orihara and Furuya (1994)
		(−)-2α-OH-1,8-cineole[c]	100	Orihara and Furuya (1994)
		(+)-3α-OH-1,8-cineole[c]	57	Orihara and Furuya (1994)
		(−)-3α-OH-1,8-cineole[c]	57	Orihara and Furuya (1994)
		(−)-2α-OH-1,8-cineole[d]	26	Orihara and Furuya (1994)
	Glomerella cingulata	(+)-2α-OH-1,8-cineole	100	Miyazawa et al. (1991a)
	Grape var. *Sauvignon*[e]	2α-OH-1,8-cineole[f]	100	Bitteur et al. (1990)
	Possum	(±)-1,8-cineol-9-oic acid	100	Flynn and Southwell (1979)
		(±)-9-OH-1,8-cineole	33	Southwell et al. (1980) Carman and Klika (1992)
		(±)-2α,9-diOH-1,8-cineole	17	Carman et al. (1994)
		7-OH-1,8-cineole	4	Bull et al. (1993)
		(±)-2α,10-diOH-1,8-cineole	3	Carman et al. (1994)
		(±)-3α-OH-1,8-cineole	1	Carman et al. (1994)
		7-cineolic acid	< 1	Bull et al. (1993)
		(±)-2α-OH-1,8-cineole	< 1	Carman et al. (1994)
		(±)-3β-OH-1,8-cineole	< 1	Carman et al. (1994)
	Pseudomonas flava	(+)-2-oxo-1,8-cineole	100	MacRae et al. (1979)
		ketolactone	71	Carman et al. (1986)
		(+)-2α-OH-1,8-cineole	36	Carman et al. (1986)
		(−)-2β-OH-1,8-cineole	14	Carman et al. (1986)

TABLE 3. Continued

Substrate	Species	Metabolite	Ratio[a]	References
	Rabbit, rat	(+)-2β-OH-1,8-cineole	100	Miyazawa et al. (1989)
		(+)-2α-OH-1,8-cineole	90	Madyastha and Chadha (1986)
		(+)-3α-OH-1,8-cineole	50	Madyastha and Chadha (1986)
		(+)-3β-OH-1,8-cineole	25	Madyastha and Chadha (1986)
	Rhodococcus sp.	(+)-2β-OH-1,8-cineole	100	Williams et al. (1989)
		(−)-2-oxo-1,8-cineole	100	Williams et al. (1989)
1,4-Cineole (**19**)	*Aspergillus niger*	(+)-2α-OH-1,4-cineole	100	Miyazawa et al. (1991b)
		(+)-3α-OH-1,4-cineole	45	Miyazawa et al. (1992a)
		9-OH-1,4-cineole	36	Miyazawa et al. (1992b)
		8-OH-1,4-cineole	30	Miyazawa et al. (1992c)
	Bacillus cereus	2α-OH-1,4-cineole	100	Liu et al. (1988)
		2β-OH-1,4-cineole	14	Liu et al. (1988)
	Rabbit	9-OH-1,4-cineole	100	Asakawa et al. (1988b)
		3β,8-dihydroxy-1,4-cineole	42	Asakawa et al. (1988b)
		1,4-cineol-9-oic acid	25	Asakawa et al. (1988b)
		8,9-dihydroxy-1,4-cineole	17	Asakawa et al. (1988b)
		9-hydroxy-1,4-cineol-8(10)-ene	8	Asakawa et al. (1988b)
	Streptomyces griseus	8-OH-1,4-cineole	100	Liu et al. (1988)
		2α-OH-1,4-cineole	33	Liu et al. (1988)
		2β-OH-1,4-cineole	16	Liu et al. (1988)

[a] Normalized against the major metabolite (100).
[b] Cell suspension culture.
[c] Isolated as the *O*-β-D-glucopyranoside.
[d] Isolated as the *O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside.
[e] Natural metabolite.
[f] Of unspecified enantiomeric purity.

cineol-9-oic acid (**25**), 3α,8-dihydroxy-1,4-cineole (**26**), 8,9-dihydroxy-1,4-cineole (**27**), and 9-hydroxy-1,4-cineol-8-ene (**28**) (Asakawa et al., 1988b). (Figure 5). 2α-Hydroxy-1,8-cineole (**5, 6**) is also a known volatile from the Sauvignon variety of grape (Bitteur et al., 1990). Cultured cells of *Eucalyptus perriniana* produce glucosides of 2- and 3α-hydroxycineole (**5, 6, 9, 10**) (Orihara and Furuya, 1994). A summary of the metabolism of 1,4- and 1,8-cineole by the above species is shown in Table 3. This is, to our knowledge, the first definitive report of a metabolite of cineole from insects. The gas chromatographic investigations of the frass of *P. atomaria* feeding on *E. blakelyi* (Ohmart and Larsson, 1989) have indicated an unidentified metabolite that may also be (+)-2β-hydroxycineole.

Although paralleling the hydroxylation of α-pinene by *Dendroctonus* to produce the repellant (*E*)-verbenol (Harborne, 1988), the ecological significance of the excretion of 1,8-cineole as (+)-2β-hydroxy-1,8-cineole (**8**) by *Paropsisterna* awaits further investigation. In addition, the oxidase source (microbial or gut enzymes) needs identification.

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ANTITERMITIC ACTIVITY OF *Lonchocarpus castilloi* FLAVONOIDS AND HEARTWOOD EXTRACTS

R. REYES-CHILPA,*[,1] N. VIVEROS-RODRÍGUEZ,[1]
F. GÓMEZ-GARIBAY,[2] and D. ALAVEZ-SOLANO[2]

[1]*Instituto de Ecología A.C.*
Apartado Postal 63
Xalapa, Veracruz, 91000, México
[2]*Instituto de Química, Universidad Nacional Autónoma de México*
Ciudad Universitaria
Coyoacán, 04510 México, D.F.

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Abstract—The heartwood of the tropical tree *Lonchocarpus castilloi* Standley (Leguminosae) is highly resistant to attack by the drywood termites *Cryptotermes brevis* (Walker); nevertheless successive extraction with hexane, diethyl ether, acetone, methanol, and water reduced its resistance to these organisms. Antitermitic properties of the extracts were bioassayed using impregnated filter paper disks. Although the five extracts reduced both feeding and survival of *C. brevis*, no significant differences among them were detected. Choice feeding tests showed that termites avoided eating the paper treated with the extracts. Two flavonoid compounds isolated from the heartwood, castillen D and castillen E, impregnated into filter paper showed concentration-dependent feeding deterrent activity, but were not toxic to *C. brevis*.

Key Words—Feeding deterrents, termites, *Cryptotermes brevis*, Isoptera, *Lonchocarpus castilloi*, Leguminosae, heartwood, flavonoids, antitermitic activity, castillen D, castillen E.

INTRODUCTION

Lonchocarpus castilloi Standley (Leguminosae) is a tree indigenous to the tropical subhumid forest in southern Mexico, Central America, and northern South America. It can reach 30 m in height and 40 cm diameter at breast height (Ortega-Escalona et al., 1991). In Mexico it is known as "machiche." Locally,

*To whom correspondence should be addressed.

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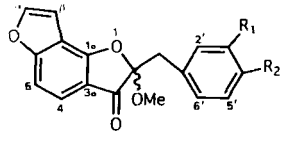
the timber is used in bridges, housing, rural wagons, and for manufacturing veneer, plywood, staves, and parquet. It is also recommended for railroad crossties, stakes, fenceposts, and post cross-arms (Echenique-Manrique, 1970).

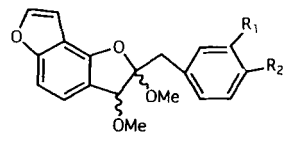
Lonchocarpus castilloi heartwood has been reported to be very durable in accelerated field tests (Bárcenas-Pazos, 1995); it is also highly resistant to several wood-rotting fungi (Reyes-Chilpa et al., 1987) and to the drywood termite *Cryptotermes brevis* (Walker) (Pérez-Morales et al., 1984).

Current evidence indicates that natural resistance to fungi (Laks, 1988; Déon et al., 1980; Hart and Shrimpton, 1979) and termites (Scheffrahn, 1991; McDaniel, 1992) is primarily determined by the concentration and type of secondary compounds found in the heartwood. In this context, it has been shown that the natural decay resistance of *Lonchocarpus castilloi* heartwood is also chemically determined. Extraction of the heartwood with a series of organic solvents reduced its decay resistance (Reyes-Chilpa et al., 1987), and the extracts showed antifungal activity in vitro (Reyes-Chilpa, 1986). Chromatographic separation of the hexane extract afforded four 2-methoxyfuran auronols named castillens A, B, C, and D, as well as one α-hydroxy-dihydro-chalcone, castillen E (Gómez-Garibay et al., 1990). These flavonoid compounds (Figure 1), inhibited the mycelial growth of the fungus *Lenzites trabea* Pers. ex Fr. The most active compound was the castillen E, followed by castillen D (Gómez-Garibay et al., 1990).

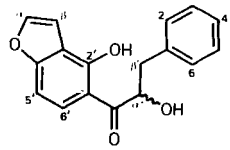
We now have examined the effect of *L. castilloi* heartwood extracts and of



1.- Castillen A: $R_1 = R_2 = H$
2.- Castillen D: $R_1 + R_2 = OCH_2O$



3.- Castillen B: $R_1 = R_2 = H$
4.- Castillen C: $R_1 + R_2 = OCH_2O$



5.- Castillen E

FIG. 1. Flavonoids from *Lonchocarpus castilloi* heartwood. Castillen A (**1**), Castillen D (**2**), Castillen B (**3**), Castillen C (**4**), and Castillen E (**5**).

castillens D (**2**) and E (**5**) on the feeding and survival of *C. brevis*. We also report the isolation of two additional compounds, pongaglabrone and 4-hydroxybenzofuran, from the heartwood.

METHODS AND MATERIALS

Lonchocarpus castilloi was collected at the "Lacandona" tropical rain forest, Chiapas, México (Bárcenas-Pazos et al., 1980). Wood samples and voucher specimens are deposited at the Ecology Institute's Herbarium (Xal) in Xalapa, Mexico.

Termite Collection. Cryptotermes brevis termites were obtained from old beams in Xalapa City. The beams were sawed and stored under controlled conditions (23°C, 75% relative humidity) until employment, and bioassays also were conducted under these conditions. Beam pieces were split on a paper; termites were taken up with entomological tweezers. Only active pseudergates above the third instar were used for bioassays. Selection of active specimens was done as follows. A group of 40 pseudergates was placed inside a 50-ml beaker, and that beaker was placed perpendicular to another. The first 30 termites that passed to the second beaker were chosen (Pérez-Morales et al., 1984).

Wood Block Bioassays. Sapwood and heartwood blocks (2 × 1 × 0.5 cm, smallest dimension being parallel to the fiber) were cut from a board. The blocks were carefully sand-papered, oven dried (105°C, 24 hr), and weighed. Heartwood blocks were successively extracted in a Soxhlet apparatus with hexane, diethyl ether, acetone, methanol, and water. After each extraction step (10 hr), an equal number of blocks were removed. Extractive yields were calculated as the percentage of oven-dried weight loss (Table 1).

Sapwood and heartwood (extracted and unextracted) blocks were tested against *C. brevis*. Each block was placed in a glass vial with 30 pseudergates. The vials were capped with filter paper. Each treatment was replicated five times. Mortality was recorded periodically, and the wood consumption was determined at the end of the test period (seven weeks) as the percentage of oven-dried weight loss.

Filter Paper Bioassays. Lonchocarpus castilloi heartwood shavings (500 g) were extracted under reflux with the same sequence of solvents described previously. The extracts were concentrated in a rotary evaporator and freeze-dried when the solvent was water. Solutions (2.5% w/v) of each extract were prepared in order to impregnate filter paper disks. This concentration was chosen since 2% was the mean extractive yield from blocks. Hexane and diethyl ether extracts were dissolved in a mixture of hexane–ethyl acetate 1:1 (HE), and acetone and methanol extracts were dissolved with a mixture of acetone–meth-

anol 70:30 (AM). Each solution was poured into a Petri dish, and filter paper disks (Whatman No. 1, diameter = 7 cm) were dipped for 30 sec and then air dried. Impregnated disks were then placed in a hood for 12 hr. Control disks were treated with the solvent mixtures only.

Two bioassays were run with the impregnated filter paper: (1) For force feeding, a disk was placed into a Petri dish and 15 termites were added. (2) For the choice test, one disk of each treatment, including the AM control, was sectioned into six equal slices. A slice of each treatment was then fixed to the bottom of a Petri dish (Figure 2) with a bit of "Blue Tac" plaster (Williams, 1980) and 15 termites were added. Five replicates per test were run. Mortality was recorded periodically. Paper consumption was quantified at the end of the test (36 days).

Isolation of Compounds. Heartwood shavings (650 g) were extracted at room temperature with hexane and then with methylene chloride. After concentration, the methylene chloride extract was chromatographed over silica gel. The compounds were obtained by preparative TLC and identified by ^{1}H NMR, ^{13}C NMR, IR, UV, and MS.

Castillens D and E were tested using impregnated filter paper and following the force-feeding protocol. The solutions tested were 0.025, 0.05, 0.1, and 0.15% w/v with acetone as solvent. Control paper disks were treated with the solvent only.

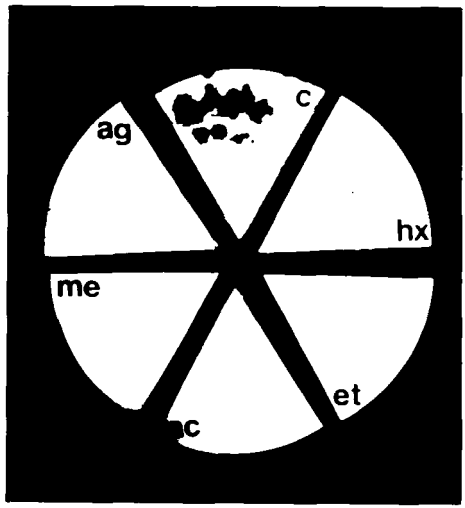


FIG. 2. Choice test. Feeding of termites on filter paper impregnated with hexane (hx), diethyl ether (et), acetone (ac), methanol (me), or water (ag) extracts of *Lonchocarpus castilloi* heartwood. Control disks (c) treated only with a mixture of acetone–methanol (7:3).

RESULTS

Extracted Wood. Cryptotermes brevis pseudergates ate over six times more of *Lonchocarpus castilloi* fully extracted heartwood blocks than of the sound heartwood (Table 1). The most efficient solvent that rendered heartwood more susceptible was methanol and then water. Heartwood blocks successively extracted with hexane, diethyl ether, and acetone were eaten equally when compared with sound heartwood. Termites consumed 1.7 times more of extracted heartwood blocks than sapwood. Sapwood seemed also to be eaten more than sound heartwood, but the difference was not significant when examined by ANOVA (Table 1); nevertheless, it was significant ($P = 0.001$) by Student's t test. At the end of the test period, termite survival was similar among all treatments (Table 1).

Effects of Extracts. All the heartwood extracts incorporated into filter paper reduced both the paper consumption and the survival of termites (Table 2). No significant differences among the extracts were detected. On average, there was a fivefold reduction in consumption when the treatments were compared with the control; there was also a twofold reduction in termite survival. The above data suggested that all the extracts contained antitermitic compounds.

In the choice feeding test, pseudergates consumed only the control and avoided all the filter paper slices treated with the heartwood extracts (Figure 2). Survival in this test was similar to that depicted for controls in the force-feeding experiment (see Table 2).

Compounds Isolated. TLC of the hexane extract of *L. castilloi* heartwood showed the presence of castillens A, B, C, D, and E as compared with authentic

TABLE 1. FEEDING AND SURVIVAL OF *Cryptotermes brevis* PSEUDERGATES ON *L. castilloi* WOOD AFTER 47-DAY EXPOSURE[a]

Treatment	Extractive yields (% w/w)	Consumption (% weight loss)	Survivors
Sapwood		1.77a	9.0a
Heartwood		0.52a	7.8a
Extracted heartwood			
Hexane	2.79	0.61a	6.0a
Ether[b]	0.36	0.87a	6.2a
Acetone	2.28	0.40a	5.8a
Methanol	3.84	2.17b	6.6a
Water[b]	0.76	3.16b	11.5a

[a]Means ($N = 5$) using 30 termites per replicate. Numbers followed by same letter are not significantly different at $P > 0.05$ (Duncan's NMRT).
[b]$N = 4$.

TABLE 2. FEEDING AND SURVIVAL OF *Cryptotermes brevis* PSEUDERGATES ON PAPER IMPREGNATED WITH HEARTWOOD EXTRACTS OF *L. castilloi* AFTER 36-DAY EXPOSURE[a]

Treatment	Consumption (mg)	Survivors
Control HA	13.7a	10.4a
Control AM	14.1a	9.8a
Hexane	1.8b	4.4b
Ether	3.2b	4.4b
Acetone	2.6b	3.8b
Methanol	4.5b	4.4b
Water	3.1b	4.2b

[a]Means ($N = 5$) using 15 termites per replicate. Numbers followed by same letter are not significantly different at $P > 0.05$ (Duncan's NMRT).

samples. The methylene chloride extract rendered castillen D (25 mg), castillen E (49 mg), pongaglabrone (6 mg), and 4-hydroxy-benzofuran (13 mg) by column chomatography and preparative TLC. Castillens D and E were then crystallized, and the spectral data from the purified compounds agreed with those previously reported (Gómez-Garibay et al., 1990).

Castillen D. Colorless solid, mp 121°C. ^{13}C NMR ($CDCl_3$, 50 MHz): 197.0 [C = 0], 166.5 [C-6], 162.2 [C-1a], 147.1 [C-3'], 146.4 [C-4'], 145.6 [C-α], 126.4 [C-1'], 123.8 [C-4], 119.7 [C-6'], 115.2 [C-3a], 113.3 [C-7], 110.9 [C-5'], 110.1 [C-2], 107.7 [C-2'], 107.2 [C-β], 103.7 [C-5], 100.7 [OCH_2O], 52.5 [OCH_3], 41.0 [Ar-CH_2].

Castillen E. Colorless solid, crystallyzed from diethyl ether, mp 72–73°C. ^{13}C NMR ($CDCl_3$, 50 MHz): 204.5 [C = 0], 159.4 [C-4'], 144.8 [C-α], 136 [C-2'], 130.8 [C-6'], 129.4 [C-3], 128.8 [C-5], 128.4 [C-2], 126.9 [C-6], 125.5 [C-4], 118.1 [C-1], 112.3 [C-1'], 111.1 [C-3'], 105.1 [C-β], 104.5 [C-5'], 77.8 [C-α'], 42.9 [C-β'].

Pongaglabrone (Khanna and Seshadri, 1963) and 4-hydroxy-benzofuran were also obtained as solids and identified by their spectral data.

Effects of Castillens D and E. Consumption by termites of filter paper impregnated with castillen D decreased as the concentration of this compound increased (Table 3). The highest concentration tested (0.15% w/v) caused a 3.7-fold reduction in the amount of paper eaten as compared to the control. Survival of termites was not affected by any concentration of castillen D tested.

Similar results were obtained with castillen E (Table 4). In this case, the 0.15% treatment caused a 2.6-fold reduction in paper consumption. Survival of termites was not affected at any concentration tested.

Since small amounts of castillen C, and a mixture of castillens A and B

TABLE 3. FEEDING AND SURVIVAL OF *Cryptotermes brevis* PSEUDERGATES ON PAPER IMPREGNATED WITH CASTILLEN D AFTER 28-DAY EXPOSURE[a]

Treatment	Consumption (mg)	Survivors
Control	9.4a	14.8a
0.025%	7.4ac	13.8a
0.050%	4.1bc	13.2a
0.100%	3.1b	13.6a
0.150%	2.5b	14.4a

[a]Means (N = 5) using 15 termites per replicate. Numbers followed by the same letter are not significantly different at $P > 0.05$ (Duncan's NMRT).

TABLE 4. FEEDING AND SURVIVAL OF *Cryptotermes brevis* PSEUDERGATES ON PAPER IMPREGNATED WITH CASTILLEN E AFTER 28-DAY EXPOSURE[a]

Treatment	Consumption (mg)	Survivors
Control	14.6a	11.8a
0.025%	13.3a	12.0a
0.050%	16.2a	13.2a
0.100%	7.0b	10.6a
0.150%	5.5b	11.0a

[a]Means (N = 5) using 15 termites per replicate. Numbers followed by same letter are not significantly different at $P > 0.05$ (Duncan's NMRT).

were available, the substances were also tested at the lower concentration (0.025% w/v) following the force-feeding protocol. Neither paper consumption nor survival of termites was significantly different from controls.

DISCUSSION

The heartwood of *Lonchocarpus castilloi* contains antitermitic compounds to *Cryptotermes brevis* pseudergates. These compounds were extracted with four organic solvents and water as indicated by the force-feeding test with impregnated filter paper (Table 2). At the concentration tested, all the extracts were equally effective. Similar results have been reported for other tropical hardwoods (Carter and de Camargo, 1983; Carter et al., 1975). Feeding-deterrent activity, but not toxicity, was detected for the previously known fungistactic flavonoids (Gómez-Garibay et al., 1990), the castillens D and E (Tables 3 and 4). These

compounds could be removed by hexane and other nonpolar solvents; nevertheless, reduction of the heartwood termite resistance was only noted after extraction with methanol and water (Table 1). The above results suggests that other compounds could be directly involved in *L. castilloi* termite resistance or could play a synergistic role.

Besides castillens D and E, other flavonoids have been previously tested against termites (Scheffrahn, 1991; Supriana, 1983). Several flavonoids were able to repell *C. brevis* attack when impregnated into a highly susceptible wood (Wolcott, 1953). Most of them showed poor activity, but taxifolin and pomiferin were considered outstanding. For instance, treatment with 0.05, 0.10, or 0.2% taxifolin solutions for 10 min was enough to protect susceptible wood for two years (Wolcott, 1953). Rotenone, a well-known insecticidal isoflavonoid, which can be isolated from the roots of several legume species, has also been found to be toxic and to deter the feeding of subterranean termites *Coptotermes formosanus* Shiraki (Křečková et al., 1988). Afzelin also has been reported to be toxic to *Heterotermes indicola* Wasmann (Supriana, 1983). At present, however, flavonoids seem to be more involved in the resistance of wood to fungi than to termites. Antifungal properties of monomeric and polymeric wood flavonoids are documented (Déon et al., 1980; Hart and Hillis, 1972). On the other hand, antitermitic compounds identified from wood have been found to be predominantly terpenoids (from monoterpenes to triterpenes), aliphatic acids, naphtho- and anthroquinones, simple quinones, stilbenes and, naphthopyrans (Scheffrahn, 1991). Therefore, in the case of *L. castilloi* heartwood resistance to *C. brevis*, it seems probable that besides the feeding deterrents castillen D and E, other active compounds, probably of a nonflavonoid nature, remain to be identified.

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REJECTION OF HOST PLANT BY LARVAE OF CABBAGE BUTTERFLY: DIET-DEPENDENT SENSITIVITY TO AN ANTIFEEDANT

J.A.A. RENWICK* and XIN PEI HUANG

Boyce Thompson Institute, Tower Road
Ithaca, New York 14853

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Abstract—Garden nasturtium, *Tropaeolum majus* (Tropaeolaceae), is an acceptable host plant for the cabbage butterfly, *Pieris rapae*. Eggs are readily laid on the plant and hatching larvae feed and develop into normal pupae and adults. However, when second- to fifth-instar larvae were transferred from cabbage to nasturtium, they refused to feed and starved to death. Similar results were obtained when larvae were transferred from other host plants to nasturtium. However, larvae that were reared on nasturtium readily accepted cabbage as a new host plant. We have demonstrated the presence of strong antifeedants in nasturtium foliage and identified the most prominent active compound as chlorogenic acid. However, larvae reared on nasturtium had limited sensitivity, and larvae reared on a wheat germ diet were completely insensitive to the antifeedants. Larvae apparently develop sensitivity to the deterrent as a result of feeding on other host plants, whereas continuous exposure to the deterrent causes habituation or suppression of sensitivity development. The results demonstrate that dietary experience can dramatically affect the response of an insect to a potentially antifeedant compound in a plant.

Key Words—Sensitization, habituation, dietary experience, feeding deterrents, *Pieris rapae*.

INTRODUCTION

Behavioral responses of phytophagous insects to chemical constituents of their host plants are known to be affected by previous learning experience, which may involve habituation, conditioning, or imprinting (Szentesi and Jermy, 1989). The most common examples of learning appear to be the result of an associative

*To whom correspondence should be addressed.

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process, whereas habituation and sensitization are generally considered to be nonassociative. Therefore, quite different neural mechanisms are thought to be involved. Most previous studies of altered feeding behavior have focused on induced food preferences as a result of initial experience. When late-instar larvae are transferred from one acceptable host plant to another, they may initially refuse or lower their consumption rate of the new food (Hanson, 1976; Schoonhoven and Meerman, 1978; Scriber, 1982; Grabstein and Scriber, 1982). However, in a no-choice situation, the new host is generally accepted after a period of temporary starvation. Similar changes in behavior have been observed for insects that were transferred from plants to artificial diet. Some insects will refuse to feed on diet after feeding on a plant (Schoonhoven, 1977; Städler and Hanson, 1978), whereas others may actually perform better (Hajek, 1989). On the other hand, larvae reared on artificial diet may accept a wider range of potential host plants, and, in the case of *Manduca sexta*, this change in behavior can be explained by changes in the sensitivity of chemoreceptors (Schoonhoven, 1967, 1969).

When late-instar larvae of *Pieris brassicae* were given a choice of food plants, they generally preferred the plant on which they were reared (Johansson, 1951). Larvae reared to fifth instar on *Tropaeolum majus* L. (nasturtium) also developed a preference for this plant, so induced preference was clearly indicated. However, when *P. brassicae* larvae were transferred from *Brassica oleracea* to *T. majus*, they refused to feed (Ma, 1972). Larvae of *P. brassicae* also refused to feed on artificial diet after feeding on cabbage, even when a feeding stimulant was added (David and Gardiner, 1966). However, no explanation for the rejection behavior was provided. Recent studies on both *P. brassicae* and *P. rapae* indicate that caterpillars reared on artificial diet have reduced sensitivity to deterrents compared to those reared on a host plant, and this could be related to changes in chemosensory activity (van Loon, 1990). However, the mechanisms responsible for induced preference or changes in sensitivity to plant constituents have not been studied.

Here we report a phenomenon of diet-induced changes in the acceptance of a host plant by larvae of the cabbage butterfly, *Pieris rapae* L., similar to that reported for *P. brassicae* (Ma, 1972) and further show that this can be explained by differential development of sensitivity to antifeedants in that plant. Larvae that were transferred from cabbage to nasturtium refused to feed and consequently starved to death. We have investigated the temporal and dietary requirements for this extreme rejection behavior and have isolated the primary deterrent constituent from nasturtium foliage.

METHODS AND MATERIALS

Insects. Pieris rapae larvae were obtained from a colony that is continuously maintained in the laboratory but is renewed annually by the addition of

field-collected butterflies. Experimental larvae were reared from eggs laid on the plant that served as the initial food. Oviposition occurred in greenhouse cages with supplemental lighting, and as the larvae developed, additional plants were provided to ensure an adequate supply of the specific food. Neonate larvae were obtained from eggs laid on a 4-cm strip of Parafilm wrapped around beakers that were covered with a cabbage leaf, so that tarsal contact provided the necessary stimulant for oviposition (Webb and Shelton, 1988; Renwick et al., 1992). The naive neonates were then transferred to either 2- to 3-week-old nasturtium or 4- to 6-week-old cabbage plants, using a small paint brush. Survival rates for neonates transferred from Parafilm to either cabbage or nasturtium were generally ca. 70%. For rearing on wheat germ diet, larvae were allowed to hatch from eggs laid on Parafilm strips that were subsequently placed in the diet cups.

Plants and Diet. All plants were grown under the same conditions, in Cornell Mix (Boodley and Sheldrake, 1977), with supplemental lighting in an air-conditioned greenhouse maintained at ca. 25°C. Seeds of nasturtium, *Tropaeolum majus* var. Double Gleam, were obtained from Thompson & Morgan, Jackson, New Jersey; *Cleome spinosa* var. Pink Queen, from The Page Seed Co., Greene, New York; and *Reseda luteola* (Weld) from Otto Richter & Sons Ltd., Goodwood, Ontario, Canada. Seeds of *Sinapis alba* were a gift from B. Gabrys and *Brassica juncea* was obtained from Agway, Inc., Syracuse, New York. All plants were 4–6 weeks old when used in experiments. Wheat germ diet was prepared according to the recipe of Bell et al. (1979) and poured into 250-ml styrofoam cups.

Bioassays. Acceptance of new food plants was tested by allowing butterflies to oviposit on a plant and rearing the hatching larvae to third instars on this plant, before transferring them to their new host plant. Acceptance of nasturtium foliage by larvae was tested by placing one larva on a nasturtium leaf contained in a 250-ml ice cream cup. The petiole of each leaf was passed through a hole in the bottom of the cup and immersed in water, so that the leaf remained fresh for the duration of the experiment. Larval transfers were made with a small paint brush, and 50 replications of each transfer were performed. Nasturtium plants were 2–3 weeks old when used for these assays. Continued feeding and frass production on test leaves within 24 or 48 hr was considered to be acceptance, whereas small nibbles followed by avoidance or a complete lack of biting was considered to be rejection.

The feeding deterrent assay (Dimock et al., 1991) consisted of a choice of four leaf discs, 13 mm diameter, cut from fresh cabbage leaves with a cork borer. Two of these were treated with the test extract and the other two with solvent (methanol) alone. Five larvae (early fourth instars) of *P. rapae* were introduced at the center of each cup and allowed to feed for a period of 6 hr (when ca. 50–70% of the control disk area had been eaten) under controlled conditions of high humidity in an incubator. Leaf area consumed was measured

using a Li-Cor 3100 area meter. A feeding deterrent index (FDI) was calculated, based on the areas consumed from all the disks. $FDI = 100\ (C - T)/(C + T)$ where C is the area consumed of control disks and T the area consumed of treated disks. The area of treated disks consumed was expressed as a proportion of the total (treated + control) disk area consumed, and the data were subjected to arcsine square-root transformation. Differences between treatments and controls were analyzed using a one-sample t test on the transformed data, under the null hypothesis that the total area consumed was distributed evenly over treated and control disks. Differences among treatments were analyzed using a Waller-Duncan K-ratio t test ($K = 100$) on the transformed data.

Extraction of Foliage. Foliage was extracted following a standard procedure for the isolation of deterrents (Renwick and Radke, 1987). Fresh foliage was dropped into boiling ethanol, followed by homogenization and filtration. The ethanolic extract was evaporated under reduced pressure and the residue was defatted with hexane before dissolving in water. The aqueous extract was partitioned 3× with 1-butanol, and all fractions were tested for deterrent activity.

Isolation of Deterrent. Butanol extracts were first fractionated by flash chromatography on a 45 × 2-cm column packed with 30 g 55–105 μm C_{18} silica, and fractions were eluted with 90 ml each of water, 10%, 20%, 50%, and 100% acetonitrile in water. The most deterrent fraction was subjected to reverse-phase HPLC, using a semipreparative C_{18} column, with a gradient of 100% water to 100% acetonitrile to further isolate fractions for bioassays. The most prominent active component was isolated by repeated HPLC and characterized by UV and NMR spectroscopy. Details of the isolation, identification, and deterrent activity will be published elsewhere (Huang and Renwick, unpublished data).

RESULTS

In an initial experiment, *P. rapae* larvae at each stage from second to fifth instar were removed from cabbage plants on which they had been reared and placed on foliage of nasturtium. Observations of possible feeding were made after 24 and 48 hr. None of these larvae initiated feeding on nasturtium. Most individuals left the leaves and all eventually died, apparently as a result of starvation. However, when neonate (naive) larvae were placed on nasturtium plants, ca. 70% of these fed successfully, and the percentage surviving was similar to that for neonates placed on cabbage plants.

The possibility of induced dependence on the first host plant as a general mechanism for refusal behavior was tested by performing the reverse transfer. *P. rapae* butterflies were allowed to oviposit on nasturtium plants, and when the resulting larvae reached third instar, they were transferred to cabbage. These larvae readily accepted cabbage as their new food plant (Table 1). Similarly,

TABLE 1. ACCEPTANCE OR REJECTION OF NEW HOST PLANTS BY *Pieris rapae* LARVAE ($N = 50$) REARED TO THIRD INSTAR ON VARIOUS PLANTS OR ARTIFICIAL DIET[a]

Initial diet	New plant	Feeding on new plant (%)
Cabbage	Brassica juncea	98
(Brassica oleracea)	Sinapis alba	100
	Cleome spinosa	98
	Reseda luteola	96
	Nasturtium	0
Nasturtium	Cabbage	100
(Tropaeolum majus)		
Cabbage	Nasturtium	0
Brassica juncea		2
Sinapis alba		6
Cleome spinosa		4
Reseda luteola		14
Wheat germ diet		94

[a]Larvae on the initial host plants hatched from eggs laid on these plants. The larvae on wheat germ diet were from eggs laid on a strip of Parafilm so that no previous contact with a plant occurred.

the nasturtium-reared larvae accepted a range of alternate hosts, including members of the Cruciferae (*Brassica juncea* and *Sinapis alba*), Resedaceae (*Reseda luteola*) and Capparidaceae (*Cleome spinosa*). Cabbage-reared larvae also fed readily when transferred as third instars to these alternate host plants. Thus, nasturtium is unique among this group of experimental plants in its unacceptability to *P. rapae* from cabbage.

The requirements for development of refusal behavior were examined by rearing larvae to third instars on each of the alternate host plants as well as wheat germ diet, and then transferring these insects to individual leaves of *T. majus*. In general, almost all larvae that had developed on the test plants refused nasturtium foliage. Only a few larvae that were transferred from *Reseda luteola* showed a tendency to accept the nasturtium. However, larvae reared on the "artificial" diet readily fed on nasturtium leaves within 24 hr (Table 1).

Since neonate larvae accept nasturtium for feeding, the onset of rejection behavior must occur during the first-instar stage of larval development. The time required to trigger this behavioral change was measured by allowing neonate larvae to feed on cabbage foliage for increasing periods of time, from 0 to 24 hr, before transferring them to nasturtium leaves. Since these neonates were initially transferred from Parafilm, ca. 30% of them did not feed successfully on either nasturtium (0 time) or cabbage, probably as a result of handling. No change in behavior was induced after 2 hr of feeding, but the number of larvae

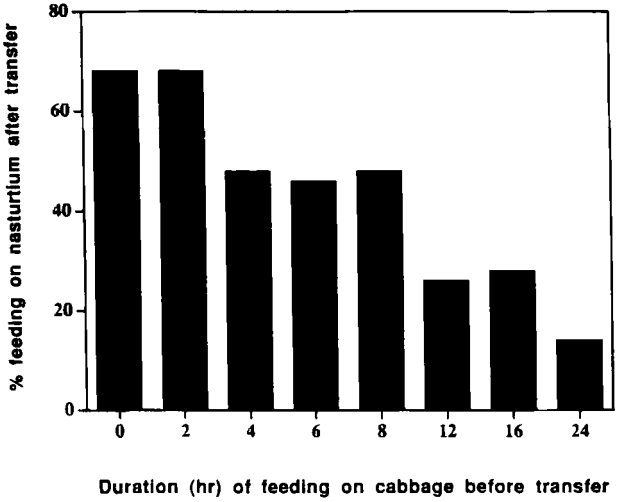

FIG. 1. Change in acceptance of nasturtium by first-instar *P. rapae* after feeding for an increasing length of time on intact cabbage plants. After each time period, 50 larvae were transferred to individual leaves of *Tropaeolum majus*, and the number feeding was recorded after 24 and 48 hr. The data are shown for 24 hr, at which time acceptance or rejection generally was clear, based on either distinct consumption of tissue or lack of damage to the leaf.

accepting the new host declined steadily thereafter with increasing time spent on cabbage (Figure 1).

The possible presence of antifeedants in nasturtium was investigated by extraction of foliage with hot ethanol, fractionation by solvent partitioning, and bioassay of the fractions. When aqueous fractions of the nasturtium extracts were partitioned with *n*-butanol, the butanol fraction proved to be highly active in standard feeding deterrent assays. At concentrations of 0.1 g leaf equivalents (gle), fourth instars that had been reared on cabbage were completely deterred from feeding on treated cabbage leaf disks. Deterrent activity was obtained at concentrations as low as 0.004 gle/disk (between treatment and control $t = 2.25$, $P < 0.05$), but disappeared at 0.001 gle/disk ($t = 0.16$, $P > 0.05$) (Figure 2). A significant difference was found between FDIs for these two treatments. Reverse-phase HPLC of the active fraction on a C_{18} column with a diode array detector revealed the presence of compounds that gave UV spectra typical of phenolic derivatives. The most prominent compound was isolated and found to be strongly deterrent on its own. This compound was subsequently

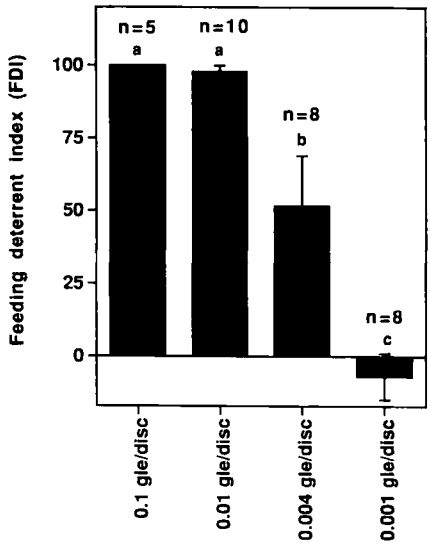

FIG. 2. Feeding deterrent activity of the butanol-soluble components of nasturtium on *P. rapae* larvae reared from hatching to early fourth instar on cabbage, and then given a choice of feeding on two cabbage disks treated with butanol extract of nasturtium at doses of 0.1, 0.01, 0.004, or 0.001 gle/disk, or feeding on two control cabbage disks each treated with solvent only. Concentrations are expressed as gram leaf equivalents (gle) based upon fresh weight of extracted foliage. The feeding deterrent index (FDI) is based on areas of discs consumed. FDI = 100 × (control − treated)/(treated + control). Means (±SE) with the same letters are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$) on the transformed data; n = number of replications of the bioassay.

identified as chlorogenic acid, on the basis of spectral analyses, acid and base hydrolysis, and comparison with a sample of the authentic chemical.

Feeding deterrent assays of the butanol extract of nasturtium were performed to compare the sensitivities of fourth-instar *P. rapae* that were reared on cabbage, nasturtium, or wheat germ diet. At a deterrent concentration of 0.01 gle/disk, the cabbage-reared larvae were strongly deterred. However the nasturtium-reared larvae were much less responsive to the deterrent (between treatment and control, $t = 3.00$, $P < 0.05$), and the diet-reared larvae were completely unaffected ($t = 0.11$, $P > 0.05$) (Figure 3). Significant differences were found between FDIs for cabbage-reared larvae and larvae reared on nasturtium or diet.

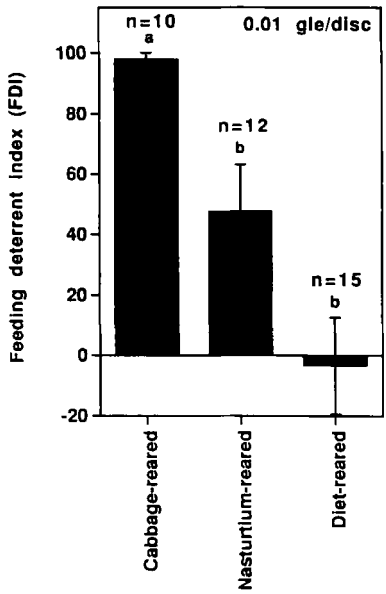

FIG. 3. Response of *P. rapae* larvae reared from hatching to early fourth instar on cabbage, nasturtium, or artificial diet, and then given a choice of feeding on two cabbage disks each treated with butanol extract of nasturtium at doses of 0.01 gle/disk or two control cabbage disks each treated with solvent only. The feeding index was calculated as in Figure 2. Means ($\pm$SE) with the same letters are not significantly different according to a Waller-Duncan K-ratio t test ($K = 100$) on the transformed data; n = number of replications of the bioassay.

DISCUSSION

Absolute rejection of a normally acceptable host plant by an insect appears to be relatively unique. This behavior differs from the more typical induced preference behavior that occurs as a result of an insect becoming conditioned on a plant (Szentesi and Jermy, 1989). However, the refusal of artificial diet after feeding on a plant has been observed for some insects (Schoonhoven, 1977; Städler and Hanson, 1978), as has the reverse situation where conditioning on artificial diet actually increases the host acceptance range of *Manduca sexta* larvae (Schoonhoven, 1967). Rejection of nasturtium by cabbage-reared larvae of *P. rapae* can now be explained by the presence of antifeedants, the most prominent of which is chlorogenic acid, in the new food plant. However, sensitivity to the deterrents is dependent on the previous experience of these larvae. Newly hatched first-instar larvae appear to be insensitive to the negative stimuli,

but, by the time they have molted to second instar on cabbage or other host plants, sensitivity to deterrents has developed. This could mean that an inducer present in these plants, but absent in nasturtium, is responsible for triggering the development of sensitivity. However, relative insensitivity of larvae reared on nasturtium might also suggest that habituation to the deterrents occurs. The lack of sensitivity of diet-reared larvae would then indicate the presence in diet of compounds that cause habituation, or suppression of development of sensitivity, to the nasturtium semiochemicals. A similar development of refusal behavior in *P. brassicae* was reported by Schoonhoven (1977), who transferred larvae to artificial diet after various periods of time feeding on cabbage. After three days, the proportion of larvae accepting the artificial diet was negligible. Schoonhoven suggested that the larvae become "fixed" to cabbage in this time, thus making the artificial diet unacceptable.

The development of deterrent sensitivity in first instars may occur gradually, but the timing appears to be highly variable. The majority of larvae required about 12 hr of feeding on cabbage, but 4 hr was sufficient for some individuals. Actual consumption by individual larvae is highly variable, so the physiological trigger responsible for the change in behavior may depend on the quantity of food consumed. However, individual differences in dose dependency may also occur, and, if changes in sensory peception are involved, individual variation in thresholds for response is likely. Another source of variation is the time taken for an individual larva to initiate feeding. More precise measurement of feeding times would require recording the time at which each larva actually begins to ingest leaf material.

The concept of sensitization to a negative stimulus may suggest new approaches to understanding nonassociative learning in insects. Previous studies of sensitization have focused on increased response to a positive cue after repeated presentation, a reaction referred to as "pseudoconditioning" (McGuire, 1984). *Manduca sexta* larvae were deterred by constituents of wheat germ diet after feeding on a host plant (Städler and Hanson, 1978). However, when reared on wheat germ diet, they were less sensitive to the deterrents. This has been interpreted as meaning that the larvae became habituated to compounds in the diet, resulting in sensitization of these larvae to feeding stimulants in plant leaves (Szentesi and Jermy, 1989). Our results would suggest an alternative explanation, i.e., that the larvae became sensitized to the deterrents in the diet as a result of their feeding on a host plant.

Possible habituation or adaptation of insects to feeding deterrents is a major concern that could limit the practical application of such natural compounds for insect pest control. The physiological mechanisms of habituation and sensitization in vertebrates as well as invertebrates have yet to be elucidated in detail. In insects, sensory adaptation or increased activity of deterrent receptors may occur, or more basic effects on the central nervous system could be involved

(Szentesi and Bernays, 1984). *Pieris rapae* may well serve as a model for future study of the physiological processes involved in the changing responses of organisms to chemical stimuli.

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EVOLUTION AND FUNCTION OF LINGUAL SHAPE IN LIZARDS, WITH EMPHASIS ON ELONGATION, EXTENSIBILITY, AND CHEMICAL SAMPLING

WILLIAM E. COOPER, JR.

Department of Biology
Indiana University-Purdue University Fort Wayne
Fort Wayne, Indiana 46805

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Abstract—Major squamate taxa exhibit extreme variation in lingual morphology, presumably due to correlated variation in trophic and chemosensory functions. Data are presented on evolution of lingual shape documenting several trends: (1) Resting lingual elongation is greatest in families specialized for lingual chemosensory sampling. (2) The greatest increase in elongation achievable by intralingual means including elasticity and foretongue retractility occurs in families with intermediate degrees of lingual specialization for chemosensory sampling. Sampling efficiency may be enhanced by the ability to extend the tongue well beyond the mouth, with resting elongation and intralingual extensibility perhaps jointly determining distance extended. In families lacking sufficient resting elongation, augmentation of intralingual extensibility may be a means of approaching optimal protrusion distances. Decreased extensibility evolved in tandem with the greatest resting elongation, suggesting that resting elongation may be more efficient for protrusion and that elasticity declines as optimal resting length is approached. The optimal shape for chemosensory sampling may be predicted to be highly elongate, as in teiids, varanids, and colubrids. The tongue should be broad at the tip for prehension (as in iguanians), fleshy for manipulation and swallowing, and broad at the base for tamping prey into the esophagus. (3) Lingual surface area relative to that of a rectangle of dimensions length × base width varies accordingly. Relative area is high in families that do not tongue-flick much while foraging because tongues are broad and fleshy throughout their length. It is low in families that have wedge-shaped tongues and intermediate specialization for chemosensory sampling. Narrowing of the anterior tongue may improve chemical sampling. Relative lingual area in chemosensory specialists is very high, with progressive narrowing toward the base as optimal sampling shape is approached in taxa lacking lingual function in swallowing, prehension or prey manipulation.

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INTRODUCTION

The squamate tongue shows dramatic structural and functional variation among families. Great and consistent interfamilial differences are evident in numerous features, including the presence and development of forking (McDowell, 1972; Schwenk, 1994), the distribution and abundance of taste buds (Schwenk, 1985), and muscular anatomy (Schwenk, 1993; Bels et al., 1994). Behaviorally, the tongue is used for lingual prey prehension (Schwenk and Throckmorton, 1989) in one of the two major clades of lizards, Iguania, but not in the other, Scleroglossa.

Functions of lingually mediated chemoreception also vary with lingual structure. All families studied appear to show some degree of pheromonal communication involving lingual chemical sampling (Mason, 1992; Cooper, 1994a). Families of scleroglossan active foragers and iguanid herbivores additionally use the tongue to gather chemical samples for locating and evaluating food prior to biting (Cooper and Alberts, 1990; Cooper, 1990a, 1994a,b). Forked tongues enhance the ability to follow scent trails (Ford and Low, 1983), and a relationship between foraging mode and development of forking is expected and has been confirmed. Tines are absent in most ambush-foraging lizards but are present in several families and higher taxa of active foragers (Schwenk, 1994).

The tongue's major role in detection and identification of prey is sampling chemicals from the environment for analysis by vomerolfaction (Cooper and Burghardt, 1990). Tongue-flicking extends the tongue beyond the mouth, where it contacts chemicals in the air and on any substrate that it touches. Molecules adhere to the moist tongue and are transported to the mouth when the tongue is withdrawn. They reach the vomeronasal ducts at the roof of the mouth by an unknown mechanism and pass through the ducts to the vomeronasal epithelium, where they contact the vomerolfactory chemoreceptor cells (Young, 1990; Halpern, 1992).

Forking is the most obvious lingual adaptation of squamates for chemical sampling, but other features of the tongue may strongly affect sampling ability. Such features might also be related to chemosensory behavior and foraging mode. For example, tine development is positively correlated with active foraging mode (Schwenk, 1994). Excluding the tines, the most obvious lingual characteristic affecting sampling ability for vomerolfaction is the degree of elongation. A long tongue that can be extended further outside the oral cavity could enhance both the area sampled during a tongue-flick and the maximal distance from which a substrate can be sampled.

Another aspect of lingual shape that might be related to the evolution of

chemosensory sampling abilities is the degree to which the tongue resembles a rectangle or a wedge. In several families the tongue is broad and roughly rectangular; in others it is wedge-shaped, tapering from its broad base to the tip; in still others it is narrow yet roughly rectangular.

Data are presented in this paper on the lingual shapes of 18 species representing 10 major squamate families. Three measures of static lingual elongation are described, in addition to measures of intralingual extensibility and the degree to which the tongue resembles a wedge or a rectangle. Variation of each variable within and between families is documented. In a cladistic context, interfamilial correlations among the variables are discussed in relation to the evolution of lingual shape. Relationships between the shape of the tongue and its chemosensory and other functions are considered.

METHODS

Several aspects of lingual shape were measured in specimens of 18 species representing major lizard families, snakes, and all of the grades of lingual structure described by McDowell (1972). Availability and cost dictated which species were chosen within families. Although major features of squamate lingual anatomy are consistent at the familial and sometimes subfamilial levels, multiple species were studied in five families to allow a preliminary assessment of intrafamilial variation in the shape of the tongue.

After a long period of relative stability, lizard taxonomy is in a state of flux, necessitating a choice between competing schemes. The families used here are taxa recognized without formal familial designation by Estes et al. (1988) supplemented by the families named by Frost and Etheridge (1989) in reclassifying Iguania, which formerly consisted of Iguanidae, Agamidae, and Chamaeleontidae, but is now divided into numerous families.

The phylogeny adopted here is shown in Figure 1 and representation of taxa in the study appears in Table 1. Frost and Etheridge's (1989) classification of Iguania affects only four species, *Anolis carolinensis*, *Holbrookia maculata*, and *Sceloporus malachiticus*, all of which were formerly in Iguanidae but now are divided between Phrynosomatidae and Polychrotidae, and *Calotes mystaceus*, formerly in Agamidae, which is now the subfamily Agaminae of Chamaeleonidae. Having 11 versus 10 families made very little difference in the outcomes of numerous statistical tests that were conducted using both taxonomies. Therefore, results at the familial level are reported for only the newer classification (Frost and Etheridge, 1989).

Prior to 1991 specimens were killed by freezing and thawed prior to measurement. Subsequently, they were euthanized using sodium pentobarbital. With one exception, only preserved specimens were available for Helodermatidae,

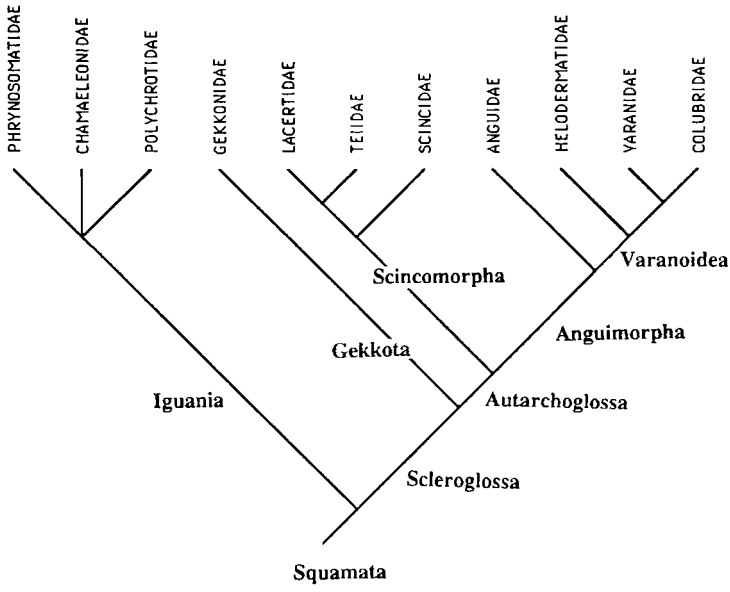

FIG. 1. Phylogeny of squamate reptiles, modified from Estes et al. (1988) to include snakes within Anguimorpha as suggested by chemosensory characters studied by Schwenk (1988, 1993) and the recent revision of Iguania by Frost and Etheridge (1989). Only families for which data are presented are included.

precluding measurement of stretched tongue length and therefore intralingual extensibility. Variables were measured to the nearest millimeter by ruler or to the nearest 0.1 mm by vernier calipers. Lingual measurements were made using a dissecting microscope or illuminated magnifying glass except for *Holbrookia maculata* and *Cnemidophorus sexlineatus*, which were measured in the field using the naked eye.

For each specimen, the following variables were measured: snout–vent length (SVL) in millimeters; resting tongue length from tip to base of the tongue (RTL) in the midline to the nearest millimeter with the tongue lying unextended in situ; stretched tongue length (STL) from tip to base (in the midline to the nearest millimeter with the tongue being grasped at the base of the notch or tines by forceps and stretched to its maximal extent), base width (BW) of the resting tongue to the nearest 0.1 mm; and tip width (TW) to the nearest 0.1 mm measured 1.0 mm posterior to the base of the tines or notch. All lingual variables were measured on the dorsal surface.

Several derived variables reflecting the tongue's intrinsic shape were cal-

Family and species	*N*	BASELONG	RELONG	SELONG	EXTEND	RELAREA
Phrynosomatidae	49	1.31 ± 0.14	1.95 ± 0.23	2.50 ± 0.35	0.29 ± 0.16	0.84 ± 0.04
Holbrookia propinqua	40	1.32 ± 0.14	1.97 ± 0.24	2.42 ± 0.28	0.23 ± 0.09	0.84 ± 0.04
Sceloporus malachiticus	9	1.23 ± 0.11	1.85 ± 0.15	2.89 ± 0.39	0.56 ± 0.14	0.84 ± 0.04
Polychrotidae						
Anolis carolinensis	12	1.63 ± 0.10	3.06 ± 0.27	5.13 ± 0.52	0.68 ± 0.16	0.77 ± 0.02
Chamaeleonidae						
Calotes mystaceus	8	1.29 ± 0.09	3.69 ± 0.34	5.72 ± 0.53	0.55 ± 0.07	0.68 ± 0.02
Gekkonidae						
Thecadactylus rapicaudus	12	1.76 ± 0.19	3.66 ± 0.36	6.13 ± 0.75	0.68 ± 0.16	0.74 ± 0.04
Scincidae	35	1.48 ± 0.19	4.18 ± 0.57	7.04 ± 1.22	0.69 ± 0.22	0.68 ± 0.03
Eumeces fasciatus	9	1.42 ± 0.12	3.90 ± 0.50	6.31 ± 0.85	0.63 ± 0.15	0.68 ± 0.02
Eumeces laticeps	26	1.50 ± 0.20	4.28 ± 0.56	7.30 ± 1.24	0.71 ± 0.24	0.68 ± 0.03
Lacertidae						
Podarcis hispanica	14	2.27 ± 0.17	5.38 ± 0.62	9.55 ± 1.06	0.78 ± 0.19	0.71 ± 0.03
Teiidae	45	4.21 ± 0.90	8.37 ± 1.52	12.36 ± 1.93	0.49 ± 0.17	0.75 ± 0.04
Cnemidophorus sexlineatus	30	4.71 ± 0.62	9.15 ± 1.17	12.88 ± 1.95	0.41 ± 0.14	0.76 ± 0.04
Cnemidophorus tigris	9	2.97 ± 0.29	6.48 ± 0.64	10.61 ± 1.32	0.64 ± 0.11	0.74 ± 0.04
Tupinambis rufescens	6	3.55 ± 0.15	7.28 ± 0.47	12.36 ± 0.88	0.70 ± 0.08	0.74 ± 0.02
Anguidae	37	1.69 ± 0.20	4.70 ± 0.50	9.56 ± 0.99	1.04 ± 0.15	0.68 ± 0.02
Elgaria coerulea	14	1.59 ± 0.10	4.59 ± 0.52	9.59 ± 0.97	1.10 ± 0.15	0.67 ± 0.02
Elgaria multicarinatus	11	1.62 ± 0.17	4.56 ± 0.40	9.40 ± 1.29	1.06 ± 0.15	0.68 ± 0.02
Ophisaurus ventralis	12	1.86 ± 0.21	4.97 ± 0.49	9.69 ± 0.74	0.96 ± 0.10	0.69 ± 0.02
Helodermatidae						
Heloderma suspectum	5	2.14 ± 0.32	4.29 ± 0.49	[a]	0.52[b] ± [a]	0.75 ± 0.01
Varanidae						
Varanus exanthematicus	8	7.39 ± 0.48	12.43 ± 1.01	18.37 ± 1.82	0.48 ± 0.12	0.79 ± 0.02
Colubridae	17	24.83 ± 5.08	22.86 ± 3.79	31.12 ± 5.60	0.36 ± 0.12	1.04 ± 0.07
Elaphe guttata	5	18.55 ± 0.96	19.58 ± 2.02	26.94 ± 2.47	0.38 ± 0.10	0.98 ± 0.04
Thamnophis sirtalis	12	27.44 ± 3.43	24.22 ± 3.53	32.86 ± 5.68	0.36 ± 0.13	1.07 ± 0.07

[a] Indicates missing value.
[b] Elasticity for Helodermatidae was based on a single specimen.

TABLE 2. DERIVED VARIABLES REFLECTING FORM OF TONGUE AND ITS MEASUREMENTS RELATIVE TO HEAD SIZE

Variable	Formula	
BASELONG	RTL/BW	Elongation of the resting tongue relative to base width
RELONG	RTL/TW	Elongation of the resting tongue relative to tip width
SELONG	STL/TW	Elongation of the stretched tongue relative to tip width
EXTEND	(STL/RTL) − 1	Elasticity in resting tongue lengths
RELAREA	(TW + BW)/(2BW)	Area of upper lingual surface relative to maximum possible rectangular area

culated from the measured variables (Table 2). The variables BASELONG, RELONG, and SELONG reflect the resting elongation relative to base width and tip width and in part, the tongue's stretchability beyond its resting length. The variable EXTEND gives the increase in length due to stretching, the units being numbers of resting tongue lengths. EXTEND indicates the proportional increase in degree of extensibility during protraction that is attributable to hydrostatic lengthening and intralingual sheathing. RELAREA gives the tongue's area as a proportion of the area of a rectangle of length RTL and width BW. It is calculated from the formula for a trapezoid having height RTL and end lengths TW and BW.

Preliminary regressions showed highly significant correlations of the variables with SVL. An attempt was made to remove allometric effects of body size by analysis of covariance using logarithmically transformed variables with SVL as the covariate, but the preliminary tests revealed pronounced heterogeneity of slope, eliminating that technique.

Another method for removing effects of body length is analysis of residuals of regressions of variables on SVL, but the heterogeneous slopes among families also eliminated that possibility. However, correlations between SVL and the derived lingual variables need not indicate allometry. Such correlations necessarily exist whenever the families differ in both SVL and the values of derived variables. Fortunately, much of the effect of body length is removed because the derived variables are ratios.

A second difficulty is that despite the statistical pooling of data for both sexes, body proportions such as head size differ between the sexes in some species. This effect might tend to obscure interfamilial differences. However, the problem was reduced by including data for only two female *Eumeces laticeps*, a species having strongly sexually dimorphic head size.

The primary statistical analyses were nested analyses of variance for unequal cell frequencies (Sokal and Rohlf, 1981), species being nested within families. Individual comparisons between families were made by using Duncan's multiple-range tests. Some tests for differences between pairs of species within families were conducted by analysis of variance at the specific level followed by Duncan's multiple-range tests.

The derived variables were tested for normality. Significant nonnormality was discovered in BASELONG, EXTEND, and RELAREA. Raising the raw data to the 0.1 power rendered the distribution of EXTEND normal. Applying a logarithmic transformation to RELAREA reduced, but did not eliminate, nonnormality. Nonnormality could not be removed for BASELONG. Differences among families in BASELONG and RELAREA were therefore tested nonparametrically by Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U tests for individual comparisons (Siegel, 1956).

Relationships between derived variables at the familial level were studied by conducting regressions using family means. Some of the derived variables show intercorrelations at the individual level due to shared defining variables. Nevertheless, the various derived variables might or might not be significantly correlated with each other at the familial level. Statistical tests were two-tailed unless otherwise indicated, with $\alpha = 0.05$, with the exception of the unprotected sign tests and Mann-Whitney U tests used for multiple comparisons with BASELONG, for which a significance level of 0.01 was adopted.

Evolutionary histories of elongation and extensibility were examined using the TRACE function of MacClade 3.01 (Maddison and Maddison, 1992). BASELONG was selected to represent elongation because that variable produced the greatest statistical resolution among taxa and the three elongation variables were highly intercorrelated. Characters were coded as unordered multistate transformation series with each character corresponding to a family or group of families for which the variable was statistically distinct. Although extensibility was treated as a single transformation series, lingual elasticity and retractility of the foretongue in Anguidae are distinct traits having effects that are summed as extensibility. Zero was used to represent the lowest quantitative character values and progressively higher integers to represent greater elongation or extensibility. The single data point of 0.52 was used for extensibility in Helodermatidae.

LINGUAL VARIABLES AND CORRELATIONS

Individual Variables: Interfamilial and Intrafamilial Variation

BASELONG. Other things such as hydrostatic elongation and hyoid structure being equal, a highly elongated tongue may be best suited to extension well

beyond the mouth during tongue-flicking. Elongation of the tongue relative to its width at the base varied dramatically among squamate families (Table 1), reaching a pinnacle in snakes over 19 times that in agamine chamaeleonids. By far the greatest elongation among lizards was found in varanids, but even these lizards had less than one third the elongation of the snakes. Among the other lizards, the most pronounced elongation occurred in Teiidae, Lacertidae, and Helodermatidae, all of which tongue-flick at high rates while active. The range of mean elongation was quite small (1.29–1.73) in the lizard families exclusive of these three.

Differences in BASELONG among families were highly significant (X^2 = 213.18, df = 10, $P < 0.001$). Given the distinctness of mean BASELONG values, it is not surprising that Colubridae, Varanidae, Teiidae, and Lacertidae all differ significantly from any of the other families (Table 3). Even with the small sample size, Helodermatidae differed significantly from all but Gekkonidae. The difference between Helodermatidae and Gekkonidae, which approached significance at $P = 0.016$, is very likely a real difference that will be detectable with a larger sample. Even the relatively small differences among the remaining families were consistent enough to be significant with two statistically indistinguishable exceptions: (1) polychrotids, gekkonids, and anguids; and (2) phrynosomatids and chamaeleonids.

Species within three families differed in BASELONG. The teiids *Cnemidophorus sexlineatus* and *Tupinambis rufescens* each had significantly greater

TABLE 3. SIGNIFICANCE OF DIFFERENCES IN BASELONG BETWEEN SQUAMATE FAMILIES[a]

	POL	CHA	GEK	SCI	LAC	TEI	ANG	HEL	VAR	COL
PHR	***[b]	NS	***	***	***	***	***	***	***	***
POL		***	NS	0.001	***	***	NS	0.006	***	***
CHA			***	***	***	***	***	0.004	***	***
GEK				***	***	***	NS	0.016	***	***
SCI					***	***	***	***	***	***
LAC						***	***	0.008	***	***
TEI							***	***	***	***
ANG								0.004	***	***
HEL									0.004	***
VAR										***

[a]Familial abbreviations are ANG, Anguidae; CHA, Chamaeleonidae; COL, Colubridae; GEK, Gekkonidae; HEL, Helodermatidae; LAC, Lacertidae; PHR, Phrynosomatidae; POL, Polychridae; SCI, Scincidae; TEI, Teiidae; and VAR, Varanidae.

[b]***$P < 0.001$; NS, not significant. Probabilities of no difference are less than or equal to the numerical values given.

elongation than *C. tigris* ($U = 1.5$; $df = 9, 30$; $P < 0.001$ and $U = 4$; $df = 6, 9$; $P < 0.007$, respectively), but did not differ significantly from each other. The intrageneric difference might be the result of the different measurement procedure used for *C. sexlineatus*. In Anguidae, *Elgaria coerulea* and *E. multicarinatus* each had significantly lower elongation relative to base width than did *Ophisaurus ventralis* ($U = 13$; $df = 12, 14$; $P < 0.001$ and $U = 22$; $df = 11, 12$; $P < 0.007$, respectively), but were themselves quite similar in elongation ($U = 51$; $df = 11, 14$; $P > 0.10$). The colubrids *Thamnophis sirtalis* and *Elaphe guttata* differed significantly in BASELONG ($U = 0$; $df = 5, 12$; $P < 0.001$), the mean being much greater in the former (Table 3). No significant differences in BASELONG occurred in Phrynosomatidae or Scincidae.

RELONG. As with BASELONG, the greatest resting elongation occurred, as expected, in those taxa that tongue-flick most extensively. Lizard families differed markedly in degree of resting lingual elongation relative to the width of the tongue's tip, elongation being greatest in varanids and teiids (Table 1). Snake tongues showed much greater resting elongation than those of any lizard family. There was considerable overlap in RELONG among several of the remaining families, but RELONG was notably lower in phrynosomatids than in the remaining families.

RELONG clearly differed among lizard families ($F = 634.76$; $df = 10, 224$; $P < 0.001$) and among species within families ($F = 18.57$; $df = 7.224$; $P < 0.001$). Differences among families and among species within families accounted for 97% of the variance in RELONG. Colubrids, varanids, and teiids had significantly higher and phrynosomatids significantly lower RELONG than any of the other families (Table 4). Lacertidae, the family with the fourth

TABLE 4. SIGNIFICANCE OF DIFFERENCES IN RELONG BETWEEN FAMILIES

	POL[a]	CHA	GEK	SCI	LAC	TEI	ANG	HEL	VAR	COL
PHR	*[b]	*	*	*	*	*	*	*	*	*
POL		NS	NS	*	*	*	*	*	*	*
CHA			NS	NS	*	*	*	NS	*	*
GEK				NS	*	*	*	NS	*	*
SCI					*	*	NS	NS	*	*
LAC						*	NS	*	*	*
TEI							*	*	*	*
ANG								NS	*	*
HEL									*	*
VAR										*

[a] Familial abbreviations are in Table 3.
[b] NS, not significant; *$P < 0.05$.

greatest mean RELONG, was statistically distinct from all families except Anguidae. Gekkonidae, Polychrotidae, and Agamidae had relatively low mean RELONG values that did not differ significantly; the same is true for Helodermatidae, Scincidae, Agamidae, and Gekkonidae. In addition, several families at intermediate levels of RELONG did not differ significantly: Anguidae, Helodermatidae, and Scincidae. Despite the numerous nonsignificant comparisons, there is a clear gradient of RELONG from low values in iguanian families to higher ones in autarchoglossan lizards and snakes. Use of preserved specimens of helodermatids may possibly have lowered RELONG artificially if the tongue shrank proportionally more in length than in width at the tip.

Individual comparisons following significant ANOVA at the specific level ($F = 381.04$; $df = 17, 224$; $P < 0.001$) revealed some differences among species within families. For the colubrids, mean RELONG was significantly greater in *Thamnophis sirtalis* than in *Elaphe guttata*. Among teiids, *Cnemidophorus sexlineatus* had significantly greater RELONG than either of the other two species, which did not differ from each other. The presence of an intrageneric difference in the absence of an intergeneric difference among teiids suggests the possibility of a measurement artifact due to lack of magnification during measurements for *C. sexlineatus*. However, the results admit several interpretations, including artifact, lower elongation in *C. tigris*, and possibly allometric differences among the taxa or some combination of these. There were no significant differences in RELONG between species within Anguidae, Scincidae, and Phrynosomatidae.

SELONG. Because SELONG combines resting elongation with any other features allowing additional extension intrinsic to the tongue, but not including any contribution by the hyoid apparatus to protraction, this variable should give the best estimate of overall extensibility during tongue-flicking. As for RELONG, SELONG varies among families by over an order of magnitude. For families other than Helodermatidae, for which data were lacking, SELONG varied significantly among families ($F = 459.88$; $df = 9, 220$; $P < 0.001$) and among species within families ($F = 8.27$; $df = 7, 220$; $P < 0.001$). Differences among families and among species within families accounted for 95% of the variance in SELONG.

The ranks of families were similar to those for RELONG in that colubrids, varanids, and teiids showed the greatest and phrynosomatids and polychrotids the least SELONG (Table 1). Comparison of the significances of differences between families for RELONG (Table 4) and SELONG (Table 5) reveals only two differences: Scincidae differs significantly from Chamaeleonidae and Anguidae for SELONG, but not RELONG. These differences reflect increasing extensibility in the order Chamaeleonidae, Scincidae, Anguidae. There were some differences in ranks of familial means between total elongation, reflected in SELONG, and RELONG, but these were minor and for the most part did

TABLE 5. SIGNIFICANCE OF DIFFERENCES IN SELONG BETWEEN FAMILIES

	POL[a]	CHA	GEK	SCI	LAC	TEI	ANG	HEL	VAR	COL
PHR	*[b]	*	*	*	*	*	*	'	*	*
POL		NS	NS	*	*	*	*	'	*	*
CHA			NS	*	*	*	*	'	*	*
GEK				NS	*	*	*	'	*	*
SCI					*	*	*	'	*	*
LAC						*	NS	'	*	*
TEI							*	'	*	*
ANG								'	*	*
HEL									'	'
VAR										*

[a] Familial abbreviations are in Table 3.
[b] NS, not significant; $***P < 0.05$.
' Missing values.

not correspond to significant differences among pairs of families having reversed ranks.

SELONG also varied significantly among species within families ($F = 262.30$; $df = 16, 220$; $P < 0.001$). The two colubrid species differed significantly, *T. sirtalis* having the greater SELONG. In Teiidae, *C. sexlineatus* and *T. rufescens* had significantly greater SELONG than did *C. tigris*, presumably for the same reasons discussed above. There were no significant differences among species in the anguids, scincids, and phrynosomatids.

EXTEND. The capacity to extend the tongue beyond the mouth for chemical sampling might be accomplished by increasing the tongue's resting length, by making the tongue more elastic, or by a combination of these and intralingual ensheathment. Because hydrostatic stretchability may be important in iguanian lizards that use the tongue to capture prey (Schwenk and Throckmorton, 1989), as well as during lingual feeding cycles (Bels et al., 1994), it was predicted that EXTEND would be greatest in families that use the tongue extensively for chemosensory sampling and in those in which great extension of the tongue occurs during lingual prey prehension.

The predictions that stretchability should occur in families that use the tongue to capture prey and rely on chemical sampling to locate food are contradicted. The tongue of an agamine chamaeleonid exhibited low stretchability despite marked protrusion during lingual prey prehension. Presumably, prey prehension depends on lingual projection (Bels et al., 1994) rather than hydrostatic elongation.

EXTEND was also low in the families most specialized for lingual che-

mosensory sampling. From these first data (Table 1), it would appear that the greatest stretchability is found in families of autarchoglossans that tongue-flick at relatively high rates, but have not evolved either great resting elongation or great proportional tine length. Enhanced stretchability may be interpreted as a possible means of compensating for relatively short resting tongue length by providing greater extensibility and access to chemicals in the external environment. However, it is unclear whether or not EXTEND reflects a real capacity for voluntary lingual elongation.

The tongue was somewhat stretchable in all families studied, but the degree of intralingual extensibility varied considerably (Table 1). It was lowest in the families having greatest BASELONG, i.e., Colubridae, Varanidae, and Teiidae, and in those having the least, Phrynosomatidae and Chamaeleonidae. The greatest intralingual extensibility occurred in Anguidae, in which the tongue was slightly over twice as long when stretched as when resting.

EXTEND differed significantly among families ($F = 75.71$; $df = 9, 220$; $P < 0.001$) and among species within families ($F = 17.16$; $df = 7, 220$; $P < 0.001$), the two factors accounting for 0.78 of the variance. The two families having lowest mean extensibility, Phrynosomatidae and Colubridae, differed significantly from each other, and both had significantly lower means than any of the other families (Table 6). Varanidae, Teiidae, and Chamaeleonidae did not differ significantly from each other, and had mean extensibilities significantly higher than Phrynosomatidae and Colubridae. Varanidae and Teiidae had significantly lower means than the remaining families.

No differences in extensibility were statistically detectable in representatives of Gekkonidae, Polychrotidae, Scincidae, and Lacertidae, but EXTEND

TABLE 6. SIGNIFICANCE OF DIFFERENCES IN EXTEND BETWEEN FAMILIES

	POL[a]	CHA	GEK	SCI	LAC	TEI	ANG	HEL	VAR	COL
PHR	*[b]	*	*	*	*	*	*	[c]	*	NS
POL		*	NS	NS	NS	*	*	[c]	*	*
CHA			*	*	*	NS	*	[c]	NS	*
GEK				NS	NS	*	*	[c]	*	*
SCI					NS	*	*	[c]	*	*
LAC						*	*	[c]	*	*
TEI							*	[c]	NS	*
ANG								[c]	*	*
HEL									[c]	[c]
VAR										*

[a] Familial abbreviations are in Table 3.
[b] *$P < 0.05$; NS, not significant.
[c] Missing values.

in these families was greater than in all other families except Anguidae, with the exceptions that no significant differences occurred between Scincidae, Gekkonidae, and Agamidae. In Anguidae, EXTEND was significantly greater than in all other families. Statistical tests were not conducted for Helodermatidae due to lack of data. However, measurement of a single fresh specimen allowed estimation of a degree of extensibility (Table 1) close to that of varanids and chamaeleonids.

Considerable intrafamilial and intrageneric variation in extensibility was found in phrynosomatids and teiids. In Phrynosomatidae, *Sceloporus malachiticus* had substantially and significantly greater extensibility than *Holbrookia propinqua*, which could be a real difference or an artifact of the lack of magnification during measurements of the latter species. Among the three teiids, the only significant differences were significantly greater extensibility in *Cnemidophorus tigris* and *Tupinambis rufescens* than in *C. sexlineatus*. This might indicate either that the tongue of *C. sexlineatus* is less elastic or that its resting elongation was overestimated relative to that of the other species. No other significant differences were found within families.

RELAREA. RELAREA was conceived as a proportion in which low values would indicate narrowing of the tip of the tongue, which may indicate specialization of the tongue for chemosensory sampling. The tongue's base was always substantially wider than its tip in the lizards. However, in the groups having tongues with the most advanced chemical sampling features, narrowing has continued past the tip of the tongue toward its base. This trend is complete in snakes. Values of RELAREA greater than 1.00 occurred in snakes because the tongue was slightly wider just posterior to the tines than at its base. As a result, lingual surface area was closest to that of a rectangle having dimensions base width and resting length in those squamate families having some of the most elongated and least elongated tongues and the most and least specialized tongues for chemosensory purposes (Table 1).

RELAREA differed significantly among families ($X^2 = 199.24$, $df = 10$; $P < 0.001$). Snakes had much higher RELAREA than any of the lizards (Tables 1 and 7). Phrynosomatids and varanids, having the second and third greatest means, had significantly greater RELAREAs than the remaining families and differed significantly from each other. For Polychrotidae, the mean was significantly greater than for Teiidae and Gekkonidae, but the other differences among Polychrotidae, Teiidae, Helodermatidae, and Gekkonidae were nonsignificant.

Three families, Chamaeleonidae, Scincidae, and Anguidae, had the identical lowest value of RELAREA, i.e., their tongues flared the most from tip to base. These families did not differ significantly from each other, but each had significantly lower RELAREA than any of the other families. Lacertidae, having a RELAREA intermediate to those of the last two groups of families, differed significantly from all families.

TABLE 7. SIGNIFICANCE OF DIFFERENCES IN RELAREA BETWEEN FAMILIES

	POL[a]	CHA	GEK	SCI	LAC	TEI	ANG	HEL	VAR	COL
PHR	***[b]	***	***	***	***	***	***	***	0.005	***
POL		***	0.004	***	***	0.027	***	NS	0.008	***
CHA			0.002	NS	0.008	***	NS	0.004	***	***
GEK				***	0.027	NS	***	NS	0.005	***
SCI					***	***	NS	***	***	***
LAC						***	***	0.010	***	***
TEI							***	NS	***	***
ANG								***	***	***
HEL									0.004	***
VAR										***

[a] Familial abbreviations are in Table 3.
[b] NS, not significant; ***$P < 0.001$. Probabilities of no difference are less than or equal to the numerical values given.

Because deviation from normality occurred in only two families and was not large, ANOVA of logarithmically transformed RELAREA were conducted for comparison, yielding nearly identical results. Differences were highly significant among families ($F = 171.00$; $df = 10, 224$; $P < 0.001$) and among species within families ($F = 3.78$; $df = 7, 224$; $P < 0.001$); they accounted for 0.89 of the variance. The only difference from the nonparametric analysis was that Polychrotidae did not differ significantly from either Gekkonidae or Teiidae.

Differences among species within families were also significant ($X^2 = 200.93$, $df = 17$, $P < 0.001$). The colubrid species had significantly different RELAREAs ($U = 1.5$; $n = 5, 12$; $P < 0.003$), *Thamnophis sirtalis* often having greater width at tip than at the base of the tongue. There were no significant intrafamilial differences in RELAREA for Phrynosomatidae, Scincidae, Teiidae, and Anguidae.

Relationships among Variables

Familial means were significantly correlated for several pairs of lingual variables (Table 8). Very strong relationships occurred between BASELONG, RELONG, and SELONG. These high correlations were expected because the three variables all indicate the degree of elongation relative to different lingual width referents and states of lingual stretching. That the greatest statistical resolution was achieved with BASELONG may merely reflect the larger variation of tip width than base width among families.

Of greater interest are the significant correlations between all three intrinsic

TABLE 8. CORRELATIONS BETWEEN FAMILIAL MEANS OF LINGUAL VARIABLES

	RELONG	SELONG	EXTEND[a]	RELAREA
BASELONG	0.97***[b]	0.94***	0.46[NS]	0.87***
RELONG		0.99***	0.21[NS]	0.80**
SELONG			0.01[NS]	0.71*
EXTEND				−0.68*

[a]For correlations involving EXTEND, $N = 10$; for others, $N = 11$.
[b]*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant at $P > 0.05$.

elongation variables and RELAREA. The relationships with RELAREA are somewhat misleading because two of the four highest values of relative area were found in the two families having the lowest elongation. The relationship is driven by the high lingual elongation in snakes, varanids, and teiids and their correspondingly high values of RELAREA due to narrowing of the tongue further toward the base than in other taxa.

Extensibility was significantly correlated only with lingual area relative to that of a rectangle. Inclusion of Helodermatidae in correlations involving extensibility by using the single measurement of EXTEND as the family mean strengthens the correlation to $r_s = -0.80$, $N = 11$, $P < 0.005$). This relationship highlights the finding that extensibility is greatest in those families that use the tongue in several contexts for chemosensory investigation but have evolved neither great anterior narrowing of the tongue nor narrowing proceeding to the base of the tongue. Intralingual extensibility is maximized in families that are intermediate between extreme vomerolfactory specialists and those that use the tongue little or not at all while foraging.

STABILITY, EVOLUTION, AND FUNCTION

Relative Stability of Shape Within and Between Families

Based on the limited number of species sampled, pronounced, reliable differences appear to occur among squamate families for each of the lingual variables. The high proportions of variance explained by differences among families indicate that several variables clearly partition the families. Differences among families are clearer for elongation than intralingual extensibility or degree of narrowing of the tongue from posterior to anterior, but even the latter variables differ substantially among some families.

Significant intrafamilial variation occurred for all of the lingual variables, but the range of this variation was quite small in comparison to that of inter-

familial variation. One indication of this is that the ratios obtained by dividing F values among species within families by F values among families were quite low, ranging from 0.02 to 0.23, being highest for the variables accounting for the lowest proportions of the total variance. Smaller F values might be expected within families in part due to the large number of families relative to species, but this can account for only a small portion of the large observed difference.

A stronger indication that variation is greater between than within families is that despite the numerous significant differences among species within families, the total range of variation within families invariably occupies only a small fraction of the total range for all families on a given variable. For example, significant differences in BASELONG occurred among species in Teiidae, Anguidae, and Colubridae. However, the total ranges of values for all species of teiids (5.56–11.67) overlapped minimally with those of anguids, but with no other families; ranges for colubrids (17.36–30.00) did not overlap with those of any other families. In addition, the range for Anguidae (3.79–5.82) overlaps with those of the families showing relatively little elongation, but does not overlap with those for Colubridae and Varanidae. Intrafamilial variation in the lingual variables does occur, but its magnitude is much less than that of interfamilial variation.

Chemosensory anatomy and behavior in squamates have long been considered phylogenetically conservative; indeed, they have been used as important taxonomic characters (e.g., Camp, 1923; Schwenk, 1988, 1993). The present findings of much less intrafamilial than interfamilial variation show that lingual shape variables also reflect phylogeny at the familial level. An equally important set of findings, some of which were predictable qualitatively from earlier studies (e.g., McDowell, 1972), is that the lingual variables differ sufficiently to clearly separate numerous squamate families.

Shape Relationships

Relationships among elongation, intralingual extensibility, and relative surface area are summarized in Table 9. The groupings emphasize the opposing trends of elongation and intralingual extensibility and association of intermediate elongation and relative area with high intralingual extensibility. Lingual chemosensory roles during foraging are minimal (Cooper, 1994a,b) in all of the taxa showing combined high relative areas, low elongation, and low to medium elasticity. The low elongation, elasticity, and relative areas of Chamaeleonidae, which lacks lingually mediated prey chemical discrimination, may be attributable to the unique lingual projection mechanism of this family.

In Scincidae the tongue is used to detect and locate prey during foraging. This chemosensory function may account for the wedged shape of the tongue and its low relative area. The scincid tongue exhibits a slightly greater resting elongation (RELONG) than BASELONG due to anterior narrowing, perhaps to

TABLE 9. PATTERNS OF LINGUAL ELONGATION, ELASTICITY, AND DORSAL SURFACE AREA

Relative area[a]	Elongation	Elasticity	Family
High	low	low	Phrynosomatidae
High	low	medium	Polychrotidae, Gekkonidae
Low	low	low	Chamaeleonidae
Low	low-medium	medium	Scincidae
Low-medium	low-medium	high	Anguidae
Medium	medium	high	Lacertidae
High	medium	low	Helodermatidae
High	high	low	Teiidae, Varanidae
Very high	very high	low	Colubridae

[a]Expressed as a proportion of a rectangle of resting tongue length and base width.

enhance chemosensory sampling. However, it has low values of BASELONG and its elasticity is no greater than the plesiomorphic squamate value. The anterior narrowing produces a wedged lingual shape.

In all of the remaining families, lingually mediated prey chemical discrimination is important to foraging and there is clear corresponding evidence of progressive adjustments in lingual morphology related to chemosensory sampling. Lacertids and anguids show intermediate lingual elongation and the highest levels of intralingual extensibility. They are the first groups of lizards mentioned in this section having forked tongues and have relatively poorly developed forking compared to several other families. Correspondingly, surface areas are low or intermediate, reflecting the wedged shape.

In Helodermatidae, lingual area is high, elongation is slightly greater than in anguids but nearly identical to that of lacertids, and development of forking is slightly greater than in lacertids (Schwenk, 1994). The moderate lowering of extensibility may have been made possible by increasing elongation and improved chemical sampling by the forked portion of the tongue. The high relative area possibly may represent no change from the ancestral condition.

In the two lizard families having the greatest lingual specialization for chemical sampling, Varanidae and and Teiidae, the relative area is high and the tongues are highly elongated. In Varanidae, and perhaps Teiidae, this is attributable to narrowing toward the base, so that the tongue has, in effect, passed its maximal wedging and is returning due to a more rectangular condition. Low elasticity corresponds to great elongation in these families. These trends are more pronounced in Varanidae, presumably due to the loss of a lingual role in swallowing.

Colubrid snakes exhibit by far the greatest specialization for lingual chem-

ical sampling. The greatest elongation is combined with very low elasticity and the greatest relative area. Relative area is great, presumably due to loss of lingual functions unrelated to chemical sampling. That elasticity is less and elongation greater in colubrids than in varanids suggests both an increased importance of lingual chemical sampling in snakes and continued evolution toward the optimal shape and resting elongation for chemical sampling.

Evolution and Functional Significance

Lingual shape may be expected to reflect important lingual functions including chemosensory behavior as well as food manipulation (prehension, reduction, transport, swallowing) and possibly drinking (Bels et al., 1994). For example, all three measures of elongation have strong positive correlations with quantitative measures of chemosensory responses to prey chemicals and with degree of activity during foraging, which is in turn related to use of the tongue to locate prey (Cooper, unpublished data). The probable evolutionary histories of the shape variables are outlined below and their possible adaptive significances are addressed.

Elongation. Based on statistically distinct ranges of character values, the evolution of lingual elongation (BASELONG) is shown in Figure 2. This portrayal suggests that the degree of elongation in polychrotids and gekkonids is plesiomorphic. Within Iguania, elongation may have decreased in Phrynosomatidae and Chamaeleonidae. Due to unresolved familial relationships in Iguania, it is unclear whether any decreases occurred independently in these families.

The state of elongation is uncertain in ancestral scincomorphans (the ancestors of Scincidae, Teiidae, and Lacertidae among others), but the tongue is less elongated than the plesiomorphic condition in Scincidae and more elongated in both Lacertidae and Teiidae. The relatively low BASELONG value of skinks may be somewhat misleading because RELONG and SELONG are both greater than in the plesiomorphic condition. It is clear that elongation increased beyond the plesiomorphic values either in ancestral scincomorphans or in the ancestors of Lacertiformes (Lacertidae, Teiidae, and Gymnophthalmidae).

All three varanoid families (Helodermatidae, Varanidae, and Colubridae) have greater lingual elongation than did plesiomorphic lizards, and this increase appeared independently of that in Lacertiformes. The condition in ancestral varanoids is shown as equivocal because it is uncertain to what degree the tongue elongated. Nevertheless, some degree of elongation occurred in ancestral varanoids and further increases may have occurred in the common ancestor of snakes and varanids and autapomorphically within Serpentes.

There is a striking correspondence between the evolutionary origins of lingual elongation and the evolution of forked tongues. Lingual tines appear to have had independent origins in Lacertiformes and Varanoidea, based on current

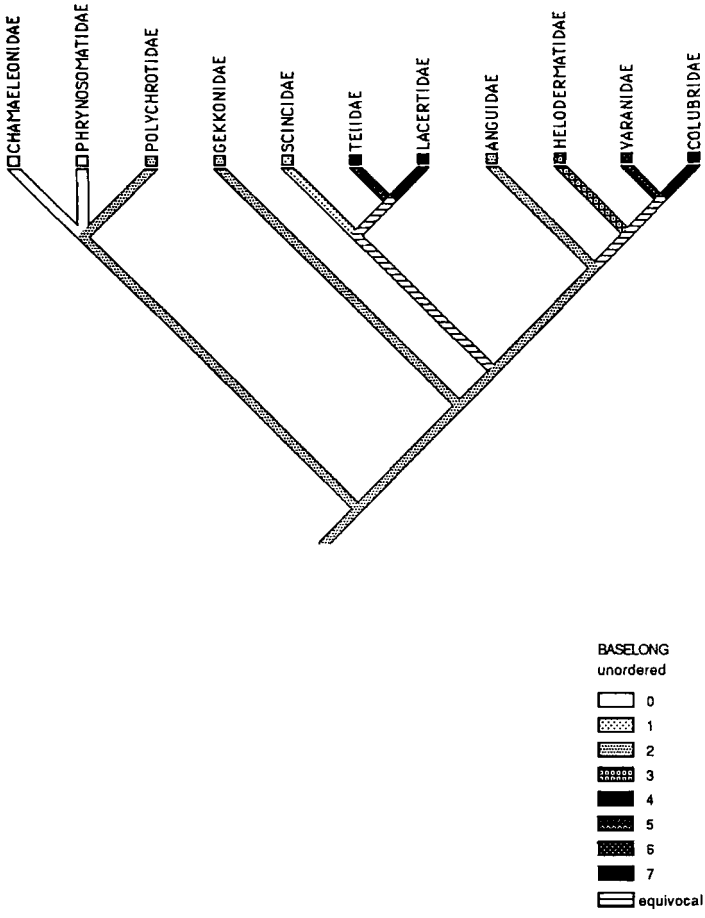

FIG. 2. Evolution of lingual elongation (BASELONG) in Squamata. Zero was used to represent the lowest quantitative character values and progressively higher integers to represent greater elongation.

views of the probable relationships of Serpentes and Amphisbaenia (Schwenk, 1994). However, up to four independent origins are possible if Amphisbaenia is not part of Lacertiformes and Serpentes is not correctly placed in Varanoidea (Schwenk, 1994). Although data are lacking on elongation in Amphisbaenia, the data do indicate the independent evolution of increased elongation in the same two taxa in which tines originated, with a possible additional independent origin in Serpentes.

The exact correspondence of origins between lingual forking and elongation strongly suggests a functional link between these traits. The obvious commonality is that both traits improve lingual chemosensory sampling ability. Forking enhances the ability to follow trails, likely by tropotaxis (Schwenk, 1994), and may also increase the surface area involved in gathering samples from substrates. Elongation presumably improves the ability to bring the tip of the tongue, including tines, into contact with substrates to be sampled. Forking and elongation appear to have coevolved as joint participants in lingual chemical sampling in those taxa that rely most heavily on the lingual–vomeronasal system in foraging and exhibit lingually mediated prey chemical discrimination and strike-induced chemosensory searching (Cooper, 1994a,b).

Using RELONG as the measure of elongation, anguids have tongues that are somewhat elongated beyond plesiomorphic values. The correspondence between origins of forking and elongation breaks down because all autarchoglossans have greater than plesiomorphic elongation. Nevertheless, the greatest elongation originates in the taxa where forking originates. This statement also requires some qualification based on the definition of forking. Whereas McDowell (1972) states that tines are present in anguids, Schwenk (1994) does not recognize the abbreviated structures of Anguids as tines. Whichever definition is used, the underlying relationships are unaffected.

Provided that elongation allows the tongue to be protruded further out of the mouth, it presumably facilitates chemosensory sampling of areas from which the head is excluded, such as narrow crevices. Any increase in the ability of the tongue to collect molecules from substrates a greater distance outside the mouth might also aid in chemosensory investigation of potential prey or other objects without approaching as closely. Greater elongation would also permit sampling volatiles from a greater volume of air.

Protrusion distance for chemosensory sampling could be enhanced in several ways. First, the resting tongue length could be augmented, possibly in conjunction with lengthening of the head. The pattern of interfamilial variation in BASELONG and RELONG to be discussed next shows that this has occurred during squamate evolution. Second, an increase in lingual elasticity (capacity for hydrostatic lengthening) might allow greater protrusion distance even if resting elongation were constant. Elasticity might be important for the alternative or additional reason that it might provide increased flexibility (see discussion of lingual musculature, flexibility, and chemosensory sampling in Bels et al., 1994), perhaps facilitating lingual sampling in crevices or at large angles to the head. Third, a portion of the tongue could be folded or the anterior portion ensheathed within the remainder of the tongue while at rest, as in anguids. Fourth, mechanisms for lingual protraction could be altered by evolutionary changes in intrinsic lingual musculature (reviewed by Bels et al., 1994) even in the absence of the other changes. All of these changes might occur simultaneously.

The hypothesized importance of elongation for chemosensory sampling is supported by the observations that: (1) Some of the least elongated tongues occur in three iguanian families lacking lingually mediated prey chemical discrimination (Cooper, 1989a, 1994c). These families have the lowest values of total possible elongation as indicated by stretched elongation. The only apparent exceptions are that the species sampled in the iguanian family Polychrotidae has slightly greater BASELONG than that measured in members of the scleroglossan family Scincidae and the sole chamaeleonid species has slightly (but not significantly) greater RELONG than the gekkonid. The resting and basal elongation in Polychrotidae does not reflect any current chemosensory adaptation related to vomerolfaction because polychrotids have very poorly developed vomeronasal organs (Gabe and Saint Girons, 1976) and show no signs of lingually mediated detection of prey chemicals (Cooper, 1989a). (2) There is consensus among all three measures that the greatest elongation is found in Colubridae, Varanidae, and Teiidae, the three families most specialized for lingual–vomeronasal chemoreception. (3) Intermediate values are found in four autarchoglossan families (Lacertidae, Scincidae, Anguidae, and Helodermatidae) that are less extreme chemosensory specialists but are capable of lingually mediated prey chemical discrimination (Cooper, 1989b,c, 1990a,b, 1991) and in Gekkonidae, a family in which the presence of a high percentage of chemoreceptor cells in the vomeronasal organ suggests a greater capacity for making vomerolfactory discriminations than in iguanians (Gabe and Saint Girons, 1976).

Extensibility. Intralingual extensibility (Figure 3) has a very different history than elongation and forking. As for elongation, the plesiomorphic condition of extensibility is an intermediate value indicating a fairly high capacity for being stretched. Elasticity may have decreased in Phrynosomatidae and Chamaeleonidae, but as for elongation, it is unclear whether the decrease represents one or two independent origins. The lowest values occur not only in iguanian families, but also in the most extreme chemosensory specialists, i.e., Colubridae, Varanidae, and Teiidae. The families having the greatest intralingual extensibility are Anguidae and Lacertidae, families in which lingually mediated prey chemical discrimination is important but in which tines are either absent or relatively small.

The only significant increase in elasticity beyond the plesiomorphic condition is seen in Anguidae, but it is uncertain whether any change in extensibility occurred in ancestral anguimorphans (anguids + varanoids). Elasticity in ancestral varanoids is less than the plesiomorphic condition. Regarding the equivocal condition in ancestral anguimorphans, some decrease might have occurred along that internode prior to a great increase in Anguidae, but the proposed function of extensibility to increase lingual protrusibility suggests that the decrease is more likely to have begun in ancestral varanoids. A further decrease occurred in snakes. The only other decrease in elasticity is in Teiidae.

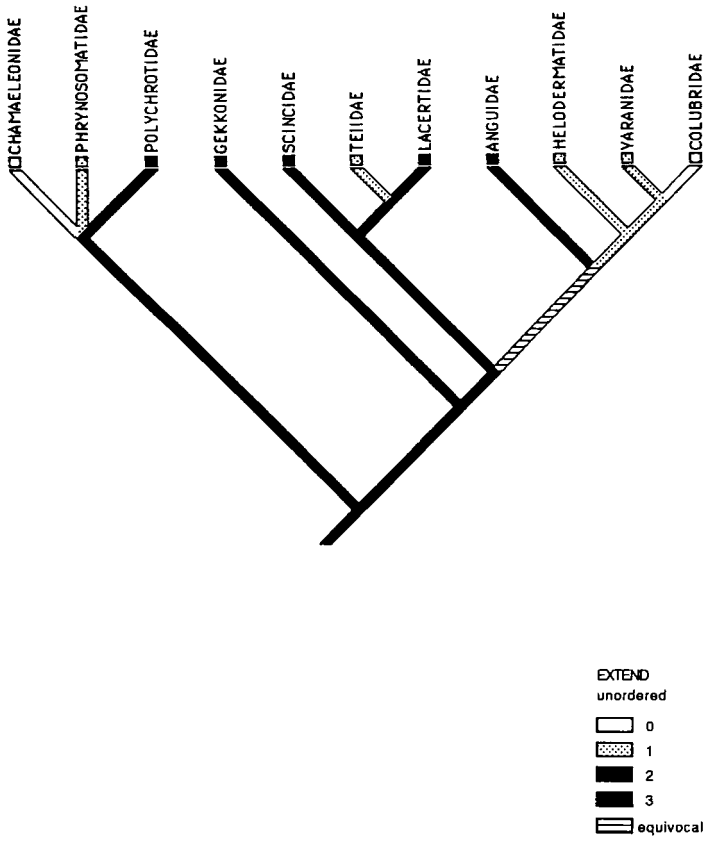

FIG. 3. Evolution of intralingual extensibility in Squamata. The primary contributing factors are elasticity and (in Anguidae) retractility of the foretongue into the anterior portion of the hindtongue. Zero was used to represent the lowest quantitative character values and progressively higher integers to represent greater extensibility.

There is a close, but imperfect relationship between changes in forking and elongation and changes in elasticity. Elasticity decreased and both forking and increased elongation evolved in Varanoidea, with further decrease in elasticity corresponding to the greatest elongation in Colubridae. In Iguania, no change in forking occurred, but both elongation and elasticity decreased in Phrynosomatidae and Chamaeleonidae. The correspondence is imperfect in Lacertiformes because forking originated and elongation increased in both Teiidae and Lacertidae, but elasticity decreased only in Teiidae.

These correlated evolutionary changes support the conjecture that lingual elasticity and elongation may be alternative, but jointly acting, mechanisms of increasing the maximum distance that the tongue may be protruded beyond the mouth. If so, the absence of a decrease in elasticity in Lacertidae despite the presence of tines and a slight degree of elongation may indicate that the resting tongue is not long enough to permit optimal chemical sampling without some degree of augmented extension afforded by elasticity.

If extensibility is a mechanism permitting greater lingual extension when the tongue is too short to permit optimal sampling, maximal extensibility should occur in those families that rely heavily on chemosensory sampling, but lack great lingual elongation. Anguids and lacertids have the most extensible tongues. In both families, the tongue is elongated slightly above plesiomorphic values and the degree of RELONG is very similar. Anguids either lack or have very short tines and lacertids have the relatively shortest tines among the remaining taxa having forked tongues (Schwenk, 1994). The prediction is thus borne out by the occurrence of greatest extensibility in the two families showing intermediate specialization for chemosensory sampling.

The great similarity of SELONG in Anguidae and Lacertidae suggests that additional elongation provided by elasticity and/or retractility of the foretongue may be especially advantageous when the total achievable elongation is about 9.5 times the width at the tip of the tongue. Teiids, varanids, and colubrids have SELONG values over 12 and the other families have SELONG values of roughly 2.5–7.0. The expectation of high extensibility in the range above 7 and less than 12 can be tested in Amphisbaenia and Xenosauridae.

Although lingual elongation and extensibility are not significantly correlated, intralingual extensibility and elongation might be importantly related as alternative means of achieving protrusibility. In squamates having the greatest resting tongue length, the needed protrusibility is achieved without exceptionally great intralingual extensibility. However, any initial evolutionary development of a highly elastic or otherwise extensible tongue for chemosensory sampling might have been effected by muscular innovations such as acquisition of a well-developed intrinsic circular muscle system, which is absent or weakly developed in iguanians but strongly developed in scleroglossans (Schwenk, 1988, 1993).

In all of the families studied here other than Anguidae, EXTEND is a measure of lingual elasticity. In anguids the foretongue is ensheathed in a region of invagination between the foretongue and hindtongue (McDowell, 1972). Because the foretongue of anguids is retractile, measures of the tongue's resting length underestimate its total length. Thus, EXTEND in anguids measures the total proportional increase in length due to both elasticity and extension of the foretongue from its sheath.

Elasticity as measured here could be a quantitative measurement of the degree of hydrostatic lengthening (Smith, 1984; Schwenk, 1986) or bear some

functional relationship to it. However, it seems unlikely that the degree of elasticity observed can be accounted for entirely by hydrostatic lengthening (Schwenk, personal communication). The extent to which elasticity permits voluntary elongation, whether by hydrostatic lengthening or other mechanisms, is unknown.

Although variation in intralingual extensibility among families could be epiphenomenal, it will likely prove to be explicable in terms of lingual functions in swallowing and chemical sampling. Competing demands for efficient swallowing, intraoral prey handling, lingual prey prehension, grooming, and chemosensory sampling presumably result in selective compromises reflected in lingual resting and extended shapes. Beyond the obvious potential benefits of increased extensibility and flexibility for lingual chemical sampling, some degree of elasticity may be important for the other lingual roles noted above.

Selective compromise might account for the restriction of highest levels of extensibility to families having short tines and relatively unelongated resting tongues. This restriction also is consistent with the hypothesis that greater extensibility evolved to permit behavioral compensation for limitations on lingual extension for chemosensory sampling in families lacking great resting elongation. These hypotheses are not mutually exclusive.

The decrease in elasticity and high resting elongation found in families having the most highly developed tines also is consistent with the compensatory interpretation. According to the hypothesis, resting elongation and elasticity initially increase as chemosensory demands result in evolution of distinct tines. As this process continues in families having very strong chemosensory orientation, tines increase in proportional length and resting elongation continues to increase. When the resting elongation in concert with means of protrusion other than intralingual extensibility becomes sufficient to meet the heightened need for chemical sampling, intralingual extensibility begins to decrease, falling to levels as low as in families not relying on the tongue to detect prey vomodors.

A reconstruction of the evolutionary history of SELONG by MacClade's trace function suggests that the initial evolutionary increase in total achievable elongation occurred in the common ancestor of autarchoglossa, which includes the vast majority of actively foraging lizards and all lizards and snakes showing forking, elongation, and behavioral specializations for lingual chemosensory sampling. This reinforces the hypothesis that the total elongation due to the combination of resting elongation and extensibility is important for chemosensory sampling.

A different interpretation might be that elasticity decreases in those families having the greatest resting lingual elongation, but for reasons unrelated to chemosensory sampling. The decrease might be thought to have occurred simply because the tongue has lost its role in swallowing (as in varanids and snakes) (McDowell, 1972). However, although retention of some degree of elasticity may be important for glutitional function, elasticity is also low in Teiidae,

Phrynosomatidae, and Chamaeleonidae, families in which the tongue does participate in swallowing (Bels et al., 1994). The unique lingual projection mechanism of chamaeleonids may explain the decreased elasticity in that family, but not in the other two families. Lingual function during swallowing cannot explain the high extensibility in Anguidae or the increase in total elongation in the common ancestor of Autarchoglossa in the absence of an increase in resting elongation.

Surface Area. The probable evolution of dorsal lingual surface area relative to that of a rectangle is shown in Figure 4. The surface area of the tongue is a

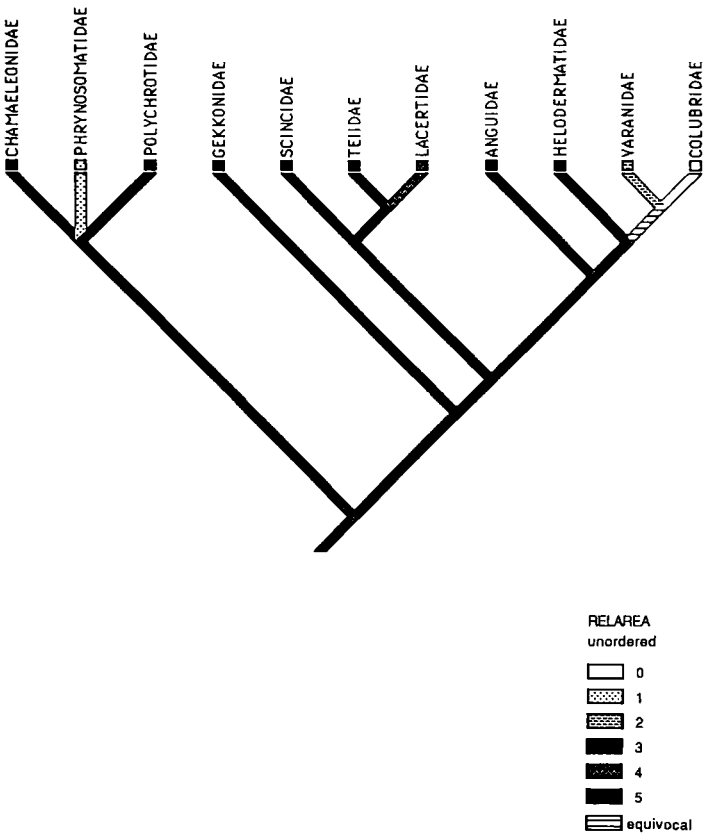

FIG. 4. Evolution in Squamata of lingual surface area relative to that of a rectangle having sides equal to resting tongue length and base width. Zero was used to represent the highest quantitative character values and progressively higher integers to represent lower relative lingual area.

relatively high proportion of that of a rectangle with sides equal to resting tongue length and base width in phrynosomatids, polychrotids, and gekkonids, families of lizards having tongues that are relatively unelongated and lack tines. Relative area may have decreased from the plesiomorphic level in Chamaeleonidae and increased in Phrynosomatidae. These changes may be related to the lingual projection mechanism of chamaeleonids or to relative widths of posterior arms of the tongue that may function in manipulation of food (McDowell, 1972; Bels et al., 1994).

The evolutionary path shown in Scincomorpha (represented here by Scincidae, Lacertidae, and Teiidae) illustrates the hazards of using shape variables that may be based on multiple underlying factors. A decrease in relative area occurred in Lacertidae and Scincidae, but not in Teiidae. As teiids have nearly the plesimorphic value of relative area, the plesiomorphic condition is depicted as present in the ancestors of Scincomorpha and Lacertiformes.

It is far more likely that RELAREA actually decreased in ancestral scincomorphans and may have decreased further in the ancestor of Lacertiformes. The decrease in Scincidae and Lacertidae primarily reflects narrowing toward the tip of the tongue, resulting in a wedged shape. As the tongue becomes progressively elongated, it also tends to become narrower posteriad. Thus, in Teiidae, in which the tongue is more elongated than in any families save Varanidae and Colubridae, the effect of the narrow tongue tip is just offset by the posterior narrowing to produce the observed relative area.

In Anguimorpha relative area appears to have decreased in anguids and increased in Varanoidea, presumably increasing in ancestral varanoids and further in colubrids. The most likely interpretation of the depicted evolutionary path is that narrowing at the tip began in the ancestor of Autarchoglossa or of Anguimorpha, then RELAREA returned to the apparently plesiomorphic condition in Helodermatidae as progressive elongation with narrowing toward the base occurred, and finally RELAREA increased above plesiomorphic levels in Varanidae and Colubridae as posterior narrowing continued.

In taxa having relatively broad tongues, tapering from base to tip might increase lingual sampling abilities by: (1) allowing access of the sampling surface to restricted spaces and (2) concentrating the sample in a narrow region having the primary responsibility for indirect transfer of chemicals to the vomeronasal ducts. Retention of a broad base might be important for swallowing or other lingual functions. In taxa such as Serpentes and Varanidae, which have narrow, rectangular tongues, the lack of participation of the tongue in swallowing or manipulation of food (McDowell, 1972) likely eliminated the need for a wide base, freeing the tongue to evolve toward the optimal shape for chemosensory sampling.

The negative correlation between extensibility and relative area may also reflect lingual chemosensory roles. The families having high extensibility, puta-

tively for chemosensory sampling, are precisely those having some anterior narrowing without exceptional resting elongation, a combination yielding low relative area. Other families have low extensibility combined with high relative areas due either to the relative unimportance of chemosensory sampling or to evolution of alternative means obviating the need for great intralingual extensibility.

Caveats

In this study I have speculated from a very narrow data base. Nevertheless, the major findings regarding lingual shape are presumably robust because lingual structure tends to be very conservative within squamate families. The generalizations that I have attempted to infer from the limited taxonomic base must be considered hypotheses requiring verification by data on a wider range of squamate families, genera, and species. This is especially clear for the preliminary reconstructions of evolutionary histories of the shape variables; these must be taken as provisional hypotheses.

I have speculated also about evolutionary and functional relationships among lingual elongation, intralingual extensibility, and lingual surface area relative to that of a rectangle. Behavioral data on the importance of these aspects of lingual shape are needed to verify or refute several of my functional assumptions. Perhaps the most conjectural topic covered is the importance of intralingual extensibility due to a combination of elasticity and extension of any folded portion. The data suggest the hypothesis that intralingual extensibility compensates for resting elongation insufficient to allow optimal protrusion of the tongue beyond the mouth for chemosensory sampling. It must be emphasized that this novel idea is not to be taken as a conclusion. It is merely a preliminary hypothesis that must be tested. Information is needed regarding intralingual extensibility in additional taxa, including the families noted earlier.

Kurt Schwenk (personal communication), who has thoroughly studied squamate lingual anatomy (e.g., Schwenk, 1984), has suggested that the lengthening caused by stretching the tongue is an artifact, not a natural ability. For the hypothesis to be valid, squamates must be able to voluntarily increase the length of the tongue. It is not necessary that they be able to do so to the maximal extent that I measured by stretching the tongue artificially, but the magnitude of voluntary extension must be proportional to the stretched extension. Data on the magnitude of voluntary intralingual lengthening are unavailable, but are crucial to the hypothesis.

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THE "RAISON D'ÊTRE" OF PYRROLIZIDINE ALKALOIDS IN *Cynoglossum officinale:* DETERRENT EFFECTS AGAINST GENERALIST HERBIVORES

NICOLE M. VAN DAM,[1,2,*] LUCIENNE W.M. VUISTER,[1] CORA BERGSHOEFF,[1] HELENE DE VOS,[1] and ED VAN DER MEIJDEN[1]

[1]*Institute of Evolutionary and Ecological Sciences*
Research Group Ecology of Plant-Animal Interactions
P.O. Box 9516, 2300 RA Leiden, The Netherlands

[2]*Present address: Department of Entomology*
University of California, Riverside, Riverside, California 92521

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Abstract—In this study we tested whether pyrrolizidine alkaloids (PAs) of *Cynoglossum officinale* serve as antifeedants against herbivores. Total PA N-oxide extracts of the leaves significantly deterred feeding by generalist herbivores. Specialist herbivores did not discriminate between food with high and low PA levels. Three PAs from *C. officinale*, heliosupine, echinatine, and 3'-acetylechinatine, equally deterred feeding by the polyphagous larvae of *Spodoptera exigua*. Although the plants mainly contain PAs in their N-oxide form, reduced PAs deterred feeding by *S. exigua* more efficiently than PA N-oxides. On rosette plants, the monophagous weevil *Mogulones cruciger* significantly consumed more of the youngest leaves, which had the highest PA level and the highest nitrogen percentage. Larvae of *Ethmia bipunctella*, which are oligophagous within the Boraginaceae, did not discriminate between leaves. All generalist herbivores tested significantly avoided the youngest leaves with the highest PA levels. In the field, the oldest leaves also were relatively more damaged by herbivores than the youngest leaves. It is hypothesized that the skewed distribution of PAs over the leaves of rosette plants reflects optimal defense distribution within the plant.

Key Words—*Cynoglossum officinale*, Boraginaceae, pyrrolizidine alkaloids, chemical defense, specialist herbivores, generalist herbivores, *Ethmia bipunctella*, *Mogulones (Ceutorhynchus) cruciger*, *Spodoptera exigua*, *Helix aspersa*, *Frankliniella occidentalis*, *Locusta migratoria*, *Lyriomyza trifolii*.

*To whom correspondence should be addressed

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INTRODUCTION

Like philosophers who search for the meaning of life, chemical ecologists look for the "raison d'être" of secondary plant substances (Fraenkel, 1959) or, more specifically, alkaloids (Wink, 1993). Pyrrolizidine alkaloids (PAs) are mainly found in the Asteraceae and the Boraginaceae (Hartmann, 1991). Although the toxicity of PAs to vertebrates has been well documented (Greimer, 1900; Mattocks, 1986; Anonymous, 1989; Wink, 1993), their role as defenses against insect herbivores is less clear. It is often assumed "intuitively" (Wink, 1993) that PAs protect the plant against insect herbivores as well, although evidence for this assumption is sparse (Hartmann, 1991) and even contradictory (see Bentley et al., 1984).

Plants containing PAs still can suffer considerable herbivore damage. In the first place, the plants can be damaged by specialist herbivores that have adapted to PAs (Prins and Nell, 1990; Van der Meijden et al., 1991). Secondly, the total PA content may not be related to herbivory, because the different types of PAs in the plant are not equally deterrent (Bentley et al., 1984; Dreyer et al., 1985; Speiser et al., 1992). Thirdly, some plant parts contain less PAs than others (Hartmann and Zimmer, 1986; Hartmann et al., 1989; Van Dam et al., 1994).

In order to generate a hypothesis on the role of PAs in the feeding behavior of herbivores on the whole plant, we have to consider both the plant's and the herbivore's point of view. Young leaves are more valuable for the plant than old leaves, because they have the highest photosynthetic rates and the longest life expectancy (Harper, 1989). Meanwhile, young leaves are more attractive for herbivores (Hodkinson and Hughes, 1982; Soldaat and van der Meijden, 1990) because they contain higher nitrogen and water percentages (Mooney and Gulmon, 1982). According to optimal plant defense theory, the young leaves should thus be protected best (Zangerl and Bazzaz, 1992). Based on theories of coevolution of plants and herbivores (Ehrlich and Raven, 1964), it can be expected that specialist herbivores will not be deterred by high chemical defense levels in the youngest leaves, while generalists will (Cates, 1980).

In this paper we test whether PAs of rosette plants of *Cynoglossum officinale* L. (Boraginaceae) act as defenses against herbivores. PA levels differ significantly between leaves on the same rosette plant; the youngest leaves have 50–190 times higher PA concentrations than the oldest leaves (Van Dam et al., 1994). *C. officinale* contains several PAs, such as echinatine, 3′-acetylechinatine, heliosupine, trachelanthamine, and viridiflorine (De Jong et al., 1990; Hartmann and Witte, 1994; Van Dam et al., 1995), which are mainly present in the N-oxide form (Sykulska, 1962; Van Dam et al., 1995).

C. officinale is a host plant for a range of herbivores (De Jong et al., 1990). Nevertheless, herbivory on *C. officinale* is usually less than 10% of the leaf

area (Prins and Nell, 1990), indicating that the plants are well defended. For our experiments, we chose two specialist herbivore species, the monophagous weevil *Mogulones cruciger* and the oligophagous larvae of *Ethmia bipunctella*. Both herbivores are commonly found on *C. officinale* in The Netherlands (Prins and Nell, 1990; Prins et al., 1992). We also tested the deterrency of PAs on five herbivore species that are not specialized on *C. officinale*. The polyphagous snail, *Helix aspersa*, is the only one of these five species that is naturally occurring in the plant population we studied (Gittenberger et al., 1970). *Lyriomyza trifolii*, *Frankliniella occidentalis*, and *Spodoptera exigua* all are imported, horticultural pest species from different orders within the Insecta. Finally, we used *Locusta migratoria*, which is an oligophagous leaf-chewer that mainly feeds on grasses (Bernays and Chapman, 1977).

METHODS AND MATERIALS

Plant Species. Cynoglossum officinale L. (Boraginaceae) is a facultative biennial that is commonly found throughout the temperate zones of Europe and Asia (De Jong et al., 1990). The plants used in these experiments originated from the calcareous dune area, Meijendel, near The Hague, The Netherlands. In addition to PAs, the leaves of *C. officinale* are known to contain several other secondary metabolites, such as flavonoids, naphthoquinones, and saponins (Hegnauer, 1964; De Jong et al., 1990).

Herbivores. Mogulones (Ceutorhynchus) cruciger Herbst (Coleoptera, Curculionidae) is monophagous on *C. officinale* (Freese, 1990). The genus *Ceutorhynchus* has recently been revised by Colonelli (1986). The name of this weevil, therefore, has been changed to *Mogulones cruciger* (Heijerman, 1993). The adults feed on the leaves, and the larvae develop in the rootcrown (Dieckmann, 1972), by which they significantly reduce plant weight and seed output (Prins et al., 1992). Adults of *M. cruciger* were sampled in the coastal dune area, Meijendel, The Netherlands, and were kept on leaves of *C. officinale* until they were used for experiments. The larvae of *Ethmia bipunctella* F. (Lepidoptera, Ethmiidae) are oligophagous and feed exclusively on leaves of Boraginaceae (Sattler, 1967). Larvae of *E. bipunctella* were also sampled in Meijendel and propagated on *C. officinale* leaves in the lab (25°C/16 hr light, 18°C/8 hr dark, 70% relative humidity). For the experiments we used second and third stage lab-reared larvae.

Helix aspersa Müller (Gastropoda, Helicidae) occasionally damages leaves of *C. officinale* plants in our experimental garden (K. Vrieling, personal communication). *H. aspersa* individuals were sampled in the garden around the laboratory and kept on wet filter paper and nettle leaves at room temperature before they were used in the experiments.

Lyriomyza trifolii Burgess (Diptera, Agromyzidae) is a polyphagous leafminer, known to feed on over 100 plant species (Harborne, 1988). The larvae feed in the leaf on the mesophyll layer, producing contorted mines (Minkenberg and van Lenteren, 1986). *Frankliniella occidentalis* Pergande (Thysanoptera, Thripidae) also is an extremely polyphagous herbivore. Both adults and larvae feed by piercing leaf cells with their mandibles and ingesting the content through the stylet (Hunter and Ullman, 1989). *Frankliniella* spp. are deterred by lupine alkaloids (Gustafsson and Gadd, 1965). Both *L. trifolii* and *F. occidentalis* were reared on chrysanthemum plants, cv. Ultra Light (20°C continuous 12 L:12 D photoperiod, 70% relative humidity, and 25°C continuous 16 L:8 D photoperiod, 70% relative humidity, respectively). The larvae of *Spodoptera exigua* Hübner (Lepidoptera, Noctuidae) are polyphagous leaf-chewing herbivores (Heath, 1983). *S. exigua* and other *Spodoptera* species are commonly used in antifeedant tests and are known to be sensitive to several types of alkaloids (Aerts et al., 1992; Krug and Proksch, 1993). The larvae were reared in our laboratories on an artificial diet (Aerts et al., 1992) at 25°C/16 hr light, 18°C/8 hr dark and 70% relative humidity. For the experiments we used second to fourth stage larvae. Adults of *Locusta migratoria* L. (Orthoptera, Acrididae) were kept on grass at room temperature before experimentation.

Choice Tests. Disks (2 cm diam.) were punched from young, fully expanded leaves and from old leaves of rosette plants of *C. officinale*. Two disks were placed in every Petri dish (9 cm diam.), which was lined with wet filter paper. One herbivore was placed in each Petri dish. All choice tests were replicated 10 to 20 times, depending on availability of the species. The choice tests with *L. migratoria* were conducted in transparent plastic cups, 9 cm diam., 7 cm high. The disks were fixed in the cups with needles.

We tested two choice situations: a disk of a young leaf versus a disk of an old leaf, and a disk of an old leaf treated with MeOH (control) versus a disk of an old leaf treated with PA N-oxide solution in MeOH. Every disk was painted with 3 μg PA/mg fresh weight (FW), which is comparable to the mean PA level of the youngest three to four leaves. The control disks were treated with the same volume of pure MeOH to rule out solvent effects. The area eaten was scored after three days, except for the *L. migratoria* adults, which were so voracious that the amount eaten had to be scored after one day.

Deterrency of Different PAs. The difference in deterrency between different types of PAs was tested with *S. exigua* larvae. For this experiment leaves of lettuce (*Lactuca sativa* L.) were used as a carrier for the PA solutions, since they are well accepted by *S. exigua* larvae and do not contain PAs. The deterrency of the PAs was tested in a no-choice situation, using lettuce disks (2 cm diam.) treated with MeOH or a methanolic solution of either heliosupine, 3′-acetylechinatine, or echinatine. Since we did not want to fully deter feeding, we applied a relatively low dose of 0.5 μg PA/mg FW. Dose–response experiments with total PA extracts showed that at this concentration the feeding

deterrency was about 50% relative to control disks (Van Dam et al., unpublished data). Four disks treated with the same solution were placed in a Petri dish lined with wet filter paper. Per Petri dish, two second or third stage *S. exigua* larvae were allowed to feed overnight. Every treatment was replicated 10 times. Leaf disks were copied onto quadrille paper and damage was scored as square millimeters area eaten.

PA N-Oxides versus Reduced PAs. Disks from lettuce leaves received 2 μg PA N-oxide/mg FW or 2 μg reduced PA/mg FW, respectively. The two treatments were offered in a choice situation to second stage *S. exigua* larva (20 replicates).

Whole Plant Experiments: Herbivory in the Lab. Seeds of *Cynoglossum officinale* plants were sampled in Meijendel in 1992, scarified, and soaked for two weeks at 20°C/16 hr light, 10°C/8 hr dark. The seedlings were potted in 1-liter pots, each containing a mix of dune sand and compost (1 : 1). Nutrients were supplied by adding 3 g Osmocote Plus (15% N, 11% P, 13% K, 2% MgO, Sierra Chem. Co., Milpitas, CA, USA) per pot. At the start of every experiment, plants of the same half-sib family that had equal numbers of leaves were selected and placed in transparent cylinders. The herbivores were released on the ground near the plant. Since the herbivores differed in voracity, the number of individuals released per plant differed. Per plant, either three adults of *M. cruciger* (six replicates), one individual of *E. bipunctella* (10 replicates), two individuals of *H. aspersa* (21 replicates), 20 females of *F. occidentalis* (12 replicates), or one larva of *S. exigua* (20 replicates) were released near every plant. The herbivores were allowed to feed until a substantial amount of damage was visible. After this period, the absolute amount of damage (area eaten) and the leaf area were scored separately for every leaf. For *F. occidentalis* the percentage of leaf area with silver damage was measured.

For the leaf-miner, eight plants were left for 1 hr in the *L. trifolii* breeding room to allow female flies to lay eggs in the leaves. Seven days later the number of large mines was counted. With this method, the combined effect of antixenotic effects on the choice of female flies and antibiotic effects on the larvae was measured.

Some control plants were kept apart to measure PA content, percentage of nitrogen, leaf toughness, and the number of hairs on individual leaves.

Herbivore Damage in the Field. On June 23, 1994, ten field populations of *C. officinale* in Meijendel were selected to measure the natural herbivory per leaf on rosette plants. Five populations were in a shaded habitat, and five were in an open habitat. In each population the percentage of area damaged per leaf of 12 randomly selected rosettes was estimated. The presence of *E. bipunctella* and *M. cruciger* was noted. The presence of blotch mines caused by *Agromyza* spp. (De Jong et al., 1990) was scored.

Extraction and Purification PA N-Oxides. In order to obtain purified PA N-oxides, dried leaves of *C. officinale* were ground in a Waring blender and

extracted overnight with 10 ml MeOH/g leaf powder. The extract was filtered over cotton wool, and the residue was reextracted twice for 1 hr. The volume of the combined extracts (±500 ml) was reduced in vacuo to ±15 ml and was placed on a Silica-60 (Merck) column. The column was eluted with 150 ml dichloromethane (DCM), followed by 200 ml 10% MeOH in DCM, and three times 200 ml MeOH. The PA N-oxides were eluted in the MeOH fraction. Purity and presence of PA N-oxides were checked by TLC on Silica gel 60 precoated plates that were developed in DCM–MeOH–NH_3 (82:15:3). PA N-oxides were detected with acetic anhydride and Ehrlich reagent (Molyneux and Roitman, 1980). If the extract was still impure, the extract was placed on a preparative Silica plate (Merck, layer thickness 2 mm), which was developed as above. The PA-containing bands were scratched off, and the silica was extracted three times with MeOH with continuous stirring. The MeOH fraction containing the PA N-oxides was evaporated and the residue dissolved in 25.0 ml MeOH. The concentration of the PA N-oxide solution was determined with a spectrophotometric color reaction (Mattocks, 1967). By omitting the oxidation step in this color reaction, we could specifically determine that the solution contained N-oxides only.

Extraction and Purification of Individual PAs. Dried leaf material was extracted with 0.5 M H_2SO_4. After 1 hr, Zn dust was added to reduce the PA N-oxides. The acid extract was filtered and extracted twice with DCM to remove apolar impurities. Subsequently the extract was made alkaline with ammonia (25%) and placed on an Extrelut (Merck) column. After 20 min, the column was washed with two times 100 ml DCM. The volume of combined DCM extracts was reduced with a rotary evaporator to ±10 ml and placed on a Silica-60 column. The column was eluted with a gradient of DCM–MeOH–NH_3 (60:40:1), DCM–MeOH (1:1), DCM–MeOH (25:75), and pure MeOH. Presence of PAs in the fractions was checked on TLC (system as above). The PA-containing fractions were evaporated, dissolved in MeOH, and the PAs were identified with GC-MS (DB-1 column, method II as described by Witte et al. (1993). The concentration of the solutions containing the PAs was determined with a spectrophotometric color reaction (Mattocks, 1967).

PA and Nitrogen Analysis. Individual leaves of five control plants were extracted for PAs as described in Van Dam et al. (1993). Monocrotaline (Sigma, 99% pure) was used as a reference compound in the spectrophotometric color reaction (Mattocks, 1967). The percentage of organic nitrogen in the leaves of two control plants was determined according to the semimicro-Kjeldahl method (Chapman and Pratt, 1961).

Physical Characteristics. Leaves of three control rosette plants were sampled separately and weighed to determine FW. After two days of drying at 50°C, the dry weight (DW) was determined in order to calculate the water content. *C. officinale* leaves are covered with thick-walled, unicellular hairs (De

Jong et al., 1990), which might serve as a barrier against small herbivores. Of three other control plants, the number of hairs was counted in two randomly chosen circles of 1 mm^2 per leaf.

Leaf toughness (in Newtons) was measured on the same leaves as the hairiness, with the use of a penetrometer (Prins and Laan, 1988). Every leaf was punctured on four places, avoiding the midvein, in order to obtain an average toughness over the whole leaf.

Statistical Analysis. Standard statistical tests were performed with Statgraphics 5.0 (Statgraphics Company, 1992). The differences in physical characteristics and damage between leaves were analyzed with the Friedman analysis of variance, since the groups (leaves) were not independent (Sokal and Rohlf, 1981)

RESULTS

Choice Tests

Leaf disks from young and old leaves differed significantly in both PA content and nitrogen percentage; the young leaves had higher levels of both (Table 1). As predicted, PAs did not deter the specialist herbivores *M. cruciger* and *E. bipunctella*. In fact, they ate more of the disks punched from young leaves, but their preference was not significant (Table 1). Both generalists, *S.*

TABLE 1. CHOICE TEST WITH DISKS OF OLD AND YOUNG LEAVES OF *Cynoglossum officinale* AND DISKS TREATED WITH METHANOL (CONTROL) AND PA N-OXIDE (PAs) EXTRACT[a]

	First experiment			Second experiment		
	Old	Young	*P*	Control	PAs	*P*
PA (mg/g FW) (SE)	0.93 (0.22)	4.54 (1.36)	0.03	0.93	3.93	
Nitrogen, % (DW) (SE)	3.30 (0.26)	4.30 (0.26)	0.01			
Specialist herbivores						
Mogulones cruciger	4	6	0.48	9	3	0.11
Ethmia bipunctella	4	9	0.33	12	8	0.18
Generalist herbivores						
Spodoptera exigua	19	0	0.0001	19	0	0.0001
Locusta migratoria	16	2	0.002	10	0	0.004

[a]The table gives the number of individuals that chose to eat more of the treatment. *P* values of Wilcoxon's signed-ranks matched-pairs test. SE = standard error of mean.

exigua larvae and *L. migratoria* adults, preferred the disks with the lowest level of PAs. In the first experiment they preferred to feed on disks from old leaves, which also had the lowest nitrogen percentage (Table 1). In the second experiment, the disks treated with PA N-oxides were totally avoided by both herbivores. This indicates that the PA N-oxides extracted from *C. officinale* had a strong deterrent effect on these herbivores, which normally do not feed on this plant species.

Different PAs

Since *S. exigua* larvae were clearly deterred by PAs, they were used as test animals to assess the difference in deterrency between individual PAs. After separation and purification of a tertiary PA extract, we obtained three different types of PAs in quantities large enough to perform the deterrency test: echinatine [RI = 2172, M^+ (m/z) = 299] 88% pure, 3′-acetylechinatine [RI = 2220, M^+ (m/z) = 341] 89% pure, and heliosupine [RI = 2557, M^+ (m/z) = 397] 87% pure. The purity and identity of the alkaloids were determined with GC-MS. For some unknown reason, we did not obtain saturated PAs such as viridiflorine, although they form 20–30% of the PAs present in the leaves (Van Dam et al., 1995). Although the PA concentration on the leaf disks was relatively low (0.5 μg/mg FW), all PAs clearly reduced feeding by *S. exigua* larvae compared to the methanol treatment (Figure 1, Kruskal-Wallis analysis of variance, H = 13.7, P = 0.004). However, there was no statistically significant difference in

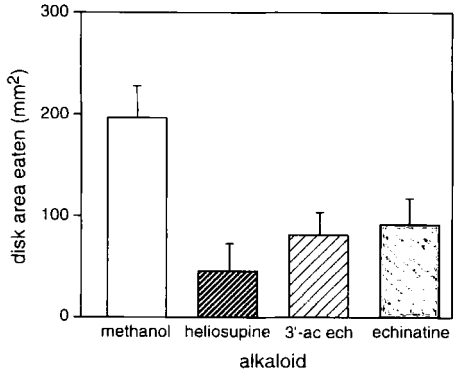

FIG. 1. Feeding deterrency of different types of pyrrolizidine alkaloids (PAs) of *Cynoglossum officinale* against *Spodoptera exigua* larvae. All PAs were applied on lettuce disks (0.5 μg PA/mg FW). Abbreviation: 3′-ac ech = 3′-acetylechinatine. Error bars denote standard error of the mean.

deterrency between the single PAs (Kruskal-Wallis on results of PA treatments only, $H = 3.90$, $P = 0.14$).

PA N-oxides versus Tertiary PAs

For the reason mentioned above, the difference in deterrency between PA N-oxides and their free bases was tested with *S. exigua* larvae. In a choice test, the larvae clearly preferred disks with PA N-oxides over disks with the same concentration (2 μg/mg FW) of tertiary PAs (Table 2). However, this does not mean that PA N-oxides are not deterrent at all. In the first choice experiment, both *S. exigua* and *L. migratoria* were deterred by high PA N-oxide concentrations in their food (Table 1).

Whole Plant Experiments

Physical and Chemical Leaf Characteristics. Again the PA levels differed significantly between the leaves (for results of Friedman analysis of variance see legend to Figure 2): they decreased from the youngest to the oldest leaf (Figure 2a; linear regression on ranks $R = -0.98$, $P < 0.001$). The percentage of nitrogen also declined with leaf age; young leaves had higher nitrogen percentages than old leaves (Figure 2b; linear regression on ranks $R = -0.98$, $P < 0.001$).

Contrary to our expectations, the water content of young leaves was significantly smaller than that of middle-aged leaves (Figure 2c) and was not significantly correlated with leaf number ($R = 0.54$, $P = 0.21$). Not surprisingly, the youngest leaves had more hairs per square millimeter than the older leaves (Figure 2d, $R = -1.0$, $P < 0.001$). Both the increase in water content and the decrease in hairs per area with age probably result from cell stretching. The toughness did not significantly differ between the leaves (Friedman, $\chi^2 = 7.4$, $P = 0.19$) and there was no correlation with leaf number ($R = 0.01$, $P = 0.98$).

TABLE 2. CHOICE TEST WITH GENERALIST HERBIVORE *Spodoptera exigua*[a]

	PA N-ox	*tert*-PA	*P*
Mean eaten (mm^2 ± SE)	42.3 (± 4.3)	13.4 (± 2.8)	0.0004
Animals that ate more of (*N*)	18	2	

[a]Pyrrolizidine alkaloid N-oxides (PA N-ox) of *Cynoglossum officinale* and their corresponding free bases (tertiary PAs) were both applied at 2 μg/mg FW, on disks from lettuce leaves. Differences were analyzed with Wilcoxon's signed-ranks matched-pairs test.

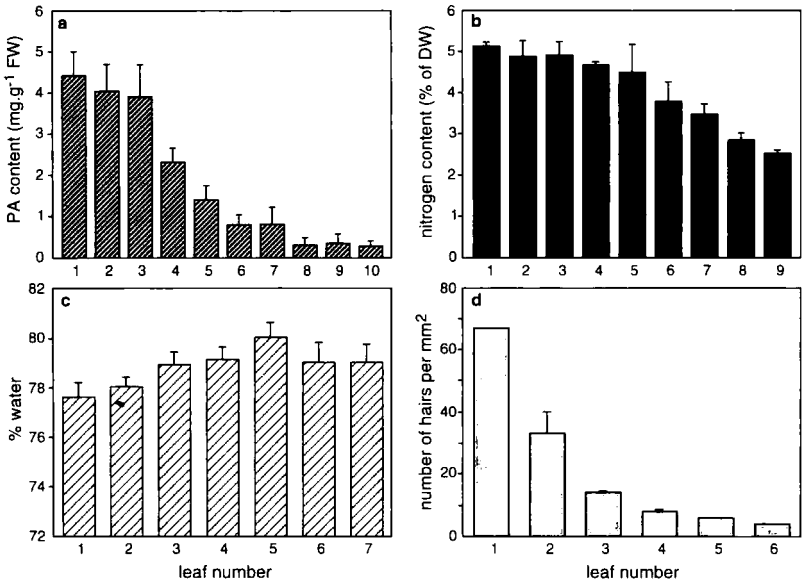

FIG. 2. Chemical and physical leaf characteristics of rosette plants of *Cynoglossum officinale*. Leaf number 1 is the youngest leaf. χ^2 and P value of Friedman analysis of variance are given in parentheses after each characteristic: (a) pyrrolizidine alkaloid (PA) level ($N = 5$, $\chi^2 = 31$, $P = 6.2 \times 10^{-5}$); (b) nitrogen percentage ($N = 2$, $\chi^2 = 14.8$, $P = 0.063$); (c) water content ($N = 8$, $\chi^2 = 13.8$, $P = 0.031$); (d) number of hairs ($N = 3$, $\chi^2 = 15$, $P = 0.01$).

Herbivory in the Lab. The amount of herbivory was measured as the area eaten (square millimeters) per leaf. We choose to express herbivory as the fraction of the leaf that was eaten (square millimeters per square centimeter or percentage). In this way we corrected for the greater chance that larger leaves have to be found by randomly searching herbivores. Without this correction, the differences in damage between leaves were even greater. The monophagous *M. cruciger* adults significantly preferred young leaves over older leaves (Figure 3a, for results of Friedman analysis see legend to Figure 3; linear regression on ranks, $R = -0.99$, $P = 0.00001$), which may be due to the higher nitrogen levels. PAs again did not deter feeding by these monophagous weevils. *E. bipunctella* larvae did not discriminate between leaves (Figure 3b). The negative correlation between damage and leaf number was not significant ($R = -0.55$, $P = 0.12$). This is consistent with our earlier findings that *E. bipunctella* larvae are not deterred by high PA levels in the younger leaves. However, unlike *M.*

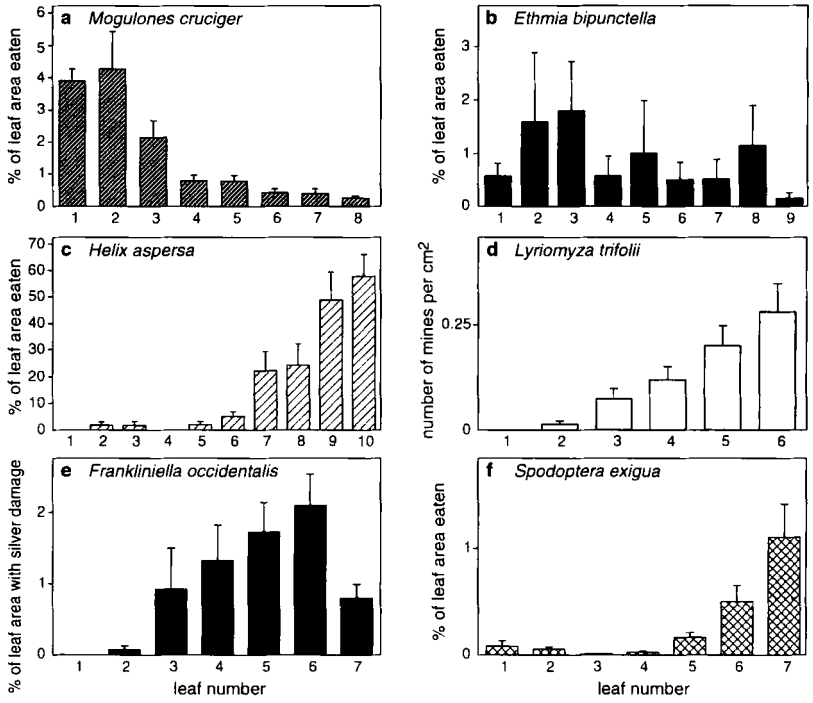

FIG. 3. Fraction of leaves eaten by different herbivores on rosette plants of *Cynoglossum officinale*. Leaf number 1 is the youngest leaf. χ^2 and P value of Friedman analysis of variance are given in parentheses after each species name. (a) *Mogulones cruciger* (N = 6, $\chi^2 = 32.7$, $P = 3.10^{-5}$); (b) *Ethmia bipunctella* ($N = 9$, $\chi^2 = 4.66$, $P = 0.79$); (c) *Helix aspersa* ($N = 21$, $\chi^2 = 86.4$, $P = 8.5 \times 10^{-15}$); (d) *Lyriomyza trifolii* ($N = 8$, $\chi^2 = 33.4$, $P = 3.12 \times 10^{-6}$); (e) *Frankliniella occidentalis* ($N = 12$, $\chi^2 = 45.5$, $P = 3.7 \times 10^{-8}$); (f) *Spodoptera exigua* ($N = 19$, $\chi^2 = 40.8$, $P = 3 \times 10^{-7}$).

cruciger, they did not feed significantly more on leaves with higher nitrogen percentages, as we would have expected.

All generalist herbivores in this experiment significantly discriminated between the leaves (Figure 3c–f). The older the leaves, the more they were consumed (linear regressions on ranks: *H. aspersa*, $R = 0.92$, $P = 0.0002$; *L. trifolii*, $R = 0.996$, $P = 0.000$; *F. occidentalis*, $R = 0.80$, $P = 0.03$; *S. exigua*, $R = 0.90$, $P = 0.006$). Consequently, these generalist herbivores strongly prefer those leaves with a low PA content, which is in accordance with the results of the choice tests (Table 1). Since *L. trifolii* fed and developed in the leaf, the

difference in number of large mines per leaf may be a combination of antixenotic and antibiotic effects of PAs. Because the leaf number differed between experiments, we were unable to correlate herbivory directly to leaf characteristics.

Herbivory in the Field. Although in each population at least one specialist herbivore species was present, the youngest leaves overall had relatively less herbivore damage than the older leaves (Figure 4). Since the Friedman analysis requires equal numbers of observations per leaf number, the differences between leaves were analyzed per group of plants with the same number of leaves. Except for the small group of plants with eight leaves, the herbivore damage was significantly different between leaves (Table 3, Friedman analysis). The amount of damage and leaf number were significantly correlated for all groups, which indicated that independent of number of leaves on the plant, the older leaves were relatively more damaged than the youngest leaves.

The average damage on the leaves, 3.8%, was comparable to earlier reports (Prins and Nell, 1990). Mines of *Agromyza* spp. caused a significant loss of photosynthetically active leaf surface. On average the mines caused a loss of 11.7% of the leaf surface, while leaves without mines suffered only 3.12% damage (Wilcoxon test, $Z = 8.36$, $P < 0.001$). Blotch mines were only found on older leaves, on average on leaf number 4. This indicates that, like *L. trifolii*, the naturally occurring leaf-miners avoid the youngest leaves with the highest PA levels.

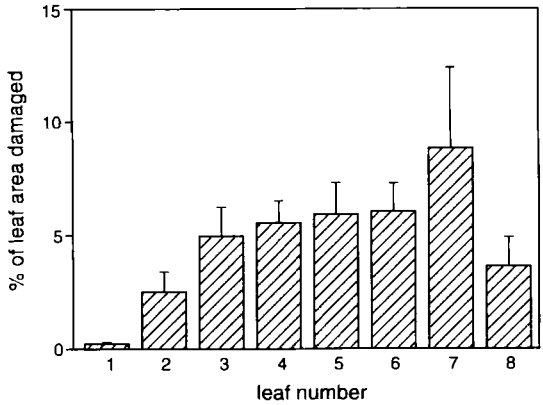

FIG. 4. Average percentage of herbivore damage per leaf, observed on rosette plants of *Cynoglossum officinale* in the dune area in Meijendel near The Hague, The Netherlands, on June 23, 1994. Leaf number 1 is the youngest leaf.

TABLE 3. RESULTS OF FRIEDMAN ANALYSIS OF VARIANCE AND SPEARMAN RANK CORRELATION ANALYSIS FOR DIFFERENCES IN HERBIVORY BETWEEN LEAVES OF FIELD PLANTS

Leaves (*N*)	Plants (*N*)	Friedman ANOVA		Spearman RC	
		χ^2	*P*	R^2	*P*
3	17	19.3	6×10^{-5}	0.54	0.01
4	41	58.3	1×10^{-12}	0.67	<0.001
5	25	53.5	6×10^{-11}	0.62	<0.001
6	19	53.0	3×10^{-10}	0.71	<0.001
7	9	37.0	2×10^{-6}	0.72	<0.001
8	3	10.3	0.17	0.77	<0.001

DISCUSSION

In this paper we showed that PAs in *C. officinale* rosettes act as deterrents against generalist herbivores. Despite different ways of feeding, all generalist herbivores avoided the young leaves, which had the highest PA levels. The results of the disk choice tests with PA N-oxide extracts indicate that PAs indeed are responsible for deterring generalist herbivores.

The specialist herbivore species we tested were unaffected by PAs in their food. The monophagous *M. cruciger* preferably consumed the youngest leaves, which are richest in nitrogen, thus maximizing intake of nitrogen. For many insect herbivores, fitness increases with nitrogen intake (Raupp and Denno, 1983; Scriber, 1984). In this case, PAs might even serve as feeding stimulants, since high PA levels coincide with high nitrogen percentages.

In contrast to *M. cruciger*, the oligophagous *E. bipunctella* does not significantly prefer the plant parts with the highest nitrogen levels. However, this has no negative effects on the fitness of *E. bipunctella*. Time to pupation and pupal weight were not significantly different between individuals reared on the oldest three leaves and on the youngest three leaves (Van Dam and Bergshoeff, unpublished data). The difference in nitrogen levels in leaves of *C. officinale* may have been too small to cause significant differences in fitness parameters. Only a factor of 1.6 existed between the youngest three and the oldest three leaves (Figure 2b).

In earlier reports on retarded development of *E. bipunctella* larvae on damaged *C. officinale* plants, it was suggested that this effect on the herbivore's fitness was due to induction of PAs (Prins and Laan, 1988). However, data on

induced changes were lacking. This makes them unsuitable for comparison. Unfortunately, data on the effect of leaf quality on the fitness of *M. cruciger* are hard to get, because these weevils spend their larval stage in the roots.

We found that low concentrations of echinatine, 3′-acetylechinatine, and heliosupine all deterred feeding by a nonadapted herbivore in a no-choice situation. Contrary to our findings, Bentley et al. (1984) reported that echinatine and echinatine N-oxide, in concentrations comparable to those we used, did not significantly deter feeding by spruce budworm. This indicates that not all nonadapted herbivore species are equally sensitive to the antifeedant effect of PAs.

Since all types of PAs we tested reduced feeding equally well, the question arises as to why *C. officinale* maintains such a variety of different PAs. The reason may be that a mixture of compounds is even more effective, because of synergistic effects (Berenbaum, 1985) or because it reduces the probability of adaptation by herbivores (Jones, 1983). Moreover, plants are attacked not only by all sorts of herbivores but also by pathogens. Specific PAs might have evolved as protection against different species of natural enemies. These three hypotheses all need further testing, which is beyond the scope of this paper.

Although the reduced form of PAs seemed more effective as a defense against herbivores (Speiser et al., 1992; this paper), the PAs in the leaves of *C. officinale* almost exclusively occur as N-oxides (Sykulska, 1962; Van Dam et al., 1995). The reason may be that PA N-oxides are better suited for specific phloem transport and safe vacuolar storage within the plant (Hartmann et al., 1989). Moreover, the difference in deterrency between the two forms of PAs is only relative. The choice experiment clearly showed that moderate concentrations of PA N-oxides also strongly deter feeding by generalist herbivores.

Furthermore, our experiments showed that the youngest leaves with the highest PA levels suffer less damage from generalist herbivores. Since generalist herbivores form the greatest potential number of enemies (Wink, 1993), we may conclude that, overall, the youngest leaves are better protected against herbivory. However, the fact that these leaves contain the highest PA levels does not preclude that other characteristics may play a role. High hair densities, which coincide with high PA levels in *C. officinale*, may also act as a defense against herbivores.

The damage we observed in the field, of course, is a combination of feeding by specialist and generalist herbivores. Although the damage pattern in the field seems more comparable to that of generalist herbivores, the difference in damage between the leaves is less extreme than in the lab experiments. Nevertheless, these data show that in the field also, the youngest leaves are the best protected. The lab data show that this is not due only to the fact that old leaves have been exposed to herbivory for a longer time. The observation that the youngest leaves are best protected, may reflect the plant's optimal defense strategy. Two aspects

of plant organs are important for optimal defense distribution: the photosynthetic value and the vulnerability of a leaf (Mooney and Gulmon, 1982; Zangerl and Bazzaz, 1992). On both aspects young leaves score higher than old leaves (Harper, 1989). If a rosette plant should have to maintain the same PA level of the youngest leaves throughout the whole plant, the investment in PA synthesis should be more than 50 times higher (see Van Dam et al., 1994). Assuming that it is too costly to do so, investing in defense for young leaves to optimize plant growth thus seems an optimal strategy for *C. officinale* rosettes.

In conclusion, the "raison d'être" of pyrrolizidine alkaloids in rosettes of *C. officinale* is that they protect the most valuable organs of the plant against generalist herbivores. Our paper also shows that to study the possible defensive functions of secondary metabolites, one should test these chemicals against several specialist and generalist herbivores to gain insight into their ecological role.

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HOST MICROHABITAT LOCATION BY STEM-BORER PARASITOID *Cotesia flavipes:* THE ROLE OF HERBIVORE VOLATILES AND LOCALLY AND SYSTEMICALLY INDUCED PLANT VOLATILES

ROEL P.J. POTTING,* LOUISE E.M. VET, and MARCEL DICKE

Department of Entomology,
Agricultural University Wageningen,
PO Box 8031, 6700 EH
Wageningen, The Netherlands.

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Abstract—The origin of olfactory stimuli involved in the host microhabitat location in *Cotesia flavipes*, a parasitoid of stem-borer larvae, was investigated in a Y-tube olfactometer. The response of female *C. flavipes* towards different components of the plant–host complex, consisting of a maize plant infested with two or more larvae of the stem borer *Chilo partellus*, was tested in dual-choice tests. The concealed lifestyle of the stem-borer larvae did not limit the emission of volatiles attractive to a parasitoid. A major source of the attractive volatiles from the plant–host complex was the stem-borer-injured stem, including the frass produced by the feeding larvae. Moreover, the production of volatiles attractive to a parasitoid was not restricted to the infested stem part but occurs systemically throughout the plant. The uninfested leaves of a stem-borer-infested plant were found to emit volatiles that attract female *C. flavipes*. We further demonstrate that an exogenous elicitor of this systemic plant response is situated in the regurgitate of a stem-borer larva. When a minor amount of regurgitate is inoculated into the stem of an uninfested plant, the leaves of the treated plant emit volatiles that attract female *C. flavipes*.

Key Words—Infochemicals, *Zea mays*, stem borer, *Chilo partellus*, parasitoid, *Cotesia flavipes*, host searching, herbivore-induced synomones, systemic effect, exogenous elicitor, caterpillar regurgitate.

INTRODUCTION

In the evolutionary arms race between herbivores and their natural enemies, herbivores have evolved several traits to reduce their vulnerability to enemies.

*To whom correspondence should be addressed.

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One of these traits can be a specific behavior that reduces the recognition and detection by natural enemies. It has been argued that the stem-boring habit of several herbivore species avoids proximity of feeding damage cues and reduces accessibility for natural enemies, which may have been one of the reasons for its evolution (Strong et al., 1984). However, absolute safety from natural enemies is virtually impossible. Although attack by enemies imposes selection in victims to reduce vulnerability, counter-selection works on enemies to improve their efficiency in finding hiding hosts (Jeffries and Lawton, 1984). Despite their hidden lifestyle, stem-borer larvae have a broad range of natural enemies, which are able to locate and attack the larvae that feed inside the plant tissue (Baker et al., 1949; Smith et al., 1993). The response of stem-borer parasitoids to contact chemicals is well described (Leerdam et al., 1985), but there are only a few studies that focus on the volatile stimuli involved in the host searching for stem-borer parasitoids (Roth et al., 1982; Ding et al., 1989a,b; Ma et al., 1992).

For other parasitoids, the use of volatile infochemicals in their long-range search for suitable hosts is well documented (Vinson, 1976; Nordlund et al., 1988; Vet and Dicke, 1992). Many natural enemies are known to discriminate between volatile chemicals emitted by uninfested and herbivore-infested plants. Chemical stimuli emanating from the plant–host complex can orginate from the herbivore, the plant, or from interactions between the herbivore and the plant. The response to volatile cues from the plant–host complex is well described for parasitoids of leaf-feeding caterpillars. For instance, the braconids *Cotesia marginiventris*, *Cotesia glomerata*, and *Cotesia rubecula* are highly attracted by odors emanating from herbivore-damaged plants, whereas odors from larval frass or the larvae themselves are far less attractive to these parasitoids (Turlings et al., 1991a; Steinberg et al., 1992; Geervliet et al., 1994). Although odors from the host or host products are very reliable indicators for host presence, their long-range detectability is very low (Vet et al., 1991). Turlings et al. (1991b) demonstrated that larvae and frass only release volatiles in very low quantities, whereas plant volatiles are released in relatively large amounts. Plant odors play an important role in the foraging behavior of larval parasitoids, especially plant volatiles that are released by plants infested by herbivores. Damage by hervibores can increase the emission of plant volatiles enormously (Dicke et al., 1990a; Turlings et al., 1990), and these plant volatiles can be specific indicators of herbivore identity (Dicke et al., 1990a,b). If these chemicals attract natural enemies whose activities are favorable to the plant, they are called herbivore-induced synomones (Vet and Dicke, 1992). Recent studies have demonstrated that the plant has an active role in the production of such herbivore-induced synomones (for review see Dicke, 1994). The release of these chemical signals is not restricted to the infested plant parts, but occurs systemically

throughout the plant (Dicke et al., 1990b; Takabayashi et al., 1991; Turlings and Tumlinson, 1992; Dicke and Dijkman, 1992).

In contrast to leaf-feeding herbivores, the larvae of stem borers have a concealed lifestyle and feed inside the plant. This present research deals with the foraging behavior of *Cotesia flavipes* (Hymenoptera: Braconidae), a gregarious larval endoparasitoid of tropical stem borers. *C. flavipes* attacks the last larval instars, by ingressing the stem through the entrance hole of the stem-borer tunnel (Smith et al., 1993). *C. flavipes* is indigenous to Southeast Asia, where it attacks several stem-borer species in maize, sorghum, rice, sugercane, and various wild grasses. This parasitoid has been extensively used in classical biological control programs against *Chilo partellus* (Alam et al., 1972; Overholt, 1994), *Diatraea saccharalis* (Gifford and Mann, 1967; Alam et al., 1971; Fuchs et al., 1979), and *Chilo sacchariphagus* (Betbeder-Matibet and Malinge, 1968). Earlier reports on the searching behavior of *C. flavipes* mainly focused on the short-range searching behavior, in particular the contact-response to larval frass (Kajita and Drake, 1969; Mohyuddin, 1971; Mohyuddin et al., 1981; Leerdam et al., 1985). Potting et al. (1993) and Ngi-Song et al. (1994) demonstrated that female *C. flavipes* are attracted to odors emanating from stem-borer-infested maize plants.

The purpose of the present research was to elucidate the sources of the volatile stimuli responsible for this attraction. This was achieved by testing the attractiveness of different components of the plant-host complex, which consisted of a maize plant with two or more feeding *C. partellus* larvae in the stem. We provide evidence that the production of volatiles attractive to a parasitoid is not restricted to the infested part of a plant but occurs systemically throughout the plant. We further demonstrate that an exogenous elicitor of this systemic plant response is present in the regurgitate of a stem-borer larva.

METHODS AND MATERIALS

Insects

The *Chilo partellus* culture originated from a colony maintained by the International Centre for Insect Physiology and Ecology in Nairobi, Kenya. The culture was maintained in temperature-controlled rooms (25°C) at 50–70% relative humidity, and a light–dark regime of 16L:8D. Adult moths were kept in an oviposition cage lined with waxed paper to provide suitable oviposition sites. Egg masses were cut from the paper daily and transferred to a closed Petri dish containing moist cotton wool to maintain high humidity. Three to four egg masses (blackhead stage) were transferred to 1-liter glass jars with 125 ml artificial diet prepared according to the procedure described by Seshu Reddy and

Davies (1978) and Ochieng et al. (1985). The covers of the jars were provided with a hole and were lined with a stainless steel screen to prevent escape of the larvae. As moths emerged they were transferred to an oviposition cage.

The larvae used in the experiments and for the maintenance of the parasitoid culture (see below) were always removed from the artificial diet as fourth instars and allowed to feed on fresh maize stems for at least 24 hr.

Cotesia flavipes was obtained from the International Institute for Biological Control in Rawalpindi, Pakistan, where it was collected from *C. partellus* in maize. In Wageningen, the parasitoid culture was maintained on *C. partellus* at 25°C, 70% relative humidity, and a 16-hr photophase. Individual, mated, females (1 day old) were offered one host larva with some fresh larval frass to stimulate oviposition. Parasitized larvae were reared on cut maize stems kept in one liter jars.

Cocoons were collected from the stem pieces one to two days prior to emergence and transferred to a 250-ml bottle with agar medium lined with a polyester top with a drop of honey. One day after emergence the adult wasps were placed in an incubator at 15°C. All experiments were conducted with naive (no oviposition experience), mated females that were 2 to 4 days old.

Plants

Maize plants (*Zea mays* cv. Anjo) were grown in a greenhouse at 20–25°C, 50–80% relative humidity, and a 16L:8D light regime. When the light intensity dropped below 500 lux in the photophase, artificial light was used. Plants were watered daily and, when necessary, treated with AA Kasaerosol (Schering AAgrunol B.V.) against aphid infestation.

Olfactometer Setup

Responses of *C. flavipes* females to volatile chemicals emitted by different odor sources were measured in a Y-tube olfactometer. The olfactometer consisted of a glass Y-shaped tube. The two arms of the Y tube were connected to a flowmeter and an odor source container. Odor sources were placed in containers, which consisted of 500-ml glass bottles. Air was blown through the odor source containers and introduced into the arms of the Y tube. By balancing the input through the arms (4 liters/min) and the output through the common tube (8 liters/min), two laminary airflows were created. For more details of the olfactometer, see Steinberg et al. (1992). The environmental conditions in the bioassay room were 25–27°C and 40–70% relative humidity.

Bioassay Procedure

Individual female parasitoids were introduced into the base tube of the Y-tube olfactometer, and their behavior was observed during a 5-min period. If

the test female walked towards an odor source and crossed the choice line (4 cm after division of the base tube) and stayed there for more than 15 sec, it was recorded as a choice for the odor source in that arm. To circumvent any asymmetrical bias in the setup, the odor sources were exchanged after testing five parasitoids. For each combination, 40–50 wasps were tested. The choice of the females was analyzed with a chi-square test ($\alpha = 0.05$).

Odor Sources

Uninfested vs. Infested Maize Plants. Uninfested plants consisted of 4- to 6-week-old plants (40–50 cm tall). An infested plant was obtained by introducing two fourth-instar *C. partellus* larvae into two holes bored 10 cm apart in the stem of a plant. The larvae were allowed to feed overnight. Prior to the bioassay, the test plant was cut into four parts: three pieces of stem and the remaining part with the leaves of the plant. Care was taken that the tunnel of the stem-borer larvae never reached the part with the leaves. The four parts of each test plant were introduced into the odor source container. Three odor sources were tested: (1) clean air, which consisted of an empty odor source container (AIR); (2) the complete plant–host complex (PHC) with two feeding larvae; and (3) an uninfested plant (UNINFEST). The combinations tested were AIR versus UNINFEST and UNINFEST versus PHC. In an additional experiment it was tested whether an artificially damaged maize plant emitted attractive volatiles. In this test the plants were not cut into pieces. An artificially damaged plant (ART-DAMAGE) was obtained by puncturing five holes in the stem. To mimic feeding behavior of the stem-borer larvae, this was repeated every 15 min. The combination tested was ART-DAMAGE versus AIR. Forty wasps were tested in each choice situation.

Stem vs. Leaves. To investigate which part of the PHC emitted attractive volatiles, the following combinations were tested: (1) stem pieces of an uninfested plant (CONTROL-STEM) versus stem pieces of an infested plant (PHC-STEM), (2) leaves of an uninfested plant (CONTROL-LEAF) versus leaves of an infested plant (PHC-LEAVES), and (3) leaves of an infested plant (PHC-LEAVES) versus stem pieces of the same infested plant (PHC-STEM). All three combinations were tested on the same day and 47 wasps were tested in each choice situation.

Stem. To determine the origin of attractive volatile chemicals, the different components of an infested stem were tested in dual-choice tests. These were infested stem pieces with or without feeding larvae, larvae, larval frass, and artificially damaged (uninfested) stem pieces. The stem of 2-month-old maize plants (flowering stage) was cut into 12-cm pieces. Five pieces of stem were used as odor source. A standard infested stem piece was obtained by boring a hole on each side of the piece and introducing one larva in each hole. The 10 larvae were allowed to feed for 60 hr. The following odor sources were tested:

INFESTED	Five stem-borer-infested stem pieces, consisting of maize stems with two larvae in their stem-borer tunnel filled with frass.
HERB-DAM	As INFESTED but larvae removed prior to experiment.
DAM-STEM	As HERB-DAM but all frass removed prior to experiment.
LARVAE	10 water-washed larvae, removed from INFESTED.
FRASS	Frass removed from INFESTED.
ART-DAM	Artificially damaged stem pieces (10 holes bored in each stem piece, 12 hours prior to the start of the experiment).

The following choice tests were made: (1) INFESTED vs. ART-DAM, (2) HERB-DAM vs. ART-DAM, (3) INFESTED vs. HERB-DAM, (4) DAM-STEM vs. FRASS + LARVAE, and (5) LARVAE vs. FRASS (in this test both odor sources were placed directly into the Y tube). The choice tests were performed on separate days and 40 wasps were tested in each choice situation.

Leaves. In this series of experiments the odor sources consisted of the leaves of treated plants. All plants used consisted of singly potted maize plants, 4–6 weeks old. In the stem of each plant two holes were bored 10 cm apart. The leaves were obtained by cutting the stem just below the whorl in such a way that all the leaves were still attached to the stem apart.

The treatments tested were undamaged leaves that were removed from a host-infested plant 24 or 48 hr after infestation (LARVAE-24 hr; LARVAE-48 hr) and undamaged leaves from a regurgitate-treated plant (REGURGITATE).

To obtain a regurgitate-treated plant, 5 μl regurgitate from fourth-instar *C. partellus* larvae was inoculated in each hole and the test plant was incubated for 24 hr at 25°C before removing the leaves. The regurgitate was collected following the method described by Mattiacci et al. (1994). A larva was held with a pair of soft forceps and teased at the mouthparts with a microcapillary tube. In this way 5–10 μl regurgitate could be obtained from one larva. In all tests the control (CONTROL) odor source consisted of the leaves of a standard treated plant (with two artificially bored holes), incubated as long as the treatment it was tested against.

In the first series the choice tests, CONTROL vs. LARVAE-24 hr and CONTROL vs. LARVAE-48 hr were tested on the same day (40 wasps tested in each combination). In a second series, the choice tests CONTROL vs. REGURGITATE and CONTROL vs. LARVAE-24 hr were tested on the same day (50 wasps tested in each combination).

RESULTS

Uninfested vs. Infested Maize Plants. Volatiles emanating from a stem-borer-infested maize plant attracted all responding parasitoids, when offered versus an uninfested plant (Figure 1A). Parasitoids were not attracted to odors

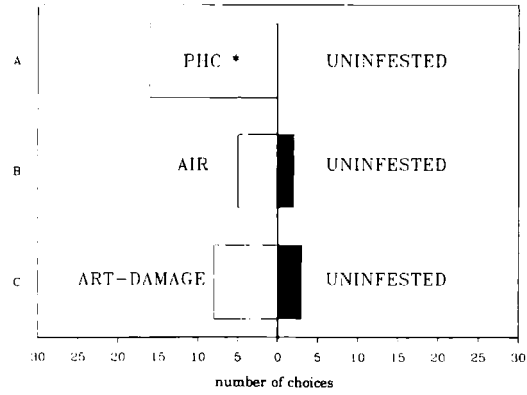

Fig. 1. The response of *C. flavipes* to volatile chemicals emitted by *C. partellus*-infested and uninfested maize plants in a Y-tube olfactometer. In each dual-choice test 40 females were tested. The bars indicate the number choosing a particular odor source. Asterisks indicate statistically significant preferences within tests (chi-square, $P < 0.05$).

emanating from uninfested maize plants or artificially damaged maize plants (Figure 1B and C).

Stem vs. Leaves. Although the response level was low, the females that made a choice preferred the odors emanating from the undamaged leaves of a stem-borer-infested plant, when offered together with undamaged leaves from an uninfested plant (Figure 2A), hence the emission of attractive volatiles is not restricted to the site of damage, but occurs throughout the plant. Volatiles emanating from the infested stem part of the plant (with the feeding larvae) attracted the majority of the parasitoids when tested against undamaged stems or the leaves of the very same infested plant (Figure 2B and C).

Stem. The parasitoids were clearly more attracted to odors emanating from stem-borer-damaged stem pieces when tested against artificially damaged stem pieces (Figure 3A). A stem-borer-damaged stem consists of feeding larvae in their tunnel filled with larval frass. The infested stems remained attractive even when the feeding larvae were removed (Figure 3B). The feeding activity of the larvae did not significantly enhance the attractiveness of the infested stems. The parasitoids did not make a distinction between infested stems with or without larvae (Figure 3C). When the larvae and their frass were removed from the infested stems and tested against the remaining stem, the latter was the most attractive (Figure 3D). Frass by itself is also an attractive component of a stem-borer-infested stem. The majority of the parasitoids was attracted to frass when tested against larvae (Figure 3E).

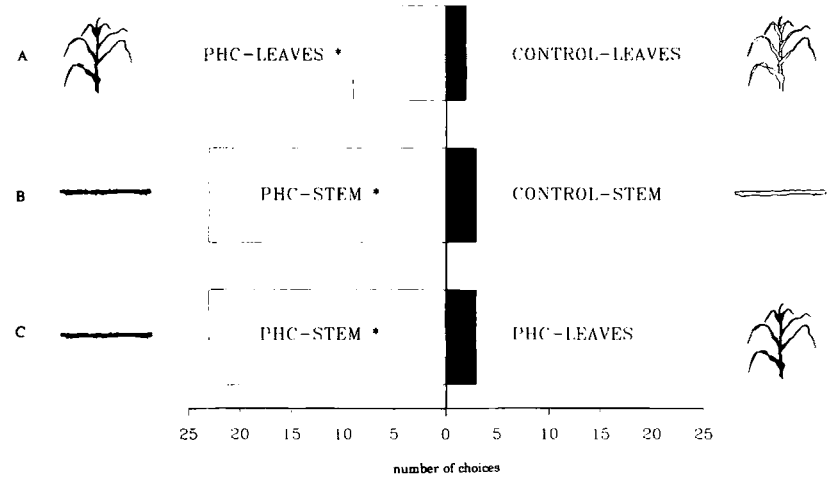

Fig. 2. The response of *C. flavipes* to volatile chemicals emitted by different parts of the plant-host complex in a Y-tube olfactometer. In each dual-choice test 47 females were tested. The bars indicate the number choosing a particular odor source. Asterisks indicate statistically significant preferences within tests (chi-square, $P < 0.05$).

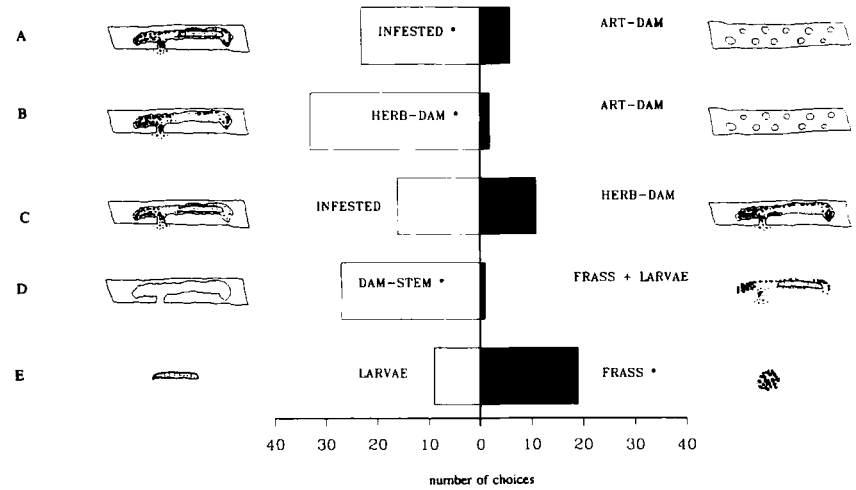

FIG. 3. The response of *C. flavipes* to volatile chemicals emitted by different components of *C. partellus*-infested stem pieces. In each dual-choice test 40 females were tested. The bars indicate the number choosing a particular odor source. Asterisks indicate statistically significant preferences within tests (chi-square, $P < 0.05$). For more information on odor sources see Methods and Materials.

Leaves. Leaves of a stem-borer-damaged plant that had not been in contact with the feeding larvae in the stem, emitted attractive volatiles (Figure 4). Leaves from a plant where two stem-borer larvae were allowed to feed for 24 or 48 hr attracted the majority of the parasitoids when tested against leaves from a plant with two artificially bored holes. The induced effect was clear after 24 hr of feeding and was not significantly enhanced by a longer feeding time (Figure 4). An elicitor of the systemic production of the plant-produced synomones seems to be present in the regurgitate of the feeding larva. If larval regurgitate was inoculated into the stem, the leaves of the treated plant started to emit attractive volatiles, and a similar response was obtained as with the leaves from a plant with two feeding larvae inside the stem (Figure 4).

DISCUSSION

Plants have evolved several characteristics to defend themselves against herbivore attack. These direct defense mechanisms include plant structures that prevent herbivore settlement, detrimental chemicals in the plant tissue, or the systemic production of defensive chemicals (Juniper and Southwood, 1986; Tallamy and Raupp, 1991). An indirect defense mechanism of plants is to promote the effectiveness of natural enemies of the herbivore (Price et al., 1980; Dicke and Sabelis, 1988). This may be achieved by mediating an interaction between the herbivore and its natural enemies. Evidence is accumulating that plants are actively involved in the release of chemical signals that attract natural enemies of the herbivore (see Dicke, 1994, for review). The emission of these volatiles by infested plants is not limited to the damaged sites but occurs throughout the plant, even in undamaged leaves (Dicke et al., 1990b, 1993; Takabayashi et al., 1991; Turlings and Tumlinson, 1992). All this research involves externally feeding herbivores. The present study provides evidence that even when a plant is injured by an herbivore that feeds inside the stem, the whole plant emits chemical signals that attract a natural enemy of the herbivore. The concealed lifestyle of the stem-borer larvae was found not to limit the emission of plant synomones. Female *C. flavipes* can discriminate between odors emitted by uninfested and stem-borer-infested maize plants (Potting et al., 1993) (Figure 1). A major source of the attractive volatiles from the plant–host complex is the stem-borer-infested stem (Figure 2). A stem-borer-infested stem consists of a feeding larva inside a tunnel filled with larval frass. The larvae feeding inside the stem appear to trigger the emission of attractants by the plant, but do not emit attractants themselves. The frass produced by the feeding larvae was an attractive component, but odors emanating from a stem-borer-injured stem are even more attractive (Figure 3). This parallels results from other studies in which herbivore-damaged plants or plant parts were more attractive to natural enemies

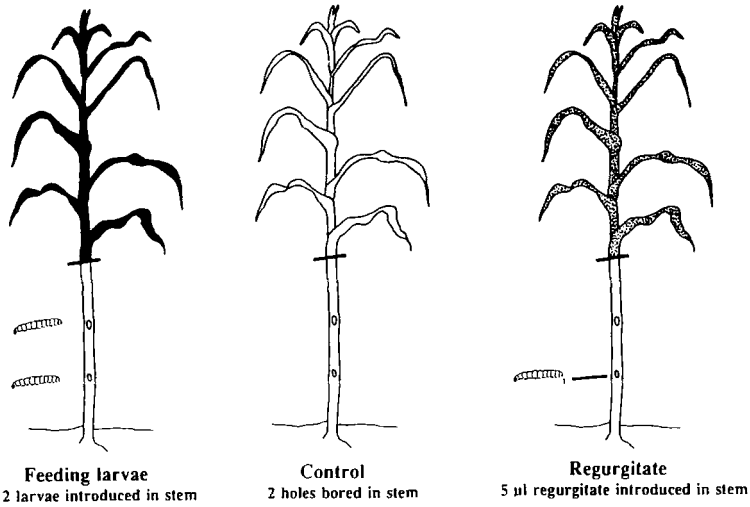

FIG. 4. The response of *C. flavipes* to volatile chemicals emanating from the undamaged leaves of stem-borer-infested maize plants vs. control leaves and to undamaged leaves from a regurgitate treated plant vs. control leaves. For more information on odor sources see Methods and Materials. The bars indicate the number choosing a particular odor source. Asterisks indicate statistically significant preferences within tests (chi-square, $P < 0.05$).

than the herbivore or its products (Roth et al., 1982; Turlings et al., 1991a; Steinberg et al., 1993; Geervliet et al., 1994).

The release of the attractive volatiles is not restricted to the infested stem part but occurs systemically throughout the plant. Female *C. flavipes* are attracted to volatiles released by the undamaged leaves of a stem-borer-infested plant (Figure 4). The systemic release of these volatiles probably enhances the detectability of the infested plant for natural enemies. However, when given a choice between the odors emanating from the leaves and from the stem of an infested plant, the majority of the parasitoids was attracted to the infested stem. This may indicate that at close range the parasitoid is able to find the infested part of the plant. A similar result was reported by Dicke et al. (1993), who found that predatory mites preferred host-damaged leaves when offered them versus undamaged leaves that emitted herbivore-induced synomones in response to an endogenous elicitor.

A factor that induces the systemic response seems to be present in the regurgitate of the larva. When regurgitate was inoculated into the stem, the leaves of the treated plant emitted volatiles that attracted female *C. flavipes*. It

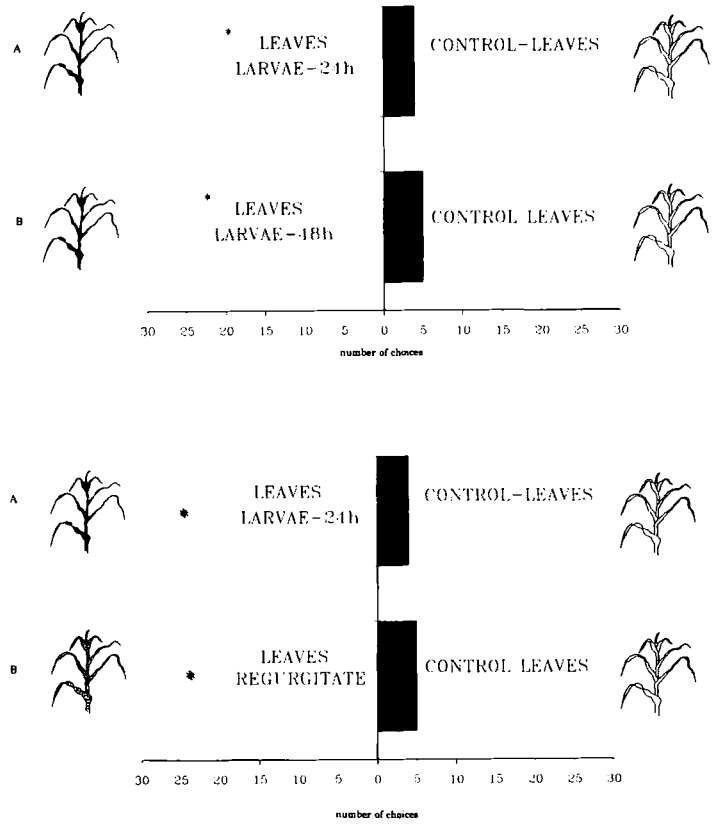

FIG. 4. Continued

remains to be investigated whether a regurgitate component itself or an endogenous elicitor induced by the regurgitate is transported through the stem to the leaves, which start to produce attractive volatiles. Some recent studies have focused on the elicitor that triggers the systemic response of herbivore-infested plants. Dicke et al. (1993) extracted an elicitor that is transported from infested leaves to uninfested leaves. Turlings et al. (1993) could induce the production of plant volatiles by placing maize seedlings in diluted regurgitate from several species of caterpillars and a grasshopper. Recently Mattiacci et al. (1995) shed more light on the elucidation of the factor responsible for the induced plant response. They showed that an elicitor present in the gut regurgitate of *Pieris brassicae* caterpillars induced the release of synomones by mechanically dam-

aged cabbage leaves (Mattiacci et al., 1994). They identified the enzyme β-glucosidase, a component of the regurgitate of *P. brassicae* caterpillars, as an elicitor of the synomone release by *P. brassicae*-infested leaves (Mattiacci et al., 1995). Glucosidases seem to be common enzymes among herbivorous insects (Mattiacci et al., 1995), and it remains to be investigated if they are responsible for the induced plant responses in different plant–host complexes, which could indicate that the plant response is a general response to attack by herbivorous insects.

A detailed analysis of the specific chemicals involved can establish whether there are qualitative and/or quantitative differences in the volatile blends emanating from the stem or the leaves of an infested plant. An identification of the specific chemicals may also reveal whether the same terpenoids are involved, as is shown for the maize–*Spodoptera exigua*–*C. marginiventris* system (Turlings et al., 1990, 1991b) and the maize–*Pseudaletia*–*Cotesia kariyai* system (Takabayashi et al., 1995).

In their search for the host habitat, parasitoids may exploit volatile cues from uninfested plants (Vinson, 1976; Nordlund et al., 1988; Vinson and Williams, 1991). For example, a response towards volatiles from uninfested maize plants was demonstrated for *Macrocentrus grandii* (Ding et al., 1989a; Udayagiri and Jones, 1992) and *Eriborus terebans* (Ma et al., 1992), two larval parasitoids of *Ostrinia nubilalis*. In the present study, female *C. flavipes* were not attracted to odors from uninfested or artificially damaged maize plants (Figure 1). However, Ngi-Song et al. (1994) reported that *C. flavipes* was attracted to uninfested maize plants in a bioassay similar to the one used in our study. The discrepancy in these results could be due to different maize cultivars or to a different method of rearing the plants. In our study plants were grown in a greenhouse and treated regularly against aphids and other herbivores when necessary. Ngi-Song et al. (1994) used plants grown under field conditions, and it is likely that they were infested with herbivores, which could have resulted in nonhost damage and subsequent release of herbivore-induced synomones. Vinson and Williams (1991) reported a similar finding: the parasitoid *Campoletis sonorensis* was attracted to uninfested plants grown in a greenhouse, which were exposed to aphids and thrips, whereas it did not respond to uninfested plants grown in a herbivore free environmental chamber.

Through their concealed lifestyle stem-borer larvae have created a partially enemy-free space, especially for predators such as birds and spiders. However, parasitoids, in particular, have evolved the ability to successfully locate and attack the larvae inside the plant tissue (Smith et al., 1993). Stem-borer larvae have to feed and defecate, and although they do this inside the plant tissue, it does not prevent the release of attractive volatiles either through the frass they produce or through the volatiles that are produced systemically by the plant as a response to the feeding activity of the larvae. This study demonstrates that a

stem-borer parasitoid can exploit these plant-produced volatiles to locate the host microhabitat.

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ODORS IN TRAPS: DOES MOST RECENT OCCUPANT INFLUENCE CAPTURE RATES FOR HOUSE MICE?

LEE C. DRICKAMER

Department of Zoology,
Southern Illinois University,
Carbondale, Illinois 62901

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Abstract—Responses of house mice (*Mus domesticus*) to odors in live traps were studied in a series of eight 0.1-ha outdoor field enclosures. It was assumed that the most recent mouse capture would provide the predominant odor in a trap for at least one week. Three different populations were tested, one in 1989 and two in 1992, involving over 800 different mice. Similar response patterns were recorded from all three groups. Two types of questions were tested: (1) Were there any biases contingent upon what had been previously caught? (2) Were there consistent responses of mice of particular age, sex, or reproductive classes to trap odors? Traps soiled by juvenile females caught adult females significantly less often than expected, but there were no consistent relationships in terms of the effects of specific residual odors on the subsequent capture at a particular trap. For various age, sex, and reproductive classes, (1) adult males preferred odors from juvenile and estrous females and avoided odors of other males significantly more than expected, (2) juvenile females selected traps with odors of other juvenile females and avoided all other types of female odors significantly more than expected, (3) nonestrous females exhibited a significant preference for adult male odor, and (4) estrous females selected traps containing odors from adult males but avoided those that had previously contained either nonestrous or pregnant/lactating females significantly more than expected. These findings have potential implications with regard to both the methods used for trapping small rodents and the social biology of house mice.

Key Words—*Mus domesticus*, chemical cues, chemosignals, pheromones, social behavior, mate finding, odor avoidance, live traps.

INTRODUCTION

Chemical cues are important for the social biology of a variety of rodents including voles (*Microtus* spp.) (Wolff and Johnson, 1979; Ferguson et al. 1986),

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deermice (*Peromyscus* spp.) (Bronson and Marsden, 1964; Doty, 1972), rats (*Rattus* spp.) (Barnett, 1963; Galef and Clark, 1971, Galef and Stein, 1985), and house mice (*Mus* spp.) (Vandenbergh, 1983; Drickamer, 1986; Vandenbergh and Coppola, 1986). House mice (*Mus musculus* and *M. domesticus*) use odor cues for recognition by sex, age, reproductive condition, or dominance status (Hurst, 1990a–c), mate choice (Drickamer, 1992), reproductive processes, particularly in females (Vandenbergh and Coppola, 1986), and spatial relations (Hurst, 1990a–c).

Because many rodents are small and nocturnal, live trapping and similar methods are often used to investigate aspects of their behavior and ecology. Several previous investigators have employed live traps to explore putative functions of chemical signals in a variety of rodent taxa with varying degrees of success. Mazdzer et al. (1976) reported that *Peromyscus leucopus* preferred traps containing odors from conspecifics of the opposite sex, in agreement with an earlier report from a laboratory study by Doty (1973). Daly et al. (1980) found seasonal differences in the tendency of *Peromyscus maniculatus gambelli* to enter traps containing conspecific odors. In a similar study, Heske (1987) reported that California voles (*Microtus californicus*) exhibited significant preferences for conspecific odors. Daly et al. (1980) showed that for *P. m. gambelli*, traps baited with conspecific odors were preferred by mice in reproductive condition, but mice not in reproductive condition preferred neutral, unbaited traps. Drickamer (1984) found that *Peromyscus leucopus noveboracensis* and *P. maniculatus gracilis* avoided heterospecific odors and preferred homospecific odors, and within each species, there was a strong preference for heterosexual odors.

Soiled traps are entered more frequently than clean traps by a variety of rodents, including *Microtus townsendii*, *M. agrestis*, *Apodemus sylvaticus*, *A. flavicollis*, and *Clethrionomys glareolus* (Boonstra and Krebs, 1976; Stoddart, 1982; Gurnell and Little, 1992). Recently, Drickamer et al. (1992) reported that house mice exhibited some odor cue preferences for traps baited with particular homospecific odors, but only when both volatile and nonvolatile cues were present. Adult, but not juvenile, house mice exhibited preferences for odor cues from their own age/sex class. One feature of this last study was that the traps were cleaned after each capture and were then provided with only the specific odor cue to be tested.

Based on previous findings with other rodents and a knowledge of house mouse biology from laboratory and field settings, I questioned the findings of Drickamer et al. (1992). I would have expected more specific preferences for particular odors and more age/sex class discrimination. Perhaps cleaning the trap after each capture was removing some significant information used by the mice. In addition, the urine odors used to bait the traps in the earlier study were from unfamiliar mice, not part of the population in that particular enclosure. This led to the current study based on data sets obtained from the enclosures

during years when odor-baiting experiments were not being conducted. My objectives were: (1) to test the importance of odor cues, particularly those that were erased when traps are cleaned after each capture; (2) to test odor preferences of mice when they respond to traps that contain both a series of odors from previous occupants and a recent capture; (3) to test which types of mice, by age, sex, and reproductive condition, are caught in traps that contain a mixture of odors, assuming the most recent capture constituted a predominant odor, which involves testing the effects of residual trap odors with regard to any bias in terms of what is caught next; and (4) to test specific odor preferences by mice of different ages, sex, and reproductive condition, under such conditions, which involves testing for the possible preferences of the mice for particular odors.

METHODS AND MATERIALS

Enclosures. Eight 0.1-ha enclosures located in an abandoned pasture were used for these experiments (see Drickamer and Mikesic, 1990, for full description of the enclosures and their construction). Each enclosure was 25 × 40 m, had wire mesh and aluminum side walls, and contained four symmetrically placed 2-m-diameter structures (called mouse cities; King, 1983) made of 2-m-high aluminum affixed to wooden posts. These mouse cities have a plywood roof, two entrances on opposite sides just above ground level, nesting cavities in cinder blocks located in the interior, and are supplied weekly with food and water. A 1.5-m strip was mowed around the interior perimeter of each enclosure and around each mouse city approximately once a month; in all areas of each enclosure the vegetation was allowed to grow uncut, attaining heights of 1–1.5 m. The mowed strips served as pathways for human travel in the enclosures and also prevented vegetation from attaining heights that would have enabled mice climbing to escape. Each enclosure contained a 6 × 9 trapping grid with 5-m intervals between trap stations. The trapping grid thus had 26 trap stations located along the walls of each enclosure and 28 in the interior.

Procedures. Data were collected in 1989 and 1992, years when the odor-baiting experiments mentioned previously (Drickamer et al., 1992) were not conducted. In late March 1989, populations were introduced in six of the eight enclosures with second- and third-generation stock from wild mice originally captured at Little Egypt Grain Co. (Crab Orchard National Wildlife Refuge, Williamson County, Illinois). Populations were introduced by placing groups of four females and one male into each mouse city (total of four males and 16 females into each enclosure). Two separate stocks of mice were used to populate the enclosures in 1992. Four enclosures (designated A, B, E, and F) were supplied with second- and third-generation mice produced from crosses of indi-

viduals captured at the Southern Illinois University Swine Farm with mice from the Little Egypt Grain Co. Eight females and two males of the Illinois stock were placed in each city (total of eight males and 32 females into each enclosure). The other four enclosures (C, D, G, and H) were supplied with mice from a stock originally captured near Fort Collins, Colorado, and cross-bred in the laboratory of Dr. Sarah Lenington at Rutgers University, Newark, N.J., with an inbred stock (LT.MA). Four females and two males were placed in each city (total of eight males and 16 females into each enclosure). Thus there were three separate test groups for analysis: 1989—Illinois mice, six enclosures; 1992—Illinois mice, four enclosures; and 1992—Colorado mice, four enclosures.

Starter mice were all 110–160 days of age at the time enclosures were stocked, and they had been held in same-sex groups of three to five per cage since weaning at 25–30 days of age. One week prior to initiating enclosure populations, each mouse was weighed to the nearest 0.5 g with a Pesola scale, reproductive condition was checked, and each was given a unique toe-clip ear-punch number. Mice were defined as juveniles until they reached 15 g body mass with the testes descended (males) or 13 g body mass with the vagina open (females); after those criteria were attained, they were defined as adults.

Populations were allowed to grow freely for 30 weeks. Beginning in mid-October, the mice were removed from the enclosures, first by continuous live trapping and then by using kill traps.

Trapping Procedure. One Sherman folding live trap was located at each of the 54 stations in each enclosure. Traps were placed inside 1.75-liter wax-coated milk or juice cartons to provide extra inclusion and protection from the elements. Each trap contained one cotton square (2.54 × 2.54 × 0.3 cm thick) called a Nestlet (Ancare Corp., Belmont, New York). From early May of each year through mid-October, the live traps were operated in each enclosure for two nights per week, with at least one night (range of one to three) separating the two trap nights. Traps were opened and baited between 1600 and 1800 hours and then were checked the next morning between 0600 and 0900 hours. Captured mice were checked for identification, body mass, and reproductive condition and were released at the site of capture. Previously uncaptured individuals were given a toe-clip and ear-punch number. Traps were not cleaned between captures and cotton squares were replaced on an as needed basis, as they disintegrated or, in a few instances, became soaked when it rained. All traps were checked once each month to ensure that they were functioning properly, and approximately 10 traps were replaced each summer due to malfunctions. No traps were moved between trap stations in any of the enclosures. While such a manipulation could be quite interesting, it would likely result in alterations of general contextual cues surrounding each trap and could have produced a prob-

lem similar to that associated with the trap cleaning and odor manipulation carried out by Drickamer et al. (1992).

Although it has been tested in previous studies of this type (Montgomery, 1987; Gurnell and Little, 1992), one potential problem with interpreting mouse responses in the current design could be a position effect. That is, mice caught in a particular trap simply because it is located within their home range rather than as a response to odors in the trap. This would be true, however, only if the number of potential traps that the mouse could enter on a given night was severely limited. I believe that the following chain of logic ensures that in this study such a position effect is unlikely. The average ($\pm$SE) home ranges for male and female house mice in the enclosures are 240 $\pm$ 21 m^2 for females and 400 $\pm$ 54 m^2 for males. With the 5-m interval trapping grid used in the enclosures that would mean that there are 10–18 traps within the average female home range and 16–24 traps within the average male home range. From the trapping data, it is possible to calculate that, on average, for a particular trap night, there were 18–22 traps (of the 54 present) in each enclosure that had caught a mouse on one of the two previous trap nights and thus served as the basis for the predominant odor. Combining the previous two items results in a determination that for females there were three to seven odorized traps in her home range on the average trap night and there were five to 10 odorized traps in the home range of each male. Thus, I would conclude that there were sufficient choices available to each mouse to indicate that, when it was captured, the result was a choice or preference by the mouse and not a position effect.

Analyses. The data used for analyses consisted of those mice caught in a trap where there had been a capture during one of the two preceding trap nights. In addition, instances where the same mouse was caught twice in a row in the same trap were eliminated from the data sets. Analyses of contingency tables for each data set were carried out using the G test or log likelihood ratio method (Sokal and Rohlf, 1981). All tests involved six response categories and, thus, there were six degrees of freedom for all tests. Because of the large number of analyses carried out ($N = 18$) on each data set, statistical significance values are reported for both $\alpha = 0.05$ and $\alpha = 0.005$; the latter value should aid in adjusting for any possible comparison-wise error problem associated with multiple tests. Calculation of the experiment-wise error rate using the Dunn-Sidák method (Ury, 1976) results in a value of 0.009.

RESULTS

Tabulated data for sample sizes revealed that 213 different adult males and 361 different adult females were captured at least once across the three data sets

TABLE 1. NUMBERS OF MICE OF TWO AGES AND BOTH SEXES CAUGHT FROM THREE STOCKS USED IN 1989 AND 1992 FOR TESTS OF RESPONSES TO CONTEXTUAL ODORS IN TRAPS

Age/sex class	1989	1992 (ABEF)	1992 (CDGH)
Juvenile female	64	90	106
Adult female[a]	78	83	200
Juvenile male	48	38	67
Adult male[a]	42	65	106

[a] Adults consist of original mice used to stock the enclosures and juveniles that matured during the field season. Thus, some mice may appear as both a juvenile and an adult in this table.

combined. In addition, 153 different juvenile males and 260 different juvenile females were captured at least once across the three data sets combined. Of the 153 mice first caught as juvenile males, 48 (31%) were later caught at least once as adults and, for females, 86 of the 260 (33%) first caught as juveniles were later captured at least once as adults.

Because some mice were caught more than once during the course of the study and because some mice matured during the course of an annual field season, there may be a question with regard to the independence of data points. I tabulated the number of different mice represented in each of the three data sets (Table 1). Calculations of the mean numbers of times male and female mice of the two ages were captured (Table 2) indicate that some mice were caught up to seven or eight times in a few of the classes in one or more years. Repeat captures were more frequent for adult males than for adult females or juveniles of either sex, and adult female repeat captures occurred more often

TABLE 2. MEAN FREQUENCY OF CAPTURES PER MOUSE AND RANGES OF NUMBERS OF CAPTURES FOR ADULT AND JUVENILE MALE AND FEMALE HOUSE MICE IN THREE DATA SETS INVOLVING MULTIPLE CAPTURES FOR SOME INDIVIDUALS

	1989		1992 ABEF		1992 CDGH	
Age/sex class	Mean	Range	Mean	Range	Mean	Range
Junveile female	1.1	1–3	1.2	1–4	1.3	1–4
Adult female	3.7	1–6	4.1	1–7	2.6	1–5
Juvenile male	1.4	1–4	1.3	1–4	1.2	1–4
Adult male	5.4	1–8	5.3	1–7	4.8	1–8

than repeat captures of either sex of juvenile. An examination of 40 mice, caught five or more times, sampled from across all three data sets indicated that the responses of each repeat capture individuals were not concentrated in a single odor-source type of trap or at a particular trap location. Both the large overall sample size and the lack of any consistent biases in terms of mice that were caught multiple times makes it unlikely that the statistical tests would be confounded by lack of independence for some of the data points involving repeat captures. In addition, the G test is relatively robust with respect to this problem (Sokal and Rohlf, 1981).

There could also be some concern about the availability of the various trap odors on a given night. If only limited numbers of odors were available, then some biases could be introduced because mice caught on those particular nights would have limited choices in terms of being caught with only certain odors in the traps. Analyses revealed that for 1989, there were, on average, 5.2 $\pm$ 0.3 odors available (range = 3–6) for 48 trap nights; for 1992 in enclosures C, D, G, and H there were, on average, 5.4 $\pm$ 0.2 odors available (range = 2–6) for 47 trap nights; for 1992 in enclosures A, B, E, and F there were, on average, 5.4 $\pm$ 0.4 odors available (range = 2–6) for 47 trap nights. These values indicate that there were, on most occasions, high percentages of the six test odors available in the traps.

Capture data for 1989, 1992 (enclosures A, B, E, and F), and 1992 (enclosures C, D, G, and H) are shown in Tables 3–5 such that two types of questions can be tested using the log likelihood ratio statistic. First, examining the tables

TABLE 3. CAPTURES BY AGE AND SEX CLASS FOR MICE IN SIX ENCLOSURES DURING 1989 ACCORDING TO CHARACTERISTICS OF PREVIOUS CAPTURE AND TYPE OF MOUSE CAPTURED NEXT[a]

	Previous capture						
Next capture	Juv M	Ad M	Juv F	NE F	ES F	PG/LA F	Total
Juv M	12	18	7	11	13	4	65
Ad M	21	57	30	36	57	27	228
Juv F	5	21	18	15	9	3	71
NE F	4	37	5	27	13	24	110
ES F	7	39	6	6	12	13	83
PG/LA F	8	38	9	13	5	23	96
Total	57	210	75	108	109	94	653

[a]Abbreviations for this and subsequent tables: Juv M = juvenile male; Ad M = adult male; Juv F = juvenile female; NE F = nonestrous female; ES F = estrous female; PG/LA F = pregnant lactating female.

TABLE 4. CAPTURES BY AGE AND SEX CLASS FOR MICE IN SIX ENCLOSURES DURING 1992 FOR ENCLOSURES A, B, E, AND F ACCORDING TO CHARACTERISTICS OF PREVIOUS CAPTURE AND TYPE OF MOUSE CAPTURED NEXT[a]

	Previous capture						
Next capture	Juv M	Ad M	Juv F	NE F	ES F	PG/LA F	Total
Juv M	5	18	7	9	6	4	49
Ad M	13	133	33	88	23	53	343
Juv F	7	45	27	18	4	6	107
NE F	10	90	20	39	4	24	187
ES F	2	34	4	2	1	5	48
PG/LA F	5	45	7	23	4	19	103
Total	42	365	98	179	42	111	837

[a]See Table 3 for explanation of abbreviations.

in terms of the previous capture, that is, from the perspective of the trap, we can ask whether there is any bias in terms of what types of mice, by age and sex, are captured next, depending upon the class of the odor source mouse. I call this a methods approach. Second, the tables can be examined looking across the rows according to respondents to determine whether mice of a particular class have any bias in terms of significant differential responses to particular types of odor sources in the traps. This examination of the data is from the mouse's perspective, and I call it a biosocial approach.

TABLE 5. CAPTURES BY AGE AND SEX CLASS FOR MICE IN SIX ENCLOSURES DURING 1992 FOR ENCLOSURES C, D, G, AND H ACCORDING TO CHARACTERISTICS OF PREVIOUS CAPTURE AND TYPE OF MOUSE CAPTURED NEXT[a]

	Previous capture						
Next capture	Juv M	Ad M	Juv F	NE F	ES F	PG/LA F	Total
Juv M	12	25	12	17	4	12	82
Ad M	25	150	66	129	56	84	510
Juv F	14	55	31	17	3	14	134
NE F	13	106	19	58	29	35	260
ES F	1	48	4	11	12	13	83
PG/LA F	10	75	9	37	18	33	182
Total	75	459	141	269	119	189	1252

[a]See Table 3 for explanation of abbreviations.

TABLE 6. RESULTS FROM G TESTS CONDUCTED ON MOUSE RESPONSES TO ODORS IN TRAPS INVOLVING DIFFERENTIAL RESPONSES ACCORDING TO PREVIOUS CAPTURE BY EXAMINING DATA FROM A METHODS PERSPECTIVE[a]

Previous capture	1989 (all)	1992 (ABEF)	1992 (CDGH)
Juv M	4.40	2.78	12.38[b]
Ad M	10.74	10.30	19.82[c]
Juv F	12.02[b]	11.80[b]	25.90[c]
NE F	5.22	10.04	12.30[b]
ES F	22.66[c]	6.46	10.80
PG/LA F	16.62[b]	5.66	1.04

[a] See Table 3 for explanation of abbreviations. $df = 5$ for all tests.
[b] $P < 0.05$ (critical value for significance = 11.07).
[c] $P < 0.005$ (critical value for significance = 16.75).

For the methods approach (trap perspective; Table 6), there are only three values of 18 log-likelihood ratios that are significant at $\alpha < 0.005$. These three significant test results are each for a different class of mouse and can be examined further by reference to Tables 3–5; (1) Traps odorized by adult males caught more nonestrous, estrous, and pregnant/lactating females than expected. (2) Traps that had previously contained a juvenile female caught fewer nonestrous, estrous, and pregnant/lactating females than expected. (3) Traps that were occupied previously by an estrous female caught more adult males than expected. There are no consistent trends with respect to whether particular odor types in the traps resulted in significant differential responses by the mice (Table 6).

For the biosocial approach (mouse perspective; Table 7), 10 of the tests were significant at the $\alpha < 0.005$ level, and there are some clear patterns of consistent differences. Adult males, as well as juvenile, nonestrous, and estrous females all exhibited significant differential responses to particular odors in the traps. Examination of the significant findings in Table 7 in conjunction with Tables 3–5 reveals the following explanations for the significant differences: (1) Adult males exhibited a preference for traps that had contained juvenile or estrous females and avoided traps that had previously contained adult males. (2) Juvenile females showed a preference for traps that had contained a juvenile female, but consistently avoided traps whose previous occupant had been an estrous female, a nonestrous female, or a pregnant/lactating female. (3) Nonestrous females exhibited a preference for traps that had contained an adult male. (4) Estrous females showed a preference for adult males and consistently avoided traps that had contained nonestrous or pregnant/lactating females.

In order to know whether some of the recorded differences were due to

TABLE 7. RESULTS FROM *G* TESTS CONDUCTED ON MOUSE RESPONSES TO ODORS IN TRAPS INVOLVING DIFFERENTIAL RESPONSES ACCORDING TO PREFERENCES OF RESPONDER MOUSE BY EXAMINING DATA FROM A BIOSOCIAL PERSPECTIVE[a]

Responder type	1989 (all)	1992 (ABEF)	1992 (CDGH)
Juv M	13.30[b]	2.74	0.56
Ad M	54.54[c]	18.36[c]	20.01[c]
Juv F	26.58[c]	21.54[c]	23.82[c]
NE F	27.02[c]	0.16	19.20[c]
ES F	14.52[b]	18.74[c]	67.60[c]
PG/LA F	8.14	2.08	9.70

[a] See Table 3 for explanation of abbreviations. $df = 5$ for all tests.
[b] $P < 0.05$ (critical value for significance = 11.07).
[c] $P < 0.005$ (critical value for significance = 16.75).

either variations in capture rates of mice or possibly differences in densities, it was necessary to calculate values for these parameters for each of the four years 1989–1992. Because most mice are captured at the interior 28 traps of each trapping grid, only those trap stations have been used to assess variations in capture rates. Rates of mouse captures at interior traps in the years with odor-baiting (1990 + 1991 = 16.6 ±1.9 mice/100 trap nights) did not differ significantly ($t = 0.40$, $df = 28$; $P > 0.20$) from the two years used for the present analyses of odors in traps (1989 + 1992 = 15.5 ± 2.0 mice/100 trap nights). Three-week intervals were used for calculating population size in the enclosures as minimum number known alive. Analysis of those interval estimates of density across the enclosures revealed no significant difference ($t = 1.67$; $df = 28$; $P > 0.10$) for the two years (1990 + 1991 = 29.2 ± 3.6 mice/enclosure) with odor-baiting compared to the two years (1989 + 1992 = 21.6 ± 2.9) used for the present analyses involving residual odors in traps.

DISCUSSION

The foregoing analyses provide the basis for two key conclusions and interpretations. (1) From a methods or trap perspective, there is minimal reason for concern about odors in traps from previously captured mice exerting a specific bias on what is captured next; these effects are not as pervasive as one might expect. (2) From the perspective of the mice, there are clear odor choices for specific odors by mice of most age, sex, and reproductive groups.

That findings revealed by the analyses of trapping data from years in which no specific odor-baiting was carried out show very clear choices suggests that

cleaning the traps, as was done in earlier experiments (Drickamer et al., 1992), resulted in the removal of contextual odor cues necessary for the mice to exhibit preferences. This effect could be due to mice avoiding clean traps or the need for the odor cues provided by a general soiling of the trap. It may also be due, at least in part, to the fact that the urine odors that were used by Drickamer et al. (1992) were, in fact, from mice not part of the test population. This would suggest that for odor-baiting experiments, the sources of the odors should come from within the test population to avoid confounding the results with unfamiliar scents.

Rates of captures at interior traps in the years with odor-baiting did not differ significantly from rates of capture for the two years used in the analyses reported here (Drickamer et al. 1992). Mean densities of mice per enclosure were also not statistically different for years when odor-baiting was conducted compared with years when traps were allowed to remain soiled throughout the season. Thus, effects recorded in the analyses reported here and the rather striking differences between the present findings and the data reported by Drickamer et al. (1992) are not, it seems, due to either variations in capture rates of mice or to some density-related difference.

It thus appears that contextual odors must play a significant role in the ability of mice to respond to traps. This finding contributes to our understanding of house mouse biology, as discussed in more detail below, and to our knowledge about methods used in rodent trapping. In attempting to avoid the sorts of biases that could occur when traps are cleaned after each capture, we actually may be biasing the findings by removing important cues. My data are in partial agreement with earlier reports for species of *Microtus*, *Apodemus*, and *Clethrionomys* (Boonstra and Krebs, 1976; Gurnell and Little, 1992). These investigators all report that rodents entered soiled traps more readily than clean traps. The results of Drickamer et al. (1992) differed in that the rates of capture do not differ between soiled and clean traps, but there is a significant change in the pattern with which traps are entered when they are left soiled between captures. The finding by Stoddart (1982) that for *M. agrestis* more mice were caught in recently cleaned traps than in traps that remained soiled seems to be unique in the literature on odors in rodent traps and is quite distinct from the present findings.

Examining the present findings from a methods or trap perspective revealed that no responder class of mouse exhibited the same significant bias in all three sets of data that were analyzed. Only three of the 18 tests were statistically significant at the $P < 0.005$ level. Perhaps the most consistent bias was that traps that had most recently contained a juvenile female were avoided by all other types of adult females, although not by other juvenile females. From these analyses, I would conclude that for house mice there is little overall concern about introducing biases into trapping data because of what was previously

captured in each particular trap. Earlier reports on other rodent species have not generally examined the data from the perspective of whether there are biases in what is captured subsequent to catching mice of a particular age, sex, or reproductive condition, although Gurnell and Little (1992) do conclude for wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*) that there should not be any real concern about biases in trapping due to resident odors. My findings do substantiate something that has been "known" by many who trap rodents, namely, the live traps should be left in the field during the course of an experiment so that they become part of the olfactory environment of the animals. In terms of a methods perspective, it is also worth noting that for some species, particularly vole species that could be cycling and at high population density, there must be sufficient traps present to avoid any bias in terms of captures with particular odors due to a paucity of available open traps. With the house mice in this study, the proportion of traps occupied after a night of trapping was consistently less than 50%, making it unlikely that any major bias of this type was introduced. I did not note any differences in responses in terms of odor selections across a range of mouse densities that ranged from only four to six mice known to be alive in an enclosure for a given trapping week to situations with approximately 50 mice in an enclosure.

If we examine the analyses reported here from the perspective of olfactory communication and social biology of the mice, several significant conclusions emerge. Adult males and females in estrous selected traps most recently occupied by estrous females and adult males, respectively. Clearly, for house mice, the two sexes apparently actively seek each other when reproduction is possible. It is noteworthy that this level of odor preference may lead to further steps in mate selection by the mice, as in the recent report from laboratory tests indicating that estrous females can discriminate dominant and subordinate males (Drickamer, 1992). Since I did not have data for dominance status of the individual males in the enclosures in either of the years analyzed here, it is not possible to test this hypothesis further. Such tests should be conducted in the future under field conditions. House mice may differ in this regard from *Apodemus* spp. and bank voles (*Clethrionomys glareolus*), where there were no significant heterospecific trap odor preferences (Gurnell and Little, 1992). The results for house mice are similar to the findings of preferences for traps containing heterospecific odors reported earlier for *Peromyscus maniculatus* and *P. leucopus* (Drickamer, 1984).

That male house mice consistently avoided traps odorized by other males may be a reflection of the function of olfactory communication in terms of the spatial relations of the mice. House mice living in commensal situations are generally territorial (Anderson and Hill, 1965; Crowcroft, 1973), but in feral settings the sociospatial system appears to be more one of overlapping home ranges (Dunn, 1960; Mikesic and Drickamer, 1992). As indicated in Methods

and Materials, males do have a tendency to be captured again in traps containing their own odor; they appear to recognize and discriminate their own odor from those of conspecific males. This is similar to findings reported by Hurst (1989, 1990a) concerning the frequency of urine marking and investigation of urinary cues.

Nonestrous females exhibited a significant preference to enter traps that had previously contained an adult male. This result may be interpreted in several ways. First, these females may simply inhabit an area that includes the trap site. Second, if some of the nonestrous females were nearing estrous, then they may have been engaging in the initial stages of seeking a mate. Third, such females may also be exposing themselves to odors from males because such odors have the effect of accelerating the onset of estrus (Whitten, 1956; McClintock, 1983). Thus, responses to odor cues by mice may have both a communication function and a physiological function in terms of affecting the reproductive endocrinology of those responding to the odor cues.

Juvenile females avoided the odors of most types of females, except those of the same age, toward which they exhibited a significant preference. It is possible that this avoidance pattern reflects resource competition. In the feral situation, such juvenile females may emigrate from the natal site, dispersing to areas where there is less competition for mates, nest sites, or food. This sort of dispersal has been hypothesized previously for house mice by Bronson (1979). A similar hypothesis may be used to explain why estrous females significantly avoided traps that had been previously occupied by other adult females, regardless of reproductive condition. Females in estrous, should they become pregnant, will need to establish and maintain a sufficient resource base to achieve successful parturition and rearing of their young. Seeking an area free of potentially competing females may be part of the reason I detected the avoidance of traps from those other adult females. That the avoidance seems strongest to nonestrous females and weakest toward pregnant or lactating females may be due to some form of mate competition.

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UPTAKE AND SEQUESTRATION OF OUABAIN AND OTHER CARDIAC GLYCOSIDES IN *Danaus plexippus* (LEPIDOPTERA: DANAIDAE): EVIDENCE FOR A CARRIER-MEDIATED PROCESS

C. FRICK and M. WINK*

Universität Heidelberg,
Institut für Pharmazeutische Biologie,
Im Neuenheimer Feld 364,
D-69120 Heidelberg, Germany

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Abstract—Larvae of *Danaus plexippus* feed almost exclusively on milkweed species of the genus *Asclepias*, whose characteristic secondary metabolites are cardiac glycosides (CGs). Aposematic last-instar larvae were fed with ouabain and other cardiac glycosides of differing polarities. Time course experiments show that ouabain is sequestered in the integument within 48 hr after feeding, whereas midgut tissue and hemolymph function as transient CG storage compartments. About 63% of ouabain was transferred from larvae to the butterflies, whereas 37% of ouabain was lost with larval and pupal exuviae and with the meconium. The main sites of storage in imagines are wings and integument. If mixtures of CGs are fed to *D. plexippus* larvae, differential sequestration can be observed: The polar ouabain contributes 58.8% of total CGs, followed by digitoxin (19.6%), oleandrin (10.6%), digoxin (4.9%), digoxigenin (4.6%) and proscillaridin A (1.5%). Thus, uptake and sequestration must be selective processes. Uptake of [^{3}H]ouabain in vitro by isolated larval midguts was time-, pH-, and temperature-dependent and displayed an activation energy of 49 kJ/mol. Furthermore, the in vitro uptake of ouabain was inhibited (probably competitively) by the structurally similar convallatoxin. These data provide first evidence that ouabain uptake does not proceed by simple diffusion but with the aid of a carrier mechanism, which would explain the differential cardenolide uptake observed in living larvae.

Key Words—*Danaus plexippus*, cardenolides, uptake, carrier, sequestration, chemical defense, midgut, integument.

*To whom correspondence should be addressed.

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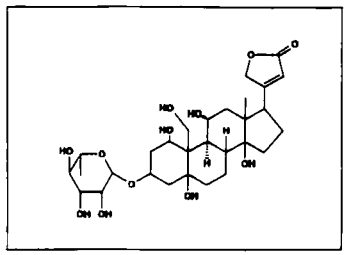
INTRODUCTION

Larvae of the monarch butterfly, *Danaus plexippus* L., feed on milkweed plants of the genus *Asclepias* that produce cardiac glycosides as characteristic secondary metabolites. *D. plexippus* is a specialized herbivore and is closely adapted to the secondary chemistry of Asclepiadaceae. Monarch larvae and imagines store CGs and are aposematic, similar to the situation in a number of other aposematic Lepidoptera, Hemiptera, and Coleoptera (Duffey and Scudder, 1972; Duffey, 1980; Malcolm, 1990; Harborne, 1993). Several experiments and observations have shown that cardenolide-rich monarch butterflies may become distasteful and toxic to predators (Reichstein et al., 1968; Brower et al., 1975, 1982; Brower, 1984; Cohen, 1985; Fink and Brower, 1981; Brower and Fink, 1985; Seiber et al., 1980, 1986; Ritland, 1991).

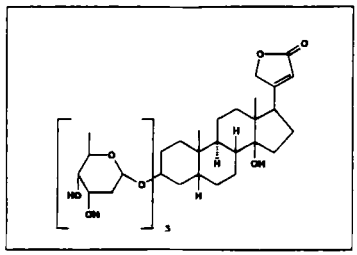
The sequestration and storage of cardiac glycosides by monarchs is a selective process in both quantitative and qualitative terms (Reichstein et al., 1968; Roeske et al., 1976; Seiber et al., 1980, 1986; Brower et al., 1982; Lynch and Martin, 1987; Martin and Lynch, 1988; Malcolm, 1990; Malcom and Brower, 1989): Monarchs appear to regulate their overall content of cardiac glycosides in such a way that an appropriate cardenolide capacity necessary for chemical defense is achieved, although a substantial part of the monarch population has a low cardenolide content (Brower et al., 1972; Brower and Mofitt, 1974; Ritland, 1991). Out of the complex pattern of cardenolides that are present in food plants, monarch butterflies selectively store polar cardenolides, but not the more lipophilic apolar glycosides (Brower, 1984; Brower et al., 1988; Malcolm et al., 1989; Groeneveld et al., 1990).

Cardiac glycosides are apparently not stored in all tissues of the monarch, but preferentially in the exoskeleton of the butterfly, i.e., the wings and the cuticle (Brower and Glazier, 1975; Seiber et al., 1986; Brower et al., 1988). This peripheral storage seems to provide a chemical defense barrier against nonadapted enemies, but adapted predators (birds and mice) can avoid the cardenolide-rich tissues (Fink and Brower, 1981; Brower et al., 1988; Glendinning, 1990).

In this communication we have further characterized the kinetics and tissue specificity of cardiac glycoside sequestration in larvae and adults of *D. plexippus* with respect to differing polarities of particular compounds such as ouabain, digitoxin, oleandrin, digoxin, digoxigenin, and proscillaridin A. In addition, biochemical transport studies with isolated larval midguts have been carried out with the polar ouabain (which resembles the naturally occurring CGs of the *Asclepias* food plants; Scheme 1), providing the first evidence for a cardenolide carrier mechanism.



Ouabain



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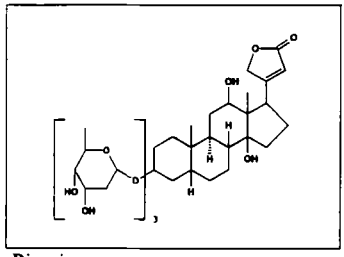

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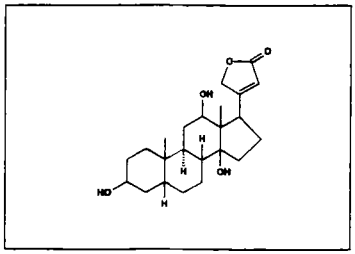
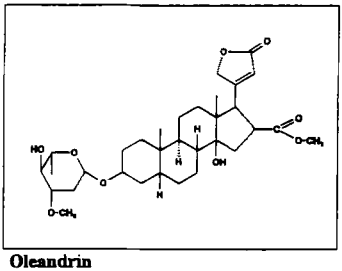
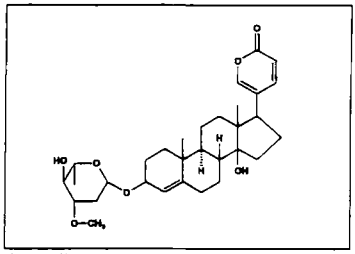


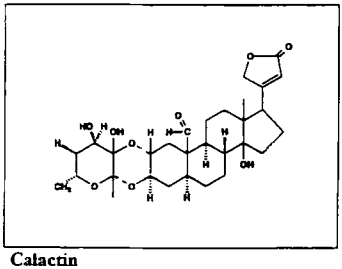
Digoxigenin



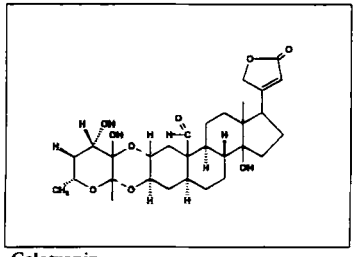
Oleandrin



Proscillaridin A



Calactin



Calotropin

SCHEME 1. Structures of the main cardiac glycosides employed in this study (ouabain, digitoxin, digoxin, digoxigenin, oleandrin, and proscillaridin A); typical cardiac glycosides of *Asclepias* are calactin and calotropin (Reichstein et al., 1968).

METHODS AND MATERIALS

Animals. Eggs of *D. plexippus* came from a laboratory colony kept at the Boyce Thompson Institute for Plant Research at Ithaca, New York (USA). In Germany, larvae were reared on *Asclepias syriaca* and a few generations of monarchs were grown in a greenhouse in the summers of 1991, 1992, and 1993. We chose *A. syriaca* (in North America *A. incarnata* or *A. tuberosa* would have been preferable) because it was locally available and HPLC analyses showed that only very low levels of endogenous cardenolides were produced. These endogenous compounds did not interfere with our experiments, because larvae that were reared on these plants were almost devoid of cardenolides.

Feeding Experiments. Concentrated solutions of ouabain and other CGs were applied on small leaf areas (12 × 12 mm) of *A. syriaca.* When solutions were dried, the leaves were fed to last-instar larvae, which were kept individually in plastic containers. Following the application of CGs (feeding was always finished after 45 min), larvae were given untreated *A. syriaca* leaves ad libitum. At time intervals given, larvae were collected (and their corresponding feces) and immediately dissected into hemolymph, guts (their contents were separately collected), fat body, and integument. After metamorphosis, larval and pupal exuviae were also collected. Meconium was taken from freshly emerged imagines.

Quantitation of Cardiac Glycosides. Larvae or dissected organs were stored at −20°C until processing. Tissues were ground in liquid nitrogen in a mortar. Then, 10 ml 90% ethanol was added and the homegenate was thoroughly mixed for 45 min at 70°C. After centrifugation (10 min at 10,000 rpm), 5 ml of the supernatant were collected. After adding 2 ml of Kedde reagent (100 mg 3,5-dinitrobenzoic acid in 10 ml methanol), samples were measured in a spectrophotometer at 540 nm (background). Then 1 ml 1 M NaOH was added and the optical density again measured after exactly 5 min at 540 nm. This value was then evaluated with a calibration curve established with respective reference glycosides.

When larvae were fed with mixtures of cardenolides, individual cardenolides were separated and quantified by HPLC analysis (Groeneveld et al., 1990; Wiegrebe and Wichtl, 1993). A Hewlett Packard 1040 with photodiode array detection was used; the column was an RP 18-100, 250 × 4 mm, 5 μm particles (Lichrocart, Merck, Darmstadt, Germany). Acetonitrile/water gradient elution: 3 min isocratically at 28% acetronitrile, 3–15 min linearly to 60% acetronitrile, then 2 min isocratically followed by 2 min with 95% acetronitrile. An elution profile is illustrated in Figure 1.

Carrier Studies. Larvae were dissected in modified Ringer solution, which consisted of 8000 mg NaCl, 200 mg KCl, 1000 mg glucose, 1000 mg Tricine, pH 8, in 1000 ml distilled water. Guts were excised and slit open to remove

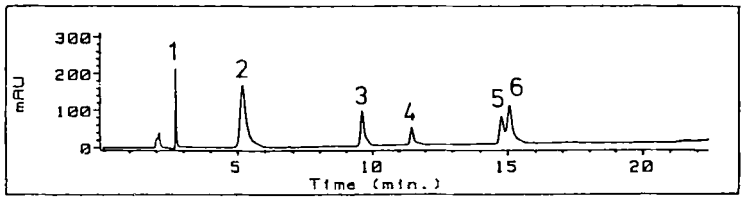


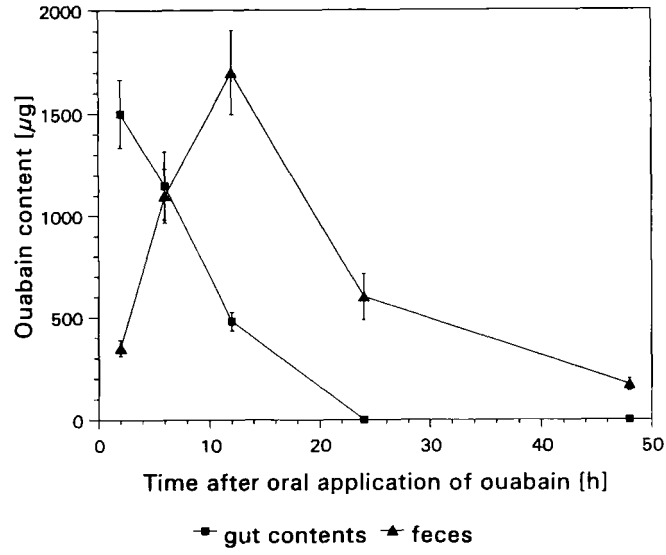
FIG. 1. Separation of a mixture of cardenolides by reversed phase HPLC. 1 = ouabain, 2 = digitoxigenin, 3 = digitoxin, 4 = proscillaridin A, 5 = oleandrin, 6 = digoxin. (For HPLC conditions see Methods and Materials).

the peritrophic membrane and the gut contents. Midguts were cut into pieces 2 mm long, which were immediately used for the uptake experiments. For each data point, three or four gut pieces were added to 200 μl buffer in Eppendorf vials and left there for equilibration for 15 min. Experiments were started by adding [^{3}H]ouabain (ca. 200,000 cpm; specific activity 28.8 Ci/mmol). Experiments were terminated by taking out the gut segments and washing them in "cold" incubation buffer for 2 min. Then they were individually transferred to Eppendorf vials containing 100 μl 1 M HCl. Tissues were homogenized by hand with a small glass rod and then in an ultrasonic disintegrator for 30 min. Homogenates were quantitatively transferred into scintillation vials containing 10 ml scintillation cocktail. The radioactivity was determined in a scintillation counter. In addition, the radioactivity of the incubation buffer was measured to calculate the percentage of radioactivity taken up by the gut segments. As a control, gut segments were incubated with tritiated water and processed in the same way as in the ouabain experiments.

RESULTS

Kinetics and Selectivity of Sequestration of Ouabain and Other Cardiac Glycosides. Whereas most authors have studied the sequestration of cardenolides present in the respective host plants (Reichstein et al., 1968; Brower et al., 1975, 1982, 1988; Brower, 1984; Cohen, 1985; Fink and Brower, 1981; Brower and Fink, 1985; Seiber et al., 1980, 1986; Roeske et al., 1976; Lynch and Martin, 1987; Martin and Lynch, 1988), we have chosen a different approach: We have applied pure cardiac glycosides from nonhost plants that differed in structure and polarity and studied their kinetics and the tissue specificity of sequestration in larvae and butterflies.

In a first time-course experiment, last-instar larvae were fed with a single dose of 3.5 mg ouabain, which was applied on leaves of the host plant, *Asclepias syriaca.* At the time intervals shown in Figure 2, larvae were collected and



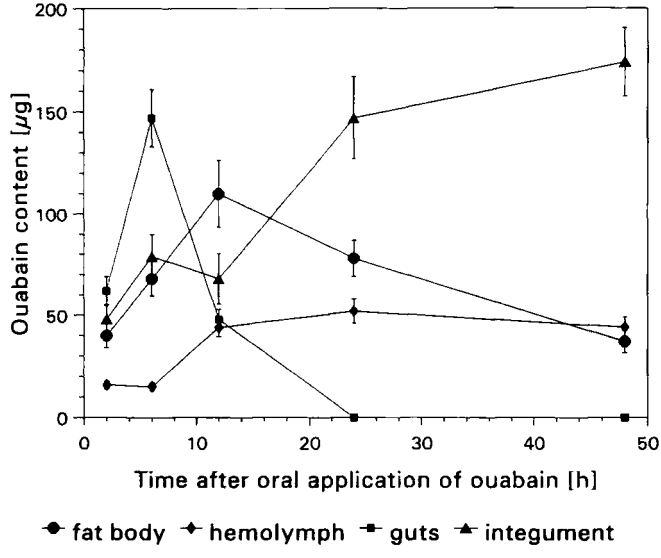


FIG. 2. Time course of ouabain uptake and sequestration in last-instar larvae of *Danaus plexippus*. (A) Kinetics in guts and feces; (B) kinetics in gut epithelia, hemolymph, fat body, and integuments. Bars represent standard deviations (N = 3–5).

dissected to separate hemolymph, guts (gut tissue separated from gut contents), fat body, and integument. Fecal pellets were also collected from the corresponding animals. Whereas the majority of the ouabain was excreted via the feces (a peak is reached 12 hr after feeding), 255 μg ouabain was retained by the larvae after 48 hr and was predominantly sequestered in the larval integument (Figure 2). Ouabain reached a transient maximum after 6 hr in the midgut epithelia. As ouabain decreased in this tissue, it increased transiently in hemolymph and the fat body and was eventually concentrated in the integument.

Another set of larvae was allowed to develop into imagines. As shown in Figure 3, the integument and wings represented the main storage sites for ouabain containing 82% of the total ouabain stored, whereas the fat body and muscles had significantly less ouabain; the ovaries and eggs, testes, and hemolymph were almost ouabain-free. Larval and pupal exuviae and the meconium contained 37% of the total amount of ouabain originally sequestered by the larvae, which qualitatively agrees with a previous study (Malcolm and Brower, 1989).

For comparison, a polar (i.e., ouabain) and several apolar cardiac glycosides [i.e., digoxin, digitoxin, oleandrin and digoxigenin (an aglycone)] were individually fed to last-instar larvae. Resorption, which was determined after 24 hr, was observed in all instances and was highest for digitoxin and digoxin, followed by ouabain, digoxigenin, and oleandrin (Table 1). The integument was again the main storage site.

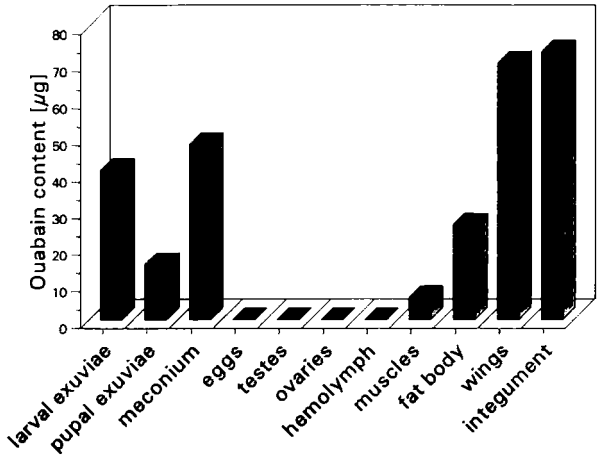


FIG. 3. Distribution of ouabain in monarch butterflies. Larvae had obtained 3.65 mg ouabain.

TABLE 1. SEQUESTRATION OF INDIVIDUALLY APPLIED CARDIAC GLYCOSIDES IN LARVAE OF *Danaus plexippus*[a]

Compound	Amount applied (μmol)	Amount taken up (μmol, %)[b]
Polar CG		
Ouabain	4.80	0.37 (7.7)
Medium polarity		
Digoxin	4.48	0.44 (9.6)
Apolar CG		
Digitoxin	4.58	0.52 (11.3)
Digoxigenin	8.97	0.55 (6.1)
Oleandrin	6.07	0.24 (3.9)

[a] Determination after 24 hr. Pure cardenolides applied on *Asclepias* leaves were injested by the larvae within 45 min.
[b] CG contents were determined photometrically.

In another set of experiments the glycosides were given as a mixture to find out whether the same distribution pattern appeared or the uptake was structure-specific. As can be seen from Table 2, the polar ouabain was clearly sequestered selectively (88% of the ouabain given was taken up) to account for 58.8% of the total resorbed CGs (Table 2). When a mixture of ouabain and its aglycone ouabagenin was applied, resorption of the aglycone (which accounted for 15.3%) was almost identical to that of ouabain (uptake = 14.6%) (Table 2).

A comparable pattern can be seen in imagines, indicating that the data in Table 2, which are derived from a 24-hr experiment only, are already representative. From the mixture of six CGs that was analyzed by HPLC (same as in Table 2, mixture I) the sequestration of ouabain was significantly favored: Ouabain contributed 51.7% of total CGs (= 0.52 μmol/animal), followed by digitoxin (15.3%), oleandrin (18.3%), digoxin (12.2%), digoxigenin (0.25%) and proscillaridin A (0%). Both integument and wings represented the main site of CG accumulation in most instances (Figure 4), except for oleandrin, which was predominantly found in fat-body tissue. Like the situation described in Figure 3, the meconium and larval and pupal exuviae also contained CGs (0.18 μmol/animal) and show a pattern that resembles that of the integument (Figure 4).

Evidence for Carrier-Mediated Ouabain Uptake. For the in vitro uptake studies, ouabain was selected because this glycoside is polar and does not diffuse across biomembranes by simple diffusion. Commercially available [^{3}H]ouabain was rapidly resorbed by larvae and a maximum of radioactivity occurred in the

TABLE 2. SEQUESTRATION OF CARDIAC GLYCOSIDE MIXTURES BY *D. plexippus* LARVAE[a]

Compound	Amount applied (μmol)	Amount taken up (μmol, %)[b]	Total CGs (%)
Complex mixture			
Ouabain	0.41	0.36 (87.8)	58.8
Digitoxin	0.39	0.12 (30.8)	19.6
Oleandrin	0.52	0.06 (12.5)	10.6
Digoxin	0.38	0.03 (7.9)	4.9
Digoxigenin	0.77	0.03 (3.6)	4.6
Proscillaridin A	0.58	0.01 (1.6)	1.5
Total CGs	3.06	0.61	100
Ouabain versus its aglycone			
Ouabain	1.23	0.18 (14.6)	42.9
Ouabagenin	1.57	0.24 (15.3)	57.1
Total CGs	2.80	0.42	100

[a] Quantitation after 24 hr. Pure cardenolide mixtures applied on *Asclepias* leaves were completely injested by the larvae within 45 min.
[b] Determined by HPLC.

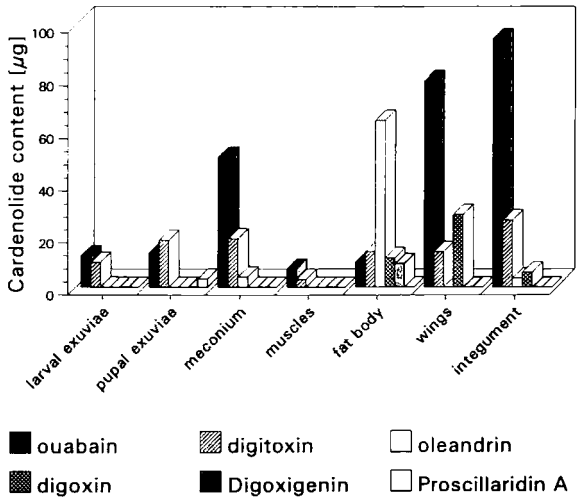


FIG. 4. Tissue-specific distribution of ouabain, digitoxin, oleandrin, digoxin, digoxigenin, and proscillaridin A in monarch butterflies. Quantitative determinations by HPLC.

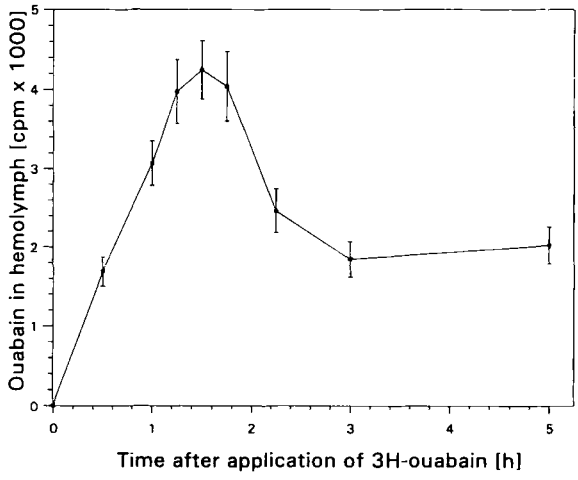


FIG. 5. Kinetics of [^{3}H]ouabain in the hemolymph. Larvae were fed 5 μCi [^{3}H]ouabain. At the time intervals given, animals were taken and their hemolymph was collected. The radioactivity of the hemolymph was measured in a scintillation counter. Bars represent standard deviations (N = 3–5).

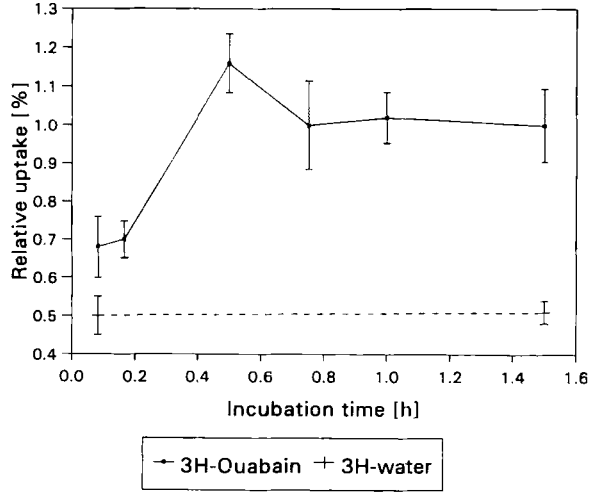


FIG. 6. Time course of the uptake of [^{3}H]ouabain and [^{3}H]H_2O by isolated gut segments of *D. plexippus*. Experiments were performed at pH 7 and 24°C. Bars represent standard deviations (N = 3–5).

hemolymph about 90 min after feeding (Figure 5). This provides some insight into the expected kinetics.

Since uptake and carrier experiments are difficult with intact larvae or complete midguts, midguts were prepared from last-instar larvae and cut into small pieces of equal size. These midgut segments were incubated with [^{3}H]ouabain for differing time intervals, and the incorporation of [^{3}H]ouabain was monitored by measuring its radioactivity. Uptake of [^{3}H]ouabain followed an almost linear increase with time for the first 30 min (Figure 6). [^{3}H]H_2O, which freely diffuses across membranes, was taken up at 0.5%, whereas [^{3}H]ouabain became enriched in the midgut epithelia and reached a maximum

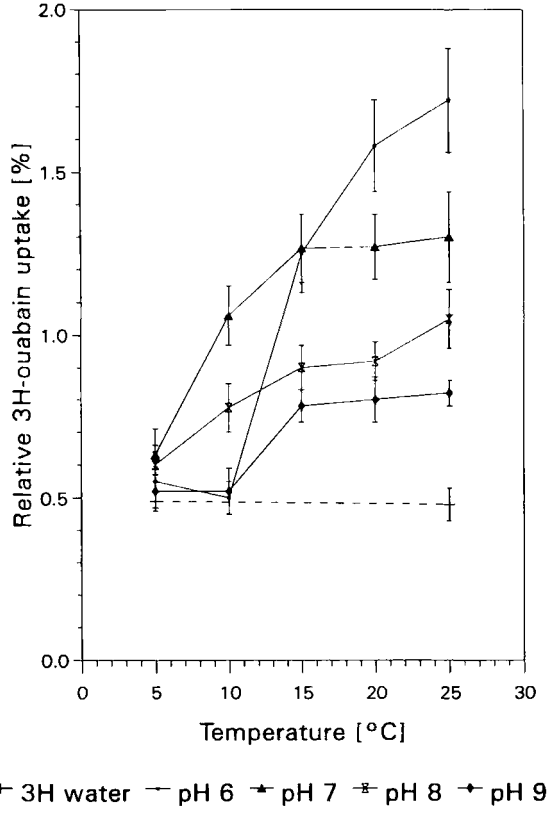


FIG. 7. Influence of hydrogen ion concentration and temperature on the uptake of [^{3}H]ouabain by midgut segments of *D. plexippus*. Reaction time is 50 min. Bars represent standard deviations (N = 3–5).

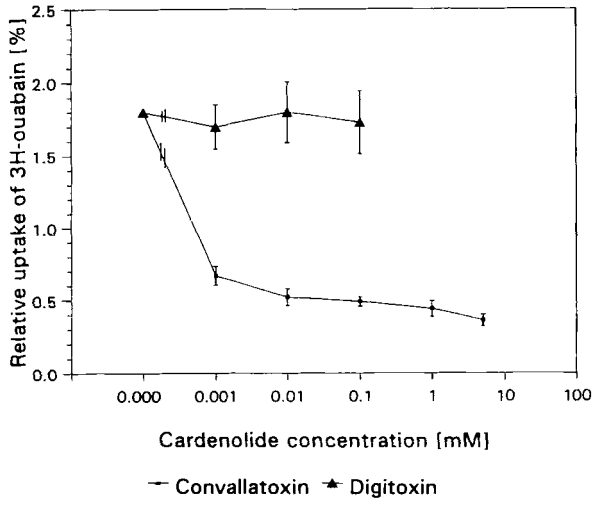


FIG. 8. Inhibition of the uptake of [^{3}H]ouabain by the structurally related cardenolide convallatoxin (final concentrations between 0.001 and 8 mM). Ouabain uptake was not influenced by the apolar digitoxin, which could not tested at higher concentrations because of solubility problems. Reactions were performed at 24°C for 50 min at pH 6.

of 1.15%. This indicates that [^{3}H]ouabain is taken up by the tissues in vitro and that it may even accumulate against a concentration gradient.

Experiments were carried out to determine the effect of temperature and hydrogen ion concentrations on uptake. As can be seen from Figure 7, uptake was optimal at pH 6 and displayed a clear temperature dependence. An activation energy of 49 kJ can be derived from an Arrhenius plot performed with data from pH 6 experiments. In addition, [^{3}H]ouabain uptake was inhibited by the structurally similar convallatoxin, but not by the apolar digitoxin (Figure 8).

DISCUSSION

If the patterns of cardenolides present in the host plant are compared with those in the monarch butterfly, it is apparent that they do not match either qualitatively or quantitatively; polar cardenolides are often present in higher amounts than in the host plant (Reichstein et al., 1968; Roeske et al., 1976; Seiber et al., 1980, 1986; Lynch and Martin, 1987; Martin and Lynch, 1988; Malcolm, 1990; Malcom and Brower, 1989). A selective cardenolide sequestration has also been reported for *Caenocoris nerii*, *Spilosthethus pandurus* (Euw

et al., 1971), *Poikilocerus bufonius* (Euw et al., 1967), and *Oncopeltus fasciatus* (Scudder and Meredith, 1982a; Scudder et al., 1986).

How is Selective Sequestration Achieved? It has been shown that *Danaus* and *Oncopeltus* are able to metabolize some of their dietary cardenolides (Scudder et al., 1986; Brower, 1984). For example, a cardenolide may be hydrolyzed to obtain the corresponding aglycone (Seiber et al., 1980), but metabolism can only partly explain the difference of CG profiles between host plants and insects. Some cardiac glycosides are highly apolar and lipophilic and can be resorbed by simple diffusion, whereas others are polar and more hydrophilic, such as ouabain. Physicochemical characteristics of the hydrophilic compounds imply that they do not freely diffuse across membranes. When feeding CGs to moth larvae (*Syntomis mogadorensis* and *Creatonotos transiens*), which do not encounter cardenolides in their diet, we found that the cardiac glyclosides were not taken up but eliminated with the feces (Wink and Schneider, 1990). Furthermore, the midguts of *Schistocerca gregaria* and *Periplaneta americana* were impermeable to ouabain and other cardenolides (Scudder and Meredith, 1982b). These data suggest that ouabain and other polar CGs do not diffuse nonspecifically across gut membranes.

In larvae of *Syntomeida epilais*, which also store CGs for chemical defense, we obtained evidence that CGs are resorbed from the guts by a carrier-mediated process (von Nickisch-Rosenegk et al. 1990). For comparison, in this study we analyzed whether ouabain is taken up by midguts of *D. plexippus* larvae using a similar mechanism.

Isolated midgut segments of late-instar larvae of *D. plexippus* took up [^{3}H]ouabain in a time-dependent way (Figure 6). Because ouabain uptake exceeded that of [^{3}H]H_2O, which can freely equilibrate between cells and the incubation medium, these results could be interpreted as ouabain accumulation against a concentration gradient. We cannot rule out, however, that the ouabain taken up was only bound to membranes or a binding protein that would mimic an accumulation against a concentration gradient. [^{3}H]Ouabain uptake depended on the hydrogen ion concentration of the incubation medium and was optimal at pH 6, which is in the range of carrier systems for secondary metabolites in other Lepidoptera: uptake of pyrrolizidine alkaloid N-oxides in *Creatonotos transiens:* pH 5 (Wink and Schneider, 1988); uptake of 13-hydroxylupanine in *Uresiphita reversalis:* pH 9 (Wink et al., 1991); and uptake of digoxin in *Syntomeida epilais:* pH 4. The hydrogen ion concentration in the gut of *Danaus plexippus* is between pH 7.2 (hindgut) and 8.4 (midgut) (Appel and Martin, 1990). Because of the apparent discrepancy between overall gut pH and the pH optimum, we assume that the carrier should have a microenvironment with a hydrogen ion concentration closer to pH 6.

[^{3}H]Ouabain uptake was temperature dependent and, similar to the situation in *Creatonotos*, *Syntomeida*, and *Uresiphita*, was highest at approximately 24°C

(Figure 7). Carrier transport (facilitated diffusion and active transport) is a catalyzed process. Temperature-dependence data can be evaluated with an Arrhenius plot to calculate the corresponding activation energy. For pH 6, we obtained an activation energy of 49 kJ/mol, which indicates that the uptake is a catalyzed process (carrier-free diffusion shows values below 20 kJ/mol) and not due to simple diffusion or any unspecific adsorption. Similar values were determined in other carrier systems: *C. transiens* 55–81 kJ/mol (Wink and Schneider, 1988), *S. epilais* 59 kJ/mol (Nickisch-Rosenegk et al., 1990a), and *U. reversalis* 55–78 kJ/mol (Wink et al., 1991). Furthermore, the uptake of [^{3}H]ouabain was inhibited (probably competitively) by adding another polar, but structurally similar CG, convallatoxin, to the incubation mixture (Figure 8). A simple diffusion process would not be influenced by such an addition.

Nonspecific adsorption (probably to biomembranes) also can be observed in Figures 6 and 7: Immediately after starting the experiments, 0.68% of [^{3}H]ouabain was bound and at 5°C when catalyzed processes do not work, "uptake" values were between 0.53 and 0.65%. Since the "real" uptake under optimal conditions was significantly higher (Figure 6 and 7), this background adsorption can be neglected as an explanation for the results obtained.

These experiments provide first evidence that a simple carrier-free diffusion cannot be responsible for the resorption of [^{3}H]ouabain in *D. plexippus* larvae. Instead, the experimental data suggest the presence of a cardenolide carrier mechanism. Although experiments with whole tissue samples are better for studying the activity of a carrier than studies with living larvae, they still leave certain questions unanswered. Certainly more experiments are required for a more complete characterization of the putative cardenolide carrier. Similar transport systems are better known for the uptake of primary metabolites (sugars, amino acids) or ions into living cells of bacteria, fungi, plants, or animals.

In 1988, we obtained queen butterflies (*Danaus gilippus*) from Florida, which were used in preliminary uptake experiments with cardenolides and pyrrolizidine alkaloid N-oxides. Isolated midgut segments resorbed [^{3}H]digoxin in a time-dependent way: uptake was almost linear for 45 min. Female midguts were 30–100% more active than those of males [a similar discrimination has also been observed in nature (Cohen, 1985; Brower et al., 1988)]. From temperature-dependence data, an activation energy between 35 and 50 kJ/mol was derived. We assume that these data also reflect the presence of a cardenolide carrier in the midguts of adult queen butterflies (M. Wink, E. von Nickisch-Rosenegk, and D. Schneider; unpublished observations). Since adult butterflies do not feed on a CG-rich diet, such a CG carrier in adult midguts seems to be superfluous but might be the result of the constitutive expression of a carrier gene.

Such a carrier system in *Danaus plexippus* and *D. gilippus* would provide a means to discriminate cardiac glycosides already in the midgut lumen and to

discharge all those compounds that do not bind to the carrier with the feces. This concept is also supported by the *in vivo* feeding experiments shown in Tables 1 and 2, which suggest that ouabain appears to have the highest affinity for the carrier and is preferentially taken up from CG mixtures, whereas the lipophilic cardenolides come second (Table 2). When cardiac glycosides were given as a single compound, the rate of ouabain uptake was nearly equal to that of the other compounds (Table 1). Considering the chemical structure of cardiac glycosides, the sugar side chain and the stereochemistry of the A/B rings seem to be of minor importance (Tables 1 and 2), whereas the five-membered lactone ring appears to be critical because proscillaridin A, a CG of the bufadienolide type, was not sequestered (Table 2). Even if the affinity of these CGs for the carrier was low, they could still use the carrier when it is not occupied by a more suitable CG, such as ouabain. However, in nature, where only CG mixtures exit, the polar CGs are preferred.

Lipophilic CGs may also be resorbed by an animal by simple diffusion, but as shown in Table 2, this process does not exceed the carrier-mediated uptake. Lipophilic CGs are either converted into more polar CGs or are eliminated via the Malpighian tubules (Seiber et al., 1980). The selective uptake and retention of polar glycosides provides the advantage that these compounds cannot easily diffuse across biomembranes and can thus be concentrated in certain tissues such as the integument.

The exact biochemical mechanisms that lead to storage of a secondary metabolite in insects are not well understood, but it is remarkable that plant-derived toxins are often deposited in the integument or cuticle: pyrrolizidine alkaloids in arctiid moths (Wink et al., 1988; Nickisch-Rosenegk et al., 1990b) and ithomiine butterflies (Brown 1984); cannabinoids in *Arctia caja* and *Zonecercus elegans* (Rothschild et al., 1977), cardenolides in *Danaus* (Brower and Glazier, 1975; Nishio, 1989; Brower et al, 1988; this study), *Syntomeida epilais* (Nickisch-Rosenegk et al., 1990a), and *Oncopeltus fasciatus* (Scudder et al., 1986); cyanogenic glycosides in *Zygaena* (Franzi et al., 1988); phenols in tree locusts, *Anacridium melanorhodon* (Bernays and Woodhead, 1982); cannabinol in *Arctia caja* (Rothschild et al., 1977); and quinolizidine alkaloids in *Uresiphita reversalis* (Wink et al., 1991). In *Oncopeltus fasciatus*, which also stores dietary cardenolides in the integument, a characteristic morphological adaptation towards CG storage was observed: This bug has a modified integument that is composed of a double-layered epidermis with an inner layer (the dorsolateral space) that constitutes the cardenolide storage compartment. On both sides of the thorax and abdomen thin cuticle areas exist that rupture when the bug is attacked. As a result, the cardenolides are released from the dorsoventral space and the predator is immediately confronted with the bitter cardenolides (Scudder and Meredith, 1982a; Scudder et al., 1986). The kinetics of uptake from the midgut via hemolymph to integument were almost identical in *Danaus* larvae sequestering

ouabain or in *Creatonotos transiens* taking up pyrrolizidine alkaloids (Nickisch-Rosenegk et al., 1990b), i.e., 48-hr after feeding the majority of allelochemicals had found their way to the integument.

Feeding experiments in three mouse species (*Peromyscus melanotis*, *P. aztecus*, and *Reithrodontomys sumichrasti*) with intact monarch butterflies of differing CG content have shown that CG-rich cuticular material (wings, integuments) was clearly rejected, whereas CG-free butterflies and tissues of low CG content were preferred (Glendinning, 1990). Mice mouthed CG-rich butterflies but immediately dropped them. It appeared that the mice tasted the cuticle and no signs of biting and chewing were observed. As a consequence, a number of CG-rich butterflies were left undamaged (Glendinning, 1990). This response is only possible if an allelochemical is deposited in the integument, which would explain an advantage for storing CGs in this part of the body of larvae, pupae, and imagines. The presence of CGs in the meconium could also be advantageous: freshly emerged imagines are able to spray the meconium towards a predator. If it contains bitter-tasting CGs, the defense response should be enhanced.

In addition, further mechanisms must be present in the monarch to avoid autotoxicity of CGs. Since CGs inhibit Na^+, K^+-ATPase in insects and other animals, *Danaus* must have evolved insensitivity at this molecular target or other detoxification mechanisms (such as storage in the integument). It has been shown experimentally that *D. plexippus* larvae can tolerate CG doses exceeding 1200 mg/kg body weight (which would kill almost any other animal) and its Na^+, K^+-ATPase cannot be inhibited by CGs (Vaughan and Jungreis, 1977; Holzinger et al. 1992). At the molecular level we found that one amino acid is different in the putative ouabain binding site (length 12 amino acids) in monarch Na^+, K^+-ATPase, and we have suggested that ouabain can no longer bind and thus insensitivity is achieved (Holzinger et al., 1992). In *Oncopeltus fasciatus* a comparable pattern emerged, indicating a high degree of molecular and physiological adaptation towards CG utilization. Besides a carrier-mediated uptake (Detzel and Wink, 1995), a predominant storage of the cardenolides in the dorsoventral space, Na^+, K^+-ATPase is ouabain-insensitive and the insect can tolerate high doses of cardenolides (Scudder and Meredith, 1982a; Scudder et al., 1986).

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HOME BEDDING MODIFIES ULTRASONIC VOCALIZATION OF INFANT BANK VOLES

JOANNA KAPUSTA,[1] ANNA MARCHLEWSKA-KOJ[1,*] and GILLIAN D. SALES[2]

[1]*Department of Mammalian Reproduction*
Institute of Zoology, Jagiellonian University
30-060 Krakow, Poland

[2]*Division of Life Sciences*
King's College, London W8 7AH, United Kingdom

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Abstract—Bank vole, *Clethrionomys glareolus*, pups, similar to many other infant rodents, emit ultrasonic vocalizations when they are removed from the nest and cooled. Infants exposed to bedding from their home cage produced fewer and shorter calls than infants tested without bedding. Sound frequencies were significantly higher in infants exposed to home bedding than in other experimental groups. These results provide evidence that infant bank voles are able to identify the presence of home bedding, probably on the basis of odor. This appears to be the first report of shifts in frequency of pup ultrasonic calls in response to olfactory cues.

Key Words—Bank vole, chemosignals, infants, pups, rodents, ultrasonic vocalization.

INTRODUCTION

Infant rodents during the first two weeks after birth depend on the warmth of the mother's body and of siblings present in the same nest. Lower ambient temperature is one of the factors that strongly influences behavior of pups. This may be manifested as emission of ultrasonic calls by animals when they are removed from the nest and cooled. The vocalization of infants can be perceived by adult females and stimulates maternal behavior. Calls of infant rats at a frequency of 40 kHz prompt lactating females to return to the nest (Jams and Leon, 1983) and in various species of voles, *Microtus*, mothers are attracted to the source of ultrasonic calls in a Y maze (Colvin, 1973).

*To whom correspondence should be addressed.

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In bank voles *Clethrionomys glareolus*, locomotory activity increases during the second week of life and infants are able to move out of their nest. However, the pelage of small rodents is still poorly developed, and they cool relatively easily. They also become less tolerant of lowered body temperature, and both rates and intensities of ultrasonic calling reach peak values at around 7–10 days (Okon, 1972; Blake, 1992). At this age bank vole pups are still blind, but their olfactory system is well developed and must therefore be a major source of sensory input. Our hypothesis is that pups can distinguish conspecifics of different familiarity, probably on the basis of odor, and this will be detectable by differences in their vocalizing. In these experiments, bedding from cages were used as a source of typical odor of animals. To test this, we elicited ultrasonic calls from pups exposed to cooling temperatures under four olfactory conditions: bedding from their home cage, bedding from a unfamiliar adult male, bedding from a unfamiliar adult female, and no bedding. Response was measured in terms of the calling rate, duration of individual calls, and the sound frequency of calls.

METHODS AND MATERIALS

Animals. The bank voles used in the experiments came from an outbred stock colony reared at the Institute of Zoology, Jagiellonian University, Krakow. The animals were maintained in metal cages (40 × 20 × 25 cm) at 18 ± 2°C, on photoperiod of 14 hr light and 10 hr dark (lights on at 6:00 AM). Standard pelleted rabbit chow diet (Motycz, Poland) and water were available ad libitum. Wood shavings were provided as bedding and nesting material and were changed once a week.

Sixty-five (32 males and 33 females) 6- and 7-day-old infants with a mass of 3.0 ± 0.16 g were selected. Two to four pups per litter were raised with both parents. The animals were blind, the external auditory meati open, and pelage was poorly developed. Approximately equal numbers of males and females were used in each treatment. As indicated by our previous results, 6- and 7-day-old males and females do not differ in calling rate (Marchlewska-Koj et al., 1995), and therefore the results obtained for both sexes have been combined.

Experimental Procedure. Animals in the home cage were transferred from colony room to the laboratory in the morning, and all tests were performed between noon and 2 PM. The influence of the presence or absence of bedding and of the presence of familiar or unfamiliar bedding was tested. Unfamiliar bedding came from nests of either adult male or female conspecifics. Pups were randomly assigned to one of four groups (no bedding, home bedding, unfamiliar male, unfamiliar female). The numbers of tested animals are given in the appro-

priate tables below. An individual pup was removed from the home cage, placed on a plastic dish, and kept warm for 10 min. After this period, it was either observed with no bedding or approximately 2 cm^3 of the appropriate bedding material placed on the dish next to the pup. The tested animal was then introduced to an acoustically isolated chamber with an ambient temperature of 18–20°C. One minute after the bedding was presented, or after a further minute for the pups observed with no bedding, the vocalization of the animal was monitored for 3 min. Calls were monitored with an ultrasound receiver suspended 5 cm above the tested animals. The microphone was connected to a QMC ultrasound detector, type S25, coupled to a cassette recorder (Sony, HX PRO). The detector was used in the frequency division mode, and the frequency of the incoming signal was divided by 16 so that the resulting output could be recorded directly onto cassette tape.

Samples of recorded ultrasonic calls were analyzed using a Kay Sonograph, type 7029A. Cassette recordings were played directly into the sonograph. Where possible samples were chosen at regular intervals throughout the tape. Calls were often emitted at a steady rate per minute. The numbers of calls analyzed are given in the appropriate tables. Duration of calls and the initial, terminal, maximum, and minimum frequencies of the calls were determined from the resultant sonograms using a specially prepared transparent graticule with axes in milliseconds and kilohertz. The number of calls in each 3-min recording was counted using a BBC B computer and a specially written program that allowed the computer to be used as an event recorder. Data were analyzed by using ANOVA and Duncan's new multiple-range test.

RESULTS

Bank vole pups produced ultrasound calls when they were taken out of the home cage and cooled. They vocalized in all four conditions, i.e., in the absence of bedding as well as in the presence of home cage bedding or of bedding from unfamiliar male or female adults (Table 1). Infants exposed to bedding from the home cage produced fewer calls per minute than animals tested without bedding. The duration of calls produced by infants tested in the presence of home bedding tended to be shorter than in other experimental groups, but differences were not statistically significant.

The calls of newborn bank voles are 20–55 kHz and 30–70 msec long (Sales and Pye, 1974; Stoddart and Sales, 1985). We found the fundamental frequency to be modulated slightly, first rising and than falling, with a bandwidth of about 3 kHz. Bedding affected the sound frequency of ultrasounds emitted by pups. With one exception, the initial frequency as well as the terminal frequency of calls were significantly higher in infants tested in the presence of

TABLE 1. INFLUENCE OF BEDDING ON NUMBER AND DURATION OF CALLS OF INFANT BANK VOLES (MEAN ± SE)[a]

Bedding conditions	Animals	Calls/min (*N*)	Calls sampled (*N*)	Duration (msec)
Without	20	92.6 ± 9.82a	73	54.03 ± 1.86c,d
Home	14	61.7 ± 10.42b	71	48.27 ± 2.44c
Unfamiliar female	14	82.0 ± 9.73a,b	72	57.67 ± 2.03d
Unfamilar male	17	77.9 ± 8.67a,b	74	54.05 ± 1.67c,d
ANOVA		$F_{(3,61)}$ 1.73 $P < 0.05$		$F_{(3,286)}$ 3.57 $P < 0.01$

[a]Means in a column followed by the same letters are not significantly different. $P > 0.05$.

home bedding than in other experimental groups. Only in pups without bedding was the initial frequency similar to those of pups with home bedding (Table 2). There were statistically significant differences between minimal and maximal frequencies (Table 2). The highest sound frequencies were recorded when animals were tested in the presence of bedding from home cages. No differences were found between the frequencies of calls of pups tested without bedding and those produced in the presence of bedding from a strange male or female.

TABLE 2. INFLUENCE OF BEDDING ON SOUND FREQUENCY OF CALLS EMITTED BY BANK VOLE INFANTS (MEAN ± SE)[a]

Bedding conditions	Samples (*N*)	Frequency (kHz)			
		Initial	Terminal	Minimum	Maximum
Without	85	28.89 ± 0.31a,b	26.16 ± 0.33a	26.11 ± 0.33a	29.12 ± 0.31a
Home	71	30.15 ± 0.84a	28.24 ± 0.8b	28.12 ± 0.76b	30.94 ± 0.92b
Unfamiliar female	72	27.89 ± 0.74b	25.07 ± 0.36a	25.07 ± 0.36a	28.27 ± 0.59a
Unfamiliar male	74	27.59 ± 0.29b	25.64 ± 0.25a	25.47 ± 0.25a	27.98 ± 0.27a
ANOVA		$F_{(3,298)}$ 4.85 $P < 0.01$	$F_{(3,298)}$ 8.21 $P < 0.01$	$F_{(3,298)}$ 5.40 $P < 0.01$	$F_{(3,298)}$ 8.48 $P < 0.01$

[a]Means in a column followed by the same letters are not significantly different. $P > 0.05$.

DISCUSSION

In adult rodents, social interaction is one of the factors that provokes vocalization at low or high frequency. Ultrasonic calls can be stimulated by olfactory signals released by conspecifics. Adult female hamsters vocalized in response to male odor (Johnston, 1992), and males produced ultrasounds when exposed to vaginal discharge (Johnston and Kwan, 1984). Vocalization in prairie voles (*Microtus ochrogaster*) also is evoked by the presence of odor of opposite sex (Lepri et al., 1988). In laboratory mice and laboratory rats, adult males emit ultrasonic calls in response to olfactory signals produced by females (Nyby et al., 1977; Barfield et al., 1979).

Infant rodents, too, modify ultrasonic calling in response to olfactory cues. Laboratory rats are capable of discriminating between the presence and absence of home odor, and cooled pups increased ultrasound production after removal of familiar nest odor (Oswalt and Meier, 1975; Hofer and Shair, 1987). They can also discriminate between different sources of conspecific odor; fewer ultrasonic calls were emitted in the presence of bedding from the home cage or from the cages of adult males than with bedding from the cages of females, either virgin or lactating (Conely and Bell, 1978; Lyons and Banks, 1982). In this study we have shown that bank vole pups can identify home odor.

The number and duration of calls were lower in the pups exposed to home bedding than in the other groups, although few of the differences were statistically significant. However, the vocalizations of animals tested with home bedding differed significantly in sound frequency and were generally at a higher frequency than those of any other experimental group. Differences were found for initial and terminal frequencies as well as for maximal and minimal frequencies. This appears to be the first report of such shifts in the frequency of pup ultrasonic calls in response to olfactory cues.

In adult rodents the effects of odor on the rate of ultrasonic calling have been documented for several species, including laboratory rats, laboratory mice, and hamsters (Nyby and Whitney, 1978). In woodmice, *Apodemus sylvaticus*, odor also has a marked effect on the frequency pattern of the ultrasonic calls elicited from males (Gyger and Schenk, 1980). Our results provide additional evidence that indicates a similarity between the high-frequency calls of infants and those of adults. In both, the rate of ultrasonic calling and, in some cases, the frequency characteristics of the calls, can be modified by sensory processes evoked by chemosignals of conspecifics. Ultrasonic calls and pheromones have complementary roles in a communication chain that regulates reproduction in hamsters (Floody and Pfaff, 1977). Possibly, acoustic and odor cues have similarly complementary roles in regulating parent–infant interactions in a number of rodent species.

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BROWNING-ASSOCIATED MECHANISMS OF RESISTANCE TO INSECTS IN CORN CALLUS TISSUE

PATRICK F. DOWD* and ROBERT A. NORTON

Mycotoxin Research Unit, U.S.D.A.
Agricultural Research Service
National Center for Agricultural Utilization Research
1815 N. University St., Peoria, Illinois 61604

(Received June 10, 1994; accepted January 30, 1995)

Abstract—Callus of the fungus- and insect-resistant corn inbred, Mp313E, of differing ages and degrees of brownness was tested for effects on neonate larvae of the corn earworm, *Helicoverpa zea*. Older, browner, 8-week-old callus caused significantly higher mortality of *H. zea* after two days compared to larvae fed 5-week-old callus (brown or not) or pinto bean diet. Larvae fed on the 5-week-old callus were significantly smaller after nine days compared to those fed on nonbrown callus of the same age. Ferulic acid, *p*-coumaric acid, and sinapic acid, corn allelochemicals associated with cell walls, were oxidized (produced a brown product) up to 10-fold more rapidly by peroxidase preparations of the brown callus compared to nonbrown callus. Peroxidase isozymes from both types of brown callus separated by conventional gel electrophoresis and isoelectric focusing showed more intense anodic/acidic bands compared to peroxidase isozymes separated from the nonbrown callus. Some additional peroxidase isozymes were also present in the brown callus and were able to oxidize ferulic acid. Conventional extraction techniques for phenolic acids indicated free ferulic acid levels were similar for same-age callus, but somewhat less for older callus; no free *p*-coumaric acid was detected. For esterified and insoluble-bound phenolic content, the level of *p*-coumaric acid was higher than that of ferulic acid and was somewhat greater in insoluble-bound fractions for the old callus compared to the younger callus. Color ratings of aqueous extracts and phenolic acid extract residues indicated much brown material remained, which was proportional in color to the original material. The differential insect response to, and peroxidase activity of, brown vs. nonbrown callus were similar to results previously noted for brown pericarps of corn compared to nonbrown pericarps. This information suggests the peroxidases are contributing to the browning through oxidation of phenolic acids and thereby enhancing resistance to insects.

*To whom correspondence should be addressed.

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Key Words—*Helicoverpa*, peroxidase, quinone, ferulic acid, callus tissue, resistance, corn.

INTRODUCTION

Tissue culture can be used to more readily investigate some physiological features associated with insect or pathogen resistance compared to the intact plant due to continuous availability of uniform plant material. For example, many of the pathways of flavonoid biosynthesis have been elucidated using various plant tissue cultures (Barz and Nicholas, 1978). Corn callus has been used to screen rapidly for resistance to insects; ratings obtained often correspond well with whole plant material, such as leaves (e.g., Williams et al., 1983).

Mechanisms of corn plant resistance to insects are diverse and include production of flavonoids such as maysin (Waiss et al., 1979), benzoxazolinones such as 6-methoxybenzoxazolinone (Meyer, 1988), and phenolic compounds such as ferulic acid (Serratos et al., 1987; Classen et al., 1990). Oxidation of phenolics appears to cause antinutritive effects in insects fed tomato foliage (Felton et al., 1989), and trichome adhesiveness in wild potato (Steffens and Walters, 1991). The pericarp of the corn inbred Mp313E has kernel-based resistance to *Aspergillus flavus* (Scott and Zummo, 1988) and the dusky sap beetle, *Carpophilus lugubris* (Dowd, 1994a). The rapid browning of the pericarp when damaged, which appears to be promoted by peroxidases, is a likely mechanism of insect resistance (Dowd, 1994b). Corn callus cultures are known to produce some of the peroxidases (Fransz et al., 1989) that appear to be the same as those reported in differentiated tissues of corn such as leaves and kernels (Brewbaker and Hasegawa, 1975) based on band positions when peroxidases were separated by gel electrophoresis. In order to further explore the browning reaction as it relates to insect resistance, we examined the ability of insects to survive on browned vs. nonbrowned callus and determined the potential involvement of peroxidases and phenolic acids in the browning reaction.

METHODS AND MATERIALS

Insects. The *H. zea* larvae (stock originally obtained from the Department of Entomology, University of Illinois, Urbana, Illinois) were reared on pinto bean-based diet at 27 ± 1°C 40 ± 10% relative humidity, and a 14L:10D photoperiod as described previously (Dowd, 1988). Neonate larvae were used for assays.

Chemicals. Bovine serum albumin, *trans*-caffeic acid, *trans*-cinnamic acid, *trans-p*-coumaric acid, coniferyl alcohol, 1-chloronaphthol, dimethyl-*p*-phenylenediamine, *trans*-ferulic acid, and MS medium salts and vitamins were obtained

from Sigma Chemical Co. *trans*-Sinapic acid was obtained from Aldrich Chemical Co. 2,4-Dichlorophenoxyacetic acid was obtained from Chem Service. The *cis* isomers of ferulic and *p*-coumaric acids were prepared as previously described (Norton, 1994). All other chemicals were of reagent grade.

Tissue Culture. Kernels of selfed Mp313E grown in Peoria, Illinois, during the summer of 1991 by conventional practices (Dowd, 1994a) were removed from ears 35 days after pollination. The kernels were sterilized by placing them in a Petri dish containing 70% ethanol for 1 min, and then for 15 min in 15% laundry bleach with a few drops of detergent, with agitation throughout the process. The kernels were rinsed four times with sterile distilled water and germinated in Petri dishes containing 1% water agar. Six days after germination, hypocotyl segments were excised and transferred to callus growth media consisting of MS medium salts and vitamins (Murashige and Skoog, 1962), supplemented with 3% sucrose, 2 mg/liter of 2,4-dichlorophenoxyacetic acid, and 7 g/liter agar, with the pH adjusted to 5.7 with 0.1 N sodium hydroxide. The callus was grown in 100-mm-diameter Petri dishes in the dark at 25°C and subcultured every six weeks.

Bioassays. Several clumps of callus tissue from 8-week-old cultures that were uniformly brown (no nonbrown material available), and both brown (<5% nonbrown over the entire clump) and nonbrown (<5% brown over the entire clump) callus from cultures that were 5 weeks old were lyophilized for 48 hr. In order to provide uniform material for the insects, all of each type of callus was weighed and powdered together using a smooth glass mortar and pestle (Figure 1). Because preliminary tests with both unlyophilized and lyophilized callus removed from cultures indicated callus would subsequently become contaminated with molds within a few days after insects were introduced, sorbic acid was used as a mold inhibitor. Sorbic acid was added as 2.4 mg in 4 ml of acetone to 600 mg of dried callus so that the final concentration, when rehydrated to 75% moisture, would be 1000 ppm [approximately the same concentration used in artificial diets for these insects (Dowd, 1988)]. Acetone was evaporated in a fume hood, and the callus rehydrated with sterile distilled water. The rehydrated callus was divided into 20 approximately equal-sized parts and added to 20 wells of a 24-well tissue culture plate. A newly hatched (ca. 1 day old) *H. zea* larva was added to each of the 20 wells containing callus (i.e., 20 larvae per treatment), and plates were sealed as described previously (Dowd, 1988) and held under the same conditions as described for rearing. An additional plate was set up with caterpillars using the pinto bean diet as a food source. All assays were set up on the same day with the source of caterpillars. Assays were examined after two, four, and nine days for mortality. Because of high mortality of insects fed on the old, brown callus at nine days, the assay was ended. Both dead insects and survivors were frozen and subsequently weighed on a Mettler

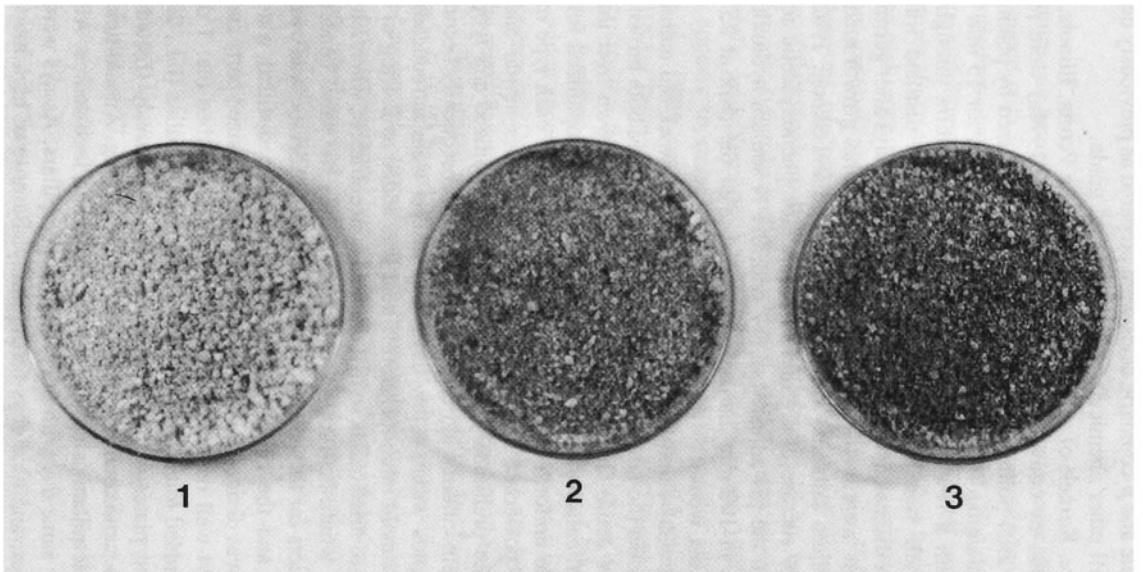


FIG. 1. Relative degree of browness of callus material used in assays. 1 = 5-week-old nonbrown (color rating of 10YR 8/2), 2 = 5-week-old brown (color rating of 10YR 6/4), 3 = 8-week-old brown callus (color rating of 10YR 5/4). Color ratings from Kollmorgan Instruments Corp. (1992).

AE 163 analytical balance to the nearest 0.01 mg in order to determine both weights of survivors and weights the dead insects had reached prior to death.

Enzyme Assays. Two intact callus clumps of each type and of the same three types and ages described above that were as close as possible in size (measuring 0.5–1.0 cm across) were homogenized individually in 2 ml of ice cold, pH 7.4, 0.1 M sodium phosphate buffer with a ground glass tissue grinder. Each homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was used in the assays. Peroxidase activity of fresh callus was examined with *p*-coumaric acid, ferulic acid, sinapic acid, and coniferyl alcohol, which are known to be present in corn and many other plants (Fry, 1986). As described previously (Dowd, 1994b), assays consisted of 700 μl of pH 7.4, 0.1 M sodium phosphate buffer, 100 μl of 0.3% hydrogen peroxide (mixed from a 30% concentrate, considered to be 100%), 100 μl of callus homogenate supernatant (diluted 1 : 10), and 100 μl of 0.1% substrate. When solvents other than water or buffer were used to dissolve the substrate, they were also used in controls. Changes in absorbance for the respective substrates were monitored with a Perkin-Elmer Lambda 4B spectrophotometer for 10 min at 30°C at the following absorbances as determined by differential scanning of generated products (Dowd, 1994b): phenolic acids, 420 nm; and coniferyl alcohol, 800 nm. All assays were run in duplicate with two separate sources of callus of each type. Protein content was determined with the Bio-Rad packaged protein assay (Bio-Rad, 1985), with bovine serum albumin as a standard.

Enzyme Separation. Differences in peroxidase type and activity were determined using nondenaturing polyacrylamide gel electrophoresis and wide-range (pH 3.5–9) isoelectric focusing. Gel electrophoresis was performed as described previously (Dowd and Sparks, 1986; Dowd, 1994b) using 1-mm-thick, 7.5% polyacrylamide gels run on a LKB Multiphor II electrophoresis system. Bromophenol blue was used as the tracking dye. Isoelectric focusing was performed as described previously (Dowd and Sparks, 1986; Dowd 1994b) using precast, pH 3.5–9, Pharmacia gels on the Multiphor system. Gels were run at 25 W constant power at 2000 V for 1.5 hr; Evan's blue was used as the tracking dye. For isoelectric focusing gels, 1-cm sections of the gel across the pH gradient were placed in 1 ml of deionized water overnight, and the pH readings were taken to the nearest 0.001 pH unit with a Corning Ion Analyzer 250. Graphical analysis indicated pH ranges were uniform within 0.1 pH unit across the gel. Each sample homogenate was run in duplicate (total of four replicates for each callus type).

Gels were stained for general peroxidase and phenolic acid peroxidase activity with a histochemical procedure (Conyers and Kidwell, 1991) modified for staining of polyacrylamide gels (Dowd, 1994b). Briefly, for general peroxidase activity, 12 ml of 0.3% peroxidase solution and 6 ml each of 0.0067% ethanol solutions of 1-chloronaphthol and DMPD were added to 100 ml of pH

7.4 sodium phosphate buffer at room temperature. Gels were incubated for approximately 15 min, and the resulting banding pattern was photographed. For detection of peroxidase oxidation of ferulic acid, both uncoupled reactions and prereacted, coupled assays were run (Dowd, 1994b). Uncoupled reactions had 0.1% ferulic acid alone. Coupled reactions had the same concentration of ferulic acid for 15 min, followed by 12 ml of dilute (1:30 in the phosphate buffer) solutions of 1-chloronaphthol—DMPD allowed to react for 15 min. Coupled reactions to detect peroxidase oxidation of *p*-coumaric acid and sinapic acid were run in an analogous manner (Dowd, 1994b).

Allelochemical Analysis. Phenolic acids were extracted from lyophilized callus according to the method of Krygier et al. (1982) with the following modifications. Instead of the initial preparation steps used by Krygier et al. (1982) with flour, ca. 0.2 g of dry callus was powdered with a mortar and pestle and extracted with 6 ml of methanol–acetone–water 35:35:30 (v/v/v), vortexed for 15 sec, centrifuged, and the supernatant removed. The residue was extracted six times. Instead of using diethyl ether–ethyl acetate (Krygier et al., 1982), ethyl acetate was used after the hexane extraction step to extract the free acids from the aqueous solutions obtained from free, esterified, and bound fractions. The extracts were dried with Na_2SO_4, evaporated with N_2, and made up to volume with ethyl acetate. To minimize the formation of *cis* isomers, all manipulations of callus and extracts were carried out with laboratory lights off. Samples were analyzed by HPLC with a Beckman Ultrasphere 5-μm, C-18 column that was 25 cm × 4.6 mm. The flow rate was 1 ml/min. The solvent system was acetic acid–tetrahydrofuran–water (2:10:88) (solvent A) and tetrahydrofuran–methanol (1:9) (solvent B). A linear gradient solvent program was used as follows: from 0% to 15% B over 20 min, from 15% to 80% B over 15 min, isocratic for 10 min, 80% B to 0% B over 15 min, and isocratic for 5 min, all at ambient temperature. The 20-μl injections were made by the filled-loop method. Standard curves for caffeic, cinnamic, *p*-coumaric, ferulic, and sinapic acids (315 nm) were derived by averaging four injections at concentrations of 0.033, 0.104, and 0.320 mg/ml. Phenolic acid levels reported are the average of duplicate callus samples run at least twice each. Portions of buffer extracts prepared for use in enzyme assays that were lyophilized and extract residue remaining from the phenolic acid extractions were color rated using soil color charts (Kollmorgan Instruments Corp., 1992). Values were analyzed for statistical significance by least significant differences after analysis of variance.

RESULTS

Survival of all *H. zea* larvae fed callus was significantly ($P < 0.05$ by chi-square analysis) reduced compared to larvae fed pinto bean diet (Table 1).

TABLE 1. EFFECT OF DIFFERENT CORN CALLUS TYPES ON *H. zea* LARVAE[a]

Material	Mortality (%)			9-day weight (mg)	
	2 days	4 days	9 days	Alive	Dead
Pinto bean diet	0.0a	0.0a	0.0a	21.8 ± 2.8a	
Nonbrown callus	0.0a	0.0a	60.0b	0.9 ± 0.1b	0.7 ± 0.1a
Brown callus	0.0a	0.0a	60.0b	0.4 ± 0.1c	0.1 ± 0.0b
Old brown callus	44.7b	86.6b	93.3c	0.02	NW

[a]Weights are means ± standard errors. Values in columns followed by the same letter are not significantly different at the 5% level of probability by chi-square analysis (mortality) or least significant differences in analysis of variance (weights). NW = due to the small size, dead insects caged with old brown callus were not weighed. Only one insect survived for nine days on the old brown callus, hence there was no variance and no analysis of variance.

However, the 8-week-old brown callus caused significantly higher mortality ($P < 0.05$ by chi-square analysis) of *H. zea* larvae compared to larvae fed on either type of the 5-week-old callus. Although mortality of larvae fed both types of younger callus was similar over the intervals examined, the weights of both dead and live larvae at nine days fed the younger, brown callus were significantly lower ($P < 0.05$ by analysis of variance) than the weights of the larvae fed the younger, nonbrown callus.

The older brown callus oxidized all phenolic acids at the highest rate, while the rate of oxidation of the phenolic acids by the younger brown callus was significantly lower (Table 2). Ferulic acid was oxidized at the highest rate. The *p*-coumaric acid had the greatest difference in oxidation rate from nonbrown to old brown callus, ca. 10-fold. No oxidation of coniferyl alcohol was detected for any of the callus homogenates at the enzyme concentration tested.

TABLE 2. OXIDATION OF ARYL ALCOHOLS BY CALLUS HOMOGENATES[a]

Compound	Change in absorbance/mg protein/min for callus		
	Nonbrown	Brown	Old
Coniferyl alcohol	ND	ND	ND
p-Coumaric acid	0.0069 ± 0.0005a	0.035 ± 0.005b	0.077 ± 0.004c
Ferulic acid	0.11 ± 0.03a	0.32 ± 0.03b	0.44 ± 0.01c
Sinapic acid	0.022 ± 0.003a	0.085 ± 0.016b	0.14 ± 0.01c

[a]Values are means ± standard errors. Values in rows followed by the same letter are not significantly different at the 5% level of probability by least-significant differences in analysis of variance.

Conventional gel electrophoresis and subsequent general peroxidase activity staining showed several different peroxidase isozymes (Figure 2). Although overall intensity of bands was higher for both browned sources, it appeared that the cathodic bands present in the nonbrown callus samples were more prevalent (based on intensity) than those from both types of brown callus. Some of the anodic bands were more intense in both ages of the brown callus compared to the nonbrown callus. Additional bands were also seen for both ages of brown callus as compared to the nonbrown callus. Differences in intensity of the bands of the two replicates of nonbrown callus corresponded to differences in protein content (and the smaller size of one of the nonbrown callus clumps used). Isoelectric focusing separated many peroxidase "isozymes" from each callus type (Figure 3A). It appeared that some acidic forms were less intense in the nonbrown vs. brown callus of both types and that some additional isozymes were present in both ages of the brown callus vs. nonbrown callus. More specific ferulic acid peroxidase staining of isoelectric-focused gels indicated most intense staining in basic and acidic regions corresponding to general peroxidase (Figure 3B). Both uncoupled (not shown) and coupled assays with ferulic acid yielded the same areas of intense staining. As was seen for general peroxidase, coupled ferulic acid peroxidase assays indicated the appearance of more intense acidic isozymes in the brown vs. the nonbrown callus material. These differences were less noticeable for isozymes detected using *p*-coumaric acid as a substrate (Figure 3C). Isozymes detected with sinapic acid were mainly limited to the two intensely staining bands in the acidic region seen for ferulic acid and were in the same proportional intensity for the different callus types as was seen for ferulic acid (data not shown).

Chemical analyses indicated that ferulic acid was the only free acid detected, at levels significantly lower in the old callus compared to the younger callus (Table 3). Low levels of esterified *p*-coumaric were detected that were similar for the three callus types, but no esterified ferulic acid was detected. Bound *p*-coumaric was approximately twofold higher in the old vs. younger callus, while bound levels of ferulic acid were approximately the same for all three types of callus. Bound *p*-coumaric acid was at least 10 times higher than esterified *p*-coumaric acid, while the levels of bound ferulic acid were two- to fourfold higher than the levels of free ferulic acid. The higher levels of bound *p*-coumaric acid were reflected in the higher overall levels of phenolics seen in the old vs. younger callus. No caffeic, cinnamic, or sinapic acid was detected, nor were *cis* isomers of *p*-coumaric acid or ferulic acid detected.

Color ratings for brown portions of lyophilized aqueous extracts were 2.5Y 8/2, 2.5Y 7/4, and 2.5Y 6/4 for nonbrown, brown, and old brown materials, respectively. Color ratings for residues of ferulic acid extractions were 2.5Y 8/3, 2.5Y 7/4, and 2.5Y 6/4 for nonbrown, brown, and old brown materials, respectively.

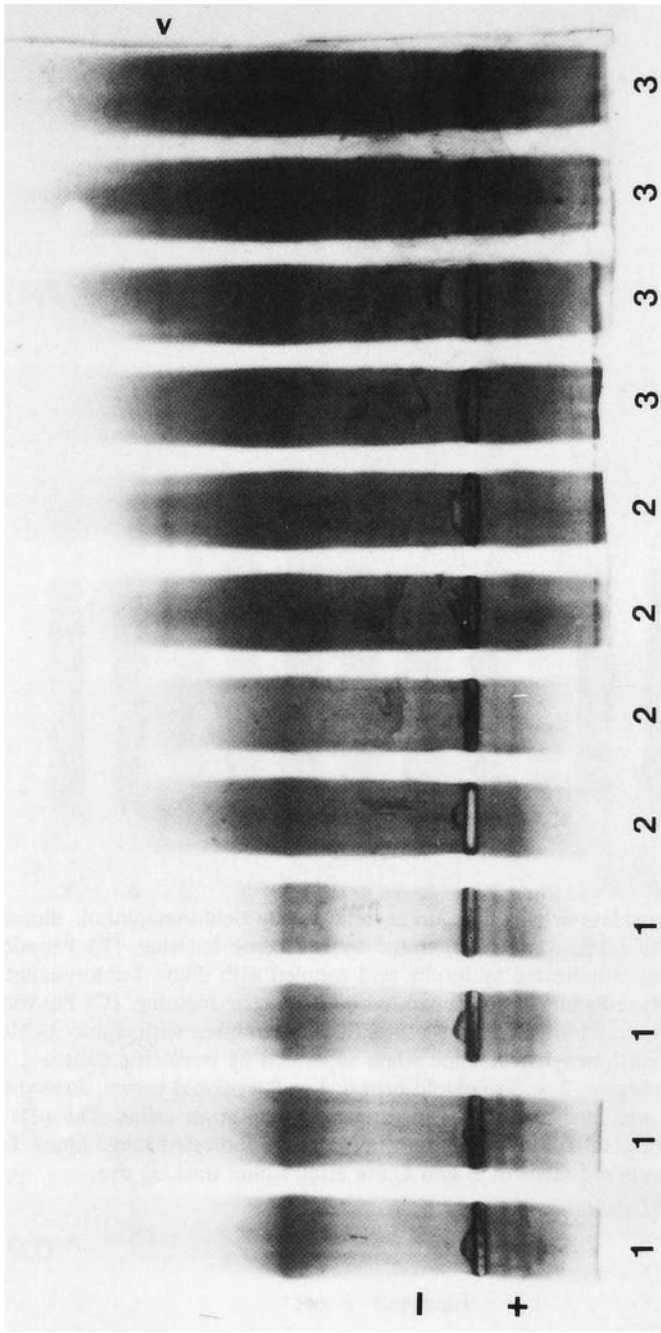


FIG. 2. Peroxidase activity of callus as indicated by 1-chloronaphthol, dimethylphenylenediamine assays when separated by nondenaturing polyacrylamide gel electrophoresis. 1 = 5-week-old nonbrown, 2 = 5-week-old brown, 3 = 8-week-old brown. Arrowhead indicates additional isozyme.

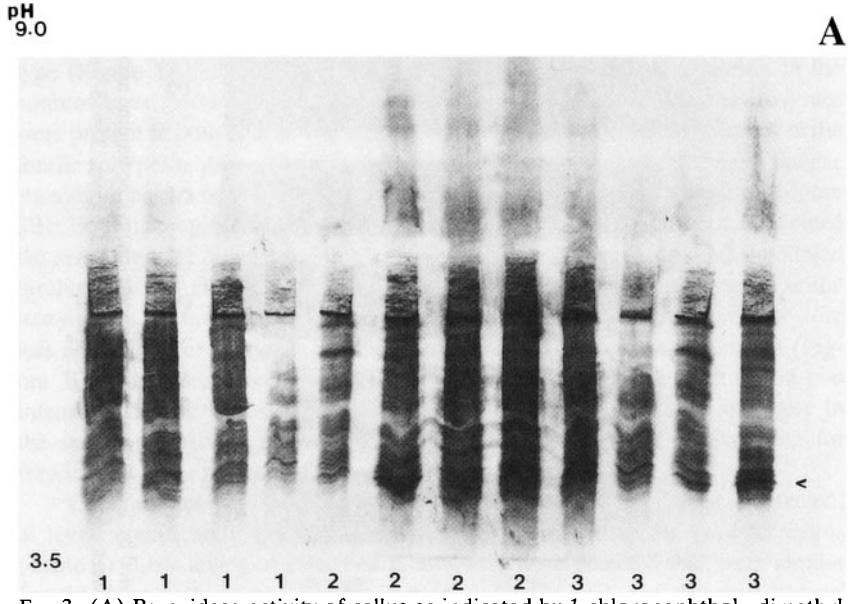
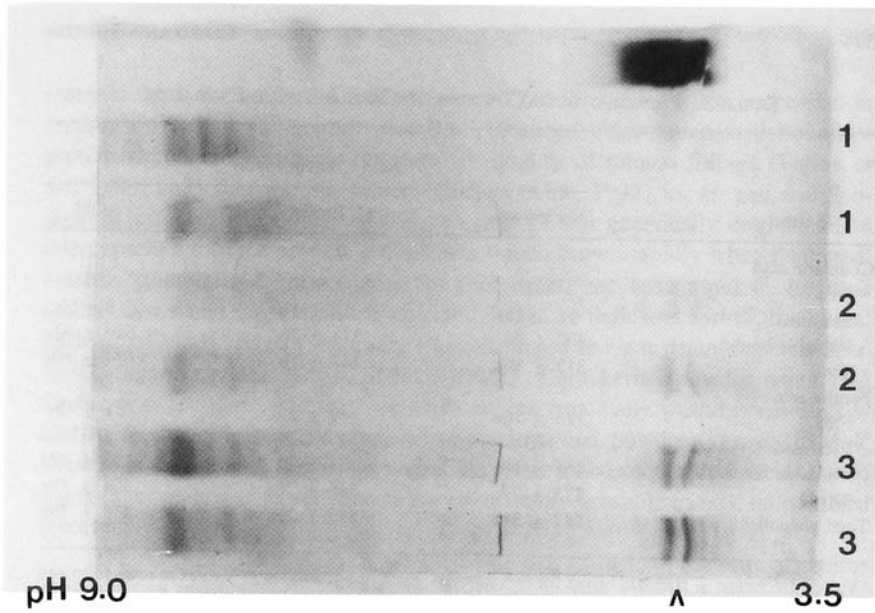


FIG. 3. (A) Peroxidase activity of callus as indicated by 1-chloronaphthol, dimethylphenylenediamine assays when separated by isoelectric focusing. (B) Peroxidase activity of callus is indicated by ferulic acid coupled with dilute 1-chloronaphthol, dimethylphenylenediamine when separated by isoelectric focusing. (C) Peroxidase activity of callus as indicated by *p*-coumaric acid coupled with dilute 1-chloronaphthol, dimethylphenylenediamine when separated by isoelectric focusing, 1 = 5-week-old nonbrown, 2 = 5-week-old brown, 3 = 8-week-old brown. Arrowheads indicate additional isozymes seen with brown vs. nonbrown callus. The pHs are uniformly distributed within 0.1 pH unit between the indicated range limits. Dark bands in unnumbered lanes of B and C are Evan's blue tracking dye.



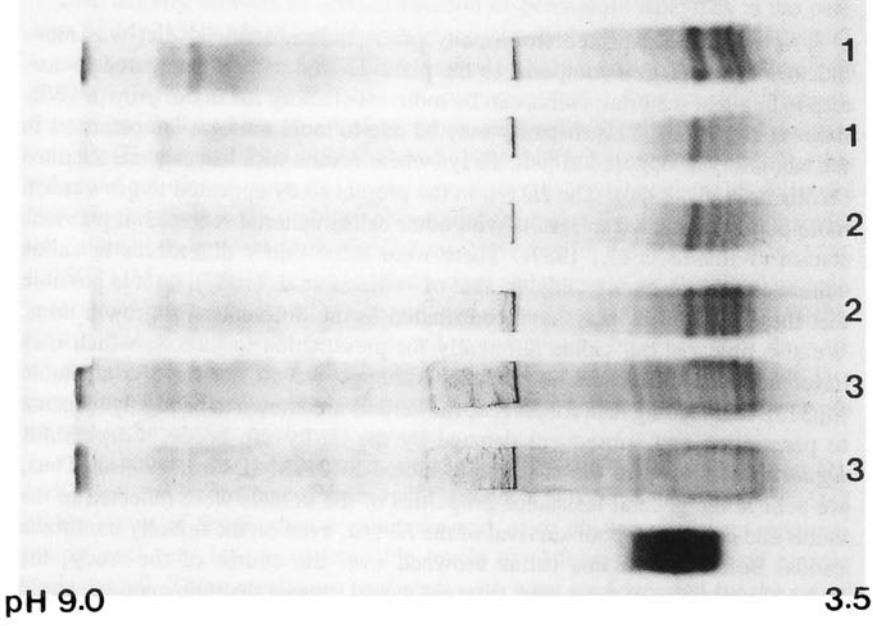


FIG. 3. Continued.

TABLE 3. PHENOLIC ACID CONTENT OF CALLUS TISSUE EXTRACTS[a]

Extract	Acid Content (ppm)		
	Nonbrown	Brown	Old
Coumaric acid			
Free	ND	ND	ND
Esterified	13 ± 1a	18 ± 1b	8 ± 3a
Insoluble bound	300 ± 9a	231 ± 2b	646 ± 62c
Total	313 ± 10a	247 ± 1b	654 ± 60c
Ferulic acid			
Free	113 ± 16a	149 ± 26a	51 ± 9b
Esterified	ND	ND	ND
Insoluble bound	161 ± 19a	137 ± 3a	153 ± 27a
Total	275 ± 27a	286 ± 26a	204 ± 19a
Total phenolics	587 ± 38a	533 ± 25a	838 ± 78b

[a] Values are means ± standard errors for two samples, each run in duplicate. Values in rows followed by the same letter are not significantly different at the 5% level of probability by least significant differences in analysis of variance. ND = not detected.

DISCUSSION

As would be expected from many prior studies, artificial diet was more satisfactory for *H. zea* compared to the plant-derived callus. Compared to corresponding leaf material, callus can be more satisfactory for insect growth (Williams et al., 1983). This disparity may be due to more uniform temperatures in the laboratory as opposed to field assays where results with leaves were obtained (Williams et al., 1983). The *H. zea* in the present study appeared to grow much more poorly compared to results with other callus material reported in previous studies (Williams et al., 1987). There were some minor differences in callus culture conditions in our study vs. that of Wiliams et al. (1987), so it is possible that these differences may have contributed to the differences in growth seen. We also prepared our callus differently for presentation to insects, which may have produced differences in growth. Although we do not have comparable Mp313E leaf feeding data for *H. zea*, the kernels are known to be highly resistant to penetration and subsequent damage by the dusky sap beetle, *Carpophilus lugubris*, compared to the susceptible inbred SC212M (Dowd, 1994a). Thus, we believe the general resistance properties of the kernels were reflected in the callus and caused the poor survival of the *H. zea*, even on the initially nonbrown callus. Because even this callus browned over the course of the study, the resistant properties of the brown material would suggest that this material would subsequently become increasingly resistant to *H. zea*.

Regardless of the reasons behind the poor survival of *H. zea* on the callus

material used, the study did indicate that *H. zea* larvae were less successful in feeding on brown vs. nonbrown callus. Oxidized chlorogenic acid bound to protein significantly reduces the nutritive quality of tomato foliage (Felton et al., 1989) and other protein sources (Felton et al., 1992) for *H. zea* and *Spodoptera exigua*. The tissues of tobacco and tomato genetically engineered to overexpress a tobacco anionic peroxidase, which brown rapidly when damaged, are less suitable as a food source for neonate *H. zea* larvae and *C. lugubris* adults (Dowd and Lagrimini, unpublished data). As indicated earlier, the kernel pericarp of Mp313E browns rapidly when damaged and is resistant to penetration by *C. lugubris* adults (Dowd, 1992, 1994a). Corn kernel powder containing ferulic or *p*-coumaric acid that was browned enzymatically was also more resistant to feeding by insects compared to nonbrowned powder (Dowd, 1994b). Thus, our results with the brown callus tissue are consistent with those obtained using other materials in that insects are consistently less able to feed on oxidized materials.

The results of the enzyme assays indicate that extracts of the browner callus material can more rapidly oxidize phenolic acids. Gel electrophoresis and isoelectric focusing indicate enhanced activity of anodic, acidic peroxidases in brown vs. nonbrown callus. Differences were especially prominent when ferulic acid was used as a substrate. This relative activity difference corresponds to the relative activity differences seen in location of peroxidase isozymes in the pericarp of Mp313E, which are absent in the slowly browning *A. flavus* and *C. lugubris* susceptible inbred SC212M; as well as brown and nonbrown Ch mutant pericarp (Dowd, 1994b). Enzymes of corn responsible for peroxidase activity appear generally to be identical to those responsible for polyphenol oxidase activity (Grison and Pilet, 1985; Dowd, 1994b). Thus our results indicate that enhanced activity of particular peroxidase isozymes is associated with enhanced browning and reduced suitability of material for insect feeding. The results with the callus in the present study parallel results seen with the pericarp of Mp313E vs. SC212M and cold-shocked (nonbrowning) and normal Ch (chocolate) mutants in terms of browning, insect resistance, and peroxidase activity (Dowd, 1994b). In this earlier study with pericarps and enzymatically browned corn flour, in nearly all cases browner material was more resistant to insects compared to less brown material. In all cases, additional peroxidase activity and isozymes were present in brown vs. nonbrown material.

Past reports on corn kernel material (Sosulski et al., 1982; Classen et al., 1990) and bm3 corn leaf (Eraso and Hartley, 1990) have generally indicated higher levels of ferulic than *p*-coumaric acid, with the *trans* isomers predominating, and very low to undetectible levels of caffeic, cinnamic, and sinapic acids overall. These results are consistent with what we have seen for the callus in terms of levels of caffeic, cinnamic, and sinapic acids. However, the higher levels of *p*-coumaric acid compared to ferulic acid are similar to results reported

for stem tissue of bm3 corn, which also has more *p*-coumaric acid than ferulic acid. Free levels of ferulic acid and *p*-coumaric acid were approximately equal in corn flour, but levels of ferulic acid were much higher in the soluble ester fraction and insoluble-bound fractions of corn flour (Sosulski et al., 1982). Our studies indicated differences in the callus material, in that free ferulic acid was higher than free *p*-coumaric acid, but the opposite was true for the esterified and bound insoluble fractions. Whether these ratios for the callus are variety specific or are a general phenomenon for all corn callus remains to be determined. However, the relatively low ratio of bound to free ferulic acids approximates what occurs in milk-stage developing wheat (McCallum and Walker, 1991) and presumably corn (Dowd, 1994b). Thus, the callus tissue may be a suitable model for some reactions that occur in a developing corn kernel. This information also suggests that the ferulic acid is being regenerated as it becomes bound (regardless of the enzymatic method and whether it is an ester or ether linkage), as indicated by similar levels in brown and nonbrown callus of the same age, so the free ferulic acid pool remains relatively constant. This is relevant not only for the bound material quantitated in this study, but also for the ferulic acid that is presumably bound to protein or lignin, which would not be quantitated in the methods we used but would be expected based on relative differences in browning seen for the aqueous extract and extracted residue. The distribution of the ferulic acid in these nonquantitated, brown fractions would also explain the higher toxicity of the old material because total differences in phenolics that were quantitated are not that different between younger and older callus. This free ferulic acid distribution is consistent with the feedback inhibition that is known for some enzymes in the ferulic acid biosynthesis pathway (Kahl, 1974; Ralph and Helm, 1993). This information supports prior hypotheses (Dowd, 1994b) that peroxidases are responsible for taking ferulic acid out of the free pool, which ultimately increases total ferulic acid content of tissue due to the continual replacement of ferulic acid in the free pool.

Portions of corn cells (e.g., Harris and Hartley, 1970), including the pericarp (e.g., Classen et al., 1990), can contain significant levels of ferulic acid in various forms. Thus, it is appropriate that the peroxidases induced during browning would be able to oxidize ferulic acid. Because coniferyl alcohol (a direct percursor, along with coumaryl and sinapyl alcohols, of lignin) was not acted on relative to ferulic acid (several biosynthetic steps from coniferyl alcohol) by the same tissue homogenates, it appears classical lignification is less important in the browning reaction than is the oxidation of ferulic acid (and perhaps analogs of coumaric and sinapic acid). Formation of dimers of ferulic acid conjugates or other forms of ferulic acid cross-linking is a likely role of the peroxidases detected (see Fry, 1986), contributing to insect resistance (Arnason et al., 1994; Dowd, 1994b), although many other reactions involving oxi-

dized phenolic acids could contribute to insect resistance as well (Dowd, 1994b). The presence of brown material in aqueous extracts and phenolic acid extract residues indicates additional interactions are occurring, few of which would be recovered using the phenolic extraction that is standard, due to intractible ether linkages. The peroxidase-catalyzed generation of quinones that subsequently react with proteins to form ether linkages is a likely source of brown material in the aqueous extracts. Prior studies have indicated oxidation products generated from phenolic acids related to ferulic acid were bound to protein and subsequently reduced digestibility (Felton et al., 1989, 1992). Ferulic acid is also known to form ligninlike polymers when acted on by peroxidases (Stafford, 1960). Peroxidase-generated ferulic acid quinones could thus form a variety of ether linkages with other materials in the callus that would not be hydrolyzable, and thus not extracted, by the conventional techniques used, and thus remain in the residue. Again, this material is likely to be tougher and/or less digestible by the insects and would occur at higher levels in the brown materials.

Resistance of oxidized plant tissue to insects has been attributed to binding of quinones to proteins, which makes them more difficult to digest (Felton et al., 1989, 1992). The quinones themselves are more toxic to bacterial (Kojima, 1931) and fungal (Byrde et al., 1960; Urs and Dunleavy, 1975) plant pathogens than are the corresponding precursors. With the exception of maysin-containing silks, silk browning was inversely correlated with resistance to *H. zea* (Byrne et al., 1989). Subsequently, oxidation of high maysin silks was shown to be important in the resistance response (Wiseman and Isenhour, 1993). Sugar-water solutions containing allelochemicals oxidized by peroxidases or tyrosinases are more toxic to the leafhopper *Dalbulus maidis* than are solutions containing the corresponding unoxidized allelochemical (Dowd and Vega, unpublished data). Thus, the browning of the corn plant tissue is often, but not always, a likely indication of production of quinones or other reactions catalyzed by peroxidases that produce material that is more toxic and/or less nutritious to insects than precursors such as ferulic acid.

Attempting to understand how phenolic oxidations affect insect growth is likely to be difficult due to the complexity of conditions involved (Appel, 1993). Our results indicate that browned corn callus is more resistant to *H. zea* than nonbrown material. The browning is associated with an enhanced ability of the tissue to oxidize phenolics, such as ferulic acid, and ultimately results in a higher accumulation of phenolics at a variety of sites. This ability is associated with an enhanced activity and presence of certain peroxidases. This work further demonstrates the potential importance of peroxidase activity in promoting plant resistance to insects through oxidation of associated allelochemicals. This peroxidase activity may also be involved in cross-resistance to plant pathogens (Dowd, 1994b).

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EFFECTS OF BIOTIC AND ABIOTIC STRESS ON INDUCED ACCUMULATION OF TERPENES AND PHENOLICS IN RED PINES INOCULATED WITH BARK BEETLE-VECTORED FUNGUS

KIER D. KLEPZIG,[1,3] ERIC L. KRUGER,[2] EUGENE B. SMALLEY,[1] and KENNETH F. RAFFA[3]

[1]*Department of Plant Pathology*
[2]*Department of Forestry*
[3]*Department of Entomology*
University of Wisconsin–Madison
Madison, Wisconsin 53706.

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Abstract—This study characterized the chemical response of healthy red pine to artificial inoculation with the bark beetle-vectored fungus *Leptographium terebrantis*. In addition, we sought to determine whether stress altered this induced response and to understand the implications of these interactions to the study of decline diseases. Twenty-five-year-old trees responded to mechanical wounding or inoculation with *L. terebrantis* by producing resinous reaction lesions in the phloem. Aseptically wounded and wound-inoculated phloem contained higher concentrations of phenolics than did constitutive tissue. Trees inoculated with *L. terebrantis* also contained higher concentrations of six monoterpenes, α-pinene, β-pinene, 3-carene, limonene, camphene, and myrcene, and higher total monoterpenes than did trees that were mechanically wounded or left unwounded. Concentrations of these monoterpenes increased with time after inoculation. Total phenolic concentrations in unwounded stem tissue did not differ between healthy and root-diseased trees. Likewise, constitutive monoterpene concentrations in stem phloem were similar between healthy and root-diseased trees. However, when stem phloem tissue was challenged with fungal inoculations, reaction tissue from root-diseased trees contained lower concentrations of α-pinene, the predominant monoterpene in red pine, than did reaction tissue from healthy trees. Seedlings stressed by exposure to low light levels exhibited less extensive induced chemical changes when challenge inoculated with *L. terebrantis* than did seedlings growing under higher light. Stem phloem tissue in these seedlings contained

*To whom correspondence should be addressed at USDA Forest Service, 2500 Shreveport Hwy., Pineville, Louisiana 71360.

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lower concentrations of α-pinene than did nonstressed seedlings also challenge inoculated with *L. terebrantis*. It is hypothesized that monoterpenes and phenolics play a role in the defensive response of red pine against insect–fungal attack, that stress may predispose red pine to attack by insect–fungal complexes, and that such interactions are involved in red pine decline disease. Implications to plant defense theory and interactions among multiple stress agents in forest decline are discussed.

Key Words—Monoterpene, phenolics, α-pinene, β-pinene, red pine, bark beetles, *Leptographium*, *Ips*, *Hylastes*, defense, stress, decline, growth-differentiation balance.

INTRODUCTION

In several coniferous tree species, monoterpenes (Russell and Berryman, 1976; Cates and Alexander, 1982; Raffa and Berryman, 1982, 1983; Cook and Hain, 1986; Paine et al., 1987; Lewinsohn et al., 1991a) and phenolics (Jorgensen, 1961; Shain, 1967; Lieutier, 1993) occur in increased concentrations in host tissue following failed attack by bark beetles (Scolytidae) and their associated fungi. Hence, these compounds have been hypothesized to exert defensive pressures against both insects and pathogens. Supporting evidence includes inhibition of mycelial growth of beetle-vectored fungi by monoterpenes, as well as toxic and/or repellent effects on bark beetles (Smith, 1963; Cobb et al., 1968; Shrimpton and Whitney, 1968; DeGroot, 1972, Coyne and Lott, 1976; Bordasch and Berryman, 1977; Raffa et al., 1985; Bridges, 1987; Delorme and Lieutier, 1990; Raffa and Smalley, 1995). Recently, Klepzig (1994) demonstrated inhibition of fungal propagule germination, mycelial growth, and beetle tunneling by monoterpenes and inhibition of mycelial growth by phenolics that are found in red pine resin.

Critical gaps remain in our knowledge of the dynamics of conifer chemical defenses. Although trees can usually resist attack by most parasitic organisms, numerous examples exist of widespread mortality caused by beetle–fungal complexes. In many of these cases, mortality appears to follow some biotic and abiotic stress (Nebeker et al., 1993). Such relationships may provide insights into basic theories of plant defense and of insect–microbial complexes that jointly encounter host resistance. Likewise understanding the relationships among trees, bark beetles, fungi, and stress may assist in our ability to manage damaging species, as well as identify important parameters in complex forest declines. The relative extents of the natural, management-related, and anthropogenic causes of forest declines have become the subject of increasing research (Klein and Perkins, 1988; Oren et al., 1988; Manion, 1991). This study concerns host–fungal interactions in a decline disease that occurs in Lake States red pine (*Pinus resinosa* Ait.) plantations.

Red pine decline disease (RPDD) is characterized by expanding, relatively circular zones of dead trees (Klepzig et al., 1991). Living trees that surround this zone of mortality show reduced growth rates. Living trees near the zone of tree mortality also have root systems exhibiting high levels of mortality and infestation with facultatively parasitic (Klepzig, 1994) fungi. Among these, the strain fungi, *Leptographium terebrantis* Barras and Perry and *L. procerum* (Kendrick) Wingfield, are most prevalent. Trees further from the area of mortality and decline have higher growth rates, lower levels of root mortality, and lower levels of fungal infection than do trees at or near the margin of mortality. Five species of parasitic root- and lower stem-infesting insects are also associated with RPDD (Raffa and Hall, 1988; Klepzig et al., 1991). These include *Hylobius radicis* Buchanan (Curculionidae), *H. pales* Herbst (Curculionidae), *Pachylobius picivorus* (Germar) (Curculionidae), *Dendroctonus valens* LeConte (Scolytidae), and *Hylastes porculus* Erichson (Scolytidae). These beetles carry the above fungi in the field and are demonstrated vectors of plant parasitic fungi (Klepzig et al., 1991; Nevill and Alexander, 1992; Klepzig, 1994). An additional subcortical insect species, *Ips pini* (Say) (Scolytidae), colonizes the aboveground portion of the stem, consistently carries the plant parasitic fungus *Ophiostoma ips* (Rumb.) Nannf., and is invariably associated with the ultimate death of declining trees (Klepzig et al., 1991, Raffa and Klepzig, 1995).

The primary objective of this study was to identify and quantify the major components of the induced chemical response of red pine to attack by the insect–fungal complex associated with RPDD. Because of the frequent association between tree mortality to bark beetle–fungal complexes and environmental stress (Lorio and Hodges, 1968; Lorio, 1986; Christensen and Ericsson, 1986; Paine and Stephen, 1987; Nebeker et al., 1993), we also compared the chemical responses of vigorous and stressed trees. In particular, we evaluated the effects of: (1) a predisposing, controlled abiotic stress (reduced light availability) on the ability of trees to induce chemical defenses, and (2) a naturally occurring biotic stress (root disease) on subsequent defensive capability against stem-invading complexes.

METHODS AND MATERIALS

Host Response to Wounding and Fungal Infection. One of three treatments were applied to healthy, 25-year-old, field-grown red-pines: mechanical wounding, wound inoculation with *L. terebrantis*, and control consisting of unwounded trees. These are hereafter referred to as "mature" trees as they are reproductively mature and nearing commercial cutting age (we recognize that "mature" is a relative term as trees undergo developmental changes throughout most of their lives). Samples of phloem from these trees were analyzed for total phenolic and monoterpene content.

Mature trees were located in a plantation in the Bakken's Pond Wildlife Management Unit, near Spring Green, Wisconsin. A modified cork-borer technique (Wright, 1933) was used to administer mechanical wounds and wound inoculations. A 12-mm-diameter disk of bark and phloem was removed at 1.5 m height with a sterile cork borer. A disk of the same size was cut from an actively growing, pure culture of *L. terebrantis* (isolate K9013) on potato dextrose agar (PDA). This culture originated from the collection of E.B. Smalley (University of Wisconsin–Madison) and is deposited in the American Type Culture Collection and the collection of Dr. Hal Burdsall (USDA Forest Products Laboratories, Madison, Wisconsin). The inoculum disk was removed from the Petri plate with a surface disinfected spatula and placed on the exposed surface of the xylem. The bark/phloem disk was returned to the wound surface to seal the inoculation site. Trees were mechanically wounded as above, but with a surface-disinfected cork borer. The cork borer and spatula were surface disinfected with 90% ethanol between treatments. Constitutive control trees remained unwounded until collection of tissue samples. At the termination of each experiment, the section of phloem containing the visibly apparent reaction tissue from inoculated and mechanically wounded trees, or an approximately equivalent portion of unwounded phloem tissue from unwounded trees, was removed.

In 1986, 24 trees were left unwounded, 24 trees were mechanically wounded, 25 trees were inoculated with living *L. terebrantis*, and 23 trees were inoculated with dead (autoclaved) *L. terebrantis*. In 1993, 23 trees were left unwounded, 17 trees were mechanically wounded, 18 trees were inoculated with living *L. terebrantis* and sampled after one week, and 24 trees were inoculated with living *L. terebrantis* and sampled after three weeks. These trees were analyzed for total phenolic content. In 1993, additional trees were sampled for monoterpene content: 22 trees were left unwounded, 24 trees were mechanically wounded, 25 trees were inoculated with living *L. terebrantis* and sampled after one week, and 26 trees were inoculated with living *L. terebrantis* and sampled after three weeks.

Analysis of Subcortical Tissue. Subsamples of phloem were finely chopped ($< 1 \times 1$-mm pieces) on a Plexiglass cutting board with a razor blade, weighed, and placed in vials. Vials were sealed and stored at $-80°C$ until extraction.

Acetone and methanol were used to extract phenolics. In 1986, tissue samples were extracted in 10 ml of 50% methanol for 24 hr at 25°C. In 1993, tissue samples were extracted in 10 ml of 70% acetone for 24 hr at 25°C. Extracts were filtered through qualitative-grade, medium-porosity paper (Fisher products) and analyzed using a modified version of the techniques of Kelsey and Harmon (1989) and Julkunen-Tiito (1985). Aliquots (200 μl) of extract were dispensed into 10-ml test tubes and diluted to 2 ml with distilled water. Folin-Ciocalteu reagent (1 ml) was added to each tube, and the tube was shaken vigorously for 5 sec. Aliquots (5 ml) of 20% Na_2CO_3 were added, and the tubes were shaken

vigorously for 5 secs. After 20 min at 25°C, percent light absorbances at 735 nm were measured with a Beckman DB-G grating spectrophotometer. Total phenolic concentrations (tannic acid equivalents, micrograms per milligram of tissue) were calculated based on comparison with a standard curve derived from control solutions containing various concentrations of tannic acid. Mean concentrations of total phenolics were calculated and compared by treatment using analysis of variance and the protected least square means (LSM) method in SuperANOVA (Abacus Concepts, 1989).

Monoterpenes were extracted in pentane. Constitutive and reaction tissues were weighed and extracted in 10 ml of 0.1% *p*-cymene in pentane solution for 24 hr at 25°C. Extracts were then filtered through qualitative-grade, medium-porosity paper (Fisher products), placed in vials, and stored at −30°C until analysis. Monoterpenes were analyzed by gas chromatography (GC). Extracts were passed through a 25-m × 0.25-mm Superox II Carbowax 20 M column (Alltech Associates, Deerfield, Illinois) in a Shimadzu GC-9A gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, Maryland) using modified methods of Raffa and Steffeck (1988). Identities of individual monoterpenes were determined by comparison of relative retention times of individual peaks within the extract profile with relative retention times of commercially available synthetic pure monoterpenes. Synthetic monoterpenes used included (±)-α-pinene (97%), (+)-camphene (80%), (*S*)-(−)-β-pinene (99%), 3-carene (95%), myrcene (99%), (*S*)-(−)-limonene (96%), and γ-terpinene (98%) (Aldrich, Milwaukee, Wisconsin). Concentrations of each monoterpene were determined by integrating peak areas with a Shimadzu C-R3A Chromatopac (Shimadzu Scientific Instruments), and comparing each peak with *p*-cymene, the internal standard. Mean concentrations of individual and total monoterpenes were calculated and compared by treatment using analysis of variance and the protected least square means (LSM) method in SuperANOVA (Abacus Concepts, 1989).

Analysis of Subcortical Tissue in Biotically Stressed Trees. To determine the effects of biotic stress on defensive chemistry, responses by healthy and root-diseased mature red pines within the same plantation were compared. Test trees were inoculated with *L. terebrantis*, sampled, and analyzed. A site undergoing RPDD (Klepzig et al., 1991) was selected within a 25-year-old plantation in the Bakken's Pond Wildlife Management Unit near Spring Green, Wisconsin. The location of every tree in relation to the margin of decline symptoms was carefully mapped. The relationship between distance from the margin of decline and level of root disease (Klepzig et al., 1991) was used to estimate the percentage of diseased roots in any given tree. At the Bakken's Pond plantation, this relationship is expressed by the linear equation:

$$P_m = 1.234 - 0.053D$$

where P_m is the arcsin [square root (proportion of mortality in root system)], and D is the distance (meters) from the decline margin.

This equation was used to determine the distance at which trees would have an estimated average root mortality of 0–25% and could thus be considered to be healthy. The same equation was used to determine the distance at which trees would have an estimated average root mortality of 90–100% and could thus be considered diseased. Twenty-one "healthy" and 20 "diseased" trees were selected in this manner. Inoculations, sampling, extractions, and analyses were conducted as described above.

Analysis of Subcortical Tissue of Abiotically Stressed Seedlings. Two experiments were conducted to determine the effects of reduced light availability and resulting variations in photosynthate on the constitutive and induced subcortical chemistry of red pine seedlings. Two-year-old seedlings were grown at four light intensities, in two separate experiments, in the University of Wisconsin–Madison Biotron. In both experiments 2-year-old seedlings were obtained from the Wilson State Nursery at Boscobel, Wisconsin, and planted in 1-liter plastic pots containing a 1:2 mixture of sand and field soil. Roots were pruned to fit into the pots, the seedlings were placed in the soil, and a 2- to 4-cm-thick layer of acid sphagnum moss was placed over the soil surface to maintain proper pH. Seedlings were released from dormancy in a greenhouse and were watered as needed with a 0.6 g/liter solution of Peters Acid Greening Special fertilizer (Grace-Sierra Horticultural Products Company, Milpitas, California). Seedlings with terminal bud expansion were transferred to a growth room. Seedlings with unexpanded terminal buds were discarded.

In the first experiment, 72 two-year-old seedlings were placed in a 3.7 × 2.6-m growth room under cool white fluorescent lamps (General Electric, Cleveland, Ohio), which provided an average photosynthetic photon flux density (PPFD) of 200 μmol/m^2/sec at the top of a metal frame covered with neutral density shade cloth, which reduced PPFD by 60% to an average of 80 μmol/m^2/sec. Growing conditions included an average day–night air temperature of 20°C relative humidity of 60%, and a 12-hr photoperiod. Soil moisture was monitored in a subsample of pots with soil tensiometers and maintained at field capacity in both treatments by watering as necessary with the fertilizer solution described above. Seedlings were grown for eight weeks, and any that died in the interim were discarded. Sixteen seedlings within each of the two light treatments were randomly assigned to one of two inoculation treatments ($N = 8$). Seedlings were either mechanically wounded with a 7-mm cork borer or wounded and then inoculated with *L. terebrantis* (isolate K9013). Seedlings were inoculated as described for the mature trees. A 7-mm-diameter cork borer was used to remove a disk of phloem and outer bark from the main tap root just below the root collar. Seedlings were then inoculated or mechanically wounded as above. Roots were covered with soil, and seedlings were destructively harvested after three weeks. Extraction and analyses of phenolics and monoterpenes were conducted using the methods described above for mature trees.

On the day before destructive harvest, needle photosynthesis and stomatal conductance were measured using an ADC LCA-2 Portable Infrared Gas Analyzer with a Parkinson Broadleaf chamber (Analytical Development Co., Hoddesdon, U.K.). Needles were measured in their growth light environments.

In a subsequent experiment, seedlings were grown in two additional light environments—one with a higher irradiance than full light in the first experiment, and the other with an irradiance intermediate between full and reduced levels in the first experiment. In the second experiment, 72 two-year-old seedlings whose terminal buds had begun to expand were transferred from the greenhouse to a growth room (3.7 m × 2.6 m) equipped with 1000-W high-pressure sodium vapor lamps (General Electric, Cleveland, Ohio) suspended 3 m from the floor. Thirty-six seedlings were placed under a metal frame covered with three layers of neutral density shade cloth (PPFD = 120 μmol/m^2/sec). The other 36 seedlings were grown without shade cloth (PPFD = 350 μmol/m^2/sec). Daily integrated PPFD in the four light treatments ranged from 3.5 to 15.1 mol/m^2, which was equivalent to 8% to 34% of full sunlight on a clear midsummer day in Wisconsin (E.L. Kruger, unpublished data). The average temperature was 20°C, the average relative humidity was 60% and the photoperiod was 12 hr. Soil moisture was maintained as before. Seedlings were grown for seven weeks, and any seedlings that died in the interim were discarded. All remaining seedlings were randomly assigned to one of three inoculation treatments. Six seedlings in reduced light and seven seedlings in full light were left unwounded and uninoculated. These seedlings were designated constitutive controls. In the second treatment, 12 seedlings in each light treatment were inoculated with *L. terebrantis* (isolate K9013). In the third treatment, 12 seedlings in each light treatment were mechanically wounded as described above. Seedlings were inoculated as described above, but with a 4-mm-diameter cork borer. Roots were covered with soil, and seedlings were destructively harvested after five weeks. Extraction and analyses of phenolics and monoterpenes were conducted using the previously described methods.

Needle photosynthesis and stomatal conductance were measured as described above at the time of inoculation and one week later. Needle water potential was measured with a pressure chamber (P.M.S. Instruments, Corvallis, Oregon) at the time of, and one and four weeks after, inoculation. All root and stem tissue within 5–10 cm (or as far as the extent of the lesion) was removed and stored at −80°C. All new shoot growth, as well as the entire root system, was clipped from each seedling, weighed, and stored at −80°C.

RESULTS

In all experiments, mature red pine trees reacted to wound inoculation with *L. terebrantis* by forming elliptical, brown to black, resin-soaked lesions within

the phloem and across the xylem surface. Lesions typically became shorter and narrower with increasing depth in the xylem. Lesions that formed in response to aseptic mechanical wounding were much lighter in color and much less resinous than those resulting from inoculation with *L. terebrantis*. Unwounded (constitutive) tissues were free of discoloration and resinosis at the time of sampling.

Analysis of Subcortical Tissue in Healthy Trees. Increased concentrations of total phenolics and monoterpenes accompanied the response of red pine to simulated insect and pathogen attack. The phenolic concentrations within red pine tissue subjected to simulated attack were higher than those found in constitutive tissue. However, this response was relatively nonspecific to the type of attack. Preliminary characterization of methanol- and acetone-extractable fractions via thin-layer chromatography indicated the presence of pinosylvin and pinosylvin monomethyl ether (Klepzig, 1994). Spectrophotometric analysis revealed significant differences in total phenolics among treatments. Lesions formed in response to wound inoculation with *L. terebrantis* contained higher concentrations of total phenolics than did constitutive tissues in both 1986 and 1993 (Figure 1).

Mechanical wounding elicited a similar response. Total phenolic concentrations in lesions resulting from inoculation with *L. terebrantis* and mechanical wounding did not differ significantly in 1986 inoculations (Figure 1a). However, in 1993, polar extracts of *L. terebrantis* inoculations contained higher concentrations of total phenolics than did extracts of mechanical wounds (Figure 1b). The concentrations of total phenolics in lesions one week after inoculation with *L. terebrantis* did not significantly differ from the concentrations in lesions three weeks after inoculation with *L. terebrantis* (Figure 1b). Differences in levels of phenolics between 1986 and 1993 may be due to variation in host response, methods, and/or amount of tissue sampled. Increased but relatively nonspecific accumulation of phenolics has been reported in Scots pine (Lieutier, 1993).

Mechanical wounding and wound inoculation with *L. terebrantis* also caused significant increases in pentane-extractable compounds. However, the monoterpene fraction of the resin formed in response to simulated parasite attack exhibited changes more consistent with a specific plant defensive response. Nonpolar extracts were comprised primarily of six monoterpenes. These were, in order of decreasing abundance, α-pinene, β-pinene, 3-carene, limonene, camphene, and myrcene (Table 1). γ-Terpinene was present only in trace quantities that did not significantly differ according to treatment. Concentrations of α-pinene, camphene, β-pinene, 3-carene, myrcene, and limonene were all higher in inoculated than in mechanically wounded or constitutive tissues. Inoculated tissues also contained significantly higher concentrations of total monoterpenes than did mechanically wounded or constitutive trees. Concentrations of α-pinene, cam-

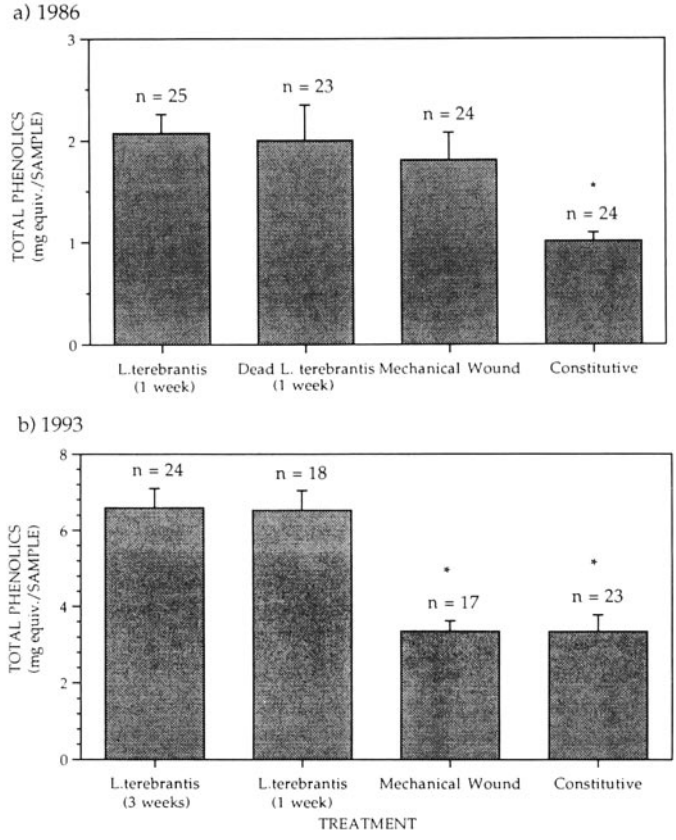

FIG. 1. Phenolic content (mean ± SE) of red pine constitutive phloem tissue and reaction tissue one week after mechanical wounding, one and three weeks after inoculation with viable *L. terebrantis*, and one week after inoculation with nonviable *L. terebrantis*. An asterisk indicates significant difference at $P < 0.05$ by protected least squares means in ANOVA. (a) $df = 3$, $F = 4.26$, $P < 0.01$; (b) $df = 3$, $F = 16.31$, $P < 0.0001$.

phene, myrcene, and limonene were higher in mechanically wounded than in constitutive tissue, but mechanically wounded and constitutive tissues did not significantly differ in total monoterpene concentrations. Total monoterpene concentrations, as well as concentrations of α-pinene, camphene, β-pinene, 3-carene, myrcene, and limonene in lesions one week after inoculation with *L. terebrantis* did not differ significantly from the concentrations in lesions three

TABLE 1. MONOTERPENE CONTENT OF RED PINE REACTION RESIN[a]

Monoterpene	Control	Mechanically wounded	Inoculated		F^b	P
			1 week	3 week		
α-Pinene	8.0 (7.5)a	113.5 (1.8)b	306.1 (34.7)c	288.6 (47.9)c	18.80	0.0001
Camphene	0.003 (0.0)a	1.1 (0.1)b	3.1 (0.3)c	2.9 (0.5)c	17.24	0.0001
β-Pinene	0.1 (0.0)a	38.9 (7.6)b	102.4 (17.0)b	118.2 (24.2)b	11.40	0.0001
3-Carene	0.01 (0.0)a	3.6 (1.0)b	9.3 (1.9)b	9.0 (2.3)b	7.02	0.0003
Myrcene	0.001 (0.0)a	0.9 (0.1)b	2.5 (0.3)c	2.6 (0.5)c	13.69	0.0001
Limonene	0.001 (0.0)a	1.5 (0.2)b	4.0 (0.5)c	4.0 (0.6)c	16.59	0.0001
Total monoterpenes	8.2 (35.6)a	159.9 (24.2)b	427.7 (50.9)c	425.5 (71.0)c	18.20	0.0001

[a] Mean concentration (mg monoterpene/g dry weight) (standard error) of reaction resin in red pine unwounded (control), mechanically wounded, wound inoculated with *L. terebrantis* and sampled after one week, and wound inoculated with *L. terebrantis* and sampled after three weeks. Means followed by different letters are significantly different from other means within the row at $P < 0.05$ level, as determined by protected least squares means in ANOVA.

[b] Degrees of freedom = 3; F and P (determined by protected least squares means procedures in ANOVA) given for each monoterpene by treatment.

TABLE 2. MONOTERPENE CONTENT OF RED PINE REACTION RESIN[a]

Monoterpene	Control	Mechanically wounded	Inoculated		F^b	P
			1 week	3 week		
α-Pinene	73.6 (2.5)	72.8 (1.8)	73.1 (1.7)	69.4 (2.0)	0.88	0.45
Camphene	0.0 (0.0)a	0.7 (0.0)b	0.7 (0.0)b	0.7 (0.0)b	99.72	<0.0001
β-Pinene	26.1 (2.5)	22.8 (1.9)	22.6 (1.7)	25.1 (2.3)	0.66	0.57
3-Carene	0.4 (0.2)a	2.4 (0.5)b	2.1 (0.4)b	3.1 (1.0)b	3.03	0.03
Myrcene	0.0 (0.0)a	0.5 (0.1)b	0.6 (0.0)b	0.6 (0.1)b	42.37	<0.0001
Limonene	0.0 (0.0)a	0.9 (0.1)b	0.9 (0.0)b	1.0 (0.0)b	81.54	<0.0001

[a] Mean percent composition (standard error) of reaction resin in red pine unwounded (control), mechanically wounded, wound inoculated with *L. terebrantis* and sampled after one week, and wound inoculated with *L. terebrantis* and sampled after three weeks. Means followed by different letters are significantly different from other means within the row at $P < 0.05$ level, as determined by protected least squares means procedure in ANOVA.

[b] Degrees of freedom = 3; F and P given for each monoterpene by treatment. Gamma-terpinene levels were less than 0.00% and are not presented here.

weeks after inoculation with *L. terebrantis*. However, 3-week-old inoculations contained significantly higher concentrations of total monoterpenes than did 1-week-old inoculations.

The composition of reaction tissue also varied somewhat with respect to treatment (Table 2). These changes were limited to the minor components.

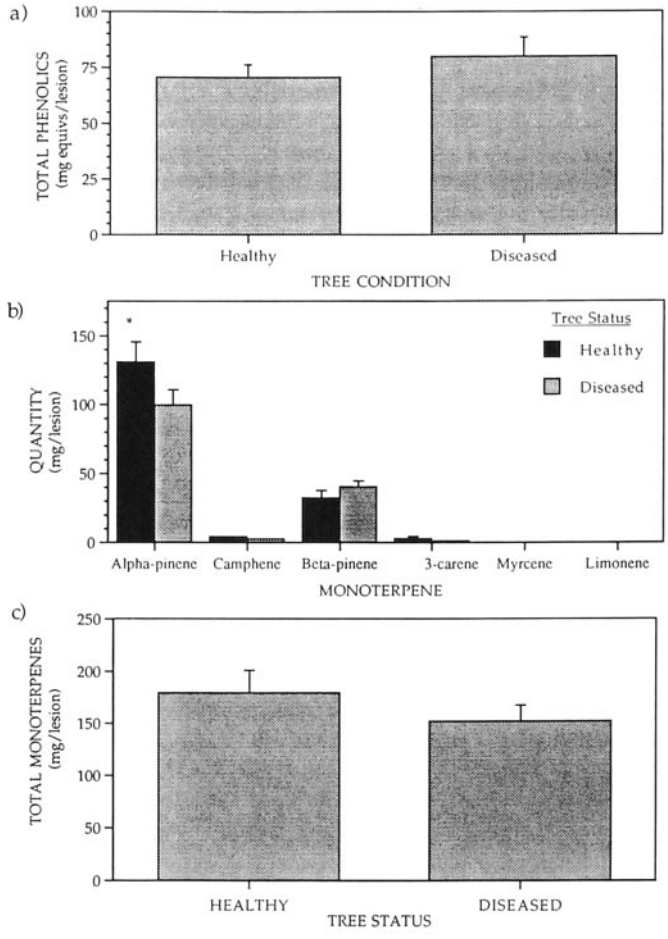

FIG. 2. Defensive chemistry of stem phloem reaction tissue of healthy and root diseased red pine one week after inoculation with *L. terebrantis*. An asterisk indicates significant difference by tree status at $P < 0.001$ by protected least squares means in ANOVA. (a) $df = 1$, $F = 1.491$, $P < 0.43$. (b) $df = 7$, $F = 2.66$, $P < 0.01$. (c) $df = 1$, $F = 0.96$, $P < 0.33$.

Camphene, 3-carene, myrcene, and limonene represented a higher proportion of the total monoterpene profile in mechanically wounded or inoculated tissue than in constitutive tissue. However, the proportions of the major monoterpenes in red pine phloem, α-pinene, and β-pinene, did not vary significantly among treatments.

Analysis of Subcortical Tissue in Biotically Stressed Trees. The effects of root disease on the subcortical chemistry of red pine varied according to the chemical fraction. Total phenolic concentrations within reaction lesions did not significantly differ between root-diseased and healthy trees (Figure 2a). However, significant differences were found in the composition of the nonpolar fraction.

α-Pinene concentrations in lesions formed in response to inoculation with *L. terebrantis* were significantly lower in diseased than in healthy trees (Figure 2b). However, concentrations of camphene, β-pinene, 3-carene, myrcene, limonene, and total monoterpenes did not vary significantly with root status (Figure 2b and c). The relative composition of the reaction resin differed between healthy and root-diseased trees (Table 3). α-Pinene comprised a higher pecentage of the total monoterpene composition within lesions from healthy trees than it did in lesions from root-diseased trees. Conversely, β-pinene comprised a lower proportion of total monoterpenes within lesions from healthy trees than in lesions from root-diseased trees. The constitutive monoterpene content did not vary

TABLE 3. MONOTERPENE CONTENT OF REACTION RESIN IN HEALTHY AND ROOT-DISEASED RED PINE.[a]

	Tree status			
Monoterpene	Healthy	Diseased	F^b	P
α-Pinene	74.2 (1.2)a	61.3 (4.6)b	7.803	0.008
Camphene	1.8 (0.3)	1.5 (0.4)	0.42	0.52
β-Pinene	16.9 (0.7)a	31.1 (4.5)b	10.08	0.002
3-Carene	1.3 (3.8)	0.6 (0.3)	0.70	0.40
Myrcene	0.0 (0.0)	0.0 (0.0)	1.05	0.31
Limonene	0.0 (0.0)	0.0 (0.0)	0.36	0.55

[a] Mean percent composition (standard error) of reaction resin in red pine located near a margin of decline and mortality (diseased), vs. far from a margin of decline and mortality (healthy) and wound inoculated with *L. terebrantis*. Trees were sampled after three weeks and extracted. Means followed by different letters are significantly different from other means within the row at $P < 0.05$ level, as determined by Protected Least Squares in ANOVA.

[b] Degrees of freedom = 1; F and P given for each monoterpene by tree status interaction. Gamma-terpinene levels were less than 0.00% and are not presented here.

significantly between healthy and diseased trees (Klepzig, 1994). These results are similar to those of Werner and Illman (1994), who reported that the relative proportions of α-pinene were lower in mature Lutz spruce that had been attacked by spruce beetles and then inoculated with *L. abietinum* (Peck) Wingfield than they were in inoculated trees that showed no bark beetle damage.

Physiology, Growth, and Subcortical Chemistry of Abiotically Stressed Seedlings. Reduced light resulted in significant differences in several physiochemical parameters. However, due to differences in seedling size and possible variation in other parameters, caution should be used when comparing the results of the two experiments. In both experiments, seedlings grown under reduced light photosynthesized at significantly lower rates per unit area (Figure 3a) and per unit mass (Figure 3b), and they had significantly lower stomatal conductance (Figure 3c) than trees grown under full light. In the second experiment only, root and shoot growth were significantly lower in seedlings grown under reduced light than they were in seedlings grown under full light (Figure 4). Xylem water potentials were highly variable in both experiments and did not differ significantly according to treatment.

Total phenolic concentrations differed between mechanically wounded seedlings grown under full light and reduced light in the first (Figure 5a) but not the second experiment (Figure 5b). Phenolics did not differ by light treatment in the second experiment. Both monoterpene concentration (Figures 6 and 7) and relative composition (Table 4, Table 5) varied with light intensity. In the first experiment, concentrations of individual monoterpenes did not differ significantly between seedlings grown under full and reduced light and then inoculated. However, total monoterpene concentrations were significantly higher in seedlings grown under full light and wound inoculated with *L. terebrantis* than in seedlings grown under reduced light and wound inoculated with *L. terebrantis* (Figure 6d). In the first experiment, the composition of reaction resin differed significantly with respect to light treatment for two monoterpenes, camphene and β-pinene (Table 4). Percent composition did not differ significantly by treatment for any of the other monoterpenes detected in the first experiment.

In the second experiment, α-pinene concentrations were significantly higher in seedlings grown under full compared to reduced light following wound inoculation with *L. terebrantis* (Figure 7c). No other differences in concentrations of individual monoterpenes between seedlings grown under full and reduced light were observed. As with the mature trees, total monoterpene concentrations were higher in seedlings wound inoculated with *L. terebrantis* than they were in constitutive or mechanically wounded seedlings (Figure 7d). Although the intensity of light under which seedlings were grown was not found to be correlated with any significant changes in total monoterpene concentrations, the relative composition of the monoterpene fraction differed according to treatment

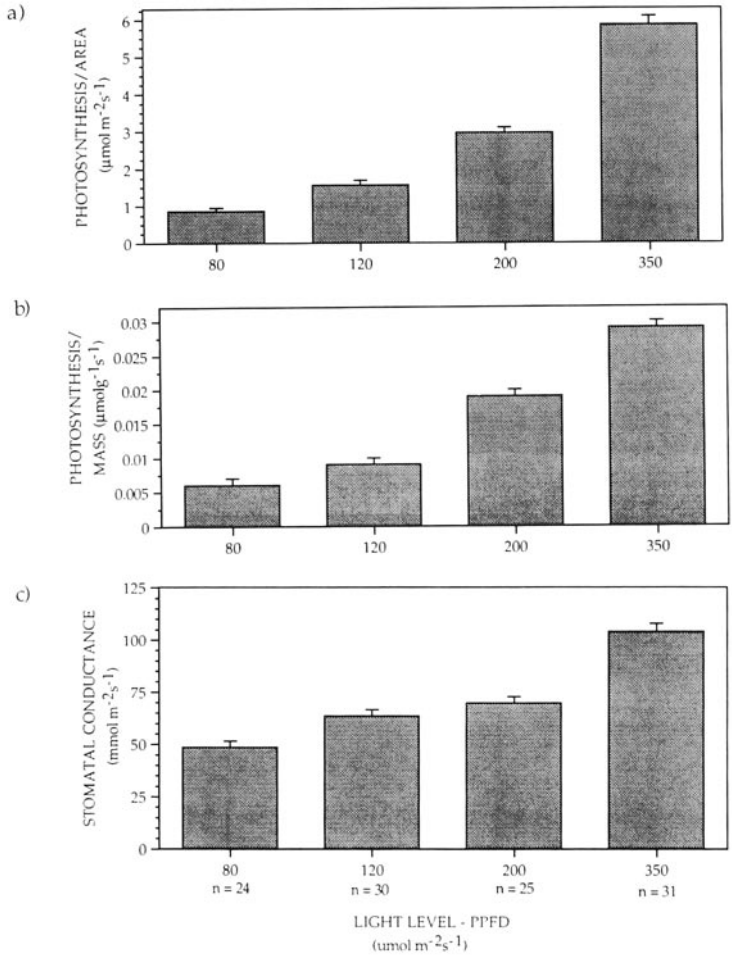

FIG. 3. Effects of irradiance on physiological parameters (mean ± SE) of red pine seedlings. (a) Photosynthesis per unit area. PPFD = photosynthetic photon flux density. First experiment, PPFD = 80 and 200 μmol/m^2/sec, $df = 1$, $F = 175.40$, $P < 0.0001$. Second experiment, PPFD = 120 and 350 μmol/m^2/sec, $df = 1$, $F = 281.0$, $P < 0.0001$. (b) Photosynthesis per unit mass. First experiment, PAR = PPFD = 80 and 200 μmol/m^2/sec. $df = 1$, $F = 87.69$, $P < 0.0001$. Second experiment, PAR = 120 and 350 μmol/m^2/sec. $df = 1$, $F = 160.61$, $P < 0.0001$. (c) Stomatal conductance per unit area. First experiment, PAR = 80 and 200 μmol/m^2/sec. $df = 1$, $F = 24.27$, $P < 0.0001$. Second experiment, PAR = 120 and 350 μmol/m^2/sec. $df = 1$, $F = 58.60$, $P < 0.0001$.

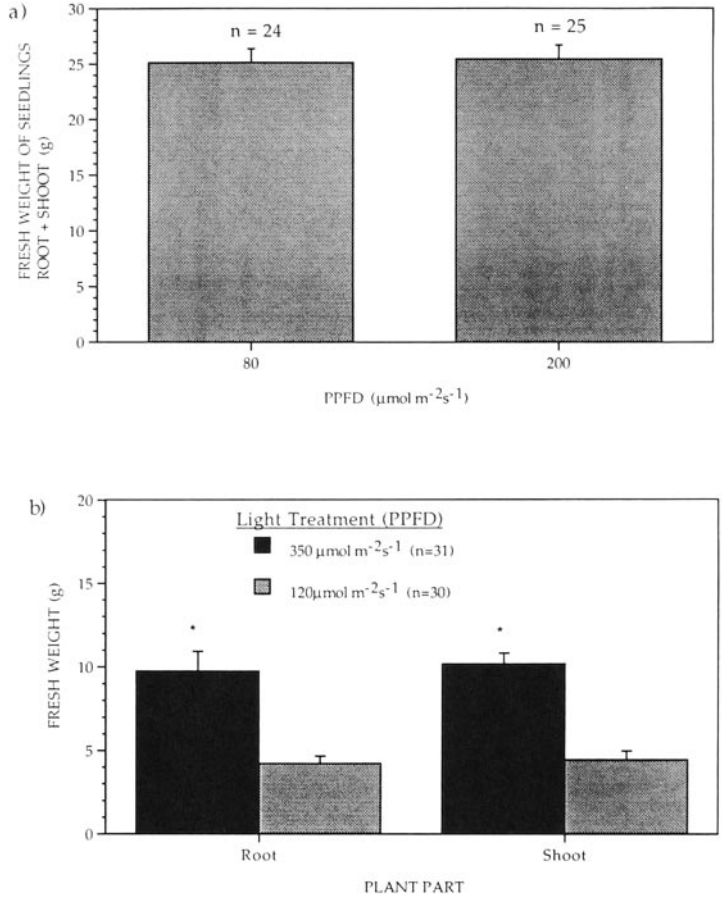

FIG. 4. Growth (mean ± SE) of red pine seedlings under various light levels. PPFD = photosynthetic photon flux density. An asterisk indicates significant difference at $P < 0.0001$ by protected least squares means in ANOVA. (a) First experiment: $df = 1$, $F = 0.0006$, $P < 0.93$. (b) Second experiment: roots—$df = 1$, $F = 19.26$, $P < 0.0001$; shoots—$df = 1$, $F = 45.36$, $P < 0.0001$.

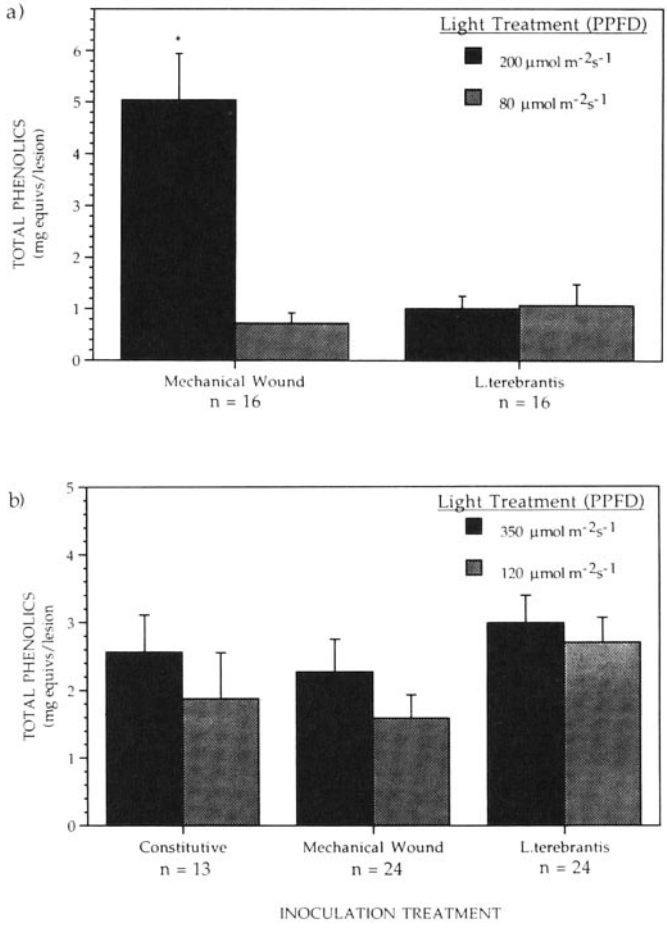

FIG. 5. Phenolic content (mean ± SE) of red pine constitutive phloem tissue and reaction tissue three weeks after mechanical wounding and inoculation with *L. terebrantis* in healthy and shade stressed seedlings. PPFD = photosynthetic photon flux density. An asterisk indicates significant difference at $P < 0.0001$ by protected least squares means in ANOVA. (a) First experiment: $df = 3$, $F = 0.08$, $P < 0.96$. (b) Second experiment: $df = 2$, $F = 0.13$, $P < 0.87$.

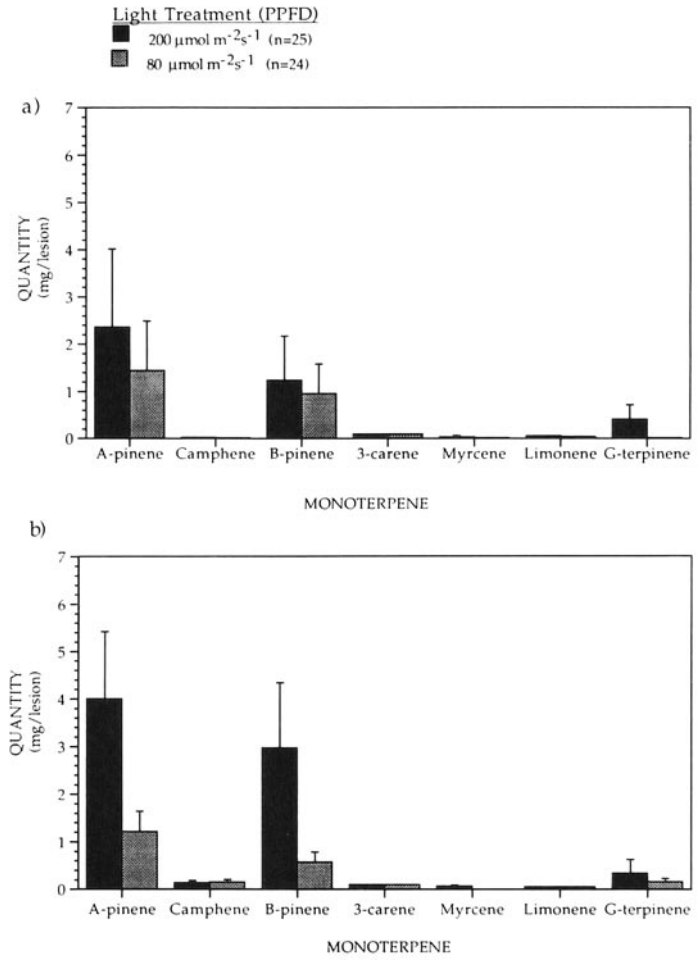

FIG. 6. Monoterpene content (mean ± SE) of red pine reaction tissue three weeks after mechanical wounding, inoculation with *L. terebrantis*, or *L. wageneri* in healthy and shade-stressed seedlings in first experiment. PPFD = photosynthetic photon flux density. No significant differences found at $P < 0.05$ by protected least squares means in ANOVA. (a) Mechanical wound, (b) inoculation with *L. terebrantis*, and (c) inoculation with *L. wageneri*. Light treatment × inoculation treatment × monoterpene: $df = 52$, $F = 2.49$, $P < 0.0001$. (d) Total monoterpenes: means followed by same letters are not significantly different at $P < 0.05$ by protected least squares means in ANOVA. $df = 3$, $F = 3.49$, $P < 0.03$.

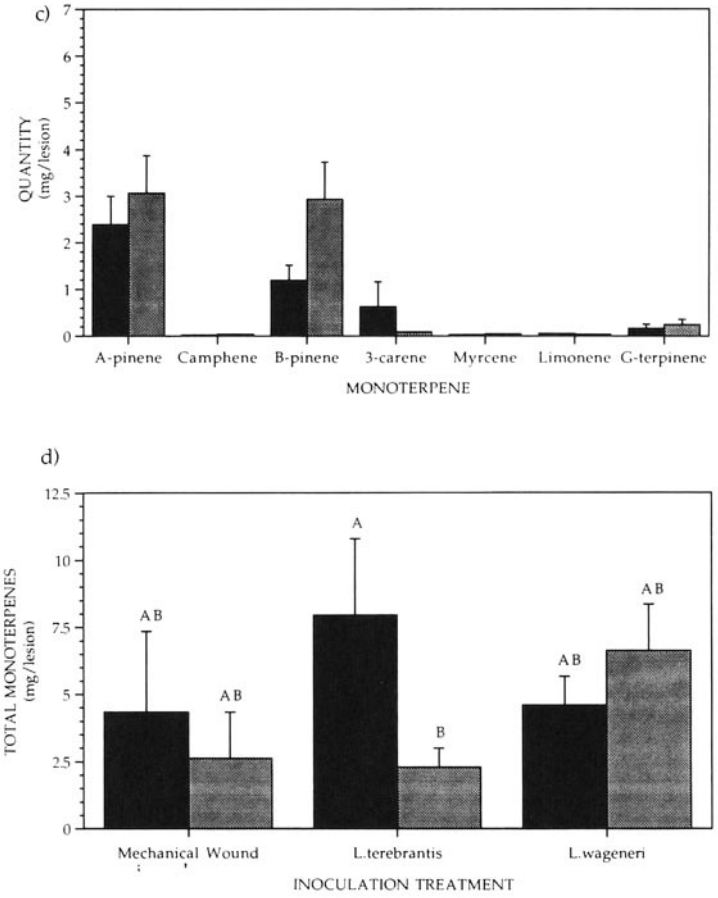

FIG. 6. Continued

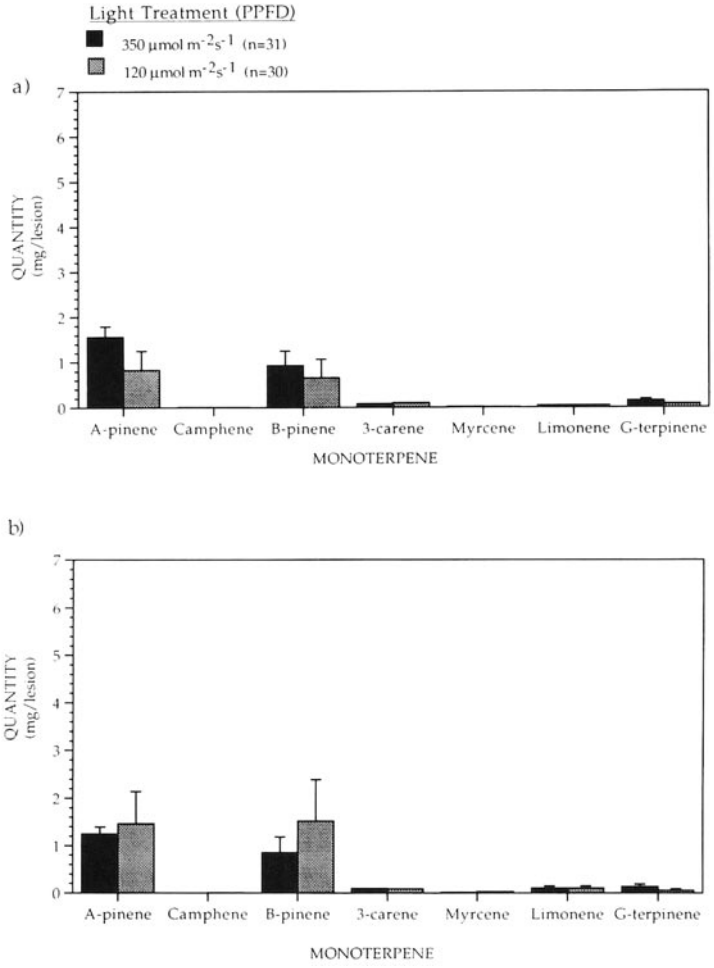

FIG. 7. Monoterpene content (mean ± SE) of red pine constitutive phloem tissue and reaction tissue three weeks after mechanical wounding or inoculation with *L. terebrantis* in healthy and shade stressed seedlings in second experiment. PPFD = photosynthetic photon flux density. An asterisk indicates significant difference at $P < 0.0001$ by protected least squares means in ANOVA. (a) Constitutive, (b) mechanical wound, (c) inoculation with *L. terebrantis*. Light treatment × inoculation treatment × monoterpene: $df = 37$, $F = 4.06$, $P < 0.0001$. (d) Total monoterpenes: $df = 2$, $F = 0.11$, $P < 0.89$.

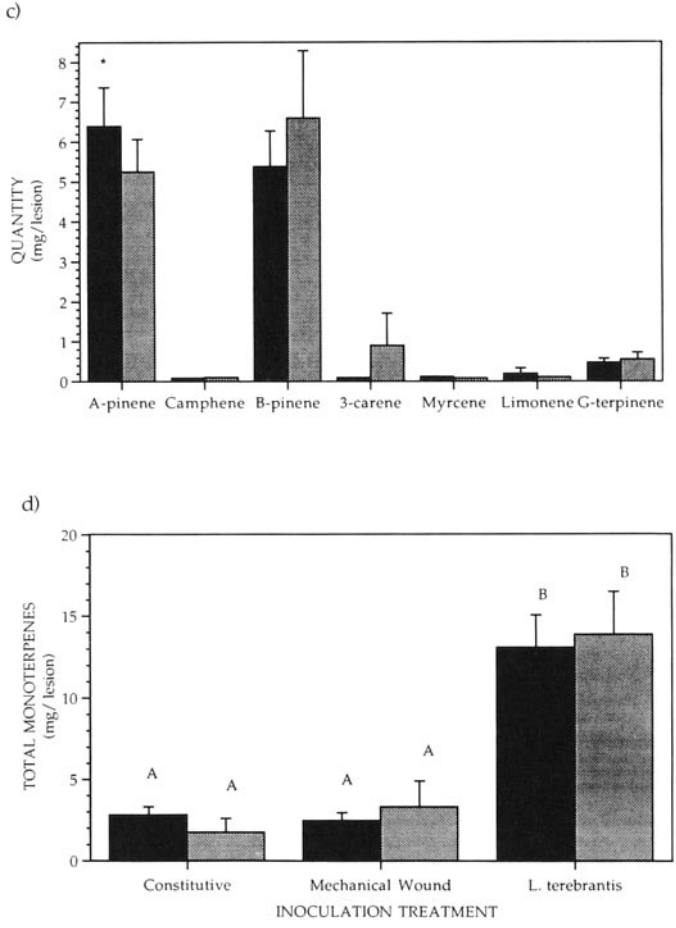

FIG. 7. Continued

TABLE 4. MONOTERPENE CONTENT OF REACTION RESIN IN HEALTHY AND SHADE STRESSED RED PINE.[a]

Monoterpene	Mechanical wound: light treatment		*L. terebrantis* inoculation: light treatment	
	Full ($N = 8$)	Reduced ($N = 8$)	Full ($N = 8$)	Reduced ($N = 8$)
α-Pinene	49.3 (7.7)	40.2 (16.1)	50.1 (2.0)	50.6 (2.3)
Camphene	0.5 (0.3)a	0.3 (0.3)a	2.0 (0.7)a	5.6 (1.6)b
β-Pinene	27.7 (3.2)ab	39.8 (4.9)b	36.7 (6.0)ab	23.5 (1.8)a
3-Carene	9.4 (7.0)	11.0 (7.2)	1.9 (1.0)	6.5 (2.1)
Myrcene	0.3 (0.3)	0.2 (0.2)	0.6 (0.3)	0.1 (0.1)
Limonene	4.8 (3.2)	5.0 (3.4)	1.1 (0.7)	3.3 (1.0)

[a] Mean percent composition (standard error) of reaction resin in red pine grown under full (200 $\mu mol/m^2/sec$) vs. reduced light (80 $\mu mol/m^2/sec$), and mechanically wounded, or wound inoculated with *L. terebrantis*. Means followed by different letters are significantly different from other means within the row at $P < 0.05$ level, as determined by protected least squares means in ANOVA. Degrees of freedom = 7; F and P for each monoterpene by inoculation × light treatment interaction: α-pinene, $F = 0.64$, $P = 0.71$; camphene, $F = 6.47$, $P = 0.0003$; β-pinene, $F = 2.35$, $P = 0.05$; 3-carene, $F = 0.62$, $P = 0.72$; myrcene, $F = 1.81$, $P = 0.13$; limonene, $F = 0.90$, $P = 0.52$.

for several monoterpenes (Table 5). α-Pinene consistently comprised a greater proportion of the total monoterpene concentration in seedlings grown under full compared to reduced light. However, these differences were not always significant at $P < 0.05$. The percentage of camphene was significantly higher in seedlings inoculated with *L. terebrantis* than in all other seedlings, and the percentage of limonene was significantly lower in seedlings grown under reduced light and wound inoculated with *L. terebrantis* than in all other seedlings.

DISCUSSION

Many of the compounds detected in these experiments can affect the attacking beetles, fungi, or both. Both the total phenolic fraction and several individual monoterpenes can inhibit insect tunneling, fungal growth, and fungal germination to varying degrees, at concentrations similar to those found previously (Klepzig, 1994). In particular, α-pinene, consistently the most abundant monoterpene in red pine phloem extracts, exhibits a high degree of biological activity towards the insects and fungi associated with RPDD. Together, these data indi-

TABLE 5. MONOTERPENE CONTENT OF REACTION RESIN IN HEALTHY AND SHADE STRESSED RED PINE[a]

	Constitutive: light treatment		Mechanical wound: light treatment		*L. terebrantis* inoculation: light treatment	
Monoterpene	Full (N = 6)	Reduced (N = 7)	Full (N = 12)	Reduced (N = 12)	Full (N = 12)	Reduced (N = 12)
α-Pinene	59.7 (9.2)a	45.3 (4.8)c	54.9 (4.5)ab	49.4 (3.5)ac	49.5 (1.5)ac	39.9 (2.6)c
Camphene	0.1 (0.1)a	0.1 (0.1)a	0.0 (0.0)a	0.1 (0.1)a	0.6 (0.0)b	0.8 (0.2)b
β-Pinene	28.6 (6.8)a	29.6 (3.8)a	29.8 (5.1)a	33.9 (6.3)a	40.3 (1.6)ab	46.1 (3.1)b
3-Carene	3.0 (0.8)ab	10.1 (1.8)a	4.7 (0.9)ab	6.4 (1.9)ab	0.9 (0.2)b	5.1 (4.1)ab
Myrcene	0.4 (0.2)ab	0.1 (0.1)a	0.4 (0.3)ab	1.5 (1.1)b	1.0 (0.1)ab	0.8 (0.2)ab
Limonene	1.4 (0.4)ab	4.3 (0.7)ab	4.8 (2.8)ab	6.2 (2.5)a	1.4 (0.9)bc	0.8 (0.1)c

[a]Mean percent composition (standard error) of reaction resin in red pine grown under full (350 μmol/m^2/sec) vs. reduced light (120 μmol/m^2/sec), and left unwounded (constitutive), mechanically wounded, or wound inoculated with *L. terebrantis*. Means followed by different letters are significantly different from other means within the row at $P < 0.05$ level, as determined by protected least squares means in ANOVA. Degrees of freedom = 5; F and P for each monoterpene by inoculation × light treatment interaction: α-pinene, $F = 2.99$, $P = 0.02$; camphene, $F = 5.29$, $P = 0.0007$; β-pinene, $F = 0.01$, $P = 0.01$; 3-carene, $F = 1.08$, $P = 0.38$; myrcene, $F = 1.46$, $P = 0.22$; limonene, $F = 3.09$, $P = 0.01$.

cate that α-pinene and perhaps other monoterpenes exert an important role in the defense of red pine against parasitic attack.

The chemical responses to stress reported here correspond to field observations and laboratory studies on RPDD and its associated organisms (Klepzig et al., 1991; Klepzig, 1994; Raffa and Smalley, 1995). Stressed red pines are significantly more susceptible to the two species of bark beetles, the stem colonizing *I. pini*, and the root colonizing *H. porculus*, and the bark beetle-vectored fungi, *L. terebrantis* and *L. procerum*, associated with declining plantations. Similarly, reduced light levels have been shown to lower concentrations of phenolics and increase susceptibility to root disease in other conifers (Entry et al., 1991). In bioassays with red pine extracts, lower α-pinene concentrations were highly correlated with increased beetle and fungal success (Klepzig, 1994). At very high concentrations, β-pinene sometimes appeared to have slightly stimulatory effects on insect feeding and fungal germination, suggesting a possible benefit to some insect-fungal complexes at certain concentrations and conditions. Thus, the dynamics of monoterpene profiles in red pine during phenological, stress-related, and wound-induced events may have a significant role in explaining changes in plant resistance to parasitic attack.

In this system, certain stresses can concurrently reduce both plant growth and allelochemical concentrations. A naturally occurring biotic stress, root infection, and an artificially administered abiotic stress, reduced light resource availability, each generated this pattern. These results are not entirely consistent with predictions based on the growth-differentiation balance hypothesis. Rather they support recent views that rapid alterations in the metabolic demand for allelochemicals (Gijzen et al., 1991; Lewinsohn et al., 1991b; Gershenzon, 1994) must also be incorporated into allocation models in systems where induced defenses are important (Lerdau et al., 1994). In red pine phloem, constitutive allelochemicals were not affected, but phytochemical induction and growth both decreased with stress (Figure 4b); (Klepzig et al., 1991). Our results also indicate that allelochemical responses to stress can vary among chemical groups. In red pine reaction phloem, for example, monoterpene, but not phenolic, concentrations were affected.

These relationships between plant defensive responses and parasitic organisms may also relate to the onset and spread of inciting factors in forest declines. In the instance of RPDD, reduced light availability may lead to the establishment of *Leptographium* spp. and its vectors within the red pine root system. Thereafter, the proposed decline disease cycle of RPDD may continue (Klepzig et al., 1991). Although these data improve our understanding of this complex decline disease, the modes of action of inhibition of propagule germination and mycelial growth by monoterpenes and the pathways by which stress interferes with defensive compound synthesis need to be addressed.

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ABSOLUTE CONFIGURATION OF SEX PHEROMONE FOR TEA TUSSOCK MOTH, *Euproctis pseudoconspersa* (STRAND) *VIA* SYNTHESIS OF (*R*)- AND (*S*)-10,14-DIMETHYL-1-PENTADECYL ISOBUTYRATES

AKIO ICHIKAWA,* TETSUYA YASUDA, and SADAO WAKAMURA

National Institute of Sericultural and Entomological Science
Owashi 1-2, Tsukuba, Ibaraki 305, Japan

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Abstract—(*R*)- and (*S*)-10,14-dimethyl-1-pentadecyl isobutyrates were synthesized from (*S*)- and (*R*)-citronellols, respectively. The *R* enantiomer was as active as the natural pheromone but the *S* enantiomer was less active in the electrophysiological analyses, which provided conclusive proof that the absolute configuration of the natural pheromone is *R*.

Key Words—Tea tussock moth, *Euproctis pseudoconspersa*, sex attractant pheromone, 10,14-dimethyl-1-pentadecyl isobutyrate, citronellol, dihydrocitronellol, chirality, absolute configuration.

INTRODUCTION

The tea tussock moth, *Euproctis pseudoconspersa* (Strand) (Lepidoptera: Lymantriidae), is a notorious pest of the family Theaceae, including *Camellia sinensis*, *Camellia japonica*, and *Camellia sasanqua*. The larvae have venomous spicules on their backs. The spicules are hazardous to human skin on contact (Ogata, 1958).

The major sex pheromone of *E. pseudoconspersa* has been identified as 10,14-dimethyl-1-pentadecyl isobutyrate (**1**) (Wakamura et al., 1994). The synthetic **1** (racemate) was found to show a potent attractant activity for the male, although the absolute configuration of C-10 remained to be determined.

Important advances in the asymmetric syntheses of insect pheromones (Mori, 1994) prompted us to synthesize *R*-**1** and *S*-**1** from (*S*)- and (*R*)-citronellols (*S*-**2** and *R*-**2**), respectively (Scheme 1). The biological activities of the

*To whom correspondence should be addressed.

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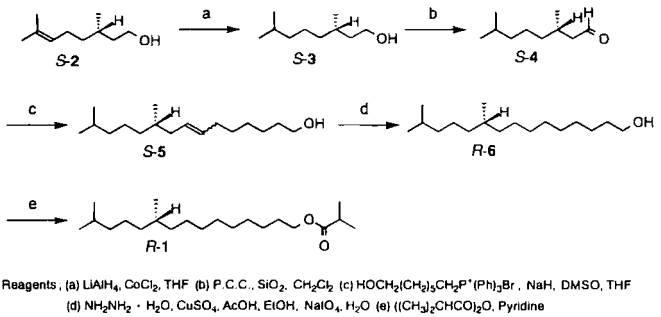


SCHEME 1. Synthetic pathway for *R*-**1**.

synthetic *R*-**1** and *S*-**1** were compared with that of the natural pheromone by a gas chromatograph equipped with an electroantennographic detector (Struble and Arn, 1984).

METHODS AND MATERIALS

General. Column chromatography was conducted employing a Wako C-100 or Wako C-200. NMR spectra were obtained on a JEOL EX90 or a JEOL GSX270 in $CDCl_3$ with TMS as an internal standard. IR spectra were recorded on a Shimadzu 8200 or a JASCO IR Report 100. GC-MS data were obtained from a Hewlett-Packard 5890 Series II gas chromatograph combined with an HP 5971A mass selective detector using DB-WAX (polyethylene glycol 20 M, 30 m × 0.25 mm ID × 0.25 mm film thickness, J & W Scientific). High-resolution mass spectra (HR-MS)(EI) were measured with a JEOL JMS-SX102A mass spectrometer. Optical rotations were determined on a JASCO DIP140 or a JASCO DIP181 digital polarimeter. CD spectra were recorded on a JASCO J-20 spectropolarimeter.

Commercially available (*S*)-citronellol (Aldrich 30,348-8) and (*R*)-citronellol (Aldrich 30,346-1) were used for the starting materials.

(S)-3,8-Dimethyl-1-octanol (S-3). *S*-**3** was synthesized from *S*-**2** in the same manner as previously reported by Mori et al. (1991) and Naoshima et al. (1991). A small amount of 1-butanol, probably derived from THF, was also obtained. We used the mixture without purification because the open column chromatography after PCC oxidation gives pure *S*-**4**.

(S)-3,8-Dimethyloctanal (S-4). Pyridinium chlorochromate (4.8 g) and SiO_2 (Wako C-100, 2.3 g) were dissolved in dry CH_2Cl_2 (15 ml) and cooled in an ice bath. *S*-**3** (500 mg) dissolved in CH_2Cl_2 (8 ml) was added dropwise to the solution. Two hours later, the mixture was directly added to a SiO_2 column and eluted by CH_2Cl_2. Further purification on the SiO_2 column gave *S*-**4** (280 mg).

(S)-10,14-Dimethyl-7-pentadecen-1-ol (S-5). *S*-**5** was synthesized in the same manner as used for *n*-alken-1-ol syntheses (Horiike et al., 1978). (7-Hydroxyheptyl)-triphenylphosphonium bromide (700 mg) in warm DMSO (2.2 ml) was added to a THF solution of methylsulfinylmethanide ion [prepared from sodium hydride (200 mg) and DMSO (1.8 ml) under nitrogen at 75°C] with cooling in an ice bath. The yellowish red solution of phosphorane was stirred at room temperature for 15 min, then *S*-**4** (180 mg) was added at 0°C. After stirring for 1 hr, the reaction mixture was poured into 20 ml of water and extracted three times with hexane. After removal of the solvent, subsequent open column chromatography gave *S*-**5** (52 mg).

Prolonged reaction time (5 hr) and increased phosphorane improved the yield. IR ν cm^{-1}: 3325 (br), 2925 (s), 2850 (s), 1465 (m), 1360 (m), 1055 (m). ^1H NMR (90 MHz): δ 5.4 (2 H, m), 3.63 (2 H, t, J = 7 Hz), 0.9–2.3 (2OH, m), 0.87 (9 H, d, J = 7 Hz). ^{13}C NMR (22.5 MHz): δ 130.43, 128.61, 62.98, 39.29, 36.90, 34.52, 33.41, 32.76, 29.68, 29.09, 27.95, 27.24, 25.65, 24.88, 22.70, 22.58, 19.60. MS: 254 (8%, M^+), 179 (1), 169 (6), 151 (12), 137 (4), 126 (46), 109 (33), 95 (57), 81 (55), 71 (89), 57 (100). HR-MS: $C_{17}H_{34}O$ (observed: 254.2611; calculated: 254.2610).

(R)-10,14-Dimethyl-1-pentadecanol (R-6). The solution of sodium metaperiodate (545 mg) in water (1 ml) was added dropwise during 1 hr to the ethanol solution (3 ml) of *S*-**5** (19 mg), hydrazine monohydrate (1 g), acetic acid (two drops), and sat. $CuSO_4$ (two drops). One week later, ethanol was removed in vacuo, and the resulting aqueous solution was extracted with hexane. The hexane solution was dried (Na_2SO_4) and concentrated in vacuo. The yield was 16 mg.

IR ν cm^{-1}: 3325 (br), 2915 (s), 2850 (s), 1460 (m), 1380 (m), 1055 (m). ^1H NMR (270 MHz): δ 3.62 (2 H, t, J = 7 Hz), 0.9–1.7 (24 H, m), 0.87 (6 H, d, J = 7 Hz), 0.82 (3 H, d, J = 7 Hz). ^{13}C NMR (67.5 MHz): δ 63.10, 39.36, 37.31, 37.09, 32.81, 32.75, 29.99, 29.62, 29.62, 29.44, 27.96, 27.07, 25.73, 24.79, 22.71, 22.62, 19.71. MS: 223 (1%, M^+-33), 210 (2), 195 (1), 182 (2), 168 (8), 153 (21), 140 (11), 126 (30), 111 (50), 97 (89), 83 (76), 71 (85), 69 (90), 57 (100), 55 (86). HR-MS: The dehydration peak was predominant. M^+-H_2O: $C_{17}H_{34}$ (observed: 238.2661; calculated: 238.2661).

(R)-10,14-Dimethyl-1-pentadecyl isobutyrate (R-1). *R*-**6** (20 mg) was dissolved in pyridine (0.4 ml) and isobutyric anhydride (0.4 ml). After 10 hr, the solution was poured into cooled 5% hydrochloric acid and extracted with hexane. After the usual work-up, the crude product was purified by open column chromatography (20.9 mg).

IR ν cm^{-1}: 2925 (s), 2850 (s), 1740 (s), 1470 (m), 1190 (m), 1155 (m) ^1H NMR (90 MHz): δ 4.06 (2 H, t, J = 7 Hz), 2.55 (1 H, sept, J = 7 Hz), 1.3–1.8 (24 H, m), 1.15 (6 H, d, J = 7 Hz), 0.87 (9 H, d, J = 7 Hz). ^{13}C NMR (22.5 MHz): δ 177.28, 64.41, 39.35, 37.29, 37.08, 34.04, 32.76, 29.98, 29.59, 29.59, 29.23, 28.64, 27.98, 27.06, 25.89, 24.79, 22.70, 22.61, 19.69,

19.00. 19.00 MS: 326 (4%, M^+), 283 (2), 239 (1), 210 (1), 183 (1), 168 (3), 153 (8), 126 (13), 111 (21), 97 (32), 89 (100), 88 (62), 83 (28), 71 (56), 69 (29), 57 (38), 55 (30). HR-MS: $C_{21}H_{42}O_2$ (observed: 326.3194; calculated: 326.3185). CD (*c*1, hexane): no measurable absorption was observed from 220 to 400 nm.

Reduction of 3,8-dimethyloctanal (4). *S*-**4** (30 mg) was dissolved in ether (1.5 ml) and cooled in an ice bath. Lithium aluminum hydride suspension (1.5 ml) in ether (1 mol/dm^3) was added to the solution. After 25 min, the reaction mixture was poured into cooled 3% hydrochloric acid. Then the solution was extracted with hexane two times. Work-up gave *S*-3 (quantitative). *S*-**3** (from *S*-**4**): $[\alpha]_D^{25}$ −5.7° (*c*0.72, MeOH). *R*-**3** (from *R*-**4**): $[\alpha]_D^{25}$ +5.6° (*c*2.0, MeOH); lit. (Mori et al., 1991) $[\alpha]_D^{20}$ +5.3° (neat, d = 0.827).

(S)-3,8-Dimethyl-1-octyl (R)-α-methoxy-α-trifluoromethylphenylacetate (S-7). *S*-**3** (1.6 mg, derived from *S*-**4**) was dissolved in 0.2 ml of dry pyridine. Then 15 μl of (*S*)-MTPA chloride was added. After 19 hr, the reaction mixture was poured into cooled 3% hydrochloric acid. Then the solution was extracted with hexane two times. Work-up gave crude *S*-**7** (7 mg). The ^{1}H NMR spectrum was measured without purification. The starting material *S*-**3** disappeared.

IR ν cm^{-1}: 2950 (s), 2925 (m), 1740 (s), 1270 (m), 1165 (s), 1120 (m), 1050 (m), 700 (m). ^{1}H NMR (270 MHz): δ 7.3–7.6 (5 H, m), 4.36 (2 H, dd, J = 8, 6 Hz), 3.55 (3 H, q, J = 0.5 Hz), 1.72 (1 H, m), 1.0–1.6 (9 H, m), 0.88 (3 H, d, J = 7 Hz), 0.85 (6 H, d, J = 7 Hz). MS: 344 (M^+-30, 1%), 189 (100), 158 (4), 141 (9), 139 (4), 127 (5), 119 (7), 105 (14), 99 (6), 91 (4), 85 (23), 71 (25), 69 (10), 57 (23), 55 (9).

(R)-3,8-Dimethyl-1-octyl (R)-α-methoxy-α-trifluoromethylphenylacetate (R-7). *R*-**7** was prepared in the same manner used for *S*-**7** synthesis. The starting material *R*-**3** also disappeared. IR ν cm^{-1}: 2950 (s), 2925 (m), 1740 (s), 1265 (m), 1160 (s), 1120 (m), 1050 (m), 700 (m). ^{1}H NMR (270 MHz): δ 7.3–7.6 (5 H, m), 4.36 (2 H, m), 3.57 (3 H, q, J = 0.5 Hz), 1.72 (1 H, m), 1.0–1.6 (9 H, m), 0.89 (3 H, d, J = 7 Hz), 0.86 (6 H, d, J = 7 Hz)., MS: 190 (M^+-184, 9%), 189 (100), 141 (9), 127 (5), 119 (7), 105 (14), 99 (6), 85 (23), 77 (6), 71 (23), 69 (8), 57 (22).

(S)-3,8-Dimethyl-6-octen-1-yl (R)-α-methoxy-α-trifluoromethylphenylacetate (S-8). *S*-**2** (2.0 mg), (*R*)-MTPA (11.0 mg), dicyclohexylcarbodiimide (15.0 mg), and pyridine (10 μl) were dissolved in 0.5 ml of dry dichloromethane. After 24 hr, the reaction mixture was directly added to the SiO_2 column and eluted by hexane–CH_2Cl_2. The yield was 4.0 mg. IR ν cm^{-1}: 2960 (s), 2920 (s), 1740 (s), 1445 (m), 1260 (m), 1180 (s), 1165 (s), 1120 (m), 1020 (m), 715 (m). ^{1}H NMR (270 MHz); δ 7.3–7.6: (5 H, m), 5.06 (1 H, br t, J = 7 Hz), 4.36 (2 H, dd, J = 7, 6 Hz), 3.55 (3 H, q, J = 0.5 Hz), 1.95 (2 H, m), 1.72 (1 H, m), 1.68 (3H, s), 1.60 (3H, s), 1.1–1.6 (4H, m), 0.90 (3 H, d, J = 7 Hz). MS: 203 (1%), 189 (78), 138 (M^+-MTPA, 51), 123 (34), 105 (30), 95 (46), 82 (40), 81 (57), 69 (100), 67 (21), 55 (32), 41 (54).

(R)-3,8-Dimethyl-6-octen-1-yl (R)-α-methoxy-α-trifluoromethylphenylacetate (R-8). *R*-**8** was prepared in the same manner used for *S*-**8** synthesis. The yield was 3.4 mg. IR ν cm^{-1}: 2960 (s), 2920 (s), 1740 (s), 1445 (m), 1265 (m), 1180 (s), 1165 (s), 1120 (m), 1020 (m), 715 (m). ^1H NMR (270 MHz): δ 7.3–7.6 (5 H, m), 5.06 (1H, br t, J = 7 Hz), 4.36 (2 H, m), 3.56 (3 H, q, J = 0.5 Hz), 1.95 (2 H, m), 1.72 (1 H, m), 1.68 (3 H, s), 1.60 (3 H, s), 1.1–1.6 (4 H, m), 0.90 (3 H, d, J = 7 Hz). MS: 203 (1%), 189 (73), 138 (M^+-MTPA, 49), 123 (33), 105 (29), 95 (46), 82 (40), 81 (58), 69 (100), 67 (22), 55 (32), 41 (56).

Gas Chromatograph–Electroantennographic Detection (GC-EAD). A Hewlett-Packard 5890 II gas chromatograph equipped with a cool on-column injector and flame ionization detector (FID) was operated using helium as carrier gas at a column head pressure of 55 kPa. Injections (0.6 μl) were made directly on a DB-1 fused silica capillary column (15 m × 0.25 mm ID, 0.25 μm film thickness; J & W Scientific, Folsom, California) at an initial column temperature of 50°C. After 1 min, oven temperature was programmed at 20°C/min to 190°C, then 10°C/min to 220°C, and held at the final temperature for 4 min.

The GC-EAD system was set up according to Struble and Arn (1984). Makeup gas (He, ca. 50 ml/min) was introduced at the end of the fused silica capillary column, and then the column flow was split into the FID and EAD lines at ca. 1 : 1 ratio.

RESULTS AND DISCUSSION

The terminal structure of **1** resembles the terpenoids such as the citronellols. Citronellol has been widely used as a chiral building block for the syntheses of optically active pheromone compounds (Mori et al., 1991; Naoshima et al., 1991).

(*S*)-Citronellol (*S*-**2**) was reduced with lithium aluminum hydride and cobalt(II) chloride to (*S*)-3,8-dimethyl-1-octanol (*S*-**3**) (Mori et al., 1991; Naoshima et al., 1991). This was oxidized with pyridinium chlorochromate to (*S*)-3,8-dimethyloctanal (*S*-**4**). Then *S*-**4** was coupled with the ylide (Horiike et al., 1978).

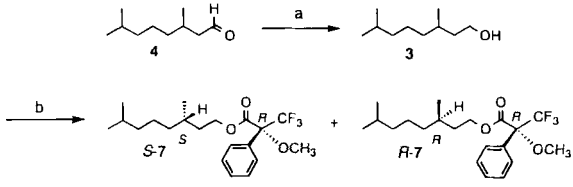
The alcohol *S*-**5** was reduced to *R*-**6** by diimide, which was generated in situ by the oxidation of hydrazine (Corey et al., 1961; Hoffman and Schlessinger, 1971; Wright et al., 1988). To avoid possible racemization (Mori et al., 1991; Naoshima et al., 1991), the catalytic hydrogenation was not adopted. Note that *R*-**6** was derived from *S*-**5**. Acylation of *R*-**6** by isobutyric anhydride in pyridine gave isobutyrate *R*-**1**. The physical properties of *R*-**1** were coincident with those of the natural pheromone and the racemic compound, which were previously reported (Wakamura et al., 1994).

S-**1**, the enantiomer of *R*-**1**, was also synthesized from (*R*)-citronellol

(*R*-**2**) in the same manner. Neither *R*-**1** nor *S*-**1** gave an $[\alpha]_D^{25}$ value (*c*1, CH_2Cl_2). Some predecessors had met similar phenomena, i.e., (*R*)- and (*S*)-15-methyltritriacontane, the sex stimulant pheromone of the stable fly *Stomoxys calcitrans* L., exhibited no measurable optical rotations at the sodium D-line (Naoshima and Mukaidani, 1987). Several other cases where the two enantiomers could not be distinguished by optical rotation were also listed in the literature. Racemic **1** showed a single GC peak on a chiral capillary column (Chirasil DEX CB, 0.25 mm ID × 25 m length, 0.25 μm film thickness) at 200°C when the carrier gas was helium at a column head pressure of 100 kPa. These circumstances compelled us to evaluate the enantiomeric purity at an earlier stage of the syntheses.

Both (*S*)- and (*R*)-3,8-dimethyloctanal (*S*-**4** and *R*-**4**) were reduced to *S*-**3** and *R*-**3** by lithium aluminum hydride in ether. *S*-**3** and *R*-**3** were then converted to the corresponding (*R*)-MTPA esters (Dale and Mosher 1973; Ohtani et al., 1991) (*R*-**7** and *S*-**7**, respectively) by treating with (*S*)-MTPA chloride in pyridine (Scheme 2). The C-1 methylene protons of *S*-**7** and *R*-**7** showed different coupling patterns. No isomer peak was observed in either sample (Figure 1), which indicated that the *S*-**4** and *R*-**4** employed for the Wittig reactions were enantiomerically pure. We therefore considered that the resulting compounds (*R*-**1** and *S*-**1**) were also optically pure because later reaction conditions suggested no isomerization reaction took place.

In the case of *S*-**7**, Figure 1 reveals that C-1 methylene protons form an A_2 pattern. On the other hand, *R*-**7**'s C-1 methylene protons form an AB pattern, and they couple with the diastereotopic C-2 methylene protons. Thus, C-1 methylene protons of *R*-**7** show a complicated coupling pattern. Most probably, the major cause of this difference is the magnetic anisotropy of the phenyl groups. We also synthesized *S*-**2** and *R*-**2** (*R*)-MTPA esters (*S*-**8** and *R*-**8**, respectively). The coupling patterns of the C-1 methylene signals of *S*-**8** and *R*-**8** (^{1}H NMR) were coincident with those of *S*-**7** and *R*-**7**, respectively. We think that we can determine the C-3 absolute configurations of some terpenoids possessing the 3-methyl-1-ol system by MTPA esterification.



SCHEME 2. Consideration of the optical purity of **4**.

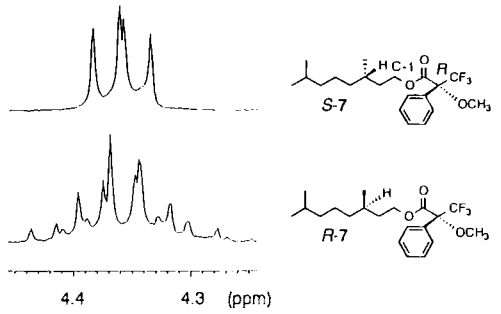


FIG. 1. ^{1}H NMR spectra of *S*-**7** and *R*-**7** (C-1 methylene region) (270 MHz, $CDCl_3$).

Electrophysiological activities of *R*-**1** and *S*-**1** were evaluated using a gas chromatograph equipped with an electroantennographic detector. The dose-response curve of *R*-**1** (3 pg–3 ng) based on the relative voltage generated by the male antenna of *E. pseudoconspersa* was well adjusted with that of the natural pheromone (Figure 2). *S*-**1** was apparently less active than *R*-**1**. We therefore concluded that the major sex pheromone component of *E. pseudoconspersa* is (*R*)-10,14-dimethyl-1-pentadecyl isobutyrate.

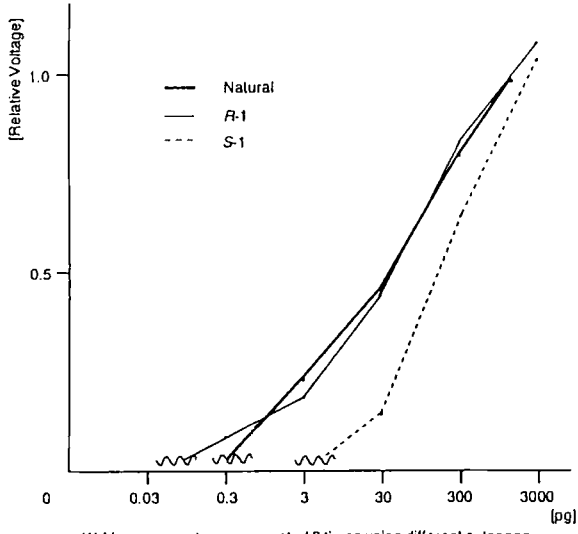


FIG. 2. Dose–response curves of GC-EAD experiment.

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EFFECTS OF MONGOOSE ODORS ON RAT CAPTURE SUCCESS

MARK E. TOBIN,[1,*] RICHARD M. ENGEMAN,[2] and ROBERT T. SUGIHARA[1]

[1]*Denver Wildlife Research Center*
US Department of Agriculture
PO Box 10880, Hilo, Hawaii 96721

[2]*Denver Wildlife Research Center*
US Department of Agriculture
PO Box 25266, Building 16
Denver Federal Center, Denver, Colorado 80225

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Abstract—Wild rats, *Rattus norvegicus*, *R. exulans*, and *R. rattus*, avoided wire-cage live traps that had previously captured mongooses, *Herpestes auropunctatus*. Replacing traps soiled by mongooses with clean traps would increase rat capture success and reduce a source of experimental bias.

Key Words—*Herpestes auropunctatus*, predator odors, *Rattus*, semiochemicals, trap success.

INTRODUCTION

Many animals utilize olfactory cues to detect potential danger from predators (Griffith, 1920; Weldon, 1990; Ylönen et al., 1992; Epple et al., 1993; Jędrzejewski et al., 1993). Recently much interest has focused on the use of predator odors to reduce damage by agricultural pests (Shumake, 1977; Sullivan et al., 1988c; Müller-Schwarze, 1990; Mason et al., 1994). Predator odors have been used to repel mountain beavers (*Aplodontia rufa*) (Epple et al., 1993; Nolte et al., 1993), voles (*Microtus* spp.) (Merkens et al., 1991; Sullivan et al., 1988a, 1990a), pocket gophers (*Thomomys talpoides*) (Sullivan et al., 1988b, 1990b), house mice (*Mus musculus*) (Coulston et al., 1993), snowshoe hares (*Lepus Americanus*) (Sullivan et al., 1985; Sullivan, 1986), woodchucks (*Marmota monax*) (Swihart, 1991), deer (*Odocoileus hemionus*) (Müller-Schwarze, 1972;

*To whom correspondence should be addressed.

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Melchiors and Leslie, 1985), elk (*Cervus elaphus*) (Andelt et al., 1992), and sheep (*Ovis aries*) (Arnould and Signoret, 1993).

Predator odors can also substantially depress rodent population estimates obtained by live trapping (Stoddart, 1976, 1982). Short-tailed voles (*Microtus agrestis*) avoided traps scented with weasel (*Mustela nivalis*) anal gland secretions (Stoddart, 1976, 1979, 1982). Capture success of Orkney voles (*Microtus arvalis*) was lower in traps soiled with red fox (*Vulpes vulpes*) fecal odors (Calder and Gorman, 1991) or stoat scent (Gorman, 1984). House mice (Dickman, 1992), wood mice (*Apodemus sylvaticus*) (Dickman and Doncaster, 1984), and voles (*Clethrionomys glareolus* and *Microtus agrestis*) (Dickman and Doncaster, 1984) shunned traps tainted with red fox fecal odors.

Personnel at the Hawaii Field Station of the Denver Wildlife Research Center, US Department of Agriculture, routinely capture rats (*Rattus norvegicus*, *R. rattus*, and *R. exulans*) for laboratory and field studies related to crop pest management. Small Indian mongooses (*Herpestes auropunctatus*) frequently enter live traps set for rats, potentially biasing trap results. We analyzed trapping records from a study conducted on a Hawaiian sugarcane plantation to determine whether mongoose captures affected subsequent capture success for rats.

METHODS AND MATERIALS

We conducted the study at the Mauna Kea Agribusiness Company, Inc., a 6000-ha sugarcane plantation on the windward coast of the island of Hawaii. We divided each of six fields with sugarcane 16–26 months of age into three comparable sections of 12–18 ha each. We set 50 steam-cleaned Haguruma wire-cage live traps (22 × 28 × 15 cm) along a 150-m transect starting from an interior road and extending into the interior of each section. At each trap site, we cleared an area approximately 30 × 30 cm to one side of the transect and secured a trap directly on the ground with a numbered wire flag. We scattered grated coconut along the traplines three days before baiting the traps with coconut chunks and setting them. Between 0630 and 1200 hr on each of the succeeding four days, we checked, rebaited, and reset traps as necessary; we collected the traps on the fourth day. We identified and released captures at their respective trap sites.

To determine whether capturing a mongoose in a trap affected subsequent rodent capture success, we compared rat capture success during nights 2–4 for traps that captured a mongoose on the first night versus traps that did not capture a mongoose on the first night. We excluded traps with mongoose captures on nights 2, 3, and 4 to avoid potential bias due to repellent effects and due to the traps already being occupied and unavailable to rodents part of the time.

TABLE 1. NUMBER OF MONGOOSES CAPTURED DURING 4 DAYS OF TRAPPING AND SUBSEQUENT NUMBER OF RATS CAPTURED IN SAME TRAPS

	Day			
	1	2	3	4
Mongooses captured (N)	17	9[a]	3	4
Rats subsequently captured in same traps (N)	1	1	0	

[a]Two traps that captured a mongoose on day 2 subsequently captured another mongoose on day 4.

RESULTS

We captured 33 mongooses in 31 different traps during 3600 trap-nights (Table 1). Two traps that captured mongooses during the second day subsequently captured another mongoose during the fourth day; no other traps captured > 1 mongoose. Four traps captured mongooses during the final 24 hr of trapping and thus had no subsequent chance to capture rats. Of the 29 traps that captured a mongoose with trap-nights remaining, only two captured a rodent during 72 subsequent trap-nights. Seventeen traps captured a mongoose on the first night, of which only one (6%) subsequently captured a rat. This is a substantially smaller proportion than the 208 of 869 traps (24%) that did not capture a mongoose but captured ≥ 1 rat on days 2–4 ($P = 0.06$, one-tailed Fisher's exact test).

DISCUSSION

Pheromones serve several functions in mammals, including regulating social behavior, marking territories, and signaling reproductive receptiveness. Predator odors generally attract other predators (Teranishi et al., 1980; Fagre et al., 1981), and many commercial predator lures and baits utilize predator-derived compounds (Blom, 1993). That only two of our traps captured > 1 mongoose may indicate that captive mongooses emitted a stress odor that repelled other mongooses.

Most rodents are under intense selective pressure to assess and avoid predatory risks (Lima and Dill, 1990). By signaling the recent presence of predators in an area, residual predator odors may provide an early warning that enables potential prey to avoid fatal encounters with predators. Such predator avoidance behavior is widespread among rodents (e.g., Epple et al., 1993; Sullivan et al., 1988a,b; Coulston et al., 1993; Swihart, 1991) and is generally resistant to

habituation (Swihart, 1991; Arnould and Signoret, 1993; Epple et al., 1993). Rats form a major portion of the diet of mongooses in and around Hawaiian sugarcane fields (Baldwin et al., 1952; Kami, 1964), and one would expect them to avoid areas frequented by these predators. Clearly most of the rats in our study avoided traps soiled with mongoose odor. This avoidance persisted for up to three days after mongooses visited the traps.

Our results indicate that replacing traps soiled by mongooses with clean traps would increase rat capture success and reduce a source of experimental bias. Further investigation is warranted to explore the use of predator odors as nonlethal repellents to reduce crop damage by rats.

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DIFFERENCES IN SEX PHEROMONE COMMUNICATION SYSTEMS OF CLOSELY RELATED SPECIES: *Spodoptera latifascia* (WALKER) AND *S. descoinsi* LALANNE-CASSOU & SILVAIN (LEPIDOPTERA: NOCTUIDAE)

LAURENCE MONTI,[1,*] BERNARD LALANNE-CASSOU,[1] PHILIPPE LUCAS,[1] CHRISTIAN MALOSSE,[1] and JEAN-FRANÇOIS SILVAIN[2]

[1]*INRA Laboratoire des Médiateurs Chimiques 78026 Versailles Cedex, France*

[2]*ORSTOM c/o CNRS Laboratoire Populations, Génétique et Evolution 91198 Gif-sur-Yvette Cedex, France*

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Abstract—*S. latifascia* and *S. descoinsi* are closely related species that occur sympatrically over limited areas in French Guiana. We examined allopatric populations, *S. latifascia* originating from Barbados and *S. descoinsi* from French Guiana. Studies on nocturnal activity cycles showed temporal partitioning of female calling behavior, male sexual activity, and mating behavior. *S. descoinsi* were sexually active in the first half of the scotophase whereas *S. latifascia* were sexually active in the second half. Seven compounds (*Z*9-14:Ac, *Z*9,*E*12-14:Ac, Z11-16:Ac, *E*9,*E*12-14:Ac, *Z*9-14:Ald, *Z*9,*E*11-14:Ac and Z11-14:Ac) were identified in females of both *S. latifascia* and *S. descoinsi* extracts. *Z*9-14:Ac was a main pheromone component for the two species. The major difference between the pheromones of *S. latifascia* and *S. descoinsi* was the proportion of *Z*9,*E*12-14:Ac in the extracts: 7% for *S. latifascia* and 42% for *S. descoinsi*. The proportion of *Z*9,*E*12-14:Ac relative to the sum of *Z*9-14:Ac and *Z*9,*E*12-14:Ac in individual gland extracts was 4 ± 1% (mean ± standard deviation) for *S. latifascia* and 44.8 ± 6% for *S. descoinsi*. Electrophysiological studies showed no major differences between species in the morphology and physiology of the pheromone receptors of males. Receptors were identified for *Z*9-14:Ac and *Z*9,*E*12-14:Ac, but no receptor was found for the other compounds. In the wind tunnel, synthetic blends with *Z*9-14:Ac and *Z*9,*E*12-14:Ac gave the same behavioral responses as conspecific female extracts for the males of the two species. Some cross-attraction was observed with synthetic blends and female extracts. Nethertheless, previous field trapping experiments in French Guiana

*To whom correspondance should be addressed

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were species-specific and suggested differences in the attractivity of males. In the laboratory, *S. latifascia* and *S. descoinsi* could hybridize in both reciprocal crosses. Female *S. descoinsi* × male *S. latifascia* mating rate was significantly lower than for the reciprocal cross, and 26.7% of female *S. descoinsi* could not separate from male *S. latifascia* after mating. These copulatory problems may involve genital incompatibilities between males and females. Several barriers against interbreeding between *S. latifascia* and *S. descoinsi* seem to combine including differences in nocturnal activity cycles, pheromone differences, and genital barriers. The study of sympatric populations will be necessary to define the role of sex pheromones in the reproductive isolation of *S. latifascia* and *S. descoinsi*.

Key Words—Lepidoptera, Noctuidae, *Spodoptera latifascia*, *Spodoptera descoinsi*, pheromones, (*Z*)-9-tetradecenyl acetate, (*Z,E*)-9,12-tetradecadienyl acetate, sexual activity rhythms, cross-attraction, electrophysiology, reproductive isolation.

INTRODUCTION

When different species occur sympatrically, precopulatory (ecological or ethological) and/or postcopulatory barriers (mechanical isolation, gametic mortality or incompatibility, inviability or sterility of hybrid zygotes) are necessary to keep them reproductively isolated (Mayr, 1942; Dobzhansky, 1970). In moths, differences in pheromone communication systems between species may be an important barrier against interspecific matings. The specificity of the pheromone blend emitted by females is the most common factor limiting reproductive interactions (Roelofs and Cardé, 1974). In some cases, this specificity is not sufficient to prevent hybridization, and other mechanisms are required to ensure complete reproductive isolation. Hence, in order to understand the role of pheromones in reproductive isolation, it is critical to study their specificity.

Spodoptera latifascia (Walker) is a noctuid moth of the *Amphipyrinae* subfamily. It occurs from Brazil to the United States where it can cause damage to vegetable crops (King and Saunders, 1984; Godfrey, 1987). Field-trapping experiments with sexual attractants in French Guiana enabled us to discover a new population of *Spodoptera* morphologically close to *S. latifascia* but attracted by a different pheromone blend (Lalanne-Cassou et al., 1994). While *S. latifascia* males were mainly caught by binary mixtures of (*Z*)-9-tetradecenyl acetate (*Z*9–14 : Ac) with 4–24% of (*Z,E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12–14 : Ac), the new population of males was attracted by mixtures containing 32–48% of *Z*9,*E*12–14 : Ac. Furthermore, males and females of this particular population could be distinguished from *S. latifascia* by small differences in wing and body coloration and by the morphology of the genitalia. The stability of these phenotypical differences, the sympatry of the two moth populations in limited areas, and the differences in sexual attractants led us to consider this population, which

seems to be endemic to French Guiana, as a new species. It was described and named *S. descoinsi* (Lalanne-Cassou et al., 1994).

The present work deals with a comparison of pheromone communication systems between allopatric populations of *S. latifascia* and *S. descoinsi*. Female calling rhythms and pheromone composition were determined for the two species. Male olfactory and behavioral responses to pheromone were studied by electroantennography and single sensillum recordings and by wind tunnel experiments, respectively. The potential role of these characters in the reproductive isolation of *S. latifascia* (SL) and *S. descoinsi* (SD) is discussed.

METHODS AND MATERIALS

Rearing of Insects. A SL culture was established from larvae collected in the field in Barbados in 1990 and a SD culture from adults caught by light traps in French Guiana in 1992. Larvae were fed on a semiartificial diet supplemented with cabbage and corn powders. They were maintained at 23°C and 70% relative humidity under a 12 : 12 hr light–dark regime. At pupation, males and females were separated in distinct rooms.

Female Calling Behavior and Mating Rhythms. At emergence, females were placed individually in 250-cm^3 plastic boxes for observation of calling behavior or placed with a male in 690-cm^3 plastic boxes for observation of mating periods. Preliminary studies showed no calling behavior during the photophase. Observations were then performed during the scotophase every 0.5 hr with a red lamp at a light intensity of 1 lux on 1- to 8-day-old females for SD and on 1- to 6-day-old females for SL. The day of emergence was designated day 1. Mating rates were estimated on groups of 15 pairs per box by dissecting females after death and searching for spermatophores transferred by males during copulation. Mating rhythms were observed during the scotophase every 0.5 hr on pairs formed with individuals of the same age. The origin of time measures for calling and mating was fixed at lights off.

Chemical Analysis. Pheromone glands of calling females were dissected during the scotophase between 3 and 7 hr after lights off for SD and between 6 and 10.5 hr after lights off for SL. Glands were individually soaked in 15 μl of hexane during 40 sec to study interindividual variation or extracted in batches of 15–30 in 250 μl of hexane during 2 hr to determine the average composition of pheromone in the populations and to detect minor compounds. Global extracts were analyzed by gas chromatography–mass spectrometry (GC-MS) in the electronic impact ionization mode on a Nermag R10-10C quadrupole interfaced with a Girdel 32 gas chromatograph. A WCOT capillary column (25 m × 0.32 mm ID Chrompack) with a polar phase FFAP-CB or a CPSil 88-CB column (60 m × 0.32 mm ID, Chrompack) was temperature programmed from 140 to 240°C

at 5°C/min. Compounds in the extracts were identified by comparison with retention times and mass spectra of synthetic standards analyzed under the same conditions. Individual gland extracts were subjected to GC analysis with a Girdel 300 or a Carlo Erba Fractovap 2900 chromatograph with flame ionization detectors and splitless and Ross injectors, respectively, for the Girdel 300 and the Carlo Erba. Helium was used as carrier gas at approximately 27 cm/sec linear velocity. A capillary column WCOT (25 m × 0.32 mm ID Chrompack) with a polar phase FFAP-CB was used. The temperature program for the Girdel 300 was 120 to 240°C at 3°C/min, with injector and detector temperatures at 240°C. The program for the Carlo Erba Fractovap 2900 was 55 to 140°C at 35°C/min, 140 to 210°C at 5°C/min with injector temperature at 225°C and detector temperature at 250°C. In each individual gland extract, the peaks for *Z*9,*E*12-14:Ac and *Z*9-14:Ac were identified by comparison with the retention times of synthetic standards analyzed under the same conditions.

Electrophysiology. Electrophysiological activity of SL and SD male antenna was measured by electroantennographic (EAG) and single sensillum recording (SSR) techniques as previously described by Lucas and Renou (1989). All experiments were carried out on whole insect preparations. Briefly, for EAGs, the apex of the antenna was excised and inserted into the recording electrode. The reference electrode was implanted in the neck of the insect. Both electrodes were connected to a Neurolog NL102 preamplifier through chloridized silver wires. The EAG responses were filtered (DC, 300 Hz) and amplified (×1000). In order to eliminate the differences in absolute sensitivity between individuals, corrected EAG data were calculated. For each individual, the response to a given compound was divided by the mean of the responses to all compounds tested (Renou et al., 1988). The responses of 10 males were measured for each species. Means of the corrected data and their confidence intervals were calculated. For SSRs, the tip recording technique of Kaissling and Thorson (1980) was used. Glass electrodes were filled with sensillar saline for the recording electrode and with hemolymph saline for the reference electrode. The reference electrode covered the tip of the antenna, the last segments of which had been cut. The tips of several sensilla were cut off using sharpened forceps, and the recording electrode was slipped over the cut end of one hair. The SSR responses were filtered (DC, 5000 Hz) and amplified (×1000). The recordings were stored on a PC-AT-compatible microcomputer via a DASH 16 (Metrabyte) analog-to-digital conversion board. Acquisition and analysis of the recordings were performed by specific programs developed in our laboratory and written in Asyst language (Macmillan Software Company).

The olfactory stimulations were delivered from Pasteur pipets containing a piece of filter paper on which 500 ng of diluted test compound had been deposited. The antenna was continuously flushed with humidified air (1.0 liter/min) and stimulations were achieved by blowing a puff of air (1 sec, 0.5 liter/min)

through the Pasteur pipet. Nineteen stimulus compounds were tested in a random order.

Male Sexual Activity and Behavioral Response to Pheromone. Male sexual activity was first tested with a simple bioassay. Four males, one to three days after the emergence, were kept together in boxes and maintained separated from females to prevent any contact with pheromone. A piece of filter paper impregnated with conspecific female gland extract at a dose of 0.5 female equivalent (FE) was introduced for 5 min into a box containing four males. A group of four males was tested every hour during the scotophase, and each group was tested only once. When sexually excited, the males behaved in a characteristic way by rapidly beating their wings (wing fanning), displaying genitalia, and displaying a pair of hairpencils situated on eversible epithelial pouches on the genital valvae. Wing fanning was chosen as a behavioral indicator of male sexual activity.

Male attraction to pheromone blends was studied in a 200 × 90 × 70-cm Perspex wind tunnel. The air speed was approximately 0.2 m/sec and the temperature 23°C. Red fluorescent tubes were placed above the tunnel to provide illumination of 0.3 lux. Tests were performed on 2- to 4-day-old males, between 3½ and 6 hr after the onset of the scotophase for SD, and between 7½ and 10 hr after the onset of the scotophase for SL. Males were placed on a wired cage at 1.5 m from the source made of a piece of filter paper impregnated with natural or synthetic pheromone. A dead male was fixed as a decoy on the source of pheromone. We chose seven phases of the behavioral response to describe male attraction to pheromone blend: (1) walks with or without wing fanning (W); (2) taking flight (Tf), males flew away from the wired cage; (3) orientation (O), males flew up the odor plume; (4) touching the source in flight without landing (T); (5) landing at the source (L); (6) precopulatory behavior (Pb), hairpencil or genital displays; (7) copulatory attempts *sensu stricto* (Ca).

Previous field-trapping experiments in French Guiana (Lalanne-Cassou et al., 1994), suggested to us to test two binary mixtures of *Z*9–14 : Ac and *Z*9,*E*12–14 : Ac at different doses: (1) SL 50 and SL 500: the ratio of *Z*9,*E*12–14 : Ac to *Z*9–14 : Ac was 5 : 95 at 50 and 500 ng, respectively, of *Z*9–14 : Ac; and (2) SD 50 and SD 500: the ratio of *Z*9,*E*12–14 : Ac to *Z*9–14 : Ac was 50 : 50 at 50 and 500 ng, respectively, of *Z*9–14 : Ac. Furthermore, female SD extracts were tested at a dose of 0.5 FE and female SL extracts at doses of 0.15, 0.5, and 1.5 FE. Each male was tested only once.

The percentage of male response was calculated for each of the seven behavioral steps as the number of males exhibiting a given behavior divided by the number of tested males. Differences in the behavioral responses to the extracts and the synthetic blends were analyzed by using χ^2 tests for each behavioral step (2 × 4 for SD and 2 × 7 for SL) with the threshold of significance set at 0.05. When heterogeneity was detected, all treatments were compared using 2

× 2 contingency tables. To minimize the risk of error arising from the increase of degrees of freedom, the threshold of significance was set to 0.01.

Chemicals. Compounds used as standards in the chemical analysis of the pheromone, as stimulus compounds in electrophysiological studies, or in wind tunnel experiments were all synthesized in the laboratory according to the procedures described by Mori (1981), Ramiandrasoa and Tellier (1990), and Renou et al. (1979, 1981). Their chemical purity was at least 98% as determined by gas chromatographic analysis.

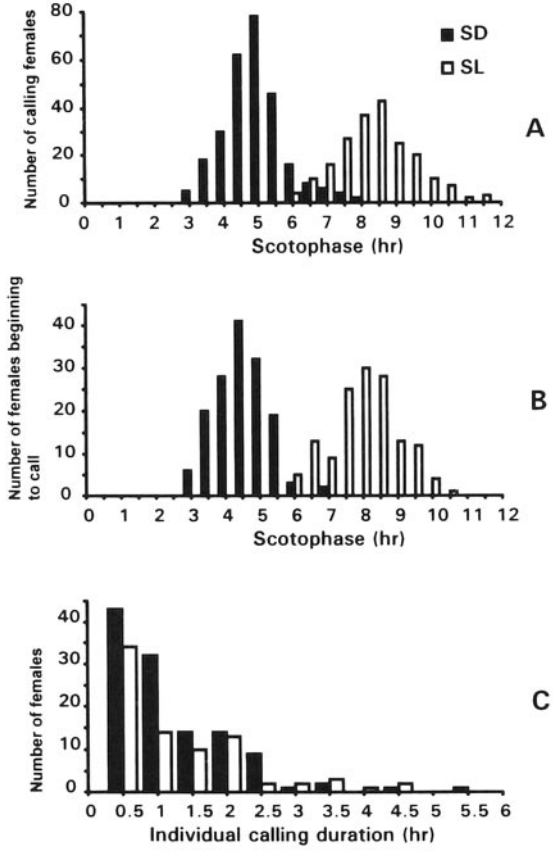

FIG. 1. Nocturnal calling periods of female SD and SL (A), beginning of calling behavior (B), and individual calling duration (C).

RESULTS

Female Calling Behavior. Calling behavior studies indicated marked temporal differences between species (Mann-Whitney test, $P < 0.001$) despite a slight overlap (Figure 1A). For SD, the maximum calling activity occurred in the first half of the scotophase, 5 hr after onset. Calling began at 4.5 $\pm$ 0.8 hr (mean $\pm$ SD) with a variation from 3 to 7 hr (Figure 1B). In contrast, for SL, the peak calling activity occurred in the second half of the scotophase (8.5 hr) (Figure 1A). Calling began at 8.2 $\pm$ 1 hr with a variation from 6 to 10.5 hr (Figure 1B). The beginning of calling was significantly different between SL and SD (Mann-Whitney test, $P < 0.001$). Individual calling durations (Figure 1C), 1.2 $\pm$ 0.9 hr and 1.3 $\pm$ 0.9 hr, respectively, for SD and SL, were not significantly different between species (Mann-Whitney test) at the 5% level of significance.

Mating Behavior. SL and SD matings occurred at significantly different times of the night (Mann-Whitney test, $P < 0.001$) despite an overlap of 2.5 hr (Figure 2A). Copulation durations (Figure 2B), 1.1 $\pm$ 0.7 hr and 1 $\pm$ 0.5 hr, respectively, for SL and SD, were not significantly different between species

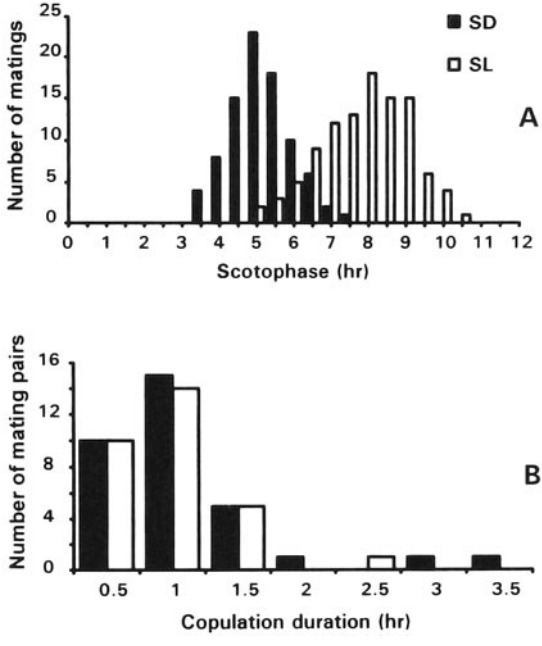

FIG. 2. Mating periods in SD and SL (A) and mating duration (B)

TABLE 1. MATING EFFICIENCY IN SL AND SD CROSSES[a]

Cross ♀ × ♂	Pairs (*N*)	Fertile matings (%)	Stuck pairs (%)
SD × SL	60	81.7b	0
SD × SD	60	83.3b	0
SL × SD	60	65.0b	0
SD × SL	60	10.0a	26.7

[a]Numbers followed by the same letter are not significantly different by χ^2 test at the 5% level of significance. Stuck pairs: male and female could not separate after copulation.

(Mann-Whitney test, $P > 0.05$). Fertile interspecific matings (giving offspring) could be obtained in both reciprocal crosses between SL and SD (Table 1). The SL × SD mating rate (65%) was not significantly different from those of the parental crosses (81.7% and 83.3% for SL × SL and SD × SD, respectively), (χ^2 test, $P > 0.05$). However, SD × SL mated less effectively than the reciprocal (SL × SD) and the parental crosses. Only 10% of pairs gave offspring and 26.7% of females could not separate from males after copulation.

Pheromone Composition. Analysis of an extract of 23 female SD glands showed the presence of six acetates and one aldehyde (Table 2). *Z*9–14:Ac [mass spectrum, *m/z* 41, 43 (100%), 53, 55, 61, 67, 81, 82, 96, 109, 194 (M-$CH_3COOH)^+$] and *Z*9,*E*12–14:Ac [mass spectrum, *m/z* 41, 43 (100%), 55, 61, 67, 68, 79, 81, 93, 95, 107, 121, 135, 149, 192 (M-$CH_3COOH)^+$] were found to be the main pheromone compounds in the extract with 41% of *Z*9–14:Ac and 42% of *Z*9,*E*12–14:Ac. Minor compounds could be identified as *Z*11–

TABLE 2. PERCENTAGES OF COMPOUNDS IN GLAND EXTRACTS FROM FEMALE SD AND SL

Compounds	SD (%) (23 females)	SL (%) (14 females)
*Z*9–14:Ald	2	8
*Z*9–14:Ac	41	75
*Z*11–14:Ac	<1	<1
*Z*9,*E*12–14:Ac	42	7
*E*9,*E*12–14:Ac	3	1
*Z*9,*E*11–14:Ac	1	1
*Z*11–16:Ac	11	8

16:Ac (11%) [mass spectrum, *m/z* 41, 43, 55 (100%), 61, 67, 81, 82, 96, 110, 123, 137, 151, 222 (M-$CH_3COOH)^+$], *E*9,*E*12-14:Ac (3%) [mass spectrum, *m/z* 41, 43 (100%), 55, 61, 67, 68, 79, 81, 93, 95, 107, 121, 135, 149, 192 (M-$CH_3COOH)^+$], *Z*9-14:Ald (2%) [mass spectrum, *m/z* 41, 43, 55 (100%), 67, 69, 81, 95, 98, 107, 121, 192 (M-$CH_3COOH)^+$], *Z*9,*E*11-14:Ac (1%) [mass spectrum, *m/z* 41, 43, 55, 61, 67 (100%), 79, 82, 93, 95, 107, 121, 135, 149, 192 (M-$CH_3COOH)^+$] and *Z*11-14:Ac (<1%) [mass spectrum, *m/z* 41, 43 (100%), 55, 61, 68, 82, 96, 110, 127, 194 (M-$CH_3COOH)^+$]. The retention times of these compounds were identical to those of synthetic references both with the WCOT column (*Z*9-14:Ac 4.3 min, *Z*9,*E*12-14:Ac 5 min, *Z*11-16:Ac 6.6 min, *E*9,*E*12-14:Ac 5.3 min, *Z*9-14:Ald 3 min, *Z*9,*E*11-14:Ac 5.9 min, and *Z*11-14:Ac 4.5 min) and with the more polar CPSil 88-CB column (*Z*9-14:Ac 11 min, *Z*9,*E*12-14:Ac 12.3 min, *Z*11-16:Ac 13.8 min, *E*9,*E*12-14:Ac 12.8 min, *Z*9-14:Ald 9.8 min, *Z*9,*E*11-14:Ac 13.7 min, and *Z*11-14:Ac 11.2 min). These same pheromone compounds were identified in the female SL extract (14 glands). *Z*9-14:Ac was the major pheromone compound (75%). *Z*11-16:Ac (8%), *Z*9-14:Ald (8%), *Z*9,*E*12-14:Ac (7%), *E*9,*E*12-14:Ac (1%), *Z*9,*E*11-14:Ac (1%), and *Z*11-14:Ac (<1%) were found as minor compounds. The pheromone compositions of SL and SD differed by the proportion of *Z*9-14:Ald in the extracts (2% for SD and 8% for SL), but the main difference was the proportion of *Z*9,*E*12-14:Ac (42% for SD and 7% for SL).

To confirm the differences found between the global extracts of female SL and SD, we analyzed individual gland extracts. The proportions and quantities of *Z*9-14:Ald were low for the two species. Therefore, we chose to calculate the percentage of *Z*9,*E*12-14:Ac relative to the sum of *Z*9-14:Ac and *Z*9,*E*12-14:Ac {[*Z*9,*E*12-14:Ac/(*Z*9-14:Ac + *Z*9,*E*12-14:Ac)] × 100}. This percentage averaged 4 ± 1% (mean ± SD) and ranged from 2 to 7.4% for SL, while it averaged 44.8 ± 6% and ranged from 29.1 to 59.2% for SD (Figure 3). Individual variation was high for the two species with coefficients of variation [CV = (SD/mean) × 100] reaching 26% for SL and 13.3% for SD. Despite this intraspecific variation, SL and SD distributions were significantly different (Mann-Whitney test, $P < 0.01$) and did not overlap. Percentages were stable among generations, allowing data pooling. *Z*9-14:Ac amounts averaged 61.1 ± 53 ng for SL and 56.5 ± 49.5 ng for SD. They were highly variable in the individual gland extracts (CV = 86.9% for SL and 87.6% for SD), but not significantly different between species (Mann-Whitney test, $P > 0.05$) (Figure 4).

Electrophysiology. *Z*9,*E*12-14:Ac was the most active compound for both SL and SD males (corrected EAG responses SD: 2.0; SL: 2.5) (Figure 5). *Z*9-14:Ac showed significant lower levels of responsiveness for the two species (one-way ANOVA, Newman-Keuls test, $P < 0.05$), whereas this compound

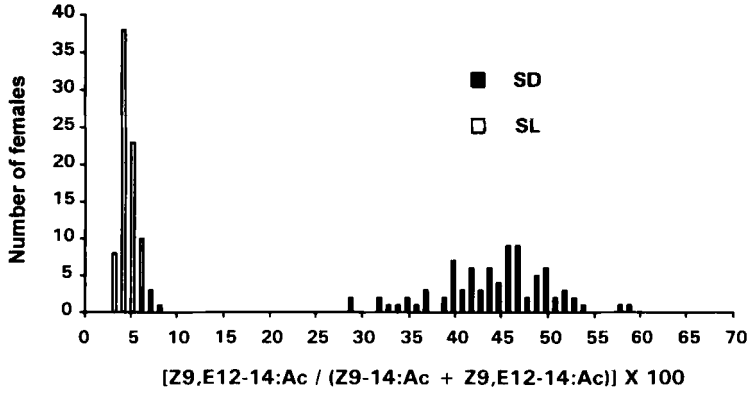

FIG. 3. Percentages of *Z*9,*E*12–14 : Ac relative to the sum of *Z*9–14 : Ac and *Z*9,*E*12–14 : Ac measured by gas chromatography in 84 individual gland extracts of female SD and 83 individual gland extracts of female SL.

was in equivalent proportion to *Z*9,*E*12–14 : Ac in the pheromone of female SD and was the major compound in the pheromone of female SL. *Z*9–12 : Ac also gave strong responses, although it is absent from the pheromone of the two species. Other compounds, except *Z*9,*E*12–14 : OH for male SD, had no significant EAG activity when compared to the control. Among them Z11–16 : Ac, present for 11% in the pheromone of female SD and for 8% in the pheromone

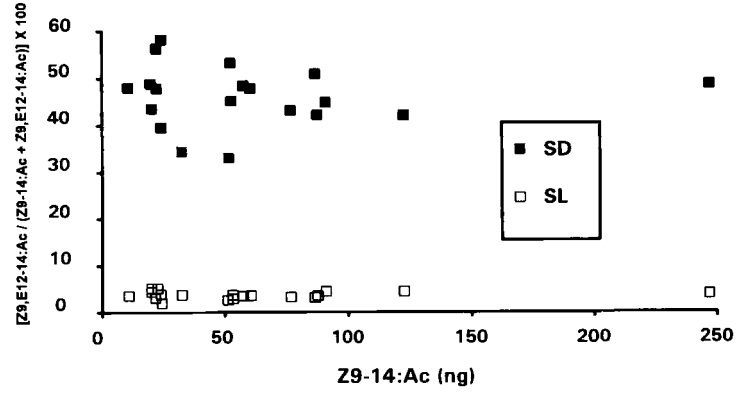

FIG. 4. Relation between the percentage of *Z*9,*E*12–14 : Ac relative to the sum of *Z*9–14 : Ac and *Z*9,*E*12–14 : Ac, and the quantity of *Z*9–14 : Ac from 23 individual female SD extracts and 19 individual female SL extracts.

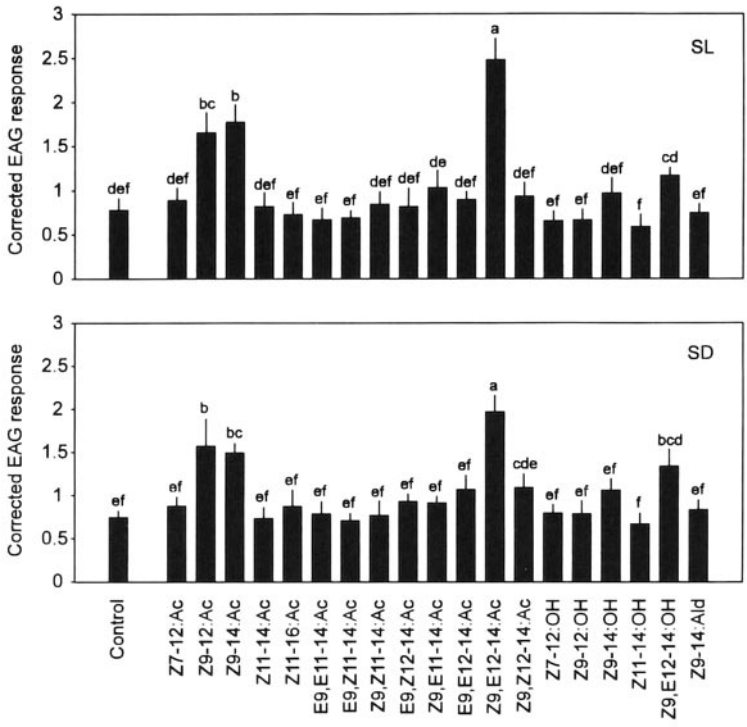

FIG. 5. EAG recordings of male SL and SD with a series of 19 pheromone compounds or analogs and pure air as control. Each compound was tested at 500 ng. Mean of the responses of 10 males. Error bars represent 95% confidence intervals. Bars associated with the same letter are not significantly different at the 5% level of significance (one-way ANOVA, Newman-Keuls test).

of female SL, showed no significant responses compared to the control (corrected EAG responses SD = 0.9; SL = 0.7).

Antennae are filiform in SD and SL. Observation of their morphology under light microscopy demonstrated the presence of at least two classes of sensilla trichodea on the unscaled ventral side of the antenna. These hairs were separated according to their length and location. Each flagellar segment exhibited the same sensillar organization. The longer hairs were located on the lateral parts of the ventral sensory area. They are referred to as the lateral hairs (LHs). Shorter hairs were located medioventrally on the flagellar segments and are referred to as medial hairs (MHs).

The activity of 30 LHs in SL and 43 in SD was recorded (Figure 6). In each LH, the firing activity clearly consisted of two amplitude classes of action potentials, indicating the presence of at least two neurons. The cell firing high-amplitude action potentials, up to 2.8 mV, is referred to as cell A. Action potentials emitted by the other cell, referred to as the B cell, were much smaller (50% of the amplitude of the A cell action potentials). In SD, the amplitude of B-cell spikes was smaller than in SL and was often hardly discriminated from the noise. In both SD and SL, A cells responded specifically to Z9,*E*12–14 : Ac. B cells were less specific since they had two key-compounds, Z9–14 : OH and Z9,*E*12–14 : OH. Moreover in SL, B cells also slightly responded to a third alcohol, Z9–12 : OH, and to a minor pheromone compound, Z9–14 : Ald.

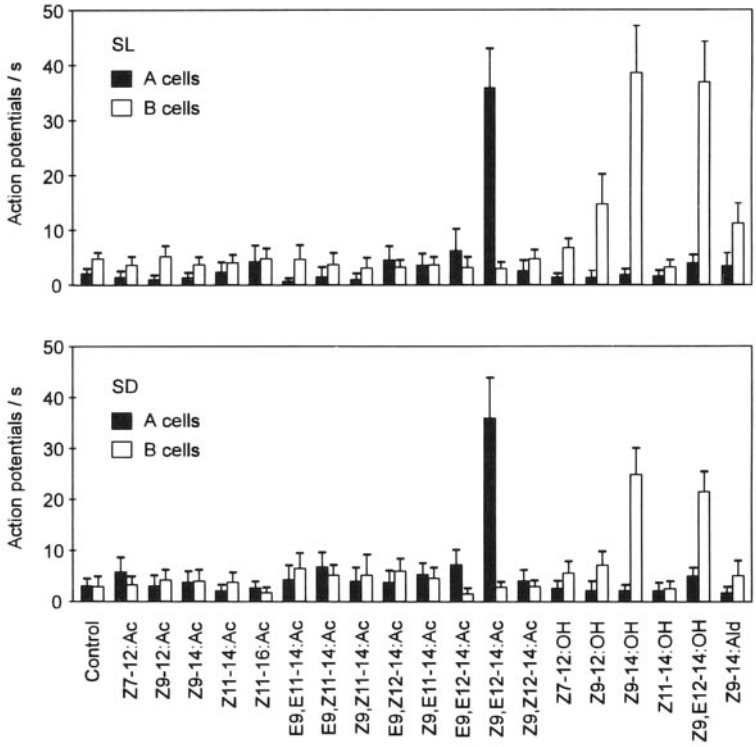

FIG. 6. Response profiles obtained by single sensillum recordings of A and B cells in long lateral hairs of SL (N = 30 hairs) and SD (N = 43 hairs) to 19 pheromone compounds or analogs. Each compound was tested at 500 ng, on 11–30 different hairs for SL and on 11–40 hairs for SD. Vertical bars represent 95% confidence intervals.

MHs appeared to be in smaller number and to be slightly shorter in SD than in SL. Electrical contacts were very unstable in SD. For these reasons, the activity of only three MHs was recorded in SD and Z9–14 : Ald, Z11–16 : Ac, and *E*9,*E*12–14 : Ac were not tested on these hairs because these compounds were not available at the time of the experiments. Analysis of the activity of MHs in both SL and SD always revealed the presence of only one class of action potential amplitudes, with a mean amplitude of 1.4 mV (Figure 7). Equal responses were recorded to the two pheromone compounds Z9,*E*12–14 : Ac and Z9–14 : Ac. None of the other compounds increased the firing activity.

No other sensillar type was discovered. In particular, unlike *S. littoralis*

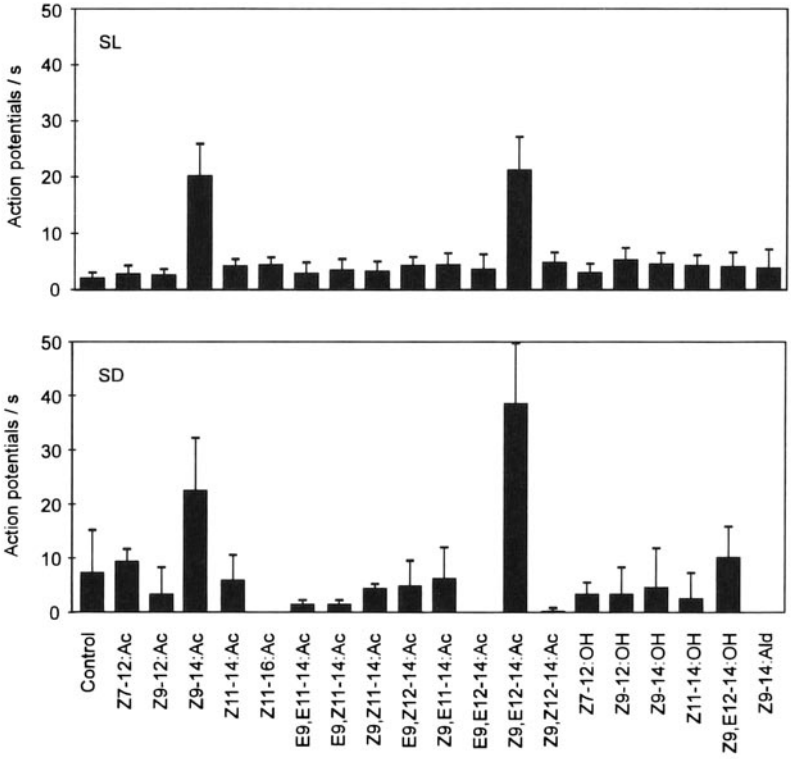

FIG. 7. Response profiles obtained by single sensillum recordings of short medial hairs of SL (N = 23 hairs) and SD (N = 3 hairs). Nineteen pheromone compounds or analogs were tested for SL and 16 compounds for SD. Each compound was tested at 500 ng, on 10–22 hairs for SL and on 2–3 hairs for SD. Z9–14 : Ald, Z11–16 : Ac and *E*9,*E*12–14 : Ac were not tested in SD. Vertical bars represent 95% confidence intervals.

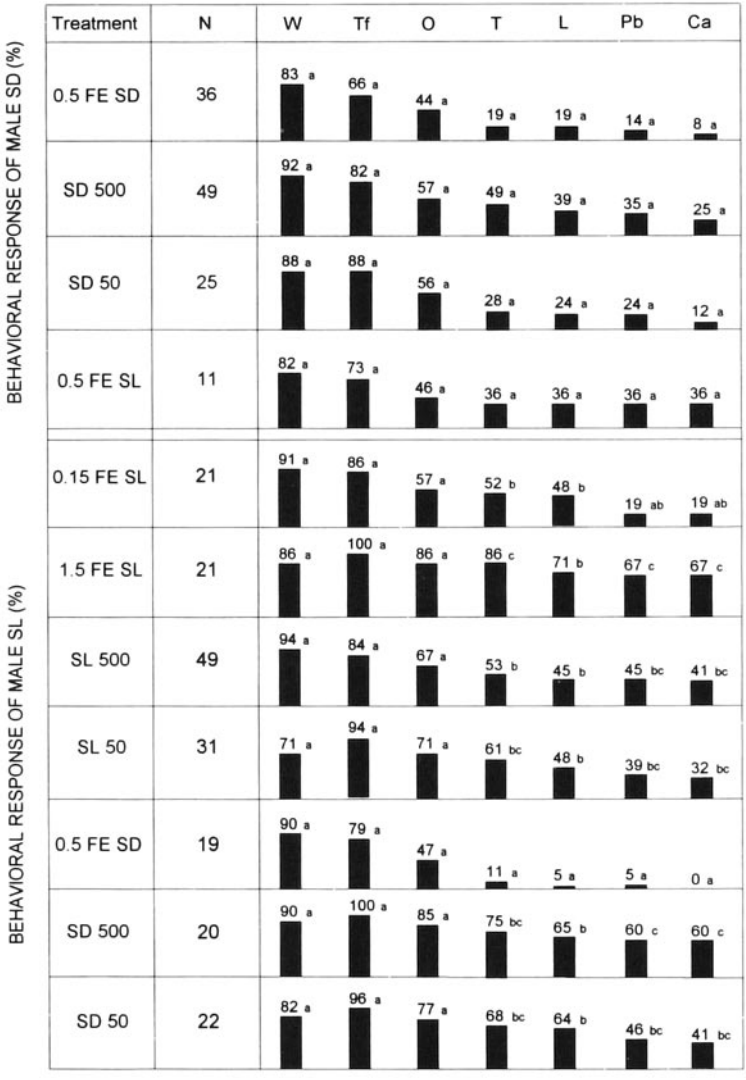

FIG. 8. Behavioral response of male SD and SL to female extracts and synthetic blends in the wind tunnel. For each species, the percentages in the same column followed by the same letter are not significantly different (χ^2 2 × 4 test, $P > 0.05$; χ^2 2 × 2 test, $P > 0.01$ for SD and χ^2 2 × 7 test, $P > 0.05$; χ^2 2 × 2 test, $P > 0.01$ for SL).

(Ljungberg et al., 1993), the most lateral LHs had the same response pattern as other LHs.

Male Sexual Activity and Behavioral Response to Pheromone. Male SL and SD showed distinct peaks of "wing-fanning" response to the pheromone of their conspecific females. The peak occurred at 4–5 hr in the scotophase for SD and at 9 hr for SL. Periods of male sexual activity were significantly different between species (Mann-Whitney test, $P < 0.001$) although they widely overlapped. SL and SD were simultaneously sexually active from 4 to 9 hr. The levels of responsiveness were high for the two species ranging from 20 to 89% for SD and from 34 to 88% for SL. However, wind-tunnel experiments showed low frequencies of behavioral response of male SD (Figure 8). Especially with the 0.5 FE SD treatment, only 8% achieved the complete behavioral sequence until "copulatory attempts." Blends with synthetic components SD 500 and SD 50 were as effective in eliciting behavior as the female extract. No significant difference was observed between these blends. SD males were also attracted by the female SL extract (0.5 FE SL) and completed the behavioral sequence until "copulatory attempts." In that case, frequencies of behavioral categories were not significantly different from all other treatments. Wind-tunnel experiments on male SL revealed that the female extract 1.5 FE SL was more effective than the 0.15 FE SL extract in the last steps of the behavioral sequence ("touching," "precopulatory behavior," and "copulatory attempts"). Blends with synthetic compounds SL 500 and SL 50 showed no significant difference and induced similar levels of behavioral response as female extracts (except SL 500, which gave less "touching" than 1.5 FE SL extract). SL males also showed behavioral response to heterospecific extracts and blends. No significant difference was observed between SD 500 and SD 50 that gave similar levels of behavioral response as conspecific blends and extracts. However, the female extract 0.5 FE SD elicited lower "touching," "precopulatory behavior," and "copulatory attempts" than the other treatments.

DISCUSSION

Female calling behavior studies in SL and SD showed temporal partitioning of nocturnal calling activities. Female SD called in the first half of the scotophase, whereas female SL called in the second half. Mating activities showed the same pattern. Peaks of male sexual activity were distinct between species and concomitant with their respective female ones, but sexual activity periods were longer than in the population of females, showing more substantial overlaps between SL and SD. Some SD males were found to be responsive at the time of the peak calling activity of female SL. Some SL males showed sexual activity at the peak calling activity of female SD. These results suggest that differences

mainly observed in the nocturnal activities of female SL and SD are not sufficient to allow a complete premating isolation between species. A similar case of temporal partitioning of mating activities was observed in *S. frugiperda* between the corn and rice strains. While corn strain females mate in the first two thirds of the scotophase, rice strain females mate in the last one third. This difference in mating rhythms seems to be a strong barrier against interbreeding between the two strains (Pashley et al., 1992).

The same compounds were identified in the female SL and SD extracts. The main difference in the pheromone composition between species was the proportion of *Z*9,*E*12–14:Ac in the extracts: 7% for SL and 42% for SD. Teixeira et al. (1989) identified *Z*9–14:Ac and *Z*9,*E*12–14:Ac as pheromone compounds of female SL. The analysis of the proportion of *Z*9,*E*12–14:Ac relative to *Z*9–14:Ac from individual gland extracts showed that in spite of intraspecific variation, the two species were completely discriminated. While the proportions ranged from 2 to 7.4% for SL, they ranged from 29.1 to 59.1% for SD. Quantities of *Z*9–14:Ac were highly variable between individuals of a given species but not significantly different between species. Differences in pheromone composition between closely related species that depend more on relative proportions of compounds than on different chemical structures have frequently been observed in moths. In the *Yponomeuta* complex, *Y. evonymellus* and *Y. cagnagellus* share the same pheromone compounds, and pheromones mainly differ by the amount of *E*11–14:Ac relative to the *Z* isomer (*Z*11–14:Ac) (Löfstedt and Van Der Pers, 1985). Similarly, in the sibling taxa *Diachrysia chrysitis* and *D. tutti*, pheromone compositions differ by the proportions of *Z*5–10:Ac and *Z*7–10:Ac almost in opposite ratios (Löfstedt et al., 1994).

Electrophysiological studies showed no major differences in the morphology and physiology of the pheromone receptors on the antennae of male SL and SD. Two size classes of sensilla trichodea were identified for both species. While longer hairs were located on the lateral parts of the ventral side (LHs), shorter hairs were distributed medioventrally (MHs). This sensillar organization, found in other noctuid moths such as *Agrotis exclamationis* (Hansson et al., 1986), *Mamestra suasa* (Lucas and Renou, 1991), or *M. brassicae* (Renou and Lucas, 1994), was correlated with sensitivity to different compounds. Receptors to *Z*9–14:Ac and *Z*9,*E*12–14:Ac were identified for SL and SD. *Z*9,*E*12–14:Ac induced the strongest EAG response even for SL, where it is a minor pheromone compound. For the two species, receptors to *Z*9,*E*12–14:Ac were found in the two sensillar types, while receptors to *Z*9–14:Ac were only identified on MHs. No receptor was found for the minor compounds of the pheromone except in SL for *Z*9–14:Ald slightly detected by B cells on LHs. Although not produced by female SL or SD, *Z*9–12:OH, *Z*9–14:OH, and *Z*9,*E*12–14:OH activated olfactory neurons of B cells on LHs. The role of these compounds in the behavior

of males is unknown. Z9–12 : Ac gave strong EAG responses for SL and SD, although it did not increase the firing activity of any of the two sensillar types.

The flight-tunnel bioassay showed that for the two species, binary mixtures with Z9–14 : Ac and Z9,E12–14 : Ac were as effective in eliciting reproductive behavior as female extracts. Hence, these chemicals were critical to the expression of male behavior. Some mutual cross-attraction was observed between SL and SD with synthetic compounds and female extracts. Thus, male behavioral response was initiated whatever the proportion of Z9,E12–14 : Ac relative to Z9–14 : Ac. However, for male SL, the female SD extract elicited significantly fewer behavioral responses than any other treatment, particularly than SD 50 and SD 500. In the SD extract, another (other) component(s), seems to be responsible for these low responses. Further tests are required to confirm this hypothesis. The high levels of mutual cross-attraction with synthetic blends were not in accordance with previous field-trapping experiments. Traps with binary mixtures of Z9–14 : Ac and Z9,E12–14 : Ac, in different proportions and in which positions were rerandomized every night, were tested on pasturelands surrounded by the forest in French Guiana (Matoury) during January 1982 and July–August 1983 (Lalanne-Cassou et al., 1994). These traps caught specifically male SL or SD. Field experiment results suggest differences in male attractivity between species. The flight-tunnel bioassay did not confirm these differences that may occur in the timing of behavioral sequences or in the way of approaching or landing on the trap. Similar cases of cross-attraction in the wind tunnel were found for several species of *Orgyia* (Grant, 1977), for the *Euxoa declarata* group (Byers et al., 1981), and for several members of the stored-product complex of phycitine moths (Phelan and Baker, 1986). The above authors supposed that these interspecific attractions were favored by the similarity of the pheromones used by the closely related species. Grant et al. (1975) observed interspecific courtship between the Indian meal moth, *Plodia interpunctella*, and the almond moth, *Cadra cautella*, but several isolating mechanisms before and during courtship prevented cross-mating. In the small ermine moths (*Yponomeuta*), wind-tunnel experiments also showed a high level of cross-attraction, particularly between *Y. evonymellus* and *Y. vigintipunctatus*, two species with very similar pheromones (Hendrikse, 1986). However, field-trapping experiments with synthetic attractants were species-specific in the natural habitat of the respective species (Löfstedt et al., 1991). The case of *Y. evonymellus* and *Y. vigintipunctatus* is, however, different from that of SL and SD because these species are reproductively isolated by their asynchronous occurrence and differences in habitats (Löfstedt and Van Der Pers, 1985).

In the laboratory, if confined in boxes, SL and SD hybridized and produced fertile offspring in both reciprocal crosses. However, mating frequency was significantly lower in the cross SD × SL than in the reciprocal one. Several

pairs SD × SL could not separate after copulation. Differences in the morphology of genital structures between SL and SD (Lalanne-Cassou et al., 1994) suggest that failure to separate could be linked to genital incompatibilities between males and females. However, further studies are required to understand the importance of morphological features in male–female mating success.

No individual with intermediary morphology between French Guiana populations of SL and SD has been found in the field so far. During the two trapping campaigns in French Guiana in 1982 and 1983, 476 SL and 254 SD of 730 males caught by sexual attractants could be identified in regard to the morphology of the genitalia (Lalanne-Cassou et al., 1994). On the several dozens of individuals caught by light traps, no hybrid form between SL and SD has been found either (Lalanne-Cassou, personal communication).

In the laboratory, with SL individuals originating from Barbados, we found a combination of several mechanisms involved in the reproductive isolation of the species: (1) differences in nocturnal activity cycles may be a partial premating barrier; (2) pheromone specificity has not been proved by flight-tunnel experiments, but it seems to be an effective isolating factor in the field (Lalanne-Cassou et al., 1994); (3) genital differences might constitute a mechanical barrier against interbreeding; and (4) other isolating factors such as differences in host plants might be involved.

Reproductive isolation is generally thought to evolve as an incidental by-product of divergence between allopatric populations. If, upon secondary contact, unfit hybrids are produced, increased isolation will occur. Butlin (1985, 1987) called this process "reinforcement." He distinguished it from the "reproductive character displacement" process of divergence in the mate recognition systems of species already reproductively isolated. In the case of SL and SD, no hybrid morph has been found in French Guiana so far, suggesting that reinforcement is less likely to occur. However, a detailed study of French Guiana sympatric populations, including both enzymatic and pheromonal approaches, is necessary to determine the potential for gene flow between populations and to draw conclusions on the role of sex pheromones in the reproductive isolation. The comparison between allopatric and sympatric populations will be critical to understanding the evolutionary history of the species.

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INTRODUCTION AND OVERVIEW

J.P. BRYANT

Institute for Arctic Biology
University of Alaska
Fairbanks, Alaska 99775-0180

This issue of *Journal of Chemical Ecology* contains three papers from a symposium on Plant Allelochemicals in Mammalian Foraging: Current Status and Future Prospects, presented at the Sixth International Theriological Congress (ITC) held in Sydney, Australia, July 4–10, 1993. The contributors to the symposium were asked to seek general patterns or mechanisms by which plant allelochemicals could influence either diet selection or the nutritional quality of different diets in mammals. Not all the papers presented were prepared for final publication. The topics discussed in this symposium were the physiological consequences of ingestion of allelochemicals and the adaptations of mammals that minimize these consequences. An ITC symposium especially directed toward these topics seemed timely, because there is good evidence allelochemicals deter herbivory by mammals. By comparison, little is known about the physiological consequences of ingesting allelochemicals or adaptations mammals have evolved to counter plant chemical defenses. The three papers presented here go a long way toward correcting this deficiency, and furthermore, I would suggest that the ideas presented in them will become paradigms in the study of chemically mediated interactions between plants and mammals that eat plants.

McArthur, Sanson, and Beal review information on the structure and function of salivary proline-rich proteins (PRPs). After reviewing information on the structure of PRPs, and discussing the benefits of binding tannin with PRPs, they provide a scenario of how increased production of PRPs evolved. Their hypothesis is that the original function of PRPs was maintenance of homeostasis, but as tannin-rich plant tissue increased in the diet, increased production of PRPs became selectively advantageous, because it minimized the negative effects of eating tannin. If this hypothesis is correct, mammals such as browsers that feed regularly on tannin-containing plant tissue should produce more salivary PRP than mammals that eat less tannin-rich plant tissue.

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The paper by Foley, McLean, and Cork focuses on the physiological consequences of absorbing plant allelochemicals. Their thesis is that detoxification of these substances by mammals results in production of organic acids, which must be buffered and excreted from the body. Since this is likely to occur in all mammals and with all allelochemicals, the hypothesis of Foley et al. provides a unifying theme for future research on chemically mediated interactions between plants and mammals.

Building on the ideas of Foley et al., Illius and Jessop have mathematically modeled the metabolic costs of allelochemical absorption by mammals. This model predicts an interaction between allelochemical dose and nutrient status, so the ratio of allelochemical to nutrient absorption rate defines the tolerance of the animal to the absorbable allelochemical concentration in foods. Since this interaction is nonlinear, the negative effect of allelochemical chemical absorption is expected to be especially high for starving animals. This prediction has important implications for interactions between plants and mammalian herbivores when the herbivore population is at or near its nutritional carrying capacity.

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SALIVARY PROLINE-RICH PROTEINS IN MAMMALS: ROLES IN ORAL HOMEOSTASIS AND COUNTERACTING DIETARY TANNIN

CLARE McARTHUR,[1,*] GORDON D. SANSON,[1] and A. MICHEL BEAL[2]

[1]*Department of Ecology and Evolutionary Biology*
Monash University, Clayton 3168 Australia

[2]*School of Biological Sciences*
University of New South Wales
Kensington 2033 Australia

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Abstract—We review information on the structure of proline-rich proteins (PRPs), their various functions related to oral homeostasis and dietary tannin, and the structural basis of these functions. Consideration of the multifunctional nature of these salivary proteins helps explain both the subtle and large variations found in structure and secretion rates both within individuals and between species. We propose that the ancestral function of PRPs is in maintaining oral homeostasis and that counteracting dietary tannins by binding with them is a derived function. PRPs are effective in oral homeostasis at low secretion levels, whereas counteracting tannin depends on high secretion levels. In the dietary habits ranging from carnivores through omnivores to exclusively plant-eaters, the dietary nitrogen level is progressively reduced, and plant allelochemical intake, including tannins, increases. We suggest that during this evolution from meat-eater to plant-eater, there was some point in omnivory at which selective pressure from nitrogen limitations, arising from a low nitrogen/high tannin diet, became sufficiently great for the evolution of increased secretion level and diversification of PRPs for dealing with tannin. If this hypothesis is correct, carnivorous mammals should secrete low levels of PRPs for oral homeostasis, but should never secrete high levels, unless they are secondarily carnivorous. Omnivores consuming a diet of very little animal tissue but higher levels of tannin-containing foliage or fruit should generally have the capacity to produce high levels of salivary PRPs. Browsers and frugivores should also produce high levels of PRPs, but grazers may have reduced secretion rates depending on the antiquity of the dietary habit. This hypothesis is consistent with the limited information available on the abun-

*To whom correspondence should be addressed.

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dance, type, and distribution of PRPs in mammals. Studies are suggested which would test the functional and evolutionary arguments presented.

Key Words—Diet, calcium, carnivore, herbivore, omnivore, oral homeostasis, proline-rich proteins, saliva, secretion, tannin.

INTRODUCTION

Many mammals secrete a group of proteins from parotid and mandibular salivary glands that are characterized by unusually high proline levels. Two main functions have been attributed to these proline-rich proteins (PRPs): maintaining oral homeostasis, and reducing digestibility and toxic costs of consuming dietary tannin. Functions in maintaining oral homeostasis include, most importantly, associations with calcium and its salts (Bennick, 1982), but also roles in lubrication and bacterial aggregation (Bennick, 1982; Hatton et al., 1985; Bergey et al., 1986). Tannin-related function involves preferentially binding tannins, possibly in large amounts per unit protein, thereby increasing the amount of dietary protein available to the consumer (Hagerman and Butler, 1981; Mehansho et al., 1983). Generally the two functions have been considered separately, the first in the human dental literature and the second in the ecological physiology literature. It is informative and necessary, however, to consider both functions together if we are to understand structural, functional, and evolutionary relationships of these proteins in mammals.

There is a wealth of detailed information on some aspects of PRPs. Overall amino acid composition has been described for PRPs from about 15 species (Kauffman and Keller, 1979; Muenzer et al., 1979; Mehansho et al., 1985, 1987; Oppenheim et al., 1985; Austin et al., 1989; Spielman and Bennick, 1989; Mole et al., 1990a; Hagerman and Robbins, 1993). Genetic coding, amino acid sequencing, and posttranslational modifications have been determined for some PRPs, mainly from humans and rats (Wong and Bennick, 1980; Ziemer et al., 1984; Clements et al., 1985; Oppenheim et al., 1985; Kauffman et al., 1991). Some relationships between structure and function have been established (Oh et al., 1980; Wong and Bennick, 1980; Bennick et al., 1981; Hagerman and Butler, 1981; Asquith et al., 1987; Hay et al., 1987). Our aim is to synthesize information on structural/functional relationships of PRPs and to attempt to place this in an evolutionary context.

CHEMICAL AND STRUCTURAL CHARACTERISTICS OF SALIVARY PROLINE-RICH PROTEINS

PRPs vary in chemical composition and associated characteristics, size, and possibly shape. These characteristics and their subtle variations ultimately determine how, and how effectively, they function.

Chemical Composition

Chemical and structural characteristics that vary include amino acid composition, residue sequence, regional and overall charge, and extent of phosphorylation and glycosylation. These properties are best described in relation to the amide (*N*) terminal and the carboxyl (*C*) terminal regions of the molecule.

PRPs are broadly classified as acidic or basic. This reflects overall charge but does not indicate variation in degree of polarity. There are some important functional differences between basic and acidic PRPs, as will be seen below, although some structurally intermediate PRPs have mixed characteristics (Kauffman et al., 1991).

Basic PRPs (BPRPs) are comprised mainly of residues that are uncharged or basic at neutral pH. They are relatively uniform, since just four amino acids make up 70–90% of the total. These are proline (Pro), glutamine (Gln), glutamic acid (Glu), and glycine (Gly) (Kauffman and Keller, 1979; Muenzer et al., 1979; Mehansho and Carlson, 1983; Mehansho et al., 1987). Overall proline levels are generally high (30–45%), but not always. Rabbit BPRPs, for example, contain only 17–21% Pro (Spielman and Bennick, 1989).

Acidic PRPs (APRPs) have two distinct structural domains. The *C*-terminal region is structurally equivalent to the whole BPRP and makes up about 70–80% of the molecule. In contrast to BPRPs, however, APRPs also have a distinct *N*-terminal extension with many highly acidic residues but very little proline (Wong and Bennick, 1980; Ziemer et al., 1984; Oppenheim et al., 1985). The overall amino acid composition of APRPs differs from BPRPs because of this second region, both in the number of acidic versus basic residues and also in the generally lower proline content (15–30%).

At neutral pH, many residues in both BPRPs and APRPs are uncharged. However, the isoelectric pH (pI) of BPRPs is generally high (pI $\geq$ 9), since most of the polar amino acids are basic. In APRPs the pI is low (4–5) (Muenzer et al., 1979), because acidic residues in the *N*-terminal extension dominate. In rats at least, BPRPs are moderately hydrophobic, more so than APRPs (Muenzer et al., 1979).

The "skeletal" PRPs may have phosphate and/or sugar groups added in posttranslational modifications. Phosphorylation, usually of serine, occurs in the acidic *N*-terminal domain and hence is generally a modification of APRPs (Hay et al., 1987, 1988). Up to five phosphoserines have been recorded in APRPs (Oppenheim et al., 1985). Glycosylation of threonine, asparagine, and/or serine may occur throughout BPRPs and in the neutral/basic *C*-terminal region of APRPs. The resulting glycoprotein may be up to 40% carbohydrate (Mehansho and Carlson, 1983; Bergey et al., 1986). To date, glycosylation appears more common in BPRPs, but some APRPs are also glycosylated (Spielman et al., 1991). The major acidic PRP in macaque parotid saliva, for example, is about 30% carbohydrate (Oppenheim et al., 1985), although this extent appears exceptional for an APRP.

Size

Accurate estimates of the molecular weight of PRPs, determined from amino acid sequencing, range from a low of 5000 (Robinson et al., 1989; Kauffman et al., 1991) to at least 25,000 (Muenzer et al., 1979). Apparent molecular weights of up to 220,000 for glycosylated PRPs, based on comparison with standards run on SDS-polyacrylamide systems (Mehansho et al., 1983), are likely to be overestimates because of abnormally slow migration rates of PRPs (Beeley et al., 1991).

Shape

A fundamental and significant feature of PRPs is their shape. In complete contrast to most proteins, whose biological activity depends on specific conformation and folding, PRPs have very little tertiary structure. In solution they are highly elongated rods or random coils (Henkin et al., 1978; Muenzer et al., 1979; Hagerman and Butler, 1981; Hagerman and Klucher, 1986). The lack of folding is largely, but not exclusively, due to proline, whose structure both prevents rotation of the N–C bond of the peptide linkage and prohibits intrachain hydrogen bonding (Lehninger, 1970, p. 113). In polar solutions the open structure of PRPs exposes hydrophobic residues, in contrast to most proteins, in which folding hides these residues. No differences have been noted in the overall shape of acidic and basic PRPs, probably because of the dominating influence of proline. However, subtle or localized differences may exist, particularly given the distinct low-proline acidic *N*-terminal extension of APRPs.

Phosphorylation, a feature of APRPs, is unlikely to have much influence on overall shape for two reasons. First, the extent of phosphorylation is generally small (one to five residues), and second, it is always confined to the acidic *N*-terminal region. Since this acidic region only makes up 20–30% of the molecule, any influence on shape should be confined to local conformation.

Glycosylation may enhance the open conformation of proteins (Hagerman, 1989), although to what extent this can occur in PRPs, which are already long and unfolded, is not clear. Hydroxyproline-rich glycoproteins are less compact than their deglycosylated forms (Asquith et al., 1987), so it is possible that PRPs are also more extended when glycosylated. Consistent with this is some evidence that affinity for tannin, which relies on an open structure (described below), is enhanced by glycosylation (Asquith et al., 1987).

FUNCTIONS OF SALIVARY PROLINE-RICH PROTEINS

Oral Homeostasis

The role of proline-rich proteins in oral homeostasis has been studied mainly in humans, but information from this work should be relevant to other animals.

PRPs contribute to three aspects: calcium homeostasis, lubrication, and bacterial attachment or aggregation.

Teeth are exposed to variable conditions of the semiexternal environment of the mouth, including the potentially wearing chemical and physical properties of food. For reptiles with teeth that are continually being replaced, tooth degradation may not be particularly costly. For mammals, with teeth that may have to last essentially a lifetime, the problem becomes significant. To emphasize the difference, a single tooth position may be replaced 50 times in the lifetime of a crocodile (Miles and Poole, 1967), but only once in a mammal. Tooth enamel became thicker in the evolution from reptiles to mammals (Poole, 1967), providing, among other factors, a greater buffer against degradation. Dental enamel of both reptiles and mammals is composed largely of hydroxyapatite, a basic calcium phosphate salt. While hydroxyapatite is relatively insoluble in aqueous solutions, it will nevertheless gradually be worn away by food and dissolve in saliva, unless counteractive mechanisms prevent this (Hay and Moreno, 1989).

In humans, and the only other toothed mammals investigated, saliva is supersaturated with basic calcium phosphate salts, including hydroxyapatite (Schlesinger and Hay, 1977; Schluckebier et al., 1989). This supersaturation acts to stabilize, protect, and repair tooth surface enamel, by maintaining an equilibrium of calcium salts in and out of solution. Equilibrium is not achieved automatically, however, but relies on inhibitory activity to prevent excess precipitation of hydroxyapatite on tooth surfaces (Hay et al., 1987). Acidic PRPs, in particular, appear to play a major role in preventing this precipitation. They achieve this through three closely related functions: calcium binding in solution, hydroxyapatite binding at the tooth surface, and inhibition of hydroxyapatite formation (Bennick, 1982). These processes have been examined in detail mainly with human salivary PRPs (Schlesinger and Hay, 1977; Hay et al., 1979, 1987; Moreno et al., 1979; Aoba et al., 1984), but structurally similar salivary PRPs from other species behave in a similar manner (Oppenheim et al., 1985; Schluckebier et al., 1989; Spielman et al., 1991).

Lubrication of food and tooth surfaces aids food processing, at the same time as reducing tooth wear by reducing friction. Mucin glycoproteins are the major lubricating molecules in saliva, but glycosylated BPRPs (gBPRPs) also have lubricating properties (Hatton et al., 1985).

Glycosylated PRPs can adsorb to bacteria in the mouth, with both positive and negative effects. Agglutination may inhibit attachment of bacteria and remove them through swallowing. Alternatively, some APRPs are bound as part of the tooth pellicle to dental enamel, and adhesion of bacteria to these APRPs may lead to enamel erosion through production of acidic products from bacterial metabolism (Bennick, 1982). There appears to be some specificity between different bacteria and PRPs (Bergey et al., 1986; Gibbons and Hay, 1988), but the significance of this is unclear. Whether the tooth pellicle, including APRPs, can play an important role in this aspect of oral homeostasis in herbivores, as

distinct from omnivores, with the much higher intakes of abrasive food in the former, is unknown.

Counteracting Dietary Tannin

Tannins are phenolic compounds found in foliage and fruits of many trees and shrubs (Goldstein and Swain, 1963; Haslam, 1979; Wrangham and Waterman, 1983). Their defining functional characteristic is a capacity to bind with proteins (Zucker, 1983). From an ecological perspective, their significance lies in their perceived role as plant defense compounds (Feeny, 1976; Swain, 1977; Mole and Waterman, 1987b). Tannin–protein complexes may be less digestible than free proteins. Animals that consume tannin-containing food, such as omnivores, frugivores, and browsers are confronted with a diet with potentially reduced levels of digestible nitrogen.

Salivary PRPs have a very high affinity for tannins, similar to polyproline polymers, an affinity at least three orders of magnitude greater than some other proteins (Oh et al., 1980; Hagerman and Butler, 1981). Hagerman and Butler (1981) suggested that salivary PRPs may protect dietary proteins and digestive enzymes from tannin, since tannins bind preferentially to PRPs even in the presence of 100-fold molar excess of other proteins of lower affinity.

Austin et al. (1989) hypothesize that on chewing tannin-containing food, salivary PRPs bind to tannin released from vacuoles (in the case of leaves), and the indigestible tannin–PRP complex, which is often soluble, passes through the digestive tract and is excreted in feces. Presumably less protein is required to bind all the tannin, providing a quantitative nitrogen saving, and the nonessential amino acids of PRPs are excreted instead of essential amino acids from dietary protein, providing a qualitative nitrogen saving (Robbins et al., 1991). There is accumulating evidence consistent with this hypothesis (Mole et al., 1990b; Robbins et al., 1991; Hagerman and Robbins, 1993).

Toxicity of tannins can be reduced in animals producing sufficient PRPs to prevent degradation and absorption of tannin itself. This is particularly likely in consumption of hydrolyzable tannins, which are often readily degraded to toxic components, but may also be relevant to consumption of some condensed tannins (Clausen et al., 1990; McArthur and Sanson, 1991).

STRUCTURAL BASIS FOR FUNCTIONS OF SALIVARY PROLINE-RICH PROTEINS

Structural Basis of Functions for Maintaining Oral Homeostasis

Activity and binding sites associated with processes maintaining equilibrium of calcium and its salts in and out of solution is confined to the acidic *N*-terminal region of PRPs (Wong and Bennick, 1980; Bennick et al., 1981;

Bennick, 1982; Hay et al., 1987). Activity therefore is almost exclusively a function of APRPs. The acidic residues, aspartic acid, glutamic acid, and serine, are particularly important; aspartic acid probably has a greater effect on activity than glutamic acid (Hay et al., 1979), but phosphorylated serine provides the major activity (Bennick, 1982; Hay et al., 1987). Subtle differences in amino acid composition, such as the substitution of a single aspartic acid residue for asparagine, can have a large effect on affinity (Moreno et al., 1982; Hay et al., 1988).

Larger APRPs may have lower activity than smaller APRPs because the proline-rich *C*-terminal region is extended rather than the acidic *N*-terminal region. The number of active sites in the acidic region is, if anything, reduced in larger PRPs (Bennick et al., 1981), probably due to increased steric hindrance from the longer proline-rich region (Wong and Bennick, 1980). In humans, small acidic phosphopeptides are produced from degradation of larger APRPs in the mouth. Activity of these phosphopeptides, measured as inhibition of hydroxyapatite formation from seeded crystals, is 6–30 times that of APRPs and 20–420 times that of any BPRPs with any activity (calculated from Madapallimattam and Bennick, 1990; Kauffman et al., 1991), again emphasizing the significance of the acidic region to activity.

No studies have directly examined the effect of glycosylation of the *C*-terminal region on calcium function in PRPs, but if anything, it may enhance activity. The major APRP of the crab-eating macaque (*Macaca fascicularis*) prevents both primary and secondary precipitation of calcium salts from supersaturated solutions. This PRP is both highly glycosylated in the *C*-terminal region and highly phosphorylated in the *N*-terminal region (Oppenheim et al., 1985). Unglycosylated APRPs in humans only inhibit secondary precipitation; primary precipitation is inhibited by another protein, statherin (Hay et al., 1984; Schwartz et al., 1992). Whether glycosylation contributes to the high activity in the macaque APRP is unknown, but comparison of calcium-related activity of the native and deglycosylated form could determine this. Similarly, although most of the calcium-related activity of human PRPs is associated with the APRPs, glycosylated BPRPs show some activity, but unglycosylated BPRPs do not (Hay et al., 1982; Oho et al., 1992), indicating that glycosylation is important if the acidic *N*-terminal region is absent. The exception is two human BPRPs, which have some activity, but since these have intermediate structural characteristics including one phosphoserine (Kauffman et al., 1991), this activity is not surprising.

Several properties appear to influence lubricating activity of PRPs. The presence of carbohydrates, the type of carbohydrates, the extent of glycosylation, and therefore the presence and abundance of certain amino acids for linkage are important factors. Glycosylated PRPs have greater lubricating activity than deglycosylated forms (Hatton et al., 1985). Acidic sugars, such as sialic acid,

repel each other, producing an extended molecule that enhances viscosity (Dawes, 1976). Neutral sugars present in some glycosylated PRPs (Keller et al., 1975; Oho et al., 1992) may contribute less to this effect. Glycosylation of PRPs occurs by linkage with asparagine (Hatton et al., 1985), threonine, and serine (Oppenheim et al., 1985). These amino acids are therefore important. The rheological properties of mucins are related to their high level of glycosylation. Similarly, extent of glycosylation in PRPs should also be important, and this varies considerably. Glycosylated PRPs are 9–19% carbohydrate (w/w) in mice (Oppenheim et al., 1985), 6–36% in rats (Muenzer et al., 1979; Mehansho and Carlson, 1983), 5–16% in rabbits (Spielman and Bennick, 1989), and 30% in the macaque (Oppenheim et al., 1985). It is not known whether there are any differences in lubricating properties of these proteins.

Glycosylation of PRPs also influences interactions with bacteria. The type of carbohydrate confers specificity towards certain bacteria. This specificity appears to result from recognition by lectins on the bacterial cell surface of particular carbohydrates (Bergey et al., 1986). Some bacteria bind with nonglycosylated APRPs, but only when these proteins are attached as part of the tooth pellicle. Possibly, segments of APRPs that are normally hidden in solution become exposed after attachment to tooth enamel (Gibbons and Hay, 1988; Gibbons et al., 1988). The structural basis for this interaction with APRPs is not known, but a longer *C*-terminal region enhances bacterial attachment (Gibbons and Hay, 1988), indicating that this region is important.

Structural Basis for Interactions with Dietary Tannin

Less is known about the specifics of interactions of salivary PRPs and dietary tannin than about PRPs and functions associated with oral homeostasis. Anticipating potentially significant structure–function relationships relies on understanding how tannins interact with proteins in general. In polar solutions, tannins interact with proteins through both hydrogen bonding and hydrophobic interactions (Oh et al., 1980; Artz et al., 1987; Hagerman, 1989). It has been suggested that tannins associate with hydrophobic regions of proteins first, followed by hydrogen bonding between hydroxyls of the tannin and polar groups of proteins, including the carbonyl in peptide linkages (Spencer et al., 1988). However, some evidence indicates that if sufficient hydrophobic regions of protein are exposed for interaction with tannins, then hydrophobic interactions predominate. If these regions are hidden, however, hydrogen bonding is more important (Artz et al., 1987). Whatever the type(s) of interaction, conformational flexibility of both the protein and the tannin seems to maximize the interaction, presumably in the case of protein by exposing more binding sites (McManus et al., 1985). Therefore, long open-structured proteins have higher affinity for tannins than compact globular proteins (Hagerman and Butler, 1981;

Butler et al., 1984). Tannin–protein interactions are generally strongest at the isoelectric point of the protein, but proteins with a high isoelectric point seem to interact with tannin over a wide pH range (Hagerman and Butler, 1978).

Structurally, a high proline content is the major feature associated with the exceptional affinity of salivary PRPs for tannin (Butler et al., 1984), as a result of its effect on the shape of the protein. Proline may also strengthen tannin–protein interactions, because hydrogen bonding to a peptide linkage with proline is strengthened by the substituted nitrogen next to the carbonyl (Hagerman and Butler, 1981).

It has been suggested that tannin binding by PRPs is not strongly amino acid dependent provided proline levels are relatively high (Mehansho et al., 1987; Kauffman et al., 1991). This makes sense because of the multiple, unspecific sites available on PRPs for hydrogen bonding and hydrophobic interactions. Furthermore, in general, it does seem that specific amino acid composition and sequence differences of various salivary PRPs have less impact on affinity for tannin than on functions related to oral homeostasis. That is, all salivary PRPs examined have a high affinity for tannin compared with many other proteins. However, there is evidence that PRPs from different species do have different affinities for different tannins (Mole et al., 1990a; Hagerman and Robbins, 1993). Presumably there is a structural explanation for this, which may include differences in amino acid composition or other characteristics.

Amino acids other than proline may alter affinity for tannin by altering the extent of local hydrophobic interactions or hydrogen bonding or by changing local or general conformation. This effect may be more important in salivary PRPs with lower proline levels, with more localized regions less influenced by proline. The affinity of acidic and basic PRPs to tannins may differ because of the acidic amino acids in the *N*-terminal extension in APRPs. Tannins interact poorly, if at all, with acidic polyamino acids, at least at pH $\leq$ 6 (Oh et al., 1980), so tannins may bind weakly or not at all to the acidic domain of APRPs. Given that this region can be 30% of an acidic PRP, its effect may be significant and deserves attention. Examining the interaction of tannins with acidic *N*-terminal and proline-rich *C*-terminal fragments from APRPs, as done for calcium-related activity (Bennick et al., 1981), would be useful.

Salivary PRPs are always high in glutamic acid/glutamine and/or glycine, not just in proline. We suggest that, as with proline, these amino acids should also exert a significant influence on tannin-binding characteristics, because of their likely effect on protein shape. Amino acids can be classified according to their impact on conformation, as α-helix and β-sheet formers or breakers (or no impact) (Chou and Fasman, 1974). Both proline *and* glycine are strong breakers of α-helices. Similarly, glutamic acid is a strong breaker at pH $\geq$ 6, illustrated by the observation that polyglutamic acid exists as a random coil at pH $\geq$ 6 (Lehninger, 1970, p. 113). At salivary pH (6.5 or greater), therefore, the influ-

ence of both glycine and glutamic acid on conformation should be similar to proline. Theoretically, proteins could be functionally similar to PRPs even if they have low Pro levels, provided the level of Glx/Gly is high. Therefore, the total amount of Pro/Glx/Gly in PRPs is probably more important than just the level of proline alone. If so, a more descriptive chemical name such as "PGGRP" (Pro/Gly/Glx-rich proteins) may be more appropriate than PRP. Differences are found in the total amount of Pro/Glx/Gly in PRPs; for example, 47% in the PRP from deer but 90% in PRP from rats (Austin et al., 1989). If a higher Pro/Glx/Gly level produces a molecule that has an extended rather than folded backbone, then it may also increase affinity for tannin. Rat PRP may therefore have higher affinity for tannins than deer PRP.

Other factors, such as total number of residues and extent of glycosylation or phosphorylation, may also confer differences in affinity for tannins. There is little information on the effect of size. It is likely that increasing molecular weight increases affinity, just as it does in polyproline peptides (Hagerman and Butler, 1981); particularly as the appropriate domain for activity, that is, the basic/neutral *C*-terminal end of the molecule, seems to be extended in PRPs rather than the acidic *N*-terminal domain.

There is also little direct information on the effect of glycosylation and phosphorylation on tannin binding. If acidic amino acids in the *N*-terminal domain of APRPs reduce affinity even locally, then phosphorylation may exacerbate this effect. Glycosylation, on the other hand, may enhance affinity and may even confer some specificity for particular tannins (Asquith et al., 1987). This may in part be due to further extension or looseness in molecular shape, exposing more binding sites. It may also result from some interaction with the carbohydrate residues themselves, since tannins interact with polysaccharides as well as proteins (McManus et al., 1985).

Another potential difference between APRPs and BPRPs, when they occur together in saliva, relates to the observation of a wide pH range over which proteins with high isoelectric points precipitate tannin. If BPRPs form complexes with dietary tannin over a similarly wide pH range compared with APRPs (even though the complexes tend not to precipitate), BPRPs may be the major group of PRPs interacting with tannins throughout a mammalian gut (pH range about 2.5–8). Competitive binding curves comparing affinity for one tannin of a BPRP and two APRPs at a given pH showed slight differences between PRPs, but this was insignificant compared with the much higher affinity they all had compared with another protein (Mehansho et al., 1987). However, different conditions, such as pH, or different tannins may alter this affinity (Martin and Martin, 1983; Asquith and Butler, 1986; Mole and Waterman, 1987a) and indicate more subtle differences between the PRPs. The ratio of BPRPs to APRPs varies between species [for example, it is 50:50 in humans (Kauffman and Keller, 1979) but

90:10 in rats (Muenzer et al., 1979)], the consequences of which may therefore be affected by any differences between the two types of PRP.

Clearly, many questions are yet to be answered regarding the significance of structural differences in PRPs on affinity for tannins.

SIGNIFICANCE OF SECRETION LEVEL OF SALIVARY PROLINE-RICH PROTEINS FOR FUNCTION

There is an absence of direct comparative quantitative information on the concentration and rates of secretion of PRPs needed for the two main functions of oral homeostasis versus tannin binding. However, from less direct evidence, it generally appears that the amount of PRPs required to function effectively in oral homeostasis is much lower than for dealing effectively with dietary tannin. Theoretically, mammals consuming tannin-free diets should be in normal oral homeostasis. Secretion of PRPs should therefore be sufficient for any roles in maintaining this homeostasis. One study using rats does indicate that levels of PRPs are both important in and adequate for oral homeostatic function on normal tannin-free diets. If salivary PRP production is inhibited in rats fed a high sucrose diet, their teeth are more prone to demineralization and caries than those of rats with normal PRP production (Watson et al., 1990). The functional implication is clear, even though these salivary and dietary conditions are extreme; that is, PRPs protect teeth under normal secretion rates. Such normal secretory levels, however, are often insufficient for dealing with dietary tannin. Evidence includes the induction of PRP production level and change in parotid gland size by some animals on tannin-rich compared with tannin-free diets, and detrimental consequences in animals in which this does not occur.

Dramatic induction of PRPs occurs in rats and mice fed tannin-rich diets, and the extent of the detrimental effects of tannin on growth and protein digestibility is consequently reduced (Mehansho et al., 1983, 1985; Mole et al., 1990b). In contrast, hamsters produce low levels of PRPs on tannin-free diets, but dietary tannin does not induce production of high PRP levels, and detrimental effects are severe, with growth impairment or death (Mehansho et al., 1987). In rabbits, APRPs make up only about 4% of the total parotid proteins [calculated from Spielman et al. (1991)]; yet since they appear to be the major type of PRP inhibiting hydroxyapatite formation, they are apparently effective in calcium-related activity. Parotid glands in domestic rabbits (presumably on tannin-free diets) are smaller than those in feral rabbits (possibly on diets containing tannin) (Mole et al., 1990a). Similarly, both parotid gland size and the concentration of PRPs for tannin binding is much higher in mountain hares on a tannin-rich diet compared with a tannin-free diet (A.E. Hagerman, G.R. Iason, and C. McArthur, 1993 unpublished data).

Some animals, including humans, produce high levels of PRPs on both tannin-free and tannin-rich diets. It has been proposed (Austin et al., 1989; Robbins et al., 1991; Hagerman and Robbins, 1993) that there is an association between levels of production of PRPs and feeding niche, with mammals that regularly consume high tannin diets producing large quantities of salivary PRPs. Such a pattern would be consistent with the argument that dealing with dietary tannin requires higher levels of PRPs than do functions in normal oral homeostasis.

We have measured salivary protein secretion levels in a number of marsupial species. Table 1 lists the nominal maximum protein secretion rates from parotid glands obtained for several marsupial species. Protein secretion rates vary considerably. Wombats (grazers) secrete very low protein levels and much of this is amylase (Beal, 1991). In contrast, swamp wallabies (browsers) secrete much higher protein levels, and a major proportion is protein with very high affinity for a number of tannins (C. McArthur, A.M. Beal, and G.D. Sanson, 1993 unpublished data). Protein secretion of the grazing parma wallaby is also low compared with swamp wallaby. This comparison would be meaningless unless the samples contain PRPs. Figure 1 illustrates that a large number of the proteins in these parotid secretions from the species that have been tested are indeed rich in Pro/Glx/Gly, although at this stage it is not possible to quantify the exact proportion. However, we conclude from the electrophoretic results (Figure 1) combined with the total protein secretion data (Table 1) that there are different levels of salivary PRP secretion between species and that this reflects potential differences in the capacity to deal with tannin. We are currently examining the salivary protein characteristics of these animals further to determine, among other factors, how much this secretion level may alter on a tannin-rich diet.

IMPLICATIONS FROM STRUCTURE-FUNCTION AND SECRETION-FUNCTION RELATIONSHIPS

Table 2 summarizes types of salivary PRPs described previously, their relative importance in oral homeostasis and counteracting tannin, and the secretion levels required to be effective. Differences in activity have been demonstrated between the various PRPs in relation to oral homeostasis. While all PRPs may have a high affinity for tannins, it is not possible at this stage to generalize about any differences in activity between them.

It is clear that salivary proline-rich proteins must be considered as multifunctional in order to understand the variety of structures and secretion rates. Subtle differences in amino acid composition at the *N*-terminal region of APRPs, for example, are difficult to explain from a tannin-binding function but are

TABLE 1. MAXIMAL FLOW RATES, ASSOCIATED PROTEIN CONCENTRATIONS, AND NOMINAL MAXIMUM PROTEIN SECRETION AS A FUNCTION OF METABOLIC BODY WEIGHT OBTAINED FROM ONE PAROTID GLAND, FOR A RANGE OF MARSUPIALS SPECIES[a]

Species	Predominant dietary habit	Animals (N)	Metabolic body weight ($kg^{0.75}$)	Maximal flow rate (ml/min)	Protein conc. (mg/liter)	Nominal maximum protein secretion ($\mu g/min/kg^{0.75}$)
Foregut fermenter						
Red kangaroo	grazer	10	12.87	3.73	286	78
(*M. rufus*)			(0.581)	(0.228)	(70.4)	(17.6)
Euro	grazer	8	13.40	3.63	223	62
(*M. robustus*)			(1.405)	(0.299)	(34.6)	(9.0)
Grey kangaroo	grazer	10	12.71	4.30	160	46
(*M. giganteus*)			(0.875)	(0.516)	(34.0)	(6.7)
Parma wallaby	grazer	2	3.39	0.61	350	62
(*M. parma*)			(0.050)	(0.020)	(160.0)	(25.8)
Red-necked	intermediate	2	4.16	1.67	590	225
pademelon	browser/grazer		(0.180)	(0.045)	(420.0)	(152.0)
(*T. thetis*)						
Swamp wallaby	browser	2	5.77	2.94	740	366
(*W. bicolor*)			(1.470)	(0.425)	(380.0)	(155.2)
Hindgut fermenter						
Wombat	grazer	12	12.18	2.01	461	78**
(*V. ursinus*)			(0.378)	(0.149)	(89.1)	(17.6)
Rufous rat-kangaroo	mixed (browse/	14	1.99	0.44	5610	1284**
(*A. rufescens*)	roots/herbs/grass)		(0.049)	(0.017)	(568.7)	(160.6)
Brushtail possum	generalist (browse/	14	1.78	0.087	2054	98
(*T. vulpecula*)	fruit/herbs)		(0.036)	(0.0040)	(293.1)	(12.9)
Koala	specialist browser	6	4.77	1.14	3347	800
# (*P. cinereus*)			(0.041)	(0.104)	(298.4)	(92.6)

[a]Results are mean (±SE). Values were obtained during maximal flow stimulated by parasympathetic stimulation in anaesthetized animals (see text for details). Values should be doubled for total parotid output. Species have been grouped on the basis of gut structure (foregut or hindgut fermenter). Predominant dietary habit is indicated, but it should be noted that dietary habits are a continuum. ** indicates that much of the protein in these samples is amylase. Data obtained from Beal (1989a, 1990, 1991, 1992), Scott and Beal (1994), and some data collected here described in Materials and Methods.

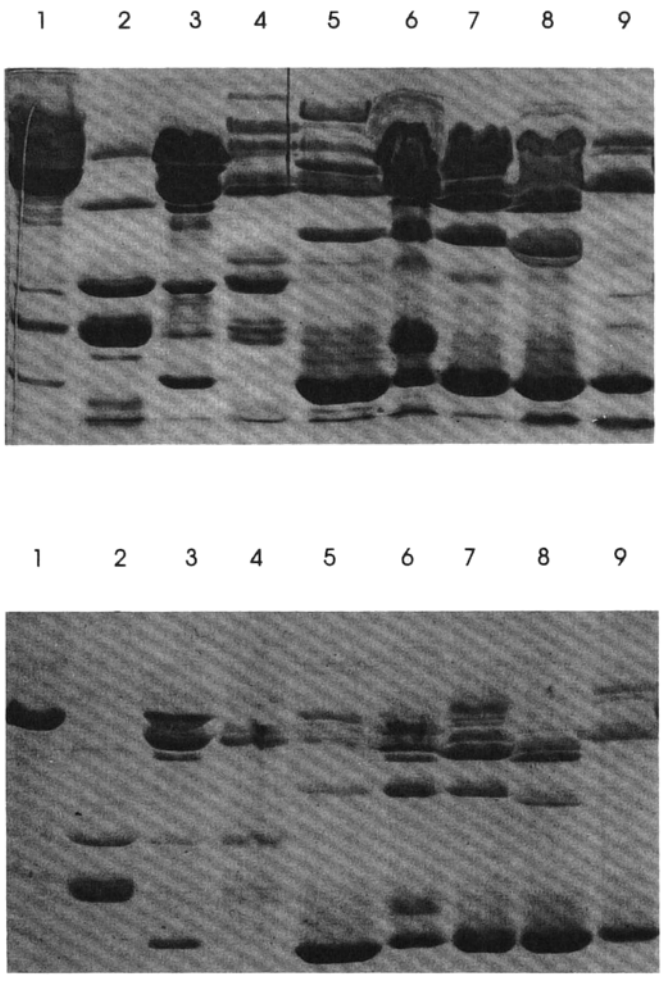

FIG. 1. PAGE analysis of salivary protein samples from eight marsupial species. Upper and lower samples and gels are identical, apart from the destaining solutions used. The upper gel is destained to detect all proteins, including those rich in Pro/Gly/Glx, whereas the destain used in the lower gel detects Pro/Gly/Glx-rich protein poorly if at all (see text for details). All samples are parotid saliva except for lane 4, which is mandibular saliva. Lane 1: wombat (*V. ursinus*), lane 2: common brushtail possum (*T. vulpecula*); lane 3: rufous rat-kangaroo (*A. rufescens*); lanes 4 and 5: mandibular and parotid samples respectively from red kangaroo (*M. rufus*); lane 6: grey kangaroo (*M. giganteus*); lane 7: parma wallaby (*M. parma*); lane 8: red-necked pademelon (*T. thetis*); and lane 9: swamp wallaby (*W. bicolor*). Note the presence of many protein bands in the upper gel which either are completely absent or stain relatively poorly in the lower gel. These bands are proteins which are Pro/Gly/Glx-rich.

TABLE 2. FOUR TYPES OF SALIVARY PROLINE-RICH PROTEINS, THEIR FUNCTIONS, AND SECRETION LEVELS REQUIRED FOR ORAL HOMEOSTASIS, AND COUNTERACTING DIETARY TANNIN[a]

	Oral homeostasis				Counteracting dietary tannin	
Type of PRP	Calcium equilibrium	Lubrication	Bacterial binding	Secretion level required	Tannin binding	Secretion level required
APRP	+++	−	+	low	+?	high
gAPRP	++	+	++	low	+?	high
gBPRP	+	+	++	low	+?	high
BPRP	−	−	−	n.a.	+?	high

[a]Unglycosylated acidic PRP = APRP; glycosylated APRP = gAPRP; glycosylated basic PRP = gBPRP; and unglycosylated basic PRP = BPRP. Under oral homeostasis, the role and relative importance of the various proteins are indicated as no perceived role (−), or a role with increasing importance (+ < ++ < +++). Under counteracting dietary tannin, tannin binding in all cases is shown as: +?, which reflects the lack of information on any differences in activity between the different types of PRPs. n.a. = not applicable.

clearly significant when considered in the context of calcium equilibrium in the mouth.

Nonglycosylated BPRPs *per se*, and differences in amino acid composition of these BPRPs, cannot be explained from a calcium-related or lubricating function, but may be related to tannin affinity. The variation in amino acid composition may reflect a weak functional constraint in residue requirements for affinity to tannin, provided proline levels are high (Kauffman et al., 1991), or they may confer differences in tannin affinity. If the latter proves correct, the apparent lack of functional differences assumed today may simply reflect a lack of studies on detailed structure–function relationships.

Similarly, secretion of high levels of PRPs in some animals, for example, deer and rats, is difficult to explain in relation to oral homeostasis alone. It is explicable, however, if the significance of PRPs in dealing with dietary tannin is considered (Hagerman and Butler, 1981; Mehansho et al., 1983; Robbins et al., 1987; Austin et al., 1989). For humans, we suggest that the high levels of PRPs reflect selective pressure from exposure to a low-nitrogen, high-tannin diet during our evolutionary history.

CONSTRAINTS IN GENERATING GENERAL CONCEPTS FROM AVAILABLE DATA

The patterns of structure, secretion, and function described above have been developed from a fairly restricted group of species, and their generality

needs confirmation from other studies. Hagerman is examining the distribution of PRPs in relation to tannin binding and feeding niche in eutherians, and we are carrying out a similar study with marsupials. Studies are also required on the detailed structure–function relationships of different PRPs in relation to tannin affinity, using better defined proteins (and tannins) than have been examined to date. Information is needed on exactly what influence characteristics such as the presence of an acidic *N*-terminal region, phosphorylation, or glycosylation have on tannin affinity, in order to determine whether these structural differences are significant in this context.

Information is also required on the significance of PRPs in the various functions associated with maintaining oral homeostasis in a broader range of carnivores, omnivores, and herbivores. Is saliva always supersaturated with calcium salts, with an unstable equilibrium potentially leading to excess precipitation of these salts, and therefore requiring inhibitory molecules? If so, are PRPs or other proteins generally present? How important are glycosylated PRPs compared with mucins in lubrication? If their role is always minor, is this function merely incidental?

Much of the information relating to oral homeostasis has been gathered on human saliva. We suggest that there are sufficient similarities in results when other species have been examined to generate hypotheses from the human data. Acidic PRPs, for example, have been found in animals as evolutionarily diverse as other primates, rodents, lagomorphs, and marsupial macropods (Ziemer et al., 1984; Oppenheim et al., 1985; Schluckebier et al., 1989; Spielman et al., 1991; Ferreira et al., 1992), and calcium-related activities have mainly been established in these groups. Similarly, the importance of tooth protection from acids has been demonstrated in rats as well as humans (Watson et al., 1990).

The importance of various functions of salivary proteins in different animals will depend partly on diet. The importance of calcium- and bacteria-related functions in oral homeostasis in humans, for example, is most relevant to other omnivores and frugivores, which consume broadly similar amounts of acidic and tannin-containing fruits or foliage. The functions may be less important to grazing herbivores, which consume much larger quantities overall, but of acid-free and tannin-free herbage. However, these functions should still be necessary, because grazers still suffer related problems of tooth wear but for different reasons; that is, much higher chewing levels combined with abrasiveness of silica bodies in grasses. Presumably they have similar salivary systems of high mineral content also requiring mechanisms to maintain an equilibrium. Certainly calcium and phosphate levels in saliva of grazing ruminants are not insignificant (Manas-Almendros et al., 1982).

Finally, there is little information on the oral microflora of mammalian carnivores, omnivores, or herbivores. Consequently its significance is unknown.

There is no reason to believe, however, that animals other than humans should be less prone to the colonization of potentially detrimental bacteria, and therefore they are just as likely to require some means of influencing both the establishment of benign bacteria and the removal of detrimental ones.

The limitations in the data now available simply mean some caution must be taken when generalizing. Provided implications of the differences as well as the similarities in, for example dietary habits, are considered, some interesting and testable hypotheses can be generated regarding the evolution of the structural and functional diversity of salivary proline-rich proteins.

EVOLUTIONARY PERSPECTIVE OF MULTIFUNCTION SALIVARY PROLINE-RICH PROTEINS

Two evolutionary hypotheses could explain the abundance, variety, and distribution of salivary PRPs in mammals. The first hypothesis is that PRPs evolved originally as proteins involved in maintaining oral homeostasis, and their role in dealing with dietary tannin is a derived feature, depending on higher secretion levels than that used in oral homeostasis, and improved by changes in, or diversification of, structure. The second hypothesis is essentially the opposite, that dealing with dietary tannin is the fundamental function of PRPs, and roles in oral homeostasis are derived. We suggest that the evidence available favors the first hypothesis.

Evolutionary Hypothesis of Functional Expansion of Salivary Proline-Rich Protein in Relation to Dietary Niche

A generalized scheme of the grades of dietary niche in mammals is shown in Figure 2. In both eutherians and marsupials, there has been an evolutionary progression from carnivory (generally insectivory) through omnivory to herbivory (including fruit-eating). Carnivores consume a nitrogen-rich diet. Omnivores eat a mixture of animal tissue and plant material. This plant material is generally much lower in nitrogen than animal tissue and contains structural and chemical defenses. Omnivores eating mainly animal tissue consume a nitrogen-rich diet, but as less animal tissue and more plant material is eaten, intake of digestible nitrogen decreases and intake of plant allelochemicals increases. Digestible nitrogen is reduced both because of lower nitrogen levels in plants and also from tannins that can further reduce digestibility. Herbivores consume a nitrogen-poor diet, with various levels of allelochemicals.

We suggest that along the omnivore gradient to mainly plant-eater, two dietary costs—nitrogen limitation and toxicity—increased, requiring counteractive mechanisms. When nitrogen is potentially unavailable due to tannin, secre-

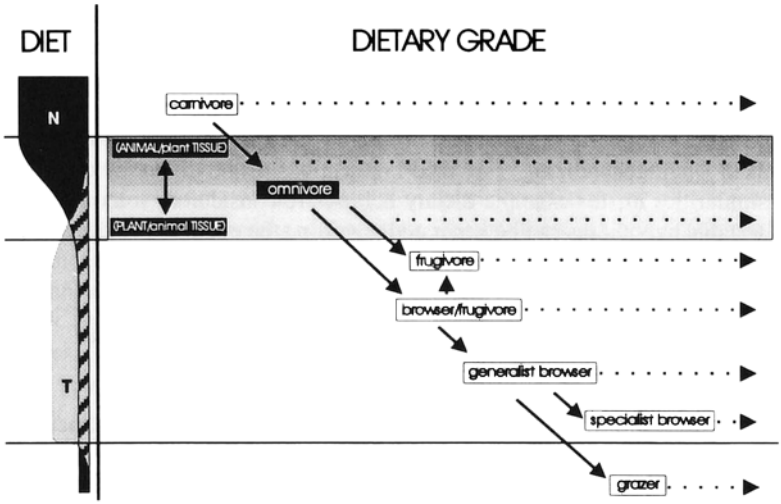

FIG. 2. A generalized scheme of the evolution of dietary grades in mammals and their associated dietary characteristics. Carnivory (insectivory) is the primitive dietary grade. Omnivores follow a gradient from eating mainly animal tissue to eating mainly plant tissue. This is associated with decreasing dietary nitrogen (N) and increasing dietary allelochemicals including tannin (T). Browsers/frugivores also consume low nitrogen, high tannin diets, whereas grazers consume low-nitrogen diets with little or no tannin. (See text for details).

tion of high levels of high-affinity tannin-binding salivary proteins (generally PRPs) reduces the loss of available nitrogen and also reduces the toxicity of tannins by preventing any degradation. Another mechanism, which will not be discussed further, is an increased capacity for degrading and eliminating absorbed toxic components. These mechanisms should be reinforced in herbivores consuming mainly browse foliage and fruits. We suggest, therefore, that one major evolutionary change required in the dietary shift to predominantly plant material was a significant increase in the secretion levels of the already-present PRPs. This enabled a functional expansion from simply maintaining oral homeostasis to counteracting dietary tannin as well.

A second evolutionary change may have been a diversification in structure of PRPs, with associated differences in affinity for tannin. Figure 3 illustrates a proposed sequence in the evolution of different types of PRPs. All PRPs, *except* unglycosylated BPRPs, function in oral homeostasis (Table 2), therefore APRPs and possibly gBPRPs probably evolved in early carnivores (if not in reptilian

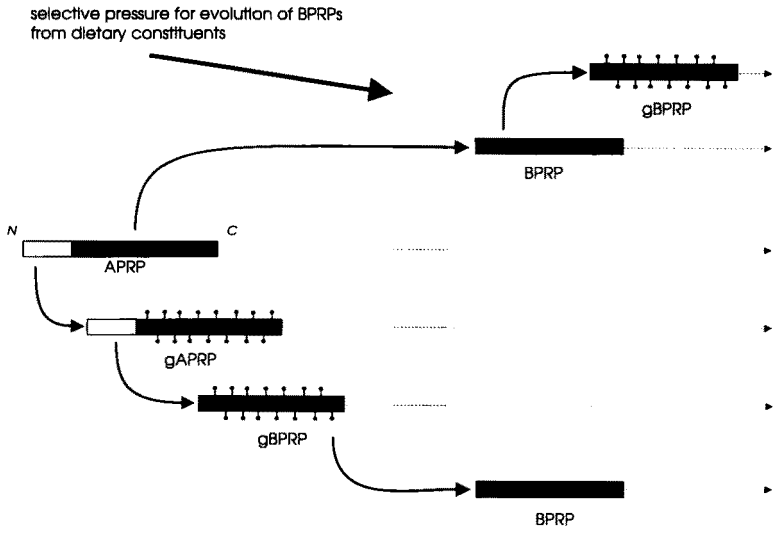

FIG. 3. Proposed pathways in the evolution of different types of proline-rich proteins (PRPs). In the lower pathway, the APRP first becomes glycosylated in the proline-rich region (bobbles attached to the *C*-terminal darkly shaded section) (gAPRP). Next the acidic domain is lost, producing a glycosylated basic PRP (gBPRP). Finally, deglycosylation leads to a simple basic PRP (BPRP). The alternative (upper) pathway has just two steps. First, the APRP loses the acidic domain to produce a BPRP. Second, the BPRP is glycosylated (gBPRP). Either or both pathways are possible. The evolution of BPRPs required selective pressure associated with costs of dietary constituents (mainly low nitrogen and high tannin levels) (See text for details).

ancestors). We suggest that BPRPs evolved when mammals began to consume a diet that was both low in nitrogen and had the additional cost of tannins. This scenario should be reflected in the variety, secretion level, and distribution of PRPs in mammalian species from different dietary niches today.

Support for Hypothesis of Changes Associated with Functional Expansion of Salivary PRPs

Given that the level of PRPs required to counteract tannin appears to be much higher than required for oral homeostasis, a proposed evolution of increased secretion of PRPs with functional expansion is understandable, particularly if

there are costs in producing high levels of PRPs unnecessarily. PRPs are relatively poorly digested, because many peptide bonds involving proline are resistant to cleavage (Muenzer et al., 1979; Wong and Bennick, 1980; Oppenheim et al., 1985). Production of excess PRP may therefore incur a nitrogen cost. Grazers consume a nitrogen poor diet that generally lacks tannins, so selective pressure should be strong for reduced secretion rates of PRPs (Robbins et al., 1987; Austin et al., 1989), even if their ancestors produced higher levels.

Evolution of PRP diversification, including BPRPs, can only be understood in a multifunctional context. Effective functioning in calcium homeostasis, lubrication, bacterial aggregation, and tannin binding require some different and sometimes slightly conflicting structural characteristics. Presumably there is variation in tannin binding associated with some structural differences in PRPs, but there is insufficient information at this stage to make any generalizations. Clearly this is an important area for future research.

There is evidence that BPRPs function specifically in dealing with tannin and are associated particularly with the parotid salivary gland, and that the parotid gland is more responsive to dietary tannin than other salivary glands. Where PRP production differs among salivary glands, BPRPs are present or induced in parotid but not mandibular glands. For example, APRPs are present in mandibular and parotid secretions in rabbits, but BPRPs and mRNA coding for BPRPs are only produced in the parotid (Ferreira et al., 1992). Exactly the same pattern is seen in humans (Robinson et al., 1989; Kauffman et al., 1991), except for a single BPRP in submandibular secretions. This single BPRP is a telling exception because it is apparently derived by postsecretional cleavage of an APRP, whereas all the other BPRPs are secreted by the parotid essentially unchanged (Kauffman et al., 1991). Furthermore, dietary tannin induces a massive increase in production of PRPs in rats and mice, but only in the parotid gland (Mehansho et al., 1983, 1985), even though the β-agonist isoproterenol increases production in both parotid and mandibular salivary glands (Muenzer et al., 1979; Mehansho and Carlson, 1983; Mehansho et al., 1985). Finally, a tantalizing piece of evidence for the evolution of BPRPs from APRPs is that in humans the decoded nucleotide sequences for BPRPs are much longer than the secreted sequences, and the extra region not appearing in the secreted BPRP corresponds to an acidic *N*-terminal region found in APRPs (Minaguchi and Bennick, 1989).

PRPs are not the only proteins involved in oral homeostasis in different animals. This is not surprising given that at least one major role (calcium-related function) relies mainly on the proline-poor end of the molecule. The implication is, however, that some mammals may not use PRPs at all, provided they have other proteins, such as histatins or cystatins (Shomers et al., 1982; Oppenheim et al., 1986; Minaguchi and Bennick, 1989) to perform the same functions. This is fascinating in an evolutionary sense, because these proteins have low

levels of proline (and Gly/Glx) and therefore are unlikely to have a high affinity for tannin. If any ancestral carnivores produced only these proline-poor proteins for oral homeostasis, then the capacity to evolve the changes required to counteract dietary tannin by salivary proteins would be difficult and may even have constrained the evolution of such groups to different dietary niches.

Predictions and Suggested Tests of the Hypothesis

The first major point of the above hypothesis is that secretion levels of PRPs increased significantly during the evolution from omnivores consuming a nitrogen-rich diet to those consuming a nitrogen-poor, allelochemical-rich diet including tannins. Carnivores should therefore secrete much lower levels of PRPs than browser/frugivores. The second point of the hypothesis is that BPRPs seem related only to counteracting dietary tannin. BPRPs should therefore only be produced by animals that, or whose ancestors, had to deal with significant levels of dietary tannin on a nitrogen-poor diet. Examining salivary characteristics of related species from a range of dietary niches would test these predictions.

Shrews, for example, which are in many ways similar to early mammals and which are always insectivorous, should never produce high levels of PRPs and should lack BPRPs. Carnivores that are secondarily derived, however, may produce high levels of PRPs if their evolutionary history includes the adaptive threshold in diet type provoking changes in PRP secretion. Polar bears (*Ursus maritimus*), which are largely but not exclusively carnivorous, may have evolved from mid-Pleistocene *Ursus arctos* (brown and grizzly bears) (Kurten and Anderson, 1980) that are omnivorous. The related omnivorous black bear (*Ursus americanus*) secretes high levels of PRPs (Robbins et al., 1991; Hagerman and Robbins, 1993), and polar bears may too.

In primates, the suborder Prosimii includes carnivorous, omnivorous, and herbivorous species that would provide a reasonably closely related group to test for functional and structural differences in PRPs. In Madagascar, a comparison of *Microcebus murinus* (gray mouse lemurs) and *Lepilemur ruficaudatus* could be made. *Microcebus* has a diet of about 35% insects, 50% gum, and only 15% leaves, a nitrogen-rich diet; while *Lepilemur* eats no animal tissue, but nearly 75% browse leaves and the rest fruits, a nitrogen-poor diet (Fleagle, 1988). *Microcebus* should produce low levels of PRPs, probably lacking in unglycosylated BPRPs. *Lepilemur* should secrete high levels of PRPs, including BPRPs.

Bats appear to have evolved from primitive insectivorous mammals similar to shrews (Anon., 1983). The order Chiroptera is divided into the suborder Megachiroptera (fruit-bars and their relatives) and the suborder Microchiroptera (insectivorous bats). A comparison of salivary characteristics between these

would also test the hypothesis. Even better, some microchiropterans have secondarily become adapted to a diet of fruit, nectar, and pollen, so a comparison between these and the insectivorous species from the same suborder would be fruitful.

SUMMARY

As indicated by this review, proline-rich proteins and their roles in maintaining oral homeostasis and counteracting dietary tannin have been examined by a number of research groups from a variety of angles. At this stage, more is known about the effects of amino acid composition, phosphorylation, and glycosylation of salivary PRPs on their function in oral homeostasis than in binding with tannin. We have synthesized the information available and proposed an evolutionary hypothesis that we believe clarifies our understanding of the variation in both type and secretion level of salivary PRPs found in mammals today. Predictions from the hypothesis are testable. However, at this stage more studies on structural and functional characteristics of saliva from species from a broader range of dietary niches are required.

Methods and Materials

Saliva Collection. Parotid saliva was obtained from eight marsupial species: common wombat (*Vombatus ursinus*), common brushtail possum (*Trichosurus vulpecula*), rufous rat-kangaroo (*Aepyprymnus rufescens*), red kangaroo (*Macropus rufus*), grey kangaroo (*Macropus giganteus*), parma wallaby (*Macropus parma*), red-necked pademelon (*Thylogale thetis*), and swamp wallaby (*Wallabia bicolor*). These herbivorous marsupials include both foregut and hindgut fermenters and provide a range of species having natural diets with different levels of tannin.

A brief synopsis of the methods used to obtain samples is given here, as the protocols for particular animals have been published in detail elsewhere [possum, Scott and Beal (1994); rat-kangaroo and other small macropods, Beal (1992); wombat, Beal (1991); and kangaroos, Beal (1987, 1989a,b)]. Anesthesia was induced either by intraperitoneal injection of sodium pentobarbitone in animals with body weights up to 3 kg (possum and rat-kangaroo) or by intramuscular injection of ketamine hydrochloride in larger species. A peripheral blood vessel was cannulated (possums, saphenous artery; wombat, saphenous vein; other species, lateral tail vein) to allow maintenance of anesthesia by vascular administration of pentobarbitone. The trachea was intubated through its pharyngeal orifice and salivary ducts were catheterized via their oral openings to facilitate collection of uncontaminated saliva.

Saliva samples were obtained under two conditions. First, samples were

collected at maximum flow rate. These were used for determination of nominal maximum protein secretion rates. Maximum salivary flow was stimulated parasympathomimetically using either bethanechol or acetylcholine chloride. In the smaller species, bethanechol was infused via the peripheral vascular cannula at rates dependent on the size and response of the particular animal. In kangaroos and wombats, acetylcholine was infused into the carotid artery, just upstream from the salivary glands, via a chronic cannulation of the artery (wombat) or temporary cannulation of the artery either *in situ* (grey kangaroo) or exteriorized in a loop (red kangaroo). This treatment produces saliva having flow rates and composition similar to that produced by natural reflex stimulation.

Second, samples were collected at maximum protein concentration. These were used for detecting PRPs by gel electrophoresis. Isoprenaline (β-sympathomimetic) stimulation was used to maximize salivary protein concentrations. Isoprenaline was infused via the vascular cannulae and was superimposed on a background level of parasympathomimetic stimulation. This method increased the protein concentration but did not alter the pattern of protein bands detected by gel electrophoresis (C. McArthur, A.M. Beal, and G.D. Sanson, 1993 unpublished data). One essentially mandibular salivary sample was obtained from a red kangaroo by collection of saliva accumulating in the mouth when the parotid glands were cannulated.

Total protein concentrations were assayed by the method of Daughaday et al. (1952). Saliva was stored at $-90°C$ until analyzed for PRPs by gel electrophoresis. No changes in protein bands were detected over months of storage.

Determination of Nominal Maximum Protein Secretion Rates. Nominal maximum protein secretion rates were determined as a function of protein concentration at maximum flow rate. Examination of available data on protein concentration as a function of different flow rates (Beal, 1987, 1989a, 1991; Scott and Beal, 1994) indicated that maximum total protein secretion was generally achieved at this maximum flow rate, rather than at some intermediate or low flow rate. Data were then expressed in relation to metabolic body weight. These secretion rate values are nominal in the sense that they were obtained under anesthetic. Differences may occur when chewing or feeding on tannin-rich food, and these could not be taken into account here. However, the values are at least comparable between individuals and species because they were all collected under essentially equivalent conditions.

Detection of Salivary Proteins by Polyacrylamide Gel Electrophoresis. Previous electrophoresis of saliva from two to four individuals from each species (except *A. rufescens*: only one individual available) had demonstrated little if any difference in the pattern of protein bands from individuals of the one species, but obvious differences among species (C. McArthur, A.M. Beal, and G.D. Sanson, 1993 unpublished data). Only one sample from each species is illustrated (Figure 1). For each sample, a volume of saliva equivalent to 80–100 μg

total protein was made up to 20 μl with water. If samples were too dilute (*M. rufus* samples), the appropriate volume was reduced on a rotary vacuum evaporator. Each sample was mixed with 4 μl β-2-mercaptoethanol (Sigma M-6250) and 8 μl of a solution containing (1 : 1 v/v) glycerol and 0.5M Tris HCl buffer (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 0.02% (w/v) bromophenol blue, and boiled for 4 min. Half of each sample (40–50 μg protein) was loaded onto each gel.

Samples were analyzed by polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970), using the Bio-Rad Mini-Protean II dual-slab electrophoresis system. Chemicals were electrophoretic grade from either Sigma or ICN Biomedicals. The resolving gels were 12% acrylamide with dimensions of approximately 50 × 80 × 0.75 mm, and the stacking gels were 3% acrylamide. Running buffer was 0.025M Tris, 0.192 M glycine, and 0.1% (w/v) SDS. Two gels were run simultaneously for 50 min at a maximum of 250 V and 35 mA (constant amperage).

Gels were fixed and stained in a solution of 0.1% (w/v) Coomassie brilliant blue R-250 in ethanol–acetic acid–water (40 : 10 : 50), for 1 hr. Destaining to detect Pro/Glx/Gly-rich proteins followed Beeley et al. (1991). Proteins rich in these amino acids stain poorly, if at all, if destained in the usual solution of ethanol–acetic acid–water (40 : 10 : 50). However, they appear as pink/violet bands if destained in acetic acid–water (10 : 90). Gel 1 was destained in acetic acid–water (10 : 90), and gel 2 was destained in ethanol–acetic acid–water (40 : 10 : 50), with several changes over two days. Comparison of gel 1 and gel 2, which are identical apart from the destaining solutions used, indicate protein bands that are Pro/Glx/Gly-rich.

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MODELING METABOLIC COSTS OF ALLELOCHEMICAL INGESTION BY FORAGING HERBIVORES

A.W. ILLIUS* and N.S. JESSOP

Division of Biological Sciences, University of Edinburgh
West Mains Rd, Edinburgh
EH9 3JT, UK

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Abstract—A mathematical model of intermediary metabolism of allelochemicals by vertebrate herbivores is presented and used to quantify the metabolic costs of detoxification. Conjugation with glucuronic acid and maintenance of acid–base status causes catabolism of amino acid and is shown to result in loss of body protein and depletion of glucose. An interaction between allelochemical dose and nutrient status is found, and the ratio of allelochemical to nutrient absorption rate defines the tolerance of the animal to absorbable allelochemical concentration in foods. The interaction is nonlinear and the ecological implications of this for foraging behavior and diet choice are discussed.

Key Words—Allelochemical, detoxification, acid–base balance, conjugation, glucuronidation, foraging behavior, metabolic cost.

INTRODUCTION

Viewed from one standpoint, life is about making economic choices. For example, animals must allocate time, which is a scarce resource, to competing activities such as foraging and resting, and compare the benefits of including food types in their diet with the costs in time and energy needed to find and process the food (see Stephens and Krebs, 1986 for a formal treatment). Recent interest in the pathways of detoxification of plant allelochemicals by herbivores has suggested that disruption of acid–base balance is an important consequence of allelochemical absorption (Foley, 1992; Foley et al., 1995; Foley and McArthur,

*To whom correspondence should be addressed.

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1993). Thus, consumption of foods containing absorbable allelochemicals will require the expenditure of energy and materials during detoxification and excretion and maintenance of acid–base balance. These represent metabolic costs that weigh against the nutrient content of the food.

It is the purpose of this paper to estimate these costs, in order to address the question of how they affect the economics of diet choice. We have chosen to approach this by modeling intermediary metabolism and the control of acid–base status. This is intended to complement whole-animal physiological studies (e.g., Foley, 1992; Foley et al., 1995) by isolating and describing the effects of specified metabolic pathways and by testing whether these pathways can actually account for observed responses and are a sufficient description of the system. Modeling also offers the opportunity to examine responses over a far wider range of allelochemical doses and nutrient intake levels than would be possible in physical experiments and, therefore, offers a better chance of detecting interactions and non-linearities in response.

METHODS AND MATERIALS

Model Description

The model is based on that of Gill et al. (1984), with some modifications and extensions. The main changes are to the description of the animal's characteristics, protein synthesis and breakdown, addition of acid–base control, and partitioning metabolic activity between two sites, liver and body. We also distinguish between blood, extracellular fluid volume, and intracellular fluid volume, rather than describing metabolic activity within a single blood volume, as in the original model. A consequence of the changes made to the model, particularly to protein metabolism, is that the model now no longer needs to incorporate an ATP degrader, which Gill et al. (1984) found to be necessary to prevent the accumulation of ATP. Details of the changes made are given below.

The model depicts metabolites, or state variables, as a series of compartments connected by fluxes arising from the appropriate metabolic transformation and fed by steady-state absorption of metabolites from the gut (Figure 1 and Table 1). Fluxes arising from metabolism are given by equations in Michaelis-Menten form. Thus fluxes are primarily dependent on the concentrations of substrates but may be inhibited or augmented by the concentrations of end products. Nomenclature and units follow Gill et al. (1984). Concentrations and quantities of state metabolite i are given by Ci and Qi, fluxes by dQi/dt, and production of j from i by Pij. All these are further identified by the physical compartment, of which there are six: blood, extracellular, and intracellular (bl, ex, in) in either liver or body (l, b); hence Qiexl is the quantity of i in the extra

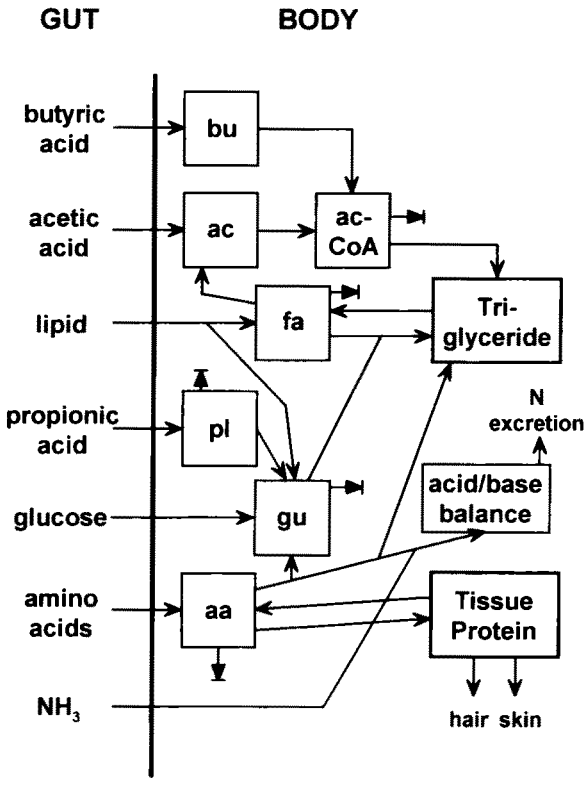

FIG. 1. Simplified compartmental structure of the model showing the metabolic reactions that occur either in liver and/or body intracellular space(s). The reactions involved in acid–base homeostasis are shown in greater detail in Figure 2.

cellular space in the liver, and so on. Mass is given in units of kilograms, volume in cubic meters, time in days, amount of substance in kilogram-moles and concentration in kilogram-moles per cubic meter. The model is programmed in BASIC, and numerical integration is performed by Euler's method. The timestep, dt, was chosen to be 4.167×10^{-5} day, (i.e., 1000 timesteps per hour). Longer timesteps were found to give unstable results, due to the wide range in the magnitudes of the rate constants producing stiffness in the set of differential equations, as noted by Gill et al. (1984). This is a problem that cannot be solved by more sophisticated methods of numerical integration, such as Runge-Kutta.

TABLE 1. METABOLITES, STATE VARIABLES, AND THEIR ABBREVIATIONS

Absorption	Liver	Body	Abbreviation
Acetic acid	Acetic acid	Acetic acid	ac
Propionic acid	Propionic acid	Propionic acid	pl
Butyric acid	Butyric acid	Butyric acid	bu
Glucose	Glucose	Glucose	gu
Lipid	Fatty acids	Fatty acids	fa
		Triglyceride	tg
Amino acids	Amino acids	Amino acids	aa
	Protein	Protein	pt
	Glutamine	Glutamine	gn
	Urea	Urea	ur
NH_3	NH_3	NH_3	ammon
	NH_4^+	NH_4^+	nh
Allelochemicals	Allelochemicals		tp1&2
		Conjugates	cg
	H^+	H^+	h
	HCO_3^-	HCO_3^-	bc
	ATP	ATP	at
	NADPH	NADPH	np
	Acetyl CoA	Acetyl CoA	cz
	O_2	O_2	ox
	CO_2	CO_2	cx

Animal Characteristics. In order to be able to apply the model to ruminants of any body weight (W, kilograms), animal characteristics are scaled with mature mass (A) (Taylor, 1980a). The composition of the live animal is estimated as follows. Gut contents of ruminants and hindgut fermenters is taken to be 0.1766W (Illius and Gordon, 1992 and unpublished) and thus empty body weight, W_{body}, is 0.8234W. Taylor and Murray (1987) suggest the mean lipid proportion of the empty body of mature animals, 0.184, may be taken as a standard, and describe the maturation of fatness by:

$$u_{fat} = u^{0.65} \tag{1}$$

where u_{fat} and u are the degrees of maturity of fatness and of the whole body, respectively. Thus, the proportion of fat in the empty body is $0.184u^{0.65}$ and the weight of adipose tissue in the body is:

$$W_{adip} = 0.184W_{body}\, u^{0.65} \tag{2}$$

Assuming triglyceride to have a molecular weight (MW) of 890, the quantity of triglyceride, Qtg, is $Q_{adip}/890$. After the animal has reached about 0.3

of its mature size, the composition of the lipid-free body remains virtually constant at 23% protein, 5% ash, and 72% water (Taylor and Murray, 1987). Therefore the weight of protein in the body, W_{pt}, is:

$$W_{pt} = 0.23(W_{body} - W_{adip}) \quad (3)$$

If the MW of protein, considered as amino acid, is taken to be 110, then the quantity of protein, Qpt, is $W_{pt}/110$. Finally, Stahl (1965) gives liver weight, W_{liver} as $0.0333W^{0.87}$.

Transport of Metabolites. Circulation of metabolites between the liver and body is achieved by blood flow, with substances absorbed from the gut passing into the portal blood. Within liver and body compartments, concentrations of metabolites in blood and extracellular fluid are assumed to be in equilibrium, while exchange with the intracellular volume is governed by diffusion, or, in the case of glucose and amino acids, by active transport. Full details are given in Appendix 1.

Maintenance of Acid-Base Homeostasis. The scheme depicting maintenance of acid/base homeostasis is shown in Figure 2. With no perturbation to acid–base homeostasis, the production of HCO_3^- from amino acid catabolism is matched by removal of HCO_3^- through ureogenesis. No distinction is made within the model as to the site of amino acid catabolism (be it within the rumen, hind gut, or body) since the net effect is assumed to be the same, i.e., production of NH_4^+ and HCO_3^- within the body. A proton load, caused by an increase in the concentration of acidic compounds, can be neutralized with HCO_3^- diverted from ureogenesis forming carbonic acid and then CO_2, which can be cleared via the lungs. Additionally, there is an increase in excretion of nitrogen in the form of NH_4^+ by the kidney, lowering the concentration of this substrate and hence the rate of ureogenesis, sparing HCO_3^- for use in maintenance of systemic

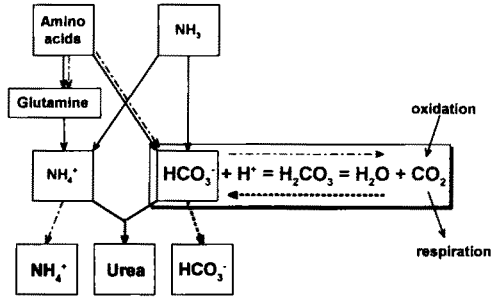

FIG. 2. The metabolic transactions concerned with maintenance of acid–base homeostasis. Fluxes that increase under acidosis are shown with a broken line and those that increase under alkalosis with a dotted line.

pH (Welbourne, 1988). Recent evidence shows that acidosis can lead to an increase in amino acid catabolism (Reaich et al., 1992) increasing buffering capacity.

Seven additional pools have been added in order to model acid–base homeostasis. Full details of these and the metabolic interactions between them are given in Appendix 2.

Conjugation. Allelochemicals absorbed into the body are metabolized in the liver to form acid conjugates to aid excretion (Caldwell, 1982). In the model it is assumed that all conjugation occurs with glucuronic acid derived from liver glucose and that conjugation is not limited by the activity of the enzyme systems involved. The acidic conjugate is exported to the liver extracellular space (Figure 3). Clearance is via the urine, assuming that the kidney receives 20% of cardiac output and of this 20% is filtered by the glomerular structure. There is assumed to be no reabsorption of conjugates from the glomerular filtrate.

Protein Metabolism. Both protein synthesis and breakdown are modeled in the two intracellular compartments. The maximum rate of synthesis and breakdown varies with degree of maturity and the absolute rates also depend on the concentrations of amino acids and ATP within the respective compartments. The pattern of protein turnover achieved is similar to that of Reeds and Fuller (1983). Further details of the description of protein metabolism are given in Appendix 3.

Partition of Metabolism. Fluxes of metabolites described in the model have the same units as the V_{max} terms: kilogram-moles per day per kilogram of tissue. The value given to V_{max} for any particular reaction is assumed to be the same wherever the reaction occurs, for example, in the liver and the body, and thus whole-tissue fluxes are determined by tissue weight and the flux rate per kilogram.

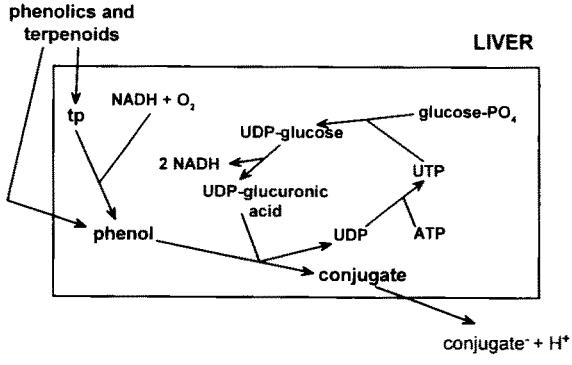

FIG. 3. Representation of conjugation in the model.

TABLE 2. PARAMETER VALUES ADDITIONAL TO GILL ET AL. (1984)[a]

Reaction	Kij(k)	Jij(k)	Yij(k)	Rij(k)	Mij(k)
aabc			1		
aacxbc		8.0×10^{-3}			4
aagn			0.5		
aapm	2.5×10^{-4}				
aapt	4.5×10^{-4}				2
aaptat	3.2×10^{-5}				
aatr	8.0×10^{-3}				4
bcur	1.0×10^{-2}				4
gnnh	2.0×10^{-3}		2		
gnur	2.0×10^{-3}				
gnurh		6.0×10^{-8}			8
gutr	1.1×10^{-2}				4
hpump				0.33	
nhgn	2.5×10^{-3}		0.5		
nhgnat				1	
nhur	5.0×10^{-4}		0.5		
ptaa	3.0×10^{-4}				

[a]For example, Yaabc is the yield of bicarbonate on catabolism of amino acid requied in equation 33, and Jaacxbc is the inhibition parameter for bicarbonate on the metabolism of amino acids to carbon dioxide (equation 37). Rhpump is the requirement of ATP for the Na^+/H^+ antiporter on the plasma membrane.

Parameters: V_{max}. It is assumed that the maximum rate of ATP use (Vat) is that which occurs at three times the rate at maintenance and that the maximal rate of triglyceride synthesis (Vtg) is such that it consumes ATP at Vat. Most other V_{max}s are set in relation to Vtg or Vat (see Gill et al., 1984). Other values for V_{max} are given in the text.

K_m values are given either by Gill et al. (1984) or are shown in Table 2.

Model Performance

Predictions from the model were obtained for a ruminant at 75% of its expected mature size of 50 kg. This was chosen to be consistent with the original model of Gill et al. (1984) and to make any departures from its results more apparent. Similarly, nutrient absorption was provided by values from the roughage diet of Gill et al. (1984), varying as required the amount but not the ratio of nutrients, and maintaining constant rates of absorption throughout each run.

The estimated efficiency of metabolizable energy (ME) use for maintenance, k_m, was 0.74, close to that expected from Agricultural Research Council (1980), while the efficiency of ME use for gain, k_g, was 0.61. This is higher

than Agricultural Research Council estimate (1980), but no higher than the estimate of Ørskov et al. (1979), obtained from lambs maintained by intragastric infusion. The composition of empty body gains during normal growth of sheep at 75% maturity is predicted by the Agricultural Research Council (1980) to be 12.3% protein and 56.7% fat (mean of male and female sheep). By comparison, the model predicts 9.3–13.8% and 71.2–57.1%, respectively, for the protein and fat content of empty body gain at intakes 1.25–2 times maintenance for an equivalent animal.

Changes in the pattern of N excretion following steady-state infusion of a proton load are shown in Figure 4. The effect of acid load on this pattern is similar to that reported previously (Häussinger et al., 1988; Foley, 1992). The slow decline in urinary urea, despite cessation of ureogenesis, can be explained by the large body pool of urea in our modeled animal (W = 37 kg) in comparison with that in the smaller (W = 1 kg) animals studied by Foley (1992).

Table 3 shows a comparison of the model's predictions with the empirical data of Pehrson (1979, 1983), and Palo et al. (1983) on mountain hares (*Lepus timidus* L.) consuming three age-classes of birch twigs (*Betula pubescens*). They found that the youngest twig sections (0–1.5 mm diameter) were eaten in lower quantities, apparently due to their higher allelochemical content and despite higher digestibility than older twig sections. Pehrson (1983) commented that the extent of weight losses by hares fed on *Betula* were partly due to the high Na losses experienced as a consequences of allelochemical intake. To generate predictions from the model, we assumed that daily rates of allelochemical absorption were some multiple of the urinary excretion of phenolics reported by Palo et al. (1983) (see Table 3), since biliary excretion may be three to four times the urinary glucuronide loss in rats (Mulder et al., 1982) and could have

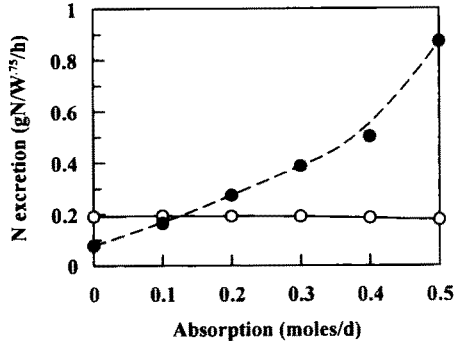

FIG. 4. Predicted changes in the pattern of nitrogen excretion as urea (○) or NH_4^+ (●) as the absorption of protons increases from 0 to 0.5 mol/day.

TABLE 3. PERFORMANCE OF MOUNTAIN HARES CONSUMING BIRCH TWIGS[a] AND COMPARABLE PREDICTIONS OF THE MODEL

Twig diameter (mm)	Digestible DM content (g/kgDM)	Phenolic glycoside content (g/kgDM)	Phenolic glycoside/ digestible DM (g/kgDDM)	Daily food intake ($gDM/W^{0.75}$)	Energy intake (multiples of maintenance)	Urinary phenol excretion ($g/W^{0.75}$/day)	Weight change (g/day)	Predicted weight change (g/day) assuming three rates of allelochemical absorption (multiples of urinary excretion):		
								1	3	10
0–1.5	339	20.4	60.2	74.3	0.92	0.22	−80	−24	−37	−60
1.5–3	269	10.0	37.1	117.7	1.06	0.17	0	+11	+ 1	−16
3–5	231	2.5	10.8	110.5	0.86	0.04	−33	−34	−36	−42

[a] Data from Pehrson (1979), Palo et al. (1983), and Pehrson (1983).

been much higher in the experiment of Foley (1992). We therefore made predictions for three rates of allelochemical absorption: 1, 3, and 10 times the observed rate of urinary phenol excretion. Despite these uncertainties, comparable model predictions show reasonably good agreement between predicted and observed daily weight losses, with a tendency to underpredict weight loss in animals feeding on high-allelochemical twigs. This may arise from effects on Na balance, which are not modeled.

Experiments Performed with the Model

We wished to estimate the changes in metabolism arising from absorption of allelochemicals. The allelochemical was represented by a phenolic or terpenoid with a MW of 150, such as terpineol, cineole, or cymene, which are typical constituents of the foliage of *Eucalpytus* (Eberhard et al., 1975). If it is assumed that 25% of the phenolics and 100% of the terpenes consumed were absorbed by animals in Foley's (1992) experiment, then dose rates of up to 0.03 mol/$W^{0.75}$/day can be estimated, equivalent to 0.4 mol/day for our modeled animal. Accordingly, doses of 0–0.5 mol allelochemical were examined using the model, in combination with nutrient absorption rates of 0.01–3 times the amounts estimated to maintain energy balance. The rate of allelochemical absorption was held constant over the course of each run, as was the case for nutrients. The rates of glucuronidation required by these doses do not exceed those implied by the data of Price et al. (1987) estimated in rats.

Two forms of experiment were performed. First, the short-term effect on metabolism was estimated from fluxes occurring 12 hr after the beginning of each run. To distinguish effects of acid-base perturbation from the effects of conjugation of allelochemical with glucuronic acid, a comparison was made of fluxes arising from allelochemical absorption and from an equimolar quantity of protons. Maintenance quantities of nutrients were supplied throughout in the ratios presented by Gill et al. (1984) to represent a forage diet. Second, the long-term tolerance to allelochemical dose was estimated at a range of nutrient intake rates. Tolerance was arbitrarily defined as the maximum dose sustainable over a four-day period, on the assumption that enzyme systems involved in cosubstrate supply and conjugation would be induced over that timescale. This would maintain indefinitely the tolerance to a dose that was at critical concentration over the initial four days. Once tolerance had been estimated over a range of nutrient intakes, three investigations were pursued. Metabolic responses to a combination of doses and nutrient intakes could be established, choosing the hour 24 after commencement as a time when steady-state solutions had been achieved in all cases. The concentration of maximum tolerable absorbable allelochemical in foods was estimated by assuming a nutrient density of 10 MJ ME/kg dry matter (DM). Last, the interaction with fiber content was examined. The

model of Illius and Gordon (1991, 1992) was used to estimate the minimum potential digestibility (calculated from the sum of the cell contents and digestible cell wall) of a browse diet required to sustain a range of nutrient intake rates (0.4–3 times maintenance) in the absence of allelochemicals. The diminution in energy yield was then calculated: (1) from the dilution effect of the presence of absorbable allelochemical (and assuming that all allelochemical was absorbed), and (2) from the energy costs of allelochemical metabolism and excretion.

RESULTS

Short-Term Responses. Steady-state amino acid breakdown arising from proton absorption is contrasted with that from allelochemical absorption in Figure 5a. The absorption of amino acids is also shown. Without either allelochemical or exogenous proton sources, the positive balance of amino acid absorption over breakdown represents the daily rate of endogenous protein loss in hair and sloughed skin. The dose–response to proton absorption shows an excess of amino acid breakdown over absorption and represents the catabolism of amino acids to bicarbonate for neutralization of the proton load. Allelochemical absorption imposes the further cost of increased amino acid catabolism in gluconeogenesis to glucuronic acid for conjugation and formation of the hydrophilic acid glucuronide which can be excreted.

The resulting changes in body protein and fat masses are shown in Figure 5b. Protein losses range up to 12 g/day from allelochemical absorption, but only 4 g/day from protons. Due to the net flux of amino acids away from protein synthesis, the energy cost of the latter is spared, causing increased fat synthesis under proton absorption, while this energy is used for glucuronic acid under allelochemical absorption. The net effects on the body weight of the whole animal, shown in Figure 5c, indicate losses of 250 g/day arising from costs of allelochemical metabolism, but only 50 g/day from the costs of maintaining acid/base status under an exogenous proton load. The majority of this weight loss is in the form of water associated with protein.

Long-Term Responses. Stable solutions to the combination of allelochemical dose and nutrient absorption rate were obtained by 24 hr after the start of each run, and the results presented are taken from them and expressed as daily rates (Figure 6). The effects of dose (x axis) and nutrient intake rate (z axis) on weight change (y axis) are depicted. This response surface is not planar, indicating an interaction between the two independent variables. At high rates of nutrient intake (e.g., three times maintenance) the dose–response to allelochemicals is less steep than at low rates (less than maintenance). The contours of response surface are isoquants of weight change, connecting combinations of

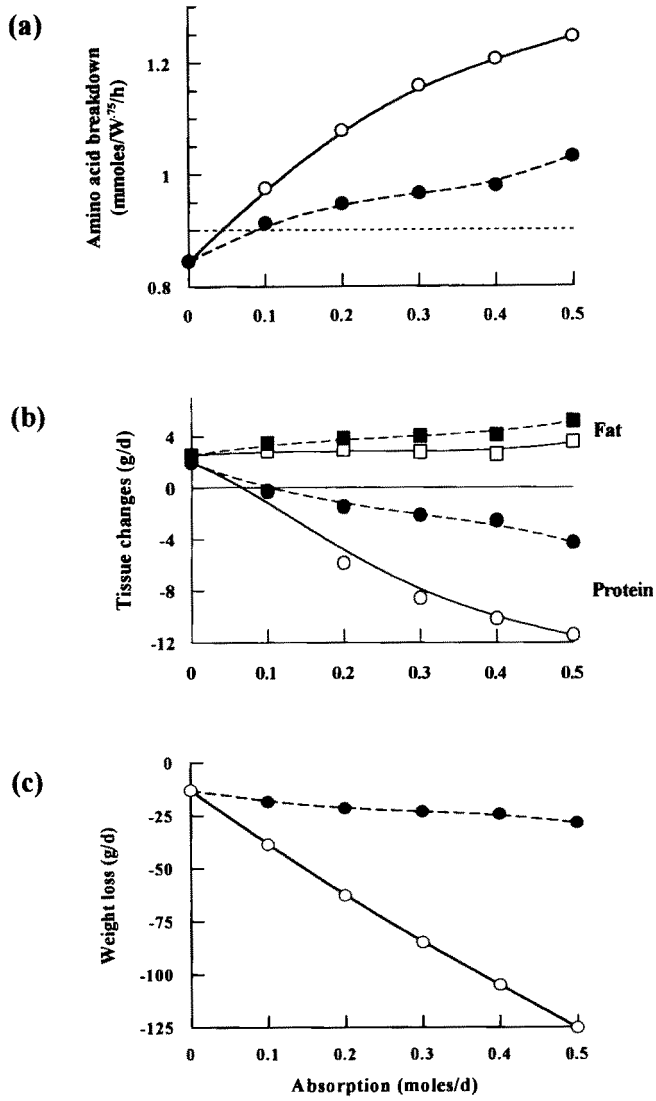

FIG. 5. (a) Predicted changes in the rate of amino acid catabolism caused by the absorption of either protons (closed symbols) or allelochemical (open symbols) at rates up to 0.5 mol/day. Amino-acid absorption (------) was constant throughout. (b) Equivalent changes predicted for body fat (□, ■) and protein (○, ●), and (c) body weight, following proton or allelochemical absorption.

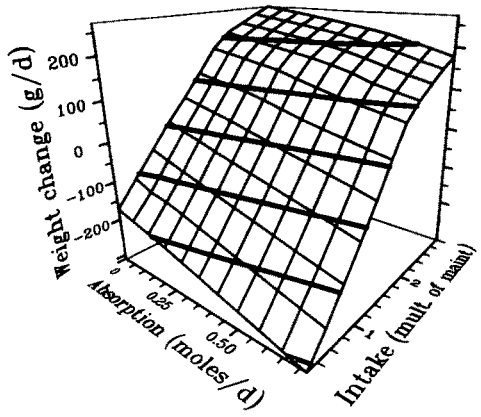

FIG. 6. The influence of allelochemical absorption across a range of intakes on the predicted weight change of the modelled animal. The contours show the combinations of allelochemical absorption and nutrient intake which result in equal weight changes.

allelochemical dose and nutrient intake which produce equal rates of weight change.

The interaction of allelochemical dose and nutrient intake rate is examined further by determining the tolerance of the modeled animal to allelochemicals (Figure 7). The maximum daily rate of allelochemical absorption sustainable over a four-day run of the model (our definition of tolerance) varies nonlinearly with nutrient intake rate. The minimum rate of nutrient intake below which no

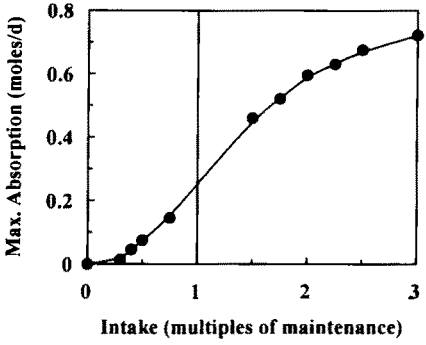

FIG. 7. Predicted allelochemical tolerance over a range of intakes from 0 to 3 times maintenance.

allelochemical could be tolerated was that equivalent to 0.25 maintenance. Above this threshold, tolerance increased exponentially until decelerating at high nutrient intakes, with an inflection approximately at maintenance nutrient intake.

By assuming that the nutrient intake is provided by a food with an energy density of 10 MJ ME/kg DM at zero allelochemical content, we can calculate the maximum tolerable concentration of allelochemicals in foods eaten at the rates supplying 0.25–3 times maintenance (Figure 8). At low nutrient intakes, especially those below maintenance, only low concentrations of allelochemical can be tolerated. Maximum tolerable concentrations are obtained when nutrient intake of around twice maintenance can be sustained. As the modeled animal approaches the limit to metabolism assumed in the model (3× maintenance), the increase in tolerance diminishes.

Contrasts between the effects of allelochemicals and fiber composition on the ability of a food to provide nutrients can be drawn from Figure 9. The solid curve shows the predicted maximum nutrient intake rate that foods varying in potential digestibility can provide the modeled animal assuming it eats to the limits imposed by gut capacity and food turnover time (Illius and Gordon, 1991). Potential digestibility is the sum of the cell contents and the digestible cell wall fractions but can also be written as 1 minus the indigestible cell wall fraction. The dashed line to the right indicates the reduction in nutrient supply rate as a consequence of the passive, diluting effect of the presence of allelochemical at the maximum tolerable concentration defined above. The dotted line shows the further diminution in nutrient supply rate due to the metabolic costs of detoxifying and excreting that dose of allelochemical. It can be seen that a food that has a potential digestibility of 0.7 provides a maintenance intake if no allelochemical is present, but the animal must choose a diet that has a potential

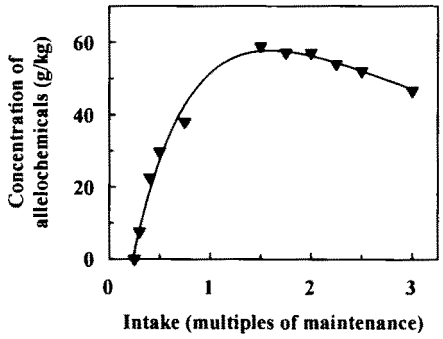

FIG. 8. The maximal tolerable concentration of allelochemical in the food over a range of intakes from 0 to 3 times maintenance.

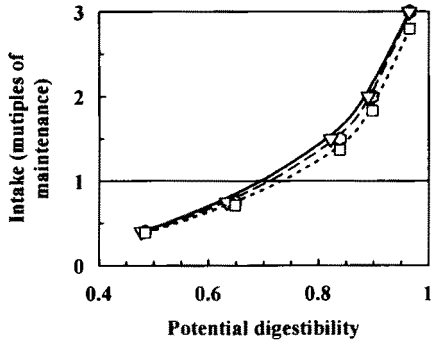

FIG. 9. The relationship between forage digestibility and maximum intake, assuming intake is limited by gut capacity (solid line, from Illius and Gordon, 1991); showing the diluting effect of including allelochemicals at the maximum tolerable concentration (dashed line) and additional effects of the metabolic costs of detoxifying and excreting that dose of allelochemical (dotted line).

digestibility of 0.75 to counteract both dilution and metabolic costs when allelochemical is present. A food with a potential digestibility (recall that the definition permits allelochemical to be part of the cell contents fraction without changing potential digestibility) of 0.7 with allelochemical present would only provide an intake of about 0.9 times maintenance, at which the concentration of allelochemical that can be tolerated is lower. The point of this example is to demonstrate the diminution in the nutritive value of a food due to dilution and detoxification costs. It also shows that this diminution may cause the allelochemical : nutrient ratio to cross the tolerance threshold, resulting in the food becoming unacceptable.

DISCUSSION

The Model. In this modeling study we have quantified the cost of allelochemical detoxification and excretion and have shown that a limit of tolerance to allelochemical absorption exists that is nonlinearly related to nutrient intake rate. Our results confirm that the metabolic pathways described by Foley (1992) and Foley et al., (1995) as being important in allelochemical excretion do indeed function at quantifiable cost. On forage diets supporting limited glucose absorption, the primary cost is expressed in terms of amino acid breakdown to maintain glucose levels for formation of glucuronide conjugates and to provide bicarbonate to neutralize excess protons. Thus, the critical step appears to be gluconeo-

genesis from amino acid precursors. High rates of allelochemical absorption deplete the glucose pool, resulting in failure of the modeled animal's obligate glucose requirement for supply of energy to nervous tissue.

The interaction of allelochemical and nutrient ingestion takes two forms. First, the model revealed that tolerance to allelochemical absorption is dependent on the rate of nutrient absorption. Tolerance of allelochemical was progressively reduced at nutrient intakes that would have been insufficient to maintain energy balance in the absence of allelochemicals. When modeled nutrient absorption was insufficient to maintain weight, the flux of amino acids via gluconeogenesis to conjugation and acid–base control was markedly curtailed. Conversely, at very high nutrient intakes, the surplus of nutrients over utilization capacity produced saturation of the ability to withstand acid load. Second, slightly less amino acid needs to be catabolized per mole of allelochemical at high nutrient intakes, because the excess of amino acid supply over requirements for net protein synthesis leads to normally high rates of amino acid breakdown. Allelochemical conjugation and excretion utilizes this "spare capacity" by diverting the flux of gluconeogenesis to conjugation rather than to lipogenesis, thus reducing the amino acid breakdown specifically for allelochemical metabolism.

The body's reduced tolerance of toxins when under nutritional stress is well known. For example, reduced dietary supply of the sulfur-containing amino acids methionine and cysteine can reduce serum inorganic sulfate in rats and reduce acetaminophen (paracetamol) conjugation with sulfate (Glazenberg et al., 1983). Fasting increases acetominophen toxicity in rats, due to reduced rates of glucuronidation and sulfation and hence reduced clearance (Price et al., 1987). Glucuronidation is partially dependent on liver glycogen stores, since treatment with acetaminophen depletes liver glycogen in normally fed rats, while significant glucuronidation capacity is maintained in fasted rats with virtually no liver glycogen. The capacity of the liver to synthesize the cosubstrate, UDP-glucuronic acid (UDPGA), is reduced in fasted rats and appears to be the rate-limiting process that determines tolerance of acetaminophen (Price and Jollow, 1988). In fasted rats, the UDPGA precursor, UDP-glucose, was lower than in fed rats both before and after an acetaminophen dose, which induced markedly higher blood glucose in both groups. Thus, reduced UDPGA synthesis is not apparently related to inadequate glucose supply from gluconeogenesis or peripheral glucose (Price and Jollow, 1989). The implication of these acute experiments is that the rate of UDPGA synthesis is limited by the rate of formation of glucose-1-phosphate from gluconeogenic precursors, but we have no evidence of whether this limitation applies under chronic conditions and have not attempted to model it. In general, the evidence of nutritional interactions with detoxification strongly supports the qualitative and quantitative behavior of the model.

Ecological Implications. There are a number of ecological implications of

our results. First, we show that the diminution in energy yield of foods to herbivores as a result of the dilution effect of the allelochemicals and the metabolic costs of detoxification is not substantial (Figure 9). It seems likely that allelochemical deterrence of vertebrate herbivory may rely more on effects of amino acid and nitrogen loss and on limited tolerance under nutritional stress. This arises primarily from the interaction between nutrient intake rate and detoxification capacity, and its effect on diet selection.

We have defined "tolerance" to be the maximum absorbed allelochemical dose sustainable by the rate of supply of cosubstrate for conjugation. It therefore comprises the balance between two variables: allelochemical intake rate and nutrient intake rate. An animal cannot consume a food that exceeds the threshold ratio of allelochemical : nutrient unless it can dilute the ratio by adding other foods to its diet. Below the threshold, detoxification ability is sustained by nutrient intake, provided that an animal can eat *enough* of such a food to maintain tolerance. For example, referring to Figure 8, a food with 40 g/kg of absorbable allelochemical is tolerable at intakes that provide more than maintenance energy intakes, but not at lower intakes. This is due to the nonlinearity shown in Figure 7. If the food is scarce enough to limit intake to below maintenance, then insufficient nutrient supply is available for detoxification and so none should be eaten. The way animals will or should modify diet selection and food intake when allelochemical dose is below the limit of tolerance is a question of how the animal weighs the costs of detoxification against the cost of alternative dietary choices. While the loss of body protein during detoxification is a cost that may not be sustainable indefinitely, it may be preferable in the short term to rates of weight loss that would ensue from reducing food intake. Moreover, reducing food intake and hence nutrient suppy will reduce tolerance. So, without alternative food sources that offer lower allelochemical/nutrient ratios, the animal must sustain its intake to supply adequate nutrients to maintain detoxification.

These inferences from the model's results could explain some of the dietary choices observed in vertebrate herbivores. Animals facing depletion of food resources during winter or the dry season have often been observed to discard or avoid current-season's growth of woody browse in preference for more mature growth (Bryant and Kuropat, 1980; Palo et al., 1983; Provenza and Malachek, 1984; Reichardt et al., 1984) (see also Table 3). Current-season's growth is less lignified than older twigs and thus is more digestible and should provide higher nutrient intakes if available in sufficient quantity. However, allelochemical concentration is also higher in current growths, and this acts as a strong deterrent to browsing (Bryant, 1981). It has therefore been concluded that herbivores avoid plant tissues with high concentrations of secondary chemical constituents and that they do not select forage purely on the basis of its proximal nutrient

content (Bryant and Kuropat, 1980). This dietary response is anomalous in the sense that the more digestible plant parts should be preferred by an animal seeking to maximize nutrient intake, but are in fact avoided.

We predict that diet breadth will vary according to the composition and abundance of the variety of forages available. First, if a food contains a high concentration of absorbable allelochemicals, it may be consumed if it is abundant enough and can be eaten at a rate sufficent to provide nutrients for detoxification. Under these circumstances, it could be the only food eaten. If the allelochemical concentration exceeds the threshold of tolerance defined by the rate of nutrient supply (see Figure 8), then the animal must supplement its nutrient supply from sources with lower detoxification requirements in order to reduce the allelochemical/nutrient ratio. Initially, this will broaden the diet (as first suggested by Freeland and Janzen, 1974). If these alternative food sources have low digestible nutrient content, or are scarce, then total nutrient supply will be reduced and tolerance of allelochemicals reduced further until none of the food sources with the higher concentration of allelochemicals can be consumed. Thus, when food supply becomes limiting, we can predict that animals will respond to declining nutrient intake by reducing their intake of current-season growths and narrowing their diet to consist of foods that have sufficiently low allelochemical concentration to be tolerable at low nutrient intake. There is indeed evidence for this narrowing of diet occurring in subarctic herbivores (J.P. Bryant, personal communication). When low abundance of foods limits intake, then the model shows that low concentrations of allelochemicals are sufficient to deter herbivory.

APPENDIX 1. TRANSPORT OF METABOLITES

Circulation is described by reference to blood volume and cardiac output. Blood volume is given by $6.55W \times 10^{-5}$ m^3 (Schmidt-Nielsen, 1984) and apportioned between the liver and body compartments by assuming that the total portal volume, Vbll, is twice that of the liver. Volumes of extracellular and intracellular fluid in liver and body (Vexl, Vinl, Vexb, Vinb) are derived from the fraction of the body water not in blood, apportioned according to the volumes of liver and body, and assuming the extracellular: intracellular volume ratio is 1:3.75 (Eckert and Randall, 1983). Schmidt-Neilsen (1984) gives cardiac output, Tbl, as a function of oxygen consumption (dQox/dt): $\text{Tbl} = a\ d\text{Qox}/dt$, where a is 20, the ratio of Tbl to dQox/dt at an arteriovenous oxygen concentration difference of 50 ml O_2 per liter of blood. Portal blood flow is taken to be 0.25 of cardiac output. The proportion of the portal blood entering the general circulation per timestep is thus 0.25 Qh/Vbll. The transport by the circulation of substance i from the liver to the body, Tilb, and from the body to the liver,

Tibl is:

$$\text{Tilb} = 0.25\ \text{Qh/Vbll Qibll} \tag{4}$$

$$\text{Tibl} = 0.25\ \text{Qh/Vbll Qiblb} \tag{5}$$

Transport by diffusion between extracellular and intracellular compartments follows the standard description, e.g., Tiinl = *ps* (Ciinl-Ciexl), where *p* is the permeability of membranes to substance i and *s* is the total surface area of the cell membrane. The membrane permeability of uncharged/lipophilic substances, such as undissociated VFA and long-chain fatty acids, was taken to be 1×10^{-3} cm/sec while that for charged/lipophobic substances, such as dissociated VFA and glucose, was taken to be 3×10^{-7} cm/sec (Hume and Thomas, 1989; Sharp and Thomas, 1981).

Additionally, for certain metabolites, there are specific transporting mechanisms on the cell surface. Those for glucose and amino acids are represented in the model. The active transport of glucose is described thus:

$$\text{Tguinx} = \frac{\text{Vgutr}}{\left[1 + \left(\frac{\text{Kgutr}}{\text{Cguexb}}\right)^{\text{Mgutr}}\right]} \tag{6}$$

where Vgutr is the sum of V_{max}s of glucose utilizing pathways. For amino acids two types of carrier are represented, the A and L types (Christensen, 1990). These are described thus:

A type:

$$\text{Taainx} = \frac{\text{Vaatr}}{\left[1 + \left(\frac{\text{Kaatr}}{\text{Caaexb}}\right)^{\text{Maatr}}\right]} \tag{7}$$

L type:

$$\text{Taainx} = \frac{\text{Kaapm} \times (\text{Caainb} - \text{Caaexb})}{[(1 + \text{Caainb}) \times (1 + \text{Caaexb})]} \tag{8}$$

where Vaatr is the sum of V_{max} values of amino acid utilizing pathways.

APPENDIX 2. MAINTENANCE OF ACID-BASE HOMEOSTASIS

Liver Periportal Glutamine (gn1inl). Glutamine reaching the liver in portal blood is taken up into the intracellular space by active transport (see above) and enters the liver periportal glutamine pool. It is deaminated to provide one source of NH_4^+ from which urea is synthesized. The rate of production of NH_4^+ from

gn1 is:

$$\text{Ugnnhl} = \frac{\text{Vgnnh}}{\left(1 + \frac{\text{Kgnnh}}{\text{Cgn1inl}}\right)} \tag{9}$$

where Vgnnh is equivalent to the maximum rate of urea synthesis, Vnhur, divided by the yield of NH_4^+ from gn1, Ygnnh. Thus the change in quantity of gn1inl due to metabolism is:

$$\frac{d\text{Qgn1inl}}{dt} = -\text{Ugnnhl} \tag{10}$$

Liver Perivenous Glutamine (gn2inl). Liver perivenous glutamine is synthesized from 2-oxo-glutamate and NH_4^+ released from either amino acid catabolism or escaping ureogenesis in the periportal region of the liver (Häussinger et al., 1988). This glutamine is released from the liver into the general circulation. The production of gn2inl is:

$$\text{Paagnl} = \text{Yaagn} + (\text{Uaacxl} + \text{Uaagul} + \text{Uaatgl}) \tag{11}$$

$$\text{Pnhgnl} = \text{Ynhgn} + \text{Unhgnl} \tag{12}$$

Thus the change in the quantity of gn2inl is:

$$\frac{d\text{Qgn2inl}}{dt} = \text{Paagnl} + \text{Pnhgnl} \tag{13}$$

Liver Ammonium (nhinl). The NH_4^+ pool within the liver is added to by diffusion of NH_4^+ from the extracellular space, or by production from gn1inl:

$$\text{Pgnhl} = \text{Ygnnh} + \text{Ugnnhl} \tag{14}$$

and is utilized either for synthesis of urea or gn2inl:

$$\text{Unhurl} = \frac{\text{Vnhur}}{\left\{\left[1 + \left(\frac{\text{Kbcur}}{\text{Cbcinl}}\right)^{\text{Mbcur}}\right] + \left(1 + \frac{\text{Knhur}}{\text{Cnhinl}}\right)\right\}} \tag{15}$$

$$\text{Unhgnl} = \frac{\text{Vnhgn}}{\left(1 + \frac{\text{Knhgn}}{\text{Cnhinl}}\right)} \tag{16}$$

where Vnhur is the sum of the V_{max}s for amino acid catabolism and Vnhgn is the maximum rate of NH_4^+ production from gn1, Vgnnh, divided by the yield of gn from NH_4^+, Ynhgn. The rate of change of NH_4^+ is:

$$\frac{d\text{Qnhinl}}{dt} = \text{Pgnnhl} - \text{Unhurl} - \text{Unhgnl} \tag{17}$$

Liver urea (urinl). The production of urea is defined by Unhurl (equation 15) together with Ynhur:

$$\text{Pnhurl} = \text{Ynhur} + \text{Unhurl} \tag{18}$$

Thus the change in the quantity of urea is simply:

$$\frac{d\text{Qurinl}}{dt} = \text{Pnhurl} \tag{19}$$

Liver bicarbonate (bcinl). Bicarbonate is formed from catabolism of amino acids, entry of NH_3 into the liver and its subsequent hydration, and is consumed during urea synthesis in equivalent amounts to that of NH_4^+:

$$\text{Paabcl} = \text{Yaagc} * (\text{Uaacxl} + \text{Uaagul} + \text{Uaatgl}) \tag{20}$$

$$\text{Pamminbc} = \text{Tammoninl} \tag{21}$$

$$\text{Ubcurl} = \text{Unhurl} \tag{22}$$

Thus the change in bcinl is

$$\frac{d\text{Qbcinl}}{dt} = \text{Paabcl} + \text{Pamminbc} - \text{Ubcurl} \tag{23}$$

If there is net HCO_3^- use, then this is replaced from hydration of CO_2 to maintain HCO_3^- levels.

Additional fluxes to the liver ATP pool account for the energy cost of synthesis of glutamine and:

$$\text{Unhgnatl} = \text{Rnhgnat} * \text{Unhgnl} \tag{24}$$

$$\text{Uhpumpatl} = \text{Rhpump} * \text{Pcbbcinl} \tag{25}$$

Acid–Base Status in Liver Extracellular Pools. Protons enter portal blood from absorption of undissociated VFA from the gut, which then exist primarily in their dissociated form at normal blood pH; from liver intracellular space in response to an acid load; and from the uptake of NH_3 formed from NH_4^+ into liver. Protons are removed from portal blood when VFA diffuse into liver intracellular space (as the undissociated molecule), on absorption of NH_3 from the gut, which exists principally as NH_4^+ at normal pH and by blood flow to the rest of the body. The change in the quantity of protons is thus:

$$\begin{aligned} d\text{Qhpo} = {} & \text{Aac} - \text{Tacinl} + \text{Apl} - \text{Tplinl} + \text{Abu} - \text{Tbuinl} + \text{Pcbbcinl} \\ & - \text{Aammon} + \text{Tammoninl} + \text{Icopv} * \text{vpo} * (\text{Chexb} - \text{Chexl}) \end{aligned} \tag{26}$$

An acid load causes HCO_3^- to be used to neutralize protons:

$$\text{Ubccbl} = d\text{Qhpo} \tag{27}$$

An alkaline load causes carbonic acid to dissociate into protons and HCO_3^-

$$\text{Ucbbcl} = -d\text{Qhpo} \tag{28}$$

so

$$\frac{d\text{Qbcpo}}{dt} = -\frac{d\text{Qhpo}}{dt} \tag{29}$$

Body glutamine (gninb). Ammonium produced from amino acid catabolism in the body is transported in the form of glutamine. Thus gn is formed:

$$\text{Paagnb} = \text{Yaagn} * (\text{Uaacxb} + \text{Uaatgb}) \tag{30}$$

Glutamine in blood can be deaminated in the kidney, resulting in NH_4^+ release into urine. This reaction has a high capacity but is inhibited at normal blood pH, increasing substantially as acidosis develops (Brosnan et al., 1988). It is assumed that the kidneys receive 20% of cardiac output, of which 20% enters the glomerular filtrate:

$$\text{Ugnurb} = \frac{\text{cardo} + 0.2 \times 0.2 \times \text{Cgnbl}}{\left\{\left(1 + \frac{\text{Kgnur}}{\text{Cgnbl}}\right) \times \left[1 + \left(\frac{\text{Jgnurh}}{\text{Chexb}}\right)^{\text{Mgnurh}}\right]\right\}} \tag{31}$$

Thus the change in body gn is

$$\frac{d\text{Qgninb}}{dt} = \text{Paagnb} - \text{Ugnurb} \tag{32}$$

Body HCO_3^- (bcinb). Bicarbonate is formed from amino acid catabolism:

$$\text{Paabcb} = \text{Yaabc} * (\text{Uaacxb} + \text{Uaatgb}) \tag{33}$$

If the concentration of HCO_3^- in blood exceeds 27mmol/liter then HCO_3^- is lost in urine (Eckert and Randall, 1983):

$$\text{Ubcun} = (\text{Qbcbl} - 0.027 * \text{Vbld}) * 0.2 * \text{carbo} * 0.2 \tag{34}$$

where Vbld is blood volume in the body. So

$$\frac{d\text{Qbcinb}}{dt} = \text{Paabcb} - \text{Ubcun} \tag{35}$$

Acid–Base in Body. Protons enter body blood from portal blood and are removed as VFA diffuse into the intracellular space:

$$\frac{d\text{Qhbl}}{dt} = \text{lcopv} \times \text{vpo}(\text{Chexl} - \text{Chexb}) - \text{Tacinb} - \text{Tbuinb} - \text{Tplinb} \tag{36}$$

Additionally the equations determining the rate of amino acid breakdown (to CO_2 in liver and body and also to glucose in liver) are modified to include an extra term in the denominator to enable the flux to respond to changes in HCO_3^- levels:

$$\ldots \times \left[1 + \left(\frac{\text{Cbcinl}}{\text{Jaacxbc}}\right)^{\text{Maacxbc}}\right] \tag{37}$$

APPENDIX 3. PROTEIN AND ENERGY METABOLISM

The daily protein requirement for maintenance (grams per day) is given by Emmans and Fisher (1986) to be $0.01W_{ptm}^{-0.27}$Qpt. The maximum rate of net protein synthesis, Pptmax, is scaled with the animal's potential growth rate. Taylor (1980b) presents data for the growth of nine mammalian species (ranging from mouse to horse) showing unrestricted growth till weaning. This can be described by a Gompertz function:

$$W = Ae^{-e(2.7-0.037\theta)} \tag{38}$$

where θ is metabolic age, given by $A^{-0.27}(t - 3.5)$, t being time since conception. Potential maturation rate at any degree of maturity is thus:

$$\frac{du}{d\theta} = 0.037u \log_e\left(\frac{1}{u}\right) \tag{39}$$

Maximum growth rate (kilograms per day) is thus

$$\frac{dW}{dt} = 0.037u\, A^{0.37} \log_e \frac{1}{u} \tag{40}$$

Combining this with the description of body composition (above) gives:

$$\text{Pptmax} = \frac{0.001565uA^{0.73}(4.475 - u^{0.65}) \log_e \frac{1}{u}}{110} \tag{41}$$

Pptmax (kilogram-moles per day) is the difference between synthesis and breakdown. In order to define the maximum rate of protein synthesis and breakdown, the fractional synthesis rate (FSR) was regressed on fractional growth rate (FGR) for data collected from sheep (Davis et al., 1981; Attaix et al., 1987), pigs (Reeds et al., 1980), rats (Goldspink and Kelly, 1984), wallaby (Nolan, 1987), wombats (Barboza et al., 1993), and humans (Reeds et al., 1985). FSR was determined to be:

$$\mathrm{FSR} = 0.036 + 2.5\mathrm{FGR} (r^2 = 0.87, P < 0.001) \tag{42}$$

The fractional breakdown rate (FBR) is the difference between FSR and FGR. Thus, the maximal FSR and FBR, Vaapt and Vptaa, respectively, that would be achieved under optimal nutritional environment were determined. Protein synthesis and protein breakdown are represented as two Michaelis-Menten fluxes:

$$\mathrm{Uaapt} = \frac{\dot{}\mathrm{Vaapt}}{\left[1 + \left(\frac{\mathrm{Kaapt}}{\mathrm{Caainx}}\right)^{\mathrm{Maapt}} + \frac{\mathrm{Kaaptat}}{\mathrm{Catinx}}\right]} \tag{43}$$

$$\mathrm{Uptaa} = \frac{\mathrm{Vptaa}}{\left[1 + \frac{\mathrm{Kptaa}}{\mathrm{Caainx}}\right]} \tag{44}$$

Kaapt and Maapt are set so that protein synthesis responds to amino acid concentration only at low amino acid concentrations, above which energy availability has a greater influence on flux (Gill and Oldham, 1993).

Energy Metabolism. The total daily energy requirement for maintenance, Ema (megajoules per day), is given by Emmans and Fisher (1986) as a function of protein mass:

$$\mathrm{Ema} = 1.63\ W_{ptm}^{-0.27} W_{pt} \tag{45}$$

where W_{ptm} is the expected protein mass in the mature animal. The model calculates the energy expended in a number of transactions that are normally included with total maintenance costs. At food intakes that maintain energy balance, the rates of utilization in the model of adenosine triphosphate (ATP) due to protein synthesis, oxidation of acetyl-CoA, and gluconeogenesis from propionate were estimated to be, resepctively, 0.397, 0.19, and 0.05 of the total ATP utilization for maintenance. The remaining expenditures (e.g., costs of maintaining ionic balances, cost of muscular activity) were thus be estimated by difference and are assumed to apply at all levels of intake.

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CONSEQUENCES OF BIOTRANSFORMATION OF PLANT SECONDARY METABOLITES ON ACID–BASE METABOLISM IN MAMMALS—A FINAL COMMON PATHWAY?

WILLIAM J. FOLEY,[1,*] STUART McLEAN,[2] and STEVEN J. CORK[3]

[1]*Department of Zoology, James Cook University Townsville 4811, Australia*

[2]*School of Pharmacy, University of Tasmania Hobart 7000, Australia*

[3]*CSIRO Division of Wildlife & Ecology Lyneham 2602, Australia*

Abstract—Regulation of acid-base homeostasis is essential for mammals and birds. Biotransformation and metabolism of absorbed plant secondary metabolites (PSMs) results in the production of organic acids that threaten acid–base homeostasis. Consequently these acids must be buffered and excreted from the body. The production of an acid load from detoxified PSMs should occur in herbivorous mammals and birds and with most PSMs and so may provide a unifying theme to explain many effects of PSMs on animal metabolism. Since the organic acids will be largely ionized at physiological pH, disposal of the hydrogen ion and the organic anion may proceed independently. Most hydrogen ions (H^+) from organic acids are eliminated by one or more of three ways: (1) when they react with bicarbonate in the extracellular fluid to form carbon dioxide and the carbon dioxide is exhaled, (2) when they bind to dibasic phosphate and are excreted by the kidney as monobasic phosphate, and (3) when they are buffered and retained in the skeletal system. The secretion of phosphate ions and ammonium excretion are two ways in which the kidney replaces bicarbonate ions that have been eliminated as carbon dioxide. Secretion in the kidney tubule is an important means of excreting excessive organic anions rapidly. This process is saturable and may be subject to competition from a variety of different metabolites. Lagomorphs have limited capacity to form new bicarbonate from ammonium excretion and may therefore be obliged to excrete other cations such as sodium to balance the excretion of organic anions from PSMs. Acidemia has wide-ranging impacts on animals but browsing mammals and birds may have to break down muscle tissues to provide for urinary ammonium in order to generate bicarbonate for

*To whom correspondence should be addressed.

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buffering. Acidemia also can affect the extent of urea recycling. Animals consuming browse diets may have to regulate feeding so that the rate of formation of hydrogen ions does not exceed the rate of disposal. The mechanisms by which this could occur are unknown.

Key Words—Plant secondary metabolites, biotransformation, detoxification, herbivore, marsupial, lagomorph, acidosis, ammonium, phosphorus, sodium, organic acids, kidney.

INTRODUCTION

There is an increasing recognition that plant secondary metabolites (PSMs) are important influences on mammalian foraging and diet selection (Palo and Robbins, 1991). To date, however, our understanding of these interactions is only rudimentary and relies largely on correlations between diet selection and PSM profiles. Some mammals avoid particular plants in their diets because of the occurrence of specific PSMs, but any mammal that feeds on browse cannot avoid ingesting substantial quantities of PSMs such as phenolics and terpenes (Bryant et al., 1992).

Accordingly, the diversity of PSMs ingested by herbivores makes it difficult to measure individual effects. For example, folivorous marsupials eating *Eucalyptus radiata* leaves may ingest up to 60 different terpenes (Foley et al., 1987; Boland et al., 1991) and at least 20–30 broadly different phenolics (Hillis, 1966). With such diverse diets, relating composition and effects is extremely difficult. Clearly some way of characterizing and evaluating the common effects of such a wide range of compounds would be useful.

One of the early dichotomies in the study of plant–animal interactions was between PSMs that exerted a supposed toxic effect and those that acted primarily in the gut to reduce digestibility of foods (Rhoades and Cates, 1976). However, the latter idea has not been supported by detailed studies of digestion and metabolism in a range of animal species. Most investigators now recognize that all classes of PSMs, including broad categories such as phenolics, usually do exert effects beyond the gastrointestinal tract if absorbed (Foley and McArthur, 1994).

Whereas some PSMs are so toxic that they can kill some herbivores quickly (Bryant et al., 1992), most animals are likely to ingest toxic plants that elicit subacute or chronic toxicosis, but the mechanisms are poorly described. Careful studies of the variety of compounds eaten by herbivores may reveal certain adaptive patterns in the metabolism. For example, several studies have now identified apparent thresholds beyond which animals are reluctant to increase their consumption of PSMs (e.g., Jakubas et al., 1993). The best explanation of these effects is that animals would exceed their capacity to biotransform and eliminate toxic PSMs (Freeland and Janzen, 1974).

In this article, we propose that in place of the traditional emphasis on the PSM content of the diet, ecologists should refocus on the biotransformed and excreted metabolites of ingested PSMs (Foley, 1992). Viewed in this way, the effects of absorbed PSMs can be directly explained and evaluated in terms of powerful, existing knowledge about physiological homeostasis and regulation. We emphasize that here we are concerned largely with the PSMs that occur in large concentrations (in particular terpenes and phenolics) that no browser can avoid, rather than alkaloids or cyanogenic glycosides, which typically occur at lower concentrations (Harborne, 1991).

CREATION OF AN ACID LOAD

The effect of PSMs on mammalian herbivores depends on the amount ingested and the degree to which they are neutralized before reaching susceptible tissues. A broader discussion of the effects of PSMs and particular countermeasures employed by mammals can be found in Foley and McArthur (1994).

Most PSMs that are absorbed from the gut must be biotransformed and excreted (Foley et al., 1987). While hydrophilic substances may be readily excreted by the kidney, lipophilic PSMs would tend to accumulate in vivo. Even relatively nontoxic PSMs would eventually produce nonspecific adverse effects at high concentrations. Enzyme systems have evolved to enable animals to transform dietary lipophilic compounds into water-soluble, excretable metabolites (Williams, 1959; Scheline, 1991; Sipes and Gandolfi, 1991).

The principal enzyme systems for metabolizing PSMs are located in the liver and gut wall (Smith, 1992), and they are characterized by an extremely low substrate specificity, in accordance with their function of metabolizing a virtually unlimited variety of substances. Thus most types of PSMs can be biotransformed, including terpenes, phenolics, steroids, and alkaloids.

Biotransformation has two phases and is similar for most vertebrates, although there can be important differences between species in specific reactions. Phase I reactions involve oxidation, reduction, or hydrolysis and introduce or uncover a functional group in the PSM. Phase II reactions are conjugations of the PSM or its phase I metabolite (via a functional group) and an endogenous molecule such as glycine, glutathione, or sulfate, or glucuronic acid. Phase I reactions usually make the PSM more polar, while phase II conjugation almost always produces a very water-soluble and highly ionized product. This greatly increases excretion by the renal or biliary–fecal routes, often involving active transport systems. The chemical change from PSM to metabolite(s) can also alter biological activity, but this is incidental to the increase in water solubility. Often toxicity is lost through metabolism, especially after conjugation, but in some cases the metabolite is more toxic than the parent PSM.

The best understood phase I enzymes are the P-450-dependent mixed-function oxidases (MFOs), which are located in the endoplasmic reticulum (Nebert and Gonzalez, 1987). Four P-450 gene families are considered to have evolved and diverged in animals during the past one billion years in response to their exposure to PSMs. About two dozen enzymes are involved, with overlapping substrate specificities. The types and amounts of P-450 present vary between and within species, and polymorphisms also occur. Glucuronide formation is a major phase II reaction, catalyzed by UDP-dependent glucuronosyltransferase, which has several forms leading to species differences in this reaction (Sipes and Gandolfi, 1991).

Glucuronosyltransferase is also located in the endoplasmic reticulum of the liver, kidney, gut wall, and other tissues, where it may be integrated with P-450 MFOs, enabling an efficient sequence of oxidation and conjugation. Most other conjugating enzymes are found in the cell cytosol. Large-molecular-weight (>350) glucuronides are secreted in the bile, while smaller ones are excreted in urine. Glutathione conjugates are also secreted in bile, while conjugates with sulfate, amino acids, mercapturic acid, and acetate are usually excreted renally.

Conjugation is a key process in our argument because, as well as making the metabolites water soluble, conjugation with glucuronide, sulfate, or amino acids can convert compounds that are neutral or weakly acidic into strong organic acids (Robinson et al., 1953; Scheline, 1991; Sipes and Gandolfi, 1991).

Some examples emphasize the importance of the process. For example, the pK_a of the terpene menthol is about 18 and of menthol glucuronide 3.7; the pK_a of benzoic acid is 4.2 and that of benzoyl glycine (hippuric acid) is 3.7. Significant species differences occur in these phase II reactions, largely related to the type of compound used for conjugation. For example, common brushtail possums (*Trichosurus vulpecula*) conjugate phenol with glucuronic acid, while carnivorous marsupials employ sulfate conjugation (Baudinette et al., 1980). However, the excreted products are strong organic acids.

Not all PSMs are prepared for excretion by being conjugated, and substantial quantities can be excreted in a nonconjugated form. For example, in common ringtail possums (*Pseudocheirus peregrinus*) fed *Eucalyptus radiata* foliage, only a minor proportion of leaf metabolites were excreted as conjugates. The bulk was excreted as polyoxygenated molecules (mainly dicarboxylic acids), which are highly polar and acidic (McLean et al., 1993). Presumably, these dicarboxylic acids are not conjugated because they are not sufficiently lipophilic to reach the site of glucuronosyltransferase in the endoplasmic reticulum membrane.

Other PSMs that may be ingested as acids are often sufficiently polar to be excreted without further metabolism. One of the best examples is salicylic acid, which in many species is largely excreted unmodified (Scheline, 1991). Whereas most alkaloids are bases, some have ester groups that can be metabolized to

acids (Harborne, 1991). Alkaloids generally occur in substantially lower concentrations compared with terpenes and phenols, and accordingly we expect their influence (whether basic or acidic) on acid–base metabolism to be minor.

In summary, the excretion of PSMs is aided by their metabolism to polar acidic compounds usually through phase II conjugation reactions but often through phase I reactions alone. Our thesis, therefore is that the processes of biotransformation and excretion of the vast majority of absorbed PSMs in mammals and birds result in the need to excrete a load of organic acids.

This means that once a compound has been absorbed and becomes a substrate for the MFO system, we can simply regard it as part of an organic acid load. Instead of being concerned with the effects of the native terpene or phenol, the effects may be viewed as those of a metabolically produced organic acid. We believe this is a useful rationalization because the effects of acid loads on animals is well studied and there are clear links between dietary acid loads and whole body protein and energy balance. This may allow nutritional ecologists to integrate better the effects of dietary PSMs with the traditional measures of dietary nutrients.

BUFFERING AND DISPOSAL OF AN ACID LOAD

All acid metabolites will be almost completely ionized at physiological pH (Robinson et al., 1953). Thus, the hydrogen ion and accompanying anion need not necessarily be disposed of together. The hydrogen ion can be either neutralized in the body by reaction with bicarbonate, buffered and retained in the body, or excreted in the urine as titratable acid. The accompanying anion is most often excreted into the urine by the organic anion transport system of the kidney and passed as ammonium or sodium salts. In the ensuing discussion of these processes, we focus on possible rate-limiting steps and, where appropriate, emphasize differences in mechanisms between species. We conclude the section with a discussion of the factors limiting the excretion of PSMs. A schematic illustration of the disposal of H^+ and organic anions is given in Figure 2 below.

Intracellular and Extracelluar Buffers

Ingestion or endogenous production of an acid results in a decline in plasma bicarbonate, which is the major extracellular buffer in mammals. The degree to which plasma bicarbonate concentrations are diminished depends on additional intracellular buffers, especially hemoglobin. In humans and laboratory animals, about half of an acute acid load can be titrated by intracellular buffers (Schwartz et al., 1957). Generally these processes are very rapid, occurring within 4–6 hr of an acute dietary acid load.

Following this initial buffering, respiratory adaptations become important.

Depletion of blood bicarbonate will eventually lead to changes in blood pH and therefore a fall in arterial P_{CO_2}. This in turn will tend to return blood pH to normal levels. Interestingly, the response in terms of plasma bicarbonate is in fact maladaptive (Madias et al., 1977) because hypocapnia induced by the respiratory response will decrease plasma bicarbonate beyond the level induced by the initial acidosis.

Buffering of Acid Loads by the Skeletal System

The skeletal system plays a role in the buffering of acid loads, but there is disagreement as to its importance under different conditions. Enormous reserves of alkaline salts are held in bone, and acidemia dissolves bone, releasing its alkaline, calcium salts. Bicarbonate is also leached from the active surface of bone and drives hydrogen ions into the bone matrix (Barzel and Jowsey, 1969).

Balance experiments over several weeks (Lemann et al., 1965, 1966) have shown that not all acid is excreted or buffered by intracellular or extracellular fluids. The remainder is believed to be buffered by the skeletal system. In humans made acidotic for six days, there was no progressive depletion of plasma bicarbonate, but a substantial excretion of calcium, magnesium, and phosphorus. These and other lines of evidence (Green and Kleeman, 1991) suggest that bone is a sink for hydrogen ions (protecting plasma bicarbonate stores) and the source of the extra urinary calcium. Although the skeletal system is accepted as a buffer of acid in the short term (days to months), there is less agreement about its role in long-term (three to five years) acidosis. Green and Kleeman (1991) suggest that skeletal sodium and potassium are more important in the defense against acute acidosis whereas efflux of calcium carbonate is augmented in the chronic acidotic state. Oh (1991) has argued that bone reserves of alkali salts are too limited to explain long-term responses, and this remains a controversial topic.

At least in the short-term chronic state, however, it is clear that metabolic acidosis leads to losses of bone calcium via an increase in urinary calcium loss. The mechanisms by which this occurs are not fully elucidated, but appear to involve an integrated response of the kidneys with an accompanying increase in the rate of bone resorption (Green and Kleeman, 1991). In lambs made acidotic with dietary ammonium chloride, no changes have been detected in the uptake of calcium from the gut (Abu Damir et al., 1991) even though urinary excretion was elevated.

Differences in the retention of calcium and phosphorus have been observed in lambs eating acid-promoting diets or which have been made acidotic by the addition of ammonium chloride to their food (Abu Damir et al., 1990). Mineral wastage in response to acidosis is important in domestic animal production, but we are aware only of anecdotal evidence to suggest that these effects are realized in browsing species. Robbins (personal communication) observed frequent bro-

ken bones in several species of deer that were maintained on browse diets in captivity but we can not be certain as to the cause. Although Pehrson (1983) and Reichardt et al. (1984) found that hares (*Lepus timidus* and *L. americanus*) were very often in negative calcium balance when fed browse, this was largely because of fecal calcium loss rather than urinary losses. Unfortunately, there are no simple markers of bone metabolism that reflect changes in acid–base status, but attempts have been made to use hydroxyproline as an index of bone mobilization during acidosis in domestic ruminants (Takagi and Block, 1991).

Given the central role played by the skeletal system in buffering acid loads, future studies of mineral balance in response to acid-loading, browse diets could be worthwhile. In such studies it will be important to distinguish between the excretion of endogenous and diet-related sources of calcium.

Excretion of Acid–Urinary Titratable Acid

Only a small percentage of the daily H^+ production can be excreted directly in the urine. In humans, dogs, and rats, the minimum urine pH is about 4.5. The limit to urine acidity is set by the gradient between blood and urine, and evidently the difference between blood hydrogen ion concentration and urine cannot be greater than 1000-fold. There is no reason to suspect that minimum urine pH is any different in wild species.

This being so, all animals need buffers so that the necessary acid load can be excreted in the urine. As with any buffer system, the capacity (in this case acid excretion) depends on the pK_a of the buffer and the total amount of buffer present. Buffers in the urine include organic anions such as citrate, acetate, hydroxybutyrate, and even creatinine (Hamm and Simon, 1987). However, all these have a $pK_a < 6$ and so can contribute little to hydrogen ion excretion over the physiological range of urine pH. Buffers are only effective one unit either side of the pK_a. In contrast, phosphate has a pK_a of about 6.8 (the exact value depends on a range of factors) and so is an ideal buffer at the typical pH values found in mammalian urine (Figure 1). At pH 7.4, 80% of phosphate is in the dibasic form (HPO_4^{2-}) and is capable of buffering hydrogen ions. At pH of 4.8, 99% will be in the monobasic ($H_2PO_4^-$) form. The bulk of the titration occurs between 6.8 and 5.8, so the phosphate can contribute to H^+ excretion at typical urine pH (Hamm and Simon, 1987).

Urinary excretion of phosphate is the major contribution to titratable H^+ excretion and changes in phosphate excretion alter urinary titratable acid (Scheiss et al., 1948). Acute dietary phosphate loading leads to a greater urinary titratable acid in direct proportion to the increase in phosphate excretion. However, this occurs because inorganic phosphate (P_i) stimulates hydrogen ion excretion, not because buffer excretion is a rate-limiting step for H^+ disposal (Gennari and Maddox, 1992). Similarly, animals that are P_i depleted retain part of an H^+

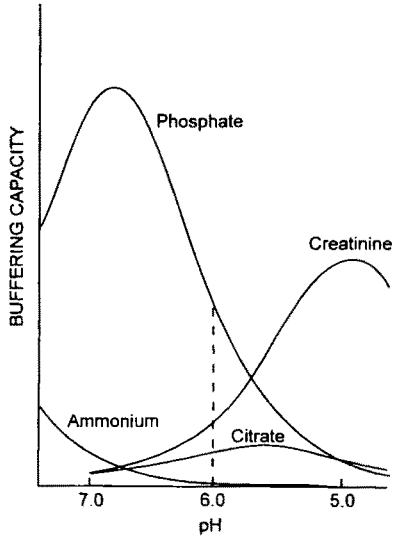

FIG. 1. Schematic representation of the relative importance of urinary buffers of H^+ in common ringtail possums. Concept and data on pK_a from Hamm and Simon (1987). Data for common ringtail possums from Foley (1992), Foley (unpublished), Johnson and Foley (unpublished). Note that ammonium is a poor buffer at typical urine pH and that phosphate is the predominant urinary buffer.

load, but again this is not because buffer excretion is limiting but because P_i depletion inhibits the secretion of H^+ ions in the distal tubules of the kidney. However, during metabolic acidosis in humans, phosphate excretion does increase and so does titratable acid excretion. The mechanisms involved are complex and interact with other factors affecting phosphate excretion, such as dietary phosphorus intake and parathyroid hormone (Hamm and Simon, 1987).

In summary, the excretion of titratable acid is an important route to dispose of excessive plasma H^+, but there is no evidence that acid excretion is regulated by changes in renal phosphate handling. An animal eating a diet that is rich in dibasic phosphate has the potential to excrete more titratable acid depending on how well that phosphate is absorbed. At present it is difficult to say how actively this is regulated.

Several studies of folivorous marsupials (Cork, 1992; Hume and Esson, 1993) and other herbivores (e.g., prairie voles, *Microtus ochragaster*) (Lindroth and Batzli, 1984) and ruffed grouse (*Bonasa umbellus*) (Jakubas and Guillon, 1991) have shown a correlation between diet choice and ratios of phenolics to nitrogen or phenolics to phosphorus in the foliage. Such correlations could be

explained by a need to maintain intakes of nitrogen and/or phosphorus sufficient to provide glutamine for production of new bicarbonate (see below) or for excretion of titratable acid in the urine. Cork and Foley (1991) and Cork (1992) suggested that ratios of nutrients to PSMs are important in relation to toxicity; if ratios are below thresholds, long-term utilization of a diet is not possible. Above the threshold, other factors might determine the selection and utilization of the diet.

Urinary Ammonium—The Measure of "New" Bicarbonate

The augmentation of urinary ammonium excretion is one of the most widespread responses to loading an animal with either exogenous or endogenous acids. For mammals, the major nitrogen-containing compound in the urine is typically urea; ammonium, although present, is a minor constituent. During acid loading, however, ammonium production is increased 10 to 20-fold and forms the dominant part of urinary nitrogen.

Until recently, the idea that ammonium was a urinary buffer of acid was firmly entrenched. Renal physiologists believed that since NH_3 diffuses into the lumen of the kidney tubules and then combines with a secreted H^+ ion, ammonium was in effect a buffer to convey acid out of the body (Pitts, 1964). There are two problems with this scheme. First, metabolism of amino acids gives rise to NH_4^+, not NH_3, so no matter how the resulting ammonium was changed in the body, urinary NH_4^+ could not represent any net transfer H^+ from the body (Atkinson and Bourke, 1987). This is shown below for alanine (equation 1).

$$CH_3-\underset{\displaystyle NH_3^+}{\underset{|}{CH}}-COO^- + 3O_2 \rightarrow 2CO_2 + HCO_3^- + NH_4^+ + H_2O \qquad (1)$$

Secondly, the pK_a of the NH_3/NH_4^+ buffer system is 9.2, so at physiological pH almost all the ammonia is in the acid form (NH_4^+) and no buffering could occur. It follows that ammonium does not in any way carry acid out of the body as many textbooks still claim. Nonetheless, ammonium does play a very special role in acid–base regulation. To understand how this is so, we need to consider the biochemistry of ammoniagenesis (Figure 2).

Urinary ammonium arises from the metabolism of glutamine (and to a lesser extent alanine and asparagine) in the cells of the proximal kidney tubules. Glutamine extracted from circulating plasma is metabolized to NH_4^+ and α-ketoglutarate. The α-ketoglutarate is then metabolized to generate HCO_3^-. The ammonium, after a somewhat elaborate transport process, ends up in the kidney's collecting duct. Therefore, each molecule of ammonium ion that appears in the urine represents a process that also generates one molecule of bicarbonate. If the ammonium from glutamine had been transported back to the liver, it

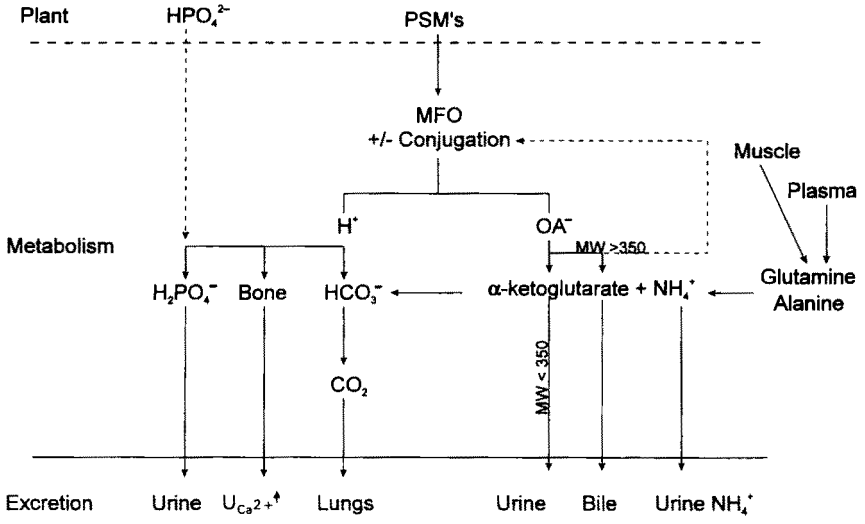

FIG. 2. Pathways of disposal of organic acids produced during the biotransformation of phenols and terpenes in mammals. Note the relationship between the urinary excretion of ammonium and the formation of "new" bicarbonate.

would have been converted into urea (equation 2):

$$2NH_4^+ + 2HCO_3^- \rightarrow H_2N{-}CO{-}NH_2 + CO_2 + 3H_2O \qquad (2)$$

This equation shows that urea formation consumes bicarbonate, and the overall process would have made no change to acid–base status. If, instead, the ammonium is excreted in the urine, then the new bicarbonate from the metabolism of α-ketoglutarate is available to titrate part of the acid load. Therefore, urinary ammonium excretion does not represent H^+ excretion per se, but it is an index of the amount of "new" bicarbonate produced and hence the amount of the acid load that is neutralized in the body.

There are several limitations to the excretion of ammonium as a metabolic response to acidemia that are worth noting. First, it takes some days for ammoniagenesis to reach full capacity. In humans and laboratory animals, this lag time between an acid load and maximum ammonium excretion is on the order of three to six days (Halperin et al., 1992). This means that if an H^+ load is to be produced every day during this adaptation period, then it must either be buffered within the body until there is sufficient ammoniagenesis or else excreted as titratable acid.

The other limitation of renal ammoniagenesis as a counter to metabolic acidosis concerns damage to the kidney that occurs during prolonged periods of

elevated ammonium production (Halperin et al., 1989). These disorders include nephrocalcinosis. Finally, circulating glutamine may be insufficient to meet the demands of ammoniagenesis and additional glutamine may have to be derived from muscle protein catabolism. We will discuss this final point in more detail later.

Renal Organic Acid Secretion—An Active and Saturable Process

Thus far we have mainly discussed the excretion of hydrogen ions arising from an endogenous acid load. Recall that, at physiological pH, organic acids will be almost completely ionized, and so disposal of the hydrogen ion is only part of the solution. It remains to dispose of the organic anion as well.

The kidney plays a major role in eliminating metabolites of biotransformation processes from the body. Many organic metabolites can be filtered from the blood and so become part of the excreted urine. However, of particular importance is the secretion of organic anions by the organic acid transport system of the kidney. There has been a large amount of research to elucidate the nature and controls on these processes (reviewed in Moller and Sheikh, 1983).

The renal clearance rate should be highest for secreted substances, and it can equal renal plasma flow when there is no reabsorption from the kidney tubules, which should be the case for most conjugates. In contrast, clearance by glomerular filtration alone will be much lower even when there is no reabsorption because only 20% of plasma is filtered.

One important feature of the renal transport system is the wide variety of substrates that are accepted—the number of compounds is unlimited and includes many (but not all) glucuronides, hippurates, mercapturic acids, dicarboxylic acids, and hydroxy acids of both endogenous chemicals and metabolites of xenobiotics. As a general rule, conjugated metabolites are far more likely to be actively secreted than their parent compounds (Caldwell, 1982). A good example is the amino acid conjugates of aromatic acids; hippuric acid is actively secreted while benzoic acid is not. Conjugates are also far less readily reabsorbed from the kidney tubule than are the free acids because of their greater polarity and lower lipophilicity (Caldwell, 1982).

Early studies show that secreted anions were capable of competitively inhibiting the secretion of other anions, and so organic acid transport was believed to be a single system (Pritchard and Miller, 1992). This concept is important to our arguments because it suggests that the system can be easily saturated and, in these circumstances, the capacity of the organic anion transporters may set some limit on the speed with which the body can rid itself of organic anions formed by biotransformation of PSMs.

Whether or not there is a single renal organic acid transport system has bearing on our argument that biotransformed PSMs can be seen to have a com-

mon effect. If saturation of the organic anion transport system does constitute a rate-limiting step to the disposal of acid loads, then it is of little importance whether the anions are derived from the PSMs of one plant or another. In other words, animals choosing a mixed diet should not be advantaged necessarily by different pathways of biotransformation unless the cosubstrates of elimination (e.g., glutathione, glycine) are in short supply.

Ullrich and Rumrich (1988) questioned the notion of a single organic anion transport system and have revealed at least three separate organic acid transporters. One system handles oxalate (and sulfates), a second handles dicarboxylates, while the third was elucidated with paraamino hippurate and so corresponds to the classical model of organic anion transport.

Puzzlingly, some mammals (e.g., common ringtail possums) excrete ingested terpenes as dicarboxylic acids rather than as conjugates of glucuronic acids, as might be expected (but see above). However, it is unlikely that this is to avoid saturating the main transport system. The dicarboxylate anion produced by common ringtail possums are all 10-carbon compounds derived from monoterpenes, and the dicarboxylate acid transporter elucidated by Ullrich and Rumrich (1988) is restricted to compounds of five to seven carbons. We would not anticipate many PSM metabolites to be less than 10 carbons, and so it seems unlikely that much capacity is spared by the use of these two alternative organic anion transports. Sulfate conjugates, however, may be able to be excreted via an alternate transporter.

Renal organic anion transport is highly complex, and there are few data of relevance to whole animal metabolism of PSMs. However, in spite of the recent research on alternative transport pathways, it seems reasonable to continue with the classical view of regarding organic anion excretion as a saturable system whose capacity may impose a rate-limiting step on the excretion of anion loads from the body.

Differences between Species—Sodium Wasting in Rabbits

Often, animals are divided into those that excrete net acid in the urine and those that excrete net base. Digestive physiology and the particular metabolism of a species affects whether it will be an acid excreter or a base excreter. The focus of our argument is that some diets may often have an overriding effect on this, but metabolic differences between species can mediate dietary effects.

In this context, the acid–base metabolism of lagomorphs is of particular interest. Many lagomorphs eat diets that expose them to significant levels of absorbable PSMs, and studies of domestic rabbits indicate that most of these are metabolized to acid end products (Williams, 1959; Scheline, 1991). Thus we might expect lagomorphs to display the responses seen in species such as common ringtail possums to endogenously produced acid loads. However,

domestic rabbits cannot normally augment urinary ammonium excretion in response to acid loads (Richardson et al., 1978) and so, even during severe metabolic acidosis, urinary ammonium remains at low levels. In contrast to laboratory rats at least, rabbits convert a greater proportion of dietary components into bicarbonate in the gastrointestinal tract, and this bicarbonate is then absorbed. Consequently, it is possible that rabbits are rarely, if ever, faced with the problem of generating new bicarbonate in the kidneys and so have no need to excrete substantial quantities of ammonium in the urine. Furthermore, there is some evidence (Cruz-Soto et al., 1982) that early exposure to acid-loading diets may alleviate this metabolic defect in domestic rabbits. Thus, browsing lagomorphs may be better able to augment urinary ammonium than their grazing kindred.

To our knowledge, there are no data on net acid excretion (or even urine pH) of any lagomorph fed browse diets. It is difficult, therefore, to evaluate whether rabbits are essentially immune to acidemia resulting from biotransformation processes. Nonetheless, the metabolism of absorbed PSMs will still result in the creation of organic anions and, even if the H^+ formed is neutralized by bicarbonate absorbed from the gastrointestinal tract, the animal must still excrete the organic anions in the urine.

These organic anions must be accompanied by some cation and, in the absence of ammonium, a highly probable alternative cation is sodium or potassium. Consistent with this suggestion is the observation that several species of lagomorphs, when fed browse or browse extracts, excrete substantial quantities of sodium in the urine (Pehrson, 1983; Reichardt et al., 1984; Iason and Palo, 1991) sufficient to ensure that they are in severe negative sodium balance for the duration of the browse diet. In addition, Iason and Palo (1991) found that mountain hares (*Lepus timidus*) lost significantly less sodium in the urine than did grazing European hares (*Lepus europaeus*) when both were fed browse.

Normally urinary losses of sodium are closely regulated by the renal system. The kidney would be expected to reabsorb sufficient filtered sodium to prevent a negative sodium balance unless that loss was obligatory. Pehrson (1983) suggested that negative sodium balance in mountain hares could occur through one of two mechanisms. Sodium imbalance could be due to the effects of a high dietary potassium intake. However, there is no evidence that the levels of potassium in any of these diets was notably high. Pehrson's second hypothesis was that many browse plants contain (unspecified) compounds that lead to sodium wasting.

We suggest that in lagomorphs, sodium is excreted in the urine in amounts equivalent to urinary excretion of organic anions and that the loss is therefore obligatory. In other words, organic anions arising from biotransformational reactions are excreted as sodium salts in lagomorphs, whereas in most other species they are excreted as ammonium salts.

However, there are alternative explanations that need to be explored. Most of the experiments in which sodium wastage has been observed were of short duration, and it may be that the sodium loss is not sustained in the longer term. In other species, urinary sodium is often elevated in the early days of a metabolic acidosis because it takes several days for renal ammonium excretion to be elevated (Halperin et al., 1992). It should also be noted that changes may have occurred in the animals' extracellular fluid volumes, and this alone may have been sufficient to account for the extra urinary sodium.

EFFECT OF ACIDEMIA ON ANIMAL METABOLISM

Disturbances of acid–base status have wide-ranging effects on biochemical and physiological processes of animals (Hills, 1973; Gennari and Maddox 1992). Very often the effects of acute and chronic acidosis differ, and so it is important to consider which state prevails in each instance. However, metabolic acidosis has wide-ranging effects on organ systems and whole body homeostasis. In particular, effects on the respiratory system, the cardiovascular system, and the skeletal system have been described. There are pronounced effects on carbohydrate metabolism (e.g., an impaired insulin response) (De Fronzo and Beckles, 1979) and on protein metabolism. All these may involve some compensatory response from the animal. However, from an ecological perspective, the effects of most interest are those on protein and nitrogen metabolism.

Protein Metabolism

We have described above the mechanisms that allow the rapid augmentation of urinary ammonium during acidosis. Urinary ammonium is derived largely from plasma glutamine and, to a lesser extent, alanine. The key question involves the source of these ammoniagenic amino acids. Previously, it was thought that there was a quantitative switch between urea excretion and ammonium excretion during acidosis so that total urinary nitrogen excretion remained constant (Hills, 1973). However, there is now clear evidence that in many species and under different conditions, this simple switch from urea to ammonium does not occur and that during acidosis urinary ammonium excretion is increased without changing net urea production. For example, in calves (Scott et al., 1971), red deer (*Cervus elaphus*) (Scott, 1971a), and pigs (Scott, 1971b) ammonium excretion was elevated during infusions of hydrochloric acid, but there was little change in urinary urea excretion.

In common ringtail possums, Foley (1992) found that animals excreting an acid urine lost more nitrogen at similar nitrogen intakes than animals excreting an alkaline urine. These possum data are confounded by the different species of leaf offered to produce the different excretory patterns, and so it is not certain

that the effects observed in other species during acid infusions occur during diet-induced acid loading. An increased loss of urinary N would be a clearly measurable cost of biotransformation and could be linked directly to measures of nitrogen requirements and the suitability of different diets.

When dietary protein levels are adequate, glutamine required for urinary ammonium can be met from circulating plasma glutamine. However, the diets consumed by browsing mammals often contain only maintenance levels of protein. When humans and laboratory animals are acidotic and are consuming insufficient dietary protein, their is insufficient plasma glutamine to meet requirements for urinary ammonium (Hannaford et al., 1975).

The maintenance of acid–base homeostasis is of such importance that animals may break down skeletal muscle to provide the necessary glutamine for ammoniagenesis. Skeletal muscle can supply the necessary glutamine in the short term, but clearly extensive muscle wasting can take place if the acidemia is prolonged and if energy balance is negative. This is why obese humans lose valuable body protein as well as body fat during starvation. The ketoacidosis that develops requires them to consume their muscle to provide for urinary ammonium (Halperin et al., 1992).

Chronic acidosis in children (Cooke et al., 1960) and rats (Williams et al., 1991) can lead to retarded growth. That the growth retardation can be corrected by giving sodium bicarbonate confirms that the effects are linked to the effect of acidosis on whole body protein metabolism. Ruminant herbivores fed silage or grain diets are particularly susceptible to acidosis, and a review of a number of studies suggests that addition of sodium bicarbonate spares body protein and improves nitrogen balance (Phillip, 1986).

In conclusion, there is growing evidence in a variety of species that metabolic acidosis changes the balance between body protein synthesis and breakdown. Studies with rats suggest that these effects are mediated by glucocorticoids (May et al., 1986), but the exact mechanisms have yet to be determined (Reaich et al., 1992).

Recycling of Endogenous Urea

In most herbivores, urea is not a waste product but a valuable source of nitrogen that can be recycled back to the gut and used by microorganisms for growth. It is likely that all mammals recycle a substantial proportion of endogenously produced urea and incorporate the nitrogen into microbial protein, but only foregut fermenting herbivores and coprophagic species can use the microbial protein as a source of essential amino acids. Therefore, the diminution of urea production and recycling could be deleterious to the protein metabolism of these species. The extent of urea recycling is most closely related to rates of fermentation in the gut (Kennedy and Milligan, 1980), but during acidemia,

urea production is substantially reduced and the quantity recycled may be independent of fermentative activity.

Foley (1992) showed that, in common ringtail possums, animals fed on foliage diets that led to net acid excretion (*Eucalyptus radiata*) synthesized and degraded in the gut far less urea than animals fed foliage that resulted in net alkali excretion (*E. ovata*)—even though the nitrogen intake of each group was the same (Figure 3).

If urea recycling is as important to the nitrogen economy of marsupial folivores, as Chilcott and Hume (1984) have argued, then clearly the consumption of acid-producing plants can have a significant impact on the process.

Ability to Concentrate Urine

Many animals fed high protein diets are able to concentrate their urine more than other animals (Jamison, 1981). This has been attributed to an effect of urea in the urinary concentrating process. This accumulation of urea in the interstitium of the kidney medulla allows a greater concentration gradient can be reached than possible when urea is absent. Most folivores eat low protein diets but, in the absence of acidosis, urinary urea concentrations can be substantial (Cork, 1981; Foley and Hume, 1987; Foley, 1992). In cases where acidosis leads to a rapid cessation of urea production in favor of ammonium production, acidotic animals may be unable to concentrate their urine as well as can those in the nonacidotic state. Controlled experiments are needed to evaluate whether the magnitude of the effects could mean a significantly greater water demand for animals during acidemia.

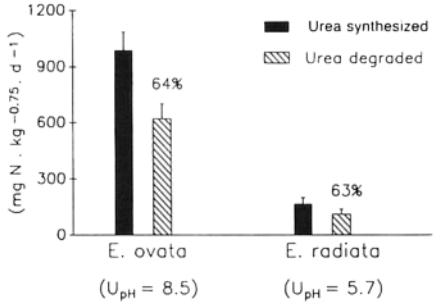

FIG. 3. Effect of diet on urea synthesis and urea degradation in common ringtail possums fed two foliage diets, *Eucalyptus radiata* and *Eucalyptus ovata*. Note the interrelationships between diet, urine pH and urea synthesis and degradation. The percentage value is the proportion of urea synthesized which was degraded in the gut (Foley, 1992).

CAN ACID-BASE HOMEOSTASIS PROVIDE FEEDBACK TO REGULATE FOOD INTAKE?

We have shown that in arboreal marsupials, at least, feeding on diets rich in absorbable PSMs leads to a large organic acid load that must be excreted. The H^+ ions are clearly diet-related and are a consequence of the metabolism and biotransformation of foliage PSMs (Foley, 1992). Given that the maintenance of acid–base homeostasis is among the most important regulatory necessities of any animal, we argue that factors that promote acid production must be controlled so that the capacity to buffer and excrete acids is not exceeded.

We have already discussed the mechanisms that allow animals to buffer and excrete acid loads from the body very rapidly. Although these processes are not a universal panacea (see discussion above on ammonium excretion), the immediate control problem is to prevent the capacity of these buffering and excretory systems from being exceeded. What then are the possible mechanisms that might provide an appropriate signal for the animal to increase hydrogen ion excretion?

Changes in Systemic pH

It is tacitly assumed that a change in systemic pH is the signal needed by the kidney to increase urinary acid excretion (Tannen, 1980). This is a controversial area because important experiments by De Sousa et al. (1974) showed that administration of some acid loads did not lower the blood pH or plasma bicarbonate content of dogs. Doses of hydrochloric acid led to a prolonged decrease in blood pH and plasma bicarbonate, whereas nitric acid had no detectable effects. These results were important in formulating a theory of H^+ excretion that is based on the nature of the accompanying anion. They suggested that since the nitrate ion is poorly reabsorbed, it will be delivered rapidly with endogenous sodium to the distal nephron, whereas anions such as chloride will be largely reabsorbed and reenter the circulation. For example, Bank and Schwartz (1960) showed that sodium-depleted subjects excreted acid urine when infused with sodium sulfate, but that sodium chloride had little effect on urine acidity.

Although these ideas appear to be largely correct, a repeat of the experiments by Madias and Zelman (1986) showed that the failure of De Sousa et al. (1974) to observe declines in blood pH and plasma bicarbonate was because they collected only a single blood sample 24 hr after acid loading. In fact, more frequent sampling showed that animals fed nitric acid did show a decrease in blood pH and plasma bicarbonate but that the effects were only transitory. It would seem, therefore, that although the anion that accompanies the hydrogen ion is an important mediator of the length of acidosis, changes in blood pH cannot be excluded in urine acidification.

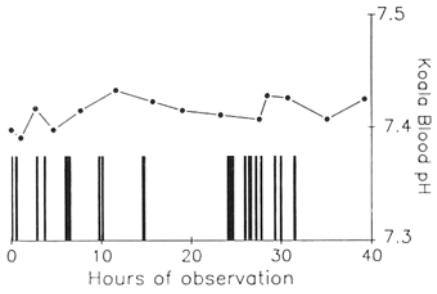

FIG. 4. Arterial blood pH in relation to feeding on *Eucalyptus robusta* by a koala. The vertical bars show duration of feeding bouts. Blood was drawn from an in-dwelling catheter in the femoral artery and analysed within four minutes (Foley and Handasyde, unpublished).

Nonetheless, this does not necessarily mean that systemic pH exerts control over renal acid excretion (Tannen, 1980). The types of anions derived from the biotransformation of absorbed PSMs are, like nitrate, likely to be poorly reabsorbed. In fact, as we have described above, many are secreted by the kidney. Thus any acidemia that results from the accumulation of organic acids, such as after a feeding bout, may itself be only transitory. There are few data to support or refute this suggestion. Foley and Handasyde (unpublished) measured the arterial acid–base status in relation to feeding bouts in a pilot study with koalas (*Phascolarctos cinereus*). Data for a single animal are shown in Figure 4. During these experiments, the animals were excreting urine of pH 5.3–5.5, which contained substantial concentrations of ammonium but the variation in blood pH and plasma bicarbonate were only minor. In addition, there was no relationship between blood pH or bicarbonate and the cessation or intensity of feeding bouts. Although these data are very limited, they suggest that changes in blood pH during the ingestion of browse diets are only small and not involved with the regulation of renal acid excretion or feeding.

Changes in Arterial P_{CO_2}

Changes in the arterial carbon dioxide tension (P_aCO_2) have a direct effect on renal hydrogen ion secretion. High P_aCO_2 depresses renal hydrogen ion secretion whereas the reverse is true under conditions of low P_aCO_2. These effects occur even within the normal day-to-day variation in P_aCO_2, but it is generally believed that they do not mediate routine adjustments in renal hydrogen ion excretion (Gennari and Maddox, 1992).

Aldosterone

The mineralocorticoid aldosterone has been implicated as a controller of renal hydrogen ion excretion because it stimulates hydrogen ion secretion in the distal nephron. An aldosterone deficiency directly depressed hydrogen ion secretion and results in a sustained metabolic acidosis. Whether or not the effects are sufficient to account for day-to-day regulation in acid excretion is uncertain because animals without adrenal glands and given constant doses of mineralocorticoids can respond to acid loads normally (Gennari and Maddox, 1992).

Potassium Status

Changes in potassium supply affect an animal's ability to produce ammonium in the kidney. In laboratory animals and in humans, ammoniagenesis is stimulated by deficiencies of potassium and depressed by potassium loading. However, the differences in ammonium excretion are countered by changes in titratable acid excretion, and there is no change in net acid excretion. Therefore it seems that the potassium status of the body is unlikely to play a major role in controlling renal acid excretion (Tannen, 1980; Gennari and Maddox, 1992).

CONCLUSION

In spite of widely varying daily acid loads that animals can encounter, plasma bicarbonate and systemic pH are kept within very narrow ranges in all homeotherms. A major question is whether these effects are sufficiently great that animals need to regulate their diets in order to control acid production. Humans eating typical diets and with normal renal function probably do not need to control their diets. However, the acid loads of mammals eating diets rich in PSMs are substantially greater. Any theory of food intake must invoke some sort of feedback signal so that intake can be modified, but at present, simple factors such as systemic pH cannot be invoked as part of the feedback process. A similar conclusion has been reached in the study of acid–base effects on silage intake in ruminants (Philip and Simpson, 1991). Nonetheless, even if the regulatory process is not known, it is clear that acidemia is a major consequence of the ingestion of diets rich in PSMs.

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A TEMPERATURE-DEPENDENT MODEL FOR PREDICTING RELEASE RATES OF PHEROMONE FROM A POLYETHYLENE TUBING DISPENSER

S.J. BRADLEY,[1] D.M. SUCKLING,[1,*] K.G. McNAUGHTON,[1] C.H. WEARING,[3] and G. KARG[1,4]

[1]*Hort Research*
PO Box 51, Lincoln, New Zealand
[2]*Hort Research*
PO Box 11 030, Palmerston North, New Zealand
[3]*Hort Research*
Clyde Research Centre, RD 1
Alexandra, New Zealand

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Abstract—A model was developed to describe release of two formulations of tetradecenyl acetates in Shin Etsu polyethylene tubing pheromone dispensers. Change in pheromone column length in the polyethylene tubing was modeled by $dl/dt = l \cdot (a + bT)/dt$ (where l is column length, t is time interval, and T is average temperature for that time interval). Regression of the natural log of relative change in liquid length against time interval and accumulated day degrees in orchards produced the coefficients a and b. The model was validated by comparing predicted and actual liquid length remaining in field-aged dispensers, as well as measuring release rates by using gas chromatography. Mean daily orchard temperatures and measurement of column length of dispensers on a single date were used to accurately predict column length within 15%, after six months. Predictions of instantaneous release rate were also made from this model, and other possible model uses and limitations are discussed.

Key Words—Pheromone, polyethylene dispenser, release rate, model.

INTRODUCTION

Mating disruption, achieved by releasing relatively large quantities of female insect sex pheromone, is becoming an important pest control technique (Cardé,

*To whom correspondence should be addressed.
[4]Present address: Department of Animal Ecology, University of Lund, 223 62 Lund, Sweden.

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1990). A wide variety of different types of dispensers for releasing these chemicals have been developed. Unfortunately, many have a short field life and are difficult to apply, release rates are often inconsistent, and the quantity of remaining pheromone is difficult to measure. In these cases, it is difficult to estimate release rates without the use of a gas chromatograph and controlled laboratory conditions (Brown et al., 1992).

Dispensers consisting of sealed polyethylene tubes containing wire fasteners have useful release characteristics for a range of pheromones (Sexton, 1989) and are finding increasing acceptance, especially for orchard use (Suckling, 1993). Their advantages include ease of application, without the need for any specialized equipment, simple construction (reducing the cost), and the potential for observing the loss of pheromone over the season by visibility of the remaining liquid. This latter attribute assists in determining the optimum replacement time. Their disadvantages include a logarithmic decline in release rate and differing release rates for different pheromone components, features they share with many other types of dispensers.

Sealed polyethylene dispensers have been studied in New Zealand orchards since 1984 (Suckling et al., 1990). Release rates have often been presented during these trials as weekly means, deduced from weekly change in liquid length. Knowledge of daily release rate would be valuable for understanding trial results, but changes in liquid length could not be measured reliably and precisely in the field over intervals shorter than one week. Estimation of release rates at a given point in time would be useful for interpretation of field trial results and could provide an indication of the probability of a mating occurring on a given night. We saw the need for a simple model, based on field data and remaining liquid length, to permit prediction of instantaneous pheromone release rates. As mating disruption of six leafroller species is being investigated in New Zealand, we also required a model where coefficients for different pheromone blends could be obtained with relative ease.

Many authors have produced descriptive models of pheromone release rate as a function of time or day degrees (Audemard and Leblon, 1987; Ioriatti and Dalla Serra, 1992; Neuman, 1992; Charmillot and Pasquier, 1993), but they have not proceeded to calculate instantaneous release rates, describe the mechanisms involved, or predict release rate under a variety of conditions. Two recent exceptions to this are the models of McDonough et al. (1992) and Brown et al. (1992), for codling moth dispensers, which, like the dispensers described here, were hollow plastic tubes filled with pheromone and sealed at both ends.

The Model. A simple physical model for pheromone release from the Shin Etsu polyethylene tubing dispenser was developed by using the observation that pheromone release from these dispensers is relatively independent of wind speed (T. Bellas personal communication and unpublished data). Freedom from wind effects implied that the resistance to transfer of pheromone through the walls of

the dispenser limited the rate of release, as opposed to the boundary-layer resistance of the air surrounding the dispenser. Furthermore, since radial resistance is large, it was inferred that very little diffusion of the contents occurred along the dispenser above the capillary. Therefore it was assumed that the active length of the dispenser was essentially that occupied by the liquid, l (millimeters). This was later tested (see below).

Our model proposes that the rate of pheromone release is proportional to the length of the capillary and the transfer conductance of the wall of the plastic dispenser. It was assumed that the same conductance is appropriate for all components of the pheromone mixture since the components in our tests were of similar chain length, polarity, and saturation. The conductance of the dispenser walls probably varies with temperature, so the conductance can be written as $g(T)$, but the exact form of the relationship is unknown. Ideally $g(T)$ would be found from laboratory studies. Whatever its exact form, $g(T)$ can be represented as a Taylor series $g(T) = a + b(T - T_m) + y(T - T_m)^2 + \ldots$, where the expansion is about a mean operating temperature, T_m. Here it is assumed that linear terms are sufficient to represent the function over the seasonal range of temperatures. That is, we propose a relationship of the form

$$\frac{dm}{dt} = l \cdot (a + bT) \tag{1}$$

where m is the mass of pheromone released, T is air temperature, and a and b are unknown constants. The mean temperature term has been absorbed into the constant a. In this paper a and b are found by regression using field data. For this we present equation 1 in integral form.

The mass of pheromone in the dispenser is proportional to the length of capillary, so the symbol m in equation 1 can be replaced by l. This gives a simple ordinary differential equation, which can be solved by separation of variables to give

$$\ln\left(\frac{l_0}{l_1}\right) = a(t_1 - t_0) + b\int_{t_0}^{t_1} T\,dt \tag{2}$$

where the first term is the time interval between readings, Δt, in days, and the second is the temperature sum accumulated between readings in degree days. This can be written in simpler notation as

$$\ln\left(\frac{l_0}{l_1}\right) = a \cdot \Delta t + b \cdot DD_0 \tag{3}$$

and used as a regression model. The coefficients a and b can then be found by fitting this model to field data. With these, equation 1 can be used to calculate

instantaneous rates of change of capillary length. Rates of pheromone mass release are then obtained using the capillary diameter and the pheromone density.

METHODS AND MATERIALS

Dispensers. Both dispensers were produced by Shin Etsu Chemical Co. (Tokyo) and consisted of 200 mm lengths of sealed polyethylene tubing, with ca. 1.4 mm outer diameter, 0.8 mm internal diameter, and 0.3 mm wall thickness (schematic diagram in Figure 5 below).

Dispensers for the light brown apple moth, *Epiphyas postvittana* Walker (LBAM), contained 54.9 mg (*E*)-11-tetradecenyl acetate (*E*11–14:OAc), 2.5 mg *E*9, *E*11–14:OAc, 19.7 mg *Z*11–14:OAc, 8.7 mg of stabilizers, and 3.1 mg of other (unknown) additives. To use this model for the mass of pheromone release, it was necessary to multiply the column length by the amount of pheromone per millimeters of liquid length. Initial liquid length was 170 mm, resulting in 0.337 mg pheromone/mm liquid length. A dispenser for the greenheaded leafroller, *Planotortrix octo* Dugdale (OCTO), contained 58.7 mg *Z*8–14:OAc, 19.6 mg *Z*5–14:OAc, 3.5 mg of stabilizers, and 6.2 mg of other additives. This yielded 0.460 mg pheromone/mm capillary length.

Dispenser liquid was shaken to one end, the tubing straightened, and suspended by thin wire at ca. 1.5 m in apple or shelter trees. Dispenser series consisted of liquid length measurements of 2–10 dispensers at 3–5 day intervals (mostly weekly). Dispenser liquid lengths were measured to the nearest 0.5 mm with a clear plastic ruler.

Twenty dispensers were also wrapped around small branches of apple trees in the Millbrook orchard, following normal commercial procedure. On June 3, 1993, 119 days after application, these dispensers were removed from around tree branches, straightened, and the liquid shaken down to a single column, which was then measured. These column lengths were later compared with those of dispensers that had been suspended and measured weekly for modeling.

Locations and Climate. Two orchard sites were used for the study of LBAM dispensers in Canterbury. The sites were Stafford Orchard (S43°34′E172°31′), which was well sheltered, and Millbrook Orchard (S43°36′E172°25′), which was less sheltered and more typical of Canterbury. Canterbury's climate and temperature are strongly correlated with wind direction, with sudden temperature changes associated with change in wind direction, and mean daily variation often not being sinoidal (Ryan, 1987). Summer temperatures are often greatly affected by a strong sea breeze. Data loggers (model CR10, Campbell Scientific, Logan, Utah) at both of these orchards recorded hourly temperatures from temperature probes placed under the canopy at 1.5 m. Daily maximum and minimum temperatures were obtained from hourly data.

The effect of temperature on release rate was studied in Central Otago at two sites for both LBAM and OCTO dispensers. The Clyde Research Centre (S45°12'E169°18') data logger (LI-1000, Li-Cor Inc., Lincoln, Nebraska) was placed in an existing open site meterological station, while the measured dispensers were in a nearby well-sheltered orchard block. The second data logger was at an orchard in Ettrick (S45°38'E169°22'), where dispensers were placed in rows of apple trees alongside the logger (model CR10, Campbell Scientific). Dispensers were also measured in three Dumbarton orchards 4 km away. Central Otago is characterized by greater seasonal extremes of temperature in summer and winter, reminiscent of a more typically continent climate, compared to Canterbury (Anon., 1980).

Analysis. Change in liquid column length, in the first week was not used, due to initial erratic patterns of release (Suckling and Shaw, 1990; McDonough et al., 1992). The multiple linear regressions were conducted by using Quattro Pro (Borland International, 1992).

Validation Experiments. Gas chromatography was used to investigate the relationship between the liquid column length and the instantaneous release rates of major components in the two dispensers. Thirty-three of each type of dispenser were suspended from one end in trees along the edge of a field in Lund, Sweden. Between August 28 and October 8, 1993, the liquid length was measured and a sample of three dispensers of each formulation was collected and frozen at −20°C until measurement of release rates. Sample dates were August 25, 26, 28, and 31; September 4, 10, 17, and 28; and October 8.

Intantaneous rates were measured after the dispensers had been removed from the freezer and suspended in the back of a fume hood for 24 hr. The dispensers ($N = 3$) were then individually suspended in a 250-ml round-bottom glass flask (Bengtsson et al., 1994) at 22°C for 1 hr, which had been shown to be much shorter than the time required for saturation of the flask (ca. 24 hr). The flasks were washed three times with redistilled heptane (5 ml altogether), and an internal standard of 10 μl of saturated tetradecenyl acetate (or dodecenyl acetate for native leafroller dispensers) was added. For quantification, we used a Hewlett Packard 5890 A gas chromatograph with a DB-Wax column and flame ionization detector, programmed at 80°C initial temperature for 2 min, followed by a rise to 230°C at a rate of 10°/min. The carrier gas was hydrogen at 30 kPa.

A further experiment was conducted to investigate the extent of capillary action, whereby pheromone enters the wall of the dispensers above the meniscus. We examined the pheromone present in the walls of aged LBAM dispensers ($N = 3$) as a function of distance from the main well, which contained 45 mm of liquid. Short sections of dispensers (10 mm) were cut at varying distances from within the pheromone column (0–10 mm) or towards the distal end (57.5–67.5 mm, 105–115 mm, 147.5–157.5 mm, and 190–200 mm). The pheromone

contents (if any) were removed by physical cleaning. The sections were then placed in 1 ml of distilled heptane and agitated for 24 hr before analysis as above.

RESULTS

Estimating Coefficients. Repeated application of the model on different data sets showed that the coefficient a, the model intercept, ranged from -0.00104 to 0.00244 for significant LBAM regressions (Table 1) and from -0.00141 to 0.00008 for significant OCTO regressions (Table 2). The model slope, b, was less variable ranging from 0.00014 to 0.00049 for LBAM (Table 1) and from 0.00028 to 0.00054 for OCTO (Table 2). The correlation coefficient, r^2, showed a wide range. Data points from all series that produced significant regressions were regressed together to give parameters used in the validation of the model, and the predictions of release rate compared with the actual data.

General Validation of the Model. The parameter estimates with greatest correlation coefficients for each region and for each dispenser type were used to determine how well the model described actual release rates (Figure 1). The initial length put into the model must be recorded a week or two after application to avoid the initial period of erratic release. An effect of compounding of error in the estimate of the volume of remaining pheromone can result in inaccuracy later in the season, although this can be readily solved by input of additional readings during the season. The size of any accumulated error of column length appears to be relatively minor in terms of the magnitude of effect on predicted release rate (15% below the actual release rate after six months). Predictions from hourly, daily, and weekly mean temperatures closely followed actual column lengths (Figure 1), although the daily maximum–minimum temperatures caused a slight underestimate in release rate.

Figure 2 shows how the use of the model can yield far more information than weekly column loss, which only provides weekly average release and may contain measurement errors. The modeled release rate follows the weekly release rate based on the measured losses in column length.

Effect of Measurement Interval and Experiment Duration. The longest series of observations (i.e., highest number of sampling occasions) for the two dispensers gave better correlations in the model, although the correlation did not change predictably with the shorter series (Tables 1 and 2). Some of the shorter series gave significant correlations (Tables 1 and 2). There was a difference between the experiment duration required between regions, and the Canterbury example indicates that at least six months should be studied to give a significant correlation. This period is similar in length to a typical growing season and would give a good variety of weekly day-degree accumulations.

TABLE 1. REGRESSION STATISTICS FROM MULTIPLE LINEAR REGRESSION OF LN (RELATIVE CHANGE IN COLUMN LENGTH) AGAINST TIME INTERVAL AND ACCUMULATED DAY-DEGREES FOR LBAM PHEROMONE DISPENSERS

Site and series	Dispensers out	Regression period	Distance (m) to data logger	r^{2a}	*df*	*a*	*b*
Stafford							
1	18 Nov	1 Dec–3 May	50	0.69***	23	0.00090	0.00047
2	18 Dec	30 Dec–3 May	20	0.25*	16	0.00076	0.00034
3	21 Jan	4 Feb–3 May	10	0.28*	11	0.00159	0.00030
4	11 Feb	25 Feb–3 May	10	0.03 NS	8	0.00550	0.00002
Millbrook	4 Feb	11 Feb–18 May	100	0.24*	11	0.00053	0.00046
Ettrick							
1	27 Nov	7 Dec–17 Jun	25	0.84***	25	−0.00104	0.00049
2	4 Jan	14 Jan–17 Jun	25	0.88***	20	−0.00011	0.00043
3	21 Jan	4 Feb–17 Jun	25	0.87***	17	0.00002	0.00042
4	19 Jan	4 Mar–17 Jun	25	0.79***	13	0.00118	0.00036
5	11 Mar	25 Mar–17 Jun	25	0.79***	10	0.00244	0.00014
6	2 Apr	14 Apr–17 Jun	25	0.79***	7	0.00097	0.00035
Clyde	20 Nov	7 Dec–28 Mar	400	0.16 NS	15	0.00619	−0.00002
All significant				0.68***	170	0.00038	0.00039

[a] * = $p < 0.05$; *** = $p < 0.001$; NS = not significant.

TABLE 2. REGRESSION STATISTICS FROM MULTIPLE LINEAR REGRESSION OF LN (RELATIVE CHANGE IN COLUMN LENGTH) AGAINST TIME INTERVAL AND ACCUMULATED DAY-DEGREES FOR OCTO PHEROMONE DISPENSERS

Site and series	Dispensers out	Regression period	Distance to data logger	r^{2a}	*df*	*a*	*b*
Ettrick							
1	4 Jan	14 Jan–2 Jun	25 m	0.85***	19	−0.00141	0.00047
2	21 Jan	4 Feb–2 Jun	25 m	0.88***	16	−0.00115	0.00046
3	19 Feb	4 Mar–2 Jun	25 m	0.81***	12	−0.00144	0.00054
4	4 Mar	18 Mar–2 Jun	25 m	0.08 NS	10	0.00104	0.00028
5	2 Apr	14 Apr–2 Jun	25 m	0.25 NS	6	0.00010	0.00047
Booth Orch.	19 Nov	30 Nov–2 Jun	5.5 km	0.48***	26	0.00008	0.00033
Kane Orch.	19 Nov	30 Nov–2 Jun	5 km	0.66***	26	−0.00072	0.00038
Preedy Orch.	19 Nov	30 Nov–2 Jun	4 km	0.78***	26	−0.00103	0.00041
Clyde	20 Nov	7 Dec–28 Mar	400 m	0.16 NS	15	0.00619	0.00020
All significant				0.67***	135	−0.00061	0.00039

[a] *** = $p < 0.001$; NS = not significant.

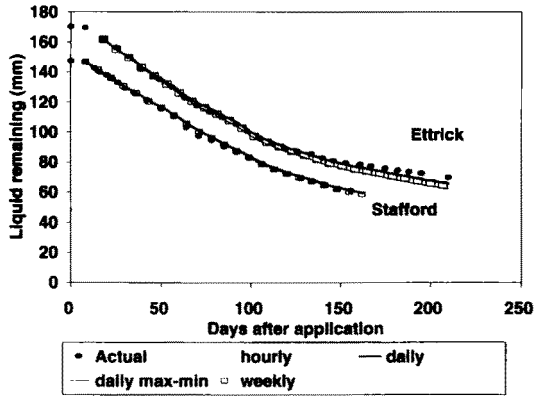

FIG. 1. Actual and predicted changes over time in pheromone column lengths in polyethylene tubing dispensers containing tetradecenyl acetates used for mating disruption of *Epiphyas postvittana* in two regions of New Zealand. Predictions from hourly temperature records were identical to those from daily records.

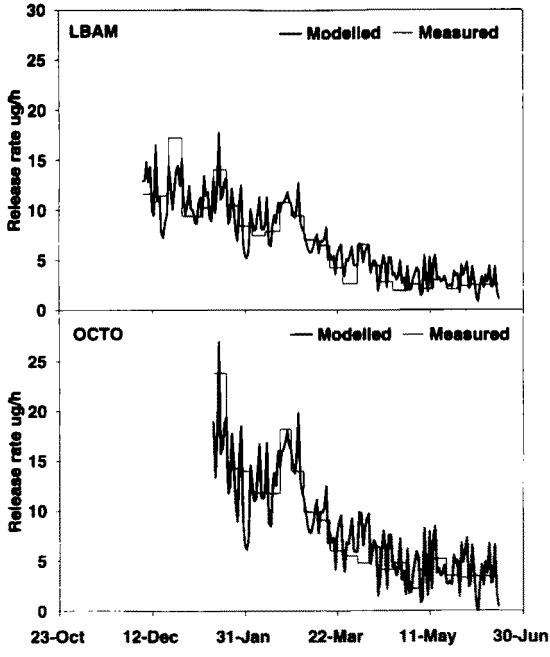

FIG. 2. Measured (weekly) and modeled (daily) release rate from polyethylene tubing pheromone dispensers containing tetradecenyl acetates used for mating disruption of *Epiphyas postvittana* (top) and *Planotortrix octo* (bottom).

In order to extend this model to new dispenser formulations, the parameters must be estimated from field data. It appears from our results that dispenser measurements at two-week intervals for six months would be sufficient to permit prediction of release rates, when accompanied by hourly temperature recordings over that period.

Effect of Data Logger Location. The prediction of column length was seen to be superior at locations where the temperature data was collected on site, compared to where it was collected at distances of several kilometers. An example of this is the measurements on dispensers in orchards at Dumbarton (Booth, Kane, and Preedy orchards) using temperature data from a site 4 km away (Table 2). While these still gave significant regressions, the correlation was lower than with the Ettrick series. Correlation decreased with increasing distance from the data logger in this situation. In an extreme situation at Clyde, where dispensers were in an overhead-irrigated, well-sheltered orchard, and temperature was recorded in an exposed, nonirrigated meterological station, the prediction of column length was not possible, as coefficients could not be estimated.

Instantaneous Release Rates. The parameters a and b were estimated for use with daily mean temperature and can be used to calculate the release rate each day (Figure 3). However, if instantaneous release rates are needed, these parameters must be divided by an appropriate time interval (dm/dt) (i.e., divide by 24 for hourly estimates). In addition, the current temperature and column length can be used to estimate the instantaneous pheromone release rate. The fluctuations over a single day can be traced more precisely with more frequent time intervals, and this can be useful for comparison with the biologically important period of insect flight (e.g., Figure 3). This figure illustrates that the bulk

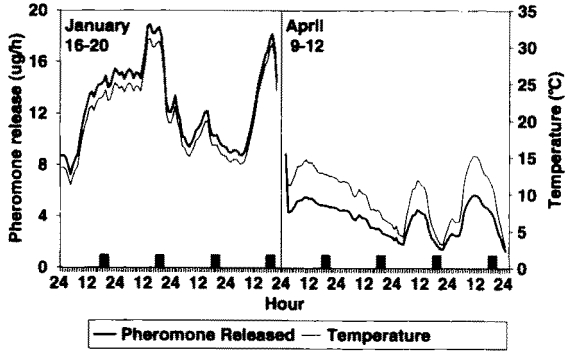

FIG. 3. Modeled hourly pheromone release rates over January 16–20 and April 9–12, 1993, compared to temperature. Bars show periods of flight activity at dusk.

of the pheromone is released during the day, when it is not needed, and that the release rate relative to temperature decreases between January and April.

Difference between Hanging and Twisted Dispensers. After 119 days, there was a significant difference ($t = 4.04$, $df = 28$, $P < 0.001$) between liquid length in dispensers suspended from one end, such as those upon which the model was based (86.3 ± 0.97 mm), and those that were twisted when applied for pest control purposes (78.5 ± 1.28 mm). The data indicated that the twisted dispensers released 9% more than the hanging dispensers. However, this difference was likely to be in part due to measurement error, because adhesion to the walls of the twisted dispensers, which were shaken down just before measurement, would have shortened the column length.

Validation Experiments. The instantaneous release rates of *E*11- and *Z*11-14:OAc decreased in proportion to the length of liquid remaining in the capillary, with a ratio that reflected the original contents. Our model gave predictions of release rates in still air that were within the experimental error, except for the initial period (Figure 4). The slower decline in release rate was better predicted by the model. The release rates of *Z*5-14:OAc and *Z*8-14:OAc were also adequately described by the model, after an initial period of higher release rate (data not shown for brevity). The proportion of *Z*5-14:OAc in the blend varied from 24 to 28%, but no trend in release was shown over time.

The most likely explanation for this early behavior is that the wall of the polyethylene capillary had a finite storage capacity and that this was more fully charged at the beginning when the dispensers had just been removed from their sealed packaging. During this period, pheromone could be conducted more rapidly to the surface. Measurements of changes in pheromone content of the

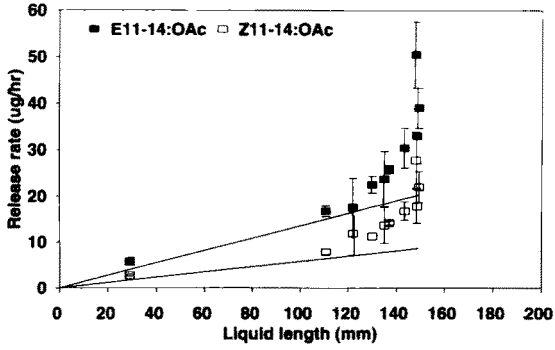

FIG. 4. Actual (data points) and predicted (lines) release rate of *E*11-14:OAc and *Z*11-14:OAc from polyethylene tubing pheromone dispensers maintained at 22°C in still air (bars show one standard error).

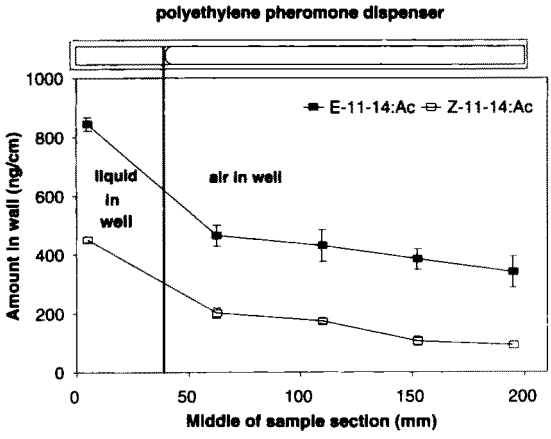

FIG. 5. Amount of *E*11–14:OAc and Z11–14:OAc in the wall of polyethylene tubing pheromone dispensers with either pheromone liquid or air adjacent to the wall.

walls above and below the capillary (Figure 5) support this view, as does the slower change in column length in the first one to two weeks (Figure 1). Walls adjacent to the pheromone liquid contain about 40 ng/mm more pheromone than that around the empty capilliary. A similar change in wall storage from initial saturation to the linear-region concentration is a sufficient source for the initial period of rapid release. Field data for the first one to two weeks were discarded so as not to include the period of rapid loss without associated column length change.

The amount of pheromone present in the wall of the dispensers adjacent to the pheromone liquid in the well was about twofold greater than the amount present in the wall when air was in the adjacent section of the well, and there was little change with distance from the meniscus (Figure 5). The amount of pheromone in the wall also declined with distance from the pheromone column, indicating limited longitudinal movement. The presence of detectable quantities of *E*- and Z11–14:OAc in the wall of dispensers may well be due to the adhesion of these materials within the polyethylene tubing, without indicating transport. Pheromone storage in the polyethylene material changes with time, so some pheromone is released from empty parts of the capillary tube. The loss from the tube wall as the capillary withdraws is about 10% as much as the loss from within the capillary. Up to a further 3% is desorbed and released later. This latter loss can be ignored. Although the model ignores the release from the portion of the dispensers with air in the well, the effect of this factor appears to

be quite small, since the model predictions quite accurately describe pheromone loss from the dispensers.

DISCUSSION

The need for a model of pheromone release rate from dispensers has arisen in the context of understanding the mechanisms and limitations associated with mating disruption of leafrollers. The model proposed here offers a practical first attempt to determine pheromone release rates, based on a minimum of inputs. In the past, weekly measurements of column length have not provided adequate resolution of pheromone release rate to explain the frequency of mating in a seven-day period, because daily fluctuations in temperature and release rate can confound the analysis (Suckling and Shaw, 1992).

This model appears to be versatile (able to predict both season-long trends and instantaneous release rates), and requires only a minimum of equipment for estimation of the inputs, in the case of stable blend components. Once the coefficients have been derived for a dispenser type and blend combination, only a single column-length and temperature reading is needed to produce estimates of current release rate. Conversely, a single column-length measurement accompanied by season-long temperature recordings provides sufficient input for at least a rough estimate of release rates over the season (although more frequent column-length measurements would improve the model prediction).

The GC analysis indicated that the release rates of individual components of the blends were not significantly different over time. In the case of LBAM (Figure 4), Suckling and Clearwater (1990) also demonstrated that the ratio of (*E*):(*Z*)-tetradecenyl acetate did not change over time. They reported a slightly faster rate of loss of the minor (3.2%) component, a diene (*E*9,*E*11:14OAc), which has been ignored in our model. Analysis of the OCTO dispensers also indicated similar release characteristics for the two components. These results were in agreement with the report of Sexton (1989), who noted the lack of effect of double-bond position on release rate in similar polyethylene tubing dispensers.

Our model may not be suitable for dispensers containing mixtures of alcohols and acetates which diffuse at different rates (e.g., codling moth) (Brown et al., 1992).

The model of McDonough et al. (1992) was developed using gas chromatographic (GC) analysis of polyethylene codling moth dispensers and showed how different components were released at different rates over time (not day-degree accumulation). They mentioned variation in day-degree accumulation between years, but they did not include this variation in their model, which was based on three constants and column length. They considered that photodegradation was the main cause of reduction in pheromone rather than liquid loss

and/or temperature accumulation. McDonough et al. (1992) did attempt to explain what was happening with dispenser release rates over the first few days, a period that our model ignores.

Complex models, such as that proposed by Brown et al. (1992), appear to have important limitations and lack portability to other linear or tubing dispenser types. The main difference between earlier models and our model involves the relationship between temperature and release. The model of Brown et al. (1992) was based on the exponential increase of pheromone release with temperature, a relationship based on the Clausius-Clapeyron equation. We considered this inappropriate for our dispenser system, since it is based on a model where release is controlled by boundary layer resistance and surface vapor pressure. The Shin Etsu dispensers that we used are relatively insensitive to wind speed (note the fit of the field model to data from still air), a factor that has been ignored by both Brown et al. (1992) and McDonough et al. (1992). Wind is also reported to have little effect on hollow-fiber dispensers, where diffusion from the liquid–vapor interface to the open end of the fiber is the rate-determining step (Bierl-Leonhardt, 1982).

Our model uses a linear release rate–temperature relationship, which seems to work well over the relevant temperature range. Pheromone release rates were found to increase exponentially with temperature by Van der Kraan and Ebbers (1990) for a polymeric or laminar dispenser, where boundary-layer resistance is much more important.

The other major difference between the model of Brown et al. (1992) and the model we present is that their decline in release rate over time was based on temperature accumulation, whereas in our model it is based on column length change. Since the ratio of dispenser components changes greatly in Brown et al. (1992), the use of temperature accumulation seems appropriate in their case. Our model, which so far only applies to the relatively stable tetradecenyl acetates, uses column length change for ease of use. Since our model is based on temperature, a relationship between column length and temperature accumulation is easily derived, once coefficients are found.

Weekly observation of dispensers for six months can give the coefficients for subsequent prediction of release rates. Temperature monitoring, often performed for other orchard applications, such as disease forecasting, can now replace regular field measurements of dispensers. However, compounding errors (15% after six months) could be reduced by occasional measurement of dispenser column lengths. Hourly modeling can provide additional information, for example, predicting release rate during evening flight periods (Figure 3) (Suckling et al., 1994).

Predictive Applications. A significant unknown in mating disruption applications is the minimum atmospheric concentration required to obtain effective

disruption. This concentration is likely to be dependent on biological factors specific to the insect species, as well as the crop growth form (e.g., green tea bushes compared to orchards), but other variables will also be very important. In addition to release rate, wind has been shown to have a large effect on atmospheric concentration (Uchijima, 1988). Crop foliage affects wind speed, as well as adsorbing and releasing pheromone (Karg et al., 1994). In future, it may be possible to use a dispenser release rate model as an input for an atmospheric pheromone concentration model to predict concentrations as a function of meteorological conditions. Such a model could provide opportunities for optimizing the effectiveness and economics of mating disruption. Once criteria such as the period and atmospheric concentration required are defined, then optimal dispenser deployment could be determined. McDonough et al. (1992) used the thresholds of other authors to recommend deployment timing, but since their model was based on time rather than temperature accumulation, their analysis was very simplistic. Our model could provide the basis for predicting the probability of dispensers lasting the required period. The risk of failure could be found through an analysis of long-term climate data, since the life of dispensers will be affected by annual changes in accumulated temperature.

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DEVELOPMENT OF A BIOASSAY TO TEST PHLOEM SAP SAMPLES FROM LETTUCE FOR RESISTANCE TO *Nasonovia ribisnigri* (HOMOPTERA, APHIDIDAE)

MAARTEN VAN HELDEN,[1,*] HANNEKE P.N.F. VAN HEEST,[1] TERIS A. VAN BEEK,[2] and W. FREDDY TJALLINGII[1]

[1]*Department of Entomology, Wageningen Agricultural University POB 8031, 6700 EH Wageningen, The Netherlands*

[2]*Department of Organic Chemistry, Phytochemical Section Wageningen Agricultural University Dreijenplein 8, 6703 HB Wageningen, The Netherlands*

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Abstract—The *Nr*-gene-based resistance of lettuce to the aphid *Nasonovia ribisnigri* (Mosley) has previously been shown to be located in the phloem. Since chemical analyses of the phloem sap had shown no differences between resistant and susceptible lines, a bioassay was developed in order to test samples from resistant and susceptible plants on aphid feeding. For this, whole-plant extracts, honeydew, and EDTA-collected phloem extracts were obtained, and a sensitive bioassay was developed using EDTA samples. The EDTA was removed, and samples were added to a simple sucrose solution or to a complex artificial diet and presented in a choice situation comparing extracts from resistant and susceptible plants. EDTA-collected phloem sap samples from susceptible plants were preferred to those from resistant plants. The resistance is probably based on a feeding deterrent activity of the phloem sap in the resistant plant.

Key Words—Aphididae, *Nasonovia ribisnigri*, *Lactuca sativa*, phloem sap, bioassay, resistance mechanism, artificial diet, aphid honeydew, EDTA chelation.

INTRODUCTION

Resistance of plants to aphids is difficult to investigate because of the highly specialized feeding behavior of these insects. In many cases the resistance is

*To whom correspondence should be addressed.

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based on an interruption of the feeding behavior after the phloem sieve element has been reached by the mouthparts of the insect (Kimmins, 1989; Padgham et al., 1990; Caillaud et al., 1992; Cole, 1993; Helden and Tjallingii, 1993). Elucidation of the (chemical) resistance mechanism(s) is very difficult (Dreyer and Campbell, 1987; Harrewijn, 1990; Wink and Witte, 1991). So far investigations into resistance to homopteran insects have often used correlations of total plant levels of certain chemical compounds with performance traits (Todd et al., 1971; Argandoña et al., 1980; Dreyer and Jones, 1981; Herrbach, 1985; Leszczynski et al., 1985; Niemeyer, 1990; Kanehisa et al., 1990; Luczak and Gaweda, 1992). Phloem sap, the only plant component ingested as food by aphids, represents only a small part of total plant extracts. When resistance is based on a chemical component of the phloem sap, it will very likely be lost in whole plant preparations.

The absolute, monogenic resistance of lettuce (*Lactuca sativa* L.) to the aphid *N. ribisnigri* (Nr gene) (Eenink et al., 1982; Reinink and Dieleman, 1989; Helden et al., 1993) is based on an early interruption of sap uptake after the aphid stylets have reached a phloem vessel (Helden and Tjallingii, 1993). Although a mechanical resistance mechanism, like blocking of the stylets or sieve elements during attempted feeding, cannot be excluded as a possible resistance mechanism, our current working hypothesis is that a chemical difference in the phloem sap may interfere with host plant recognition or acceptance in the resistant plants (Helden and Tjallingii, 1993; Helden et al., 1994a,b; Wink and Witte, 1991).

Phloem sap samples were obtained by stylectomy, facilitated excudation [using ethylene diamine tetraacetate (EDTA) chelation], and honeydew collection and analyzed for sugars, amino acids, proteins, and UV-absorbing secondary plant compounds, which showed no consistent differences between resistant and susceptible lettuce lines (Helden et al., 1994a,b). Lettuce contains many secondary compounds (Gonzalez, 1977), but little is known about their presence in the phloem and their biological activity. Exhaustive analysis of secondary compounds in the phloem sap of lettuce, without knowledge of the biological activity of the active substances (allomones), is very time consuming.

The development of a bioassay using EDTA-collected phloem sap extracts of the plants, as described here, could make a "classical" bioassay-guided fractionation, isolation, and identification of the allomones involved in the resistance feasible.

METHODS AND MATERIALS

Plants and Aphids

Plants were grown and aphids reared as described earlier (Helden et al., 1993, 1994). Larvae were used when 5 days old (L3 stage) unless otherwise

stated. Two sets of near-isogenic lettuce lines were used: SUS (susceptible, genotype *nrnr*) and RES (resistant, *NrNr*) (SET1), SUS2 (*nrnr*) and RES2 (*NrNr*) (SET2), lettuce cv. Taiwan (susceptible, *nrnr*) and oilseed rape cv. Olymp (*Brassica napus* L.) (Helden et al., 1994a,b). The two near-isogenic sets 1 and 2 have genetically different backgrounds (except for the source of the *Nr* gene, Helden et al., 1994a).

Plant and Phloem Extracts

Whole-Plant Extracts. Whole plants were heated in a microwave oven for 20 sec to destroy all enzymatic activity in the sap. Sap was pressed from the leaves, filtered, and used directly. Methanol extracts were prepared from 0.2 g lyophilized leaves in 10 ml of MeOH, followed by ultrasonic mixing for several minutes, centrifugation, filtration, and vacuum drying.

Honeydew. Honeydew from oilseed rape and Taiwan lettuce was collected using *Myzus persicae* Sulzer (biotype WM1). For the collection of honeydew from the lines RES and SUS, *Macrosiphum euphorbiae* Thomas (biotype WMe1) had to be used because the *Nr* gene also causes partial resistance to *M. persicae.* Honeydew was collected in hexadecane (Helden et al., 1994a), stored at −80°C, and lyophilized prior to use. Occasionally honeydew was collected directly on Petri dishes that were placed underneath large quantities of aphid larvae on leaves. This "dry-collected" honeydew was dissolved in a water–MeOH (1 : 1) mixture, MeOH was evaporated in vacuo and the aqueous extract was lyophilized.

Sample Preparation from Crude EDTA Extracts. Phloem sap exudate was collected by placing excised leaves in an aqueous solution with 8 mM EDTA and 5 mM phosphate buffer at pH 6 for 20 hr (King and Zeevaart, 1974; Groussol et al., 1986; Helden et al., 1994a). Initially 5 mM sodium metabisulfite ($Na_2S_2O_5$) was used as an antioxidant, but later this was omitted because of toxicity to the aphids. The crude EDTA extract was stored at −20°C. To remove the EDTA from the crude EDTA extract, the following procedure was used: 0.5 g of a weak cation exchange column (Varian Bond Elut CBA 500, carboxylic acid) was activated with 5 ml ammonium buffer (pH 10), washed with H_2O, and loaded with 10 ml of a 0.01 M lead nitrate solution. After washing with H_2O, 10 ml of crude EDTA extract was added and 2-ml fractions were collected. Fractions were tested for the presence of EDTA (see below). EDTA-free fractions (usually the first four) were combined, lyophilized, and stored at −20°C until use. These EDTA-free samples will be referred to as Ed samples (EDTA-derived samples) throughout this paper. After cleaning with 10% formic acid in water, the columns were reused.

Control Treatments. Together with the Ed-samples, two different control treatments were used. The first was a blank control (Blank) (nothing added). The second control treatment (Control) was used to compensate for the amino

acids and sugars in the Ed samples and to counterbalance the possible artifacts introduced by the EDTA extraction procedure. A complex artificial diet (consisting of 15% sucrose, amino acids, vitamins and salts) (Harrewijn and Noordink, 1971) was diluted around 30 times in the original EDTA chelation solution (8 mM EDTA and 5 mM phosphate buffer). This solution was then treated identically to the crude EDTA extracts (Pb-EDTA extraction, lyophilization, storage). The resulting samples are referred to as Ed diet. To make the Control treatments, this Ed diet was dissolved in the sugar (sugar tests) or in a complex diet (diet tests) in the same amounts as the Ed samples (see below).

EDTA Detection. EDTA was detected using a color reaction with Eriochrome black T as an indicator. Two droplets of indicator (0.1% w/v), 1 ml H_2O, and two droplets of ammonium buffer were mixed in a test tube. Lead nitrate solution was added until the color changed to purple. To test for the presence of EDTA, five (or more) droplets of a fraction were added to a test tube. A color change to blue indicated the presence of EDTA (detection limit $\pm$ 2 μM of EDTA).

Preparation of Tests

All tests were prepared in a laminar flow cabinet. Materials were cleaned with 70% ethanol prior to use. All test slutions were filtered through a 0.22-μm micropore filter.

No Choice Tests. Test solutions consisted of 15% sucrose in water with one of the following additions: whole plant sap (5 or 10% v/v); methanol extracts (50 mg/ml); crude EDTA extracts (10% v/v); EDTA at 0, 5, 10, 25, 100, or 200 mM; or $Na_2S_2O_5$ at 0, 5, 10, 25, or 100 mM. Test solutions were presented between two layers of Parafilm, stretched over a 26-mm-diameter plastic ring to form a test cage. Eight freshly molted adult aphids were introduced, and survival and reproduction were scored after 48 hr.

Multiple Choice Tests. These were prepared according to Dorschner and Kenny (1992). Parafilm sachets with different sucrose concentrations (0, 5, 10, 20% w/v in H_2O) in different combinations were presented through six holes in the lid of a 9-cm Petri dish and a large number (>100) of aphids of mixed age were introduced into the dish. Distribution of the aphids over the six sachets was determined after 24 and 44 hr. Plant extracts were not tested.

Dual Choice Tests. For the honeydew tests, sugar tests, and artificial diet tests, we constructed sachets with two adjacent test solutions separated by only a few millimeters (Figure 1). The two layers were firmly pressed together by rolling a 2-mm-wide rubber wheel over them.

Twenty larvae of *N. ribisnigri* were introduced, and the tests were placed in a 24°C incubator with weak illumination from the top. Transparent yellow plastic foil was placed below the light source to stimulate settling on the Parafilm surface. Aphid distribution was determined several times (after 8–72 hr). Usu-

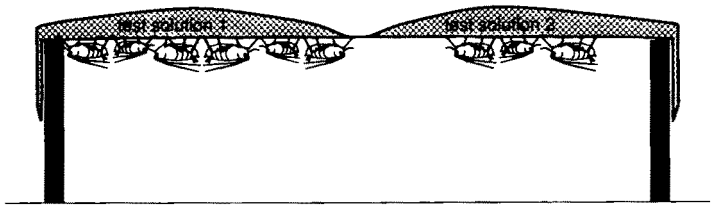

FIG. 1. Cross section of the test cage with a dual-choice sachet. Twenty larvae of *N. ribisnigri* can freely select a feeding site on a test solution.

ally at least six replicates (minimum for statistical differences) were included in each test (see results).

Honeydew Tests. Test solutions for these dual-choice tests were made by adding freeze-dried honeydew (10 mg/ml) to a 15% aqueous sucrose solution. Dual-choice tests also included 15% sucrose solution (Blank).

Testing of Ed Samples

Acceptability Test. Ed diet was dissolved at 20 mg/ml in artificial diet and tested against artificial diet to which 20 mg/ml of lyophilized artificial diet (not treated on the column) was added.

Sugar Tests. Tests were performed using Ed samples from RES and SUS (treatment RES and SUS) dissolved at 5 mg/ml in 15% aqueous sucrose solution. These were compared with two controls: a blank control (Blank, 15% sucrose with nothing added) and a second control (Control) consisting of a 15% sucrose solution to which 5 mg/ml of Ed diet was added. These four treatments make six possible pairs for a dual-choice test. The same combinations were also tested at 20 mg/ml. The extracts of RES2 and SUS2 were also tested in all possible combinations with Blank and Control at 5 mg/ml and 20 mg/ml.

Artificial Diet Tests. For these tests, all samples were dissolved in a complex artificial diet. There was no Blank treatment. The control treatment (Control) consisted of complex artificial diet to which 5 (or 20) mg/ml of Ed diet was added. For comparison of SET1, this gives RES, SUS, and Control (so only three possible dual-choice combinations) at 5 mg/ml, and the same at the higher concentration of 20 mg/ml. The same concentrations and combinations were tested for the near-isogenic SET2 (RES2, SUS2, and Control).

Statistics

Choice test results were compared by Wilcoxon's signed-rank tests at the 5% significance level.

RESULTS

No Choice Tests. Tests with whole plant sap or methanol extracts showed 10–50% mortality of the adults and very low reproduction as compared to complex artificial diet. No differences were observed in mortality or reproduction between test solutions containing the resistant or susceptible whole-plant extracts.

EDTA affected survival at concentrations over 10 mM, and at 100 mM or higher most aphids died within 24 hr. Little mortality occurred at lower concentrations but reproduction was low (< 1 larvae per adult per day). The antioxidant sodium metabisulfite caused high mortality, even at concentrations below 10 mM.

Tests with crude EDTA extracts (containing EDTA and antioxidant) showed low acceptance of the test solutions, low reproduction, and high mortality. When the antioxidant was omitted during collection and the EDTA extracted, no mortality was observed and more than 80% of the aphids settled on the test solutions. Multiple choice tests gave no discrimination among sucrose solutions of different strengths. Aphids settled poorly and appeared restless (data are not given). In the dual choice tests, observations after 8, 20, or 28 hr showed no or less pronounced differences, whereas during later observations (>48 hr) microbial contamination of the test solutions was sometimes visible. Figures 2–4 only show the results after 40 hr.

Acceptability Test. The acceptability tests suggested that the lyophilized diet was slightly preferred to the Ed diet (distribution after 40 hr diet–Ed diet $= 10.1:6.6$, $N = 11$), but this difference was not statistically significant.

Honeydew Tests. Honeydew of host (Taiwan lettuce) and nonhost plants (oilseed rape) was strongly favored over the sucrose treatment Blank (Figure 2a), and honeydew from lettuce was preferred to honeydew from oilseed rape. When testing honeydew of near-isogenic resistant (RES) and susceptible (SUS) lines, these were both selected over sucrose only and honeydew of resistant plants was preferred to susceptible plants (Figure 2b).

Ed Samples in Sugar Tests. In the sugar test, comparing Ed samples of the SET1 aphids showed a slight, but significant, preference for SUS to RES at low concentration (5 mg/ml, $N = 16$) while all other combinations showed no significant difference (Figure 3a). At the higher concentration, Ed samples of SET1 were preferred to Blank but not to the Ed diet (Control), and SUS was selected over RES (Figure 3b). Ed diet was selected over Blank at the high concentration (Figure 3b and d).

The tests of SET2 showed that RES2 was preferred to SUS2 at 5 mg/ml (Figure 3c), while none of the other combinations showed significant discrimination by the aphids at this low concentration, not even Ed diet against Blank. At higher concentrations (Figure 3d), RES2 was not favored over SUS2, but all

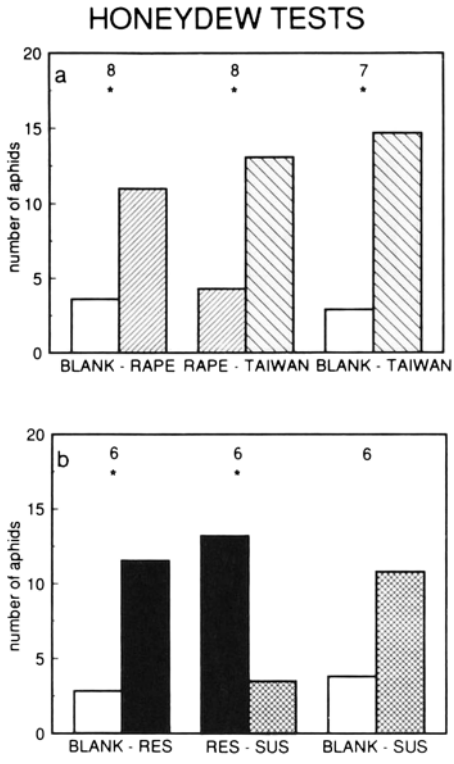

FIG. 2. Honeydew tests. Results of dual-choice tests with *N. ribisnigri* using lyophilized honeydew at 5 mg/ml in 15% aqueous sugar solution. (a) Comparison of honeydew produced by *M. persicae* on lettuce cv. Taiwan (susceptible), oilseed rape (nonhost), or Blank (just sugar); (b) comparison of honeydew produced by *M. euphorbiae* on *Nasonovia*-resistant (RES) and susceptible (SUS) lettuce and sugar-only (Blank). Bars represent mean number of *N. ribisnigri* aphids on each of the test solutions after 40 hr. Numbers above bars are number of replicates. *significant difference at 5% level.

of the other combinations showed a significant deterrence of Ed samples compared to Blank or even Control.

Ed Samples in Artificial Diet Tests. The tests based on the addition of extracts to a complex artificial diet (Figure 4) showed very distinct discrimination of different test solutions by the aphids, except for the low concentration of the SET1 Ed samples (Figure 4a). At 5 mg/ml samples of SET2, there was a preference of SUS2 to RES2 but SUS2 was not selected over Ed diet (Figure

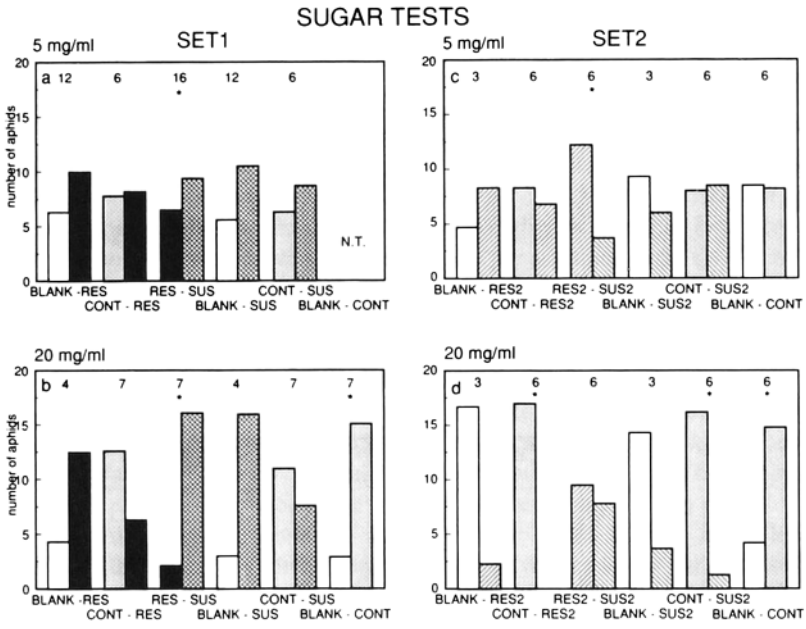

FIG. 3. Sugar tests. Results of the dual-choice tests using Ed samples from different lettuce lines, dissolved in 15% aqueous sugar solution. Blank = 15% sucrose with nothing added. See text for details on processing of extracts. (a) SET1 (RES, SUS) lines or Ed diet (Control) at 5 mg/ml and Blank; (b) at 20 mg/ml; (c) SET2 (RES2, SUS2) or Ed diet (Control) at 5 mg/ml and Blank; (d) at 20 mg/ml. (d) Bars represent mean number of aphids on each of the test solutions after 40 hr. Numbers above bars are number of replicates. *significant difference at 5% level, N.T. = not tested.

4c). At the high concentration, Ed diet was selected over Ed samples and the Ed samples from susceptible plants over resistant plants (Figure 4b and d).

DISCUSSION

Whole-plant extracts were toxic, probably due to secondary compounds present in cell vacuoles and released or activated upon the crushing of the cells during extraction (Matile, 1984). We did not succeed in designing a test using whole-plant samples that resulted in a discrimination.

Of the methods available to collect phloem sap from lettuce, only honeydew and EDTA chelation yield sufficient quantities (Helden et al., 1994a,b). The

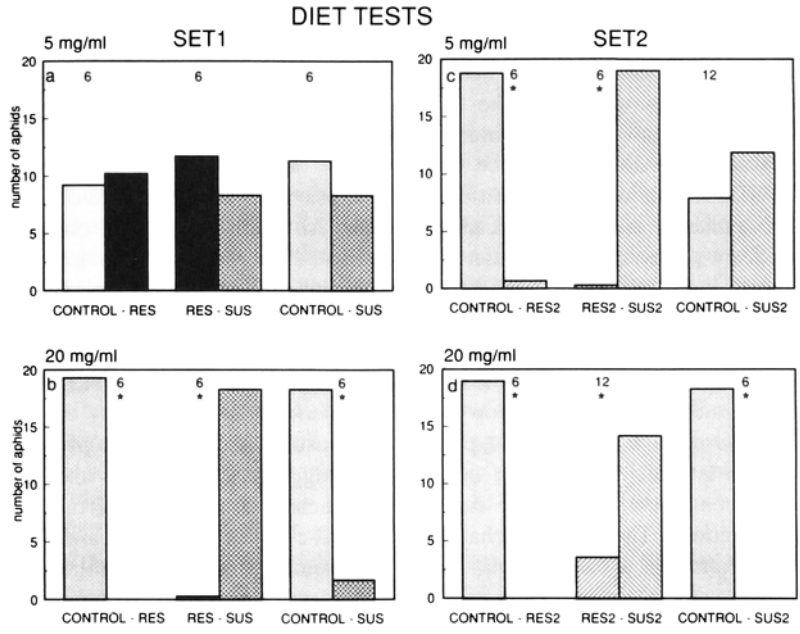

FIG. 4. Diet tests. Results of the dual-choice tests using Ed samples from different lettuce lines dissolved in complex artificial diet. See text for details on processing of extracts. (a) SET1 (RES, SUS) lines or Ed diet (Control) at 5 mg/ml; (b) at 20 mg/ml; (c) SET2 (RES2, SUS2) at 5 mg/ml; (d) at 20 mg/ml. Bars represent mean number of aphids on each of the test solutions. Numbers above bars are number of replicates. *significant difference at 5% level.

honeydew is bioprocessed (by the aphids) and different species (*M. persicae* and *M. euphorbiae*), which might differ in their bioprocessing, had to be used. EDTA chelation exudates are considered to be reliable representations of phloem sap. Contamination with substances from other plant parts and from the cut (wounded) surface of the petiole might occur, but this has not been shown to be of major importance (Fisher and Frame, 1984; Weibull et al., 1990; Girousse et al., 1991; Helden et al., 1994a,b). The crude EDTA extracts were not accepted by the aphids because of the presence of EDTA and sodium metabisulfite. Omission of the antioxidant during EDTA exudation increased the browning of the extracts. Although we cannot check it, this might have affected the biological activity against *N. ribisnigri* in the tests.

During the processing of the samples the EDTA was retained by the lead

ions on the column as a Pb–EDTA complex. It is possible that other components of the crude EDTA extracts also may have been retained on the column. Some column components may have been released (lead ions or Pb–EDTA complex), and EDTA removal during the EDTA extraction procedure may have been incomplete (below detection limit). In initial experiments, traces of lead (precipitation test with Kl) could be shown in the "EDTA-free samples" when a pure EDTA solution was processed. In the Ed samples we sometimes observed a precipitate, presumably of lead phosphate. Although we did not observe a significant preference for Ed diet over nontreated diet (acceptability test), this may still have affected the aphids. However, since the treatment of all samples included the step of EDTA removal, artifacts will occur in all Ed samples and Ed diet in roughly equal amounts, so this does not affect our conclusions.

Design of the Test. The bioassay of Dorschner and Kenny (1992), which demonstrated preference for hop (*Humulus lupulus* l.) honeydew by the hop-aphid *Phorodon humili* Schrank, proved to be unsuitable for *N. ribisnigri*, which feeds on lettuce. *N. ribisnigri* often showed aggregation behavior and accumulated on one or two of the six available sachets (irrespective of the sugar concentration). Therefore we changed to a dual-choice test (Mittler and Dadd, 1964; Harrewijn and Noordink, 1971). Our setup, using two parafilm membranes pressed together in the middle, uses very little solution to fill the two compartments and has no border between the adjacent compartments that can act as a barrier (Figure 1). Therefore, the aggregation behavior will not influence the results strongly. Still an individual aphid cannot be considered as independent from the others in the same test. The simplicity of the dual-choice test makes it possible to make many (at least six) replicates, necessary to show statistically significant differences.

Unequal distribution of the aphids over the two sides became apparent only after 20 hr or more. This is much later than observed by Mittler and Dadd (1964) on artificial diets and also much later than expected from the rejection of the phloem sieve element once this is reached by the aphid (Helden and Tjallingii, 1993). The latter may be explained by the level of compounds in the choice tests, which will be around 10 times lower than in the real phloem sap. Only 20 mg/ml of Ed sample is added, while normal lettuce phloem sap has a dry weight of around 20% (200 mg/ml). It cannot be excluded that the test solutions changed over time, e.g., because of continued enzymatic activity (Helden et al., 1994b), but this would also mean that there is a chemical difference between fractions.

Test Results. The preference for lettuce honeydew (susceptible cv. Taiwan) over the nonhost plant (oilseed rape) suggests that the host-plant honeydew has a feeding stimulant effect or the nonhost a feeding deterrent effect (Dorschner and Kenny, 1992). Preference for the honeydew to sucrose only (Blank) (Figure 2) is probably caused largely by the amino acids present in the honeydew (Dor-

schner and Kenny, 1992; Mittler and Dadd, 1964; Helden et al., 1994b). The choice for RES honeydew over SUS honeydew (Figure 2b) shows that there is a chemical difference, but this is surprising since the inverse would be expected (discussed below).

The sugar tests at 20 mg/ml (Figure 3b and d), just like the honeydew tests, clearly show that attractiveness of a combination of sucrose with amino acids. In the test combinations that include a Blank treatment, the small quantity of amino acids present in the Ed samples or the Ed diet makes these treatments strongly preferred to Blank, which lacks amino acids. At the low concentration (5 mg/ml) most choices show no significant preference. The absence of response in Blank versus Ed diet at 5 mg/ml (Figure 3c) suggests that this might be caused by the low quantity of the compounds added.

The comparison of Ed samples with Ed diet (Control) gives more valuable results since little interference due to amino acid concentration differences (or EDTA extraction artifacts) will occur. Ed samples (Figure 3b and d) are not preferred to Ed diet, and in some cases (higher concentration test) not even to Blank (Figure 3d), suggesting the presence of feeding deterrents in the Ed samples.

We decided to use a complex artificial diet as a base for the tests in order to decrease the sensitivity to variations in amino acids and sugars in the Ed samples, which is not related to the resistance (Helden et al., 1994b). This could also increase the sensitivity to other compounds that might only show biological activity in a "phloem-like" environment. The results of these artificial diet tests (Figure 4) show a profound increase in the response of the aphids.

The artificial diet test demonstrated significant preferences for Ed diet (Control) to plant extracts (except for SET1 at 5 mg/ml). This suggests that all Ed samples (of both sets and for both resistant and susceptible plants) contain a certain feeding deterrence. The preference for SUS and SUS2 to RES and RES2, respectively, proves that there is a chemical difference between these extracts, which (at 20 mg of Ed sample/ml) causes a stronger feeding deterrence in the Ed samples from resistant plants.

The total absence of significant differences in the diet tests of SET1 at 5 mg/ml (Figure 4a) compared to SET2 (Figure 4c) suggests that the concentration of the feeding deterrents in SET1 are lower than, and in this case under, the behavioral threshold for the aphid, as for the low concentration sugar tests (Figure 3a and c). This suggests that modifications in the level of the resistance do occur in spite of the supposed monogenic nature of the resistance. This could be caused by minor genes that influence the resistance caused by the *NR* gene, or other genes that provide additional (partial) resistance.

In a few cases, extracts from resistant plants were preferred to susceptible extracts (honeydew tests, Ed samples in sugar tests of SET2 at 5 mg/ml). Feeding deterrents have been reported to act as feeding stimulants at low con-

centrations (Bodnaryk, 1991). Deterrence of the resistant extracts at 20 mg/ml could change to a feeding stimulant at 5 mg/ml, and allomones could also be present in the susceptible plant but at lower concentration. To test this hypothesis more test series should be performed, comparing different concentrations of Ed samples from the same or different plants, and in combination with appropriate controls. However, possible interactions with other biologically active substances will not make this easy.

When honeydew is used, a feeding deterrent could be metabolized and excreted in the honeydew, with its degradation products acting as feeding stimulants. The reverse might be true in the case of a feeding stimulant. Since little is known about the fate of secondary plant chemicals in aphids (Wink and Witte, 1991, Givovich et al., 1992, Helden et al., 1994b), this remains speculative.

The sensitivity of the test might be improved by adjustment of the amino acid composition of the complex artificial diet (and the Ed diet) to that of lettuce phloem sap (Helden et al., 1994b) and total removal of possible interfering compounds from the Ed samples. The use of higher concentrations of the samples would require large quantities of plants.

Although the results of the honeydew and sugar tests cannot be explained easily, the diet test results show that there is a clear chemical difference between extracts from resistant and susceptible plants. This allows some optimism that this bioassay can be used to isolate the active substances through bioassay-guided fractionation. Still, the physical and chemical resemblance (pressure, concentration, etc.) of the test to the in vivo system remains very approximate, and the EDTA sample processing needs to be improved. Direct extrapolations of the in vitro test results to the in vivo situation are hazardous. The phloem sap origin of any isolated compound needs to be confirmed and the mode of action should be studied in the plant.

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A CHEMICAL BASIS FOR DIFFERENTIAL ALLELOPATHIC POTENTIAL OF SORGHUM HYBRIDS ON WHEAT[1]

MONCEF BEN-HAMMOUDA,[2] ROBERT J. KREMER,[3,*] HARRY C. MINOR,[2] and MUHAMMAD SARWAR[2]

[2]*Department of Agronomy*
University of Missouri
[3]*USDA-ARS Cropping Systems & Water Quality Research Unit*
Columbia, Missouri 65211

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Abstract—The basis for differential allelopathic potentials among sorghum (*Sorghum bicolor* L. Moench) hybrids was investigated by conducting quantitative and qualitative studies of their phenolic contents. Total phenolic content in sorghum plant parts varied within hybrids, among hybrids, and between growing seasons. Inhibition of wheat (*Triticum aestivum* L.) radicle growth was positively associated ($r = 0.66$) with concentrations of total phenolics contained in plant parts. Extracts from culms contributed the highest proportion of toxicity from sorghum plants, inhibiting radicle growth up to 74.7%. Concentrations of five phenolic acids, *p*-hydroxybenzoic (POH), vanillic (VAN), syringic (SYR), *p*-coumaric (PCO), and ferulic (FER), differed in all plant parts of the three sorghum hybrids. Concentrations of POH, VAN, and SYR were consistently higher than PCO and FER. PCO and FER were absent from some plant parts, with FER being the most frequently missing. Inhibition of wheat radicle growth was found to be positively associated with the concentration of each phenolic acid. Vanillic acid was most highly associated ($r = 0.44$) with inhibition. Thus, above-ground sorghum tissues contained phenolic acids that contributed to allelopathic potential. Additionally, sorghum roots exuded POH, VAN, and SYR that may enhance the overall allelopathic potential of sorghum during growth and after harvest when residues remain on the soil surface or are incorporated prior to planting a subsequent crop.

[1]Journal article No. 12245 of the Missouri Agricultural Experiment Station. Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the products, and the use of names by USDA implies no approval of the products to the exclusion of others that may be suitable.

*To whom correspondence should be addressed.

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INTRODUCTION

Plants produce a large variety of secondary products containing hydroxylated aromatic rings. These substances are classified as phenolic compounds, most of which are synthesized from phenylalanine, a product of the shikimic acid pathway (Taiz and Zeiger, 1991). Sorghum (*Sorghum bicolor* L. Moench) contains phenolic compounds at all stages of growth, with higher levels in leaves and glumes compared to culms and caryopses (Waniska et al., 1988). Within the same plant part, bird-resistant hybrids contain more soluble phenolics than non-bird-resistant hybrids (Reed and Tedla, 1987). In four sorghum genotypes, phenolics such as coumaric and ferulic acids were found to be more concentrated in culms than in leaf blades or leaf sheaths (Cherney et al., 1991).

Plant polyphenols known as tannins are important in the defense mechanisms that plants develop to resist predators. This is largely due to the ability of tannins to bind proteins, which is characterized in the mouth of herbivores as a feeling of constriction, dryness, and roughness (Haslam, 1988). Levels of phenolic acids in healthy plants of sorghum differ considerably among cultivars and always decrease as the plants mature. However, attack by insects and pathogenic fungi increases the phenolic content of plants by varying degrees depending on cultivar and stage of growth (Woodhead, 1981). Sorghum contains a mixture of hydrolases that can transform "inactive" phenolic esters and glycosides into "active" phenolic acids at concentrations high enough to significantly reduce feeding by *Locusta migratoria* (Woodhead and Cooper-Driver, 1979).

In addition to phenolic acids in aerial plant parts, roots of allelopathic crops actively exude phenolics (Abdul-Rahman and Habib, 1989; Alsaadawi et al., 1986; Einhellig and Souza, 1992; Tang and Young, 1982). Caffeic, chlorogenic, isochlorogenic, *p*-coumaric, *p*-OH-benzoic, and ferulic acids were detected in alfalfa root exudates and vegetative residues (Abdul-Rahman and Habib, 1989). Bioassays of trapped root exudates of bigalta limpograss (*Hemarthria altissima*), an allelopathic tropical forage, showed that the inhibitors were mainly phenolic compounds (Tang and Young, 1982). Root exudates of sorghum consist primarily of sorgoleone, a hydroquinone that is quickly oxidized to a benzoquinone, which at extremely low concentrations (0.01–0.125 mM) can potentially inhibit weed growth, suggesting a strong contribution to sorghum allelopathy (Einhellig and Souza, 1992). Water extracts from decaying residues of four sorghum cultivars revealed that all cultivars significantly reduced redroot pigweed (*Amaranthus retroflexus* L.) growth, with greatest inhibition achieved by cultivars having toxic root exudates (Alsaadawi et al., 1986).

The realistic concentrations of phenolics necessary for inhibitory action under field conditions are estimated to be 3.3 mM for ferulic, *p*-coumaric, and vanillic acids. At this concentration, a synergistic effect on germination of sorghum has been reported (Rasmussen and Einhellig, 1979). Phenolic compounds such as *p*-hydroxybenzoic, vanillic, *p*-coumaric, and ferulic acids were found in the soil solution at 0.003–0.39 mM. These compounds are produced by decomposing straw or degradation of humic acids (Whitehead, 1964). Analysis of sorghum residues revealed that *p*-coumaric acid was present in the highest concentration followed by ferulic, syringic, vanillic, and *p*-hydroxybenzoic acids at variable concentrations. Under ideal conditions for decomposition, *p*-coumaric acid could be released in sufficient quantities in localized soil microsites to affect plant growth (Guenzi and McCalla, 1966).

At a concentration of 1.0 mM, *p*-coumaric, ferulic, and vanillic acids significantly reduced dry matter production, leaf expansion, leaf production, height, and net assimilation rate of soybean [*Glycine max* (L) Merr.]. Furthermore, net photosynthetic rate and stomatal conductance were severely reduced, and marked losses of chlorophyll were registered (Patterson, 1989). Leaf diffusive resistance of sorghum seedlings increased and water potential decreased following treatments with ferulic acid, *p*-coumaric acid, and extracts from several allelopathic weeds. Water potential changes resulted from reduction of both osmotic potential and turgor pressure (Einhellig et al., 1985). Treatments with 0.25 mM ferulic acid reduced phosphorus content of roots and shoots of 2-week-old sorghum seedlings. Roots of treated plants also had lower concentrations of potassium and magnesium. In some cases, shoot potassium and iron were lower but magnesium and calcium were higher (Kobsa and Einhellig, 1987). Roots of alfalfa inhibited by water extracts of alfalfa shoots had 46% fewer and 54% shorter root hairs compared to the control, but an anatomical study showed no clogging of root xylem vessels of treated plants (Hedge and Miller, 1992).

The objective of this study was to investigate the chemical basis of the differential allelopathic potential among three sorghum hybrids (Asgrow Topaz, Taylor Evans Y-101G, and Warner W-744DR) reported previously (Ben-Hammouda, 1994). Total phenolic content and concentrations of phenolic acids that have been previously implicated in allelopathic activity of sorghum (Guenzi and McCalla, 1966) were determined.

METHODS AND MATERIALS

Collection and Processing of Sorghum Plant Parts

Randomly selected sorghum plants grown at the University of Missouri Agronomy Research Center in 1991 and 1992 were collected intact at harvest

maturity. Sorghum plants collected both years were free of disease and insect infestations. Roots were washed with tap water to remove the soil, and whole plants were stored frozen until analysis. Sorghum hybrids studied included Asgrow Topaz, Taylor Evans Y-101G, and Warner W-744DR and represented the range of suspected allelopathic potential identified in previous field and laboratory studies (Ben-Hammouda, 1994). Briefly, field studies involved planting wheat after sorghum harvest and using wheat grain yield as a measure of response to potential allelopathy. Subsequent laboratory studies relied on the response of wheat seedling radicle growth to extracts of sorghum plant components in agar bioassays.

Extraction

Sorghum plants were washed gently with distilled water, blotted, and separated into heads, leaves, culms, and roots. Heads were hand-threshed and separated into seeds and glumes. Leaves, culms, and roots were chopped into 1-cm-long pieces. All sorghum plant parts were dried at 50°C for 24 hr. A 2.5-g portion of each plant part was placed in 50 ml distilled water and agitated on a rotary shaker for 24 hr at 200 rpm. Extracts were filtered (Whatman No. 2 filter paper) under vacuum, centrifuged at 12,500 rpm for 20 min at 8°C, and filter-sterilized (0.2-μm membrane) under vacuum prior to total phenol analysis.

Soil samples from a controlled study in which one plant of each sorghum hybrid was grown to full maturity in a separate 20-liter container were extracted for total phenol analysis. Soil extraction followed the procedure described by Read and Jensen (1989). Briefly, a sample of 250 g dry soil was extracted in 250 ml of distilled water by shaking for 24 hr at 200 rpm. Soil suspensions were filtered (Whatman No. 2 filter paper) under vacuum prior to total phenol analysis.

Determination of Total Phenolics

The Folin-Denis method was used for total phenol analysis (A.O.A.C., 1990) except that ferulic acid was used as the standard, since it is an allelopathic agent (Haslam, 1988) and has been previously isolated from sorghum residues (Guenzi and McCalla, 1966).

Folin-Denis Reagent. A mixture of 10 g Na_2WO_4, 2 g phosphomolybdic acid, and 5 ml H_3PO_4 in 75 ml distilled water was refluxed for 2 hr, cooled, and diluted to 100 ml with distilled water.

Sodium Carbonate-Saturated Solution. Anhydrous Na_2CO_3 (4 g) was added to 10 ml distilled water and dissolved for 1 hr at 70–80°C, cooled overnight, and filtered through glass wool.

Ferulic Acid Standard Solution. Ferulic acid (10 g) was dissolved in 100 ml distilled water. Aliquots of 0, 0.2, 0.4, 0.6, 0.8, and 1 ml of the standard

ferulic acid solution were dispensed into tubes containing 0.5 ml Folin-Denis reagent and 1 ml saturated Na_2CO_3 solution. The standards were diluted to 10 ml with distilled water and quickly shaken. Absorbance was determined after 30 min at 750 nm (Blum et al., 1991) on a spectrophotometer (Pharmacia Ultrospec III).

Determination of Total Phenolics. Each sorghum or soil water extract (1.0 ml) was prepared by adding Folin-Denis and Na_2CO_3 reagents. Absorbance was determined, and the total phenolic content was obtained using the standard curve. Units of total phenolics were expressed in milligrams of ferulic acid equivalents per milliliter extract. For soil extracts, total phenolics in ferulic acid equivalents per milliliter of extract were expressed on a per gram basis since the ratio of extraction was 1 : 1 (w/w). For sorghum extracts, ferulic acid equivalents were multiplied by 20 based on an extraction ratio of 1 : 20 (w/w).

Determination of Phenolic Acids by HPLC Analysis

Water extracts of sorghum plants and soils used for estimation of total phenolics were analyzed for five individual phenolic acids. The pH of each water extract sample was lowered to 2.6–3.0 with dilute H_3PO_4. An aliquot of 5 ml from each sample was partitioned with an equal volume of diethyl ether two times by shaking the mixture for approximately 1 min and allowing it to stand until diethyl ether partitioning occurred. The pooled partitioned volumes of diethyl ether from each sample were dispensed into a 50-ml Erlenmeyer flask and evaporated to near dryness in a suction chamber. Residues in the flask were dissolved in 2 ml methanol and filtered through a 0.2-μm sterile membrane.

Methanol extracts were analyzed for phenolic acids using a Beckman model 338 HPLC system. The system consisted of two model 110B pumps operated at a 1 ml/min flow rate, a model 507 autosampler with a 100-μl sample loop, and a model 166 variable-wavelength UV detector set at 280 nm. A Beckman octadecyl (C18) reversed-phase column (Ultrasphere ODS, 5 μm) with dimensions at 250 $\times$ 4.6 mm (ID) was used. The mobile phase was methanol–water (30:70, v/v).

Five different phenolic acids (POH, VAN, SYR, PCO, and FER) were identified in the samples by comparing their relative retention times with that of the standard chromatogram. The identified peaks were then quantified by preparing standard curves using peak areas for each phenolic acid.

Bioassay

Potential allelopathic activity of water extracts of sorghum plant parts was assessed using a wheat seedling bioassay (Ben-Hammouda, 1994). Water extracts (2.5 g plant tissue per 50 ml water shaken for 24 hr) were incorporated in 1.0% agar and dispensed into test tubes. Pregerminated Cardinal wheat seeds were

placed on the solidified extract-agar (one pregerminated seed per tube) and incubated in the dark at 28°C for 48 hr. Radicle lengths were measured and compared to controls containing no plant extracts. Each determination consisted of measurements from 10 tubes per treatment, including controls, replicated four times.

Statistical Analyses

Root length data from bioassays were subjected to analysis of variance followed by the LSD test to determine significant differences among mean values at the 0.05 level of probability. Regression and correlation analyses of wheat radicle growth inhibition were conducted with total phenolics, individual phenolic acid (POH, VAN, SYR, PCO, FER) concentrations estimated from sorghum plant parts, and total phenolic content as quantitative variables and the sources of phenolics (hybrid, growing season, plant parts, and plant parts within hybrid) as qualitative variables.

RESULTS AND DISCUSSION

The three sorghum hybrids were presumed to be allelopathic on wheat based on wheat grain yield depression in field tests over two years and on wheat seedling radicle reduction in laboratory bioassays of sorghum extracts. Relative to water controls, extracts of one or more plant parts from each hybrid reduced wheat radicle growth (Table 1). Wheat radicle inhibition was positively correlated ($r = 0.66$) with total phenolic content (Table 2) in individual plant parts (Figure 1). When inhibition of wheat radicle growth was regressed on total phenolics as a quantitative variable and the source of phenolics as qualitative variables, significant factors in the best fitting regression were total phenolics and the plant parts glumes and culms. Parameters in this best fitting equation were: $b_0 = -4.3$, $b_1 = 267.0$, $b_2 = -14.8$, and $b_3 = 28.0$ with $Y =$ inhibition (dependent variable), $x_1 =$ total phenolics, $x_2 =$ glumes, and $x_3 =$ culms (independent variables). Phenolics from glumes reduced the inhibition by an average of 14.8 times more than any other source of phenolics. However, phenolics from culms increased the inhibition 42.8 times more than phenolics from glumes and 28 times more than any other source of phenolics.

Ranges of total phenolics in seeds, glumes, leaves, and roots were small (Table 2) but inhibition varied significantly (Table 1). This suggested that inhibition may be associated with qualitative rather than quantitative components of phenolics.

Three phenolic acids (POH, VAN, SYR) were found in different amounts in all plant parts of the three sorghum hybrids during both growing seasons (Table 3). Concentrations varied among plant parts within and between sorghum

TABLE 1. INHIBITION OF WHEAT RADICLE GROWTH BY WATER EXTRACTS FROM PLANT PARTS OF THREE SORGHUM HYBRIDS

Sorghum hybrid	Inhibition of wheat radicle growth (% of control)[a]				
	Seeds	Glumes	Leaves	Culms	Roots
1991					
Asgrow Topaz	−0.5[b]	12.1	8.9*	25.2*	50.8
Warner W-744DR	13.4*	10.0	66.6*	32.6*	45.5*
Taylor Evans Y-101G	6.7	20.6*	56.5*	74.4*	63.2*
1992					
Asgrow Topaz	5.7	40.2*	70.0*	74.7*	31.4*
Warner W-744DR	4.4	16.7*	68.5*	73.3*	37.0*
Taylor Evans Y-101G	2.7	36.9*	65.9*	65.2*	41.6*

[a]Values within a column within year followed by asterisk (*) differ significantly from the water control (LSD test, $P < 0.005$).
[b]Negative sign indicates increase over control.

TABLE 2. TOTAL PHENOLIC CONTENT IN SORGHUM PLANT PARTS

Sorghum hybrid	Total phenolics (mg ferulic acid equivalents/g)[a]				
	Seeds	Glumes	Leaves	Culms	Roots
1991					
Asgrow Topaz	0.033	0.120	0.217	0.060	0.166
Warner W-744DR	0.074	0.160	0.208	0.105	0.189
Taylor Evans Y-101G	0.035	0.135	0.214	0.202	0.189
1992					
Asgrow Topaz	0.015	0.160	0.239	0.140	0.215
Warner W-744DR	0.051	0.193	0.246	0.122	0.203
Taylor Evans Y-101G	0.007	0.171	0.232	0.132	0.182

[a]Total phenolics determined by Folin-Denis method (A.O.A.C., 1990) expressed as milligrams ferulic acid equivalents per gram sorghum tissue.

hybrids. Of the five phenolic acids analyzed, only PCO or FER was absent from any plant part, with FER absent in 53% of the cases. In 1991, Taylor Evans Y-101G did not have any detectable FER. Wheat radicle inhibition was found positively associated with each of the five phenolic acids, the highest correlation occurring with VAN (Table 4). While significant individually, asso-

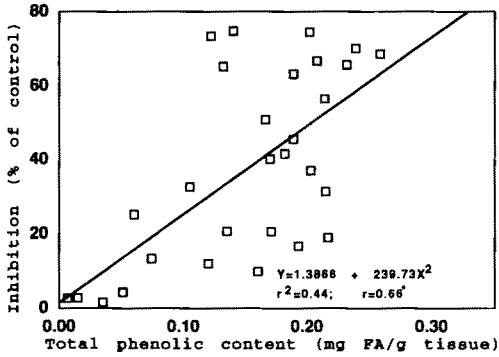

FIG. 1. Relationship of wheat radicle inhibition to total phenolic content in water extracts of plant parts from sorghum hybrids grown in 1991 and 1992. Total phenolic content expressed in ferulic acid equivalents per gram of sorghum tissue.

ciations were small, accounting for less than 20% of the variation in inhibition. Together, the five phenolic acids accounted for 72% of the variability in inhibition of wheat radicle growth due to total phenolics. Plant species are known to contain many phenolic acids in addition to those assayed, and many are phytotoxic (Chou and Patrick, 1976). Total inhibitory effects on sorghum may therefore involve other phenolic compounds and/or allelochemicals of different chemical types. Sampling intensity did not permit detection of statistically significant relationships between total phenolic content or individual phenolic acid levels and inhibition within individual plant parts, hybrids, or years.

Bioassays of water extracts from soils on wheat radicle growth showed that soil with no plants, and soils planted to Warner W-744DR and Asgrow Topaz were relatively equal in allelopathic potential (Ben-Hammouda, 1994). The minimal differences among total phenolic contents in these soils likely account for the low inhibitory activity (Table 5). However, inhibition by soil extracts from Warner W-744DR and Asgrow Topaz were significant ($P \leq 0.05$) compared to control soil, suggesting that allelochemicals other than the detected phenolics were released by sorghum roots. Extracts from Taylor Evans Y-101G soil contained the highest total phenolic content and were significantly higher in allelopathic potential, indicating a relationship between quantity of phenolics released by roots and inhibitory activity, similar to that illustrated for plant extracts (Figure 1). Other phytotoxins exuded from roots have been reported, including sorgoleone from sorghum (Einhellig and Souza, 1992) and phenolic acids from alfalfa roots, a known allelopathic crop (Abdul-Rahman and Habib, 1989; Read and Jensen, 1989). Identification of individual phenolic acids in sorghum root exudates has not been previously reported.

Soil extracts analyzed by HPLC contained only POH, VAN, or SYR (Table

TABLE 3. PHENOLIC ACID CONTENT IN SORGHUM PLANT PARTS

		Phenolic acid content (mg/kg tissue)									
		Seeds		Glumes		Leaves		Culms		Roots	
Sorghum hybrid	Phenolic acid	1991	1992	1991	1992	1991	1992	1991	1992	1991	1992
Asgrow Topaz	POH	4.1	4.3	22.5	1.7	88.8	613.2	26.8	104.2	39.8	70.5
	VAN	1.9	0.6	7.4	12.9	22.2	99.8	14.4	9.2	13.7	15.0
	SYR	0.4	1.0	1.0	4.3	1.8	6.8	1.7	2.3	3.8	4.7
	PCO	2.0	0.0	3.7	0.0	6.1	3.9	4.3	0.5	0.0	2.8
	FER	2.0	0.0	1.4	0.0	0.1	3.9	0.0	0.5	1.6	0.0
Warner W-744DR	POH	2.8	5.0	4.6	38.1	20.6	13.7	3.2	1.4	24.8	2.8
	VAN	2.5	2.8	3.7	6.6	60.5	3.4	3.8	10.0	13.4	0.4
	SYR	3.7	0.5	3.0	1.9	83.4	2.6	1.6	1.0	4.3	51.3
	PCO	0.0	0.1	0.5	0.0	0.4	25.6	0.6	0.0	0.0	0.0
	FER	0.0	0.0	0.0	0.7	3.4	0.9	0.0	0.0	0.0	0.8
Taylor Evans	POH	0.4	9.4	0.4	54.5	2.7	278.1	0.3	93.1	0.7	172.1
	VAN	1.9	1.7	0.4	15.7	2.7	49.9	0.3	1.4	0.7	25.7
	SYR	11.2	0.4	10.7	2.3	86.0	22.6	9.4	1.6	23.6	0.4
	PCO	1.5	0.5	2.2	6.0	7.5	13.0	7.5	0.0	1.0	4.5
	FER	0.0	0.0	0.0	0.5	0.0	1.6	0.0	1.4	0.0	2.0

TABLE 4. SIMPLE CORRELATION COEFFICIENTS BETWEEN WHEAT RADICLE GROWTH INHIBITION (% OF CONTROL) AND INDIVIDUAL PHENOLIC ACIDS FROM PLANT PARTS OF THREE SORGHUM HYBRIDS OVER TWO YEARS[a]

Phenolic acid	Correlation coefficient[b]
POH	0.35
VAN	0.44*
SYR	0.32
PCO	0.41*
FER	0.33

[a] $df = 29$.
[b] Values followed by (*) are significant at $P < 0.05$; remaining values are significant at $P < 0.10$.

TABLE 5. TOTAL PHENOLIC CONTENT OF SOILS PLANTED TO SORGHUM

Sorghum hybrid	Total phenolics (μg ferulic acid equiv/g)[a]	Seedling inhibition (% of control)[b]
No plants	0.07	3.7
Asgrow Topaz	0.09	6.9*
Warner W-744DR	0.08	5.9*
Taylor Evans Y-101G	0.20	27.8*

[a] Total phenolics expressed in ferulic acid equivalents per gram of soil.
[b] Inhibition of wheat seedling radicle growth from bioassay (Ben-Hammouda, 1994). Values followed by (*) differ significantly from the water control (LSD test, $P < 0.05$).

6). The highest phenolic acid content, which was predominantly SYR, was found for Taylor Evans Y-101G soil extract. Taylor Evans Y-101G soil extract also exhibited the highest inhibition toward wheat seedling growth (Table 5), suggesting that SYR may be a major source of allelopathic activity in this sorghum hybrid. SYR was detected in the other soil extracts at about 47% lower concentrations and only slightly inhibited wheat seedling growth. Antagonism of activity of SYR by other phenolic compounds in these extracts may partially account for the lower inhibitory activity (Patterson, 1989).

Phenolic acids detected in control soil extracts may have originated from microbial synthesis (Waniska et al., 1988) or humic acid degradation (Kobsa and Einhellig, 1987). Phenolic acids released from above-ground plant parts were prevented from reaching soil, thus phenolic acids detected were of root or soil origin. Higher amounts of POH and SYR in Asgrow Topaz and Taylor Evans Y-101G soil extracts, respectively, compared to control soil indicated root exudation. High concentrations of POH and SYR in vegetative tissues of

TABLE 6. PHENOLIC ACID CONTENT OF SOILS PLANTED TO SORGHUM

Sorghum hybrid	Phenolic acid[a]	Concentration (mg/kg soil)
No plants	POH	0.007
	VAN	0.000
	SYR	0.020
Asgrow Topaz	POH	0.032
	VAN	0.015
	SYR	0.020
Warner W-744DR	POH	0.000
	VAN	0.000
	SYR	0.020
Taylor Evans Y-101G	POH	0.000
	VAN	0.000
	SYR	0.038

[a]FER and PCO were not detected in any soil extract.

Asgrow Topaz and Taylor Evans Y-101G suggest that excess production of these phenolic acids may be released by roots into soil. VAN was not detected in control extracts, consequently VAN detected in Asgrow Topaz soil extracts suggests that root exudates are also a primary source of VAN in soil.

In conclusions, the allelopathic potential of sorghum plant parts was positively correlated with total phenolic content. Within plant parts, the relationship was more qualitative than quantitative. Three phenolic acids (POH, VAN, SYR) were found in all sorghum plant parts, while PCO and FER were detected inconsistently. Individual phenolic acids were significantly associated with inhibition of wheat radicle growth. The variability of allelopathic potential of plant parts within a sorghum hybrid was difficult to attribute to individual phenolic acids or a combination of phenolic acids. Allelopathic potential may be a result of either antagonism or synergy, depending on the presence of a specific phenolic acid, its proportion relative to other phenolic acids, or a combined action of several other allelochemicals present in plant tissues and exudates.

Sorghum roots exuded phenolics including POH, VAN, and SYR. Under favorable field conditions, exudates may contribute to the overall allelopathic potential of sorghum during growth and after harvest when vegetative and root residues remain in the field prior to planting a subsequent crop.

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PRODUCTION AND RELEASE OF (*Z*,*E*)-9,12-TETRADECADIENAL BY SEX PHEROMONE GLANDS OF FEMALES OF *Plodia interpunctella* (LEPIDOPTERA: PYRALIDAE).

P.E.A. TEAL,[1,*] R.R. HEATH,[1] B.D. DUEBEN,[1] J.A. COFFELT,[1] and K.W. VICK[1,2]

[1]*Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA ARS 1700 SW 23 Dr. Gainesville, Florida 32604*

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Abstract—Extracts of sex pheromone glands obtained from females of *Plodia interpunctella* contained detectable amounts of (*Z*,*E*)-9,12-tetradecadien-1-ol acetate (*Z*9,*E*12–14:Ac) and (*Z*,*E*)-9,12-tetradecadien-1-ol (*Z*9,*E*12–14:OH) 4 hr prior to the first scotophase after adult emergence. The amount of pheromone increased during the first 4 hr of the scotophase and then declined to low levels during the subsequent photophase. Decapitation of females immediately after emergence, prior to expansion of the wings, inhibited production of pheromone during the subsequent 48 hr. Injection of extracts of the heads of 1-day-old females of *P. interpunctella* of partially purified extracts of the cephalic ganglia of females of the corn earworm moth into decapitated females stimulated production of both *Z*9,*E*12–14:Ac and *Z*9,*E*12–14:OH as well as production of (*Z*,*E*)-9,12-tetradecadienal (*Z*9,*E*12–14:Al). This aldehyde was subsequently identified from extracts of pheromone glands obtained from naturally calling females as well as from volatiles emitted by calling females. Studies on the terminal steps in biosynthesis of the pheromone showed that *Z*9,*E*12–14:OH was produced from the corresponding acetate and that *Z*9,*E*12–14:Al was produced from the alcohol via the action of an oxidase(s).

Key Words—*Plodia interpunctella*, sex pheromone, pheromone biosynthesis, Indian meal moth.

*To whom correspondence should be addressed.
[2]Current address: National Program Staff, USDA, ARS, Bldg. 005, BARC-West, Beltsville, Maryland 20705

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INTRODUCTION

Plodia interpunctella, the Indian meal moth (IMM), is a worldwide pest of stored products. This insect has been the target of numerous studies aimed at development of semiochemical-based monitoring and control programs because of the limited number of pesticides available for control of pests in food storage facilities (Vick et al., 1981). The principal component of the sex pheromone produced by IMM females, (*Z*,*E*)-9,12-tetradecadien-1-ol acetate (*Z*9,*E*12-14:Ac) was identified concomitantly by Kuwahara et al. (1971) and Brady et al. (1971). Although this compound was sufficient to stimulate attraction of males, Brady et al. (1971) hypothesized that additional pheromone components were probably also used for communication. This led to the identification of the corresponding alcohol analog, (*Z*,*E*)-9,12-tetradecadien-1-ol (*Z*9,*E*12-14:OH) from rinses of containers that housed females (Sower et al., 1974). Although initial studies indicated that *Z*9,*E*12-14:OH did not enhance the effect of *Z*9,*E*12-14:Ac in bioassays using IMM males, this compound inhibited the response of males of the almond moth, *Ephestia cautella* (Sower et al., 1974). Subsequent studies by Soderstrom et al. (1980) and Vick et al. (1981) demonstrated that both *Z*9,*E*12-14:Ac and *Z*9,*E*12-14:OH were important components of the pheromone communication system of the IMM.

Studies have been conducted on the diel periodic production of these two pheromone components by IMM females (Coffelt et al., 1978). These studies showed that only small amounts of pheromone were present in gland washes obtained during the first photophase after emergence, although a significant number of females exhibited calling behavior during this period (Coffelt et al., 1978). Smithwick and Brady (1977) showed that extracts obtained from ethanol homogenates of abdomen tips as well as a fraction obtained from gas chromatographic (GC) separation of the ethanolic extracts from newly eclosed females stimulated male wing fluttering and resulted in attempted copulation by 50% of the males at concentrations of 6.4×10^{-4} gland equivalents (ethanol extracts) and 9.8×10^{-4} gland equivalents (GC fraction). This, plus evidence that a significant behavioral response by males could be evoked from ethanolic extracts obtained from pharate adults (Smithwick and Brady, 1977), suggested that females were biosynthesizing pheromone even as pupae.

We were interested in determining if pheromone was present in extracts of pheromone glands of newly emerged females and in determining the periodicity of production and release of pheromone by females. In addition, we were interested in the biosynthetic relationship between *Z*9,*E*12-14:Ac and *Z*9,*E*12-14:OH. The studies reported here were conducted to address these questions. We also report the identification of a new compound, produced and released from pheromone glands of females during the reproductive period, that is biosynthetically related to previously identified sex pheromone components.

METHODS AND MATERIALS

Insect Rearing and Holding. Insects were reared using standard procedures described by Silhacek and Miller (1972). After pupation, females were segregated and held in vented 19.0 (long) × 14.0 (wide) × 7.5 cm (deep) clear plastic boxes at 27 ± 2°C and 60 ± 4% relative humidity in a 16L:8D reversed photoperiod. Newly emerged females were transferred to new containers prior to expansion of the wings on a daily basis and were provided with cotton balls soaked in a solution of 10% sugar in water. Adults were maintained under the same environmental conditions as pupae. For most studies females from a colony maintained at our laboratory since 1972 (Silhacek and Miller, 1972) were used as test individuals. Additional experiments were conducted using insects obtained from a colony maintained at the Stored Product Insect Research Unit, USDA, Madison, WI. This colony has been in culture for less than five generations. Insects from this colony were reared under the same conditions as those of the Gainesville colony.

Periodicity of Pheromone Production. In initial studies the terminal abdominal segments, which contain the sex pheromone gland, were excised from pharate adults (black stage of Smithwick and Brady, 1977) or from females immediately after emergence and prior to wing expansion and from females at 2-hr intervals for the first 8 hr after emergence as well as from females at 4-hr intervals throughout the subsequent 40 hr. Excised abdomen tips from three females were extracted for 15 min in 10 μl of hexane containing 5 ng each of octadecane and nonadecane (internal standards) in the same vial, and the extracts were stored at −60°C until analysis by capillary gas chromatography (GC) and GC-mass spectroscopy (GC-MS).

Volatile Collections. Volatile pheromones released by females were collected using the push–pull system described by Heath et al. (1992). Compressed air was purified through two charcoal filters and passed through a glass frit near the upwind end of glass chambers (5 cm OD × 30 cm long) to provide a laminar airflow. Airflow through the chamber was pulled through a collector trap by vacuum. Each collection apparatus was fitted with three collector traps, any one of which could be selected for collection of volatiles without disturbing the insects by using manually activated external valves. Airflow through the system was set at 0.9 liters/min with a slight positive pressure of 0.2 cm H_2O established in the chamber using the vacuum (Heath et al., 1992). Volatile collections were obtained from groups of 20 females with collector traps that were changed at 2-hr intervals throughout the scotophase. After collection of volatiles, the Super Q collection traps (see Heath et al., 1992) were extracted with three 20-μl portions of CH_2Cl_2 and 20 ng of the above internal standards in hexane were added. The extract was then concentrated to ca. 25 μl under argon and analyzed by GC and GC-MS.

Endogenous Regulation of Pheromone Production. The heads were removed immediately after emergence in studies to assess the effect of decapitation on pheromone production. Pheromone glands were excised at 2-hr intervals thereafter. Females decapitated at emergence and aged for 4 hr were also used in studies to determine if neurohormones were involved in regulation of induction of pheromone production. These females were injected with 5 μl of saline (Christensen et al., 1991) or saline containing one equivalent of an extract of the heads of 12-hr-old IMM females or one head equivalent of a partially purified extract from females of the corn earworm moth, *Helicoverpa zea*. Injections were made by inserting a 0.1-mm-ID fused silica needle attached to a 10-μl syringe through the front of the thorax and into the abdomen. Insects were not used if fluid escaped from the injection site after removal of the needle. The pheromone glands of females were excised and extracted as described above at 30 min intervals after injection of test samples.

Extracts of the heads of females were prepared by homogenizing 20 heads in 50 μl of saline as described by Teal et al. (1989). The homogenate was centrifuged at 18,000*g* for 10 min and the supernatant removed. The pellet was then rehomogenized in 50 μl of saline and centrifuged. The volume of the combined supernatants was adjusted to 100 μl with saline. Extracts of the cephalic ganglia of females of the corn earworm moth were obtained by homogenizing the cephalic ganglia in H_2O containing 0.1% trifluoroacetic acid (TFA) as described elsewhere (Teal et al., 1993). These extracts were partially purified by reverse-phase solid-phase extraction using a Bakerbond SPE octadecyl (500 mg) column. The aqueous extract was applied to the preconditioned column (see Teal et al., 1993) and the column washed with 6 ml of H_2O containing 0.1% TFA. Peptides, including pheromone-biosynthesis-activating neuropeptide were then eluted with 4 ml of 80:20 acetonitrile–H_2O containing 0.1% TFA. This fraction was concentrated to dryness with a Speed Vac concentrator, and the residue was dissolved in sufficient saline to yield a concentration of 1 head equivalent/5 μl.

Terminal Steps in Pheromone Biosynthesis. All experiments conducted on the terminal steps involved in biosynthesis of pheromone were conducted with females that had been decapitated immediately after emergence. The terminal abdominal segments, containing the pheromone glands, were exposed and excised from the abdomens 4 hr after decapitation. In initial experiments five isolated abdomen tips were placed in a conical microvial (300 μl capacity), and 5 μl of hexane containing 20 ng of the internal standard with or without 200 ng of either *Z*9,*E*12–14:OH or (*Z*)-11-tetradecen-1-ol (*Z*11–14:OH) was added. The vials were capped with screw-cap GC autosampler caps having Teflon septa and incubated at room temperature for 1 hr prior to the addition of 10 μl of hexane and removal of the solvent. The extract was analyzed by GC. In other experiments the same procedure was followed, except that the vials were purged with N_2 for 15 min prior to placing the glands, substrate, and internal standard

into the vials. Some samples were held continuously in a N_2 saturated environment while others were held in air. After 30 min, 10 μl of hexane was added to the vials to extract pheromone and the extract was analyzed by GC.

Experiments to determine if acetates were converted to alcohols were conducted using (*Z*)-11-tetradecen-1-ol acetate (*Z*11–14:Ac) or *Z*9,*E*12–14:Ac as the substrate. A 2-μl drop of hexane containing 1 μg of acetate was placed in a conical microvial, the hexane was evaporated under a stream of N_2, 25 μl of saline containing 0.5% acetone was added, and the vial was sonicated for 15 min. After sonication the excised terminal abdominal segments from five females were added to the capped vial and the sample was sonicated for 15 min. After sonication the sample was incubated for an additional 45 min prior to extraction of the sample two times with 25-μl aliquots of hexane-containing internal standard. The hexane extracts were concentrated to 10 μl prior to GC analysis.

Chemical Analysis. All GC analyses were conducted using Hewlett-Packard 5890 GCs equipped with split/splitless and cool on-column injectors and flame ionization detectors. For routine analyses of gland extracts, samples were analyzed using split/splitless injectors and 30-m × 0.25-mm-ID (0.25-μm film thickness) fused silica columns coated with SE 30 or Carbowax 20 M (Altech, Econocap columns). Samples were injected in the splitless mode at an oven temperature of 60°C with the injector being purged at 1 min. After 2 min, the oven temperature was increased at 10°C/min to final temperatures of 185°C (SE 30) and 180°C (Carbowax). Helium was used as the carrier gas at a linear flow velocity of 19 cm/sec.

Samples obtained from collection of volatiles emitted by females were analyzed using the cool on-column injector. The injector was attached to a 10-m × 0.25-mm-ID deactivated fused silica retention gap column as described by Murphy et al. (1993). The retention gap column was interfaced to a 30-m × 0.25-mm-ID fused silica column coated with Carbowax 20 M. Conditions of chromatography were the same as those described above. Data from all GC analyses were acquired using Nelson Analytical 900 Intelligent Interfaces and analyzed with Nelson Analytical 2600 software. Chemical ionization (methane reagent gas) MS analyses were conducted using a Nermag R10-10 MS interfaced to a Hewlett-Packard 5790 GC. Columns and conditions of chromatography used for GC-MS analyses were the same as those used in GC analyses. The spectra and retention times of compounds eluting during the analyses of natural product samples were compared with those obtained from analyses of synthetic standards.

RESULTS AND DISCUSSION

Periodicity of Pheromone Production. Initial studies quantitated the total combined amounts of *Z*9,*E*12–14:Ac and *Z*9,*E*12–14:OH in extracts obtained from abdomen tips of groups of three females at emergence and at intervals

during the subsequent 48 hr. However, because Z9,E12–14:Al was subsequently identified from extracts, the amounts of this compound were also included in the data presented in Figure 1. Extracts from newly emerged females did not contain detectable amounts of pheromone. Pheromone was detected in extracts obtained from 4-hr-old females, and the amounts of pheromone increased to a peak at 12 hr after emergence (Figure 1). This corresponded to the fourth hour of the first scotophase after emergence. The amount of pheromone declined during the subsequent 12 hr and remained low during the second photophase. The periodicity of production was repeated during the next scotophase (data not shown). Extracts obtained from groups of 10 pheromone glands from pharate adults (black stage pupae) did not contain detectable amounts of pheromone (five replicates). The inability to detect pheromone in extracts obtained from pharate and newly emerged females agrees with results obtained by Coffelt et al. (1978) but does not account for the positive bioassay results obtained using extracts from pupae and newly emerged females by Smithwick and Brady (1977). This discrepancy could result from differences in techniques used to extract the pheromone components. Smithwick and Brady (1977) used ethanolic homogenates of the pheromone glands, which probably resulted in much more efficient disruption and extraction of lipid-containing vesicles than was achieved in our

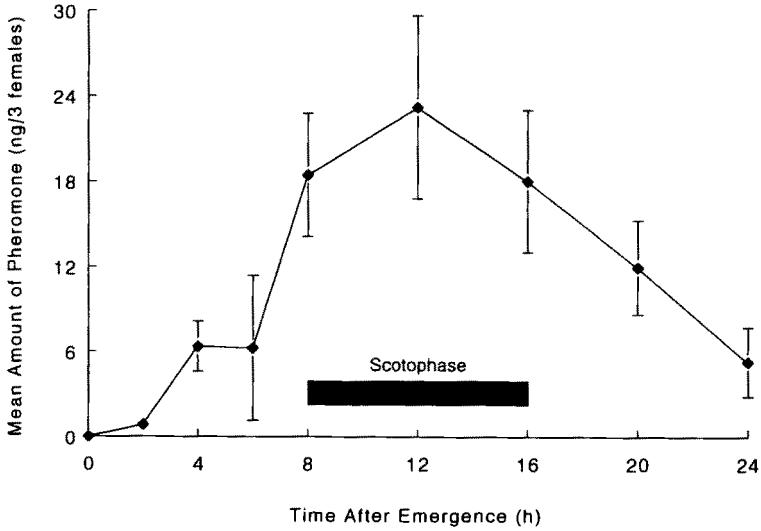

FIG. 1. Mean amounts of pheromone recovered from combined extracts of three glands of IMM females obtained at intervals (N = 13 groups/interval) during the first 24 hr after adult emergence. Means reflect the combined amounts of the three compounds identified (Z9,E12–14:Ac, Z9,E12–14:OH, and Z9,E12–14:Al) ± SE.

study using hexane. Thus, although we did not find pheromone in these extracts, it, or precursive compounds, could be present in subcellular structures that were not extracted using hexane.

Inhibition and Regulation of Pheromone Production. Decapitation of females at emergence resulted in complete inhibition of production of pheromone during the subsequent 24 hr. Injection of extracts of heads of IMM females (1 equivalent/female) into decapitated females stimulated production of pheromone, injection of saline had no effect. The amount of pheromone in gland extracts obtained at 30-min intervals after injection of the cephalic extracts increased over the first 2 hr and then declined (Figure 2). No pheromone was present in extracts obtained 6 hr after injection. Injection of partially purified extracts obtained from the cephalic ganglia of the corn earworm moth also stimulated production of pheromone. The maximum amount of *Z*9,*E*12-14 : Ac in extracts of three glands (3.67 ng ± 2.26, $N = 5$) was obtained from extracts 2 hr after injection. These results show that IMM females regulate the diel periodic production of pheromone with neurohormones, as do most other moth species (see Raina, 1993), and that pheromonotropic neuropeptides, present in the partially purified extracts of the corn earworm moth, will mimic the pheromonotropic effect of homogenates of IMM female brain extracts.

Interestingly, another compound was also detected with regularity in extracts obtained from insects that had been injected with homogenates of the cephalic ganglia. The amounts of this compound increased over the first 2 hr after injection of cephalic extracts and then declined (Figure 2). GC analyses conducted

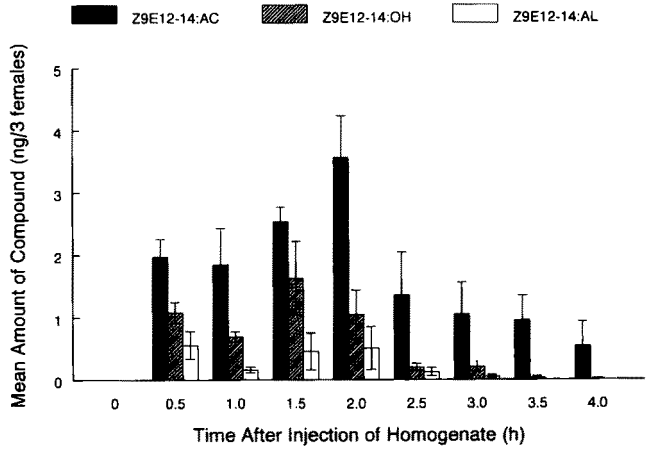

FIG. 2. Mean amounts of pheromone extracted from groups of three glands extracted at intervals after injection of extract of the cephalic ganglia ($N = 5$ groups/interval, ±SE).

using both polar and apolar capillary columns indicated that the compound had the same retention index as (*Z*,*E*)-9,12-tetradecadienal (*Z*9,*E*12–14:Al). Subsequent analyses of pheromone gland extracts from groups of 10–20 females obtained during the peak period of production of pheromone during the first scotophase (12 hr after emergence) showed that this compound was also present in these extracts (Figure 3a). Structural confirmation for *Z*9,*E*12–14:Al was provided by GC-MS analyses using both polar and apolar capillary columns (Figure 3b). The mean amounts of the three compounds present in pooled extracts of five females obtained at the peak of pheromone production during the sco-

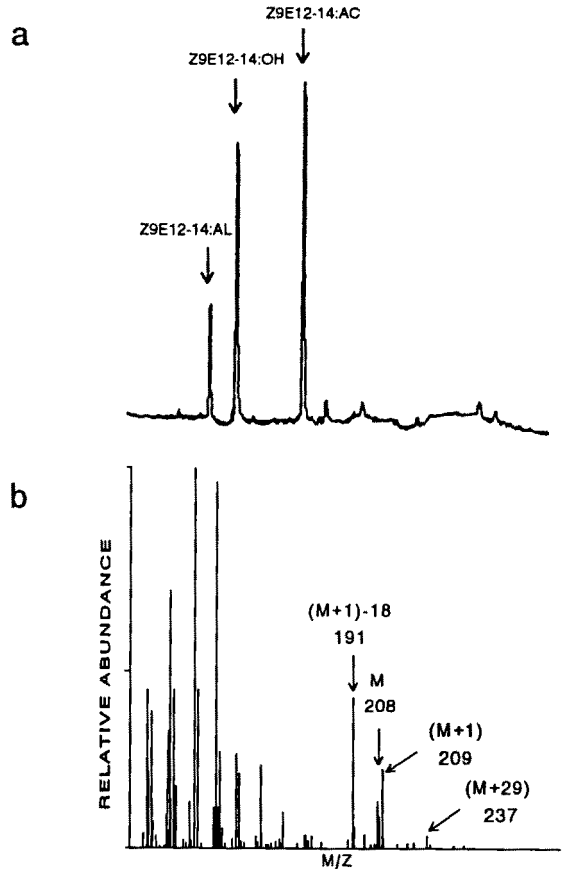

FIG. 3. GC-MS analysis of an extract of 20 glands of IMM females obtained 12 hr after emergence chromatographed on a 30-m × 0.25-mm-ID SE 30 fused silica column. (a) Total ion current chromatogram. (b) CI Mass spectrum of *Z*9,*E*12–14:Al.

tophase was determined to be (± SE): 19.38 ± 3.17 ng Z9,E12-14:Ac, 4.02 ± 0.46 ng Z9,E12-14:OH, and 1.42 ± 0.18 ng Z9,E12-14:Al (five replicates). The ratio present in extracts obtained from groups of three glands obtained from females 2 hr after injection of the cephalic extract was 69.6% Z9,E12-14:Ac, 20.8% Z9,E12-14:OH, and 9.6% Z9,E12-14:Al (Figure 2) while that in the extracts obtained from females at the peak of production was 78.1% Z9,E12-14:Ac, 16.2% Z9,E12-14:OH, and 5.7% Z9,E12-14:Al.

Analysis of Volatiles. Both GC and GC-MS analysis of volatiles emitted by females during the 2-hr collection intervals in the scotophase indicated that Z9,E12-14:Ac, Z9,E12-14:OH, and Z9,E12-14:Al were all released by calling females. Pheromone was released by females during the first 2-hr sample period but significantly more was released between 2–4 hr and 4–6 hr in the scotophase (Figure 4). Very little pheromone was released by females after the photophase began. Interestingly, the ratio of the three components determined from analysis of volatile collections was distinctly different from that present in extracts obtained from glands of calling females. This difference was not due to the ratio of Z9,E12-14:Ac to Z9,E12-14:OH, which was approximately the same as that determined from gland extracts (79% Z9,E12-14:Ac and 21% Z9,E12-14:OH in volatiles during hours 2–4 and 83% Z9,E12-14:Ac and 17% Z9,E12-14:OH in extracts obtained at hour 4), but was due to release of relatively large amounts of Z9,E12-14:Al.

The identification of Z9,E12-14:Al from both extracts of pheromone glands and volatiles emitted by calling females and the relatively large amounts of this compound found in volatile collections was somewhat surprising because, although a large number of studies have been conducted on the pheromones produced by IMM females, Z9,E12-14:Al had not been identified. To determine if Z9,E12-14:Al was produced by other colonies we obtained pupae from a colony of IMM maintained at Madison, Wisconsin. As indicated in Figure 4b these females also released significant amounts of Z9,E12-14:Al as well as the other two components. The ratio of aldehyde to acetate in these insects was not different at any time interval from the ratio found in volatiles collected from females of the laboratory colony.

Terminal Steps in Biosynthesis. The relatively high ratio of Z9,E12-14:Al found in volatile collections and the structural similarities between the three compounds suggested that the aldehyde might be the final product of the biosynthetic process involved in pheromone production. This situation has been found to occur in females of *Heliothis subflexa*, a noctuid moth that uses acetate, alcohol, and aldehyde analogs for pheromone communication (Teal et al., 1981; Klun et al., 1982; Heath et al., 1991). Extracts of the pheromone glands from calling females of *H. subflexa* contain a disproportionately low amount of aldehyde relative to the ratio present in volatiles released by females. In addition to the fact that the acetate and alcohol analogs are less volatile that the aldehydic components, both the acetates and alcohols are biosynthetic precursors of the

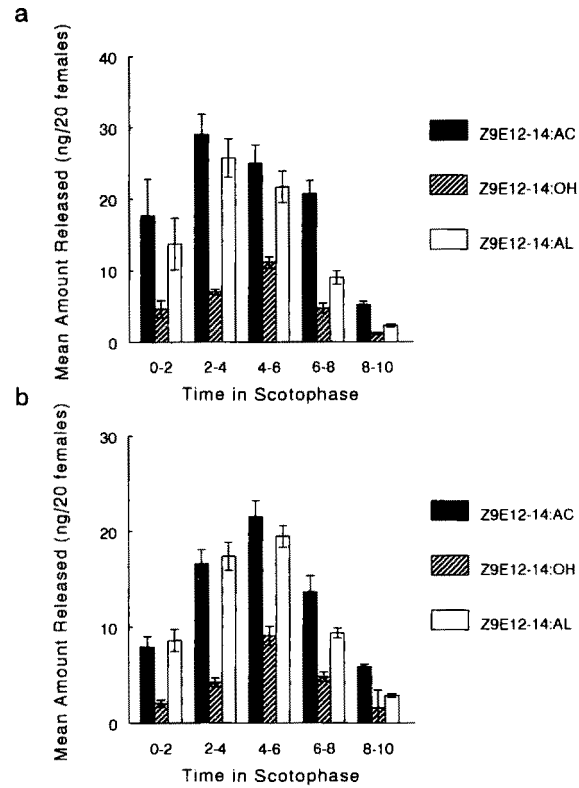

FIG. 4. Mean amounts of *Z*9,*E*12-14:Ac, *Z*9,*E*12-14:OH, and *Z*9,*E*12-14:Al detected in volatile collections obtained from groups of 20 females at 2 hr intervals during the scotophase. (a) Collections from females of the laboratory colony ($N = 5$, $\pm$ SE). (b) Collections from females of the Madison, Wisconsin, colony ($N = 5$, $\pm$ SE).

aldehydes, in addition to being pheromone components (Teal et al., 1989; Heath et al., 1991). Thus, the pheromone gland must contain relatively more acetate and alcohol in order to allow for release of the correct amount of aldehyde in the pheromone blend. To determine if a similar situation was occurring in the IMM, we conducted experiments to determine if exogenously applied alcohols would be converted to aldehyde by IMM females. Incubation of glands obtained from five decapitated females in hexane containing 200 ng of *Z*9,*E*12-14:OH and extraction of the samples after 1 hr resulted in recovery of 43.38 $\pm$ 7.77 ng ($N = 4$) *Z*9,*E*12-14:Al. Extracts of glands that had been incubated only in hexane did not contain any *Z*9,*E*12-14:Al ($N = 4$). Similar experiments con-

ducted using Z11-14:OH, an alcohol that has not been identified from pheromone glands or in volatile collections using IMM females, resulted in recovery of 48.25 ± 6.07 ng (N = 4) of (Z)-11-tetradecenal (Z11-14:Al) after a 1-hr incubation. Extracts from control experiments did not contain any Z11-14:Al. Incubation of glands in hexane containing Z11-14:OH for 30 min under N_2 did not completely inhibit the conversion of alcohol to acetate but did result in a threefold decrease in production of aldehyde with respect of samples obtained from parallel experiments in which glands were incubated under air. This strongly suggests that the conversion of alcohol to aldehyde results from the action of an alcohol oxidase, as has been described for *Heliothis* moths (Teal et al., 1989).

To determine if Z9,E12-14:Ac was the precursor for the alcohol analog, we first attempted to incubated isolated glands from decapitated females in hexane containing Z9,E12-14:Ac. However, no alcohol was recovered from any of the five replicate samples. In as much as the esterase responsible for converting acetates to alcohols in *Heliothis* species is a cellular enzyme and will not function in organic solvents (Teal et al., 1989), we repeated our experiment using Z9,E12-14:Ac that had been distributed into buffer by dissolving the acetate in acetone and then sonicating in buffer. Incubation of groups of five glands under these conditions for 1 hr and subsequent extraction with hexane resulted in recovery of 160 ± 18.84 ng (N = 5) of Z9,E12-14:OH and 10.41 ± 0.97 ng (N = 5) of Z9,E12-14:Al. Extracts from control experiments in which the glands were incubated in the same medium, but which did not contain Z9,E12-14:Ac, did not contain any Z9,E12-14:OH or Z9,E12-14:Al (N = 5). This study was repeated using Z11-14:Ac as a substrate. As expected, extracts from controls did not contain Z11-14:OH or Z11-14:Al, whereas extracts from glands incubated with Z11-14:Ac contained 187.1 ± 19.2 ng (N = 5) of Z11-14:OH and 12.22 ± 2.02 ng (N = 5) of Z11-14:Al. Results of these studies indicate that the pheromone glands of IMM females can convert Z9,E12-14:Ac to the corresponding primary alcohol and that this is subsequently converted to Z9,E12-14:Al by the pheromone gland. This implicates the action of acetate esterases and alcohol oxidases in the terminal steps of biosynthesis of pheromone components by IMM females and corroborates the identification of Z9,E12-14:Al from both gland extracts and volatiles released by calling females.

In summary, we have shown that IMM females regulate production of pheromone with neurohormones and that females can be stimulated to produce pheromone under circumstances in which pheromone would not normally be produced by injection of pheromonotropic substances. Using this technique to stimulate pheromone production, we were able to identify Z9,E12-14:Al from extracts of pheromone glands and then identify this component in extracts from naturally calling females as well as from volatiles released by calling females. Results of our studies on the terminal steps of biosynthesis have shown that

IMM females have the ability to produce *Z*9,*E*12–14:Al and that biosynthesis can proceed by converting *Z*9,*E*12–14:Ac to *Z*9,*E*12–14:OH, which is then converted to *Z*9,*E*12–14:Al. Although behavioral studies aimed at determining the biological effect of *Z*9,*E*12–14:Al on conspecific males or males of other competing species have not been completed, we believe that *Z*9,*E*12–14:Al could be an important component of the semiochemical communication system of this insect.

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INSECTICIDAL DEFENSES OF PIPERACEAE FROM THE NEOTROPICS

C.B. BERNARD,[1] H.G. KRISHNAMURTY,[2] D. CHAURET,[1] T. DURST,[1] B.J.R. PHILOGÈNE,[1] P. SÁNCHEZ-VINDAS,[3] C. HASBUN,[3] L. POVEDA,[3] L. SAN ROMÁN,[3] and J.T. ARNASON[1,*]

[1]*Institutes of Biology and Chemistry*
University of Ottawa
Ottawa Ontario, Canada K1N 6N5

[2]*Department of Chemistry*
University of Delhi
Delhi, India

[3]*Facultad de Ciencias de la Tierra y el Mar*
Universidad Nacional
Heredia, Costa Rica

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Abstract—Insecticidal and growth-reducing properties of extracts of 14 species of American neotropical Piperaceae were investigated by inclusion in diets of a polyphagous lepidopteran, the European corn borer, *Ostrinia nubilalis*. Nutritional indices suggested most extracts acted by postdigestive toxicity. *Piper aduncum*, *P. tuberculatum*, and *P. decurrens* were among the most active species and were subjected to bioassay-guided isolation of the active components. Dillapiol was isolated from the active fraction of *P. aduncum*, piperlonguminine was isolated from *P. tuberculatum*, and a novel neolignan from *P. decurrens*. The results support other studies on Asian and African *Piper* species, which suggest that lignans and isobutyl amides are the active defence compounds in this family.

Key Words—Piperaceae, botanical insecticide, isobutyl amides, lignans, dillapiol, conocarpan, piperlonguminine, European corn borer.

INTRODUCTION

The role of plants from the pepper family (Piperaceae) in traditional pharmacopeia is well established in tropical areas where these vines and shrubs are

* To whom correspondence should be addressed.

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particularly abundant (Atal et al., 1975; Schultes and Raffauff, 1990). Throughout the world, *Piper* plants have traditionally also been used against pests. For example, the Amazonian species, *Piper rotundistipulum*, is used locally as an insecticide and a fish poison (Schultes and Raffauf, 1990). *P. guineense* and *P. nigrum* are used as insecticides and molluskicides in several parts of Africa (Su and Horwat, 1981; Ivbijaro and Bolaji, 1990). The Indian species *P. longum*, *P. betle*, *P. peepuloides*, and *P. cubeba* have demonstrated insecticidal activity against mosquitos and flies (Srivastava, 1970; Miyakado et al., 1989) and were shown to repel grain pests (Kokate et al., 1980). The leaves of *P. futokatsura* from Taiwan and Japan are known as a feeding deterrent to the larvae of *Spodoptera litura* (Matsui and Munskata, 1975), and the leaves of *P. umbellatum*, *P. hispidum*, *P. auritum*, and plants reported only as *Piper* spp., which are native to Central America and the Northwest Amazonian basin, are used by indigenous peoples to prevent malaria and to remove head lice (Schultes, 1975, 1980).

Our knowledge about the compounds responsible for all of these insecticidal activities is limited. However, it is known that amides are frequently found in the Piperaceae (Greger, 1988), in particular olefinic or alkyl isobutylamides such as piperine, piperettine, trichostachine, peepuloidin, piplartin, and trichonine (Atal et al., 1975). They are toxic to fruit flies, azduki bean weevils, cockroaches, and several other insect species (Gbewonyo et al., 1993; Miyakado et al., 1983, 1989; Su and Horwat, 1981), and they act as neurotoxins (Gbewonyo et al., 1993).

Phenylpropanoids are another group of potentially active chemicals of common occurrence in *Piper* spp. They include monolignols, such as apiol, dillapiol, myristicin, eugenol, and safrol, and dimers of phenylpropanoids, such as the lignans sesamin, cubebin, yangambin or diaeudesmin. They often constitute a large part of the essential oil of the plant, as for instance, 85% safrol in the oil of the leaves of *P. auritum* (Castro and Poveda, 1983). However, the toxicity of these substances, with a few exceptions such as safrol, which has long been used as a natural insecticide (Coats, 1994), has not been established.

On the other hand, several of these compounds are known to synergize natural and synthetic insecticides. Sesamin and sesamolin, for instance, synergize the activity of natural and synthetic pyrethrins (Singh et al., 1976), and their synthetic analog, piperonyl butoxide, is currently included in most commercial preparations of pyrethrum (Worthing and Walker, 1993). Dillapiol, related to piperonyl butoxide, synergizes not only pyrethrins but also several carbamates and organochlorates (Handa and Dewan, 1974; Mukerjee et al., 1979; Parmar and Tomar, 1983). In addition, lignans and their monomers are known to repel or act as antifeedants to several grain pests (Harmatha and Nawrot, 1988; Nawrot and Harmatha, 1994; Pelter, 1986; Tomar et al., 1979). Kobusin and dillapiol reduce the growth of larvae of insect pests (MacRae and

Towers, 1984), including the larvae of a major pest of maize in North America, the European corn borer, *Ostrinia nubilalis* (Bernard et al., 1990).

These observations, together with our interest in the bioactivities of lignans in insects (Bernard et al., 1989, 1990), prompted us to investigate the activities of a group of *Piper* spp. from the neotropics (Costa Rica), a group largely unstudied from the point of view of their chemical ecology, against our model insect, the European corn borer. One African (*P. guineense*) and one Asian species (*P. betle*) were included for comparison with previous studies. We also report here the structure of a neolignan isolated for the first time from this family and the occurrence of piperlonguminine as the component responsible for the insecticidal activity of another newly investigated *Piper* species.

METHODS AND MATERIALS

Instrumentation

Melting points were determined on a Kofler hot stage and are uncorrected. ^{1}H NMR spectra were obtained on a Bruker 500 MHz spectrometer, and chemical shifts (δ) are reported in parts per million downfield from TMS. The number of protons, multiplicity (s = singlet, d = doublet, q = quartet, quint = quintet, m = multiplet, non = nonet), coupling constant, and assignment are presented in parentheses.

Mass spectrometry was carried out by a VG analytical 7070E spectrometer. UV visible and IR absorption spectra were both recorded on Perkin-Elmer spectrophotometers.

Plant Material

Plants collected in Costa Rica by C. Bernard, L. Poveda, and C. Hasbun in February 1992 included: *Piper aduncum* L., *P. auritum* Kunth, *P. aequale* Vahl, *P. hispidum* Sw., and *P. reticulatum* L. (E.A.R.T.H. station in Guapiles); *P. decurrens* C.DC, *P. marginatum* Jacq., and *P. pseudo-lindenii* C.DC. (C.A.T.I.E. in Cartago); and *P. tuberculatum* Jacq. in the city of Colon. *P. lanceiaifolium* Kunth was collected by J.T. Arnason, L. Poveda, and P. Sanchez in July 1993 near Colon; *P. obliquum* Ruiz & Pav. near Wilson botanical garden; *P. guanacastense* C.DC. near Mirador, Guanacaste; and *P. carrilloanum* C.DC. and an unidentified *Piper* sp. on the Peninsula de Osa in October 1993 by J.T. Arnason, K. Downum, L. Poveda, N. Moreno, and P. Sanchez. The fruits of *P. guineense* Schum & Thom came from Nigeria and were provided by F. Ewete, while the leaves of *P. betle* L. were collected in 1991 in Java, Indonesia, by J.T. Arnason and Y. Razali. Identification of species was carried out by L. Poveda and P. Sanchez, and voucher specimens were retained at the Universidad Nacional de Heredia and the University of Ottawa.

Fresh leaves or fruits were immediately immersed on site in 95% ethanol (EtOH), and were stored in this state two weeks or more, until processed. This first EtOH extract was set aside, and leaves were blended in a similar volume of EtOH and macerated for another two days. Both EtOH extracts were combined, filtered, and concentrated to dryness on a rotary evaporator under reduced pressure at 30°C. Water was further removed by freeze-drying the material for 12 hr, which resulted in a powder or a gum, i.e., crude extract, depending on the plant species. Dry weight of plant material was calculated from the plant solids remaining on the filter paper and the dry crude extract.

Insect Bioassays

Larvae of *O. nubilalis* Hübner (Lepidoptera: Pyralidae) from a laboratory colony were maintained according to procedures described previously (Arnason et al., 1985). The freeze-dried crude EtOH extract of each *Piper* spp. (leaves, or fruits) was incorporated into a meridic diet at 30°C at final concentrations of 4% and 0.4% (w/w). Twenty naïve second instars (6-day-old larvae weighing 8–12 mg) per *Piper* diet were individually distributed in 12-ml glass vials, which were closed with a cotton plug, and returned to the rearing conditions of the colony culture. The weights (wt) of each larva, the diet deposited, the diet remaining, and the frass in each vial were determined at five-day intervals. The relative growth rate (RGR = wt gain/mean wt during the period of study), and nutritional indices were calculated by the following equations as described in Reese (1979).

Consumption index (CI) = wt of food ingested/wt of larvae

Approximate digestability (AD)
= [(wt of food ingested − fecal wt)/wt of food ingested] × 100

Efficiency of conversion of ingested food (ECI)
= (wt gained/wt of food ingested) × 100

Efficiency of conversion of digested food (ECD)
= [wt gained/(wt of food ingested − wt of feces)] × 100

All statistical analysis were performed using Tukey's general linear models procedure (Tukey, 1949) with SAS for PC.

A second insect species, the rock hole breeding mosquito, *Aedes atropalpus* L. (Diptera: Culicidae), was used to monitor the isolation of chemicals from the plants. The small amount of material needed for the bioassays with larvae of these species when compared to the ECB, as well as the rapidity of the toxic response, made it a system of choice to follow the activity of the successive fractions of the plant extracts. Rearing conditions were previously reported (Watt et al., 1981). Ten second instars were pipetted into each of the glass vials containing the plant crude extract at concentrations of 10, 50, and 100 ppm.

TABLE 1. INSECTICIDAL ACTIVITY OF *Piper* SPP. CRUDE EXTRACTS TO MOSQUITO LARVAE

	Concentration of extract		
Pipp spp.	10 μg/ml	50 μg/ml	100 μg/ml
P. aduncum	7(5)[a]	2(2)	0
P. aequale	9(1)	8(1)	3(2)
P. auritum	8(1)	1(1)	0
P. betle	10(0)	10(0)	9(1)
P. carrilloanum	10(0)	9(1)	9(1)
P. decurrens	7(1)	5(2)	0
P. guanacastensis	8(1)	7(1)	7(1)
P. guineense	0	0	0
P. hispidum	8(0)	6(1)	2(1)
P. marginatum	9(1)	8(1)	2(1)
P. obliquum	9(1)	7(2)	2(1)
P. pseudo-lindenii	9(1)	7(1)	6(1)
P. reticulatum	1(1)	0	0
P. tuberculatum	8(1)	7(2)	4(3)
P. not identified	9(1)	8(2)	8(2)

[a]Number of surviving larvae after 24 hr: mean (± SD) from three trials of 10 larvae/trial.

After 24 hr at room temperature, the surviving larvae were counted. The experiment was repeated three times. The pattern of toxicity of the 16 *Piper* extracts to mosquito larvae generally paralleled that to the ECB, confirming the suitability of these insect species as assay guides for the isolation of compounds with activity to both species (Table 1). For assays on fractions of the crude extracts, a concentration of 100 ppm was arbitrarily selected. The activity of the fraction was expressed as the percentage of the toxicity of the corresponding crude extract at the same concentration (Table 2).

TABLE 2. DISTRIBUTION OF INSECTICIDAL ACTIVITY TO MOSQUITO LARVAE OF FRACTIONS FROM THREE *Piper* SPP

	Mortality (%) at 100 ppm[a]			
Piper spp.	HEX	CH_2Cl_2	EtOAc	MeOH
aduncum	26(3)	72(7)	2(1)	0
decurrens	88(3)	8(2)	3(1)	1(1)
tuberculatum	54(6)	44(7)	2(1)	0

[a]Values in parentheses are SE.

Phytochemical Isolations and Identifications

P. aduncum. Fifteen grams of dry crude leaf extract were suspended in minimal volume of acetone, adsorbed on 30 g of silica gel 70–230 mesh, and the solvent evaporated under reduced pressure. The resulting powder (silica–crude extract) was deposited in a Buchner funnel. Five hundred milliliters of the following solvents were added successively: hexanes (HEX), methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), and methanol (MeOH). Each fraction was then evaporated to dryness, weighed (0.2 g, 1.02 g, 0.01 g, and 0.2 g, respectively), and redissolved in the water of the mosquito bioassay to achieve the proper concentration. Most of the activity was found in the first two fractions (Table 2). These two were combined, and a pale yellow oil, dillapiol (Figure 1a), the major component, was isolated following the method described by Orjala et al. (1989). Its identification was made by comparison with the spectral data of a sample of pure compound provided by one of us (H.G.K.).

P. decurrens. The chemistry of this species had not been previously reported. Sixty grams of the crude dried extract were adsorbed on silica gel and washed successively with four solvents (HEX, CH_2Cl_2, EtOH, and MeOH), as described above. This resulted in the repartition of the toxicity shown in Table 1. The HEX and CH_2Cl_2 fractions were combined and passed through a column of Silica gel (70–230 mesh; 200 g) with HEX and EtOAc as eluents (100:0 to 0:100). From the resulting fractions, a fine white solid (92 mg) was isolated by crystallization from cold HEX. Its physical and spectroscopic data allowed it to be identified as the neolignan, conocarpan (Figure 1b). IR (KBr pellet, cm^{-1}) 3410(br), 3242(br), 2963, 1607, 1607, 1520, 1476, 1239, 925, 830; ^1H Nmr ($CDCl_3$, ppm) δ 1.40 (3H, d, J = 6.8 Hz, CH−C$\underline{H}_3$), 1.87 (3H, dd, J = 6.5 Hz, 1.5 Hz, CH=CH−C$\underline{H}_3$), 3.40 (1H, apparent quint, J = 7.3 Hz, C$\underline{H}$−CH_3), 5.09 (1H, d, J = 8.8 Hz, O−C$\underline{H}$), 6.45 (1H, dq, J = 15.7 Hz, 6.5 Hz, CH=C$\underline{H}$−CH_3) 6.37 (1H, dd, J = 15.7 Hz, 1.5 Hz, C$\underline{H}$=CH−CH_3), 6.77 (1H, D, J = 8.2 Hz, Ar−H ortho to OR), 6.83 (2H, d, J = 8.5 Hz, Ar−H ortho to OH), 7.13 (1H, d, J = 8.2, Ar−H ortho to CH=CH), 7.30 (2H, d, J = 8.5 Hz, Ar−H meta to OH); MS (EI, *m/z*) 267(28), 266(100), 251(17), 233(10), 159(12), 133(12), 131(11), 121(12), 119(18), 107(17); Anal. Calcd. for $C_{18}H_{18}O_2$: C, 81.17; H, 6.81.

P. tuberculatum. Seventy grams of crude EtOH extract from fresh leaves were treated as for the two former species. The bioactivity of the four basic fractions is shown in Table 1. The HEX and CH_2Cl_2 fractions were combined and chromatographed on a large silica gel column (70–230 mesh, 500 g) that was eluted successively with pure HEX, mixtures of HEX–EtOAc of increasing polarities, pure EtOAc, and finally with EtOAC–MeOH (1:1, v/v). The largest fraction contained one major compound, white silky needles, recrystallized from cold benzene–hexane mixtures. The physical (mp = 116–118°C) and spectro-

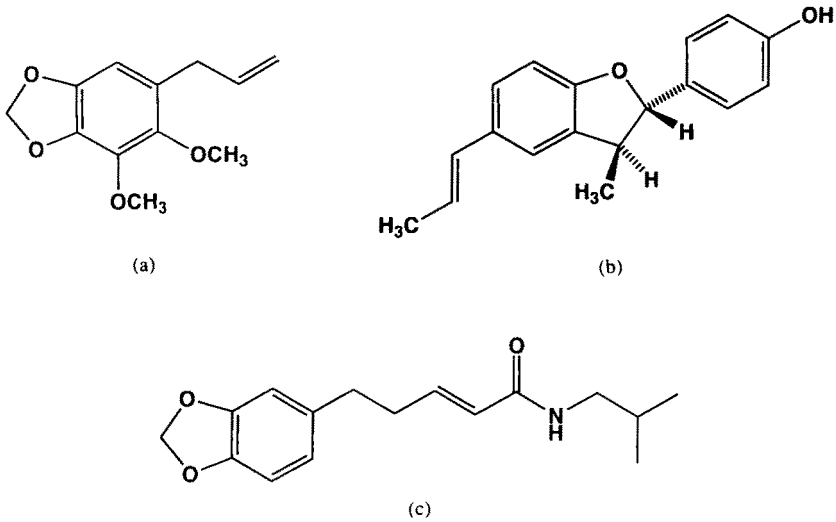

FIG. 1. Compounds isolated in this study: (a) dillapiol, (b) conocarpan, (c) dihydropiperlonguminine.

scopic data were as follows: IR (KBr pellet, cm^{-1}) 3300, 2941, 1667, 1624, 1550, 1490, 1444, 1249, 1041, 928, 812; ^1H NMR (acetone-d6, ppm) δ 0.89 [6H, d, J = 6.9 Hz, $CH(C\underline{H}_3)_2$], 1.77 [1H, non, J = 6.9 Hz, $C\underline{H}(CH_3)2$], 2.44 (2H, dq, J = 7.5 Hz, 1.5 Hz, $C\underline{H}_2-CH=CH$), 2.69 (2H, t, J = 7.5 Hz, $Ar-CH_2$), 3.16 (2H, t, J = 6.9 Hz, $NH-C\underline{H}_2$), 5.95 (2H, s, $O-C\underline{H}_2-O$), 5.96 (1H, dt, J = 15.2 Hz, 1.5 Hz, $CH_2CH=CH$), 6.69 (1H, dd, J = 7.9 Hz, 1.7 Hz, Ar—H meta and para to $O-CH_2O$), 6.75 (1H, dt, J = 15.2 Hz, 7.5 Hz, $CH_2-C\underline{H}=CH$), 6.75 (1H, D, J = 7.9 Hz, Ar—H ortho to $O-C\underline{H}_2O$ and meta to R), 6.76 (1H, d, J = 1.7 Hz, Ar—H ortho to $O-CH_2-O$ and R); HR-MS (m/z M+275, $C_{16}H_{21}O_3N$). These data for this compound matched those given for dihydropiperlonguminine (Figure 1c).

RESULTS AND DISCUSSION

Activity of Crude Extracts to European Corn Borer Larvae. The present study establishes the growth-reducing activity to the European corn borer (ECB) of the majority of the leaf extracts of the *Piper* species collected (Figure 2). *Piper aduncum* (ad), *P. tuberculatum* (tu), and *P. guineense* (gi) had the highest activity in the diet (Figure 2). The mortality data (not shown) generally paralleled the growth-reducing data, and all larvae in the *P. aduncum*, *P. tubercu-*

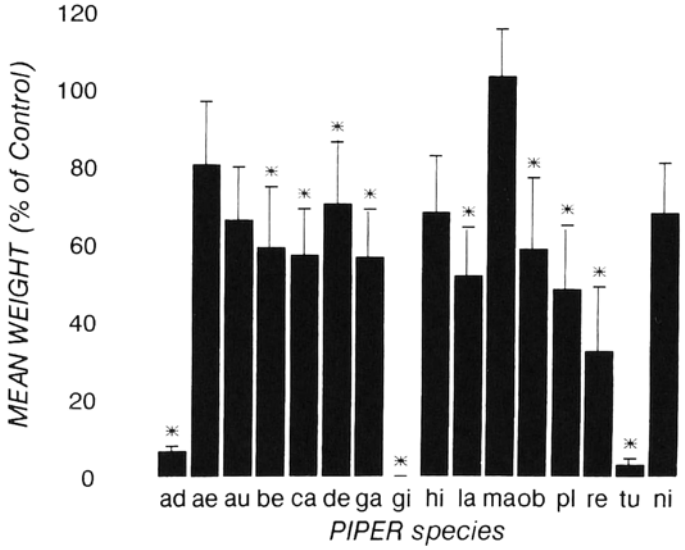

FIG. 2. Growth reducing activity of *Piper* extracts (0.4% in diet) to European corn borer larvae. ad: *P. aduncum*; ae: *P. aequale*; au: *P. auritum*; be: *P. betle*; ca: *P. carrilloanum*; de: *P. decurrens*; ga: *P. guanacastensis*; gi: *P. guineense*; hi: *P. hispidum*; la: *P. lanceiaifolium*; ma: *P. marginatum*; ob: *P. obliquum*; pl: *P. pseudo-lindenii*; re: *P. reticulatum*; tu: *P. tuberculatum*; ni: *P.* not identified. (*Piper* species with an asterisk are significantly different from their control, $P = 0.05$ in Tukey's general linear models procedure). Twenty second-instar larvae (day 6) were fed treated diets and weights were determined on day 20.

latum, and *P. guineense* groups died by day 12 of treatment. In addition, seven other species, *P. betle* (be), *P. decurrens* (de), *P. guanacastensis* (ga), *P. lanceiaifolium* (la), *P. obliquum* (ob), *P. pseudo-lindenii* (pl), *P. reticulatum* (re), and *P. carrilloanum* (ca) significantly reduced the growth of the larvae (Figure 2), with larval weights after a period of 10 days on these diets ranging from 27% to 68% of controls. For the larvae that survived the treatment, the percentage of pupation was decreased in all cases except for *P. marginatum*.

Nutritional indices (Table 1) showed limited effect of the extracts on the food consumption (CI) of the larvae, indicating little feeding deterrency (Table 3). In fact, the three most insecticidal species induced three to eight times higher consumption of food. The digestion of the insect was also little affected by the allelochemicals, but the ECD was lowered, indicating toxicity of the digested food components.

Phytochemical Isolations of Pure Compounds. Dillapiol was the main active

TABLE 3. EFFECT OF PIPERACEAE EXTRACTS (0.4%) ON INSECT GROWTH AND FOOD MANAGEMENT

Piper spp.	Percent of control				
	RGR	CI	ECI	AD	ECD
P. aduncum (leaves)	3.63[a]	591.11[a]	5.23[a]	148.62[a]	3.43[a]
P. aequale (leaves)	78.93	127.33	85.12	100.00	100.00
P. aequale (twigs)	59.26[a]	162.91[a]	58.45[a]	102.61[a]	56.02[a]
P. auritum (leaves)	68.58	164.48	55.42	109.63	49.83[a]
P. auritum (twigs)	56.17	161.69	63.37	101.24	62.66
P. betle (fruits)	55.56[a]	171.29	61.70	119.62	59.82[a]
P. decurrens (leaves)	68.31	155.73	78.55	119.80	64.87[a]
P. guanacastensis (leaves)	75.42	86.62	90.50	98.87	90.69
P. guineense (fruits)	0.18[a]	336.32[a]	0.35[a]	104.11[a]	0.33[a]
P. hispidum (leaves)	73.36	123.63	73.00	106.38	71.74
P. marginatum (leaves)	101.86	100.45	119.38	91.37	214.30[a]
P. pseudo-lindenii (leaves)	40.83[a]	256.15[a]	36.81[a]	113.62	32.48[a]
P. reticulatum (leaves)	30.65[a]	274.84[a]	29.46[a]	116.32[a]	25.72[a]
P. tuberculatum (leaves)	0.26[a]	831.24[a]	0.28[a]	121.85[a]	0.23[a]

[a]Data are significantly different from their control in Tukey's multiple range test ($P = 0.05$). The mean RGR, CI, ECI, AD and ECD (+SD) values of control groups were: 1.7(0.4), 2.7(0.6), 5.3(1.2), 0.7(0.1) and 0.07(0.03).

component of *P. aduncum* and caused 92% mortality of mosquito larvae at 0.1 ppm (Table 4). High concentrations of dillapiol were also obtained from *P. aduncum* from Brazilian species (74–88% of the leaf essential oil) (Gottlieb et al., 1981), and this monolignan occurred in the leaves, fruits, or wood of at least five other Piperaceae distributed worldwide—*P. banksii*, *P. hispidum*, *P. marginatum*, *P. novae-hollandiae*, and *P. umbellatum* (Loder and Nearn, 1972; Burke and Nair, 1986; Ramos et al., 1986; Loder et al., 1969; Bernhard and Thiele, 1978).

Dihydropiperlonguminine was isolated from the active fraction of *P. tuberculatum*. This isobutylamide has been isolated from other Piperaceae (Tabuneng et al., 1983), along with longuminine and other derivatives. Dihydropiperlonguminine is toxic to mosquito larvae (Table 4).

Conocarpan was isolated from the active fraction of *P. decurrens* extract and could account for part of the insecticidal activity of this plant (Table 4). Conocarpan has been previously isolated from the wood of *Conocarpus erectus* (Combretaceae) (Hayashi and Thomson, 1975), but this is its first isolation from the Piperaceae. Large quantities of sitosterol (118 mg) were also purified from this fraction, as well as a number of less abundant lignans of a similar type.

TABLE 4. ACTIVITY OF NEWLY ISOLATED PURE COMPOUNDS TO MOSQUITO LARVAE

Species	Mortality (%)[a]
P. aduncum	
Dillapiol	92(4)
P. decurrens	
Conocarpan	47(5)
Sitosterol	6(3)
P. tuberculatum	
dihydropiperlonguminine	47(2)

[a]Percent mortality (±SE) from three assays of 10 larvae assay. Concentrations in bioassays were 0.1 ppm for *P. aduncum* and *P. decurrens*, and 0.01 ppm for *P. tuberculatum*.

These are presently being analyzed and will be described elsewhere (Chauret, unpublished data).

Insecticidal Lignans and Amides of Piperaceae. More than 15 species of *Piper* have been reported in the literature to have insecticidal activity. According to Marquis (1991), the highly insecticidal activity of most of the *Piper* spp., which he observed in the field in relation to two phytophagous insects, could not be correlated with the leaf characteristics, the soil, or other environment factors, "leaving the individual chemistry as a possible factor to investigate."

The two species of *Piper* with the highest toxicity to the ECB, *P. tuberculatum* and *P. guineense*, contain large amounts of amides. The isobutylamide, dihydropiperlonguminine, is a major component of *P. tuberculatum* leaves, as described in the present study. In the only other chemical investigation reported on this species, the pyridone alkaloid piplartine and its dimer (Filho et al., 1981), were isolated from the root bark.

P. guineense is, by contrast, well studied. A series of isobutylamides, including pipercide, guineensinamide, guineensine, pellitorine, and kalecide, have been isolated from the roots of *P. guineense* and shown to be responsible for the insecticidal activity of this species (Gbewonyo et al., 1993). The first three amides were the most active against adults of *Musca domestica*. Each of these contain a methylenedioxyphenyl (MDP) structural moiety.

The isobutylamide newly isolated from *P. tuberculatum*, dihydropiperlonguminine (Figure 1c) also has an MDP ring and is the main component of the active fraction of the leaf extract. Greger (1988) pointed out that these types of amides, including olefinic and alkyl isobutylamides, are common to a restricted number of related plant families, namely the Piperaceae, the Asteraceae, and the Rutaceae. All three families are abundant in the tropics, particularly in the

humid tropics in the case of the Piperaceae, where herbivory is a potent selective force. The isobutylamides found in the *Piper* family have low molecular weights, contain a single nitrogen, and presumably are not "expensive" to biosynthesize. The pungent, numbing sensation caused by some of the psychoactive species, such as *P. methysticum*, may be a reason for their avoidance by herbivores, as observed by Marquis (1991), in particular with *P. auritum*. Piperaceae are mostly vines or small shrubs in the tropics, and short life-cycle species such as these would be expected to protect themselves with simply synthesized, low-cost, molecules. The isobutylamides could be described as belonging to this category of defense compounds.

The most active insecticidal species in our bioassays was *P. aduncum*. Surprisingly, no amides have been reported from this species. It does, however, contain several lignans, including dillapiol, first found in the Indian dill, *Anethum sowa* (Fabaceae), where it constitutes up to 35% of the essential oil of the seeds of Indian cultivars (Tomar et al., 1979), and also in the leaves of *P. aduncum* from Panama, where it is the only major component (Gupta and Arias, 1983). Dillapiol can synergize several natural insecticides, including pyrethrum (Mukerjee et al., 1979), azadirachtin (Bertrand, 1992), and tenulin (Bernard et al., 1990), at the concentration of 100 ppm in the diet of the European corn borer, a concentration well below the level in the leaves of *P. aduncum* (approximately 60% of the oil). It previously was found to be toxic to the ECB (Bernard et al., 1989). It is the main insecticidal component of this *Piper* sp. Monolignans and lignans with the MDP moiety are characteristic chemicals from the Piperaceae. Lignan toxicity differs from that of classical neurotoxic insecticides, as it is chronic rather than acute. Several lignans inhibit a vital biochemical defense of herbivores, the polysubstrate monooxygenase (PSMO) detoxification enzymes (Ahmad, 1986; Bernard et al., 1989; Berenbaum and Neal, 1985). Thus, several potentially toxic components in the food of the insect that are usually gradually eliminated could accumulate to a toxic level. These specific lignans, myristicin, dillapiol, epiyangambin, ashantin, and eugenol, are distributed in most of the *Piper* spp. and often occur in large quantities in the plants.

The elucidation of the structure of conocarpan, a neolignan, as the main, but not highly, insecticidal component of *P. decurrens*, further suggests the importance of lignans in the Piperaceae. Only lignans have been isolated from extracts having a growth-reducing rather than an acute toxic effect on insect larvae, i.e., *P. marginatum*, *P. decurrens*, and *P. hispidum* of the present study. These produced the lowest larval mortality. This leads to the possibility that *Piper* lignans not associated with amides, excluding monolignols, may have a long-term effect and low toxicity, as further suggested by the low incidence of pupation in the groups fed on these three species. This possibility warrants further investigation.

In any case, isobutylamides and lignans combined in the same plant constitute an inexpensive and effective means of defense elaborated by a limited number of highly successful plants from the neotropics.

The present study confirms that tropical American *Piper* spp. have insecticidal activities comparable to previously studied African and Asian species. Bioassay-guided isolation suggests the importance of neolignans and amides as the active components.

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ALKYL ETHER AND ENOL ETHER ANALOGS OF (Z)-5-DECENYL ACETATE, A PHEROMONE COMPONENT OF THE TURNIP MOTH, *Agrotis segetum*: PROBING A PROPOSED BIOACTIVE CONFORMATION FOR CHAIN-ELONGATED ANALOGS

ANNA-LENA GUSTAVSSON,[1] TOMMY LILJEFORS,[2,*] and BILL S. HANSSON[3]

[1]*Department of Organic Chemistry 1*
Chemical Center, Lund University
P.O. Box 124, S-221 00 Lund, Sweden

[2]*Department of Medicinal Chemistry*
The Royal Danish School of Pharmacy
DK-2100 Copenhagen, Denmark

[3]*Department of Ecology*
Lund University
S-223 62 Lund, Sweden

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Abstract—In order to test a previous conclusion that chain-elongated analogs of (Z)-5-decenyl acetate (**1**), a pheromone component of the turnip moth, *Agrotis segetum*, adopt a loop conformation of the terminal alkyl chain in the bioactive conformation, a series of alkyl ether and enol ether analogs of **1** and (Z)-5-dodecenyl acetate (**2**) have been synthesized and tested using single-cell electrophysiology. In these analogs a methylene group in positions 7 and 9 of **1** and in positions 7 and 11 in **2** have been replaced by an oxygen atom in order to energetically facilitate the formation of a loop conformation in the chain-elongated analogs. The electrophysiological results in combination with molecular mechanics (MM2 and MM3) calculated conformational energies show that the activity decreases of the chain-elongated ether analogs are significantly smaller than that for **2** and that these activity decreases parallel the conformational energies for a loop formation of the terminal chains in the analogs. The results support our previous conclusion that the terminal chain of chain-elongated analogs of **1** adopts a loop conformation in their bioactive conformations.

*To whom correspondence should be addressed.

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Key Words—Lepidoptera, Noctuidae, *Agrotis segetum*, (Z)-5-decenyl acetate, pheromone analogs, structure–activity, chain-elongated analogs, enol ethers, alkyl ethers, single-sensillum recordings, receptor interaction, molecular mechanics, MM2, MM3.

INTRODUCTION

In a series of papers we have studied structure–activity relationships for analogs of (Z)-5-decenyl acetate (**1,** Figure 1), a pheromone component of the turnip moth, *Agrotis segetum* (Liljefors et al., 1985, 1987; Bengtsson et al., 1987, 1990; Jönsson, 1991; Jönsson et al., 1991, 1992, 1993). Based on these studies, we have developed a model for the interaction between **1** and its receptor (Liljefors et al., 1987; Bengtsson, 1988; Jönsson, 1991). Three pharmacophore elements have been identified: the terminal methyl group, the double bond, and the acetate group. In a previous study (Liljefors et al., 1987), we found that

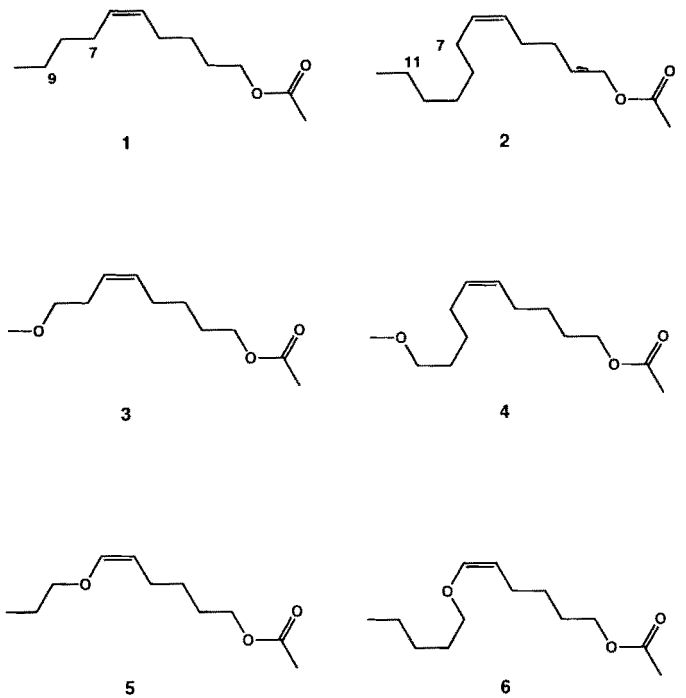

FIG. 1. Compounds studied. Compounds **2, 4,** and **6** are shown with the terminal chain in the loop formation discussed in this paper.

single-cell electrophysiological activities of chain-elongated analogs of **1** were determined by the conformational energies required for the analogs to mimic the proposed bioactive conformation of **1** with respect to the location in space of the pharmacophore elements. For instance, the chain-elongated analog (*Z*)-5-dodecenyl acetate **(2)** was shown to be able to mimic the active structure of **1** provided that the terminal alkyl chain in **2** adopts a loop conformation as shown in Figure 2. Our model is based on the hypothesis that the dimensions of the receptor cavity are such that a close fit between terminal methyl groups of analogs and that of **1** is required. In particular, we propose that the end of the terminal chain of an analog cannot, without excessive steric repulsions, be extended beyond that of the terminal methyl group of **1** in the direction of the terminal C—C bond.

The proposed biologically active conformation of **1** (Liljefors et al., 1987; Bengtsson, 1988) is shown in Figure 3a. Figure 3b shows a molecular least-squares superimposition of the active conformations of **1** and **2** based on the

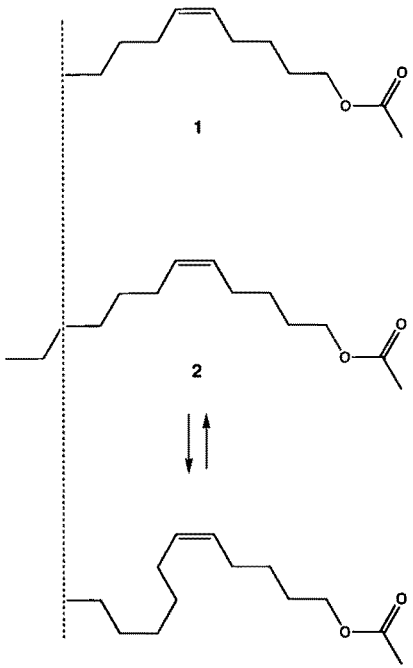

FIG. 2. Loop formation required for (*Z*)-5-dodecenyl acetate **(2)** in order to mimic the active structure of **1** with respect to the position of the terminal methyl group.

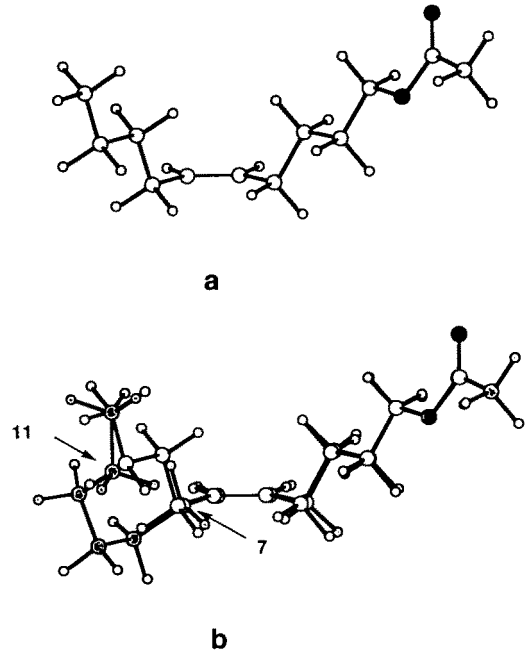

FIG. 3. (a) Compound **1** in the proposed bioactive confirmation. (b) Superimposition of the proposed bioactive conformations of compounds **1** and **2**.

concept of a loop formation of the terminal chain of the chain-elongated analog **2**.

The purpose of the present study is to test our conclusion concerning the loop conformation of the extended chain in the bioactive conformation of chain-elongated analogs of **1**. Preliminary molecular mechanics calculations (J. Palm and T. Liljefors, unpublished results) indicate that the conformational energy of the loop conformation of **2** is largely determined by repulsive van der Waals interactions between the methylene groups at positions 7 and 11 (Figure 3b). Thus, replacement of one of these methylene groups by an oxygen atom implies that the energy requirement for loop formation should be lower for the oxygen analog because of the smaller size of an oxygen atom compared to a methylene group. Consequently, our receptor-interaction model predicts that such ether analogs should display a significantly smaller decrease in activity on chain elongation than is the case for a corresponding chain elongation of **1**.

To test this prediction, the alkyl ether and enol ether analogs **3–6** (Figure 1) have been synthesized, and their electrophysiological single-cell activities

have been determined. The results, in relation to the corresponding results for **1** and **2,** have been interpreted by the use of molecular mechanics calculated conformational energies. Compounds **3** and **5** may also give information on the effect of methylene group replacement by an oxygen atom in positions 7 and 9 in **1.**

Very few ether analogs of moth sex pheromone components have been reported. Hoskovec et al. (1993) recently reported two alkyl ether analogs of (*E*,*Z*)-2,13-octodecadienyl acetate, a pheromone component of the currant clearwing moth, *Synanthedon tipuliformis*. Unfortunately, the activity of these analogs was only determined by the EAG method. To our knowledge, no single-cell electrophysiology measurements of ether analogs of moth sex pheromone components have been reported previously.

METHODS AND MATERIALS

Synthesis. All reactions of air- and water-sensitive materials were performed under a nitrogen atmosphere with oven-dried glassware. Anhydrous diethyl ether (ether) and tetrahydrofuran (THF) were distilled from dark blue solutions of the sodium benzophenone radical anion. *N*,*N*′-Dimethyl-*N*,*N*′-propylene urea (DMPU), purchased from Fluka Chemie AG, was distilled under vacuum from calcium hydride prior to use. The dimethyl sulfide complex of cuprous bromide was prepared by the method of House et al. (1975). Propargyl bromide was distilled prior to use and potassium *tert*-butoxide was purified by sublimation. 2-Bromoethyl methyl ether was purchased from Aldrich-Chemie GmbH. The crude pheromone products were purified by flash chromatography on TLC silica gel 60H. Petroleum ether–ethyl acetate (10 : 1) was used as eluent for compounds **3** and **4** and dichloromethane–ethyl acetate (50 : 1) for compounds **5** and **6.** Flash chromatographed products were further purified by argentation chromatography (Houx et al., 1974). Compounds **3** and **4** were then finally purified by preparative GLC using a 6-m × 4-mm OV-351 column. Compounds **5** and **6** were purified by preparative high-performance liquid chromatography [HPLC, normal-phase column SH-44-20 S-10 120A sil with petroleum ether–ethyl acetate (95 : 5) as eluent and a differential refractometer as monitor].

Analytical GLC was performed with a Varian 3400 capillary GLC fitted with a DB-Wax 30-m column. The purity of the isolated final products was >99%. No sample contained any detectable amount (<0.05%) of the natural pheromone component. ^{1}H and ^{13}C NMR on the final products were recorded on a Varian XL-300 spectrometer. High-resolution mass spectra were recorded on a Jeol JMS-SX 102 spectrometer. The synthetic schemes used are shown in Figures 4 and 5.

Preparation of 4-Bromobutyl Methyl Ether. 4-Bromobutyl methyl ether was

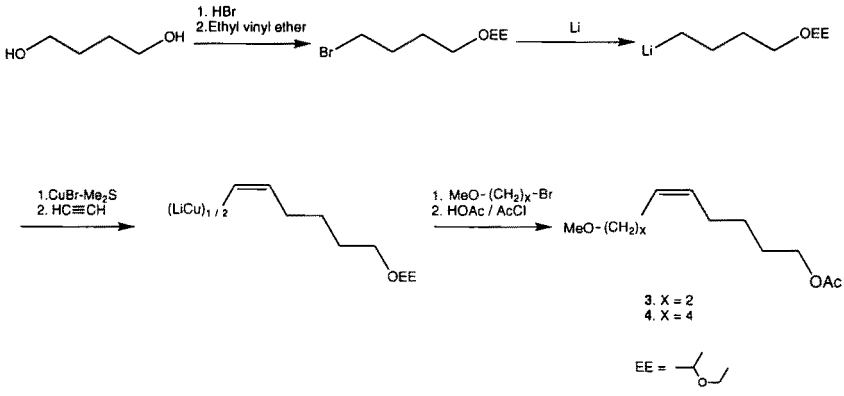

FIG. 4. Synthetic routes to compounds **3** and **4.**

prepared by Williamson's ether synthesis using a phase-transfer catalyst according to the method described by Burgstahler et al. (1977). To a solution of 20 g of sodium hydroxide and 40 ml of water, cooled to 10°C, were added 4 ml (0.1 mol) of methanol, 59.8 ml (0.5 mol) of 1,4-dibromobutane, and 1.7 g (5 mmol) of tetrabutylammonium hydrogen sulfate. After stirring over night at room temperature, the mixture was poured into 500 ml of water, extracted with pentane, and the dried extracts were evaporated. The crude product was distilled given 16 g of product, bp 64–68°C/9 mm Hg (lit: 60°C/15 mm Hg) (Kirrman and Hamaide (1957). According to capillary GLC the product was a mixture of 4-bromobutyl methyl ether and 1,4-dibromobutane in a ratio of 35:65 (yield of about 40%). The mixture was flash chromatographed with dichloromethane as eluent and gave >95% pure product according to capillary GLC.

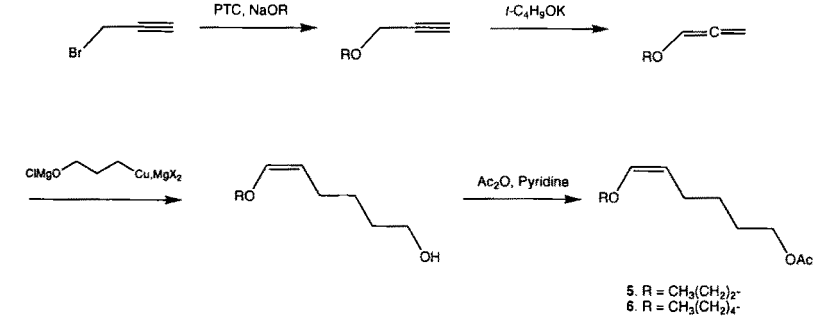

FIG. 5. Synthetic routes to compounds **5** and **6.**

Preparation of 4-(1-Ethoxyethoxy)-butyl Lithium. 4-Bromo-1-butanol was prepared according to the method of Kang et al. (1985). A mixture of 44.6 ml (0.5 mol) of 1,4-butandiol and 62.5 ml (0.55 mol) of 48% HBr in 800 ml benzene was heated at reflux for 7 hr, while the water formed (60 ml) was trapped with a Dean-Stark separator. The mixture was evaporated, and the residue was desolved in anhydrous CH_2Cl_2 (200 ml) and cooled to $<18°C$. Then ethyl vinyl ether (48 ml, 0.5 mol) was added dropwise over 45 min, while the solution temperature was kept between 10 and 16°C. The colorless solution was stirred for another hour at room temperature, washed with saturated Na_2CO_3 solution, dried over Na_2CO_3, and finally the solvent was evaporated. The crude product was distilled in base-washed glassware to give 41 g (37% yield, bp 60–69°C/2 mm Hg) of 4-(1-ethoxyethoxy)-butyl bromide. δ_H (60 MHz) 1.0–1.4 (6H, m, CH_3), 1.5–2.4 (4H, m, CH_2), 3.1–4.0 (6H, m, CH_2Br, CH_2O), 4.7 (1H, q, OCHO).

To freshly cut small chips of lithium (1.7 g, 0.25 mol) in 20 ml ether, a solution of 4-(1-ethoxyethoxy)-butyl bromide (22.5 g, 0.1 mol) in 60 ml ether was added dropwise at −15°C during 1 hr. After the addition, the solution was stirred for 1 hr at 0°C. The greyish turbid solution was transferred to a 100-ml graduated glass cylinder that was carefully purged with argon. The cylinder was stored at −15°C allowing cloudy material to precipitate. The clear pale yellow solution was titrated by the method of Watson and Eastham (1967) and found to be 0.7–0.8 N (yield 70–80%).

Preparation of Lithium Bis[(Z)-6-(1-ethoxyethoxy)-hex-1-enyl]cuprate. This compound was prepared by carbocupration of acetylene according to Gardette et al. (1985). To a well-stirred suspension of CuBr−Me_2S complex (House et al., 1975) (1.23 g, 6 mmol) in ether (25 ml) at −50°C, 12 mmol of 4-(1-ethoxyethoxy)-butyl lithium solution was slowly transferred by using a double-tipped needle. The solution was warmed to −40°C and stirred for 30 min at this temperature. The almost clear solution was cooled to −50°C and acetylene (0.3 liters, 12 mmol) was bubbled slowly into the reaction mixture. Stirring of the resulting solution was maintained for 30 min at −25°C.

9-Oxa-(Z)-5-decenyl Acetate (3). To a stirred solution of lithium bis[(Z)-6-(ethoxyethoxy)-hex-1-enyl]cuprate (12 mmol) at −30°C, DMPU (6 ml) in 40 ml THF and 2-bromoethyl methyl ether (1.69 g, 12 mmol) in 5 ml THF were successively added. The mixture was slowly allowed to reach room temperature and stirred over night. It was then cooled to −20°C and quenched with 5 N HCl (40 ml). After stirring for 15 min with air bubbling into the mixture, 50 ml of petroleum ether (40–60) was added and the precipitate was filtered off with celite. The aqueous layer was extracted once with ether (50 ml), and the combined organic extracts were washed once with 17% NH_3 (25 ml), then with saturated NH_4Cl solution (25 ml), and finally dried over $MgSO_4$. The solvents were removed and the residue treated with glacial acetic acid (8 ml) and acetyl

chloride (1 ml). This mixture was stirred over night at 30–40°C and then poured onto ice (40 ml) in saturated NaCl solution. The acetate was extracted three times with pentane (15 ml). The organic phase was washed with $NaHCO_3$ solution and dried over $MgSO_4$. After evaporation, the crude product was purified by flash chromatography to give 0.4 g (yield 17%) of compound **3**, 63% pure by GLC. δ_H (300 MHz) 1.35–1.47 (m, 2H, $=CCC\mathbf{H}_2$), 1.55–1.68 (m, 2H, $CC\mathbf{H}_2C-O$), 2.03 (s, 3H, $COC\mathbf{H}_3$), 2.07 (dt, 2H, J = 7.3 Hz, J = 6.8 Hz, $=CHC\mathbf{H}_2CH_2$), 2.33 (dt, 2H, J = 6.8 Hz, J = 6.4 Hz, $OCH_2C\mathbf{H}_2CH=$), 3.33 (s, 3H, CH_3O), 3.37 (t, 2H, $OC\mathbf{H}_2CH_2$), 4.04 (t, 2H, $CH_2C\mathbf{H}_2O$), 5.33–5.50 (m, 2H, J = 10.9 Hz, $C\mathbf{H}=C\mathbf{H}$). δ_C (75.4 MHz) 21.0, 25.9, 26.9, 27.8, 28.2, 58.6, 64.4, 72.3, 126.1, 131.2, 171.2. High-resolution CI mass spectrum for $C_{11}H_{21}O_3$ ($M+H^+$): calcd. 201.1491, found 201.1494.

11-Oxa-(Z)-5-dodecenyl Acetate ***(4).*** This compound was prepared from 4-bromobutyl methyl ether (1 g, 6 mmol) in the same manner as compound **3** to give 0.28 g (20% yield) of flash chromatographed product, 80% pure by GLC. δ_H (300 MHz) 1.33–1.46 (m, 4H, $=CCC\mathbf{H}_2$), 1.52–1.68 (m, 4H, $CC\mathbf{H}_2C-O$), 1.95–2.13 (m, 4H, $CC\mathbf{H}_2C=$), 2.03 (s, 3H, $COC\mathbf{H}_3$), 3.32 (s, 3H, $C\mathbf{H}_3O$), 3.36 (t, 2H, $CH_2C\mathbf{H}_2O$), 4.05 (t, 2H, $CH_2C\mathbf{H}_2OCO$), 5.26–5.43 (m, 2H, J = 10.9 Hz, $C\mathbf{H}=C\mathbf{H}$). δ_C (75.4 MHz) 21.0, 26.0, 26.2, 26.8, 27.0, 28.2, 29.3, 58.6, 64.5, 72.7, 129.4, 130.1, 171.2. High-resolution CI mass spectrum for $C_{13}H_{25}O_3$ ($M+H^+$): calcd. 229.1804, found 229.1806.

Preparation of Propyl Propargyl Ether. This ether was prepared according to Mikami et al. (1984), using a phase-transfer catalyst (Dockx, 1973). 3-Bromo-1-propyne (31.0 g, 0.26 mol) was added dropwise to a rigorously stirred mixture of propanol (15 ml, 0.2 mol), 50% NaOH solution (35 ml), and n-Bu_4NHSO_4 (3.5 g, 0.01 mol) at 20–30°C. The resulting mixture was stirred over night at room temperature. The solid formed was removed by filtration. After dilution of the mixture with water and ether, the water phase was extracted three times with ether and the combined organic extracts were washed with brine and dried over $MgSO_4$. Evaporation and distillation of the product gave 14.4 g (70% yield), bp 40–60°C/155 mm Hg. δ_H (60 MHz) 1.0 (t, 3H, $C\mathbf{H}_3C$), 1.3–2.0 (m, 2H, $CC\mathbf{H}_2C$), 2.5 (t, 1H, J = 2 Hz, $\mathbf{H}CCCH_2$), 3.6 (t, 2H, $OC\mathbf{H}_2CH_2$), 4.2 (d, 2H, J = 2 Hz, $HCCC\mathbf{H}_2O$).

Preparation of Propyl Allenyl Ether. The rearrangement of the propargyl ether to give the corresponding allenyl ether was done according to Hoff et al. (1968). To 7.5 g (75 mmol) of propyl propargyl ether was added 0.9 g (7.5 mmol) of potassium *tert*-butoxide. The mixture was stirred for 2–3 hr at 70°C. After cooling to room temperature, the brown reaction mixture was poured onto ice water and extracted with ether. The organic layer was washed with water and dried over K_2CO_3. After evaporation, the product was distilled, giving 1.6 g (22% yield) bp 58–59°C/160 mm Hg. δ_H (60 MHz) 0.9 (t, 3H, $C\mathbf{H}_3CH_2$), 1.1–1.3 (m, 2H, $CC\mathbf{H}_2C$), 3.5 (t, 2H, $OC\mathbf{H}_2CH_2$), 5.4 (d, 2H, J = 6 Hz, $C\mathbf{H}_2=C=CH-O$), 6.7 (t, 1H, J = 6 Hz, $CH_2=C=C\mathbf{H}-O$).

7-Oxa-(Z)-5-decenyl Acetate **(5).** This compound was made via the addition of a magnesium organocopper reagent to an allenyl ether, according to Klein et al. (1976) and Alexakis and Normant (1984), giving predominantly a Z-enol ether. The magnesium organocopper reagent was prepared according to Gardette et al. (1985). CuBr, 1.1 g (8 mmol), and 0.65 g (15 mmol) of LiCl were mixed in 15 ml THF and stirred at room temperature until a clear solution was obtained. The mixture was then cooled to −40°C and 7 mmol of γ-magnesium-protected 3-hydroxypropyl magnesium chloride, prepared in THF according to Cahiez et al., (1978), was added. After the reaction mixture was stirred 30 min at −30°C, it was cooled to −50°C and the propyl allenyl ether (0.7 g, 7 mmol) in THF (5 ml) was added dropwise. After stirring for 1 hr at −15°C, the mixture was hydrolyzed with 14 ml of saturated NH_4Cl and 2 ml of aqueous ammonia. After filtration of the salts, the organic phase, to which 100 ml pentane had been added, was washed once with saturated NH_4Cl-solution, dried over Na_2CO_3, and finally evaporated. The crude product (0.9 g) was added to a mixture of pyridine (5 ml) and acetic anhydride (1 ml) and was stirred for 4 hr at room temperature. The reaction mixture was poured on water, extracted with pentane, and dried over $MgSO_4$. After evaporation, the crude product was flash chromatographed giving 0.4 g of the final product (25% yield, 86% pure by GLC, *Z*:*E* 87:13). Further purification (see Synthesis section above) gave pure *Z* isomer, compound **5.** δ_H (300 MHz) 0.92 (t, 3H, $\mathbf{CH_3}C$), 1.36–1.46 (m, 2H, $CC\mathbf{H_2}C$), 1.56–1.70 (m, 4H, $CC\mathbf{H_2}C$), 2.03 (s, 3H, $CO\mathbf{CH_3}$), 2.10 (dt, 2H, J = 7.3 Hz, $=CHC\mathbf{H_2}CH_2$), 3.66 (t, 2H, $CH_2\mathbf{CH_2}OC=$), 4.05 (t, 2H, $CH_2\mathbf{CH_2}OCO$), 4.30 (dt, 1H, $J(=CHCH_2)$ = 7.3 Hz, J(HC=CH) = 6.3 Hz, $CH=\mathbf{CH}CH_2$), 5.93 (dt, 1H, J = 6.3 Hz, $O\mathbf{CH}=CH$). δ_C (75.4 MHz) 10.2, 21.0, 23.0, 23.4, 26.0, 28.1, 64.6, 73.7, 105.9, 145.3, 171.2. MS (high resolution) for $C_{11}H_{20}O_3$: calcd 200.1413, found 200.1420.

Preparation of Pentyl Propargyl Ether. This compound was prepared by the method described above for propyl propargyl ether. However, the reaction temperature was kept at 50–60°C. The product was distilled giving 19.8 g (78% yield), bp 64–86°C/75 mm Hg. δ_H (60 MHz) 0.9 (t, 3H, $\mathbf{CH_3}C$), 1.1–1.9 (m, 6H, $-\mathbf{CH_2}-$), 2.4 (t, 1H, J = 2 Hz, $\mathbf{H}CCCH_2$), 3.5 (t, 2H, $O\mathbf{CH_2}CH_2$), 4.1 (d, 2H, J = 2 Hz, $HCCC\mathbf{H_2}O$).

Preparation of Pentyl Allenyl Ether. This allenyl ether was prepared as described above for propyl allenyl ether from 9.5 g (75 mmol) of pentyl propargyl ether. For the preparation of pentyl allenyl ether, 12.5 mmol of potassium butoxide was used. The product was distilled, giving 6.6 g (70% yield) bp 66–68°C/35 mm Hg. δ_H (60 MHz) 0.9 (t, 3H, $\mathbf{CH_3}C$), 1.1–2.0 (m, 6H, $-\mathbf{CH_2}-$), 3.6 (t, 2H, $O\mathbf{CH_2}CH_2$), 5.5 (d, 2H, J = 6 Hz, $\mathbf{CH_2}=C=CH-O$), 6.8 (t, 1H, J = 6 Hz, $CH_2=C=\mathbf{CH}-O$).

7-Oxa-(Z)-5-dodecenyl Acetate **(6).** This compound was prepared in the same manner as compound **5** but from pentyl allenyl ether (1.26 g, 10 mmol).

The crude alcohol (1.7 g) was treated with a mixture of 5 ml of pyridine and 2 ml of acetic anhydride for acetylation. Flash chromatography gave 1.1 g (43% yield, 90% pure by GLC, *Z*:*E* 76:24). δ_H (300 MHz) 0.89 (t, 3H, CH_3C), 1.28–1.45 (m, 6H, $-CH_2-$), 1.53–1.70 (m, 4H, $-CH_2-$), 2.03 (s, 3H, CH_3CO), 2.10 (dt, 2H, J = 7.3 Hz, $=CH-CH_2CH_2$), 3.67 (t, 2H, $CH_2CH_2OC=$), 4.05 (t, 2H, CH_2CH_2OCO), 4.30 (dt, 1H, $J(=CH-CH_2)$ = 7.3 Hz, $J(HC=CH)$ = 6.3 Hz, $C=CHCH_2$), 5.93 (dt, 1H, J = 6.3 Hz, $OCH=CH$). δ_C (75.4 MHz) 14.0, 21.0, 22.4, 23.4, 26.0, 28.0, 28.1, 29.4, 64.6, 72.2, 105.9, 145.4, 171.2. MS (high resolution) for $C_{13}H_{24}O_3$: calcd. 228.1726, found 228.1719.

Electrophysiology. The activities of compounds **1–6** were determined by single-cell electrophysiology (Kaissling, 1974). The olfactory receptor cell specifically tuned to (*Z*)-5-decenyl acetate, present in the antennal sensilla type SW1 of *Agrotis segetum*, was used (Hallberg, 1981; Löfstedt et al., 1982; van der Pers and Löfstedt, 1983). The method was modified according to van der Pers and den Otter (1978) and has been previously described (Liljefors et al., 1987; Bengtsson et al., 1990).

The stimulus amounts used for pheromone component **1** were 10^{-4}–1 μg, for compound **3** 10^{-2}–1 μg, and for compound **5** 10^{-2}–10 μg, in decadic steps. For the chain-elongated analogs **2, 4,** and **6,** the amounts used were 10^{-1}–100 μg, in decadic steps. For each stimulus loading, 10 replicates were recorded and the mean value of the number of action potentials generated during 1 sec from the onset of stimulation was used in the construction of the dose–response curves. The errors were expressed as standard errors of the mean (SEM). The electrophysiological activity of each compound in relation to that of **1** is expressed as the reciprocal of the relative quantities required to elicit the same response from the receptor as the natural pheromone component **1.**

Differences in volatility for the compounds were taken into account by correcting the activities by using relative vapor pressures as previously described (Liljefors et al., 1985; Bengtsson et al., 1990). The correction factors for the alkyl ether analogs are based on vapor pressure data (Dykyj and Repas, 1979) for hydrocarbons (pentane to heptane) and their ether analogs (methyl propyl ether, diethyl ether, ethyl butyl ether, dipropyl ether, methyl butyl ether, ethyl propyl ether). The factors for the enol ether analogs are based on data for 1-butene, 1-pentene, 1-heptene, 2-pentene, and their enol ether analogs.

The logarithm of the quotient of the vapor pressure for a hydrocarbon compound and the vapor pressure for its analog (alkyl or enol ether analog) gives the correction factor. The correction factors for carbon chain-elongated compounds were obtained in an analogous way (Liljefors et al., 1985). The mean value of the quotient for each class of compounds (alkyl ethers, enol ethers, chain-elongated) was used. The correction factors were added to the logarithm of the relative activities. A positive factor indicated a lower vapor

pressure for the ether analog than for the corresponding hydrocarbon compound, and the real relative activity for the ether analog was higher than the measured activity. If the factor was negative, the analog was more volatile than the corresponding compound and the relative activity should be lowered. The correction factor for two-carbon chain-elongated analogs is 0.82, for enol ether analogs 0.12, and for alkyl ether analogs −0.03. For those analogs that are both chain-elongated and ether analogs of **1,** an additive scheme was used. The correction factors used were as follows: for compound **2,** 0.82; for **3,** −0.03; for **5,** 0.12; for **4,** 0.79 (−0.03 + 0.82); and for **6,** 0.94 (0.12 + 0.82).

Molecular Mechanics. Energy minimized geometries and conformational energies were calculated by using the molecular mechanics program MM2(91), developed by Allinger and coworkers (Burkert and Allinger, 1982), and implemented in the molecular modeling package MacMimic/MM2(91) version 2.0 (Instar Software AB, Ideon Research Park, S-223 70 Lund, Sweden) and the MM3(92) program (Allinger et al., 1989). The construction of input structures and the molecular superimposition studies were done using the MacMimic program.

RESULTS

Chemicals. The alkyl ether analogs **3** and **4,** were synthesized by carbocupration of acetylene, giving a *Z* double bond with high selectivity (Gardette et al., 1985), followed by alkylation of the cuprate by an alkyl bromide as shown in Figure 4. This method has previously been used for the synthesis of alkyl substituted pheromone analogs by Jönsson et al. (1991).

The enol ether analogs, compounds **5** and **6,** were synthesized via a carbocupration route employing allenyl ethers, as shown in Figure 5. Also in this type of reaction, the (*Z*)-enol ether is formed with high selectivity (Alexakis and Normant, 1984; Klein et al., 1976). The *Z*:*E* ratio for compound **5** was 87:13 and for compound **6,** 76:24. Purification with argentation chromatography gave the pure *Z* isomers. The allenyl ethers were obtained from propargyl bromide in two steps. Propargyl bromide was reacted with a suitable alcohol under phase transfer catalyst conditions giving the alkyl propargyl ether. This compound was then rearranged under basic conditions, to give the allenyl ether.

Receptor Cell Responses. Relative single-cell activities for compounds **1–6,** including corrections for differences in volatilies determined as described in the Methods and Materials section, are shown in Figure 6. All analogs display lower activities than the natural pheromone component **1.** However, there is a significant difference in the reduction of the activity for the two ether analogs **3** and **5.** The alkyl ether **3** is less active than **1** by only a factor of 6, while the enol ether **5** is 150 times less active than **1.** In the chain-elongated series (**2, 4,**

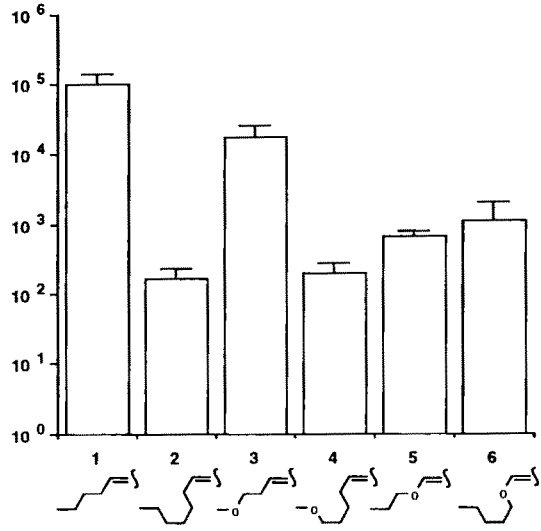

FIG. 6. Relative electrophysiological single-cell activities (+SEM) for compounds **1–6**. The data are corrected for differences in volatility.

and **6**) the effect of a replacement of a methylene group by an oxygen atom differs from that in the series **1, 3,** and **5.** The compound with an oxygen atom in position 11 **(4)** is equipotent with the parent compound **2.** Thus, in this case the replacement of a methylene group by an oxygen atom has no effect on the electrophysiological activity. Interestingly, in this series the chain-elongated enol ether **6** has slightly higher activity than the parent compound **2.**

The effects of chain elongation of compounds **1, 3,** and **5** are also very different. Chain-elongation of **1** by two methylene groups, giving compound **2,** decreases the activity by a factor of about 600, in agreement with previously reported results (Liljefors et al., 1985, 1987). The corresponding chain elongation of compound **3** to **4** gives an activity decrease by a factor of 86. In contrast to these activity decreases, chain elongating the enol ether **5** to **6** does not significantly affect the electrophysiological activity.

Conformational Energies. The main objective of the present study was to investigate if the electrophysiological activities of chain-elongated analogs were determined by the conformational energies required by a loop-formation of the terminal chain. In particular, we wanted to investigate the relationships between the effect of the activity of chain elongation of **1** and of the ether analogs **3** and **5.** Thus, we calculated the conformational energies required by the three pairs **1–2, 3–4,** and **5–6** to acquire the proposed active conformations (Liljefors et

al., 1987) as exemplified by compounds **1** and **2** in Figure 3b. For each compound, the calculations were performed as follows: (1) the energy of the preferred (lowest energy) conformation was calculated and the preferred conformation was identified by energy minimizing a large number of trial structures; and (2) the energy of a conformation was calculated in which the carbonyl carbon, the carbonyl oxygen, the carbon atom of the acetate methyl group, and the carbon atom of terminal methyl group superimposed exactly with the corresponding atoms of the proposed active conformation of the natural pheromone component **1** as shown in Figure 3a. For the chain-elongated analogs this conformation has a loop conformation of the terminal chain, as in Figure 3b. In the superimpositions, it was further required that the *Z* double bond of all analogs must be located in the plane defined by the C—C=C—C fragment of compound **1** in Figure 3a (Liljefors et al., 1987). The conformational energies required for each compound to acquire the proposed bioactive conformation were calculated finally as the difference of the energies calculated according to (2) and (1). In order to investigate the force-field dependence of the results, the calculations were performed using two different force fields, MM2(91), and the more recent MM3(92) (see Methods and Materials section). The results are shown in Table 1.

The chain-elongated analogs **2, 4,** and **6** have higher conformational energies of their bioactive conformations (ΔE) than those of their parent compounds **1, 3,** and **5.** Furthermore, the differences in conformational energies for the bioactive conformations ($\Delta\Delta E$) of the three pairs of chain-elongated analog vs. parent compound vary significantly, as shown in Table 2. Chain elongation of **1** (to **2**) was calculated to require an additional conformational energy of 5.6

TABLE 1. CALCULATED CONFORMATIONAL ENERGY DIFFERENCES (ΔE) BETWEEN PROPOSED BIOACTIVE CONFORMATION (E_{active}) AND PREFERRED CONFORMATION (E_{lowest}) OF COMPOUNDS **1–6**[a]

Compound	$\Delta E = E_{active} - E_{lowest}$		Relative single-cell activity
	MM2(91)	MM3(92)	
1	1.1	0.6	1
2	6.7	6.8	1/603
3	0.8	0.4	1/6
4	4.3	4.0	1/513
5	3.4	2.2	1/151
6	4.4	2.8	1/91

[a]The energies are in kcal/mol. Electrophysiological activities in relation to compound **1** are given in the last column.

TABLE 2. CALCULATED DIFFERENCES ($\Delta\Delta E$) BETWEEN CONFORMATIONAL ENERGIES OF BIOACTIVE CONFORMATION OF CHAIN-ELONGATED ANALOGS **2**, **4**, AND **6** AND OF THEIR PARENT COMPOUNDS **1**, **3**, AND **5**[a]

	$\Delta\Delta E$		
	MM2(91)	MM3(92)	Factor of activity decrease
2 compared to **1**	5.6	6.2	603
4 compared to **3**	3.5	3.6	86
6 compared to **5**	1.0	0.6	0.6

[a]The energies are in kcal/mol. The last column shows electrophysiological activity decreases due to chain elongation.

kcal/mol in order for **2** to acquire the bioactive conformation. The corresponding energies for **3** (to **4**) and **5** (to **6**) were significantly lower. The results in Table 2 for the two different force fields are very similar, demonstrating that the results were not dependent on the particular force field used.

DISCUSSION

The effects on the electrophysiological activity of replacement of the methylene group in either position 7 or 9 in **1** by an oxygen atom are highly dependent on the position of the replacement. The 9-oxa compound **3** is only six times less active than **1**, while the 7-oxa compound **5** is about 150 times less active than **1** (Figure 6). The calculated conformational energy required for the bioactive conformation of **3** is very similar to the corresponding energy required for **1** (Table 1). This implies that the activity decrease of **3** compared to **1** cannot be due to a conformational effect. The small but significant activity decrease of **3** is most probably due to the lower lipophilicity of the terminal chain of **3** compared to that of the hydrocarbon chain of **1**. The logarithms of the octanol–water partition coefficient (log P) for alkyl ethers are significantly lower than those for the corresponding hydrocarbons (Hansch and Leo, 1979). Previous structure–activity studies have demonstrated the importance of the lipophilic interactions between the terminal alkyl chain of **1** and the receptor site (Wenqi et al., 1993).

Compound **5** requires a significantly higher conformational energy than **1** (and **3**) to acquire the bioactive conformation (Table 1). A large part of the activity decrease of **5** is most probably due to this conformational energy effect. Thus, the significant difference in the activities of compounds **3** and **5** can be rationalized on the basis of different conformational energies for their bioactive conformations.

Our electrophysiological result for compound **3** is quite different from that of an EAG study of a 17-oxa analog of (*E*,*Z*)-2,13-octodecadienyl acetate, a pheromone component of the currant clearwing moth, *Synanthedon tipuliformis* (Hoskovec et al., 1993). In this compound, a methylene group of the natural pheromone component is replaced by an oxygen atom at a position corresponding to the ether oxygen position in **3.** Interestingly, in the EAG study of Hoskovec et al. (1993) the ether analog is virtually inactive, while the corresponding compound **3** in our study displays only a small activity decrease compared to **1** (Figure 6). Although it is not possible to directly compare results obtained by single-cell electrophysiology and EAG, this may indicate that the receptor sites interacting with the terminal chains of the pheromone components of the two species, which belong to different families of lepidoptera (Noctuidae and Sesiidae), have different properties.

The ether analogs of **2** (**4** and **6**) have lower calculated conformational energies for their bioactive conformations than that of **2** (Table 1). Replacement of a methylene group in positions 7 and 11 of **2** lowers the energy of a loop conformation due to less severe steric repulsions between a methylene group and an oxygen atom, compared to those between two methylene groups (Figure 3b). Thus, conformational energy considerations imply that these ether analogs should have higher activities than **2.** However, the lower lipophilicities of the ether analogs work in the opposite direction by lowering the activities. Consequently, the balance of the conformational energy effect and the lipophilic effect makes compound **4** equipotent with **2,** while the conformational energy effect dominates for compound **6** due to the higher lipophilicity of enol ether compared to alkyl ethers (Hansch and Leo, 1979), making this compound more active than **2.**

To be able to investigate if the activities of chain-elongated analogs are determined by the conformational energies required for a loop conformation of the terminal chain, it was necessary to compare calculated conformational energy differences and relative electrophysiological activity factors for the chain-elongated analogs **2, 4,** and **6** with respect to their parent compounds **1, 3,** and **5.** These energy differences and activity factors are given in Table 2. By discussing the factor of activity decrease for **2** vs. **1, 4** vs. **3,** and **6** vs. **5,** differences in lipophilicity and electronic effects on the relative activities of the three pairs of compounds should essentially cancel. Thus, the factors of activity decrease in Table 2 reflect the conformational energy effects of chain elongation on the activities. Consequently, these factors can be compared to the calculated conformational energies for a bioactive conformation of the chain-elongated analogs, in which the elongated chain has the proposed loop conformation, in relation to the calculated energies for the parent compounds ($\Delta\Delta E$ in Table 2).

The calculated conformational energies $\Delta\Delta E$ are highest for **2** compared to **1** and lowest for the enol ether analog **6** compared to **5.** This implies that if the chain-elongated analogs adopt a loop conformation of the terminal chain in their

bioactive conformations, as assumed in the calculations, the experimental factors of activity decrease should be largest for **2** compared to **1** and smallest for **6** compared to **5,** with the factor for **4** compared to **3** in between. This is exactly what is found. The experimental data in Table 2 show that the factor of activity decrease for **2** compared to **1** is 603; for **4** compared to **3** 86, and for **6** to **5,** it is 0.6. The activity decrease on chain elongation parallels the calculated increase of conformational energies for loop formation of the terminal chain in compounds **2, 4,** and **6.** Thus, our previous conclusion (Liljefors et al., 1987) that the terminal chain of chain-elongated analogs of **1** adopts a loop conformation in the bioactive conformation is strongly supported by the present study.

CONCLUSIONS

Replacement of the methylene group in the 7 position of **1** by an oxygen atom results in a much larger activity decrease than the corresponding replacement in the 9 position. In contrast, replacement of the methylene group in the 11 position of **2** by an oxygen atom has no effect on the activity, while the corresponding replacement of the 7-methylene group of **2** gives small increase of the activity. Molecular mechanics calculations indicate that these results are due to a combination of different conformational and lipophilic properties of the parent compound and its alkyl ether and the enol ether analogs.

Chain elongation of an alkyl ether **(3)** and an enol ether **(5)** analog of the natural pheromone component **1** results in significantly smaller decreases of the electrophysiological single-cell activity than is found for chain-elongation of **1.** Molecular mechanics calculated conformational energies for a loop conformation of the terminal chain in the chain-elongated analogs **2, 4,** and **6** closely parallel the experimental activity decreases. The results support our previous conclusion that the terminal chain of chain-elongated analogs of **1** adopts a loop conformation in the bioactive conformation.

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MECHANISM BY WHICH NONINHIBITORY CONCENTRATIONS OF GLUCOSE INCREASE INHIBITORY ACTIVITY OF *p*-COUMARIC ACID ON MORNING-GLORY SEEDLING BIOMASS ACCUMULATION[1]

K.J. PUE,[2] U. BLUM,[2,*] T.M. GERIG,[3] and S.R. SHAFER[4]

[2]*Department of Botany*
[3]*Department of Statistics*
[4]*United States Department of Agriculture*
Agricultural Research Service and Departments of Plant Pathology and Soil Science
North Carolina State University
Raleigh, North Carolina 27695

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Abstract—Noninhibitory levels of glucose-C [≤ 72 μg carbon (C)/g soil] increased the inhibitory activity of *p*-coumaric acid on morning-glory seedling biomass accumulation in Cecil B_t-horizon soil. The amount of *p*-coumaric acid required for a given level of inhibition of shoot and seedling biomass accumulation decreased as the concentration of glucose increased. Soil extractions with neutral EDTA (0.25 M, pH 7) after addition of combinations of *p*-coumaric acid and glucose (concentrations ranging from 0 to 1.25 μmol/g soil) to the soil showed that utilization of *p*-coumaric acid by microbes decreased linearly as the concentration of glucose increased. The increased inhibitory activity of a given concentration of *p*-coumaric acid in the presence of glucose was not due to a reduction in soil sorption of *p*-coumaric acid or effects of nitrogen-limited microbial growth. Noninhibitory levels of phenylalanine and *p*-hydroxybenzoic acid slowed the utilization of *p*-coumaric acid by microbes in a similar manner as glucose. The presence of methionine, however, did not affect the rate of *p*-coumaric acid utilization by microbes. These observations suggest that differential utilization of individual molecules in organic mixtures by soil microbes can modify, and in this case increase, the effectiveness of a given concentration of an inhibitor such as *p*-coumaric acid on the inhibition of seedling growth such as morning-glory.

To whom correspondence should be addressed.
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Key Words—Morning glory, *Ipomoea hederacea*, *p*-coumaric acid, glucose, methionine, phenylalanine, *p*-hydroxybenzoic acid, allelopathic interactions, microbial substrate utilization.

INTRODUCTION

Studies of allelopathy have emphasized the identification and quantification of inhibitors in soils (Rice, 1984; Kuiters, 1990; Siqueira et al., 1991). Few research studies, however, have been directed towards understanding how other organic molecules in the soil (e.g., carbohydrates, amino acids, etc.) may modify plant responses to these inhibitors. In one study (Blum et al., 1993), addition of low concentrations of glucose or methionine to soil in conjunction with inhibitory levels of *p*-coumaric acid reduced the concentration of that phenolic acid required to inhibit morning-glory seedling growth. Blum et al. (1993) hypothesized that carbon sources such as glucose may have reduced the sorption of *p*-coumaric acid onto soil particles and/or reduced microbial metabolism of *p*-coumaric acid. Either mechanism would result in higher available concentrations of *p*-coumaric acid in the soil solution. The objective of this study was to test this hypothesis by determining the rates of soil sorption and soil microbial utilization of *p*-coumaric acid in the presence and absence of low concentrations of glucose and several other organic molecules.

METHODS AND MATERIALS

Seedling Studies

General Procedures. Cecil B_t-horizon soil material (Typic Kanhaludults, clayey, kaolinitic, thermic) was obtained from the Piedmont of North Carolina, sieved (4 mm), air-dried, and stored at room temperature (Dalton et al., 1987). A soil–sand mixture (1 : 2 by weight) of Cecil B_t soil and river sand rinsed with deionized water and air-dried was used as growth medium. Morning-glory seeds (*Ipomoea hederacea* L. Jacquin.; F&J Seed Service, Woodstock, Illinois) were germinated in the dark at 34°C in 155-ml cups (five seeds per cup) containing 150 g of the soil–sand mixture. After 24 hr, cups with seedlings were placed under light banks with a 12-hr photoperiod (140 $\mu E/m^2/sec$). Cups were inserted in holes in a plywood sheet to prevent direct irradiation to the cups below the soil surface. Seedlings were thinned to one seedling per cup four or five days after seeding. For a more detailed description of the cup system see Blum et al. (1993).

Nutrient Requirements for Morning-glory Seedling Growth. Quadruple-strength Hoagland's solution (Hoagland and Arnon, 1950) (pH 5, without micronutrients) in 0.5-ml increments from 0 to 3.5 ml was added every other

day starting with day 6 from seeding. Water was added daily to achieve a maximum combined cup, soil, water, nutrient solution, and seedling weight of 185 g (160 g initially for cup and soil). Seedlings were harvested 21 days after seeding, oven-dried (105°C), and dry weights of root, shoot, and total plant biomass were determined.

Effects of p-*Coumaric Acid and Glucose Mixtures on Seedling Biomass.* Solutions (pH 5) of glucose of *p*-coumaric acid (Sigma Chemical Co., St. Louis, Missouri) ranging from 0 to 1 μmol/g soil or various combinations of these two compounds were added every other day starting eight days after seeding. Treatments were applied on five days. Solution acidity was adjusted to pH 5 with NaOH or HCl. Quadruple-strength Hoagland's solution (1.5 ml, pH 5, without micronutrients) was added every other day starting seven days after seeding. Seedlings were harvested 18 days after seeding, oven-dried, and weights of root, shoot, and total plant biomass were determined.

Soil Studies

General Procedures. Sieved (0.25 mm) Cecil B_t soil material (1 g) was added to 15-ml Corex tubes (Corning Inc., Rochester, New York). Tubes were capped (Bacti-Capall, Sherwood Medical Industries) and autoclaved (15 min at 1.2 kg/cm^2) three times at three-day intervals. At the start of an experiment, each tube received 0.5 ml double-strength Hoagland's solution (pH 5) without micronutrients, 0.5 ml soil extract (see below), 0.5 ml of phenolic acid and/or other carbon source, and 0.5 ml water. In all cases, final volume in each tube was 2 ml for a single addition or 4 ml for a double addition. Reagent-grade *p*-coumaric acid, glucose, methionine, *p*-hydroxybenzoic acid, and phenylalanine were used (Sigma). Details of specific experiments are provided in Table 1.

Soil Extracts for Reinfestation of Soils with Microbes. Soil extracts to reinfest autoclaved soil materials with microbes were obtained from a cup containing 150 g of a Cecil B_t soil–sand mixture (1:2 by weight) treated with 5 ml *p*-coumaric acid (0.5 mM), 10 ml double-strength Hoagland's solution without micronutrients, and 15 ml water. After four to five days in a dark incubator at 34°C, the soil mixture was combined with 300 ml water in a beaker. The soil particles were allowed to settle in the beaker before the supernatant was decanted and filtered through Whatman No. 1 filter paper in a Buchner funnel. This extract was used to reinfest soil samples. A portion of this extract was also filter sterilized (0.2-μm membrane filter) for addition to soil material that was to remain sterile.

Extraction of p-*Coumaric Acid from Soil.* Soil samples in the tubes were extracted with 0.25 M EDTA (ethylenediamine tetraacetic acid, pH 7; for preparation of solution see Blum et al., 1994) or water at different time intervals by

TABLE 1. EXPERIMENTAL PROTOCOLS FOR SOIL EXTRACTIONS

Experiment[a]	Presence (+)/ absence (−) of microbes	Strength of nutrient solution[b]	Concentrations (μmol/g) of additional carbon sources in soil[c]				
			PCO	GLU	METH	PHENYLAL	P-OH B
1	+/−	2×	1.25	0 or 1.25			
2	+	2×	1.25	0 to 1.25			
3	+	2×, 4×	1.25	0 or 1.25			
4	+	2×	1.25	0 or 1.25	0 or 1.25	0 or 1.25	0 or 1.25
5[d]	+	2×	0 or 1.25	0 to 1.25			

[a] For each experiment, carbon sources in aqueous solutions were added to soil (2 ml total volume) on day 0. In experiment 5, another 2 ml were added on day 6. Two soil samples were extracted with 0.25 M EDTA (pH 7) for each treatment and time interval.

[b] Hoagland's nutrient solution without micronutrients adjusted to pH 5.

[c] Only two-way mixtures were used. Molecular weights of *p*-coumaric acid (PCO), glucose (GLU), methionine (METH), phenylalanine (PHENYLAL), and *p*-hydroxybenzoic acid (P-OH B) are 164.2, 132.1, 149.1, 165.09, and 138.1, respectively. Actual concentrations of *p*-coumaric acid initially in tubes varied somewhat since the soil extracts added to the tubes to reinfest the soil with microbes also contained *p*-coumaric acid. Percentage of carbon in these compounds is as follows: 65.83, 40.00, 40.25, 65.43, and 60.88, respectively. The percentage of nitrogen in methionine and phenylalanine is 9.39 and 8.48, respectively.

[d] *p*-Coumaric acid expressed as millimolar soil solution = 0 or 0.625; glucose mM = 0, 0.3125, or 0.625.

adding 2 ml of 0.5 M EDTA or water to tubes containing 1 g soil material and 2 ml of treatment solution. Tubes were vortexed immediately after addition of the EDTA or water extractant. EDTA tubes were stored in the dark for 5 hr, and vortexed again just before centrifugation (10 min at 12,100*g*). The supernatants were filter-sterilized (0.2-μm membrane filter) for quantification of *p*-coumaric acid (EDTA extracts) by HPLC analysis (Blum et al., 1994). Water tubes were vortexed, centrifuged, and the supernutrients were analyzed for glucose by anthrone (Dische, 1962).

Statistical Analysis

Data were analyzed by PROC ANOVA, PROC GLM, or PROC NLIN of the SAS Institute (1988). For the seedling experiment, seedlings were distributed under three light banks (Blocks) in a randomized complete block design with two replicates per block for each treatment. Morning-glory seedling response was modeled using the modified logistic equation described by Gerig and Blum (1993). For the soil experiments, two soil samples were extracted for each treatment and time period.

RESULTS

Seedling Studies

Nutrient Requirements for Morning-glory Seedling Growth. Maximum morning-glory shoot, root, and seedling biomass was obtained with 1.5-ml applications of quadruple-strength Hoagland's nutrient solution without micronutrients every other day (Figure 1). This nutrient solution treatment was therefore chosen for the seedling biomass experiment.

Effect of p-*Coumaric Acid and Glucose Mixtures on Seedling Biomass of Morning glory.* The relationships between morning-glory seedling biomass (grams), Y, and the concentrations of *p*-coumaric acid (micromoles per gram), X_1, and glucose (micromoles per gram), X_2, *were* described by:

$$Y = \beta_1/(1 + \exp\{\beta_3[\ln(Z) - \beta_2]\}) \qquad \text{for } Z > 0$$

$$= \beta_1, \qquad \text{for } Z = 0$$

where $Z = X_1 \exp(-\beta_4 X_2)$. The estimated values of β_1, β_2, β_3, and β_4 are listed in Table 2. A graphical display of the data and the results from the model are presented in Figure 2. Morning-glory seedling biomass was inversely related to the concentration of *p*-coumaric acid added to the soil–microbe–seedling system. Shoot biomass was more sensitive than root biomass to *p*-coumaric acid. The 50% inhibition dose for root biomass was approximately 13 times that for shoot biomass (Table 2). Glucose alone had no statistically significant effect on morn-

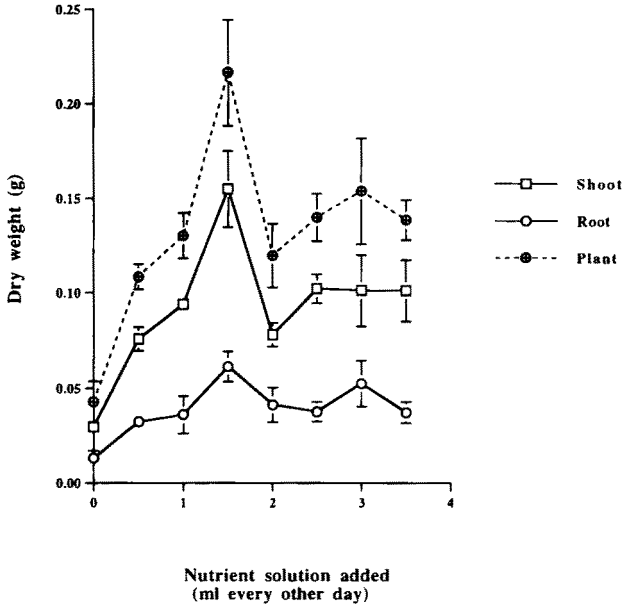

FIG 1. Effect of various amounts of 4× Hoagland's nutrient solution without micronutrients on biomass of morning-glory seedlings (one seedling per cup). Means ± standard error.

ing-glory biomass. However, the presence of glucose modified the level of inhibition on seedling biomass by *p*-coumaric acid (Table 2, Figure 3). The amount of *p*-coumaric acid required for a given level of inhibition of shoot and seedling biomass decreased as the concentration of added glucose increased. For example, a 20% inhibition of seedling biomass required 0.42 μmol/g of *p*-coumaric acid in the absence of glucose and 0.33 μmol/g in the presence of

TABLE 2. SUMMARY OF LOGISTIC MODEL DESCRIBING INTERACTIVE EFFECTS OF *p*-COUMARIC ACID AND GLUCOSE ON MORNING-GLORY SEEDLING BIOMASS

Model parameters	Shoot	Root	Plant
β_1 (control values; g dry weight)	0.07	0.05	0.12
β_2 (dose required for 50% inhibition; μmoles/g)	1.05	13.18	2.89
β_3 (rate parameter)	1.04[a]	1.20[a]	1.03[a]
β_4 (glucose modifier)	−0.75[a]	−0.89	−0.74[a]

[a]Significantly different from zero at $P < 0.05$.

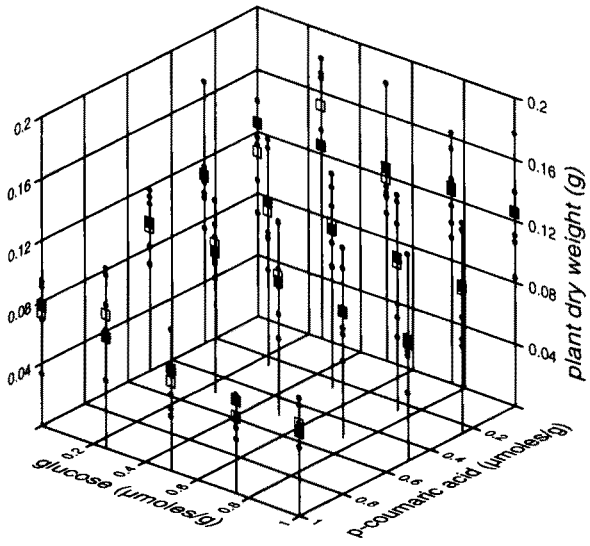

FIG 2. Observed (solid dots), means (open squares), and predicted values (solid squares) for seedling biomass from the modified logistic equation for *p*-coumaric acid and/or glucose treated morning-glory seedlings.

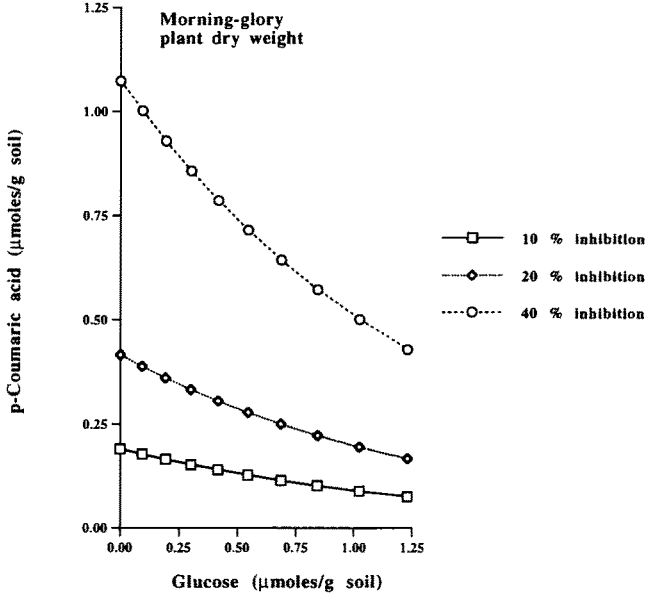

FIG 3. Morning-glory plant biomass isolines for 10, 20, and 40% inhibition of seedlings treated with *p*-coumaric acid and various concentrations of glucose.

0.30 μmol/g of glucose. Addition of glucose did not modify the effects of *p*-coumaric acid on root biomass.

Soil Extractions

Recovery of p-*Coumaric Acid from Soil in Presence and Absence of Microbes and Glucose–Experiment 1.* Soil samples were extracted with neutral EDTA (0.25 M, pH 7) because Blum et al. (1994) observed that this was an effective extractant for estimating the levels of free and reversibly bound phenolic acids available to soil microbes. Recovery of *p*-coumaric acid from Cecil B_t soil in the absence of soil microbes was not significantly affected by the presence of glucose (Figure 4). In the presence of microbes, recovery of *p*-coumaric acid was greater in the presence of glucose than in the absence of glucose. For example, between day 1 and 4 recovery of *p*-coumaric acid from nonsterile soil was reduced by 30% in the absence of glucose but only 5% in the presence of glucose. In sterile soil, recovery of *p*-coumaric acid declined only 5% regardless of glucose addition. Between days 4 and 8, recovery of *p*-coumaric acid in nonsterile soil was reduced by 84% in the absence of glucose

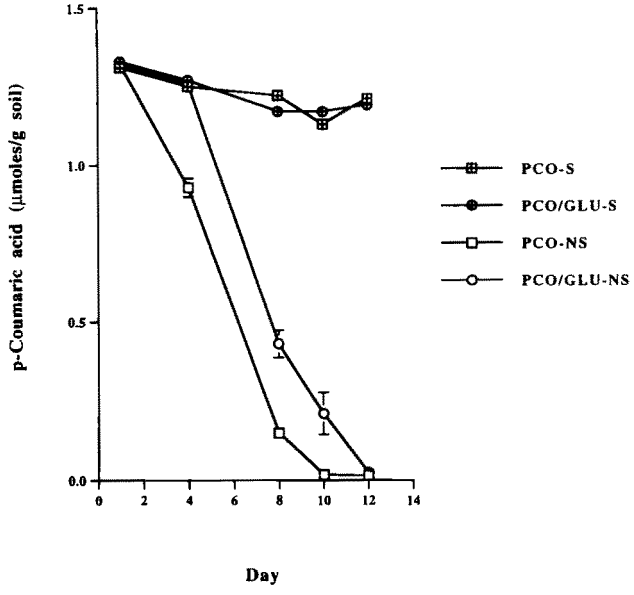

FIG 4. Recovery of *p*-coumaric acid (PCO) from Cecil B_t soil material soil by 0.25 M, pH 7, EDTA in the presence (NS) and absence (S) of microbes and glucose (GLU). Means ± standard error.

but 66% in the presence of glucose. During the same time interval, *p*-coumaric acid was reduced by only 3% in sterile soil.

Recovery of p-*Coumaric Acid from Soil in Presence of Microbes and Various Concentrations of Glucose—Experiment 2.* Once *p*-coumaric acid utilization by microbes was evident (day 3), recovery of *p*-coumaric acid on day 3 and subsequently was related directly to the concentration of glucose added (Figure 5). Effects of glucose on *p*-coumaric acid recovery declined over time (Table 3).

Effects of Glucose and Nutrients on Recovery of p-*Coumaric Acid—Experiment 3.* The glucose effect on recovery of *p*-coumaric acid from nonsterile soil was not significantly affected by doubling the concentration of the nutrient solution (2× or 4× strength Hoagland's solution minus micronutrients; Figure 6).

Recovery of p-*Coumaric in Presence of Methionine, Phenylalanine, or* p-*Hydroxybenzoic Acid—Experiment 4.* The addition of methionine to Cecil B_t soil had little effect on the rate of *p*-coumaric acid recovery in the presence of microbes (Figure 7). The addition of phenylalanine or *p*-hydroxybenzoic acid resulted in greater recoveries of *p*-coumaric acid, similar to that of glucose.

Recovery of p-*Coumaric acid and Glucose—Experiment 5.* Since solution volumes for day 0 to day 6 (2 ml) and day 6 to day 11 (4 ml) were different

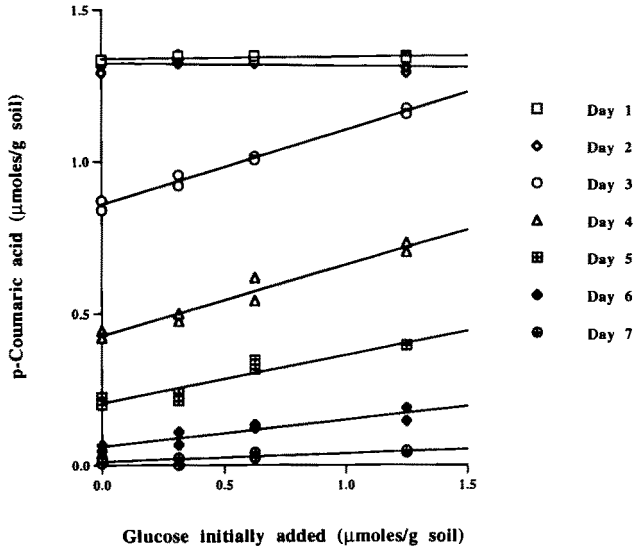

FIG 5. Regression lines for recovery of *p*-coumaric acid from Cecil B_t soil material by 0.25 M, pH 7, EDTA in the presence of microbes and various concentrations of glucose. Significant regression models are presented in Table 3.

TABLE 3. REGRESSIONS FOR RECOVERY OF *p*-COUMARIC ACID (MICROMOLES PER GRAM SOIL) BY EDTA FROM CECIL B_t SOIL IN PRESENCE OF VARIOUS CONCENTRATIONS OF GLUCOSE

Day of extraction	Intercept	Linear[a]	R^2	P
3	0.858	0.247	0.99	0.00001
4	0.428	0.232	0.96	0.00001
5	0.205	0.158	0.90	0.0003
6	0.062	0.088	0.85	0.0012
7	0.012	0.028	0.60	0.0231

[a]Where Y = *p*-coumaric acid recovered in micromoles/per gram and X = glucose initially added in micromoles per gram soil.

for this experiment, concentrations of the two compounds have been expressed as millimolar of soil solution instead of micromoles per gram of soil (Table 4, Figures 8–10). Once *p*-coumaric acid utilization by microbes was evident (day 4), recovery of *p*-coumaric acid on day 4 and for subsequent time intervals

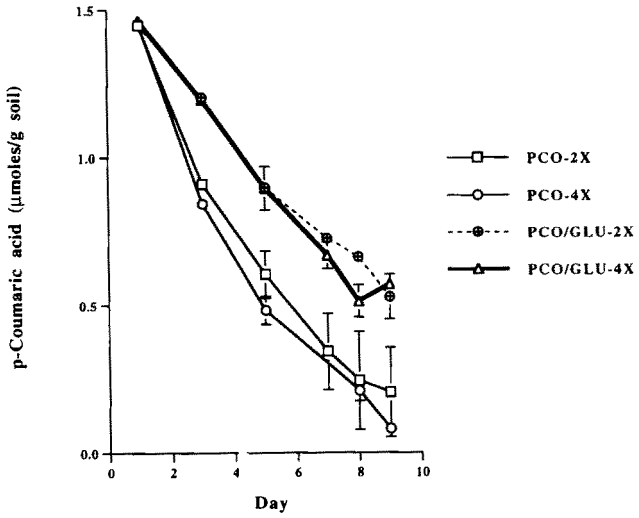

FIG 6. Recovery of *p*-coumaric acid (PCO) from Cecil B_t soil material by 0.25 M, pH 7, EDTA in the absence or presence of glucose (GLU) and two concentrations (2× and 4× strength) of Hoagland's solution without micronutrients. Means ± standard error.

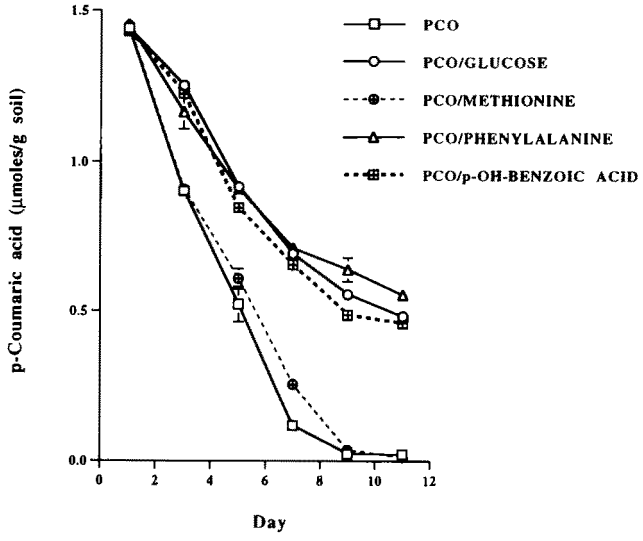

FIG 7. Recovery of *p*-coumaric acid (PCO) from Cecil B_t soil material by 0.25 M, pH 7, EDTA in the absence or presence of glucose, methionine, phenylalanine, and *p*-hydroxybenzoic acid. Means ± standard error.

increased linearly as the concentration of glucose added increased (Table 4; Figures 9 and 10). The initial delay in microbial utilization of *p*-coumaric acid seen for the first addition (days 0–4), however, was not observed for the second addition. Furthermore, the effects of glucose on the recovery of *p*-coumaric acid were greater for the second addition than for the first addition (Table 4).

TABLE 4. REGRESSIONS FOR RECOVERY OF *p*-COUMARIC ACID (MILLIMOLAR) BY EDTA FROM CECIL B_t SOIL IN PRESENCE OF VARIOUS LEVELS OF GLUCOSE (MILLIMOLAR)

Day	Intercept	Linear[a]	R^2	*P*
4	0.337	0.157	0.77	0.0209
5	0.202	0.204	0.87	0.0063
6	0.139	0.117	0.82	0.0125
24 hr after second treatment (given on day 6)				
7	0.643	0.190	0.92	0.0025
9	0.489	0.306	0.78	0.0187
11	0.338	0.266	0.94	0.0012

[a]Where Y = *p*-coumaric acid recovered (millimolar) and X = glucose initially added (millimolar).

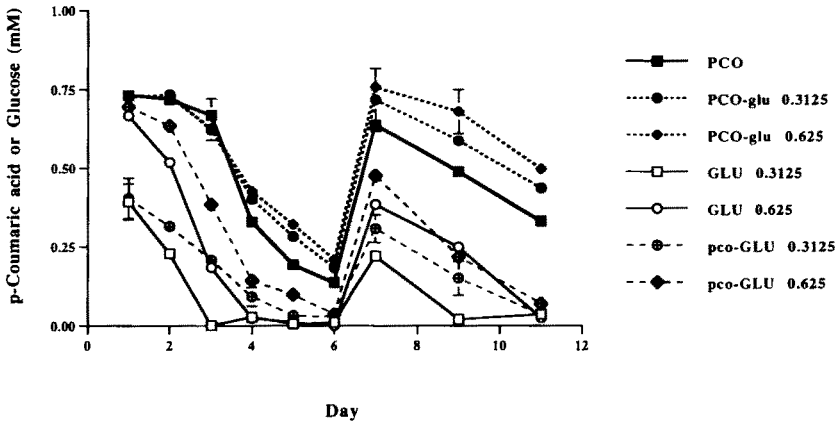

FIG 8. Recovery of *p*-coumaric acid (PCO), glucose (GLU), *p*-coumaric acid in the presence of two concentrations of glucose, and glucose in the presence of *p*-coumaric acid from Cecil B_t soil material. Additions of PCO and GLU occurred on day 0 and day 6. Means ± standard error.

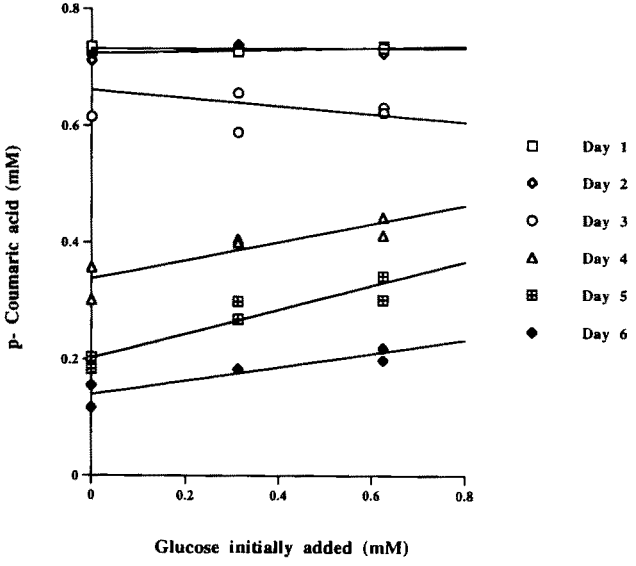

FIG 9. Regressions for the recovery of *p*-coumaric acid from Cecil B_t soil material by 0.25 M, pH 7, EDTA in the presence of microbes and various concentrations of glucose. Significant regression models are presented in Table 4.

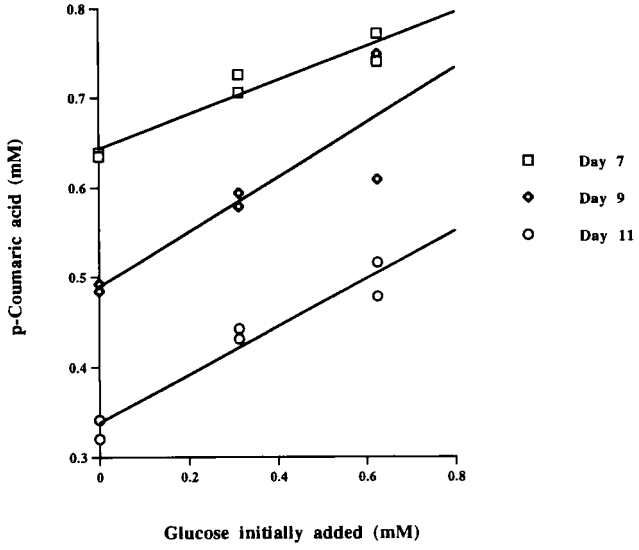

FIG 10. Regressions for the recovery of *p*-coumaric acid from Cecil B_t soil material by 0.25 M, pH 7 EDTA in the presence of microbes and various concentrations of glucose after a second addition of *p*-coumaric acid and/or glucose. Significant regression models are presented in Table 4.

In general, recovery of glucose in the absence of *p*-coumaric acid was lower than in the presence of *p*-coumaric acid (Figure 8). This difference in recovery, however, was statistically significant for only days 2 and 3 for both glucose concentrations, and days 5 and 11 for the 0.625 mM glucose concentration. For example, between days 1 and 3, recovery of 0.325 mM glucose added was reduced 99.75% in the absence of *p*-coumaric acid and 22% in presence of *p*-coumaric acid, and recovery of 0.625 mM glucose was reduced 72% in the absence of *p*-coumaric acid and 45% in the presence of *p*-coumaric acid.

DISCUSSION

In earlier work (Blum et al., 1993), addition of glucose reduced the amount of *p*-coumaric acid required for a given level of inhibition of morning-glory seedling biomass accumulation. They hypothesized that the addition of glucose C below 72 μg C/g soil reduced the microbial utilization and/or sorption of *p*-coumaric acid in the soil and thus increased the inhibitory activity of *p*-coumaric acid on seedling growth. Unfortunately, only two noninhibitory concen-

trations of glucose were included in that study, so the modification of the action of *p*-coumaric acid by such concentrations of glucose could not be adequately determined. Using a Cecil B_t-horizon soil and combinations of four noninhibitory glucose concentrations and three inhibitory *p*-coumaric concentrations, we have demonstrated, as suggested by Blum et al. (1993), that noninhibitory levels of glucose do increase the inhibitory activity of *p*-coumaric acid on morningglory biomass accumulation. Furthermore, our results show that sorption of *p*-coumaric acid onto soil particles of Cecil B_t soil material was not reduced in the presence of glucose. Thus, the increased inhibitory activity of *p*-coumaric acid in the presence of glucose is not attributable to a reduction in sorption of *p*-coumaric acid onto soil particles in the presence of glucose. We have also been able to demonstrate that the microbial utilization of *p*-coumaric acid in the range of glucose concentrations used was reduced in a linear manner and that this occurred in soil systems that were not nutrient limited. The initial C/N ratios for these soil systems were all less than 0.5. Such low initial C/N ratios indicated the addition of C by these compounds did not result in nitrogen-limiting conditions for microbial metabolism. This suggests that the effects of glucose on recovery of *p*-coumaric acid were not attributable to nitrogen immobilization.

Microbial utilization of glucose and *p*-coumaric acid as a carbon source in mixtures was reduced, but the reduction in utilization was far greater for *p*-coumaric acid than for glucose. This preferential utilization of one carbon source over another is consistent with bacteria grown on two different carbon sources in vitro. For example, microbial utilization of one C source in batch cultures is often prevented or inhibited by the presence of a second C source (Harder and Dijkhuizen, 1982; Papanastasiou, 1982; Bachmann et al., 1988). In pure cultures, the resulting sequence in C resource utilization can thus lead to diauxic growth of the microbe. Diauxic growth is characterized by two exponential phases of growth separated by a phase of no net increase in microbial biomass. Different carbon sources are being used for the two growth phases. In multispecies microbial cultures such as ours, one would not expect to see patterns of diauxic growth since different species of microbes would have different C source preferences. Nonetheless, the earlier and more rapid utilization of glucose compared to *p*-coumaric acid suggested sequential C utilization. Therefore, this may be one mechanism by which noninhibitory glucose concentrations in the soil may increase the inhibitory activity of a toxin such as *p*-coumaric acid. Differential C utilization by soil microbes and associated changes in inhibition of toxins such as *p*-coumaric acid may be a more general phenomenon, since the effects of phenylalanine and *p*-hydroxybenzoic acid were similar to those of glucose. This suggests that levels of inhibition of germination and seedling growth after desiccation of a cover crop may be much greater than expected, when this expectation is based on soil toxin levels only. This may also be true for complex organic mixtures in soil resulting from root exudates,

leaf leachates, and leachates from plant debris. Other mechanisms by which noninhibitory C sources may modify the activity of toxic phenolic acids are also possible. These may include effects on the rate of uptake of the toxin by plant roots, metabolic conversion of a substance to a more toxic product, and/or the creation of anaerobic soil conditions that inhibit plant growth. Based on these and previous observations (Blum et al., 1993), we suggest that soil bioassays of individual toxins identified as allelopathic compounds may not provide adequate information on the concentrations required for the inhibition of plant germination and growth in soils.

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CHIRALITY OF ISRAELI PINE BAST SCALE, *Matsucoccus josephi* (HOMOPTERA: MATSUCOCCIDAE) SEX PHEROMONE[1]

EZRA DUNKELBLUM,[2,3,*] REGINE GRIES,[4] GERHARD GRIES,[4] KENJI MORI,[5] and ZVI MENDEL,[2]

[2]*Institute of Plant Protection, Volcani Center*
Bet Dagan 50250, Israel

[4]*Centre for Pest Management, Department of Biological Sciences*
Simon Fraser University
Burnaby, British Columbia Canada V5A 1S6

[5]*Department of Agricultural Chemistry, The University of Tokyo*
Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

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Abstract—The absolute configuration of the sex pheromone of the Israeli pine bast scale, *Matsucoccus josephi*, was determined as (2*E*,5*R*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one, designated here as *R-E* with 10% (2*E*,5*S*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one, designated as *S-E*. The chirality of the quantitatively minor *Z* isomer was (2*E*,5*R*,6*Z*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one (*R-Z*). Chiral assignments were made by comparative gas chromatographic (GC) analysis of natural *M. josephi* pheromone with stereoselectively synthesized stereoisomers on a chiral Cyclodex-B column, which separated the enantiomers with baseline resolution. In gas chromatographic-electroantennographic detection (GC-EAD) analysis of the racemic *Z* and *E* isomers, the latter elicited the stronger antennal response by male *M. josephi*. In GC-EAD of all four stereoisomers, employing the chiral column, *R-E* was the most active stereoisomer. In field tests *R-E* attracted 10 times more males of *M. josephi* than did *S-E*. The racemic *E/Z* pheromone mixture, containing all four stereoisomers in approximately equal amounts, attracted as many male *M. josephi* as did an equivalent amount of *R-E*, indicating that the other stereoisomers are not inhibitory. The same keto-diene moiety with the same chiral center and configuration in all three known Matsucoccidae

*To whom correspondence should be addressed.

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sex pheromones implies a common biosynthetic pathway and phylogenetic relationship.

Key Words—*Matsucoccus josephi*, Coccoidae, Homoptera, Matsucoccidae, pine bast scales, sex pheromone, absolute configuration, (2*E*,5*R*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one.

INTRODUCTION

All known female scale sex pheromones are chiral compounds, except for the pheromone of the San Jose scale, *Quadraspidiotus perniciosus* (Comstock) (Fletcher and Bellas, 1988). Sex pheromones of three pine bast scales, *Matsucoccus matsumurae* (=*resinosae*) Bean & Godwin, *Matsucoccus feytaudi* Ducasse, and *Matsucoccus josephi* Bodenheimer and Harpaz, have been identified recently (Lanier et al., 1989; Einhorn et al., 1990; Dunkelblum et al., 1993). All three pheromones share a common $C_9H_{13}O$ keto-diene moiety with a chiral center in the same position. The *M. matsumurae* and *M. feytaudi* pheromones have very similar structures and, unlike the *M. josephi* pheromone with one chiral center, have two chiral centers. Stereoselective syntheses and field bioassays resolved the absolute configuration of the *M. matsumurae* (=*resinosae*) (Cywin et al., 1991; Mori and Harashima, 1993b) and *M. feytaudi* (Cywin and Kallmerten, 1992; Mori and Harashima, 1993a; Jactel et al., 1994) pheromones. We report here the determination of the chirality of the *M. josephi* pheromone by GC and GC-EAD analyses of natural and synthetic pheromones on a chiral column and by field bioassays of synthetic pheromone stereoisomers.

METHODS AND MATERIALS

Chemicals. The racemic *M. josephi* pheromone components (2*E*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one and (2*E*,6*Z*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one were available as a mixture (56% *E* + 44% *Z*) or separately from previous work (Zegelman et al., 1993). The two *E* enantiomers, (2*E*,5*R*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one (*R*-*E*) (96.6% ee) and (2*E*,5*S*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one (*S*-*E*) (99.0% ee), were synthesized from a chiral epoxide (Mori and Amaike, 1994). Natural pheromone was collected on Porapak Q and purified as described previously (Dunkelblum et al., 1993).

Insects. Male *M. josephi* pupae were reared in Israel (Mendel et al., 1990) and shipped by special delivery to Simon Fraser University in Canada. The pupae were kept there at room temperature and emerging adults were used in the morning hours.

Instrumental Methods. Racemic, *E* and *Z* isomers, *R*-*E* and *S*-*E* enantiomers

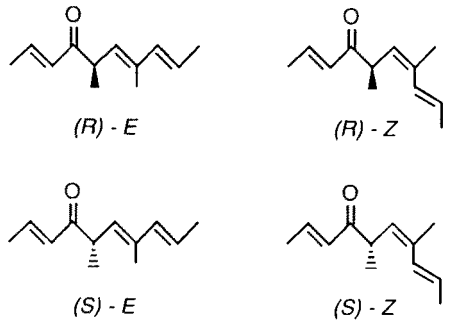

FIG 1. Stereoisomers of *Matsucoccus josephi* sex pheromone.

(Figure 1), and natural *M. josephi* pheromone were analyzed on a Hewlett Packard (HP) 5890 gas chromatograph equipped with a chiral Cyclodex-B column (30 m × 0.25 mm ID) (J & W, Folsom, California). All pheromone samples were also subjected to gas chromatographic–electroantennographic-detection (GC-EAD) analyses (Arn et al., 1975) using an HP 5890 GC equipped either with a DB-23 column (30 m × 0.25 mm ID) (J & W) or with the chiral Cyclodex-B column. As in the case of male moth antennae (Gries et al., 1991), male scale antennae were suspended between glass capillary electrodes for GC-EAD analysis. Preparing the exceedingly small antennae was difficult and may explain the variation in antennal responses.

Field Bioassay. Six-replicate field tests were set up in randomized complete blocks in the Harel forest (Judean foothills) plantations of Aleppo pine, *Pinus halepensis* Mill., and brutia pine, *Pinus brutia* Ten. Variations in topography, plot size limitations, and finding protected locations required testing in two different areas. Means were calculated from both areas despite the fact that their population levels differed. Triangular sticky traps baited with rubber septa impregnated with synthetic pheromone (Dunkelblum et al., 1987) were used. Traps were suspended from tree branches, 1.8 m above ground at least 25 m apart. The trapped scale males and the predatory bug, *Elatophilus hebraicus* Pericart, were counted using a stereomicroscope.

The first experiment tested dosages of the racemic stereoisomeric mixture (10–200 μg) as compared to *R-E* (2.5–50 μg) and *S-E* (2.5–50 μg) enantiomers. The second experiment compared a medium dosage of 25 μg of the two enantiomers. The third experiment retested attractancy of *R-E* and *S-E* at 50 μg, and trapped males were counted in four consecutive days.

Statistical Analysis. Trap catch data were transformed to $\sqrt{x} + 0.5$ and then subjected to analysis of variance followed by the Student-Neuman-Keuls multiple range test (at $P < 0.05$) to determine significance between means.

RESULTS

Laboratory Analysis. Gas-chromatographic analyses on the chiral column allowed separation of the enantiomers of the *E* and *Z* pheromone isomers with baseline resolution. The stereoselectively synthesized *R-E*, but not the *S-E* enantiomer (Mori and Amaike, 1994), coincided with the major female-produced enantiomer, whereas the antipode was present at 10% (Figure 2). Small amounts of the *R-Z* stereoisomer in one of the *R-E* samples (formed in a Wittig step of the synthesis coincided both with the first component in the racemic *Z* isomer and with the minor female-produced component. Thus, the chirality of the major *E* and *Z* pheromone components was assigned as (2*E*,5*R*,6*E*,8*E*)-5,7-dimethyl-

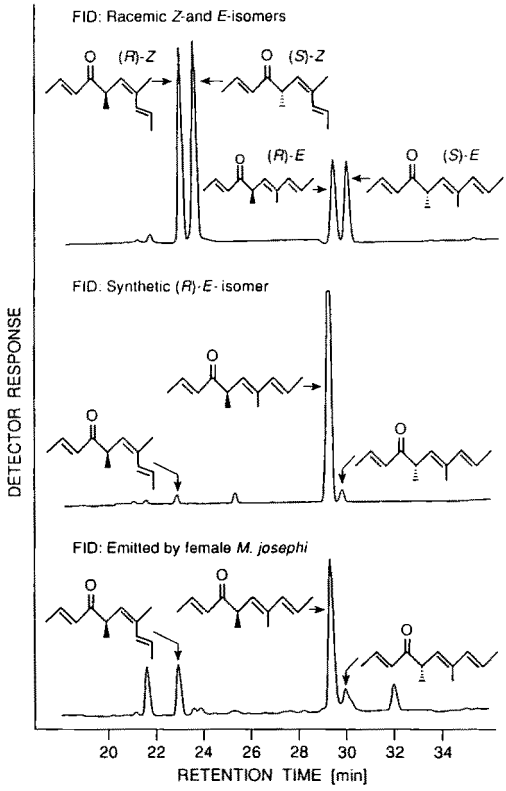

FIG 2. Chiral separation of synthetic and natural *Matsucoccus josephi* sex pheromone stereoisomers (Cyclodex-B column, isothermal at 120°C).

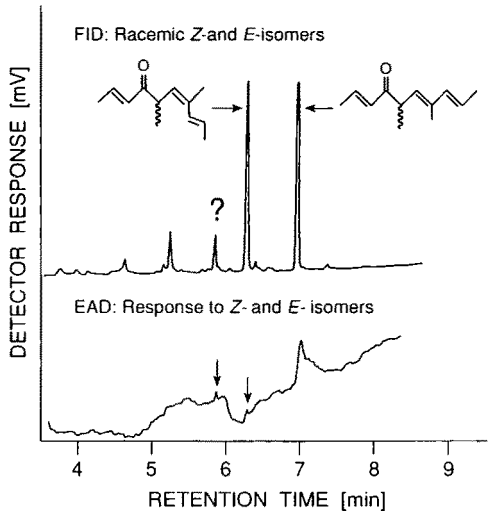

FIG 3. FID and EAD male *Matsucoccus josephi* antenna responses to the racemic *E* and *Z* isomers of the sex pheromone (DB-23 column; 1 min at 100°C, 10°C/min to 200°C).

2,6,8-decatrien-4-one (*R-E*) and (2*E*,5*R*,6*Z*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one (*R-Z*), respectively (Figure 2).

In GC-EAD recordings, male scale antennae elicited potentials in the range of 0.2–0.5 mV. In the analysis of the racemic *E* and *Z* pheromone mixture, the *E* rather than the *Z* isomer elicited a strong antennal response. An additional EAD-active isomer of unknown structure, having an identical mass spectrum to those of the *E* and *Z* isomers, eluted prior to the *Z* isomer (Figure 3). In GC-EAD analysis of the stereoisomeric pheromone mixture on the chiral column, *R-Z* and *S-Z*-enantiomers elicited weak responses, whereas the *R-E* enantiomer evoked a strong antennal response (Figure 4). The latter stereoisomer was also the most EAD-active compound in Porapak-Q extracts of female *M. josephi* volatiles (Figure 4).

Trapping Experiments. The *R-E* enantiomer and the racemic *E/Z* pheromone mixture with an equivalent amount of *R-E* were equally attractive, and they were 10 times as active as the *S-E* enantiomer. These results were obtained for a wide range of dosages (Table 1). The high activity of the *R-E* enantiomer as compared with the *S-E* enantiomer was confirmed in a second field test with 25 μg of each enantiomer. In this experiment, the *R-E* enantiomer attracted 7.2 males/trap/day as compared with only 0.8/trap/day caught with the *S-E* enantiomer. The proportion of *M. josephi* males trapped in *R-E*- and *S-E*-baited traps

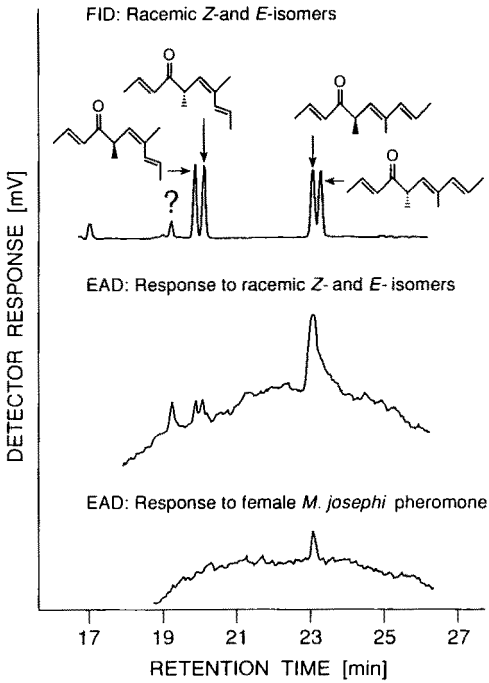

FIG 4. FID and EAD male *Matsucoccus josephi* antenna responses to synthetic pheromone stereoisomers and natural sex pheromone (Cyclodex-B column; isothermal at 135°C).

TABLE 1. CAPTURES OF *Matsucoccus josephi* (Mean Number of Males/Trap/Day) IN TRAPS BAITED WITH RACEMIC PHEROMONE AND *R-E* AND *S-E* ENANTIOMERS[a]

Racemic pheromone, μg, and enantiomers (μg)[b]	Racemate, mixture of 56% *E* + 44% *Z*	Enantiomer	
		R-E	*S-E*
control	0	0	0
10 (2.5)	24.0 a; y	35.0 a; yz	3.2 b; y
25 (6.25)	25.8 a; y	24.0 a; z	3.7 b; y
50 (12.5)	37.9 a; x	51.2 a; xy	3.6 b; y
100 (25)	50.0 a; x	37.7 a; y	4.8 b; xy
200 (50)	56.6 a; x	68.3 a; x	6.9 b; x

[a]Test was conducted April 7-26, 1994.
[b]Means followed by the same letter for rows (a, b) and columns (x, z) are not significantly different at $P < 0.05$ according to Student-Newman-Keuls test.

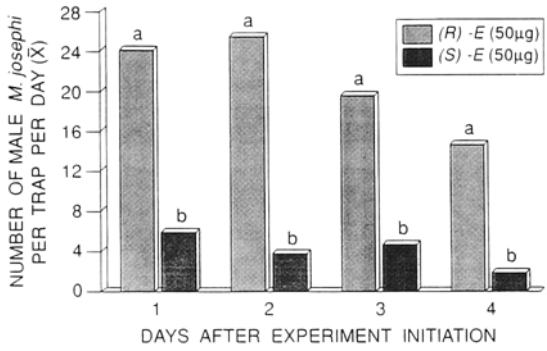

FIG. 5. Captures on four consecutive days of *Matsucoccus josephi* males in traps baited with (*R*)-*E* or (*S*)-*E* enantiomer. Means followed by the same letter are not significantly different at $P < 0.05$ according to Student-Newman-Keuls test.

remained constant during four days (Figure 5), indicating that isomerization of enantiomers in the rubber septa did not occur or was negligible. All pheromone lures also attracted the predator, *E. hebraicus*. Trapping and EAD responses of *E. hebraicus* will be reported separately.

DISCUSSION

The recently identified sex pheromone of *M. josephi* (Dunkelblum et al., 1993) consists of two geometric *E* and *Z* isomers, each with one chiral center of previously undetermined stereochemistry (Figure 1). This chiral center is part of a keto-diene moiety, present also in the pheromones of *M. matsumurae* (Lanier et al., 1989) and *M. feytaudi* (Einhorn et al., 1990) (Figure 6). Stereoselective synthesis, NMR analyses, and bioassays of stereoisomers established the chirality of this center as *R* in both *M. matsumurae* and *M. feytaudi* pheromones (Cywin et al., 1991; Cywin and Kallmerten, 1992; Mori and Harashima, 1993a,b; Jactel et al., 1994). The major pheromone component of *M. feytaudi* has two chiral centers (Figure 6); therefore, four stereoisomers are possible. Synthesis and bioassay of all four stereoisomers (Mori and Harashima, 1993a; Jactel et al., 1994) have revealed that the stereochemistry only of the chiral center in the keto-diene moiety was critical for attractancy. Because *M. matsumurae* and *M. feytaudi* pheromone stereoisomers do not separate on currently available chiral GC columns, it remains unknown whether these female-produced pheromones contain only *R* or also some S enantiomers. Conversely, enantiomers of racemic *M. josephi* pheromone could be readily separated on the

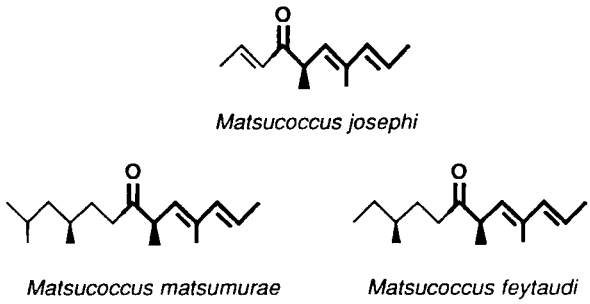

FIG 6. The keto-diene moiety (bold) common to sex pheromones of *Matsucoccus matsumurae*, *Matsucoccus feytaudi*, and *Matsucoccus josephi*.

chiral Cyclodex-B column (Figure 2). When the corresponding *R-E* and *S-E* enantiomers became available (Mori and Amaike, 1994), the chirality of the major female-produced *M. josephi* pheromone could be directly determined as *R* with 10% of the *S* enantiomer (Figure 2).

The use of GC-EAD analyses (Arn et al., 1975) are most efficacious in the identification of insect pheromones and have recently been successfully employed for chiral determination of palm weevil pheromones (Perez et al., 1994). Low responses and enhanced background noise complicated electroantennogram recordings from scale antennae. Moore (1981) obtained EAG responses to crude natural *M. josephi* pheromone (Dunkelblum and Sternlicht, unpublished results) by connecting four male antennae in series for signal amplification. This procedure was particularly difficult with the very fine *M. josephi* antennae and has not been tried since then with other scale insects. No scale antennae have ever been used in coupled GC-EAD analyses. With a GC-EAD system fine-tuned for moth antennae (Gries et al., 1991), and moth and filiform scale antennae being morphologically similar, GC-EAD analysis of *M. josephi* pheromone became possible (Figures 3 and 4). The female-produced *R-E* component elicited the strongest antennal response, whereas the *S-E* antipode was either EAD-inactive or had only slight activity (Figure 4). Interestingly, both *R-Z* and *S-Z* stereoisomers elicited weak but distinct responses (Figure 4).

In field bioassays, the major and most EAD-active (*R-E*) pheromone component was the strongest attractant (Table 1). In a dose–response test it was as attractive as the racemic *E/Z* mixture containing approximately the equivalent amount of the *R-E* stereoisomer (Table 1). Slight to moderate field activity of the *S-E* stereoisomer (Table 1, Figure 5) may be intrinsic or due to its progressive epimerization to the behaviorally very active *R-E* enantiomer. However, similar proportions of males captured in *R-E*- and *S-E*-baited traps during four consecutive days indicated that progressive epimerization did not occur (Figure 5).

Some of the attractancy of the *S-E*-baited traps is certainly due to the trace amounts of *R-E* in the synthetic *S-E*.

It is difficult to decide if the *R-E* enantiomer is the sole component of the *M. josephi* sex pheromone, but it is certainly the major one. The *S-E* enantiomer, present in the pheromone mixture, also has slight activity. Some epimerization during the isolation and purification of the female-released pheromone can not be ruled out. The *Z* component has no activity, and it may have another function. It has been shown previously that large amounts of the nonreactive racemic geometrical *Z* isomer do not interfere with the attractancy of the active *E* isomer (Mendel et al., 1995). Equal reactivity of *R-E* and a racemic mixture, containing the same amount of *R-E*, will facilitate development of a pheromone-based monitoring system for this most serious pest of pine stands in Israel and surrounding countries (Mendel et al., 1994). The lack of inhibition by other stereoisomers that was found in *M. josephi* has also been reported for *M. feytaudi* (Jactel et al., 1994), the California red scale, *Aonidiella aurantii* (Gieselmann et al., 1980), and the white peach scale, *Pseudaulacaspis pentagona* (Heath et al., 1979).

The same keto-diene moiety with the same chiral center and configuration in all three known Matsucoccidae sex pheromones (Figure 6) implies a common biosynthetic pathway and phylogenetic relationship. Pheromone diversification by modification of one chain of the ketonic compounds may have contributed to, or may be associated with, speciation in the Matsucoccidae. It will be interesting to see whether other Matsucoccidae sex pheromones, such as that of the widespread *Matsucoccus pini*, have the same structure frame.

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CHEMICAL NATURE OF LARVAL OSMETERIAL SECRETIONS OF PAPILIONID BUTTERFLIES IN THE GENERA *Parnassius, Sericinus* AND *Pachliopta*

KEIICHI HONDA* and NANAO HAYASHI

Study of Environmental Sciences
Faculty of Integrated Arts and Sciences
Hiroshima University, Higashihiroshima 739, Japan

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Abstract—Volatile components of the larval osmeterial secretion of *Parnassius glacialis* (Parnassiinae, Parnassiini) consisted of isobutyric acid, 2-methylbutyric acid, and their methyl esters. In contrast, the osmeterial exudate of *Sericinus montela* (Parnassiinae, Zerynthiini) was characterized as monoterpene hydrocarbons comprising β-myrcene (Major), α-pinene, sabinene, limonene, and β-phellandrene, whereas that of *Pachliopta aristolochiae* (Papilioninae, Troidini) was composed of numerous sesquiterpene hydrocarbons, including α-himachalene, α-amorphene, and germacrene-A, and a few oxygenated sesquiterpenoids. In these three species, the chemical nature of the secretions of the last and the penultimate instars was essentially of similar quality, suggesting that the three genera, *Parnassius*, *Sericinus*, and *Pachliopta*, are assigned to homogeneous types.

Key Words—Osmeterial secretion, Lepidoptera, Papilionidae, Parnassiinae, Papilioninae, *Parnassius glacialis*, *Sericinus montela*, *Pachliopta aristolochiae*, aliphatic acid and ester, monoterpene, sesquiterpene.

INTRODUCTION

When disturbed, papilionid larvae extrude, from the mid-dorsal intersegmental surface between the head and the prothorax, two-pronged eversible glands (osmeteria) that secrete a characteristic odoriferous liquid. The exocrine secretion is considered to be a chemical weapon to defend themselves against predators, as has been demonstrated by earlier work (Eisner and Meinwald, 1965; Honda, 1983a).

*To whom correspondence should be addressed.

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Chemical investigations on larval osmeterial secretions have been conducted for several genera of papilionid butterflies. Isobutyric and 2-methylbutyric acids, and their methyl and ethyl esters are known to be major components secreted by the larvae of *Graphium* (Honda, 1980b) and by the last (fifth) instar of *Papilio* species (Crossley and Waterhouse, 1969; López and Quesnel, 1970; Honda, 1980a, 1981; Burger et al., 1985). The two acids are also encountered in the secretions of *Baronia* and *Eurytides* larvae (Eisner et al., 1970). In contrast, various mono- and/or sesquiterpenic compounds have been identified from *Battus* (Eisner et al., 1971), *Luehdorfia* and *Atrophaneura* larvae (Honda, 1980b), and from the young larvae of *Papilio* species (Honda, 1980a, 1981). Recent reports have described the presence of a few aristolochic acid analogs in the osmeterial exudates of *Atrophaneura*, *Troides*, and *Pachliopta* (Nishida and Fukami, 1989; Nishida et al., 1993).

The derivation of such secretions has also been studied, and it has been substantiated in several species of *Papilio* and *Luehdorfia* that larvae are able to biosynthesize de novo aliphatic acids from amino acids (Seligman and Doy, 1973) and terpenoids from acetate precursors (Honda, 1983b, 1990). However, the chemical properties of osmeterial secretions of many papilionid genera still remain unknown. We wish to report here on the volatile constituents of osmeterial secretions of the last and the penultimate larval instars of three species belonging to distinct genera, *Parnassius* (Parnassiini), *Sericinus* (Zerynthiini), and *Pachliopta* (Troidini).

METHODS AND MATERIALS

Insects. Eggs of *Parnassius glacialis* (a univoltine species), obtained from adult females collected in Kanagawa prefecture, were maintained outdoors until hatching. The larvae were raised on a papaveraceous host plant, *Corydalis incisa*, under a 16L:8D photoperiod throughout the larval development at 15°C during scotophase and 20°C during photophase. Larvae of *Sericinus montela* and *Pachliopta aristolochiae*, which had originated from Kanagawa and Okinawa populations, respectively, were reared at 25°C on a 16L:8D regime. Larvae of *S. montela* were fed with *Aristolochia debilis* (Aristolochiaceae), and those of *P. aristolochiae* with *A. kaempferi*.

Collection of Secretions. The secretion was milked separately from fourth (third for *P. glacialis*) and fifth (fourth for *P. glacialis*) instars of respective species on the third day of larval ecdysis according to the method of Honda (1980a). Each sample for chemical analyses (dichloromethane solution of osmeterial exudate) was prepared from 20–30 larvae and was directly subjected to subsequent examinations.

Chemical Analyses of Volatile Components. The secretion components were

examined by GC and GC-MS. The major part of these analyses was carried out with a fused-silica capillary column (0.25 mm ID × 50 m) chemically bonded with FFAP (column A). Another column of similar dimensions bonded with OV-101 (column B) was also utilized when necessary. GC analyses were conducted with a HP 5890A gas chromatograph equipped with a flame-ionization detector. The injection temperature was 200°C, and the oven temperature was programmed from 50 to 180°C (or 200°C) at 5°C/min. The flow rate of carrier gas (He) was regulated at 1 ml/min. Identification of the components was based on comparison of GC retention data and mass spectra (EI mode) with those of authentic compounds, unless otherwise noted. Mass spectra were recorded at an ionization potential of 25 eV on a Jeol JMS-D300 mass spectrometer interfaced to a Jeol JGC-20KP gas chromatograph. CI mass spectra (reactant gas: isobutane) were also recorded for several constituents to confirm their molecular weight. The percentage composition of each component was calculated by estimating its peak area vs. total peak area on gas chromatograms.

RESULTS

Larvae of *P. glacialis* possess relatively short osmeteria, yellow in color, that secrete a colorless liquid with a slightly offensive odor. Larval osmeteria of both *S. montela* and *P. aristolochiae* are also short and yellow to orange in color. Unlike *P. glacialis*, their secretions are conspicuously colored orange and give out a faint odor. The secretions were found to contain pigmented substance(s) that were sparingly soluble in dichloromethane and appeared not to be amenable to GC. Such substance(s), therefore, were not pursued further in this study.

P. glacialis. GC analysis of the secretion of fourth (last) instar on column A showed the presence of three major components (peaks 2–4, Figure 1). They were assumed to be two aliphatic acids of low molecular weight and a methyl ester of one of the acids from the following mass spectral information. Relative intensity (%) of each ion is given in parentheses except for QM^+ in CI-MS measurement: peak 2: M^+, *m/z* 116(1) and *m/z* 101(18), 88(68), 85(23), 69(7), 59(22), 57(100), and 41(47); peak 3: M^+, *m/z* 88(5) and *m/z* 73(25), 45(8), 43(100), and 41(40); and peak 4: QM^+, *m/z* 103 and *m/z* 87(23), 74(100), 57(75), 45(10), and 41(60). Peaks 2–4 were thus identified as methyl 2-methylbutyrate, isobutyric acid, and 2-methylbutyric acid, respectively. Further GC examination of the acid-free fraction of this sample on column B disclosed an additional component that had overlapped the solvent peak when analyzed on column A. This compound (peak 1), giving M^+ at *m/z* 102(12) and other diagnostic fragment ions at *m/z* 87(21), 71(44), 59(32), 43(100), and 41(21), was readily determined to be methyl isobutyrate. In contrast, from the secretion of

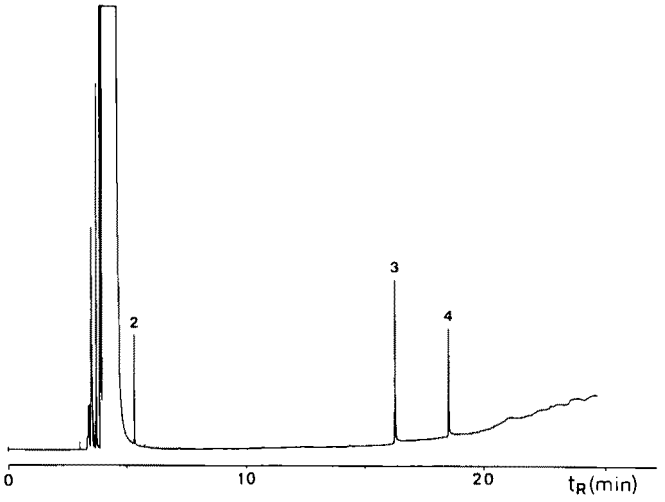

FIG. 1. Gas chromatogram of the osmeterial secretion of fourth (last) instars of *Parnassius glacialis* on a FFAP fused-silica capillary column; 50–180°C at 5°C/min.

third (penultimate) instar only two components were detected, which, upon examination by a similar method, turned out to be isobutyric and 2-methylbutyric acids. Although *P. glacialis* larvae may secrete other substances in trace amounts (Figure 1), these were not examined further because of the meager quantity of osmeterial fluid secreted. The results are summarized in Table 1.

S. montela. The osmeterial exudate of fifth instars was found to contain a major (peak 3) and four minor (peaks 1, 2, 4 and 5) components (Figure 2),

TABLE 1. VOLATILE COMPONENTS OF LARVAL OSMETERIAL SECRETION OF *Parnassius glacialis*

Peak[a]	Component	Composition (%)	
		Third instar	Fourth instar
1	Methyl isobutyrate		69.5
2	Methyl 2-methylbutyrate		6.5
3	Isobutyric acid	64.2	15.0
4	2-Methylbutyric acid	35.8	9.0

[a]Refer to Figure 1 (peak 1 is superimposed upon the solvent peak).

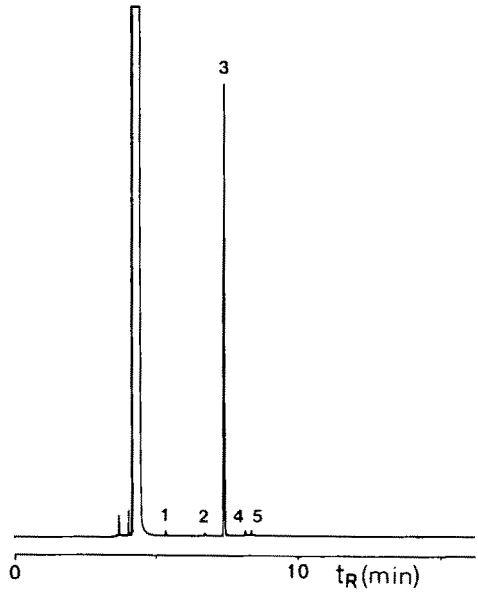

FIG 2. Gas chromatogram of the osmeterial secretion of fifth instars of *Sericinus montela* on a FFAP fused-silica capillary column; 50–180°C at 5°C/min.

and the gas chromatogram of the secretion of fourth instar showed a very similar profile to that of fifth instar. Peak 3, exhibiting a mass spectral pattern characteristic of a monoterpene hydrocarbon [M^+, *m/z* 136(6) and *m/z* 121(4), 93(100), 79(11), 77(10), 69(82), 41(83)], was identified as β-myrcene. All of the other components were also assumed to be monoterpene hydrocarbons from their mass spectral and GC data: peak 1: M^+, *m/z* 136(14) and *m/z* 121(17), 107(5), 105(10), 93(100), 91(25), 79(14), 77(18), and 41(5); peak 2: M^+, *m/z* 136(18) and *m/z* 121(5), 93(100), 91(25), 79(19), 77(31), 69(10), and 41(15); peak 4: M^+, *m/z* 136(31) and *m/z* 121(22), 107(20), 93(58), 79(19), 77(10), 68(100), 53(12), and 41(9); and peak 5: M^+, *m/z* 136(22) and *m/z* 121(6), 93(100), 91(25), 79(15), 77(26), and 41(8). These compounds were identified as α-pinene, sabinene, limonene, and β-phellandrene, respectively. The chemical nature of fourth instar secretion proved to be qualitatively the same as that of fifth instar as shown in Table 2.

P. aristolochiae. The secretion of fifth instar presented a complicated GC profile, consisting of more than 40 constituents (Figure 3). Their mass spectra suggested that they were all sesquiterpene hydrocarbons or oxygenated sesquiterpenes. Peak f, giving M^+ at *m/z* 204(30) and other salient ions at *m/z* 189(40),

TABLE 2. VOLATILE COMPONENTS OF LARVAL OSMETERIAL SECRETION OF *Sericinus montela*

Peak[a]	Component	Composition (%)	
		Fourth instar	Fifth instar
1	α-Pinene	0.7	0.7
2	Sabinene	0.5	0.5
3	β-Myrcene	97.1	97.2
4	Limonene	0.8	0.8
5	β-Phellandrene	0.9	0.8

[a]Refer to Figure 2.

161(37), 134(33), 133(31), 119(67), 105(59), 93(100), 79(63), 69(54), 55(38), and 41(74), and peak h, M^+, *m/z* 204(25) and *m/z* 189(10), 161(48), 133(15), 119(28), 105(100), 94(53), 93(49), 81(23), 55(15), and 41(26), were concluded to be α-himachalene and α-amorphene, respectively, upon comparison with published mass spectra (Stenhagen et al., 1974) and retention indices (Andersen

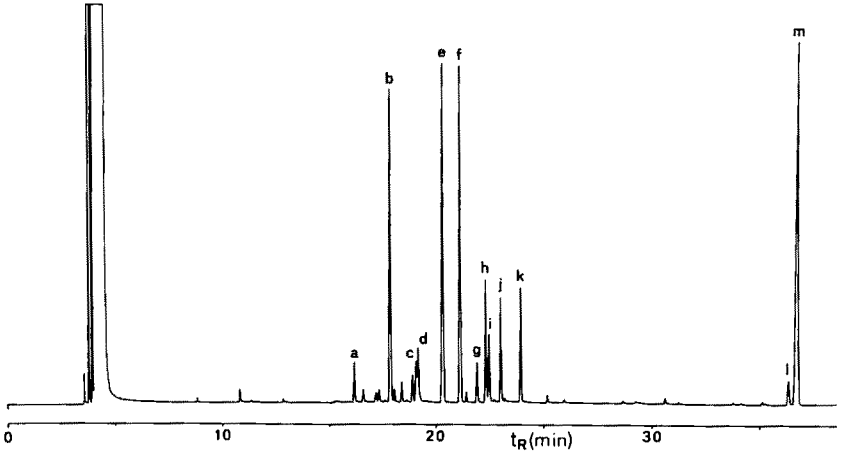

FIG 3. Gas chromatogram of the osmeterial secretion of fifth instars of *Pachliopta aristolochiae* on a FFAP fused-silica capillary column; 50–200°C at 5°C/min.

and Falcone, 1969). The mass spectrum of peak k, M^+, *m/z* 204(2) and *m/z* 189(8), 161(9), 147(19), 121(27), 107(35), 93(64), 81(50), 68(100), 53(36), and 41(55), was quite similar to that of germacrene-A previously reported from the osmeterial secretions of *Papilio* larvae (Honda, 1980a, 1981). Thus, comparison of GC retention data corroborated the identity of peak k with germacrene-A.

Attempts at structural elucidation of other components were unsuccessful; however, other peaks whose relative compositions exceeded 1.0% gave the following molecular ion and base peak: a, 204, and 119; b, 204 and 107; c, 204 and 41; d, 204 and 161; e, 204 and 93; g, 204 and 107; i, 202 and 132; j, 204 and 161; l, 220 and 159; m, 220 and 159. For the molecular weight of 204, which corresponds to representative sesquiterpene hydrocarbons, the molecular formula is assignable to $C_{15}H_{24}$. Likewise, formulae of $C_{15}H_{22}$ and $C_{15}H_{24}O$ seem relevant for 202 and 220, respectively. The secretion of fourth instar showed substantial similarities in both quality and relative quantity to that of fifth instar. The relative proportion of selected components (more than 1.0%) is given in Table 3.

TABLE 3. VOLATILE COMPONENTS OF LARVAL OSMETERIAL SECRETION OF *Pachliopta aristolochiae*

Peak[a]	Component[b]	Composition (%)	
		Fourth instar	Fifth instar
a	$C_{15}H_{24}$	1.1	1.4
b	$C_{15}H_{24}$	9.7	11.7
c	$C_{15}H_{24}$	1.3	1.7
d	$C_{15}H_{24}$	1.9	2.3
e	$C_{15}H_{24}$	13.6	15.9
f	α-Himachalene	12.7	14.5
g	$C_{15}H_{24}$	1.5	1.7
h	α-Amorphene	4.7	4.5
i	$C_{15}H_{22}$	3.1	2.7
j	$C_{15}H_{24}$	6.0	3.8
k	Germacrene-A	3.9	4.3
l	$C_{15}H_{24}O$	1.9	1.7
m	$C_{15}H_{24}O$	34.4	26.8

[a]Refer to Figure 3.
[b]Components less than 1.0% of the total are not listed.

DISCUSSION

In each of the three species presently studied, belonging to different genera, the osmeterial exudates of the last instar were very similar in both quality and quantity to those of the penultimate instar. Although we investigated only one species for each genus, our results strongly suggest that the three genera, *Parnassius* (Parnassiinae, Parnassiini), *Sericinus* (Parnassiinae, Zerynthiini), and *Pachliopta* (Papilioninae, Troidini) are of homogeneous types as are *Luehdorfia* (Parnassiinae, Zerynthiini), *Graphium* (Papilioninae, Graphiini), and *Atrophaneura* (Papilioninae, Troidini) (Honda, 1980b).

The secretion of *S. montela* larvae qualitatively resembles that of *Luehdorfia* larvae in that both species secrete only monoterpene hydrocarbons with β-myrcene as a predominant component (Honda, 1980b). This conforms well to the acknowledged taxonomic disposition of the two genera (tribe Zerynthiini). The larval secretion of *P. aristolochiae* was chemically marked by sesquiterpenes, and previous studies have documented that the osmeterial secretions of *Battus polydamas* (Eisner et al., 1971) and *A. alcinous* (Honda, 1980b) are composed exclusively of sesquiterpenes. The genus *Pachliopta* is considered to be closely related to the genus *Battus* and subgenus *Atrophaneura* (tribe Troidini) (Miller, 1987). The present study provides further evidence for close relationships among these taxa. A subsequent reexamination of the osmeterial secretion of *A. alcinous* revealed the presence of a small amount of germacrene-A (Honda, unpublished). Therefore, *P. aristolochiae* and *A. alcinous* share α-himachalene and germacrene-A, although these compounds are minor constituents of *A. alcinous*. As shown by these findings, comparative studies on the chemistry of larval osmeterial secretion, which seems to reflect phylogenetic relationships among species, would permit chemotaxonomic approaches to the systematics of the Papilionidae.

On the other hand, *P. glacialis* secreted compounds quite different from those of *S. montela*, which is classified in the same subfamily (Parnassiinae). The secretion of *P. glacialis*, containing aliphatic acids and esters, is extremely similar in quality to those of *Graphium* species (Honda, 1980b). In addition, both genera are of homogeneous types. Taking account of the cladistic relationships among tribes in the Papilionidae proposed by Miller (1987), a feasible explanation for such similarity between the two distant genera is that it might perhaps be a result of parallel cladogenesis. However, this hypothesis requires careful and comprehensive verification.

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PUTATIVE CHEMICAL SIGNALS FROM WHITE-TAILED DEER (Odocoileus virginianus). URINARY AND VAGINAL MUCUS VOLATILES EXCRETED BY FEMALES DURING BREEDING SEASON

B. JEMIOLO,[1,*] K.V. MILLER,[2,**] D. WIESLER,[1] I. JELINEK,[1] M. NOVOTNY,[1] and R.L. MARCHINTON[2]

[1]*Department of Chemistry*
Indiana University
Bloomington, Indiana 47405

[2]*D.B. Warnell School of Forest Resources*
University of Georgia
Athens, Georgia 30602

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Abstract—Urine and vaginal mucus samples from female white-tailed deer in estrus and mid-cycle were analyzed by combined gas chromatography–mass spectrometry (GC-MS). Forty-four volatiles were found in mucus and 63 in urine. The volatiles common to both vaginal mucus and urine included alcohols, aldehydes, furans, ketones, alkanes, and alkenes. Aromatic hydrocarbons were present only in the vaginal mucus, whereas pyrans, amines, esters, and phenols were found only in urine. Both estrous mucus and estrous urine could be identified by the presence of specific compounds not present in mid-cycle samples. Numerous compounds exhibited dependency on ovarian hormones.

Key Words—Chemosignals, deer, *Odocoileus virginianus*, urine, vaginal mucus, volatiles.

INTRODUCTION

White-tailed deer (*Odocoileus virginianus*) have a complex scent communication system that is insufficiently understood at present. Whitetails form two basic types of social units, doe (matriarchal) groups and buck groups. Usually there

*Present address: Department of Biology, Ball State University, Muncie, Indiana 47306.
**To whom correspondence should be addressed.

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is little or no contact between adult does and adult bucks except during the autumn breeding season (Marchinton and Hirth, 1984). Various aspects of marking behavior performed by males during the breeding season have been extensively examined (Moore and Marchinton, 1974; Miller et al., 1987a,b; Marchinton et al., 1990). White-tailed deer behavior suggests that urine could be a source of chemical signals indicating estrous status of the female (Moore and Marchinton, 1974). Female urine elicits a flehmen response from males. Additionally, Whitney et al. (1992) have presented evidence that vaginal mucus or other secretions from the reproductive tract are the source of a signal(s) indicating female reproductive status. They argue that urinalysis via vomeronasal stimulation likely affects the animal's reproductive physiology, whereas volatiles from the reproductive tract that are detected by the main olfactory system elicit approach and courtship behaviors.

Clearly, chemosignals from urine and vaginal secretion play important roles in the reproductive physiology of white-tailed deer. In this study we have investigated the urinary and vaginal mucus volatile compounds excreted by female white-tailed deer during the breeding season. Qualitative changes and concentrations of volatiles at estrus and mid-cycle as well as concentration changes in relation to the presence of ovarian hormones were investigated.

METHODS AND MATERIALS

Subject and Sample Collection. Samples of urine and vaginal mucus were collected from four adult (≥ 2 years old) female deer, housed in individual stalls (3×6 m) at the University of Georgia's Whitehall Deer Research Facility. Females were fed ad libitum a commercial feed (Omolene 300, Purina Mills, Inc.). Bermuda grass (*Cynodon dactylon*) or alfalfa (*Medicago sativa*) hay was also provided ad libitum.

Voided urine and vaginal secretions from estrous and mid-cycle females and urine from ovariectomized females were collected during the breeding season (i.e., October–January). Each female was allowed to associate daily with a mature, sexually experienced, epididymectomized male. Behavioral patterns of white-tailed deer indicative of estrus have been described by Warren et al. (1978) and Brown and Hirth (1979). Estrus was identified as the day(s) on which the male displayed courtship behaviors toward the female and the female was receptive to mounting by the male. Mid-cycle was identified as the midpoint between consecutive periods of estrus during the 26-day estrous cycle (Knox et al., 1988).

Mucus was collected by flushing the vagina/cervical area with 10 ml of sterile physiological saline. Voided urine was collected from four intact and three ovariectomized females. Studied animals were sedated using a mixture of

xylazine hydrochlorine and ketamine hydrochloride as described by Mech et al. (1985) and placed in a modified transport crate fitted with a screened bottom. Anesthesia was reversed with yohimbine hydrochloride and the females were held in the crate until urination. The mean time until urination was 3 hr 10 min. Immediately after urine and mucus collection, three 1.5-ml samples of each type were frozen and stored at −20°C until analyzed.

Procedure. Urinary and vaginal mucus volatiles were concentrated on porous polymer, Tenax GC, using the modified headspace technique developed by Novotny et al. (1974) and McConnell et al. (1979). To give each sample (1.5 ml) the same high ionic strength, 0.4 g of $(NH_4)_2SO_4$ was added. Next, the sample was absorbed on 0.6 g of glass wool. Purified helium gas at a flow of 30 ml/min was then passed through the glass wool for 30 min to purge the volatiles from it. The headspace sampling was performed at 50°C. After passing through a cooled condenser, installed to reduce water mist originating from the sample, the volatiles were trapped on a 4-mg Tenax GC precolumn packed into the injection port glass liner of a gas chromatograph.

After the headspace sampling procedure, the precolumn with trapped volatiles was inserted into the modified injection port of a Hewlett-Packard model 5980A gas chromatograph–mass spectrometer (GC-MS). The volatiles were desorbed at 200°C and retrapped cryogenically for 15 min to compress the desorbed sample at the column inlet. The column used was a glass capillary column (30 m × 0.25 mm ID) that had been statically coated with UCON-5-HB-200 (Supelco Inc., Bellefonte, Pennsylvania). Column temperature was programmed from 30 to 160°C at a rate of 2°C/min. Volatile constituents were identified through their mass spectra, using electron impact ionization at 70 eV. Peak areas were evaluated using a GC-MS data station. The identification of volatile components was verified by retention time measurements of authentic compounds.

Statistical Analysis. Statistical comparison of the concentration of excreted volatiles (expressed by integrated peak areas) was performed by using one-way analysis of variance (F at $P < 0.05$) with the Tukey test for equal sample size ($P < 0.02$). Because of the heteroscedastic data, the inequality of variance was "corrected" by logarithmic transformation of the original X values (integrated peak areas in arbitrary units) to $X' = \log X + 1$ values (Zar, 1984).

RESULTS AND DISCUSSION

Headspace analysis of the volatile compounds from the test materials revealed a large number of common compounds. Some varied considerably in concentration among samples. Others, present at constant levels, commonly

accounted for more than half of the total number of compounds observed in the samples. Only a few tended to peak consistently in relation to reproductive state and hormonal change.

Volatiles identified in both vaginal mucus and urine of female deer include alcohols, aldehydes, furans, ketones, alkanes, and alkenes. Pyrans, amines, esters, and phenols were present in the urine but not in the vaginal mucus. Conversely, aromatic hydrocarbons were characteristic of the vaginal mucus only. Several classes of volatiles that we observed in white-tailed deer have been reported from the urine of other mammals including mice, voles, and humans (Albone, 1984; Novotny et al., 1990).

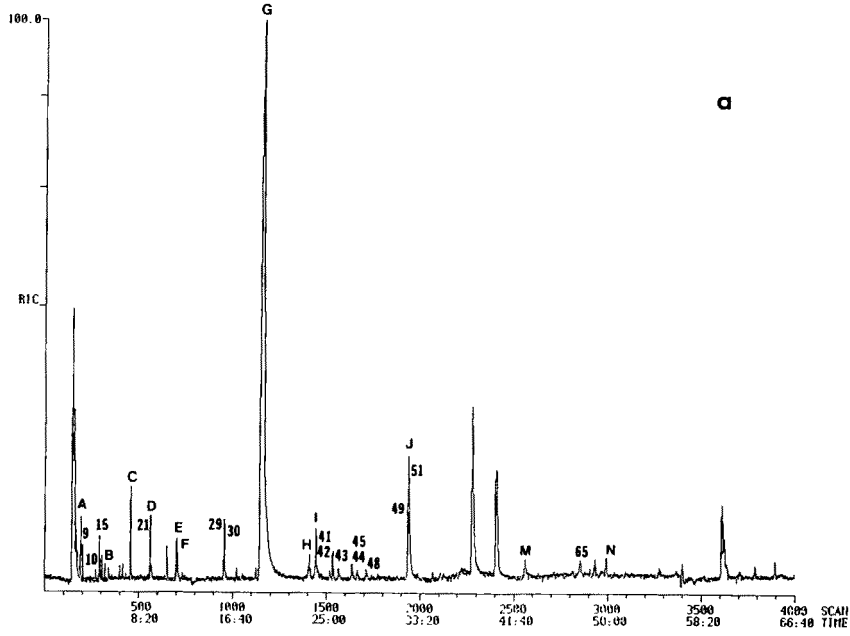

FIG. 1. GC-MS profiles of volatile compounds from (a) mucus and (b) voided urine of white-tailed deer females. (a) Not all peaks representing the identified compounds were numbered. The numbers in Tables 1 and 2 correspond to the numbers in the chromatogram. The peaks marked by letters represent compounds characteristic for estrous mucus only. The numbered peaks represent compounds common for mucus and urine. (b) Not all peaks representing the identified compounds were numbered. The numbers in Tables 3 and 4 correspond to the numbers in the chromatogram. All numbered peaks in the chromatogram represent ovary-dependent compounds. The numbered peaks marked additionally with filled dots represent compounds characteristic for estrous urine only.

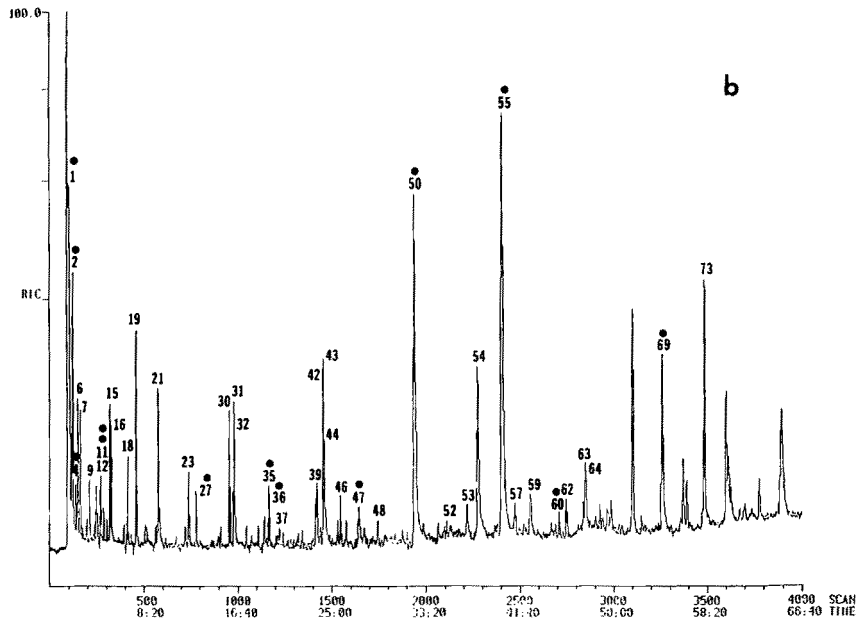

FIG. 1. Continued

The GC-MS recording shown in Figure 1a is typical of the volatile profiles obtained from the vaginal mucus of estrous females. Forty-four volatiles (Tables 1 and 2 and Table 5 below) occurred in vaginal mucus in measurable quantities; the majority of them were tentatively identified. Fourteen of these compounds, however, were found to be characteristic of the vaginal mucus only (Table 1). The composition of these characteristic compound mixtures depended upon the phase of the females' reproductive cycle. Estrous females discharged a mucus that was richer in the characteristic volatiles than that of the females in mid-cycle (Table 1). Nine of the 14 characteristic compounds (64%) were produced only by estrous females, whereas only two (14%) were produced by the females exclusively during mid-cycle. Certain aromatic hydrocarbons, whose presence may or may not be significant, along with two alcohols (2-propanol and 2-propyl-1-decanol) and two ketones (2-hexanone and methylacetophenone) occurred only in estrous vaginal samples (Table 1). Since estrous vaginal secretion, but not mid-cycle secretion, has been shown to elicit courtship behavior in male white-tailed deer (Whitney et al., 1992; Murphy et al., 1994), these compounds may represent behaviorally important chemosignals. Behavioral

TABLE 1. VOLATILE COMPOUNDS CHARACTERISTIC OF VAGINAL MUCUS

Class of compounds	Peak number	Structure	Integrated peak areas (mean ± SEM)[a]	
			Estrus	Mid-cycle
Alcohols	A	2-propanol	0.78 ± 0.04	0[b]
	B	2-butanol	1.83 ± 0.01	1.82 ± 0.01
	I	cyclohexanol	2.77 ± 0.02	2.97 ± 0.01
	K	M.W. 156	0	2.75 ± 0.01
	M	2-propyl-1-decanol	1.88 ± 0.01	0
Aromatic hydrocarbons	D	*p*-xylene	0.50 ± 0.00	0
	E	*m*-xylene	1.61 ± 0.01	0
	F	*o*-xylene	1.47 ± 0.06	0
	H	phenylacetylene	0.71 ± 0.02	0
	J	indene	1.11 ± 0.02	0
Furans	L	2-acetyl-5-ethylfuran	0	2.01 ± 0.04
Ketones	C	2-hexanone	1.35 ± 0.05	0
	G	cyclohexanone	4.60 ± 0.00	4.74 ± 0.02
	N	methylacetophenone	0.39 ± 0.04	0

[a]The value of integrated peak areas X (in arbitrary units) was transformed to $X' = \log (X + 1)$.
[b]Compound not present in the sample even in trace quantities.

experiments on these compounds should be conducted to evaluate their role, if any, in white-tailed deer communication.

Three of the 14 characteristic compounds occurred at similar levels in both estrous and mid-cycle mucus samples. These included two alcohols (2-butanol, cyclohexanol) and a ketone (cyclohexanone). Interestingly, cyclohexanone was present in both estrous and mid-cycle samples and at far higher concentration (40–92%, $P < 0.02$), than other compounds (Table 1).

The remaining 30 vaginal mucus volatiles also were found in urine samples (Table 2 and Table 5 below). Concentration of these compounds did not vary significantly between estrus and mid-cycle urines ($P > 0.05$; Table 2 shows the mean urinary value for each compound). In the mucus, 15 of these compounds varied depending on whether the mucus was discharged by estrous or mid-cycle females and tended to be lower than in the urine (Table 2). These volatiles included alcohol, aldehyde, alkane, and ketone structural classes, and all were ovarian hormone-dependent. Concentrations of 11 compounds in the estrous mucus were lower ($P < 0.02$) when compared to mid-cycle mucus, but concentration of only four mid-cycle mucus compounds was lower ($P < 0.02$) when compared to estrous mucus (Table 2). Only 1-octen-3-ol (peak 51) from

TABLE 2. DIFFERENCES IN CONCENTRATION OF VOLATILES COMMON FOR MUCUS AND URINE

Class of compounds and peak number	Structure	Integrated peak areas (mean ± SEM)[a]		
			Mucus	
		Urine[b]	Estrus	Mid-cycle
Alcohols				
44[c]	3-methyl-1,3-butanediol	1.99 ± 0.01b	0.86 ± 0.13c	2.83 ± 0.02a
51	1-octen-3-ol	1.67 ± 0.03c	2.15 ± 0.19a	1.87 ± 0.01b
65	alpha-terpineol	2.44 ± 0.01a	1.39 ± 0.01b	2.13 ± 0.01a
Aldehydes				
15[c]	valeraldehyde	2.20 ± 0.02a	1.04 ± 0.02c	1.39 ± 0.01b
21	hexanal	2.70 ± 0.00a	2.42 ± 0.02a	1.74 ± 0.05b
30	heptanal	2.72 ± 0.01a	1.99 ± 0.00b	2.69 ± 0.00a
41	octanal	3.67 ± 0.01a	2.41 ± 0.04c	2.68 ± 0.02b
49	nonanal	4.62 ± 0.04a	3.01 ± 0.01c	3.50 ± 0.02b
Alkanes				
9[c]	octane	2.25 ± 0.00a	1.09 ± 0.01c	1.41 ± 0.04b
10	1,2,4-trimethyl-cyclopentane	2.80 ± 0.04a	1.75 ± 0.01c	2.00 ± 0.01b
45	dodecane	2.26 ± 0.02a	2.12 ± 0.01a	1.02 ± 0.01b
Ketones				
29	2-heptanone	1.99 ± 0.02a	0.57 ± 0.01c	0.89 ± 0.03b
42[c]	7-methyl-4-octanone	1.90 ± 0.01a	1.39 ± 0.03b	1.00 ± 0.01c
43	6-methyl-5-hepten-2-one	2.41 ± 0.01a	1.25 ± 0.01b	1.45 ± 0.02b
48[c]	2-nonanone	1.65 ± 0.01a	0.45 ± 0.00c	0.72 ± 0.01b

[a] The value of integrated peak areas X (in arbitrary units) was transformed to $X' = \log(X + 1)$. Means in the same row not followed by the same letter are significantly different [$F(2,6)$; $P < 0.02$)].

[b] Concentration of the urinary compounds listed in this column did not vary significantly between estrus and mid-cycle samples ($P > 0.05$); thus for comparison of urine with mucus samples, the mean urinary value was used for each compound.

[c] These volatiles were not present in the urine of ovariectomized females even in trace quantities.

estrous mucus and 3-methyl-1,3-butanediol (peak 44) from mid-cycle mucus were significantly greater (45% and 43%, respectively; $P < 0.02$) in concentration than urinary level (Table 2).

In addition to the 15 volatiles previously described, common for mucus and urine (Table 2), we found 33 more compounds that were consistently present only in urine (Tables 3 and 4). The GC-MS recording shown in Figure 1b is typical of the volatile profiles obtained from estrous female urine. Of the total 63 investigated urinary compounds (Tables 2–5), 46 occurred in both reproductive stages; 13 were present in estrous urine only and four in mid-cycle samples

TABLE 3. VOLATILE COMPOUNDS[a] CHARACTERISTIC OF URINE

Class of compounds	Peak number	Structure	Integrated peak areas (mean ± SEM)[b] Estrus	Mid-cycle
Alcohol	50	2-octanol	3.92 ± 0.00	0[c]
Aldehydes	4	acrolein	0.48 ± 0.00	0
	55	2-nonenal	4.49 ± 0.00	0
	69	dodecanal	2.44 ± 0.00	0
Alkanes	1	hexane	2.16 ± 0.01	0
	2	2-methylhexane	1.77 ± 0.01	0
	27	identity uncertain	1.21 ± 0.01	0
Alkenes	11	4-octene	0.91 ± 0.01	0
	12	MW 112	1.26 ± 0.01	0
	35	limonene	1.59 ± 0.01	0
	47	identity uncertain	1.95 ± 0.01	0
Furans	36	2-pentylfuran	1.65 ± 0.01	0
	66	2-(4-hydroxybutyryl) furan	0	1.75 ± 0.01
Ketones	56	acetophenone	0	0.75 ± 0.01
	60	2-cyclohexenone	1.33 ± 0.01	0
Pyran	3	2-methyl-2-3-dihydropyran	0	2.28 ± 0.01
Unidentified	58	identity uncertain	0	2.43 ± 0.01

[a] Urinary volatiles listed in this table were not present in the urine of ovariectomized females even in trace quantities.
[b] The value of integrated peak areas X (in arbitrary units) was transformed to $X' = \log (X + 1)$.
[c] Compound not present in the sample even in trace quantities.

only (Table 3). Volatiles occurring exclusively in estrus urine represented a variety of chemical groups including alcohols, aldehydes, alkanes, alkenes, furans, and ketones. Those characteristic of mid-cycle urine belonged to the furan, ketone, and pyran groups (Table 3).

Many of the urinary compounds appeared to be hormone-dependent. Thirty-eight of 63 compounds (60%) were absent, not found even in trace quantities, from urine of ovariectomized females (Tables 2–4). The only compounds with significantly greater concentration in ovariectomized females were an alcohol [1-octen-3-ol (peak 51)] and an alkane [1,2,4,-trimethylcyclopentane (peak 10)]. Those compounds are common for both mucus and urine samples (Table 2).

Among the volatiles identified in mucus (total 44) and urine (total 63), several compounds were present in all investigated samples. Concentrations of these volatiles were similar in all samples (i.e., 1.24–2.73 log of integrated peak areas in arbitrary units), were relatively high, and were not dependent on ovarian hormones or animals' reproductive stage (Table 5).

TABLE 4. OVARY-DEPENDENT URINARY VOLATILES[a]

Class of compounds	Peak number	Structure
Alcohols	31	1-penten-3-ol
	46	1-hexanol
	52	2-octen-1-ol
	62	a monoterpene alcohol
Aldehydes	6	methacrolein
	7	butyraldehyde
	57	methylbenzaldehyde
	63	undecanal
	64	2,4-nonadienal
Alkanes	23	identity uncertain
	59	tetradecane
Alkene	18	identity uncertain
Ketones	32	6-methyl-2-heptanone
	39	2-octanone
Phenol	73	p-ethylphenol
Unidentified	37	identity uncertain

[a]Urinary volatiles listed in this table were not present in the urine of ovariectomized females even in trace quantities.

TABLE 5. VOLATILE COMPOUNDS COMMON TO BOTH VAGINAL MUCUS AND URINE SAMPLES[a]

Class of compounds	Peak number	Structure
Alkenes	67	pentadecane
	70	hexadecane
	72	heptadecane
Amine	68	*N*-ethylaniline
Ester	71	propylene dibutyrate
Ketones	13	2-pentanone
	14	3-pentanone
	17	2,3-pentanedione
	20	2-hexanone
	24	cyclopentanone
	25	4-heptanone
	28	3-methylcyclopentanone
	34	2-methyl-4-heptanone
	38	3-ethylcyclopentanone
	61	phenylacetone

[a]They demonstrated relatively constant concentration in samples of all investigated females.

Limited research has been conducted on the urinary volatiles of cervids. Bakke and Figenschou (1990) reported 70 volatile substances in adult red deer (*Cervus elaphus elaphus*) from gas chromatography of a methylated acidic fraction of urine, a technique different from the methods used in the present study. Their substances were mainly carboxylic acids and their derivatives, in addition to some aromatic compounds. They did not observe any systematic variation in volatile excretion during different seasons. In black-tailed deer (*O. hemionus hemionus*), Muller-Schwarze et al. (1978) presented evidence that a urinary γ-lactone, (Z)-6-dodecen-4-olide, is involved in social communication. Thus, the results presented here provide additional chemical background information for the future testing of animal behavior.

CONCLUSIONS

Certain compounds present in vaginal mucus are not paralleled in the urine of white-tailed deer. In addition, several compounds found in estrous mucus were not found in mid-cycle samples. Other volatiles were found in both urine and vaginal mucus, but their concentration tended to be lower in mucus samples. These overlap compounds found in both mucus and urine (for both phases of the female reproductive cycle) suggest that some compounds in mucus sample may have originated from urine contamination.

Our study documents for the first time that: (1) the composition of volatiles in white-tailed female deer changes according to the stage of the reproductive cycle; (2) estrous mucus and urine have more characteristic compounds (9 and 13, respectively) then mid-cycle mucus and urine samples (2 and 4, respectively), and these compounds may be used to identify a particular stage of the deer's reproductive cycle; and (3) numerous urinary compounds exhibit strong dependence on ovarian hormones.

Further biological and behavior research is needed to determine which chemical compounds identified in this study (if any) are important in communicating sexual/reproductive status.

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Editors' Note

The papers in this issue were presented at the 11th Annual Meeting of the International Society of Chemical Ecology, Inc. (ISCE), held in Syracuse, New York, June 4–8, 1994. The meeting was organized and hosted by R.M. Silverstein, John Simeone, and Francis X. Webster, and it marked the 20th anniversary of the founding, and editorship, of the *Journal of Chemical Ecology* by Milt Silverstein and John Simeone.

We thank Fran Webster for serving as the Guest Editor for these papers. Fran took charge of soliciting typed copies, having each paper peer reviewed, requesting revisions, and attending to most of the little details of preparing manuscripts for the printer and publisher. We are sure that Fran would want us to recognize and thank Eleanor Zaccaria, who helped with the editorial duties of this issue, and served as secretary for the journal for Milt and John for 20 years. In addition we thank our secretary here in Gainesville, Lori Clark, who also contributed to this issue.

Milt and John were very forward looking when they founded the *Journal of Chemical Ecology* in 1974. They subsequently were major forces in the development of the field of chemical ecology, especially in maintaining high standards of scholarship and excellence in the papers published for 20 years in the *Journal of Chemical Ecology*. We are confident the 12 papers in this issue are in that tradition of excellence, and are a worthy tribute to honor Robert Milton Silverstein and John B. Simeone, Founding Editors of the *Journal of Chemical Ecology*.

James L. Nation
David A. Jones
Editors, Journal of Chemical Ecology

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ONTOGENIC DEVELOPMENT OF CHEMICAL DEFENSE BY SEEDLING RESIN BIRCH: ENERGY COST OF DEFENSE PRODUCTION

JOHN P. BRYANT[1,*] and RIITTA JULKUNEN-TIITTO[2]

[1]*Institute of Arctic Biology, University of Alaska, Fairbanks Fairbanks, Alaska 99775-7000*
[2]*Department of Biology, University of Joensuu Joensuu SF-80101 Finland*

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Abstract—Whether production of chemical defenses by plants is or is not an energetically costly process is an important, but unresolved, question in chemical ecology. We suggest studies of the ontogenetic development of plant defense systems can help resolve the question. As an example of this approach to the cost question, we explore the problems associated with production of immobile chemical defenses that defend juvenile resin birches against browsing by mammals. From this exploration we draw two conclusions: (1) Shortly after germination, production of chemical defenses by small-seeded species, such as birch, is energetically costly. (2) Opposing selection for defense versus competitive ability in the seedling stage of birch has resulted in a trade-off in allocation of carbon to production of immobile chemical defense versus allocation of carbon to production of storage reserves. We suggest this trade-off results in a large indirect cost of defense because carbon used for production of immobile chemical defenses is unavailable for support of growth in the future, but stored carbon can be used to support future growth.

Key Words—Plant chemical defense, storage, seedling, cost, ontogeny.

INTRODUCTION

Research on plant chemical defense has increased dramatically in the past two decades. From the early 1970s to the mid-1980s the number of primary publications dealing with chemical defense against insect herbivory increased from about 20 per year to about 200 per year (Scriber and Ayers, 1988), and recent review of this topic (Feeny, 1992; Roitberg and Isman, 1992; Bernays, 1994)

*To whom correspondence should be addressed.

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indicate this rate of growth is continuing. Study of chemical defense against vertebrate herbivory in terrestrial ecosystems and chemical defense against herbivory in marine ecosystems started later, but recent reviews of these subjects indicate a similar rate of growth (Hay and Fenical, 1988; Palo and Robbins, 1991; Bryant et al., 1992; Hay and Steinberg, 1992).

This research explosion has been fueled by a succession of theoretical models (coevolution, optimal defense, resource availability, carbon/nutrient balance, growth/differentiation balance) that attempt to explain the evolution and ecology of plant antiherbivore defense systems (Ehrlich and Raven, 1964; Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1979; Bryant et al., 1983a; Coley, 1983; Coley et al., 1985; Lorio, 1986; Herms and Mattson, 1992). Although each model has increased our knowledge of plant antiherbivore defense, empirical research has now identified deficiencies in all models. Thus, plant defense theory is beginning a period of revision. The objective of this symposium was to identify research topics that are likely to provide direction to this revision.

The general area of research we have selected for discussion is the early ontogenetic development of plant defense systems. We suggest that research on this subject will further development of plant defense theory for the following reasons. The seedling stage is generally considered to be of paramount importance in understanding plant population dynamics and adaptations (Harper, 1977), and studies of plant population of dynamics have shown the direct negative effect of herbivory on survival of plant genets is generally greatest when herbivory occurs during juvenile stages of development (Watkinson, 1986). Thus, selection for chemical defense by herbivores should be comparatively strong in juvenile developmental stages (Bryant et al., 1983a; Langeheim and Stubblebein, 1983; Kearsely and Whitham, 1989). Yet, in comparison to what we know of chemical defense systems of mature plants, we know virtually nothing about the early ontogenetic development of plant defense systems (Simms, 1992).

The specific research problem we focus on is the energy cost paid by juvenile plants to produce chemical defenses. In this paper we suggest three hypotheses that could help resolve this question of cost, which is central to plant defense theory (Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1979; Bryant et al., 1983a; Coley et al., 1985; Bazzaz et al., 1987; Lorio, 1986; Herms and Mattson, 1992; Zangerl and Bazzaz, 1992):

Hypothesis I. In many species, juvenile developmental stages are more chemically defended than the mature stage.

Hypothesis II. In the specific case of species that reproduce by small seeds, the energy cost paid by young seedlings for formation of chemical defense is very high. Thus, production of immobile chemical defenses, sensu Coley et al. (1985), is minimal shortly after seed germination.

Hypothesis III. Opposing selection for chemical defense versus competitive ability (Herms and Mattson, 1992) in the seedling stage of small-seeded species

has resulted in a biochemical trade-off in allocation of carbon to formation of immobile antiherbivore defenses versus allocation of carbon to formation of storage reserves sensu Chapin et al. (1990). The outcome of this trade-off is a large indirect cost of defense (Bloom et al., 1985; Gulmon and Mooney, 1986; Herms and Mattson, 1992; Zangerl and Bazzaz, 1992) because carbon used for formation of immobile defenses cannot be used to support growth in the future, but carbon allocated to storage reserves can be used to support growth for defense in the future (Kozlowski and Keller, 1966; Kozlowski, 1971; Chapin et al., 1990).

These hypotheses are explored in the following sections.

HYPOTHESIS I

It has long been known that seedlings, saplings, and the mature developmental stages of woody plants differ predictably in one or more distinctive morphologic, anatomic, physiologic, and biochemical traits (Knight, 1795; Schaffalitzky de Muckadell, 1954, 1969; Waring, 1959; Sax, 1962; Kozlowski, 1971; Borchert, 1976). Among traits that change predictably during ontogeny as a result of meristem-based changes in gene expression (Kozlowski, 1971) are traits that have been assigned a defensive function. For example, woody species that produce thorns or spines are usually most thorny or most spinescent when juvenile (Schaffalitzky de Muckadell, 1969; Kozlowski, 1971). Similarly, the secondary chemistry of juvenile stages often differs either qualitatively or quantitatively from the secondary chemistry of the conspecific mature stage (Kozlowski, 1971).

The best documentation that chemical antiherbivore defenses of woody plants can change dramatically during ontogeny comes from studies of the interaction between woody plants and hares (*Lepus*) in subarctic forests (Bryant, 1981, 1987; Bryant et al., 1983a,b, 1985a,b, 1989, 1994; Reichardt et al., 1984, 1990; Tahvanainen et al., 1985; Clausen et al., 1986; Sinclair et al., 1988; Swihart et al., 1994). In the taiga and boreal forest of North America and Eurasia, winter-dormant twigs of juvenile trees and juvenile shrubs are less palatable to hares (*L. americanus* and *L. timidus*, respectively) than winter-dormant twigs of their conspecific mature stage. In every case that has been well-studied chemically, the low palatability of twigs of the juvenile stage has been related to increased production of specific feeding deterrent secondary metabolites (reviewed by Bryant et al., 1991, 1992). For example, Reichardt et al. (1984) found that winter-dormant internodes of juvenile Alaska paper birch (*Betula resinifera*) are defended against hare browsing by high concentrations of terpene resins such as papyriferic acid that are not even present in internodes of mature *B. resinifera*.

Other studies have provided evidence that chemical defense of the juvenile stage against browsing is widespread among both mammals and biomes. Basey et al. (1988) found juvenile quaking aspen (*Populus tremuloids*) in the Great Basin of North America is more defended against beaver by phenolics than is mature quaking aspen. Circumstantial evidence for this hypothesis comes from studies of willow (*Salix* spp.) and microtine rodents (Danell et al., 1987) and those of birch and moose (*Alces alces*) (Danell et al., 1990) in the boreal forest of Sweden, Monterey pine (*Pinus radiata*) and jackrabbits (*Lepus*) in California Mediterranean woodlands (Libby and Hood, 1976), Douglas fir (*Pseudotsuga menzesii*) and black-tailed deer (*Odocoileus hemionus columbianus*) and snowshoe hare in the coastal evergreen forest of northwestern North America (Dimock, 1974; Dimock et al., 1976), several birches and aspens and snowshoe hare in the temperate deciduous forests of eastern North America (Swihart et al., 1994), and several trees and elephant (*Loxodonta africana africana*) in the miombo woodland of Africa (Jachman, 1989).

Juvenile woody plants and mature woody plants can also differ in chemical defense against insect herbivory. Langenheim and her students have demonstrated seedlings of some Brazilian trees have better constitutive defenses (terpene resins) than the conspecific mature stage (Langenheim and Stubblebine, 1983; Macedo and Langenheim, 1989), and Wagner (1988) found the short-term inducible defense of Ponderosa pine (*Pinus ponderosa*) to be restricted to saplings. Results of Kearsely and Whitham (1989) suggest chemical defenses of *Populus angustifolia* change during ontogenetic development, but defenses of different developmental stages have different efficacies against different insects.

In comparison to woody species, virtually nothing is known about the ontogenetic development of chemical defense of herbaceous species, but what is known indicates chemical defenses of herbs also change during ontogeny. For example, in a particularly elegant study of *Plantago lanceolata*, Bowers and Stamp (1993) found young plants to be more defended by iridoid glycosides against both generalist insects and specialists insects than the mature stage, and plant age explained about twice as much of the variation in leaf iridoid glycoside concentration as did plant genotype. Gershenzon (unpublished data) has found young mint (*Mentha*) contain less monoterpene than does older mint.

These data suggest two generalizations. First, chemical defenses change during plant ontogenetic development. Second, in many cases, juvenile stages of development are more defended than the conspecific mature stage. They further suggest enhanced chemical defense of the juvenile stage may be more consistent in the case of mammalian herbivory than in the case of insect herbivory. We suggest comparative studies of effects of mammalian herbivory versus insect herbivory on plant recruitment in pristine ecosystems will be needed to determine the relative contributions of these two groups of herbivores to the evolution of chemical defenses of juvenile plants.

HYPOTHESIS II

The energy cost of producing antiherbivore defenses should be highest in periods of the life cycle when competition for carbon between chemical defense and other plant functions is intense. It is generally recognized that the onset of reproduction may be such a period (Bazzaz et al., 1987). In the case of small-seeded plants, it is likely that another such period is when energy reserves of the seed have been exhausted, and the plant first has to rely totally on photosynthesis for growth (Bryant et al., 1991, 1992). In these plants, growth of the radicle depletes energy reserves of the seed to such an extent that when the epicotyl emerges above the soil further growth is almost entirely dependent on the seedling's rate of carbon acquisition (Ingestad, 1962; Kozlowski, 1971). Thus, at this stage of seedling development, allocation of photosynthate to substances such as immobile chemical defenses that do not immediately support growth would be selectively disadvantageous, because it would greatly limit seedling establishment (Bryant et al., 1991, 1992). This is the reason we have predicted production of immobile chemical defenses by plants that grow from small seeds will be minimal when the epicotyl emerges from the soil.

We are testing this hypothesis by studying the ontogeny of chemical defense against browsing by mammals in birch. The birches we are focusing on are *B. resinifera* and *B. pendula* family E1970 × E1980 (Finnish Foundation for Tree Breeding, Helsinki). By the sapling stage, *B. resinifera* is more defended by terpene resin than is *B. pendula* (Bryant et al., 1989), and the resin concentration of juveniles of *B. pendula* family E1970 × E1980 is the lowest yet reported for a *B. pendula* family (Rousi et al., 1991, 1993).

We are studying these birches and their chemical defenses against browsing in winter for the following reasons: (1) Mammal browsing in winter is a major cause of death of recruitment of these birches (Rousi, 1990; Bryant, unpublished data), so juveniles of these birches should have evolved defenses against winter browsing. (2) Birch produces small seeds (mass about 0.6 mg), so energy limits the growth of young birch seedlings (Ingestad, 1962, 1970, 1971, 1979; Ingestad and Lund, 1979; Ingestad and McDonald, 1989) for reasons given above. (3) *B. resinifera* and *B. pendula* use triterpenes such as papyriferic acid and sesquiterpenes to deter browsing in winter (Reichardt, 1981; Reichardt et al., 1984; Bryant et al., 1989; Lapinjoki et al., 1991; Vainiotalo et al., 1991; Rousi et al., 1991, 1993; Taipale and Lapinjoki, 1992). These defensive substances are energetically expensive to produce for two reasons. First, the mass of glucose (Glc) used to produce a gram of defense is comparatively high for secondary metabolites: In the case of papyriferic acid it is 2.72 g Glc/g product, and in the case of sesquiterpenes it ranges from 3.34 to 3.54 g Glc/g product. By comparison, the glucose cost of producing tannin ranges from 1.28 g Glc/g product to 2.08 g Glc/g product (Gershenzon, 1994). Second, the resin glands

that secrete these defensive substances onto the epidermis of birch seedlings (Lapinjoki et al., 1991) are also energetically costly to produce (Gershenzon, 1994). Moreover, because these substances can occur in high concentrations in internodes of juvenile birch, their cost of production per gram tissue can be very high (Table 1). For example, Gershenzon (1994) has calculated *B. resinifera* saplings use 307 g Glc/g tissue to defend current-year-growth internodes with papyriferic acid, and papyriferic acid is only one third of the defensive resin found in current-year-growth internodes of sapling *B. resinifera* (Reichardt et al., 1984). (4) These substances appear to have no other function than defense against browsing by mammals in winter (Bryant et al., 1992), so the energy cost of producing them is not offset by use for other functions (Seigler and Price, 1976; Simms, 1992), (5) Finally, these defenses are immobile, sensu Coley et al. (1985), because they are deposited by resin glands on the surface of the epidermis (Reichardt et al., 1984; Lapinjoki et al., 1991).

In our experiments, seedlings were grown in pots in a mix of vermiculite and perlite (1 : 2 v/v) in a glasshouse. Light and temperature were characteristic of the environments in which these birches grow, which are essentially the same and seedlings were fed a nutrient solution developed by Ingestad specifically for growing birch seedlings in pots (Ingestad, 1962). This solution provided nitrogen at an optimum level (140 ppm N as NH_4NO_3). Other macronutrients and micronutrients were adjusted to N as recommended by Ingestad. Pots were sufficiently large to prevent root binding, and pots were drenched daily with nutrient solution to prevent desiccation and salt accumulation.

TABLE 1. HIGHEST RAW MATERIALS (GLUCOSE) COSTS ESTIMATED BY GERSHENZON (1994) FOR PRODUCTION OF CLASSES OF DEFENSE COMPOUNDS[a]

Chemical class	Plant species	Defense compound	Cost per gram of plant tissue (mg glucose/g)
Terpenoids	*Betula resinifera*	Papyriferic acid	307
Phenolics	*Isocoma acradenia*	Apigenin	103
Alkaloids	*Camellia sinensis*	Caffine	72
Cyanogenic glycosides	*Amelanchier alnifolia*	Prunasin	7.8
Glucosinolates	*Cleome serrula*	Methyl glucosinolate	20
Nonprotein amino acids	*Leucaena leucocephala*	Mimosine	42
Protinase inhibitors	*Lycopersicon esculantum*	Inhibitor 1 from tomato	0.27

[a]Estimates take into account all the starting materials and cofactors needed for biosynthesis.

For seven weeks after the epicotyl emerged above the soil, we followed production of resin glands in the upper 2 cm of internodes for 50 seedlings of each birch. These measurements were made for the first 2 cm below the apical meristem, because in these birches internode resin glands differentiate from the primary epidermis just below the apical meristem (Lapinjoki et al., 1991). To avoid destructive sampling before final harvest, we made rank estimates of the density of resin glands in weeks 1, 2, 3, 5, and 7. A rank of zero indicated no observable resin glands, and each higher rank indicated an increase of about 25–30 glands per square centimeter of internode epidermis. In week 7 we measured the seedlings' net photosynthetic rates, and then harvested them. After harvest, for each seedling we measured its height, its total leaf area, and the dry masses of its leaves, shoot, and roots. We also used the photographic procedure of Rousi et al. (1991, 1993) to count the number of resin glands in the top 2 cm of internode epidermis.

The epicotyls of both birches emerged above the soil at the same time, about one week after planting. At this time, neither birch produced internode resin glands (Figure 1A). *B. resinifera* began producing resin glands in week 2, and *B. pendula* began producing resin glands three weeks later. For both birches, resin gland production increased as the birches increased in size and their leaf area increased. This result was expected, because as the leaf area of a seedling birch increases, so does its ability to acquire carbon (Ingestad, 1962; Ingestad and Lund, 1979; Ingestad and McDonald, 1989). Thus, production of resin glands by both birches was minimal shortly after germination (Figure 1A). Furthermore, *B. resinifera* seedlings always produced more resin glands than did *B. pendula* seedlings (Figure 1A and B; $P < 0.0001$).

Similar results have been obtained for other birches and other small-seeded species. In a set of comparisons of eight birch species based on 12 secondary metabolites (96 total comparisons), R. Julkunen-Tiitto (unpublished data) found the concentrations of the 12 metabolites in CAG internodes of seedlings were less than those found in CAG internodes of saplings in 94 of the 96 comparisons. Reichardt and Chapin (unpublished data) found production of condensed tannin by CAG internodes of *Salix alaxensis* seedlings, production of pinosylvin and pinosylvin methyl ether by CAG internodes of *Alnus crispa* seedlings, and production of monoterpenes by CAG internodes of *Picea glauca* seedlings was low in comparison to production of the same substances by CAG internodes of conspecific saplings, respectively. In the case of herbaceous species, D. Bowers (personal communication) has found production of iridoid glycosides by *Plantago lanceolata* is minimal shortly after seed germination, and J. Gershenzon (personal communication) has obtained the same result for production of monoterpenes by mint (*Mentha*).

Our interpretation of these results is that the direct energetic cost of producing defensive resins is high enough that seedlings of small-seeded plants

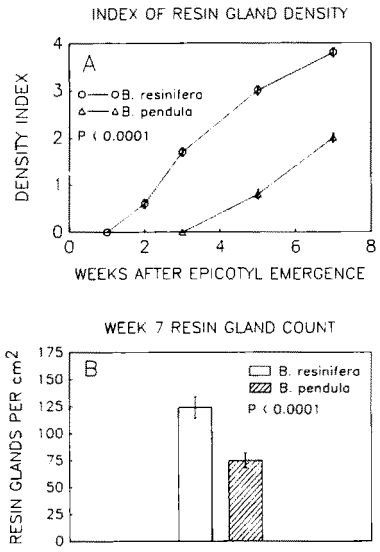

FIG. 1. Production of resin glands by seedlings of two birches, *B. resinifera* and *B. pendula* family E1970 × E1980. Mean ± 1 SE presented. $N = 50$ seedlings for each birch.

such as birch are unable to produce much of these substances. This conclusion contrasts with the suggestion of Rousi et al. (1991, 1993) that in the case of seedling resin birch, the direct energetic cost of producing terpene resins is negligible. Since this conclusion, and recent similar conclusions by others (e.g., Simms and Rausher, 1987, 1989; Simms, 1992; Karban, 1993), challenge the widely accepted hypothesis that production of chemical defense has an energetic cost, it is useful to examine the basis for the conclusion of Rousi et al. (1991, 1993).

In their experiments, Rousi et al. (1991, 1993) correlated the density of resin glands on internodes of 1-year-old birch seedlings with two measures of seedling growth, height (Rousi et al., 1991) and biomass accretion (Rousi et al., 1993). In both studies they are unable to demonstrate a negative correlation between defense (density of resin glands on internodes) and seedling growth. Similarly, in our experiments with birch seedlings, we also failed to find a negative correlation between resin gland density (Figure 1) and these two measures of growth (Table 2). Such failures to find negative correlations between simple measures of plant growth such as plant size or biomass accretion have often been interpreted to mean that production of chemical defense has no energy cost (Simms, 1992). Thus, it is not surprising that Rousi et al. (1991, 1993) reached their conclusion.

TABLE 2. SEEDLING HEIGHT, TOTAL LEAF AREA, DRY BIOMASS, AND NET PHOTOSYNTHETIC RATE[a]

Birch	Height (cm)	Leaf area (cm^2)	Biomass				Photosynthesis ($\mu mol/m^2$ sec)
			Leaf (g)	Shoot (g)	Root (g)	Total (g)	
B. resinifera	26 ± 0.9	433 ± 23	1.3 ± 0.1	0.4 ± 0.1	1.1 ± 0.1	2.8 ± 0.1	18.8 ± 0.5
B. pendula	27 ± 0.1	414 ± 23	1.4 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	2.9 ± 0.1	18.6 ± 0.5

[a]Mean ± 1 SE presented.

We suggest conclusions drawn from this approach to testing the "energy cost of defense hypothesis" should be viewed with caution for three reasons. These simple estimates of growth do not take into account: (1) differences in the ability to acquire carbon, (2) differences in maintenance respiration, or (3) biochemical trade-offs in partitioning of carbon between chemical defense and competing plant functions. Yet these differences are the physiological and biochemical basis for the energetic cost of producing chemical defense (Gulmon and Mooney, 1986; Chapin et al., 1990; Bryant et al., 1991, 1992; Herms and Mattson, 1992; Zangerl and Bazzaz, 1992).

Our experiment with *B. resinifera* and *B. pendula* indicates a difference in the ability to acquire carbon is unlikely to explain why birch seedlings that differ in defense can accrete biomass at the same rate. We found that seedlings of *B. resinifera* and seedlings of *B. pendula* that differed in production of resin glands (Figure 1A and B; $P < 0.001$) and that had the same biomass after seven weeks of growth (Table 2), also had the same ability to acquire carbon (Table 2). We further suggest a difference in maintenance respiration is unlikely to explain why birch seedlings that differ in defense can accrete biomass at the same rate, because maintenance respiration has a minimal effect on patterns of carbon allocation by birch seedlings (Ingestad and McDonald, 1989; Ingestad and Agren, 1991).

HYPOTHESIS III

These conclusions suggest that if birch seedlings pay an energy cost to defend themselves against browsing in winter, this cost is an indirect cost resulting from trade-off in allocation of carbon between defense and storage. Two observations from common garden experiments support this hypothesis: (1) In contrast to 1-year-old *B. pendula* seedlings, when Rousi et al. (1991) examined

8-year-old *B. pendula* saplings they found a negative correlation between defense (resin gland density) and growth (sapling height). This result is expected if there is a trade-off between defense and storage in juvenile stages of *B. pendula*, and juvenile *B. pendula* is polymorphic for this tradeoff. (2) After two to three years of growth in common gardens in Finland (M. Rousi, personal communication) and Michigan (M. Herms, personal communication) the comparatively heavily defended saplings of *B. resinifera* are growing at less than half the rate of saplings of the less-defended *B. pendula*.

RECOMMENDATIONS FOR FUTURE RESEARCH

Two further experiments are needed to verify that seedlings of small-seeded plants subject to differences in the intensity of selection for defense and the intensity of selection for competitive ability are polymorphic for partitioning of carbon between immobile chemical defense and storage reserves. In the one experiment, mass balance measurements should be used to document biochemical carbon partitioning by seedlings of congeners or conspecifics grown in a common environment. In this experiment, masses of carbon allocated to production of immobile defenses and to production of storage compounds (e.g., starch, storage protein, fatty acids) as well as masses of carbon allocated to growth and maintenance (e.g., sugars, amino acids, enzymes), and to structure (e.g., cellulose, lignin) should be determined using the biochemical approach suggested by Chapin et al. (1990). Assuming no differences in carbon acquisition, maintenance respiration, and allocation of carbon to other functions, evidence for a trade-off between defense and storage would be a negative correlation between the amount of glucose used to produce immobile chemical defense and the amount of glucose used to produce storage substances. Furthermore, this trade-off should become more noticeable in later stages of seedling ontogeny, because according to our second hypothesis, shortly after germination allocation of carbon to any function that competes with immediate growth should be minimal. Thus, for later stages of seedling ontogeny we expect "defense-selected" seedlings will have comparatively small storage reserves, and "competition-selected" seedlings will have comparatively poor chemical defenses.

In the other experiment, $^{14}CO_2$ pulse labeling should be used to measure the flow of carbon competing plant functions within a few hours of acquisition. In this experiment, evidence for a "defense-storage" polymorphism would be provided by the following two observations: (1) After production of defenses and storage reserves begins, the short-term flow of carbon into immobile chemical defense will be greatest for "defense-selected seedlings." (2) Conversely, the short-term flow of carbon into storage reserves will be greatest for "competition-selected" seedlings.

CONCLUSIONS

Competition and herbivory are two of the greatest challenges faced by juvenile plants (Harper, 1977; Tilman, 1982; Watkinson, 1986; Huntly, 1991). Increased ability to deter herbivory is often achieved by increased allocation of resources to chemical defense, and increased competitive ability is generally associated with rapid growth. Thus, in the juvenile stage, plants that have evolved in environments with varying levels of herbivory and competition should be polymorphic for partitioning of carbon between defense and growth (Herms and Mattson, 1992). In this paper we have suggested that studies of the early ontogenetic development of defense systems by plants are needed to test this hypothesis. We have further suggested the focus of this research should be a search for a trade-off in allocation of carbon to immobile defense versus formation of storage reserves in the seedling stage of species that grow from small seeds. This research will help resolve a central question of current plant defense theory: when is production of chemical defense energetically costly?

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PLASTICITY IN ALLOCATION OF NICOTINE TO REPRODUCTIVE PARTS IN *Nicotiana attenuata*

IAN T. BALDWIN* and MICHAEL J. KARB

Department of Biological Sciences
SUNY, University at Buffalo
Buffalo, New York 14260

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Abstract—Although little is known about the patterns of chemical defense allocation in reproductive tissues, optimal defense theory predicts a high constitutive allocation due to the tissues' high fitness value. To examine this prediction, we quantified the short- and long-term changes in the nicotine pools of reproductive tissues in response to both floral and leaf damage. Recently opened flowers (stage 5 capsules) do not alter their nicotine pools within a day in response to herbivory by *Manduca sexta* larvae or mechanical damage to the corolla. Similarly, leaf damage during both vegetative and reproductive growth does not influence the nicotine pools of the first three stage-5 capsules produced. However, the nicotine pools of capsules produced later in reproductive growth were significantly larger (1.2- to 1.9-fold) on plants with leaf damage. These differences in floral nicotine pools were a result of both increases in nicotine pools of capsules on damaged plants and decreases in the nicotine pools of capsules on undamaged plants during reproductive growth. Leaf damage did not affect the rate of capsule maturation or the mass of stage-5 capsules at any time during reproductive growth. An allometric analysis of nicotine pools and biomass of reproductive parts in all stages of development from damaged and undamaged plants demonstrates that damaged plants allocated a significantly larger quantity of nicotine to reproductive parts in all stages of development than did undamaged plants. Given that nicotine is thought to be synthesized in the roots and transported to leaves and reproductive parts, nicotine could be allocated to reproductive parts in proportion to the number of developing capsules on a plant. We excised the first 27 stage-5 capsules on plants with and without leaf damage, with the expectation that plants with fewer capsules would allocate a larger amount of nicotine to the remaining capsules. In contrast to the prediction of this passive allocation model, floral excision did not affect nicotine pools on plants with or without leaf damage. These results demonstrate that the allocation of nicotine to reproductive parts is more strongly influenced by damage to vegetative rather than reproductive tissues. Reproductive parts are constitutively defended

*To whom correspondence should be addressed.

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over the short term, but the set points for defense allocation are apparently increased by damage to vegetative tissues during reproductive growth. The decrease in allocation of nicotine to reproductive parts in undamaged plants during reproductive growth suggests an optimization of resource allocation as plants realize their potential fitness.

Key Words—Nicotine, defensive allometry, *Nicotiana attenuata*, flowers, reproductive parts, optimal defense theory.

INTRODUCTION

Leaves are functionally plastic in the face of herbivory: after attack by herbivores, they change their allocation of resources to both their primary function, carbon gain, and to chemicals used in defense (Welter, 1989; Baldwin, 1993). Few studies have examined the coordination of herbivory-induced changes in defense allocation and photosynthetic capacity (Baldwin and Ohnmeiss, 1994; Zangerl, 1986, Zangerl and Bazzaz, 1992), but the pattern that has emerged is consistent with the predictions of optimal defense (OD) theory (Rhoades, 1979, 1983; McKey, 1974, 1979). OD theory holds that plant tissues contributing more to plant fitness and those more likely to encounter herbivore attack should be allocated higher levels of resources for defense than other plant tissues. For example, the increase in photosynthetic capacity induced by damage and the induced increase in nicotine concentrations are highly coordinated in the leaves of *Nicotiana sylvestris*, and the coordination changes with nitrogen supply rates (Baldwin and Ohnmeiss, 1994).

In contrast to leaves, reproductive tissues are decidedly more determinate in growth form, and for these tissues, OD theory predicts a high constitutive allocation of defense metabolites, given the importance of reproductive parts to the realization of a plant's fitness. OD theory also predicts that the chemical defenses present in immature fleshy fruits should be neutralized in order to increase the attractiveness of mature fruit to dispersers. The latter prediction has received substantially more attention than the former; the changes in palatability that accompany ripening are legend and well described. For example, as a tomato ripens, glycoalkaloids are degraded (Heftmann and Schwimmer, 1972) and the production of proteinase inhibitors (Pearce et al., 1988) ceases, resulting in fruits that are less well defended than their immature counterparts. Much less is known about the allocation of defenses to the reproductive parts of plants that do not produce fleshy fruits, and wild parsnip (*Pastinaca sativa*) is one of the better studied plants in this regard. The concentrations of furanocoumarins in fruits are not affected by floral damage in either damaged flowers or in flowers produced after damage (Nitao, 1988). This lack of variation in floral furanocoumarin concentrations contrasts with the dramatic increases in leaf furanocoumarin concentrations that are induced locally by damage (Zangerl, 1990).

Hence, despite wild parsnip's ability to compensate for floral damage by producing more secondary umbels (Hendrix and Trapp, 1981), its allocation of defense appears to be unchanged in response to damage. Here we examine whether the allocation of nicotine to reproductive parts is similarly unaffected by damage in a species, *Nicotiana attenuata*, in which leaf damage is known to increase the concentrations of nicotine both locally in damaged leaves and systemically throughout the plant (Baldwin and Ohnmeiss, 1993).

However, first we need to consider how best to quantify changes in defense allocation in growing tissues. These issues of quantification are particularly germane to the analysis of the predictions of OD theory, which weighs the costs of producing secondary metabolites against the benefits of resistance resulting from the use of the secondary metabolite. For growing plants, costs can be envisioned as the diversion of resources from growth because reproductive output is frequently size-dependent (Samson and Werk, 1986). Hence, rigorous tests of the predictions of OD theory require secondary metabolite production to be quantified in the context of growth.

Changes in allocation of secondary metabolites in growing tissues can be described by an allometric analysis: the metabolite pool in the plant or plant part is regressed against the metabolite-free biomass of the plant or plant part from a series of harvests taken at many stages during development. If the metabolite pool reflects metabolite production (e.g., there is little or no metabolite turnover), then, borrowing the terminology from the literature on heterochronic growth (McNamara, 1986), metabolite production can be envisioned as having onset and offset times. The slope of this relationship describes how a plant partitions resources to defense during growth. Changes in allocation to defense and growth could therefore be quantified as changes in slopes and onset and offset times in the allometric relationship, and allometric relationships found in undamaged plants can serve as a null model against which damage-induced plasticity can be compared. Ohnmeiss and Baldwin (1994) used an allometric analysis to describe the changes in nicotine pools of damaged and undamaged *N. sylvestris* plants during vegetative growth. Although damage did not significantly affect plant growth, it did increase the accumulation of nicotine for approximately 10 days after damage; consequently, the slope of allometric relationship increased. The slopes of plants harvested more than 10 days after damage were the same as those of undamaged plants. The larger intercept of this allometric relationship reflected the earlier period of increased production (Figure 1). We can conclude that leaf damage causes vegetatively growing plants to move from one allometric relationship to another.

In this study we examined the plasticity in nicotine allocation to reproductive parts in *N. attenuata* Torr. ex Watson. This plant is a herbaceous annual native to North America found in dry washes and recently disturbed or burned areas throughout the Great Basin desert. It produces a central racemose or nar-

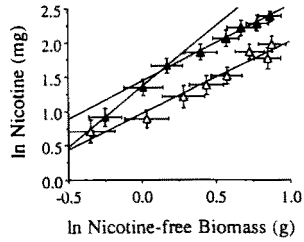

FIG. 1. An allometric analysis of nicotine pools in vegetatively growing *N. sylvestris* plants that were either undamaged (open symbols) or damaged once (closed symbols). Damaged plants increased their accumulation rates for the first 10 days after the damage event (three harvests) but then returned to the same nicotine accumulation rate as undamaged plants (data from Ohnmeiss and Baldwin, 1994).

rowly peniculate indeterminate inflorescence with self-compatible perfect flowers that mature into green capsules that contain 10–300 small (120–160 μg/seed) wind- and water-dispersed seeds (Goodspeed, 1954). The calyx is covered by many trichomes and contains approximately 80% of the nicotine of the entire reproductive part at all developmental stages. A large proportion of the nicotine in the calyx is located in the trichomes, and a single large trichome (of which there may be 27 per capsule) contains between 1.3- and 3.6 μg of nicotine (I. Baldwin, M. Karb, E. Schmelz, N. Blenk and M. Euler, unpublished data). In contrast to the calyx, seeds contain very little nicotine. We found a range of 0–1.2 ng nicotine/seed from seeds collected from pods at different stages of development and grown under a variety of conditions. Hence, the amount of nicotine found in all the seeds of a pod represents a maximum of only 18% of nicotine found in a single large trichome.

We ask the following two questions in two experiments: (1) Does the amount of nicotine in a mature flower increase rapidly in response to floral damage, as has been demonstrated for leaves? (2) Does damage to leaves influence the quantity of nicotine allocated to reproductive parts? These questions examine the prediction from OD theory that reproductive parts will be constitutively defended and examine the plasticity in defense allocation both over the lifetime of a flower and the lifetime of the plant. Few studies have examined the effect of damage to vegetative tissues on the defensive allocation in reproductive tissues despite the clear physiological reliance of reproductive tissues on the resources acquired during vegetative growth. The changes in the allocation over a plant's lifetime deserve attention because most of the nicotine in a reproductive capsule is stored in trichomes on the calyx. Trichomes are cell types that are active early in capsule development (Gershenzon and Croteau, 1991), and damage-induced changes may be constrained to the capsules initiated

after damage. Additionally, because nicotine is synthesized in the roots and transported in the shoot in all nicotine-producing *Nicotiana* species studied to date (Baldwin, 1989), we examined the possibility that the allocation of nicotine to reproductive parts may occur simply as a function of the number of capsules on a plant and the amount of nicotine produced by the roots.

METHODS AND MATERIALS

Plant Growth. N. attenuata seeds were soaked in an aqueous extract of wood smoke in 9.8 mM KNO_3 in order to stimulate germination (Baldwin et al., 1994b). Seeds were from the third generation of glasshouse-grown plants grown from seed collected from plants in 1988 on the DI Ranch in southwestern Utah (T40S R19W section 9). Approximately one week after germination, seedlings were transferred to Jiffypots (Jiffy Products Ltd., Canada), which were subsequently planted into 4- and 0.75 liter plastic pots containing Cornell mix A potting soil (Boodley and Sheldrake, 1977) for plants used in experiments 1 and 2, respectively. All plants were grown in a glasshouse and received supplemental lighting from 400-W high-pressure sodium vapor lamps for 14 hr/day.

Extraction and Quantification of Nicotine. Reproductive parts were cut from the pedicel at the base of the calyx with a razor blade, weighed, frozen at −40°C, and extracted in alkaloid extraction solution (40% methanol in 0.1% HCl) for a minimum of 2.5 days (the time necessary for complete nicotine extraction from frozen flower samples). The corollas of reproductive parts in developmental stage 5 (Table 1) were analyzed separately from the rest of the flower and are reported on in a companion paper (Euler and Baldwin, unpublished data); here we report the nicotine pools of the entire reproductive part. Twenty-four capsules from each developmental stage from one plant were

TABLE 1. CLASSIFICATION OF DEVELOPMENT IN *Nicotiana attenuata* CAPSULES

Stage	Description
1	Corolla completely within calyx
2	Corolla greenish and beginning to emerge from calyx
3	Corolla greenish and fully elongated
4	Corolla white, fully elongated but closed
5	Corolla open
6	Corolla senesced and pistil swollen

weighed, dried at 45–50°C, and reweighed for the calculation of stage-specific percentage dry mass conversion factors. Nicotine was separated by HPLC (Baldwin, 1988), quantified with external standard curves run every 50 samples, and expressed as micrograms of nicotine per capsule.

Experiment 1. This experiment examined the effect of mechanical damage and herbivory to the corolla on capsule nicotine pools. Each afternoon we chose capsules in stage 4 (Table 1) on five plants that would open their corolla tube that evening (and enter stage 5) and haphazardly assigned them to the following three treatment groups on each plant: undamaged, razor damage, and caterpillar damage. Capsules in the razor-damaged treatment received one incision half the length of the corolla tube. One second- or third-instar *Manduca sexta* was placed on the corolla of each capsule in the caterpillar herbivory treatment. Caterpillars remained on the corolla until they had initiated feeding at least once before being removed. Caterpillars that did not feed within the first 30 min were replaced with others until the corolla was damaged. Capsules were damaged in the afternoon and harvested 26–28 hr later. Capsules that aborted before this time were not included in the analysis. Undamaged capsules aborted 7% of the time (3 of 46); 23% of the razor-damaged capsules aborted (11 of 48); and 36% of the caterpillar-damaged capsules aborted (19 to 53).

Experiment 2. This experiment examined the changes in nicotine pools in reproductive parts induced by damage to leaves both over the course of capsule ontogeny and over the reproductive phase of growth for the plant. We harvested capsules at the same stage in development (stage 5) at specified intervals of capsule production on each plant (capsules 1–3, 14–16, and 27–29) in order to track the developmental stage of each plant. Moreover, we included an additional pair of treatments in which the first 26 maturing capsules were excised and predicted that if nicotine is allocated to reproductive parts passively as a function of the total amount of nicotine produced by the vegetative tissues, the nicotine pools of capsules 27–29 of these plants would be larger than those from treatments that did not have their capsules excised.

Forty plants in the early stages of stalk elongation were randomly assigned to the four following treatment groups: (1) both leaf damage and capsule excision, (2) capsule excision only, (3) leaf damage only, and (4) neither leaf damage nor capsule excision. Leaves were damaged every day throughout the period of stalk elongation and flowering. One row of evenly distributed pattern-wheel (Dritz, Spartanburg, South Carolina) damage was made across the length of the lamina on either the left or right side of the midrib on every other leaf of plants in the leaf-damage treatment groups. On average, pattern-wheel damage resulted in 4.5 1-mm^2 holes/cm of leaf. Holes were produced by crushing leaf tissue between the blunt spokes of the pattern wheel and a plastic card, and resulted in undetectable leaf dry mass loss (Ohnmeiss and Baldwin, 1994). Pattern-wheel damage was applied to all fully expanded leaves except the senescing leaves.

Corolla tubes of stage-5 capsules open in the evening and remain open for two to three nights. Plants were inspected every evening between 8:30 and 11:30 PM for capsules with recently opened corrola tubes. The number of stage-5 capsules was recorded and the first 26 stage-5 capsules produced on plants in the flower excision treatments were removed. The first three and the fourteenth to sixteenth stage-5 capsules produced on the two nonexcision treatments were harvested for nicotine quantification. The twenty-seventh, twenty-eighth, and twenty-ninth stage-5 capsules of plants in all four treatments were harvested for nicotine analysis.

An allometric analysis of nicotine contents in reproductive parts in all stages of development was conducted on the first five plants to produce 29 stage-5 capsules in treatments 3 and 4 (e.g., the two nonexcision treatments). Five capsules from each of six ontogenetic stages, for a total of 30 capsules per plant, were harvested for nicotine quantification.

Statistical Analysis. Two-way ANOVAs with damage and plant as the main effects were performed on micrograms of nicotine per capsule and gram capsule mass data of experiment 1. Two-way ANOVAs with damage and excision as the main effects were performed on the third harvest of stage-5 capsules (numbered 27–29) of experiment 2. A repeated-measures one-way ANOVA with leaf damage as the main effect was performed on the three harvests of stage-5 capsules from the two nonexcision treatments of Experiment 2. The ln-transformed amounts of nicotine per capsule were analyzed with an analysis of covariance with ln-transformed amounts of nicotine-free biomass per capsule as the covariate and with individual regressions on the data from each plant. A test of homogeneity of slopes (parallelism) was conducted to determine whether the slopes of these relationships differed between plants with and without leaf damage. Data analysis was performed with the MGLH module of the SYSTAT statistical package (Evanston, Illinois).

RESULTS

Experiment 1. The amount of nicotine in stage-5 capsules with caterpillar (37.4 ± 3.4 μg nicotine/capsule) or mechanical damage (36.3 ± 3.5 μg nicotine/capsule) to their corollas did not differ from that found in undamaged capsules (37.9 ± 3.1 μg nicotine/capsule) as determined by two-way ANOVAs (damage treatment $F_{2,98} = 0.061$; $P = 0.94$). Significant differences between plants were found ($F_{4,98} = 2.66$; $P = 0.037$), and the damage $\times$ plant interaction term was not significant ($F_{8,98} = 0.16$; $P = 0.99$). No significant differences were found in any of the flower biomass data (Ps > 0.40; two-way ANOVA).

Experiment 2. Neither the leaf damage treatment nor the floral excision treatment significantly influenced the biomass of any of the stage-5 capsules

from any of the harvests as determined by the one-way repeated-measures ANOVA or the two-way ANOVA on the twenty-seventh to twenty-ninth stage-5 capsules produced on each plant (Table 2; Figure 2). Similarly, the floral excision treatment did not significantly affect the amount of nicotine in the twenty-seventh to twenty-ninth stage-5 capsules produced on plants as determined by a two-way ANOVA (Table 2; Figure 2). Leaf damage had no significant effect on the amount of nicotine found in the first three stage-5 capsules ($F_{1,17} = 1.15$; $P = 0.30$; one-way ANOVA; Figure 2) produced on each plant; however, leaf damage dramatically increased the amount of nicotine in capsules produced later during reproductive growth as determined by the repeated-measures one-way ANOVA and the two-way ANOVA (Table 2; Figure 3). By the third harvest (capsules numbered 27–29), stage-5 capsules on plants with leaf damage had nicotine pools that were three times those produced by undamaged plants at the same stage of development. This difference in nicotine pools is due in part to a significant decrease in nicotine of capsules produced on undamaged

TABLE 2. TWO-WAY ANOVAS ON NICOTINE AND BIOMASS POOLS OF STAGE-5 CAPSULES ON THIRD HARVEST (CAPSULES 27-29) FROM ALL FOUR TREATMENT GROUPS (FIGURE 2) AND ONE-WAY REPEATED-MEASURES ANOVAS BETWEEN-SUBJECTS EFFECTS ON NICOTINE AND BIOMASS POOLS OF STAGE-5 CAPSULES FROM ALL THREE HARVESTS FROM DAMAGED AND UNDAMAGED PLANT TREATMENTS (FIGURE 2).

	df	MS	*F*	*P*
Third Harvest				
Nicotine (μg/capsule)				
Leaf damage treatment (*D*)	1	13700.2	43.094	0.000
Floral excission treatment (*E*)	1	29.5	0.093	0.763
D * *E*	1	28.6	0.090	0.766
Error	33	317.9		
Dry mass (mg/capsule)				
Leaf damage treatment (*D*)	1	0.835×10^{-5}	2.444	0.127
Floral excission treatment (*E*)	1	0.278×10^{-5}	0.814	0.374
D * *E*	1	0.221×10^{-7}	0.007	0.936
Error	33	0.342×10^{-5}		
Harvests 1–3				
Nicotine (μg/capsule)				
Leaf damage treatment	1	8524.3	14.6	0.0013
Error	17	582.0		
Dry mass (mg/capsule)				
Leaf damage treatment	1	0.3799×10^{-5}	0.8521	0.369
Error	17	0.4458×10^{-5}		

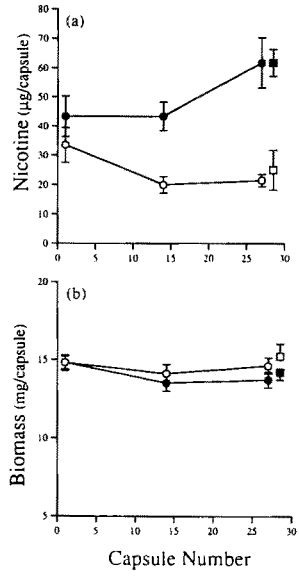

FIG. 2. Mean (±SEM) capsule (a) nicotine and (b) biomass at three stages during reproductive growth of 10 undamaged (open circles) plants and nine plants with leaves damaged during vegetative and reproductive growth (solid circles). Samples are of stage-5 capsules (see Table 1) numbered 1–3, 14–16, and 27–29 on all plants where capsule 1 was the first capsule to reach stage 5. The nicotine and biomass pools of stage-5 capsules numbered 27–29 from an additional nine undamaged (open squares) and nine plants with leaves damaged during vegetative and reproductive growth (solid squares) are also depicted. These plants are from the floral excision treatment which had their first 1–26 stage-5 capsules excised.

plants ($F_{1,27} > 4.56$; $P < 0.042$; one-way ANOVA contrasts between the first and second and first and third harvests). Neither leaf damage nor flower excision affected the plants' phenologies; the rates of flower production as determined by the time to produce 3, 14 or 27 stage-5 capsules did not differ among the treatment groups ($F_{1,33} < 2.62$; $P > 0.11$; two-way ANOVAs).

Reproductive parts at all stages in development from plants with and without leaf damage used in the allometric analysis had significantly ($F_{1,286} = 180.33$; $P < 0.00001$; one-way ANOVA on ln micrograms of nicotine) more nicotine than those from undamaged plants (Figure 3). Biomass values were not significantly different ($F_{1,286} = 0.013$; $P = 0.91$; one-way ANOVA on capsule ln dry mass). The relationship between nicotine pools and capsule biomass as determined by a test of homogeneity of slopes (parallelism) did not differ sig-

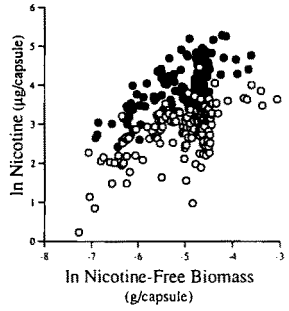

FIG. 3. Natural log transformed nicotine pools plotted against the ln-transformed nicotine-free biomass of five capsules from each of the six developmental stages described in Table 1. Capsules were sampled from five undamaged (open circles) plants and five plants with leaves damaged during vegetative and reproductive growth (solid circles). All plants were sampled after producing 30 stage-5 capsules and hence were at the same stage in their reproductive phase as those plants in the third harvest of Figure 1. See Table 3 for the regression statistics for each individual plant and the text for the ANCOVA analysis.

TABLE 3. ESTIMATES (±SEM) OF REGRESSION COEFFICIENTS (R^2, y INTERCEPT, AND SLOPE) FOR ALLOMETRIC REGRESSIONS (FIGURE 3) FOR EACH PLANT[a]

	R^2	y-intercept	Slope
Undamaged plants			
1	0.116	4.212 ± 0.753	0.285 ± 0.148
2	0.575	5.288 ± 0.391	0.452 ± 0.076
3	0.515	5.498 ± 0.474	0.499 ± 0.093
4	0.423	5.693 ± 0.622	0.536 ± 0.120
5	0.382	5.875 ± 0.806	0.647 ± 0.156
Damaged plants			
1	0.282	5.707 ± 0.654	0.410 ± 0.126
2	0.797	8.154 ± 0.358	0.719 ± 0.070
3	0.664	6.917 ± 0.369	0.546 ± 0.075
4	0.840	6.678 ± 0.294	0.644 ± 0.057
5	0.505	5.694 ± 0.414	0.422 ± 0.081

[a] Natural log-transformed nicotine pools in five capsules for each of the six developmental stages described in Table 1 were regressed against ln-transformed nicotine-free dry biomass for each plant. Plants were either undamaged or had their leaves damaged throughout the reproductive phase of growth.

nificantly ($F_{1,288} = 0.115$; $P = 0.735$) between damaged and undamaged plants. Because the assumption of homogeneity was not violated, an analysis of covariance (ANCOVA) with ln nicotine as the dependent variable and ln nicotine-free biomass as the covariate was performed, revealing that undamaged and damaged plants differed significantly ($F_{1,289} = 72.3$; $P < 0.001$). Within-plant regressions revealed the same pattern as the ANCOVA (Table 3). The *y*-intercept values were significantly higher for damaged plants ($F_{1,8} = 5.93$; $P = 0.041$; one-way ANOVA on ln-transformed values), while the slopes of the individual plant regressions were not significantly different ($F_{1,8} = 0.58$; $P = 0.47$; one-way ANOVA on ln-transformed values). In addition, the relationships between ln-transformed nicotine pools and biomass tended to have less scatter with damaged plants than with undamaged plants (mean $R^2 = 0.618$ and 0.402 for damaged and undamaged plants, respectively; Table 3).

DISCUSSION

The results of these experiments revealed some of the "rules" that govern how nicotine is allocated to reproductive parts. First, nicotine is not passively allocated to reproductive parts as a function of the amount of nicotine produced in roots and the number of capsules on a plant. The nicotine content of stage-5 capsules on plants that had the vast majority of their maturing capsules excised did not differ from those which did not have capsules excised (Figure 2). The excised stage-5 capsules contained between 20 and 60 μg of nicotine, depending on whether they developed on plants with or without leaf damage. Had these capsules remained on the plant through maturity, they would have accumulated 60–160 μg of nicotine, respectively. Hence, the excision treatment should have doubled or tripled the nicotine pools or stage-5 capsules if the passive allocation model was correct. This deduction assumes that nicotine accumulation reflects nicotine production in *N. attenuata* as has been demonstrated in *N. sylvestrius* (Baldwin et al., 1994a) and that nicotine was not stored in other plant parts for allocation to capsules later in reproductive growth. Second, the allocation of nicotine to a capsule is not influenced by herbivory or mechanical damage to the corolla (experiment 1) or to the removal of other reproductive parts (experiment 2). Third, in contrast to damage to reproductive parts, damage to leaves dramatically increases the allocation of nicotine. Fourth, the allocation of nicotine to reproductive parts follows an allometric relationship that has less scatter in damaged plants, as indicated by the higher R^2 of the individual regressions (Table 3), and has a higher intercept. The effect of leaf damage on the nicotine–biomass allometry appears similar to that found in vegetatively growing *N. sylvestrius* plants (Figure 1). The long-term effect is to move plants from one allometric relationship to another with similar slopes but different intercepts.

The *N. sylvestris* data set clearly showed that damaged plants moved from one allometric relationship to another by briefly increasing their rate of nicotine production during growth and, hence, increasing the slope of the relationship. A similar mechanism could be operating in *N. attenuata* reproductive parts, although leaf damage may have initiated the production of capsules with higher nicotine accumulation set points at the beginning of capsule growth. Fifth, leaf damage does not appear to alter the allometric set points for nicotine accumulation for the first reproductive parts produced by a plant, and these apparent set points appear to decline during the later stages of reproductive growth in undamaged plants. These patterns of nicotine allocation underscore the important regulating influence that vegetative tissues have on the defense of reproductive tissues.

Many of these patterns are broadly consistent with the predictions of OD theory. First, defense is constitutively allocated to individual reproductive parts as a function of capsule mass. OD theory predicts that defensive plasticity should be reserved for those tissues that are functionally plastic, such as leaves that are capable of physiologically rejuvenilizing and tissues whose contribution to plant fitness vary over ontogeny. Second, defense allocation to the first capsules produced on an undamaged plant appear to be higher than those produced later (Figure 2). For plants growing in unpredictable environments, the first offspring produced represent a larger proportion of realized fitness than those produced later and hence would be predicted to receive a larger defensive allocation. Moreover, if inducible defense allocation provides a means of conserving resources that could be otherwise used for offspring production, the inducible allocation of defense to offspring produced later during reproductive growth, as plants realize their potential fitness, is also consistent with the cost–benefit paradigm of OD theory. Whether or not plants that produce more defense in herbivore-free environments consequently produce fewer offspring of lower quality remains to be demonstrated.

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TRACKING VARIABLE ENVIRONMENTS: THERE IS MORE THAN ONE KIND OF MEMORY

FREDERICK D. PROVENZA

Department of Rangeland Resources
Utah State University
Logan, Utah 84322-5230

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Abstract—Three kinds of memory help herbivores track changes in the environment. The first is the collective memory of the species with genetic instructions that have been shaped by the environment through millennia. This includes skin and gut defense systems. Auditory and visual stimuli and sensations of pain impinge upon the skin defense system that evolved in response to predation. The taste of food and the sensations of nausea and satiety are an integral part of the gut defense system that evolved in response to toxins and nutrients in plants. The second kind of memory in social mammals is represented by the mother, a source of transgenerational knowledge, who increases efficiency and reduces risk of learning about foods and environments. The third kind of memory is acquired by individual experience. Postingestive feedback from nutrients and toxins enables individuals to experience the consequences of food ingestion and to adjust food preference and selection commensurate with a food's utility. The three memories interact, each linking the past to the present, and collectively shape the present and future of every individual. Thus, the dynamics of foraging involves appreciating the uniqueness of individuals and subgroups of animals, each with their own genetic and behavioral history, and recognizing that foraging behaviors may not be stable, optimal, or even predictable in the conventional sense.

Key Words—Evolution, ecology, learning, memory, foraging, habitat, spotted owl.

INTRODUCTION

Organisms rely on experience and memory to cope with hazards in the environment. In the absence of memory, every facet of the environment would ceaselessly be experienced for the first time and immediately forgotten. As Luis Bunuel lamented while in the process of losing his memory: "You have only

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to begin to lose your memory, if only in bits and pieces, to realize that memory is what makes our lives. Life without memory is no life at all . . . . Our memory is our coherence, our reason, our feeling, even our action. Without it, we are nothing . . . (I can only wait for the final amnesia, the one that can erase an entire life, as it did my mother's . . .)" (in Sacks, 1990, Chapter 2).

There are three kinds of memory. The first is the collective memory of the species, the genetic instructions that have been shaped by the environment throughout millennia. The second is represented in social mammals by the mother, who is a source of transgenerational knowledge. The third kind of memory is acquired by individual experience. The three memories interact and link consequences of behavior from the past to the present and shape the present and future of every individual. This paper discusses the three types of memories and how they are related.

EVOLUTION AS MEMORY

Genes are the cumulative memory of how environment has shaped the species through millennia. Skin and gut defense systems are part of these genetic instructions in species are diverse as fruit flies, snails, fish, lizards, tiger salamanders, pigeons, hawks, rats, cats, dogs, sheep, baboons, and humans (Garcia et al., 1985; LeDoux, 1994; Provenza, 1995). Auditory and visual stimuli and sensations of pain associated with the skin defense system evolved in response to predation. The taste of food and sensations of nausea and satiety are part of the gut defense system evolved in response to toxins and nutrients in plants. As Garcia (1989) suggests "all organisms evolved coping mechanisms for obtaining nutrients and protective mechanisms to keep from becoming nutrients" too soon.

Evolution has created skin and gut defense mechanisms that facilitate interactions with a virtually infinite variety of objects in the environment. Each system is underlain by two kinds of memory; one provides the emotional content of the experience, whereas the other renders specific details about objects and events. Collectively, these systems are designed to interact with and to be shaped by the environment during each animal's lifetime. This flexibility increases the likelihood of survival of the individual and of the species, given the fact that the environment is ever-changing and that the average lifetime of a species is several million years (Wilson, 1992). This plasticity is clearly one of the most important outcomes of evolution.

The way skin and gut defense systems work is illustrated in experiments in which hawks were fed distinctively colored or flavored mice (Brett et al., 1976; Garcia and Garcia y Robertson, 1985). Hawks fed white mice with impunity, but occasionally given a black mouse followed by an injection of the toxicant lithium chloride (LiCl), would not eat either black or white mice,

because both mice tasted the same. But when a distinctive taste was added to black mice, hawks learned to avoid black mice on sight after a single black mouse toxicosis event. The taste cue potentiated the color cue.

Such experiments show that not all cues are readily associated with all consequences. Cue–consequence specificity refers to the fact that when animals are made ill following exposure to audiovisual and taste cues, they show much stronger aversions to the taste than to the audiovisual stimuli. In contrast, if they receive foot shock following the same cues, they show much stronger aversions to the audiovisual than to the taste cues (Garcia and Koellin, 1966). The same kind of response has been demonstrated for food and place aversions. Toxins like LiCl cause strong food aversions, but they do not cause rats to avoid the place where they ate a particular food (Lett, 1985; Coil et al., 1978). Conversely, compounds such as gallamine, which causes muscular paralysis, causes rats to avoid the place where they ate but they do not stop rats from eating the food (Lett, 1985).

The incorrigible nature of such responses is illustrated further by the fact that animals acquire aversions even when they know the food did not cause the illness (Garcia and Garcia y Robertson, 1985; Provenza et al., 1994a). For instance, many of us remember acquiring an aversion to a particular food, even though we knew the illness (e.g., nausea caused by flu, reaction to a vaccine, seasickness, chemotherapy) was not related to food. This knowledge does not alter the newly acquired aversion to the taste and odor of the food.

Such seemingly irrational responses occur because gustatory and visceral afferent nerves converge at common central neural pathways that are generally distinct from audiovisual and somatosensory integrative neural systems (Garcia et al., 1985; LeDoux, 1994). Olfactory pathways have access to both skin and gut defense systems. Odor cues are associated with taste cues (i.e., taste potentiates odor) during a meal, and these two senses provide information concerning the flavors of particular foods. Conversely, odors of predators serve as warnings for the skin defense system. Thus, evolution arranged the central nervous system in a manner that affords relatively direct and independent integration of skin defense and gut defense systems.

Skin and gut defense systems were designed differently to accomplish different objectives. The skin defense system functions on a scale of milliseconds to seconds; rapid responses are required to avoid predation (LeDoux, 1994). Conversely, the gut defense system integrates information about tastes (and potentiated odors) of foods and the postingestive effects of nutrients and toxins over time frames of minutes to hours; digestion and absorption are relatively slow processes (Garcia et al, 1985; Provenza, 1995).

Skin and gut defense systems are also mutually inhibitory. Fear abolishes appetite and gastric motility, whereas a large meal is followed by drowsiness and muscular relaxation. As Robert Capa (*Newsweek*, May 23, 1994) noted in

his diary on D-Day: "The mess boys of the *U.S.S. Chase* wore immaculate white jackets and served hot cakes, sausages, eggs and coffee with unusual zest and politeness. But the pre-invasion stomachs were preoccupied, and most of the noble effort was left on the plates . . . ."

Two kinds of memory underlie skin and gut defense systems, one renders specific details about objects and events, whereas the other provides the emotional or affective content of the experience (LeDoux, 1992, 1994). Declarative (cognitive) memory is the process by which earlier conscious experiences are recalled. The original learning and remembering are conscious events mediated by the hippocampus. Memories of snakes (skin defense) and various foods (gut defense) and locations where they have been encountered are examples. In contrast, emotional (noncognitive) memory operates independently of conscious awareness, and it is mediated by the amygdala. Fear (skin defense), nausea and satiety (gut defenses) are examples. Emotional memory may be stored within declarative memory, which can activate emotional responses through the amygdalic system. Thus, emotion is not merely unconscious memory, rather it exerts a powerful influence on declarative memory and on behavior.

Emotional and declarative memories provide insights into the distinction between food preferences and food selection. Neurally mediated interactions between the senses (i.e., taste and smell) and the gut (i.e., nausea and satiety caused by toxins and nutrients) cause changes in food preferences that do not involve cognitive processes (i.e., they constitute an emotional memory). The noncognitive aspect of this phenomenon has been verified in a variety of species under various conditions, e.g., animals acquire food aversions when anesthetized (Roll and Smith, 1972; Bermudez-Rattoni et al., 1988; Provenza et al., 1994a), tranquilized (Forthman Quick, 1984), and when their electrocortical activity is depressed (Davis and Bures, 1972; Buresova and Bures, 1973). In contrast, animals use declarative memory to select (avoid) particular foods and foraging locations.

THE MOTHER AS MEMORY

The mother as a memory shapes food and habitat selection by her offspring and increases their likelihood of survival. The importance of this transgenerational link is clearly illustrated in instances when wild and domesticated ruminants are moved to unfamiliar environments. Compared with animals reared in the environment, naive animals spend more time foraging but ingest less forage; they spend more time walking and walk for greater distances; they suffer more from predation, malnutrition, and ingestion of toxic plants (reviewed in Griffith et al., 1989; Provenza and Balph, 1990).

The mother increases efficiency of learning about nutritious foods (Provenza, 1994). Lambs exposed to wheat with their mothers for as little as 1 hr/day for five days ingested more wheat than did lambs exposed to wheat without their mothers; intake of wheat was nearly 10 times higher as many as three years later with no intermittent exposure (Lynch et al., 1983; Green et al., 1984). Likewise, lambs exposed to the shrub *Amelanchier alnifolia* and to barley with their mothers ingested considerably more of these foods than did lambs who were exposed without their mothers (Thorhallsdottir et al., 1990). Finally, lambs who ate either of two palatable shrubs (*Cercocarpus montanus* or *Amelanchier alnifolia*), showed a marked preference for the shrub they ingested with mother (Nolte et al., 1990).

In the same manner, the mother reduces the risk of overingesting toxic foods (Provenza, 1994). For instance, a lamb learns quickly to avoid harmful foods and to select nutritious alternatives, when with its mother who exhibits this behavior (Mirza and Provenza, 1990, 1992). As a result of such interactions, a lamb acquires preferences for foods its mother eats, and avoids foods its mother avoids (Mirza and Provenza, 1994).

Experiences early in life with the mother also shape habitat preferences of offspring. Wild herbivores of the same species often occupy different home ranges within an area (Gruell and Papez, 1963; Geist, 1971; Fiesta-Bianchet, 1986a,b, 1988; Cederlund and Okama, 1988; Cederlund et al., 1987; Porter, 1992). This phenomenon also occurs in livestock (sheep: Hunter and Milner, 1963; Lawrence, 1990; cattle: Yeates and Schmidt, 1974; Martin, 1979; Reinhardt and Reinhardt, 1981; Roath and Krueger, 1982; Hall, 1988; Howery et al., 1995a; goats: O'Brien, 1984). Cross-fostering experiments with sheep (Key and MacIver, 1980) and cattle (Howery et al., 1995b) show that habitat selection preferences are transferred from mother to offspring. For instance, lambs whose mothers preferred native pastures were cross-fostered with lambs whose mothers preferred seeded pastures. After weaning, the lambs strongly preferred the location where their foster mothers had foraged. Individuals reared in different environments often have disparate dietary habits (Biquand and Biquand-Guyot, 1992), which in turn affects their preferences for foraging locations (Scott et al., 1995).

As a result of selecting particular foods and foraging in certain locations, the preferences of individuals can become quite rigid, to the degree that they appear to be genetically fixed. This phenomenon is illustrated in field studies of Criollo goats grazing savannas of Martinique, French West Indies (Biquand and Biquand-Guyot, 1992). The diets of mature female goats, which came from four different locations, strongly reflected their area of origin on the island, despite grazing a common area for four years. These mothers produced four lots of female offspring. Diets of the cohorts born in successive years were strongly affected by experiences early in life, which were a reflection of the

diets of their mothers and peers, as well as differences in plant species that were available the year they were born.

Research on moose provides another example of the persistence of behaviors learned from the mother (Andersen, 1991; Histol and Hjeljord, 1993). A population of moose in central Norway migrates from low-lying summer areas to winter areas situated at higher altitudes, which is contrary to the general pattern of migration to lower elevations near the sea. Archeological evidence along the migration route shows that the migratory behavior of this population has not changed since 5000 BP, despite deterioration in the quality of their winter range. The low calf production of the population (0.6 calves/cow) compared with neighboring populations (0.8–1.3 calves/cow) evidently reflects the poor winter ranges. Migratory traditions passed on from mother to offspring are inflexible, and changing migratory routes to utilize better quality range may take many generations, if it ever occurs.

THE INDIVIDUAL AS MEMORY

Experience is the memory of the consequences of behavior to the individual. The importance of individual experience in foraging is illustrated in experiments with the mother and her offspring. For instance, when a lamb experiences mild toxicosis from eating a food its mother prefers, the lamb acquires an aversion to the food even though its mother continues eating the food avidly (Provenza et al., 1993a). Thus the mother may facilitate the acquisition of foraging behaviors by her offspring, but continuation of the behaviors depends on the consequences to the individual (Galef, 1988).

Postingestive feedback enables individuals to experience the consequences of food ingestion, and it causes changes in preference for the flavor (taste and odor) of particular foods (Provenza, 1995). Animals acquire preferences for flavors of foods that cause satiety, the pleasant sensation that occurs when animals ingest adequate kinds and amounts of nutritious foods. Conversely, animals acquire aversions for the flavors of foods that cause malaise, the unpleasant feelings of physical discomfort that occur from ingesting excesses of nutrients and toxins and from nutrient deficits. What constitutes excesses, deficits, and satiety depends on the animal's nutritional requirements, which vary with age, physiological state, and environmental conditions. Thus, postingestive feedback adjusts food preferences commensurate with the food's utility. In the absence of flavor–feedback interactions, animals are not likely to select a nutritious diet because they generally can not directly taste and smell nutrients and toxins in foods.

Flavor–feedback interactions cause changes in preference (affective value), and changes in preference cause changes in food selection (Garcia, 1989; Provenza, 1995). Taste plays the pivotal role in both cases. Affective processes

integrate the taste of food and its postingestive consequences, aversive or positive, thereby causing changes in incentive to eat particular foods; they involve neurally (i.e., primarily brain stem and limbic system) mediated interactions between the sense of taste and the body, and they are noncognitive. Cognitive processes involve use of the senses of smell, sight, and hearing to select foods that cause positive consequences and to avoid foods that cause malaise; they involve interactions between higher cortical centers and the limbic system. Affective and cognitive processes function in parallel to regulate the internal environment. The taste of food is continuously calibrated according to its effect on the internal environment, and on that basis animals select foods that are nutritious and avoid those that are toxic.

Affective and cognitive processes both operate when animals forage, for instance when goats choose between current season's (CSG) and older growth (OG) twigs from the shrub blackbrush (*Coleogyne ramosissima*). Even though CSG contains more nitrogen (1.04% vs. 0.74%) than OG and is more digestible (48% vs. 38%), goats prefer older twigs containing lower levels of a condensed tannin that causes a learned food aversion (Provenza et al., 1990). When both types of twigs are offered to goats who have never consumed blackbrush, goats do not exclusively choose either type, but when they finally eat more CSG than OG in a meal (44 g and 16 g) and eat enough CSG to acquire an aversion (about 44 g), they ingest less CSG from then on (Provenza et al., 1994b). Thus, the relative amount of food eaten within a meal controls the acquisition of an aversion to CSG. The change in preference for CSG involves the brain stem and limbic system and requires no cognitive ruminations on the part of the goat, whereas the change in food selection by the goat involves the senses of sight and smell and higher cortical centers.

An animal's experiences, particularly early in life, exert a profound influence on food preference (Provenza and Balph, 1990; Provenza, 1994). As a result of eating particular foods and not eating others, young animals acquire dietary habits. They prefer familiar foods, and they become reluctant to eat novel foods. Accordingly, familiarity and novelty come to control taste-feedback interactions within meals. For instance, when animals eat a meal of novel and familiar foods and experience malaise, they avoid the novel foods (Revusky and Bedarf, 1967; Burritt and Provenza, 1991), and they exhibit the same response when the flavor of a familiar food changes (Launchbaugh and Provenza, 1993; Provenza et al. 1993a, 1995). Furthermore, when they eat a meal of only novel foods and experience malaise, they avoid the foods that are most novel (Kalet and Rozin, 1970, 1971; Kalat, 1974; Provenza et al. 1994b). For instance, lambs fed rolled barley (familiar food), with a low and a high concentration of a flavor, consume small amounts of both foods, regardless of flavor concentration (Launchbaugh et al. 1993); but after they eat a meal of both foods, and then receive a mild toxin dose, they avoid the barley with the highest concen-

tration of the flavor (i.e., the barley that is most different (novel) from plain barley). Conversely, when animals experience mild malaise following a meal of familiar foods, they avoid foods eaten too frequently or in excess (Provenza et al. 1993b, 1994b; Phy and Provenza, 1995; Burritt and Provenza, 1995). Thus, eating any familiar food to satiety is apt to cause mild malaise, which will cause animals to eat a varied diet (Provenza, 1995).

Young ruminants remember foods that provided either aversive (Lane et al., 1990; Burritt and Provenza, 1990) or positive (Green et al., 1984; Squibb et al., 1990) consequences for at least one to three years without intermittent exposure, and they sample novel foods cautiously (Chapple and Lynch, 1986; Chapple et al., 1987; Thorhallsdottir et al., 1987; Burritt and Provenza, 1989, 1991). The ability to discern novel from familiar foods is illustrated in experiments when lambs were given 10 different foods during a seven-month period (Provenza et al., 1995). Any time lambs were offered a food they had previously eaten, their initial intake was high (e.g., >600 g in 30 min). Conversely, when they were offered a novel food, their intake was low initially (e.g., <50 g in 30 min) and gradually increased over several days. Intake decreased (increased) any time a novel (familiar) flavor was added to a familiar (novel) food. Thus, lambs remembered at least 10 foods, a number similar to that typically comprising the diets of animals foraging on rangelands. For instance, goats encounter over 100 species/ha in tropical savannas, but they eat only seven species in significant amounts (10% or more of the diet), and five species constitute 65% of their diet (Biquand and Biquand-Guyot, 1992).

Animals discriminate among novel foods to the degree dictated by the environment. Goats first introduced to blackbrush-dominated rangelands sample all foods in the area during the first few hours, including potentially toxic plants like *Juniperus osteosperma* (bark and green leaves), *Gutierrezia microcephala* (a forb), and *Marrubium vulgare* (a forb), as well as nontoxic shrubs like *Prunus fasciculata* and *Purshia tridentata*. They also sample and learn to distinguish among different plant parts, for instance, current season's and older growth twigs from the shrub blackbrush. Within a few days, however, goats discriminate among and ingest different amounts of various foods, evidently on the basis of neurally mediated interactions between taste and postingestive feedback (Provenza et al., 1994b; Provenza, 1995). Considering the importance of food for survival, the design of the central nervous system, and the fact that ruminants spend as many as 8–12 hr foraging every day, it should not be surprising that herbivores can learn to discriminate among several novel foods on the basis of flavor–feedback interactions.

CONCLUSION

One of the most important outcomes of evolution is the plasticity to be shaped by the environment during an animal's lifetime. Beliefs concerning evo-

lution should be tempered with the realization that food and habitat selection are dynamic processes, adapted to environmental change. Learning is vital for adaptation, and it involves elements of history and change such as prior experiences; social interactions; neuro-, morpho-, and physiological feedbacks and feedback time lags; individual variability in responses to nutrients and toxins; forage sampling; and random events. It is prudent to recognize that learning can activate a self-perpetuating series of consequences that are nonoptimum, sensitive to perturbation, difficult to predict, and resistant to change (Provenza and Cincotta, 1993). Accordingly, food and habitat selection should be studied as dynamic behavioral processes rather than as static events that reside at some optimum.

Conservation and management programs should operate with this kind of dynamic in mind. The spotted owl is an excellent example. There is growing evidence that the spotted owl is not endangered. Instead, it occurs from British Columbia to Mexico in habitats as disparate as young- and old-growth forests and desert scrub (Easterbrook, 1994). Recent studies suggest that the DNA is no different in individuals in any of those populations. What differ are the experiences of individuals with the environment, but that does not make them different species. As Easterbrook points out: "If local variation in climate and diet convert creatures to different species, a black man who lives in Seattle, gets rained on and eats salmon would be a different species from a white man who lives in stifling humidity in Louisiana and dines on gumbo. By this theory the human race contains hundreds of species." The distinction between what has been shaped by the environment during an animal's lifetime and what has been shaped by evolution over millennia makes all the difference in the world concerning understanding of the status of spotted owls.

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TURNABOUT IS FAIR PLAY: SECONDARY ROLES FOR PRIMARY COMPOUNDS

MAY R. BERENBAUM

Department of Entomology
University of Illinois at Urbana-Champaign
320 Morrill Hall, 505 S. Goodwin,
Urbana, Illinois 61801-3785

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Abstract—Chemically based resistance of plants to herbivorous insects is today essentially synonymous with allelochemically based resistance; the importance of plant secondary compounds in determining patterns of host-plant utilization has been established in a wide variety of insect-plant interactions. In contrast, primary metabolites, those involved in fundamental plant physiological processes, are rarely considered to be major determinants of host-plant resistance despite the fact that, as insect nutrients, they can have profound effects on behavior and physiology. The degree to which variation in plant primary metabolism results from the selective impact of herbivory may be greatly underestimated in that the biosynthetic and structural diversity of primary metabolites and the consequences of that diversity on herbivores are rarely taken into account in most studies of insect preference and performance. Qualitative and quantitative variation in the production of primary metabolites can result from herbivore selection pressure if production of primary metabolites is under genetic control and if plant fitness in the presence of herbivores is associated in a predictable way with genetically based primary metabolite variation. Variation in primary metabolism is likely to be particularly effective as a defense against highly oligophagous herbivores with limited mobility, especially those confined to structures containing allelochemicals that could neutralize the benefits associated with compensatory feeding.

Key Words—Secondary compounds, primary compounds, plant resistance, herbivory.

INTRODUCTION

Over the last 40 years, as chemical techniques for the separation, identification, and quantification of secondary chemicals have become increasingly more sophisticated and accessible, interest on the part of ecologists in the chemistry

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of primary constituents of plants has virtually disappeared. With few exceptions, chemical ecologists have, for the most part, relegated the precise identification and quantification of primary plant metabolites (with the exception of elemental nutrients) to physiologists. In point of fact, however, amino acids, vitamins, sterols, and other herbivore nutrients are chemical constituents of plants that are synthesized, sequestered, or metabolized as are alkaloids, coumarins, phenylpropanoids, and the other more "secondary" metabolites. Moreover, considerable evidence exists that they play an essential role in determining host-plant suitability for herbivores and that herbivores detect and process them in ways very similar to the manner in which secondary metabolites are detected and processed.

There is a clear need, then to differentiate between the terms "secondary substance" and "allelochemical"—the two terms are not really synonymous or interchangeable. Just as some secondary substances may have primary physiological functions (Seigler, 1977), some primary substances may function as allelochemicals. By the same token, it must be emphasized that the terms "nutrient" and "allelochemical" are not necessarily exclusive and opposite (Slansky, 1992); whether a substance is nutritive or toxic is entirely dependent upon context (Reese, 1979; Janzen, 1979) and not upon biosynthetic origin. Although it is now widely accepted that allelochemicals may function in some instances as nutrients (Bernays and Woodhead, 1982; Rosenthal and Bell, 1979), the allelochemical function of erstwhile nutrients is rarely considered.

To a great extent, the relative lack of emphasis on primary metabolites of plants in current discussions of plant–insect interactions is largely the result of historical precedent; methodologies and technologies now exist, however, that make the analysis of primary metabolism in an ecological context more tractable. Such analyses stand to make substantial contributions to understanding the chemical bases for host-plant choice in insects and the evolutionary responses of plants to herbivory.

HISTORICAL BACKGROUND

The chemical characterization of plants began in earnest in the nineteenth century, and it rapidly became apparent that, while certain plant constituents, such as chlorophyll or sucrose, are virtually universal in distribution, other types of plant constituents are restricted in distribution to individual families, individual genera within families, individual species within genera, and even individual populations within species. Such idiosyncratic distributions suggested that compounds that are not universally distributed are not essential to the physiological function of the plant. Kossel (1891, as translated by Mothes, 1980) proposed "calling . . . essential components of the cell primary components, and those which are not found in every cell capable of developing, secondary."

TABLE 1. DATA FOR CHEMICAL CHARACTERIZATION OF REPRESENTATIVE PRIMARY AND SECONDARY METABOLITES (WINDHOLZ, 1983)

Primary metabolites		Secondary metabolites	
Tryptophan	1901	Cocaine	1885
Vitamin A	1913	Coniine	1886
Methionine	1922	Nicotine	1893
Vitamin E	1936	Mescaline	1896
Vitamin B_{12}	1948	β-Pinene	1896

This dichotomy was rapidly embraced and prevailed for the next century, although variously modified over the years; as more constituents were characterized, they were, for the most part, readily characterized as either primary or secondary metabolites. Surprisingly, the chemical characterization of many nutritive substances lagged behind that of many secondary metabolites (Table 1), at least in part because some essential nutrients, such as vitamins, are present in plants in concentrations even lower than are most secondary compounds; the word "vitamin," for example, was not even coined until 1912. In some ways, the choice of the word "secondary" was unfortunate; although it for the most part accurately depicts the biosynthetic dependence of secondary metabolites on intermediates of primary metabolism (referred to by Bonner and Galston (1952) as the byways and highways of plant metabolism, respectively), it also connotes inutility or expendability. Over the next century, various unflattering epithets, including "excretory products" (Goris, 1921, cited by Robinson, 1930), "metabolic waste products" (Muller, 1969), "unnecessary metabolites" (Robinson, 1974), even "flotsam on the metabolic beach" (Mothes, 1980), were applied to these compounds to emphasize the fact that they did not appear to play a role in the daily physiological demands of plant life.

Relatively early on, however, Stahl (1888) suggested that such compounds play a vital role in protecting plants against biological stress agents, referring to them as *Schutzexcrete*, or protective excretions. Stahl's suggestion that the function of the so-called secondary compounds is primarily ecological was not widely accepted until 1959, when Fraenkel (1959) reintroduced the idea to the scientific community, accompanied by a compelling body of evidence attesting to the behavioral and physiological effects of these compounds on herbivorous insects. In full recognition of the fact that secondary compounds are essential in the sense that they allow plants to survive in their biotic milieu, Whittaker and Feeny (1971) coined the less judgmental term allelochemic (from *allelo*, Greek for "one another") to describe compounds with primarily ecological functions; "most allelochemical interactions involve the diverse secondary sub-

stances . . . which do not occur in all living matter but appear sporadically throughout the living world. They are contrasted with the primary subtances, such as various proteins, carbohydrates, nucleic acids, and fats, which are of general occurrence." It is interesting to note that Whittaker and Feeny (1971) did not equate "allelochemical" with "secondary substances" but carefully noted that secondary substances do function as such in "most" cases.

Dissatisfaction with the notion that a clear dichotomy of function exists between primary and secondary compounds has arisen many times over the last century; indeed, even Kossel (1891, cited in Mothes, 1980), in proposing the terms primary and secondary metabolite, acknowledged the difficulties of distinguishing between them. Seigler (1977) and Seigler and Price (1976) suggested that secondary compounds may well play a role in primary physiological processes by serving as biosynthetic intermediates, as growth regulators, or as rather elaborate storage molecules for elements in short supply. Consistent with this function is the phenological, ontogenetic, and diurnal variation in abundance that characterizes virtually all secondary compounds. The idea that secondary compounds can function as storage molecules for elements in excess supply is, in fact, at the foundation of the carbon–nutrient balance hypotheses currently in vogue (according to which excesses of nutrients in the form of either carbon or nitrogen allow for the accumulation of either carbon-based or nitrogen-based allelochemicals, respectively) (Bryant et al., 1983; Coley et al., 1985).

Primary constituents, however, were not completely forgotten in all of this discussion. Because many biochemical and physiological processes are identical in plants and animals, it was assumed from the beginning that primary compounds produced by plants serve as nutrients for plant-feeding animals (including humans) when they are ingested. That the nutrient content of plants determines in part the suitability of plants to herbivorous insects was clearly recognized by Painter (1936), who stated that the "possible lack of specific food materials, especially proteins" could be critical to plant food quality, leading to, among other things, changes in longevity, size, fecundity, and death rates in herbivorous insects. He also ruefully noted that, at that time, "the development of insects with respect to specific food substances or mineral elements is almost an untouched field." The development of defined artificial diets and aseptic rearing techniques in the next decade allowed for tremendous gains in identifying the essential amino acid, vitamin, and other dietary requirements of insects in general and phytophagous insects in particular (Lipke and Frankel, 1965).

By mid-century, some investigators were proposing that both nutrients and secondary chemicals were important in determining patterns of host-plant utilization. This idea was perhaps best stated by Kennedy (1953, 1958), in his "dual discrimination hypothesis," according to which secondary chemicals are involved principally in host-plant recognition and nutrients principally in feeding-site selection within a plant. That both nutrients and secondary chemicals

should play a role in determining host-plant preferences was not an unreasonable supposition, according to Thorsteinson (1958), because "physiologically there is little basis for distinguishing between perception of token stimuli and nutrients since both are manifestations of the chemotactic sense." In his work with *Plutella xylostella*, Thorsteinson himself had demonstrated that the feeding-stimulant properties of sinigrin, a secondary compound typical of crucifers, were greatly enhanced in the presence of sucrose (Thorsteinson, 1960).

This synthetic view was largely forgotten, however, when Fraenkel proposed that allelochemicals, rather than nutrients, are exclusively responsible for determining plant utilization patterns by herbivorous insects—stating boldly that "there is little reason to suppose that differences in chemical composition with respect to the 'primary' substances (which occur in all living matter) can be responsible for the choice of food plant on the part of the insect" (Fraenkel, 1959). Although Fraenkel eventually softened this view (somewhat) in a later paper (Fraenkel, 1969, ". . . it would be illogical to expect that extreme changes in nutrients would leave the insect entirely unaffected"), the later paper never had the impact of the first, which went on to become a classic cited over 200 times between 1959 and 1984 (Current Contents, March 12, 1984). During that critical period in the development of insect–plant interaction theory, discussions of the impact of insect herbivory on plant evolution focused primarily on plant secondary metabolism.

LOW NUTRIENT QUALITY AS DEFENSE

Plant nutritional quality reemerged as an important element of coevolutionary studies in 1976, when both Feeny (1976) and Rhoades and Cates (1976) described allelochemicals with antinutritive, or digestibility-reducing, properties. The suggestion was made that low nutrient quality, arising either through antinutritive alleochemicals, such as tannins and resins, or through reduced amounts of essential nutrients, such as proteins, could function as a plant defense. There were perceived theoretical shortcomings with this position, however, articulated by both Moran and Hamilton (1980) and Price et al. (1980)—namely, that reduced nutrient quality could potentially lead to increased damage to plants due to the propensity of certain herbivores to compensate for low nutrient levels by ingesting some food. Moran and Hamilton (1980) conceded that low nutritive quality could function as a defense under special conditions—if herbivores are able to detect low nutrient quality and are sufficiently mobile to move to avoid it, if successive generations of extremely sedentary herbivores on the same host experience reductions in size as a result of reduced nutrient quality, or if prolonged development in early stages as a result of low nutrient quality increases the risk of predation or disease.

Over the past decade, although the effects of nutrient variation on herbivore preference and performance have been studied extensively (Slansky and Rodriguez, 1987; Slansky, 1992; Simpson and Simpson, 1990), the idea that low nutrient quality can evolve as a plant defense has garnered little attention. Neuvonen and Haukioja (1984) attempted to examine the hypothesis in the context of delayed inducible resistance in *Betula pubescens* in response to repeated defoliation by *Epirrita autumnata*; resistance in this system took the form of increased levels of foliar phenolics and decreased amounts of foliar nitrogen. These authors concluded that, due to the presence of multiple confounding variables, "one cannot say that low nutritive quality in itself is defensive." Lundberg and Astrom (1990) elaborated upon the idea of low nutritive value as a plant defense in a theoretical paper focused on repercussions of variable nutrient quality on movements of vertebrate herbivores. These authors assumed for purposes of discussion that low nutritive quality could be defined as "N primarily total nitrogen weight per unit of biomass," which they considered to be "a positive currency common to both the plant and the herbivore."

In none of these more recent studies, however, is nutrient quality ever assessed in a rigorous manner. Purported tests of the idea that low nutritive quality can confer resistance against herbivores have completely ignored the tremendous chemical variability that exists among primary metabolites; that secondary chemicals, such as tannins, phenolics, or resins, may have antinutritive effects is almost beside the point. Plant nutritional quality in ecological studies is almost invariably measured as or equated with total protein (or, even less definitively, total nitrogen content) and water content (Scriber, 1984). While these values may well be correlated with herbivore performance, it is extremely unlikely that herbivores are capable of responding physiologically to "total nitrogen." Nitrogen is at best a surrogate for nutritional value (Simpson and Simpson, 1990) and, for nitrogen in particular, it can be argued that it is not at all the same currency as far as plants and herbivores are concerned, since plants take in nitrogen in forms that are completely unutilizable by herbivores. Even for plants, which can utilize atmospheric nitrogen with the assistance of symbiotic microbes, the form in which nitrogen enters the system affects its utilizability (Fitter and Hay, 1983). As for herbivores, the efficiency with which nitrogen can be utilized depends critically on the form in which it is ingested; free amino acids, for example, are for more readily utilized than are complex proteins (Cockfield, 1988) and ureides, frequently found in legumes symbiotically associated with nitrogen-fixing bacteria, are even less readily utilized by insects (Wilson and Stinner, 1984). Due to differential needs, the efficiency with which amino acids can be utilized depends critically on their relative abundance and proportional representation (Rock, 1972; Reese, 1979). As well, vitamins, carbohydrates, lipids, and a host of other synthesized materials are as important

as, if not more important than, nitrogen per se for insect nutrition. The hypothesis that plant primary metabolism can be influenced by herbivore selection pressure effectively remains untested.

TESTING THE HYPOTHESIS

That plant primary metabolites can confer resistance against herbivores, or, alternatively, that insect selection pressure can effect changes in the content and composition of plant primary metabolites, is an eminently testable hypothesis. In order to demonstrate that plant defense is a "raison d'être," as it were, for quantitative or qualitative variation in the production of particular primary metabolites, certain conditions must be met: (1) primary metabolite content must be variable among potential host plants; (2) variation in primary metabolite content must be at least partly genetically based and thus available for selection; and (3) plant fitness (reproductive success) in the presence of the herbivore must be associated in a predictable way with that genetically based primary metabolite variability (Haukioja et al., 1991).

If these three conditions are met, then the potential exists for insects, by differential herbivory, to effect changes in the content or composition of primary metabolites in their host plants. There is one other criterion to consider as well. Insect herbivores may act as selective agents on plant primary metabolism only indirectly if variation in the content of a particular primary metabolite is genetically correlated with variation in the content of a secondary metabolite, which is the trait under direct selection, for example. Direct selection can act efficiently on plant primary metabolites only if those metabolites are themselves responsible for a change in herbivore physiology or behavior that in turn alters plant fitness.

Although it may seem a daunting task to identify particular nutrients likely to respond to herbivore pressure, it is probably less daunting than identifying particular allelochemicals likely to respond to herbivore pressure, at least in part because there may be fewer primary metabolites of ecological relevance to insects in plants than there are secondary metabolites. According to Fraenkel (1969), "Thirty to forty such substances [necessary nutrients] (according to whether one counts the amino acids individually, and making allowance for the discovery of new factors) are involved in the nutrition of insects"; by contrast, essential oils of individual plants may contain hundreds of terpenoid and phenylpropanoid constituents (Guenther, 1948). The task may be simplified further because many phytophagous insects appear to have similar nutritional requirements. All phytophagous insects, for example, have an absolute sterol requirement, although there is variation in the extent to which different plant sterols can be utilized (Chapman, 1972; Grunwald and Kogan, 1981). Although carbohydrates occur in many forms throughout the plant kingdom, simple sugars

tend to be of greater importance for folivorous insects than are complex sugars or starches (Dadd, 1963). Although not universal by any means, the dietary requirement for ascorbic acid is practically a defining trait for phytophages, among other insects (as is the case in mammals, in which frugivorous species and other species with high dietary intakes of ascorbic acid have apparently lost the capacity to synthesize it). All insects also share a dietary requirement for unsaturated fatty acids, which, for phytophagous insects, is generally satisfied by unsaturated C_{18} fatty acids, particularly linolenic acid (Thompson, 1973).

Proteins and their constituent amino acids are among the primary metabolites most likely to be influenced by insect herbivory. The protein content and composition of plants are dramatically different from that of herbivores and, in fact, this difference is widely regarded as a major nutritional barrier to utilizing plant food (Hinton, 1976). Most phytophagous insects have an absolute requirement for the same 10 amino acids that are required by the vast majority of animal species: these are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Chen, 1966). According to Chen (1966), some insects contain levels of amino acids upwards of 30 times higher than levels recorded from other animals. Why their amino acid demands are so great is unclear—amino acids appear to play a role in osmoregulation, in detoxification (e.g., conjugation with glycine for export), and in energy conservation. Certain activities may impose higher amino acid demands on certain insects, relative to other species. The parsnip webworm *Depressaria pastinacella*, for example, invests approximately 20% of its nitrogen intake into the production of a silken web. The amino acid composition of the silk is radically different from that of parsnip fruits, the principal food of the webworms; levels of glutamic acid, serine, and glycine in silk far exceed proportionately the levels in fruits. In turn, the composition of silk is radically different from that of the body of the webworm itself (Figure 1). The quantities of histidine, arginine, and methionine, all essential amino acids, are notably low

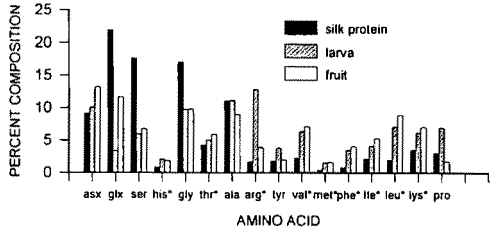

FIG. 1. Amino acid composition of parsnip webworm silk, webworm bodies with silk glands removed, and green fruits of wild parsnip. Amino acids essential for insects are marked with an asterisk (Rock, 1972).

in parsnip fruits relative to the amino acid profile of the webworm. While larval body contents may not necessarily reflect dietary needs, these disproportions between insect and plant proteins may impose a considerable nutritional stress on the insect and leave it vulnerable to deficiencies in its food plant.

The effects of variability in the amino acid composition of protein on plant suitability to insects have been demonstrated by Broadway and Duffey (1988), who found a 16-fold variation in larval weights of *Spodoptera exigua*, depending on the source of dietary protein. The most significant influence on larval growth rates was amino acid profile (in particular, the content of lysine + arginine and of sulfhydryl-containing amino acids). As storage molecules, many plant proteins appear to be effectively physiologically inactive—it is only when they are hydrolyzed to component amino acids that they enter into plant primary physiological processes (Mothes, 1980). It seems likely, then, that the amino acid composition of these storage proteins could vary among genotypes with little physiological impact on the plant. Moreover, genetic variations is known to exist for protein concentration (Moser and Frey, 1993; McFerson and Frey, 1991; Hansen et al., 1992; Sullivan and Bliss, 1983; Caradus, 1992) and composition (Axtell, 1981) and thus would potentially be available for selection by herbivores.

Evidence also exists that there is a predictable association between quantitative variation in amino acid content and plant resistance to insects in some systems. Maltais and Auclair (1957) demonstrated that *Pisum sativum* lines resistant to *Acyrtosiphon pisum* were 11.5–37.1% lower in amino acids and 16–47% higher in free sugars (such that the ratio of sugar to nitrogen in resistant lines was 23–64% higher, a critical factor for a phloem-feeding insect that relies on high throughput for extracting sufficient amounts of protein from a protein-poor food resource). Auclair et al. (1957) also demonstrated that amino acid content tended to be higher in susceptible varieties; Weibull (1994) showed that *Hordeum vulgare* resistance to *Rhopalosiphum padi* is associated with variation in glutamic acid content in the sense that low glutamic acid levels are associated with reduced aphid weights. Amino acid imbalance was also implicated by Janzen (1977) in the inability of *Callosbruchus maculatus* to thrive on diets high in tryptophan, cystine, and methionine, relative to levels in their normal host *Vigna unguiculata*. While these studies involve effectively sedentary specialists [stipulated by Moran and Hamilton (1980) as particularly prone to nutritional defenses], Waldbauer et al. (1984) have shown that in at least one more mobile species, *Heliothis* (=*Helicoverpa*) *zea*, insects can detect and respond to protein deficiencies in their diet by self-selection and avoidance of nutritionally inadequate food.

The content and composition of plant sugars appear to play an important role in resistance of plants to insects as well. In studies of Douglas fir, *Pseudotsuga menziesii*, resistance to *Choristoneura occidentalis*, Clancy (1992)

showed in artificial diet studies that optimal sugar concentrations were about 6% and survivorship actually declined when sugar levels exceeded this amount even slightly; natural levels range from 5.7 to 18.4%, thus potentially exceeding optimal concentrations by a large margin. Excess amounts of sugar may hinder growth because its processing diverts energy away from growth (Waldbauer et al., 1984). In this system, sugar composition plays a role as well; Zou and Cates (1994), upon noting that 78% of total sugar in Douglas fir was galactose, determined that 6% galactose increased mortality and decreased growth relative to comparable amounts of glucose and fructose in diets. Sugar content and composition are known to be under genetic control in certain crop plants (Simon et al., 1982, Freeman and Simon, 1983; Poehlman, 1979) and have been extensively modified by artificial selection (Stommel and Simon, 1989).

Of all plant primary constituents, vitamins are probably among the most variable in terms of distributive and quantity. Ascorbic acid content, for example, differs by an order of magnitude or more among cultivars of squash, peppers, tomatoes, muskmelons, apples, pears, and mangoes (Mozafar, 1994), and β-carotene varies by almost two orders of magnitude among varieties of carrots, sweet potato, and cassava (Mozafar, 1994). Many cases can be cited of successful breeding programs designed to increase the vitamin content of fruits or foliage of crop plants (Bittenbender and Kelly, 1988). There is at least one example of an association between vitamin content of plant tissue and susceptibility to an insect herbivore—ascorbic acid content of fruits is positively correlated with amount of damage inflicted by *Heliothis armigera* (Singh et al., 1982, cited in Mozafar, 1994)—and numerous other studies demonstrate an association between ascorbic acid content and resistance to microbial pathogens. Felton and Summers (1993) have cogently argued that ascorbate oxidase, a plant enzyme that can convert ascorbic acid into the unstable and potentially toxic oxidized derivative dehydro-L-ascorbic acid in the insect gut, is effectively a defense-related protein in that it renders ascorbic acid unusable by the insect as a dietary nutrient or antioxidant. Low levels of ascorbic acid in plant tissues, independent of the activity of ascorbate oxidase, may serve a similar defensive function. That phytophagous insects are capable of responding behaviorally as well as physiologically to vitamin imbalances in their food was demonstrated by Schiff et al. (1988), who showed that *Heliothis zea* larvae can self-select optimally between diets complete except for lipids or vitamins.

INTERACTIONS BETWEEN PRIMARY AND SECONDARY METABOLITES

In that a herbivorous insect almost inescapably ingests both primary and secondary metabolites when it feeds, the potential exists for toxicological and and physiological interactions between these classes of compounds. Examples

of mitigation of allelochemical toxicity by primary metabolites are numerous. High levels of protein, for example, can reduce the toxicity of trypsin inhibitors of glycoalkaloids (Slansky, 1992); antioxidants such as vitamins A, C, and E can reduce the phototoxicity of photosensitizing allelochemicals (Aucoin et al., 1990; Green and Berenbaum, 1994). Allelochemicals can also exacerbate the effects of low nutrient concentrations. Diet dilution can elicit compensatory feeding behavior, which in the presence of a toxin can lead to ingestion of a lethal dose (Slansky and Wheeler, 1992). In addition, the vitamin content of the diet can influence the detoxification capacity of an herbivore; in other systems, for example, there have been suggestions that vitamin C inhibits or otherwise alters metabolic transformations catalyzed by cytochrome P-450 (Sinclair et al., 1993; Suzuki et al., 1993), the principal enzyme system involved in insect detoxification of plant secondary metabolites (Brattsten, 1992).

BENEFITS AND COSTS OF NUTRIENT-BASED DEFENSE TO THE PLANT

One conceivable advantage to a plant of a defense system based on altered levels of plant primary metabolites is that autotoxicity may not pose as great a risk as it does in defense systems based on secondary metabolites. Secondary metabolites often target fundamental physiological processes, and, as such, absent a system for localization or sequestration, pose as much of a danger to the plants producing them as they do to insects consuming them. Presumably, systems for the rapid synthesis or degradation of primary metabolites are already in place to meet the physiological demands of the plant; such systems may require little modification to permit plants to store comparatively high levels of a nutrient that, if ingested by a herbivore, due to its differing physiological needs, could create a lethal nutritional imbalance. Nutritional defenses can be expected to arise most frequently in those cases in which the nutritional needs of plant and animal diverge most dramatically. Nutrient-based defenses would be unlikely to arise in developing seeds, for example. Developing seeds utilize plant nutrients in much the same way that heterotrophic organisms do, whereas photosynthetic source tissues can synthesize needed components. Indeed, seedling success in terms of competitive ability, growth rate, or germination rate is often correlated with nutrient content of the seed (Varis and George, 1985; Rahman and Goodman, 1983; Parrish and Bazzaz, 1985).

Even in those cases in which plant and animal nutritional requirements differ dramatically, plant nutrient content cannot decrease to such a point that plant function is impaired. Plants require proteins, lipids, carbohydrates, vitamins, and micronutrients to grow and reproduce, and reductions in the amounts of these constituents may compromise a plant's physiological capabilities at the same time they decrease attractiveness to herbivores. To date, there is abundant

evidence of the impact of quantitative variation in plant elemental nutrients such as nitrogen on photosynthesis (Field and Mooney, 1986; Sinclair and Horie, 1989) and seed phosphorus on seedling success (Austin and Longden, 1965; Bolland and Paynter, 1990; Zhang et al., 1990), but little information exists as to whether similar limitations exist with respect to primary metabolites synthesized by the plant.

All things considered, low nutrient value is unlikely to evolve as a defense under many, possibly even most, circumstances, and it is certainly unlikely to be selected for by herbivores that can compensate for low nutrient content by increasing consumption rates (in the absence of toxins) or by herbivores whose susceptibility to predators or pathogens is unaffected by growth rates or nutritional state. However, low nutrient value may well be selected for by highly oligophagous herbivores with physiological requirements for certain nutrients that differ dramatically from the requirements of their host plants or with limited mobility such that they must complete development on a single plant, or even a single plant part, with no opportunity to compensate for nutritional deficiencies by switching host plants. Primary metabolites acting as nutrients under these circumstances may be most effective in conferring resistance against herbivores in the presence of toxic secondary metabolites, in that such toxins may offset compensatory feeding responses by increasing toxicity as intake increases (Slansky and Wheeler, 1992). There are undoubtedly innumerable plant–insect relationships in which these conditions pertain, but in the absence of any systematic investigation of either the primary metabolite content of host plants or nutrient requirements of herbivores it is impossible to speculate whether nutrient-based defenses are rare or common.

APPLICATIONS

Demonstrations of the efficacy of nutrient-based defense have implications beyond theories of insect–plant interactions. There is currently tremendous interest in developing novel mechanisms to reduce pest problems in agroecosystems and at the same time reduce chemical inputs (both synthetic and natural) without reducing yields. Because humans do not feed exclusively on a single plant species for sustenance, as do many phytophagous insects, selective breeding for altered nutrient levels may lead to increased resistance to insects in the absence of chemical inputs with little or no adverse impact on human consumers (Slansky, 1990). This strategy has already been exploited in controlling pests of stored products that, because they are consumed by humans, are not ideally protected with synthetic organic insecticides. Prepared food, such as pudding mixes, have been formulated such that key vitamins, fatty acids, and other insect nutrients are in short supply; this strategy has proved successful in causing mortality of

flour beetles (Mansingh, 1981). Whether such a strategy will work under field conditions, for a suite of herbivores that may have differing nutrient requirements, remains to be seen.

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BIOACTIVITY OF BEAVER CASTOREUM CONSTITUENTS USING PRINCIPAL COMPONENTS ANALYSIS

BRUCE A. SCHULTE,[1,*] DIETLAND MÜLLER-SCHWARZE,[1] RONG TANG,[2] and FRANCIS X. WEBSTER[2]

[1]*Department of Biology*
[2]*Department of Chemistry*
State University of New York
College of Environmental Science and Forestry
Syracuse, New York 13210

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Abstract—North American beaver (*Castor canadensis*) were observed to sniff from the water and make land visits to some synthetic chemical components of castoreum placed on experimental scent mounds (ESM). In previous analysis, the elicitation (presence/absence), completeness, and/or strength (number, duration) of these key responses served as separate measures of biological activity. In this paper, we used principal components analysis (PCA) to combine linearly six related measures of observed response and one index of overnight visitation calculated over all trials. The first principal component accounted for a majority of the variation and allowed ranking of the samples based on their composite bioactivity. A second PCA, based only on response trials (excluding trials with no responses), showed that responses to the synthetic samples, once elicited, did not vary greatly in completeness or strength. None of the samples evoked responses as complete or strong as the castoreum control. Castoreum also elicited more multiple land visits (repeated visits to the ESM by the same individual or by more than one family member) than the synthetic samples, indicating that an understanding of the castoreum chemosignal requires consideration of responses by the family unit, and not just the land visit by the initial responder.

Key Words—Beaver, *Castor canadensis*, castoreum, communication, neutral compounds, phenolic compounds, principal components, social odors, territory.

*To whom correspondence should be addressed at Department of Biology, Providence College, Providence, Rhode Island 02918-0001.

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INTRODUCTION

The North American beaver (*Castor canadensis*) is a large, monogamous rodent that lives in family units. A beaver family typically consists of an adult pair and two to four offspring from two years of births (Bergerud and Miller, 1977; Novak, 1977; Svendsen, 1980b, 1989). Beaver are primarily nocturnal and are considered territorial. Through the construction of mud mounds marked with a chemically complex secretion from their castor sacs, called castoreum, beaver may advertise their defended area. This functional role for scent marking by beaver has been supported by two types of studies. First, resident beaver will destroy and often overmark scent marks from nonresident beaver (Hodgdon, 1978; Müller-Schwarze and Heckman, 1980; Svendsen, 1980a). Second, vacant beaver sites marked with castoreum by human researchers are colonized to a lesser degree than unmarked sites (Welsh and Müller-Schwarze, 1989). Thus, castoreum appears to play an important role in intraspecific communication between individuals from different family units. The roles that particular chemicals serve in this territorial signal are not understood.

The chemical composition of castoreum has been examined (Walbaum and Rosenthal, 1927; Stevens, 1943; Lederer, 1946, 1950; Valenta et al., 1960) but not in conjunction with biological investigations. In separate behavioral bioassays, progressive fractionation of castoreum evoked steadily decreasing responses from beaver (Müller-Schwarze et al., 1986). The phenolic and neutral components of castoreum elicited responses, but the amines and carboxylic acids did not (Svendsen and Huntsman, 1988). Recently, we initiated a combined study of the chemical make-up of these active fractions of castoreum (Tang et al., 1993, 1995) and the biological activity of the identified components (Müller-Schwarze and Houlihan, 1991; Schulte et al., 1994).

Our behavioral assay focuses on the response of resident beaver to foreign scent marks placed within their home site by humans. Resident beaver will sniff the experimental scent mound (ESM) from the water, go on land to the mound, and sniff it. Beaver may then reenter the water or paw at the mound, walk over it, and often deposit their own castor fluid on the ESM (Hodgdon, 1978; Bollinger, 1980; Müller-Schwarze and Heckman, 1980; Svendsen, 1980a; Houlihan, 1989; Schulte, 1993). Behavioral responses are composed of several stages (i.e., "sniff from water," land visit, overmarking), and vary in their likelihood of occurrence, as well as their duration, number of responses, and the number of beaver in a family that respond during one trial. Therefore, creating a single measure of biological activity by which to compare different chemical compounds and mixtures (broadly referred to as samples) is problematic. In a companion paper (Schulte et al., 1994), we presented the analysis of the individual measures of response to 10 different single compounds (nine phenolic compounds and one neutral compound, borneol) present in castoreum (Tang et al.,

1993, 1995) and 10 mixtures, composed of 4–26 compounds. Using these same data, we present the results and interpretation of our multivariate analysis of bioactivity.

Multivariate analyses have been used to study complex chemical communication systems (Smith et al., 1985; Natynczuk, 1990; Singer et al., 1992; Sunnerheim-Sjöberg and Hämäläinen, 1992). Our application involved creating one or two uncorrelated response variables from seven intercorrelated measures of beaver responses to synthetic chemical samples. These uncorrelated variables would provide a relative measure of overall bioactivity among the samples.

Our second objective in this study was to integrate repeated responses by one individual and responses by more than one animal of a social group into our measure of bioactivity. Social odors (or components thereof) that evoked repeated responses by one individual and/or responses by more than one member of the social unit may be particularly important elements for communication. Signals sent between social units may have both multiple senders and multiple recipients. For instance, beaver mounds may be marked by more than one family member (Hodgdon, 1978), and different age/sex classes of beaver may potentially receive this signal. Therefore, a measure of response made at the individual and the family level was incorporated into our data compilation.

METHODS AND MATERIALS

The field methods were described in detail in an earlier paper (Schulte et al., 1994) and are only summarized here. A more complete description is provided for the format of the data and statistical analyses.

Study Area, Field Procedures, and Chemical Samples. All bioassays were conducted during the months of May–September during 1989–1992 at 16–20 beaver sites each year in Allegany State Park, Cattaraugus County, New York State. The beaver population at Allegany State Park has been unharvested for 20 years. The average number of beavers per site over the four years was 5.5 (SD = 2.9). Beavers have been live-trapped, ear-tagged, and observed since 1985. Captured beavers were sedated; sexed; ear-tagged; weighed; measured for body length, girth, and appendage dimensions; and assigned to an age class (adult, 2-year-old, 1-year-old, kit) based on their weight and size. As of spring 1992, 245 beavers had been processed and individually marked using this procedure. During observations, untagged beavers were identified by distinguishing physical marks and assigned to an age class based upon their relative size.

Over the four years of field bioassays, 10 different single compounds (one at four concentrations) and 10 different mixtures of compounds (three at two concentrations) were tested for biological activity. Each of these compounds has been identified as a major component of castoreum (Tang et al., 1993, 1995).

The chemical identity of single compounds, concentration assayed, and composition of mixtures are shown in Table 1. The ratio of compounds in mixtures was based on their ratio in a sample of 16 pooled, male beaver castor sacs (Tang et al., 1993). Ethanol was used as the solvent for all samples. An experimental scent mound (ESM) was constructed (30 cm diameter, 10 cm high) approximately 50 cm from water's edge using mud from the pond bottom. A size 18 cork was placed in the center of the mound, and the sample was applied to the cork with a disposable pipe. As done by Svendsen (1980a), half a tongue depressor (applicator) was placed adjacent to the cork in order to detect subtle disturbances to the mound overnight. The placement of the applicator does not interfere with beaver inspecting or re-marking the mound. Observations began immediately after building the ESM at approximately 17:00 hr and ended with fading daylight (20:00 to 21:30 hr except in September 1989, 19:00–20:00 hr). The behaviors of all beavers were recorded continuously with a Tandy 102 portable computer. When one or more beavers approached the sample, observations were focused on these individuals. The responses by beaver to a single ESM with one applied sample were observed such that a trial was equivalent to a single-day, single-site assay. Each sample was tested at 8–16 different beaver sites in a given year.

Except for the neutral compound, borneol, and the controls, all tested compounds were phenolics. Castoreum was presented either dissolved in ethanol (1989) or in a dried, ground form known as quill (1990–1992). Responses to these two forms of castoreum did not differ significantly. A control using ethanol only on the ESM was included each year. Blank ESMs with no sample applied were used as an additional control in 1991 and 1992.

In addition to direct observations, the ESM was checked the next morning for signs of visitation during the night, then removed and destroyed. These signs were divided into four categories as follows: (1) intact; (2) paw prints on an otherwise undisturbed ESM; (3) scratch marks, displacement of the applicator, or removal of the cork; and (4) flattening of, or fresh castoreum odor on the mound. For the purposes of data analysis, a trial was assigned to only the category of most complete response. Except when overmarked with castoreum by beaver, the observer could detect the sample's odor on the cork during this inspection.

Measures of Response. Sniffing from the water and land visits were measured in three ways: response elicitation (yes or no), completeness, and strength. A complete response was defined as a flattening/overmarking of the ESM and numerically assessed by determining the proportion of trials in which a complete response was observed for each sample. Response strength was measured by the duration and number of responses and proportion of beavers responding. In 68% of the trials, the beaver did not respond at all, even though they passed near the samples. This high percentage of zero values affects the measures of completeness and strength of sniffing and land visits. Since many zero values

TABLE 1. CONTROLS AND SYNTHETIC SAMPLES FROM BEAVER CASTOREUM, ABBREVIATIONS, AND ASSAY YEAR AT BEAVER SITES IN ALLEGANY STATE PARK, NEW YORK, 1989–1992

	Name	Abbreviation	Assay year
A.	Single compounds (mg/trial)	s	
	Acetovanillone	AC	
	Borneol (0.56; 0.48)	BO	1990;1992
	2-Catechol (0.014)	CT	1989
	2,6-Dimethoxy-4-methylphenol (0.11)	DC	1990;1992
	3,5-Dimethyoxyphenol	DP	
	4-Ethylguaiacol (0.83)	EG	1990
	4-Ethylphenol (0.69)	EP	1989
	4-Ethylphenol (0.032)	EH	1990
	4-Ethylphenol (0.32)	EH	1990
	4-Ethylphenol (3.2)	EH	1990
	4-Ethylphenol (32)	EH	1990
	4-(4′-Hydroxyphenyl)-2-2-butanone	HB	
	Indanol	IN	
	m-Cresol (0.023)	MC	1989
	4-Methylguaiacol (0.033)	MG	1990
	o-Cresol (0.11)	OC	1989
	4-Propylguaiacol (0.025)	PG	1990
	Phenol	PH	
	4-Propylphenol (0.37)	PP	1989
	Salicylaldehyde (0.025)	SA	1989
B.	Mixtures[a]	m	
	EG + MG + PG (3 guaiacols)	G	
	BO + G (borneol + 3 guaiacols)	BG	1990
	OC + MC + CT + SA (4 phenols)	FP	1989
	FP + EP + PP (6 phenols)	SP	1989
	SP + PH + HB + IN + AC (10 phenols)	TP	1990
	TP + G (13 phenols)	TG	1992
	TG + BO (13 phenols + borneol)	TBG	1990;1992
	TG + DC (14 phenols)	P	1991;1992
	P + BO (14 phenols + borneol)	PB	1992
	BO + 11 other neutrals[b]	N	1991
	P + N (1 : 1 ratio)	PN	1991
	P at 5-fold concentration	5P	1991
	N at 5-fold concentration	5N	1991
	5P + 5N (1 : 1 ratio)	5P5N	1991
C.	Controls	c	
	Castoreum (expected maximum response)	CA	1989–1992
	Ethanol (expected minimum response)	OH	1989–1992
	Blank (no applied odor)	BL	1991;1992

[a] Assayed amounts of mixtures were 1/30 gland equivalents, except for three fivefold concentrates, with the ratio of compounds based on those in 16 pooled, male castor sacs (Tang et al., 1993).

[b] The 12 neutral compounds were borneol, benzaldehyde, benzyl alcohol, 6-methyl-1-heptanol, *trans*- and *cis*-linalool oxide, 4,6-dimenthylheptane-1-ol, *trans*-pinocarveol, isopinocamphone, verbenone, (1*R*)-myrtenol, and 3,4-dimethyoxy-acetophenone.

not only lower the means for the responses, but also result in means that primarily reflect elicitation, we analyzed the data first with, and then without, the "no response" trials. For instance, in a hypothetical example, if a sample was assayed for 16 trials and in 10 trials beaver did not respond, then $N_1 = 16$ (all trials) and $N_2 = 6$ (only trials with responses).

In 1992 beavers passed within 5 m of the ESM in 58.6% of the no-response trials. Within this distance directed sniffing towards the ESM was easily observed when it occurred. The lack of sniffing or land visits for these trials appeared to indicate true absence of a response. In 20.7% of the trials, the ESM was less than 15 m from the active lodge under favorable weather and wind conditions for detection by beavers. Hence, in at least 79% of the trials with no responses in 1992 beavers probably could detect the odor source, but failed to respond to it visibly. Distance of detection will vary depending on wind direction, velocity, humidity, and other factors.

Format of Data and Statistical Analysis. We used six response measures from the observations and an index of response completeness for the overnight visitation data. Two types of responses were observed: sniffs from the water that were directed towards and within 5 m of the ESM, and land visits to the ESM. Three response variables for each response type were generated: average duration and average number of responses per trial, and the proportion of the total number observed of beaver responding. Only land visit durations from the first beaver visit per trial were used for the analysis. This was done because scent deposition by the first beaver may affect the likelihood or duration of response by subsequent beavers. The average response per trial was calculated for all six measures, first for all trials, then for only those samples and only those trials with a response (sniff from the water, land visit, or both). In addition, two overnight indices were generated: The first is the proportion of trials with any overnight response for each sample (overnight index 1). The second overnight index was the proportion of trials with overnight visits in which the ESM was flattened/overmarked, representing a complete land visit response (overnight index 2).

To create a single, relative measure of bioactivity, principal components analysis (PCA) was utilized. PCA does not test hypotheses, but it can be used to reduce multiple independent variables to one or a small set of variables (Dunteman, 1989). A first PCA used the mean responses to each sample for the three sniff-from-water and three land-visit variables for all trials along with overnight index 1. The mean of each response variable for each sample was used to prevent confounding of the within- and between-group variation (James and McCulloch, 1990). This PCA included all of the samples assayed. The responses to each control (castoreum, ethanol, blank) were combined across years. The four concentrations of 4-ethylphenol tested in 1990 were treated as one sample (EH, Table 1) because previous analysis indicated no significant

differences among the concentrations (Schulte et al., 1994). A second PCA was performed using only those samples that evoked sniffs from the water, observed land visits, and/or flattening/overmarking of the ESM during overnight land visits (index 2) in at least one trial each. Only 17 samples fulfilled this criterion. Plots of the first two principal components for each PCA were made to spatially separate samples based on their relative bioactivities.

Because beaver live in family units, responses at the level of the social unit were measured as "observed multiple land visits." A multiple visit occurred when: (1) one beaver visited an ESM more than one time, or (2) more than one beaver visited an ESM in a single trial. A contingency table was formed from a subset of these data. These categories are not mutually exclusive, and the number of times both types of multiple visits occurred during a single trial was also tabulated.

The Statistical Analysis System (SAS, Cary, North Carolina) and the BIOM Package of statistical programs (Rohlf, 1987) were used for all data analyses.

RESULTS

Responses to All Samples for All Trials. The principal components analysis successfully reduced the number of response variables conducive to characterizing the general bioactivity of the samples. The proportion of trials with an overnight land visit (index 1), the three sniff-from-water and the three observed-land-visit variables were linearly combined to yield a first principal component that accounted for 75% of the variation in the data (Table 2). The correlation of the response variables with the first principal component (the component loading) was high for each variable. Therefore, principal component 1 may be interpreted as a general measure of response. The second component, accounting for 12.6% of the variation, contrasted sniff duration and number with the observed land visits.

The relative bioactivity of the samples can be determined by comparing the distribution of samples along the first component axis (Figure 1). Note that the scales of the two axes are not identical and the position of the samples along the first component is the most relevant in terms of bioactivity. As expected, the castoreum control has the largest positive value (high activity) along component 1. The ethanol and blank ESMs, along with several other samples, are positioned toward the negative (low activity) end of component 1. The 26-compound mixtures (5P5N and PN) and the high concentrations of the 14-phenolics (5P) and 12-neutrals (5N) mixtures are somewhat separate from the other samples along component 1. A second group is evident, comprised of the lower concentration of the 14 phenolics (P91; P92) and 14 neutrals (N), a four (FP) and a six (SP) phenolic mixture, and two single compounds, 4-ethylphenol

TABLE 2. PRINCIPAL COMPONENT ANALYSIS OF CONTROLS AND SYNTHETIC SAMPLES KNOWN FROM BEAVER CASTOREUM FOR ALL TRIALS (RESPONSE AND NO RESPONSE): LOADINGS AND CUMULATIVE PERCENT VARIANCE EXPLAINED[a]

Variables	PC 1	PC 2
Overnight index 1	0.78	0.25
Sniff		
Duration	0.84	0.37
Number	0.81	0.46
Response proportion	0.92	0.17
Land visit		
Duration (first)	0.90	−0.35
Number	0.90	−0.38
Response proportion	0.89	−0.41
Cumulative variation (%)	75.0	87.6

[a]Only two factors were retained by the mineigen criterion in SAS. Principal component 1 (PC 1) correlates positively and highly with all the response variables and is interpreted as an index of responsiveness [PC 1 = response (+) to no response (−)]. PC 2 contrasts "sniff from water" number and duration (+) with the observed land visit responses (−). A plot of the first two canonical variables is shown in Figure 1. The response variables are described in the text.

(EP) and 4-methylguaiacol (MG). The remaining samples are rather tightly grouped, although their positions along the first component would permit a linear ranking based on overall bioactivity. The spread among the samples along the second principal component is exaggerated in Figure 1 because of the scale, but sniffing from the water relative to low levels of observed land visits was most prevalent to the six-phenol (SP) mixture and 4-ethylphenol (EP).

Responses to 17 Samples for Response Trials Only. In an attempt to rank the samples based upon the strength (observed variables) and completeness (overnight index 2) of the elicited responses, a second PCA was performed using means calculated from response trials only (Figure 2).

Of extreme interest is that of the original 32 samples, only 17 elicited sniffing from the water, observed land visits, and overnight flattening/overmarking of the ESM in at least one trial (but not necessarily the same trial). Most of the retained samples are positioned to the right (more active) of the dropped samples along component 1 in Figure 1 (retained samples are demarcated by an asterisk in Figure 1). For instance, the blank (BL) mounds never elicited an overnight visitation in 29 trials over two years. While the ethanol (OH) control did elicit all three types of responses, these never occurred together during the same trial or even in the same year.

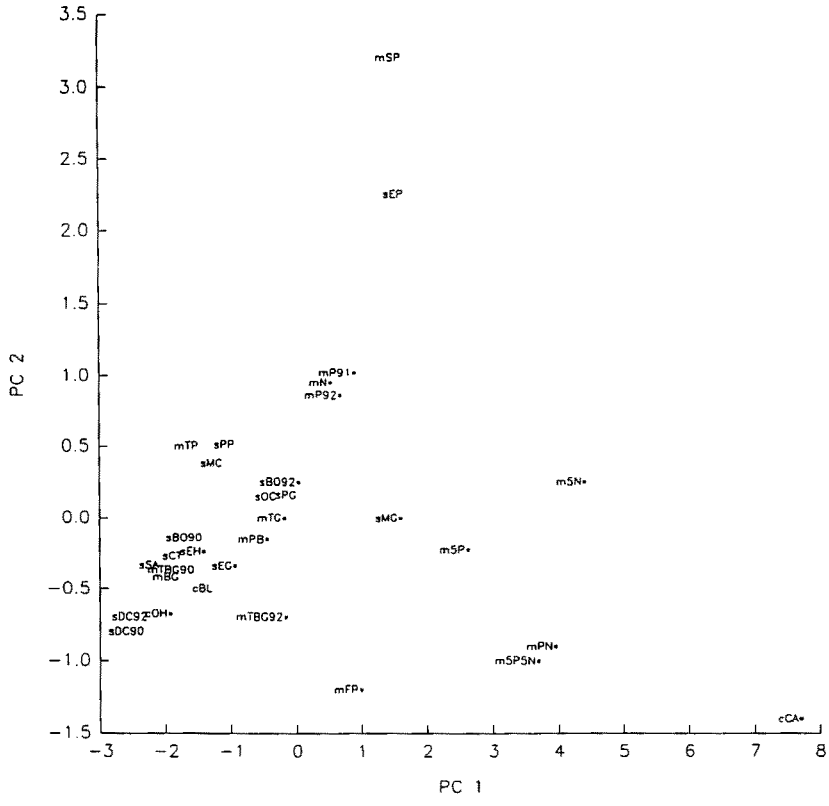

FIG. 1. Plot of first two principal component canonical variables: controls and synthetic compounds and mixtures, 1989–1992, all trials. The location of each sample is at the center of its abbreviation (see Table 1 for key to abbreviations of samples). The lowercase letter (s = single compound; m = mixture; c = control) designates the type of sample. The asterisk demarcates those samples that met the response criterion and were retained for the second PCA (Figure 2). Principal component 1 (PC 1) correlates positively with all seven response variables and represents an index of responsiveness. PC 2 contrasts sniff from water number and duration (+) with the observed land visit responses (−) (Table 2).

PCA was performed with the 17 samples that satisfied the response requirement. The six observed response variables and the values from overnight index 2 were linearly combined to yield seven principal components. The first two components accounted for 60.5% of the variation in the data (Table 3). The third component explained an additional 12.7% of the variation, but only two

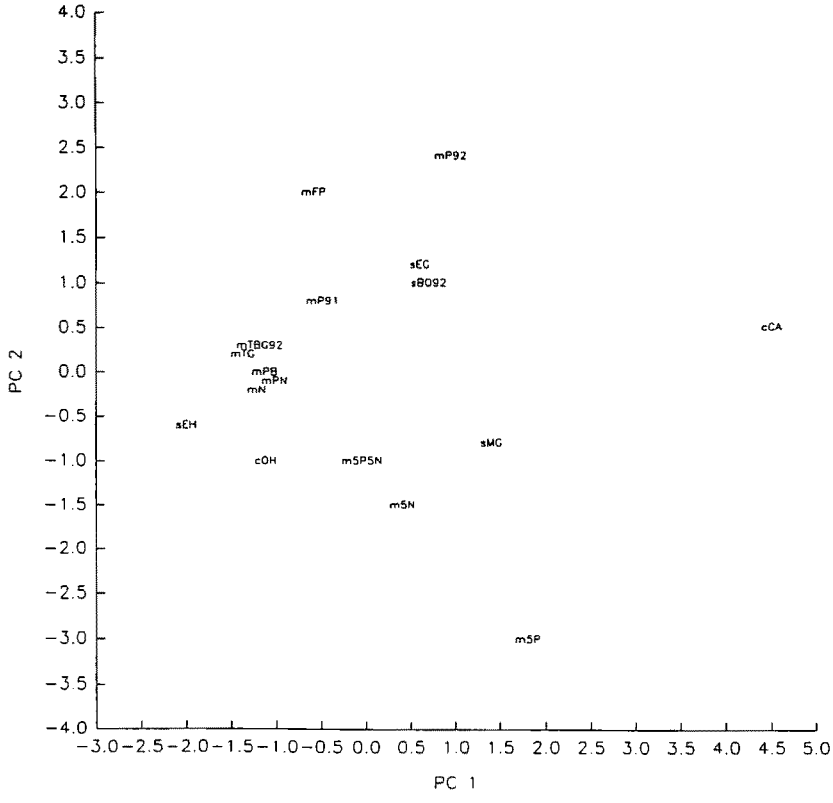

FIG. 2. Plot of first two principal component canonical variables: controls and synthetic compounds and mixtures, 1989–1992, response trials only. The location of each sample is at the center of its abbreviation (see Table 1 for key to abbreviations of samples). The lowercase letter (s = single compound; m = mixture; c = control) designates the type of sample. Principal component 1 (PC 1) correlates positively with all seven response variables and represents an index of response strength. PC 2 contrasts sniff from water proportion and overnight visitation (+) with the observed land visit duration and number and with sniff duration (−) (Table 3).

components were retained using the mineigen criterion in SAS. The correlations between the components and the response variables (loadings) permitted interpretation of the components. Principal component 1 was positively correlated with all of the response variables, especially the six observed measures (Table 3). This axis can be interpreted as an index of response strength, from weak to strong. Component 2 contrasts overnight visitation and the proportion of beaver

TABLE 3. PRINCIPAL COMPONENT ANALYSIS OF ONLY THOSE CONTROLS AND SYNTHETIC SAMPLES KNOWN FROM BEAVER CASTOREUM THAT ELICITED SNIFF, LAND VISIT, AND OVERNIGHT RESPONSES: LOADINGS AND CUMULATIVE PERCENT VARIANCE EXPLAINED[a]

Variables	PC 1	PC 2
Overnight index 2	0.12	0.67
Sniff		
Duration	0.55	−0.40
Number	0.59	0.29
Response proportion	0.46	0.68
LV		
Duration (first)	0.64	−0.52
Number	0.77	−0.42
Response proportion	0.78	0.39
Cumulative variation (%)	35.5	60.5

[a]Two components were retained based on the mineigen criterion in SAS. Principal component 1 (PC 1) correlates positively with all the response variables and represents a continuum of response strength from weak to strong. PC 2 contrasts overnight flattening/overmarking of the ESM and sniff proportion (+) with land-visit duration and number and with sniff duration (−). A plot of the first two canonical variables is shown in Figure 2. The response variables are described in the text.

that sniffed from the water (+) with land visit duration and number, and sniff duration (−). Of importance in this second PCA is the comparably low percentage of variation explained by only two components (60.5% vs. 87.6% in the first PCA) and the more even contribution of both the first (35.5%) and second (25%) components in explaining this variation.

The spatial distribution of the samples along these two components is shown in Figure 2. The castoreum control was distinct from all the samples along component 1, the index of response strength, and centered along component 2. Many of the less-active samples (OH, EH, TG, TBG92, PB), as determined from their location on component 1 in Figure 1, are located toward the less active (−) end of component 1 in Figure 2. However, the lower concentration of the 26-compound mixture (PN) and the 12-compound neutral mixture (N) are grouped with these largely inactive samples in Figure 2. The high concentration of the 26-compound mixture (5P5N) is spatially quite distant from castoreum (CA) in Figure 2 compared to Figure 1.

Multiple Land Visits. Most samples were visited only once per trial (Table 4). Beavers that visited the ESM treated with castoreum returned to the ESM in 15 of all 45 trials (33.3%), and in 15 of the 28 trials (53.6%) with an

TABLE 4. MULTIPLE OBSERVED LAND VISITS (LV) TO ESM BY BEAVER AT ALLEGANY STATE PARK IN FIELD BIOASSAYS OF CONTROLS AND SYNTHETIC SAMPLES KNOWN FROM BEAVER CASTOREUM[a]

Sample[b]	>1 LV by same beaver	>1 beaver making LV	Both types of multiple visits	Occurrence (%) of any multiple visit
cOH, cBL	0	0	0	0 (68)
SGA[c]	0	0	0	0 (164)
sMG	0	1	0	7.7 (13)
sBO92	0	1	0	10 (10)
MXA[d]	0	0	0	0 (87)
mTBG92	0	1	0	10 (10)
mPN	0	1	0	11.1 (9)
mFP	0	2	0	25 (8)
m5P	2	1	1	22.2 (9)
m5N	1	2	1	22.2 (9)
m5P5N	1	2	1	22.2 (9)
cCA	15	20	13	48.9 (45)

[a]The values in columns 2–4 are number of trials. The values in the final column are percentages with the total number of trials for that row given in parentheses. Multiple visits occurred when one beaver visited the ESM more than one time (exit water to ESM, visit ESM, reenter water = one visit), or more than one beaver visited the ESM during the course of observations. The columns are not mutually exclusive and the final column's values may be greater than any value in the other columns (e.g., cCA 22/45 trials = 48.9%).

[b]Abbreviations explained in Table 1, where prefix s = single compound; m = mixture; c = control.

[c]SGA = all single compounds assayed (see Table 1), except those listed separately in this table.

[d]MXA = all mixtures assayed (see Table 1), except those listed separately in this table.

observed-land-visit response. The high concentration of the mixture of 14 phenolics (5P) had the next greatest "multiple visits by one beaver" value (22.2%, two of nine trials, but 66.7% of those with a land-visit response). Only two other samples, both mixtures (5P5N and 5N) and in both of these the higher of the two concentrations assayed, were revisited by the first beaver to make a land visit. In 20 trials at least two different beavers visited the castoreum ESM (44.4% of all trials and 71.4% of trials with land visit responses). Only two single compounds, 4-methylguaiacol and borneol, and six mixtures received visits by more than one beaver in a single trial. For castoreum, both types of multiple visits were recorded in 13 of 45 total trials (28.9%) and 46.4% of trials with a land visit. The three high concentrations of the mixtures (5N, 5P, and

5P5N) had one trial each of both types of multiple visits (11.1%). These were the only samples to have both types of multiple visits occur in the same trial.

DISCUSSION

Principal components analysis can be utilized to provide an overall picture of relative bioactivity of single compounds present in castoreum and their mixtures. The two PCAs performed in this study revealed important differences in the spatial distributions of the samples. When all trials (and all samples) were included in the analysis, the mean values for the six observed response measures were heavily weighted by the absence of responses (numeric value of zero). For many samples, there were no observed responses in a majority of the trials. Thus, the individual response measures, such as mean duration of first land visits, were influenced greatly by whether land visits were elicited at all. Yet, using the mean values, PCA provided a reasonable separation of the samples based upon their position along the first principal component (Figure 1), which was positively correlated with all of the response measures (Table 2). By evaluating only those trials in which responses were recorded, the sniff-from-water and land-visit variables now reflected response strength. Stronger responses were defined as those that lasted longer, occurred in greater number, and by a larger proportion of the beaver in the family. In the second PCA (Table 3), the first component is still an indicator of responsiveness, but it accounts for much less of the variation in the data than in the first PCA. The spatial distribution of the samples in this plot of the first two components (Figure 2) cannot be as easily interpreted in terms of their bioactivity.

Our previous analysis (Schulte et al., 1994) revealed that the 26-compound mixture elicited responses similar in completeness, yet weaker, and evoked less often than responses to castoreum; in general, mixtures elicited greater responsiveness than single compounds. This trend may be illustrated by comparing the presence/absence of mixtures and single compounds and their spatial positions in Figures 1 and 2.

The implication from the results of principal components analysis is that particular chemicals (and possibly mixtures relative to single compounds) may influence the likelihood of a beaver responding more than they affect the extent of the response elicited. A probable explanation is that single compounds or mixtures of interest to beaver trigger an investigatory response that shows little variation (the ethological notion of "typical intensity"). Only when more complete cues are available, such as from the castoreum control, are longer or more elaborate responses evoked. Therefore, it appears reasonable to examine separately and together the elicitation of general responses (e.g., land visits), the completeness of that response (sniff the ESM, paw, overmark), and the strength

of response (duration, number, proportion of beaver visiting). Here, we have attempted to combine these various measures of bioactivity, while in a companion paper we dealt with them separately (Schulte et al., 1994). Clearly, the operational definition of bioactivity is critical to its evaluation. Bioactivity, as defined by the likelihood of eliciting a response (see Figure 1), may differ from activity determined from the strength of the response (see Figure 2).

A further consideration in investigating the chemically complex structure of many mammalian chemosignals is targeting the key behaviors in the bioassay. For beaver, responses have been measured primarily at the level of the individual, and generally only the first response by an individual (Hodgdon, 1978; Svendsen and Huntsman, 1988; Müller-Schwarze and Houlihan, 1991). By also looking at repeated land visits by the same individual and land visits by the entire beaver family, we were able to evaluate the bioactivity of chemical components of castoreum. Multiple visits to any one sample were never observed in more than 25% of the trials. However, multiple visits to castoreum were observed in 49% of the trials. Thus, the synthetic samples, even the 26-compound mixture, appeared to lack components that would evoke a strong family-level response. Only the fivefold higher concentrations of the three mixtures elicited both types of multiple visits within a single trial (once each). A more thorough study of the effect of concentration of mixtures on the responsiveness of beaver is currently being planned.

Interestingly, multiple visits do not parallel ESM destruction or overmarking during the first visit. Beavers may overmark foreign, their own, or other family members' scent marks to eliminate the scent, to freshen the odor, or to create a family scent (Hodgdon, 1978; Houlihan, 1989). Other possibilities may also exist. In response to the ESM, once a resident beaver obliterated and/or overmarked the foreign scent, the ESM became another resident scent mound. If this stimulated further land visits by the same or other beaver, an endless succession of land visits could theoretically follow. This system apparently does operate in wolves (*Canis lupus*). In some situations fresh scent marks stimulated re-marking at greater rates than older marks (Mech and Peters, 1977).

Additional land visits by beavers, or re-marking by wolves, may be in response to the first visitor's behaviors and not the sample or scent mark's odor. Multiple land visits were not examined by Hodgdon (1978) based on this reasoning. In the current study, flattening or overmarking of the castoreum-marked ESM was observed in nine of the 22 trials with multiple visits. For the eight synthetic samples that elicited multiple visits (Table 4), flattening or overmarking was observed in six of the 12 trials with multiple visits. For the synthetic samples and castoreum, repeated land visits occurred even in the absence of overmarking or flattening of the ESM during the first land visit. This does not prove that family members are not responding to the ESM as a result of the first

beaver's visit, but they are certainly not always responding to a scent mark deposited by this first visitor.

Furthermore, flattening/overmarking of the ESM often transpired during an ensuing land visit. For castoreum, flattening/overmarking was observed in only nine of 28 trials with observed land visits. In another eight of these trials and in six trials in which land visits were not observed, the ESM was eventually flattened/overmarked. Why is scent marking not an immediate but commonly an eventual outcome when a beaver visits a foreign castoreum scent?

Two related explanations are offered. Following a land visit to the castoreum ESM, a beaver usually appeared excited. Typically, this individual would begin to swim rapidly around the perimeter of the pond (termed patrolling behavior). The beaver would usually be lost from view during much of this activity. Upon reappearance, the beaver would often revisit the ESM in a very directed manner. The beaver may have been searching for further signs of an intruder before overmarking the scent. A second possibility, not mutually exclusive of the first, may explain visits by additional beavers. Leaving the foreign scent intact, until other members of the family have investigated it, may be advantageous. This may alert the entire family to a stranger's presence and "identity." Scent matching (Gosling, 1982, 1986, 1990) could then occur if the strange beaver were encountered.

Thus, a scent mark by a social mammal may truly be a social odor both in its creation and its intended addresses. In the case of beavers, an appropriate measure of scent response behavior (and thus bioactivity) should be determined at the individual and family level, as was done in this study. This may be relevant in investigations of other mammalian social odors.

In cases where social behavior is regulated by chemical cues, it has to be determined whether species-wide compounds control specific responses, or multicomponent odor images (Albone, 1984) characterize individuals. The former are the traditional "pheromones," while the latter serve in recognition and scent matching. These alternatives may not be mutually exclusive. The chemical images in the secretions of social mammals may contain functional pheromones. Information about the individual may modify an otherwise typical response to a pheromone. Wilson (1975), in discussing the numerous substances found in the castoreum of beavers, stated, "although no behavioral function has yet been demonstrated, it is likely that a deliberate testing of the idea will reveal some of the substances to be pheromones" (p. 188). The continued research on the composition of beaver scent marks and the elicited responses may assist in the general understanding of signal structure in mammalian chemical communication.

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PHEROMONE-MEDIATED MATING IN THE APHID PARASITOID, *Aphidius nigripes* (HYMENOPTERA: APHIDIDAE)

JEREMY N. McNEIL[1,*] and JACQUES BRODEUR[2]

[1]*Département de biologie*
[2]*Département de phytologie*
Université Laval
Ste-1 Foy, P.Q., Canada G1K 7P4

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Abstract—Mating in the aphid parasitoid, *Aphidius nigripes*, is mediated by a female sex pheromone that stimulates both upwind flight and courtship behavior by males. There is no evidence of any overt behavior associated with the release of the pheromone, but bioassay results, using female extracts, suggest that the pheromone is produced in the abdomen, although it may be actively released from the entire body surface. Age does not appear to have a major influence on either pheromone production by the female or on male responsiveness. However, field data suggest the presence of a strong diel periodicity in the emission of and/or receptivity to the pheromone. Laboratory bioassay results indicated diel changes in the male response but more refined techniques are needed to determine if such periodicity exists in pheromone production by females. Following mating, female pheromone production is significantly reduced. The potential use of the parasitoid sex pheromone in integrated management of aphids is discussed.

Key Words—Aphid parasitoid, pheromone, mating.

INTRODUCTION

Mackauer and Völkl (1993) postulated that the inefficacy of primary aphid parasitoids is, to a large extent, associated with certain aspects of their foraging behavior. If true, then the rational deployment of infochemicals that modify foraging offers a possible approach for increasing the impact of aphid parasitoids on host densities. There are several avenues of aphid parasitoid chemical ecology

*To whom correspondence should be addressed.

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that could be exploited from a multitrophic perspective, particularly with respect to establishing parasitoid populations in agroecosystems early in the growing season before the pest populations become well established (Pickett et al., 1991; Lilley et al., 1994). Infochemicals of plant origin may have a significant impact on parasitoid foraging behavior (Vet and Dicke, 1992; Tumlinson et al., 1993), and there is evidence that this is the case for aphid parasitoids (Powell and Wright, 1992; Hardie et al., 1994; Pettersson et al., 1994). At the level of the herbivore, both aphid honeydew and the aphid sex pheromone may serve as kairomones for parasitoids (Bouchard and Cloutier, 1984, 1985; Budenberg, 1990; Hardie et al., 1991, 1994a; Budenberg et al., 1992; Powell et al., 1993; Lilley et al., 1994).

Aphidius nigripes (Ashmead), the most abundant of the parasitoids attacking the potato aphid, *Macrosiphum euphorbiae* (Thomas) in northeastern North America is not considered to have a significant impact on the density of its host (Shands et al., 1965) despite having many attributes of an effective biological control agent (Cloutier et al., 1981). Host–parasitoid asynchrony was proposed as an explanation for the low impact of *A. nigripes*, based on the assumptions that the parasitoid was unable to overwinter and immigrants arrived after the aphid populations had already become well established in the potato ecosystem (Spencer, 1926; Shands et al., 1965). However, recent research has clearly shown that *A. nigripes* enters diapause as a prepupa and is well adapted to survive the prevailing weather conditions in Québec, Canada (Brodeur and McNeil, 1989, 1994a). Furthermore, both parasitoid and host colonize potato fields at the same time in spring (Brodeur and McNeil, 1994a), indicating that asynchrony is really not a plausible explanation for the low impact that *A. nigripes* has on *M. euphorbiae*. More probable causes relate to parasitoid foraging and/or the levels of hyperparasitism (see Brodeur and McNeil, 1994b).

An interdisciplinary project on the chemical ecology of this multitrophic system has been initiated to determine the feasibility of using different infochemicals to increase the efficacy of *A. nigripes*. There is clear evidence that aphid honeydew influences *A. nigripes* female foraging behavior (Bouchard and Cloutier, 1984, 1985), and these studies, together with the work of Brodeur and McNeil (1994a), have demonstrated the existence of a female parasitoid sex pheromone. In the present paper we present a description of *A. nigripes* mating behavior, together with data from laboratory and field experiments examining certain aspects of female pheromone production and male receptivity. This work is the first step towards the identification of the female sex pheromone, which may be used to monitor parasitoid populations (Brodeur and McNeil, 1994a), attract males into the agroecosystem, and possibly modify foraging behavior if, as in certain Lepidoptera (Palaniswamy and Seabrook, 1978; Noguchi and Tamaki, 1985), females are able to detect and react to the pheromone of conspecifics.

METHODS AND MATERIALS

All insects used in these experiments were from laboratory colonies, established and renewed annually using *M. euphorbiae* and *A. nigripes* collected from commercial potato fields near Québec City. Aphids, kept on Norland variety potato seedlings, and parasitoids were reared at 20 ± 1°C, 60 ± 10% relative humidity under a 16L:8D photoperiod. The *A. nigripes* used in the experiments were obtained by exposing potato plants, infested with third-instar aphid nymphs, to parasitoids for a 24-hr period. Mummies were collected and held in individual gelatin capsules so that all adults were virgins, of known age, and, in the case of males, naive with respect to the female sex pheromone.

In order to eliminate any visual effects in pheromone bioassays, 1 μl of a methylene chloride extract of individual virgin females was applied to a 0.38-cm^2 filter paper disk, air dried for 20 sec to allow the solvent to evaporate, and then introduced into a 8-dram shell vial containing one virgin male. Two variables were measured in all laboratory experiments: the proportion of males that respond and the time that receptive males take to respond to the stimulus. Males were considered responsive if there was evidence of rapid wing-fanning (the first step in the courtship behavior) within 60 sec following the introduction of the disk. Unless otherwise stated, each extract was obtained by soaking an individual 0- to 24-hr-old virgin female in 100 μl of methylene chloride and testing 1 μl [0.01 female equivalent (FE)] on a minimum of 20 virgin, naive, 24-to-48-hr-old males during the first 3 hr of the photophase. Room temperature remained at 20–22°C for all experiments. Newly emerged females were used for extracts, as all previous laboratory observations showed that females generally mate soon after emergence, shortly after the onset of the photophase.

The first experiments were carried out to determine optimal extraction time by soaking one female in 100 μl of methylene chloride for periods of 10–600 sec. The highest response, and the shortest reaction time, was observed with 480-sec extracts (Figure 1), and all subsequent experiments were carried out using this extraction time.

Laboratory Work. A description of *A. nigripes* mating behavior was obtained by continuous observations of individual couples, comprised of virgin adults 0 to 48 hr old, held in #000 gelatin capsules. The duration of different components of the mating sequence were also obtained.

To determine if the pheromone originated from a specific zone or was released from the entire body, newly emerged females were sectioned, and extracts obtained from the head/thorax and abdomen were tested. Three replicates were carried out.

Variability in pheromone production by different individuals was examined by testing the level of response of males to the extracts of 15 different 0 to 24-hr-old virgin female extracts.

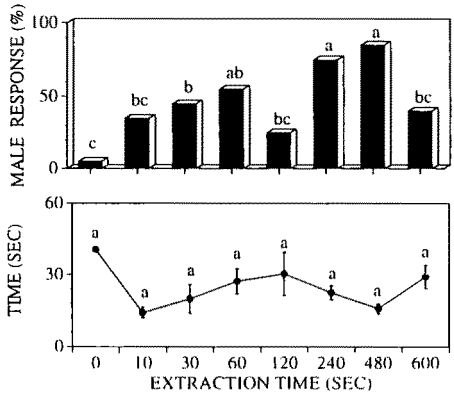

FIG. 1. The effect of extraction time on the proportion of *Aphidius nigripes* males responding and the reaction time of responding males to an 1-μl (0.01 FE) methylene chloride extract of 0- to 24-hr-old virgin females.

The diel periodicity of pheromone emission and/or male receptivity was examined by extracting 0 to 24-hr-old females 1 hr (09:00) and 7 hr (15:00) after the onset of the photophase. In this experiment, in contrast to all others, we used three females to obtain each extract, thereby minimizing any effect of individual variability in pheromone content. However, as in all other cases, bioassays were carried out using 0.01 FE. These times of extraction were chosen as copulations are most frequently observed soon after the lights-on signal. Each extract was tested at both hours of extraction, giving four combinations. Extracts to be tested at times other than at the actual hour of extraction were kept at −20°C until required.

The effect of female age on pheromone production was evaluated using extracts of individual 0-, 2-, 4-, 6-, 8-, 10-, and 12-day-old virgin females. A similar experiment was carried out to test the effect of age on male responsiveness to a female pheromone extract. Twenty 0-, 2-, 4-, 6-, 8-, and 10-day-old virgin males were tested using a 0.01-FE extract obtained from three 0- to 24-hr-old females. In the interval between emergence and time of extraction (females) or testing (males), individuals were held in isolated cages and provided 20% sugar water daily.

Field Work. Eight Delta sticky traps, each containing one newly emerged virgin female (Brodeur and McNeil, 1994a), were placed in a commercial potato field at Ste-Catherine, Québec, approximately 50 m apart and 40 cm above the crop canopy at 20:00 hr. The following day, trap catches were determined at 2 hr intervals from 04:00 to 22:00 hr (observations were terminated at sundown as preliminary experiments had indicated that no males were captured during

the scotophase) to determine if there was any diel periodicity in parasitoid mating. In addition, the total number of males captured by each female was noted, to compare the relative attractiveness of different females. This experiment was repeated over three days.

To examine the effect of mating on the attractiveness of females, eight Delta traps were placed in the field, four containing virgins and four with females mated in the 24 hr following emergence. The number of males captured by each female was recorded every two days over a 12-day period. In all cases, females were provided with a 20% sugar water solution.

Statistical Analyses. Data on the reaction time of receptive males and the number of males captured by different virgin females were analyzed by one-way ANOVA and means compared using the Tukey-Kramer unplanned test. For the comparison of different aged virgin and mated females, the data were analyzed using a two-way ANOVA. When necessary data were transformed ($\sqrt{X + 0.5}$ or $1/\sqrt{X}$) to stabilize variance. All values are presented as $\bar{X} \pm$ SE. The proportion of males responding was analyzed using chi-square and, in the case of significant differences, the contingency tables subdivided using the STP procedure ($\alpha = 0.05$).

RESULTS

In the presence of a virgin female, the first evident sign of short-distance male response to pheromone is a series of wing vibration bouts and an increase in general locomotory activity. The frequency of wing vibrations increases as the male approaches the female and is more or less continuous by the time contact occurred. The male contacts the female, usually from the side or from behind, and unreceptive females often fly away at this point. If she remains, the male mounts the female, moves forward, and grasps her around the thorax and abdomen with his fore- and mid-legs. He places his antennae between those of the female and moves them rapidly from side to side, bringing them repeatedly into contact with the female's antennae. There is also an up–down movement of the male's antennae, and on the downward stroke they touch the female on the head. If the female does not accept the male, she continues moving her antennae, flits her wings, and pushes the male with her hind legs. This is most often accompanied by the female lowering her head and curving her abdomen forward between her mid- and forelegs. In contrast, a receptive female lowers her antennae and holds them immobile lateral to her head, while raising her abdomen. When the female takes the acceptance position, the male moves backwards, curving his abdomen downwards to make contact with the female's genitalia. At this time his forelegs are either wrapped around the female's thorax or supported on her hind legs while his mid-legs are in contact with either the

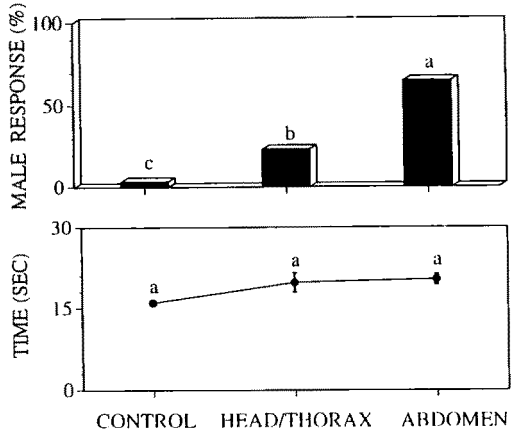

FIG. 2. The proportion of *Aphidius nigripes* males responding and the reaction time of responding males to an 1-μl (0.01 FE) methylene chloride extract from different body parts of 0 to 24-hr-old virgin females.

females wings or hind legs. In cases where the female was receptive ($N = 21$) the initial antennal interaction lasted 16.7 ± 0.7 sec and the actual copulation for 82.5 ± 2.0 sec. At the beginning of the copulation period, the male exhibits a rhythmic antennal movement, with his antennae coming into contact with the female's head at the base of her antennae, but for the last 41.7 ± 4.2 sec the male holds his antennae together, immobile above his head. Once mating has terminated, induced by the female walking forward and moving her antennae, the male dismounts and leaves.

The site of pheromone production would appear to be located in the abdomen (Figure 2). There was, however, some response to the head/thorax extract, and the mean reaction of responding males was similar for all extracts (Figure 2). There was considerable interfemale variability in pheromone production, as evidenced by differences in the proportion of males responding to the individual female extracts in the laboratory bioassay (Figure 3) and the number of males captured by different virgins in the field (Figure 4). The highest variability in the male reaction time in laboratory bioassays was observed with extracts from those females that elicited responses from the fewest males, although there was no evident trend between the proportion of males responding and the mean response time (Figure 3).

The proportion of males responding to female extracts changed with the time of the day that the bioassay was carried out, indicating the possibility of a diel periodicity in male receptivity to the female sex pheromone (Figure 5). The

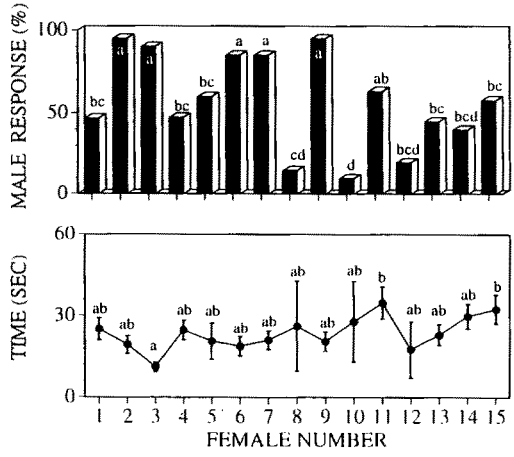

FIG. 3. The proportion of *Aphidius nigripes* males responding and the reaction time of responding males to an 1-μl (0.01 FE) methylene chloride extract from different 0 to 24-hr-old virgin females.

time of day at which the female extract was obtained did not have any significant effect on the results of the bioassay (Figure 5). There was clear evidence of a diel periodicity in the number of males captured by virgin females on the day when weather conditions permitted high levels of parasitoid activity, with the majority being caught between sunrise and 10:00 hr (Figure 6). On the two

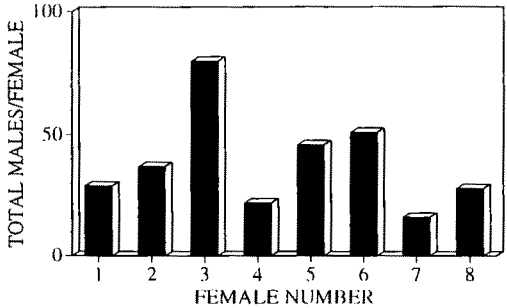

FIG. 4. The total number of *Aphidius nigripes* males captured in sticky traps baited with individual 0 to 24-hr-old virgin females during a 24-hr trapping period at Ste-Catherine, Québec.

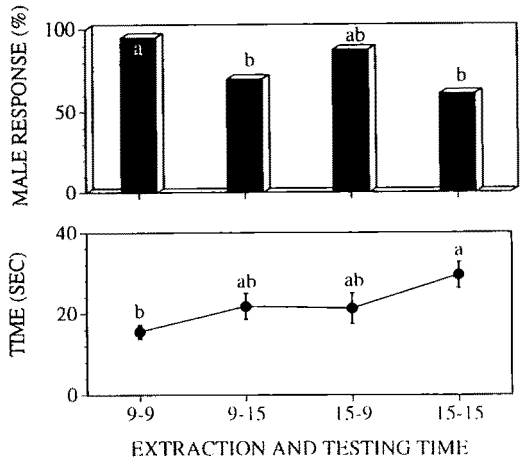

FIG. 5. The proportion of *Aphidius nigripes* males responding and the reaction time of responding males 1 (09:00) or 7 (15:00) hr after the lights-on signal to 1-μl (0.01 FE) methylene chloride extracts from 0 to 24-hr-old virgin females at the same hours. On the x axis the first value of each trial refers to the hour that females were extracted and the second to the hour that males were tested.

other days, there was nearly continuous rain, and while more males were captured in the early morning, the numbers were insufficient to carry out any meaningful treatment of the data.

In the laboratory bioassay, the attractiveness of extracts from different aged females remains reasonably high for the first eight days, after which there is a

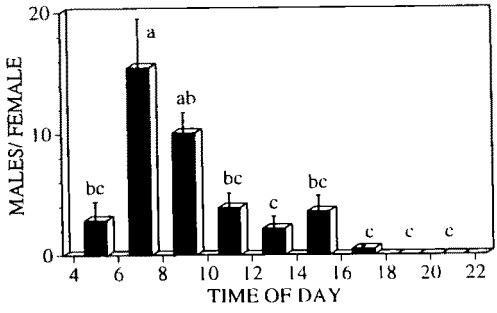

FIG. 6. The number of *Aphidius nigripes* males captured every 2 hr in sticky traps baited with individual 0 to 24-hr-old virgin females during a 24-hr trapping period at Ste-Catherine, Québec.

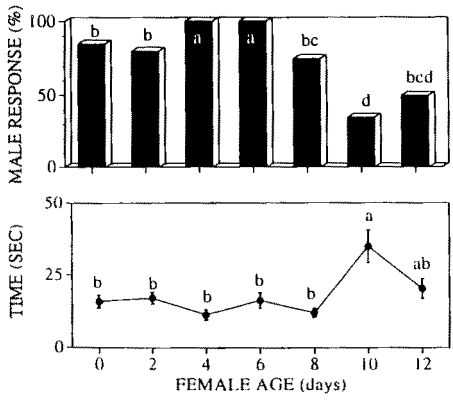

FIG. 7. The effect of female age on the proportion of *Aphidius nigripes* males responding and the reaction time of responding males to 1-μl (0.01 FE) methylene chloride extracts from virgin females.

slight decline, suggesting that pheromone production may decrease with age (Figure 7). This idea is supported by the slight increase in the mean reaction time of males to extracts obtained from older females (Figure 7). However, in the field, while there was considerable variability (probably the result of variable weather conditions), there was no significant age effect with respect to the number of males attracted to virgin females in the field (Figure 8). On the other hand, there was a significant decrease in female receptivity following mating ($F = 42.8$; $P = 0.0001$) with virgins attracting an average of 18.6 $\pm$ 2.4 males

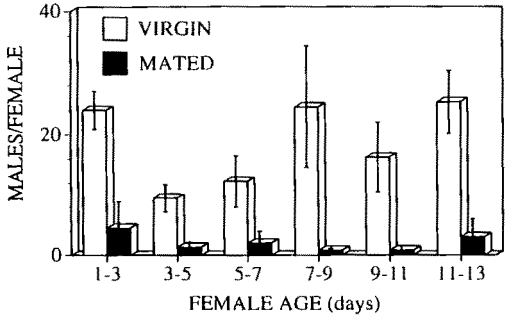

FIG. 8. The number of *Aphidius nigripes* males captured every 48 hr in sticky traps baited with either individual virgin or mated (once in the 24 hr following emergence) females over a 12-day trapping period at Ste-Catherine, Québec.

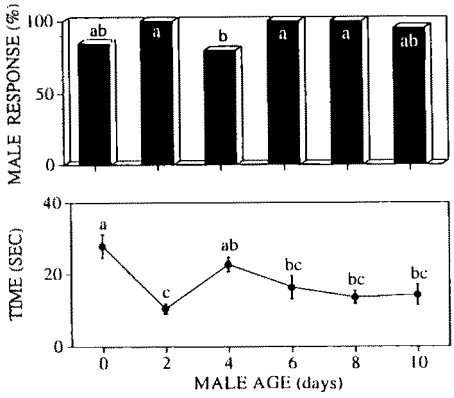

FIG. 9. The effect of male age on the proportion of *Aphidius nigripes* males responding and the reaction time of responding males to 1-μl (0.01 FE) methylene chloride extract from 0 to 24-hr-old virgin females.

per trapping period compared with 2.0 $\pm$ 0.9 by mated individuals during the whole period.

No consistent pattern of change was noted in the responsiveness of different aged virgin males to a standard female extract (Figure 9). Again, the differences observed in mean reaction times did not show any clear relationships to the number of males responding in the different assays (Figure 9).

DISCUSSION

The results of both field and laboratory experiments clearly demonstrate that mating in *A. nigripes* is modulated by a female sex pheromone, as reported for several other species of aphid parasitoid (Decker et al., 1993; Reed et al., 1994). It was clear from direct field observerations during our trapping experiments that males exhibit upwind flight towards virgin females placed in sticky traps and the bioassays show that at short distance the pheromone elicits male courtship behavior. There is no evidence of any specific behavior associated with the release of the pheromone, either a specific calling position or the extrusion of a specialized gland, as seen in the calling behavior of many Lepidoptera. This suggests that the pheromone may be released from the cuticular surface, as reported for the short-distance pheromone of certain flies (Carrière and McNeil, 1990) and the contact pheromone in the German cockroach (Schal et al., 1990). The strongest response was obtained with abdominal extracts, suggesting that this is the site of pheromone production, although release may

occur from the entire body surface, as there is some response to the head/thorax extracts. However, it is possible that the response elicited by the head/thorax extract may be the result of pheromone being moved from the abdominal surface to other parts of the body during the frequently observed female grooming behavior. Obviously, additional work is required to locate the sites of both pheromone production and emission and to determine the factors determining the high interfemale variability observed.

In the laboratory, males exhibit courtship behavior when in close proximity of freshly killed females. However, in the field, three sticky traps baited with live females captured 34.3 $\pm$ 2.7 males, while those containing dead females captured none. This would suggest that pheromone release is an active rather than passive process. There is, however, an alternative explanation: that there are two pheromones, one that serves to attract males over considerable distances and a second short-range one that elicits the actual courtship behavior. Once female specific compounds have been identified, this matter will be clarified.

The field results clearly demonstrated a diel periodicity with a peak early in the morning that coincides with the time that we observe most matings in the laboratory. Reed et al. (1994) also reported that the majority of *A. colemani* found in female-baited traps were captured before noon. While the bioassay data would suggest that this periodicity is mainly the result of changes in male receptivity, this conclusion should be viewed with a certain degree of caution. The extraction technique may remove pheromone from sites within the body (Blomquist and Dillworth, 1985; Carrière and McNeil, 1990) and thus not reflect the amount released by the female at the time extraction. Again, once the identification of the sex pheromone has been completed, then the question of periodicity will be resolved by quantifying both the amount of pheromone released by females and the response of males to known doses of pheromone at different times during the day. This will be examined under a range of different conditions, as it is very clear from our laboratory studies that mating success of pairs reared under identical conditions vary considerably from one day to the next. One possible cause is changing atmospheric pressure, as suggested for the spruce budworm (Miller and McDougall, 1973), a factor known to influence parasitoid oviposition behavior (Roitberg et al., 1993).

There appears to be no marked effect of age on either male receptivity or pheromone production by females, although in the laboratory bioassays a slight decline was noted in older females. Again, it is possible that the whole-body extraction technique used may mask age-related changes in the actual amount of pheromone released. However, based on the field trapping data, high pheromone production by virgins is maintained during the period of the female's life when oviposition is at its maximum (Cloutier et al., 1981). Mating results in a marked decrease in female attractivity and of the 49 males captured in traps baited with mated females, 46 were attracted to one female. Thus, further work

is required to determine the factors that modify pheromone production following mating and the duration of the refractory period. The effect of previous mating history on male receptivity must also be evaluated. The mean reaction time will also be reexamined, as the results of the present study suggest that this parameter remains relatively constant for males that respond.

As suggested by Decker et al. (1993), and demonstrated by Brodeur and McNeil (1994a), a female sex pheromone provides a potential means of estimating parasitoid populations. In addition, it may be used to enhance the impact of parasitoids on aphid populations. The sex pheromone of aphids appears to attract female parasitoids but not males (Hardie et al., 1994a), so its use in management may result in a very strong female-biased sex ratio if parasitoids are drawn in from the surrounding habitat. If this bias reduces the probability of females mating and results in a high production of male progeny, then there would be a lower number of effective biological control agents in the next generation. However, the use of the parasitoid sex pheromone simultaneously with that of the aphid may ensure that both sexes are attracted into the habitat, thereby increasing parasitoid efficacy. Obviously, as is the case with aphid sex pheromones (Hardie et al., 1994a), we need a much better basic understanding of the factors affecting both female production of, and male receptivity to, the sex pheromone of parasitoids such as *A. nigripes* before we can meaningfully evaluate their potential in management programs.

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MULTIFUNCTIONAL COMMUNICATION IN *Riptortus clavatus* (Heteroptera: Alydidae): CONSPECIFIC NYMPHS AND EGG PARASITOID *Ooencyrtus nezarae* USE THE SAME ADULT ATTRACTANT PHEROMONE AS CHEMICAL CUE

WALTER SOARES LEAL,[1,*] HIROYA HIGUCHI,[2] NOBUO MIZUTANI,[2] HIROAKI NAKAMORI,[3] TOSHIHIRO KADOSAWA,[4] and MIKIO ONO[5]

[1]*Department of Insect Technology*
National Institute of Sericultural and Entomological Science (NISES)
1-2 Ohwashi, Tsukuba-city 305 Japan
[2]*Kyushu National Agricultural Experiment Station*
Nishigoshi, Kumamoto 861-1 Japan
[3]*Okinawa Prefectural Agricultural Experiment Station*
4-222 Sakiyama-cho, Shuri, Naha-city 903 Japan
[4]*Chugoku National Agricultural Experiment Station*
6-12-1 Nishifukatsu, Fukuyama, Hiroshima 721 Japan
[5]*Fuji Flavor Co. Ltd.*
3-5-8 Midorigaoka, Hamura-city, Tokyo 190-11 Japan

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Abstract—The bean bug, *Riptortus clavatus* lays scattered eggs (as opposed to the egg masses of pentatomids) on host as well as nonhost plants. Therefore, the first feeding stage (second-instar) nymphs emerging from eggs laid on nonhost plants need a signal that enables them to locate a food source at the lowest energy cost. Male-released (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*Z*)-3-hexenoate, and myristyl isobutyrate play the double role of attractant pheromone for adults as well as aggregation pheromone, which enables the second-instar nymphs to find the host food plant. These male-specific semiochemicals are released only when foodstuff is available. On the other hand, females of *Ooencyrtus nezarae*, the most effective parasitoid of the host in Kumamoto, Japan (where the field experiments were conducted), utilize these semiochemicals as kairomones in order to locate the potential host community. Field experiments revealed that the synthetic pheromone rivaled 10 live males in the attraction of adults and second-instar nymphs. Captures of the egg parasitoid *O. nezarae* females in cylindrical sticky traps were signif-

*To whom correspondence should be addressed.

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icantly higher in traps baited with the synthetic semiochemicals than in control traps. The number of females captured was significantly higher than the number of males, although the captures in the sticky suction trap system revealed that the populations of male and female were not significantly different.

Key Words—*Riptortus clavatus*, *Ooencyrtus nezarae*, Alydidae, Encyrtidae, attractant pheromone, aggregation pheromone, kairomone, (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-hexenyl (*Z*)-3-hexenoate, myristyl isobutyrate.

INTRODUCTION

The bean bug, *Riptortus clavatus* (Thumberg), is an economically important soybean pest in Japan. Females lay single eggs on nonhost as well as host plants. The selective pressure of egg parasitoids probably contributes to this habit of scattering eggs. It is important to the first feeding stage (second-instar) nymphs to have a signal to locate a food source plant. Surveys conducted inside soybean fields indicated that the population of second-instar nymphs is much higher than both the number of eggs and the population of first-instar nymphs because second-instar nymphs moved from nonhost to host plants (Nakamori et al., 1994).

It has been demonstrated by field experiments that males induced aggregation of adult bugs (Kadosawa, unpublished data) and that this aggregation was semiochemically mediated (Numata et al., 1986). In the course of a study of the attractant pheromone of the bean bug, we found that the same semiochemicals are utilized by the second-instar nymphs to locate their host plants. In addition, the signal is used as a long-range kairomone by an egg parasitoid of the bean bug, *Ooencyrtus nezarae*.

METHODS AND MATERIALS

Chromatographic, Mass, and Infrared Spectral (MS, IR) Analyses

Gas chromatographic (GC) analyses were performed on a Hewlett-Packard 5890 equipped with one of the following capillary columns: DB-23 (30 m × 0.25 mm; 0.25 μm), DB-wax (30 m × 0.25 mm; 0.25 μm), or an HP-1 (12 m × 0.2 mm; 0.33 μm), operated in splitless mode either at 50°C for 1 min programmed at 4°C/min to 180°C, held at this temperature for 1 min, programmed again at 10°C/min to 230°C and held at this temperature for 20 min [in short, 50(1)-180(1)/4-230(20)/10 or 50(1)-210(10)/10]. Mass spectra were recorded on a Hewlett-Packard 5891 mass selective detector using the same type of capillary columns under the same conditions as described for the GC. This was operated either in the electron impact (EI) or chemical ionization (CI) mode

using ammonia. Gas chromatography–Fourier transform infrared (GC-FTIR) was done on a Hewlett-Packard 5965B equipped with a DB-23 capillary column operated at 50(1)-210(10)/8. The light pipe and the transfer line were operated at 250°C.

Gas Chromatography–Electroantennographic Detector (GC-EAD)

The responses of *R. clavatus* antennae were recorded with a GC-EAD system with silver wire electrodes (Struble and Arn, 1984). Separations were achieved on a DB-23, DB-wax, or HP-1 capillary column operated at 50(1)-210(10)/10, 50(1)-180(1)/4-230(20)/10, and 70(1)-170(1)/3-250(10)/10, respectively.

Extracts

The airborne volatiles of adult bugs confined in an all-glass aeration apparatus and fed on soybean seeds (or deprived of food) were collected on a Tenax TA column, eluted with hexane, and concentrated. Whole-body extracts of adults and nymphs were obtained by immersing at least five individuals in hexane for 3 min. Hexane extracts of soybean and horse bean seeds were prepared by macerating the seeds before immersing in hexane.

Isolation of Pheromone

Crude airborne extracts of male bugs were separated on a silica gel column (Wako C-200) by successively eluting with hexane–ether (100:0, 95:5, 90:10, 80:20, 50:50, and 0:100) mixtures. Further purification was done by collecting small fractions from a silica gel–silver nitrate (10%) column eluted with hexane–ether (95:5 and 90:10).

Chemical Derivatizations

Dimethyl disulfide (DMDS) adducts of the active unsaturated esters were obtained by reaction with dimethyl disulfide (DMDS, 100 μl) catalyzed by iodine. The mixture was stirred for 50–60 min at 65°C in a 1-ml conical vial containing a magnetic spin vane and closed with a Teflon-lined cap. After the mixture was cooled to room temperature, hexane (200 μl) was added, and iodine was removed by washing with sodium thiosulfate. The organic phase was dried over anhydrous sodium sulfate, concentrated to 50 μl, and analyzed by EI-MS using either a DB-wax or an HP-1 capillary column operated at 100(1)-230(20)/10 and 100(1)-230(30)/5, respectively.

Hydrolysis was done with potassium hydroxide in methanol at 60°C for 2 hr. The solvent was distilled off, the residue redissolved in ether, washed with

hydrochloric acid 2 M, dried over anhydrous sodium sulfate, concentrated, and analyzed by EI-MS.

Hydrogenation (catalyzed by PtO_2) was carried out in a 1-ml conical vial containing a magnetic spin vane and equipped with a Claisen head fitted with a rubber balloon and Teflon-lined cap.

Synthesis

(E)-*2-Hexenyl* (E)-*2-hexenoate.* To a solution of (*E*)-2-hexenoic acid (34 g) in dry benzene (90 ml), oxalyl chloride (75 g) was added dropwise at 45–50°C. After stirring at 65°C for 2 hr, the solvent was evaporated and the crude material was distilled to yield (*E*)-2-hexenoyl chloride (32.5 g; bp 62–63°C/20 mm Hg). This acid chloride (30.3 g) was added dropwise to an ice-cooled mixture of (*E*)-2-hexenol (20 g) and pyridine (23.7 g) in dry chloroform (100 ml). The reaction mixture was refluxed for 1 hr, cooled to room temperature, water was added, and the organic layer was washed with sodium carbonate, dried over anhydrous sodium sulfate, evaporated, and distilled to give (*E*)-2-hexenyl (*E*)-2-hexenoate (28 g, bp 79–80°C/0.7 mm Hg).

(E)-*2-Hexenyl* (Z)-*3-hexenoate.* (*Z*)-3-Hexenoyl chloride (bp 50–51°C/19 mm Hg) was prepared by the reaction of oxalyl chloride with the corresponding acid. The reaction of this acyl chloride with (*E*)-2-hexenol yielded (*E*)-2-hexenyl (*Z*)-3-hexenoate (bp 82–83°C/1.7 mm Hg).

Myristyl Isobutyrate. Reaction of myristyl alcohol with isobutyryl chloride gave myristyl isobutyrate (bp 127–128°C/0.7 mm Hg). The other esters were prepared in small scale by the reaction of acid chlorides and alcohols without solvent, followed by distillation of the products.

Insects

R. clavatus adults were collected in soybean fields and kept in cages (15 × 12 × 10 cm) in the laboratory (25°C, 70% relative humidity, and 16:8 LD photoperiod) and fed on a mixture of soybean and horse bean. Adults were transferred to other cages after a reasonable number of eggs were laid. Nymphs obtained from these eggs were kept together, but the emerged adults were segregated by sex.

Field Experiments

Preliminary field tests were conducted in Tsukuba (latitude 36°05′N, longitude 140°10′E) in the fall of 1992. Further evaluation of the synthetic attractant pheromone was carried out in Kumamoto (latitude 32°53′N, longitude 130°45′E) from September 7 to November 11, 1993.

The chemical lure (100 mg), incorporated into plastic pellets (4–5 mm in diameter) made of polyethylene–vinyl acetate, was placed in plastic cages (13 × 9.5 × 8 cm), set at 3 cm above the water surface of a hand-made, blue water-pan trap (16 cm × 35 cm ID). Insect baits were prepared with males (10 individuals) placed in plastic cages supplied with water and dry soybean seeds. Control and baited traps were randomly distributed around a soybean field inside Kyushu National Agricultural Experiment Station (Kyushu Noshi).

Kairomonal response of egg parasitoids to the synthetic attractant pheromone was investigated with cylindrical sticky traps made from two sticky plates (30 × 30 cm; Shin-Etsu Chemical Co., Tokyo) folded to form a cylinder (ca. 15 cm ID). Plastic pellets incorporated with the chemical lure (50 mg) were placed inside small plastic bottles (Fuji Flavor Co., Japan) and set on the top of the sticky cylinder traps. These traps were set on plastic plates placed randomly in the ground around a soybean field inside Kyushu Noshi. Number and sex of the egg parasitoids, *O. nezarae*, captured were determined under a binocular microscope. The population of the egg parasitoid was monitored in a nearby soybean field using a sticky suction trap system (Tokyo A&S Co., Tokyo).

Statistical Analyses

Field capture data were transformed to log $(x + 1)$ before differences between means were tested for significance by the Tukey-Kramer honestly significant difference test at a 5% level. Throughout this paper, treatments labeled with the same letters are not significantly different.

RESULTS AND DISCUSSION

Identification of Sex Pheromone

GC-EAD analyses of the airborne volatiles collected from *R. clavatus* males (using female or male antenna as a sensing element) showed the occurrence of four EAD-active peaks, which appeared not only on the polar columns, DB-23 (Figure 1) and DB-wax, but also on a nonpolar HP-1 column. After separation on a silica gel column, the four compounds were recovered in the hexane–ether (95:5) fraction. Peak I has been previously identified as (*E*)-2-hexenyl hexanoate, the alarm pheromone of the bean bug (Leal and Kadosawa, 1992) and was found in the airborne volatiles of male and female bugs. The other three EAD-active peaks (II, III, and IV) were detected in the (airborne or whole-body) extracts of male bugs, but they were not present in the female extracts or in the foodstuff. Collection of volatiles from the same group of males for a whole day with foodstuff followed by one-day collection without foodstuff (and vice versa) revealed that the three compounds were released only by males provided with

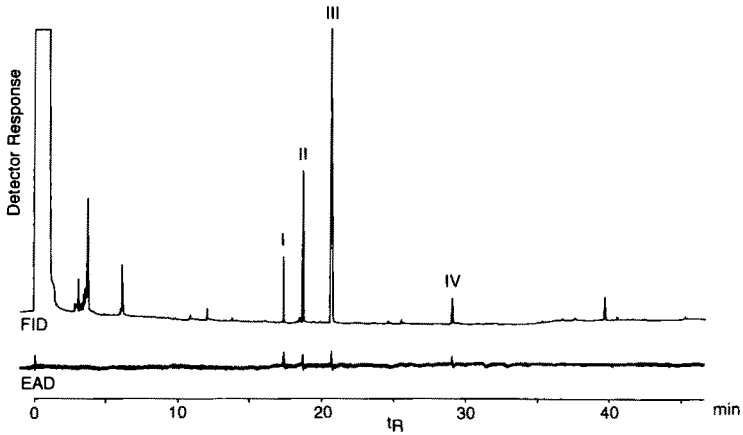

FIG. 1. Coupled GC-EAD response of female *R. clavatus* antenna to the airborne volatiles of male bugs separated on a DB-23 capillary column. The same response was detected with male antenna as a sensing element.

foodstuff. It is unknown, however, whether the bugs release the semiochemicals only when they are feeding. Compounds I, II, and III were not detected either by FID or EAD in the airborne extracts from starved males. These data suggested that compounds II, III, and IV were constituents of the attractant pheromone of *R. clavatus*.

EI-MS analyses of the male-specific, EAD-active compounds II, III, and IV gave their base peaks at *m/z* 55, 97, and 89, respectively (Figure 2). Although the molecular ion of compound II was missed in EI measurements, it was confirmed to be 196 from the quasi-molecular ion obtained at *m/z* 214 by chemical ionization with ammonia. Likewise, the molecular ions of compounds III and IV were corroborated to be *m/z* 196 and 284, respectively.

Hydrogenation of II and III gave hexyl hexanoate, suggesting that these two compounds were isomers. Vapor-phase IR (Figure 3) of III gave a profile of a conjugated ester (νC=O, 1740 and νC=C, 1655 cm^{-1}). The profiles of II (νC=O, 1755 cm^{-1}) and IV (νC=O, 1751 cm^{-1}) indicate that these compounds were nonconjugated esters. The fact that the C—H stretching band of IV was predominant over the carbonyl stretching band suggested that this ester had at least one long carbon chain.

Alkaline hydrolysis of II gave 2-hexenol and 3-hexenoic acid, whereas the products of the hydrolysis of III were 2-hexenol and 2-hexenoic acid.

EI-MS of the DMDS adduct of II displayed a base peak at *m/z* 89 along with the molecular ion M^+ 290, indicating that the unsaturation of the

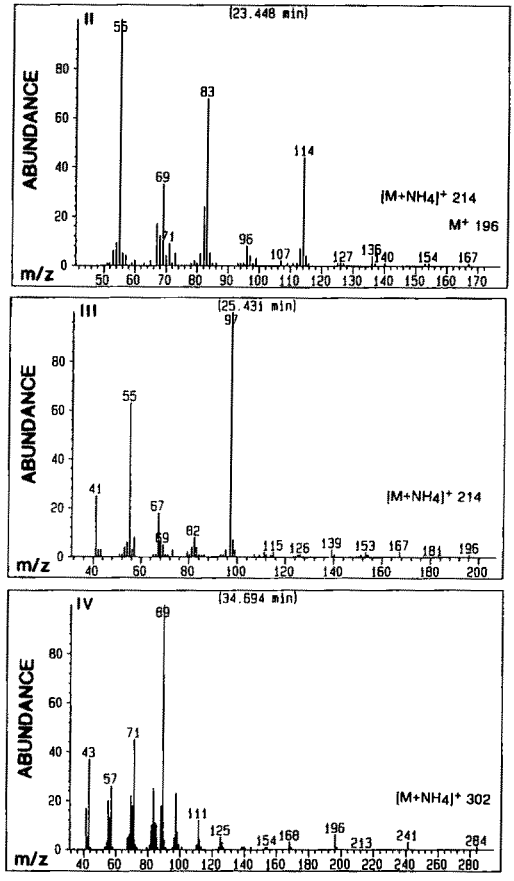

FIG. 2. Electron impact-mass spectra of the three constituents (II, III, and IV) of *R. clavatus* attractant pheromone. The quasi-molecular ions $[M+NH_4]^+$ obtained by chemical ionization with ammonia are also displayed.

acid moiety of II was in position 3 [$CH_3CH_2CHSMe^+$, *m/z* 89; $CH_3CH_2CH(SMe)CH(SMe)CH_2CO^+$, 191(5%)]. It remained to be determined what the configuration of the two double bonds was. The IR spectrum had a band characteristic of the *E* configuration, which suggested that at least one of the double bonds would be in that configuration. Synthetic (*E*)-2-hexenyl (*Z*)-3-hexenoate gave the same retention times as the natural product on three different capillary columns and showed identical MS. On the other hand, (*E*)-2-hexenyl (*E*)-3-hexanoate had longer retention times on polar capillary

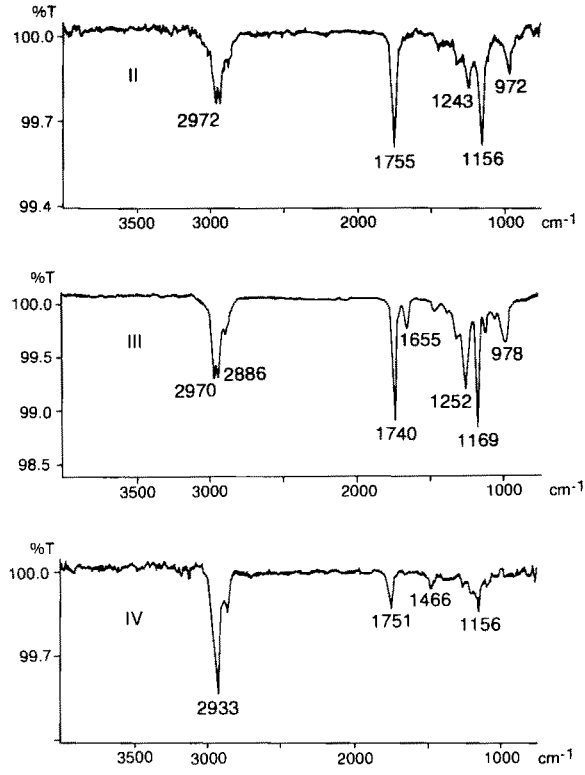

FIG. 3. Vapor-phase IR data for the three components (II, III, and IV) of the attractant pheromone system of *R. clavatus*.

columns and gave an MS with the base peak at *m/z* 55, but having a different profile: 114 (72%), 83 (55), and 69 (50). Therefore, compound II was identified as (*E*)-2-hexenyl (*Z*)-3-hexenoate.

The partial DMDS derivative of peak III gave the base peak at *m/z* 97 ($CH_3CH_2CH_2CH{=}CHCO^+$), the second peak (75%) at *m/z* 103 ($CH_3CH_2CH_2CHSMe^+$) and the molecular ion at M^+ 290. These data and the occurrence of an *E* configuration band (978 cm^{-1}) in the IR spectrum (Figure 3), suggested that compound III was (*E*)-2-hexenyl (*E*)-2-hexenoate. The synthetic ester was identical to the natural product in the retention times on three capillary columns and spectral data. Although (*Z*)-2-hexenyl (*E*)-2-hexenoate showed an MS profile very close to that of the natural product, it had a shorter retention time on polar capillary columns. Thus, compound III was identified as (*E*)-2-hexenyl (*E*)-2-hexenoate.

The facts that hydrolysis of IV gave myristyl alcohol, that IV did not undergo hydrogenation, and that the MS of IV had a base peak at *m/z* 89 (M^+ 284), suggested that this compound was either myristyl *n*-butyrate or myristyl isobutyrate. These two esters were almost indistinguishable from their MS, but myristyl *n*-butyrate showed much longer retention times on polar capillary columns, whereas myristyl isobutyrate was identical to the natural product. Therefore, the attractant pheromone of *R. clavatus* was identified as a mixture of (*E*)-2-hexenyl (*Z*)-3-hexenoate, (*E*)-2-hexenyl (*E*)-2-hexenoate, and myristyl isobutyrate, in short E2HZ3H, E2HE2H, and MI. These three compounds were found in an average ratio of 1:5:1.

Field Evaluation of Attractant Pheromone

Preliminary experiments in Tsukuba indicated that the major component (E2HE2H) alone was inactive and that there was no significant difference between catches of *R. clavatus* in traps baited with males and those in traps using the synthetic attractant pheromone mixture (100 mg) as a lure. Further experiments in Kumamoto corroborated that the attractancy of the synthetic attractant pheromone (E2HZ3H, E2HE2H, and MI: 1:5:1; 100 mg) to *R. clavatus* was not significantly different from that of 10 caged males (Figure 4). In addition, the field results with synthetic chemicals showed that there was a significant difference in catches of male and female *R. clavatus*.

Despite the fact that in the field male and female adults are attracted to the male-released pheromone of *Nezara viridula* (Harris and Todd, 1980; Aldrich et al., 1987), the semiochemicals involved are often referred to as "sex pheromones," probably because a correlation between attraction and mating was

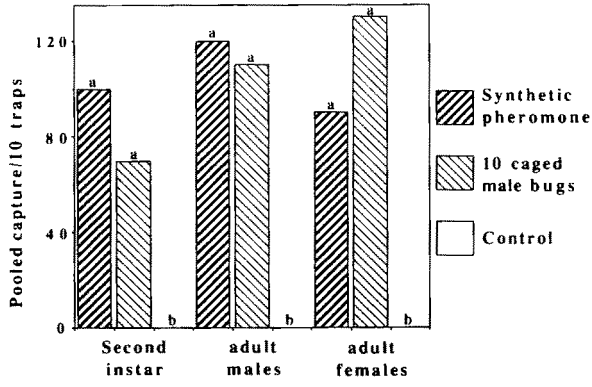

FIG. 4. Captures of second instars and male and female adults of *R. clavatus* in traps baited with synthetic attractant pheromone and 10 caged males (control = no lure).

found in the laboratory (Borges, 1987). Recently, it has been reported that the male-released volatiles act as a sex pheromone mediating mate finding and that aggregation observed in the field may be mediated by other factors (Brézot et al., 1993). The male-released pheromone system of *R. clavatus*, however, was confirmed with synthetic chemicals (as opposed to live insects or volatiles collected from them) to be an attractant pheromone.

Ecological Significance of Semiochemicals to Second Instars

Field tests showed that traps loaded with the synthetic mixture (E2HZ3H, E2HE2H, and MI; 1:5:1; 100 mg) were equally effective in attracting second-instar nymphs of the bean bug as male-baited traps. The significance of these chemicals to the ecology of *R. clavatus* is not limited to their involvement in the aggregation of male and female bugs, which is relevant for reproduction of the bean bug.

As this species lays eggs on host and nonhost plants, it is important to the first feeding stage (second-instar) nymphs to have a signal that enables them to locate a food source at the lowest energy cost. The facts that the attractant pheromone is only released by feeding males and specifically attracts the second instars (the other stages are not attracted) substantiate its involvement in food source location. Whether the later stages (third, fourth, and fifth instars) lose the ability to respond to these semiochemicals is not known, but nymphs in these stages may not need the information as they would have already reached the host food plant, or they would have starved to death.

GC-MS analyses revealed that none of these semiochemicals is present in the whole-body extracts of nymphs. In the extracts of first, second, and third instars only 2-(*E*)-octenal was detected, whereas fourth and fifth instars had a second component tentatively identified as 4-oxo-2-hexenal. These chemicals may be involved in the alarm and defensive systems of *R. clavatus* nymphs.

The use by the second instars of semiochemicals emitted by male bugs to locate host plants is an important feature of the biology of the bean bug. This is novel in the chemical ecology of hemipterans and seems to have evolved in response to selective pressure from the parasitoid. Due to the lack of terminology, we use the term "aggregation pheromone" to refer to the use of these semiochemicals in this unique context.

Kairomonal Activity of Attractant Pheromone

The possible attraction of egg parasitoids to the host's attractant pheromone was investigated in the field. In order to rule out the possibility that stimuli from bugs initially captured were used by egg parasitoids as cues for host location, we used cylindrical sticky traps in which the bean bug could not be captured. This type of trap, however, could catch the most effective egg parasitoid of the

bean bug in Kumamoto (Japan), *O. nezarae* Ishii (Hymenoptera: Encyrtidae). Experiments conducted from September 21 to October 17, 1993, revealed (Figure 5) that, although the numbers of male and female *O. nezarae* captured in sticky suction trap were not significantly different, significantly more female parasitoids were caught in the traps baited with the synthetic attractant pheromone (E2HZ3H, E2HE2H, and MI; 1 : 5 : 1; 50 mg) than in control traps. Catches of female *O. nezarae* were also significantly higher (5.5-fold) in the traps baited with the attractant pheromone than in the sticky suction trapping system. The synthetic bait was not attractive to male *O. nezarae*.

These findings confirm that the attractant pheromone of *R. clavatus* is utilized by females of the egg parasitoid *O. nezarae* as a long-range attractant kairomone. It is also likely that the bean bug has developed the strategy to lay eggs on nonhost plants to escape egg parasitoids. *O. nezarae* has evolved the ability to respond to semiochemicals produced by males that allow second instars to locate host food plants. Because the semiochemicals are released only by fed (or feeding) males, eggs laid on nonhost plants are probably less prone to attack by *O. nezarae* than those laid on host plants, unless the parasitoid can also detect other stimuli associated with females, hosts, or eggs.

Parasitoids have been reported to respond (as reviewed in Whitmann, 1988) to the host's body odor; the sex, epideitic, and aggregation pheromone; salivary constituents; excretory products; webbing; honeydew; body scales; and eggs. Recently, it has been reported that the egg parasitoid *Trissolcus basalis* utilizes a defensive substance produced by its host bug, *Nezara viridula*, as a long-range attractant kairomone (Mattiacci et al., 1993). The experimental evidence,

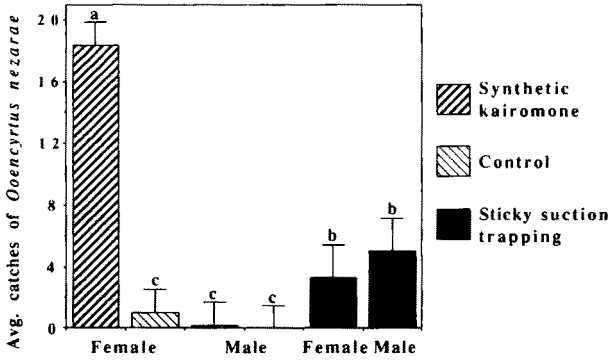

FIG. 5. Response of the egg parasitoid *O. nezarae* to the attractant pheromone of the host *R. clavatus* compared to catches of the egg parasitoid in a sticky suction trapping system.

however, for the long-range attraction is based only on the fact that female *T. basalis* spend significantly more time in the arm of an Y-olfactometer baited with the defensive substance. Attraction of parasitoids to the pheromone of host bugs is not novel. In fact, it has been previously reported that an egg parasitoid, *Telonomus* sp., was attracted to the male-released pheromone of the predatory spined soldier bug, *Podisus maculiventris* (Aldrich et al., 1984).

Field observations demonstrated that significantly more eggs of *Piezodorus hybneri* have been parasitized by *Telonomus triptus* when egg masses were placed close to caged adult bugs than egg masses alone (Higuchi, unpublished), leading to the conclusion that egg parasitoids are attracted to the vicinity of the eggs in response to a cue emanating from the host. Nevertheless, egg parasitoids usually appear later in the field than their hosts, and thus the host's first-laid eggs usually escape parasitoid attack (Higuchi, 1993). Therefore, the use of kairomones to synchronize the parasitoid population at the beginning of the host flight season may pave the way for the development of invaluable tools in integrated pest management programs.

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QUANTITATIVE HIGH-RESOLUTION GAS CHROMATOGRAPHIC DETERMINATION OF STEREOISOMERIC COMPOSITION OF CHIRAL VOLATILE COMPOUNDS IN THE PICOGRAM RANGE BY EC-DETECTION

ANN-BRITT WASSGREN and GUNNAR BERGSTRÖM*

Department of Chemical Ecology
Göteborg University
Reutersgatan 2 C, S-413 20 Göteborg, Sweden
(Received February 14, 1995; accepted March 10, 1995)

Abstract—A gas chromatographic method using electron capture detection for determination of the stereochemical composition of chiralolatile compounds, e.g., pheromones, at the picogram level is described. Substances of interest are analyzed as pentafluorobenzoate derivatives on fused silica capillary columns coated with CP Sil-88. This method was developed primarily for the quantitative analyses of stereoisomerism of the pheromone precursor, diprionol, in female sawflies, *Neodiprion sertifer*. It was possible to separate the stereoisomers showing (2*S*, 3*S*, 7*R*) and (2*S*, 3*S*, 7*S*) configurations. Resolution of the (2*S*, 3*S*, 7*S*)- and (2*S*, 3*R*, 7*R*)-diastereomers was high enough to allow the quantification of these stereoisomers. A quantitative analysis of the diprionol production in individual *N. sertifer* females yielded 7.5–9.7 ng/female.

Key Words—Chiral separation, HRGC, stereoisomers, diastereomers, enantiomers, electron capture detection, pentafluorobenzoates, pheromones, volatile compounds.

INTRODUCTION

Methods for identifying substances in biological material can be greatly facilitated by the conversion of a functional group to a suitable derivative according to the mode of determination. For example, detection of various phenols and carboxylic acids as pentafluorobenzoyl derivatives has been satisfactory using a tritium foil ECD, and analyses at trace concentrations have been successfully

*To whom correspondence should be addressed.

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performed by Kawahara (1968). Methods for determining the composition and concentration of carboxylic acids and phenols in water using derivatives have been described by Fogelqvist et al. (1980). Esterification with halogenated alcohols has been used for the identification of pesticides at nanogram levels (Satter and Paasivirta, 1979). The determination of trace amounts of different estrogens, in the form of heptafluorobutyric esters, has made it possible to measure picogram amounts by using ^{63}Ni ECD, according to Kern and Brander (1979).

Selective analysis of enantiomers of chiral compounds in biological material presents an interesting problem. In the oldest and most commonly used GC procedures for enantiomeric separations, an optically pure chiral reagent is employed to convert enantiomers into their diastereomeric derivatives, which are then separated on a nonchiral stationary phase. These methods were described by, among others, Ställberg-Stenhagen (1970). Today it is customary to perform determinations of enantiomeric compositions by chiral GC analysis on modified cyclodextrin phases, and in some cases it is possible to derivatize the compounds to trifluoroacetyl (TFA) derivatives (König et al., 1990). Separations of underivatized enantiomers and diastereomers on chiral metal chelate dissolved in an achiral stationary phase is described by Schurig and Weber (1984).

In the present report, pentafluorobenzoate was chosen as a derivatizing agent. It introduces an electron capturing moiety into the molecule, which provides good response to a ^{63}Ni detector and is suitable for gas chromatographic separation at the picogram level, as exemplified by the analysis of diprionol (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-pentadecanol, the pheromone precursor in *Neodiprion sertifer* (Geoffr.) (Hymenoptera: Diprionidae) females. Since some of the stereoisomers of diprionol occur in picogram amounts only, their determination requires sensitive methods. The gas chromatographic separation of diprionol has been performed earlier using two different methods, which we have described in Högberg et al. (1990). In both methods, the substances were derivatized, either to isopropyl carbamates or to benzoates. The isopropyl carbamates were separated on a chiral column CP-XE-60-(*S*)-valine-(*S*)-2-phenylethylamide (König, 1982; König et al., 1982) and detected by N/P detection (Wassgren and Bergström, 1984; Bergström, et al., 1992), while the benzoates were separated on a fused silica column coated with Sil-88 and detected by a flame ionization detector. Previously, we separated the diastereomers showing (2*S*, 3*S*, 7*R*)-configuration from the (2*S*, 3*S*, 7*S*) pair in synthetic diprionol as benzoates on a Sil-88 column, but problems arose when identifying small amounts in biological materials, i.e., in the picogram range (Högberg et al., 1990).

The objective of this work was to develop a convenient, sensitive method for the identification and quantification of the enantiomeric composition of diprionol from natural material in the picogram range and the quantification of the production of diprionol in single females. We think that this method may be of general interest in connection with separations of small amounts of many different chiral natural compounds.

METHODS AND MATERIALS

Biological Material. Females of *N. sertifer* were collected in 1991 in Värmland, Sweden, from two populations.

Chemical Materials. All synthetic references were synthesized in high stereochemical purity (see Högberg et al., 1990). The substances are the following: (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-tridecanol, (C_{15}-*SSS*), (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-tetradecanol, (C_{16}-*SSS*), (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-pentadecanol, (C_{17}-*SSS*), (2*S*, 3*R*, 7*R*)-3,7-dimethyl-2-pentadecanol, (C_{17}-*SRR*), (2*S*, 3*S*, 7*R*)-3,7-dimethyl-2-pentadecanol, (C_{17}-*SSR*), and (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-hexadecanol, (C_{18}-*SSS*). The solvents were of high analytical quality, containing no halogenated compounds. Ethyl acetate was obtained from American Burdick & Jackson, Fluka AG, Switzerland; pentane was distilled in our laboratory, and the water was Millipore-distilled. Other solvents and reagents, such as pyridine, methanol, pentafluorobenzoyl chloride, and isopropyl isocyanate were of Fluka analytical reagent grade. Chromabond polypropylene columns containing silica gel (SiOH) (Skandinaviska GeneTec AB), 1 ml volume, were used for sample preparation.

Preparation of Extracts. Single female *N. sertifer* were extracted in about 100 μl ethyl acetate at room temperature for 72 hr. The sample was transferred to a test tube and 50 ng of (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-hexadecanol was added as internal standard. In addition, two standards were made with 10 and 20 ng, respectively, of (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-pentadecanol, with 50 ng internal standard in each. Two blank samples containing only pentane were treated in a similar manner. The solvent was concentrated to about 20 μl by evaporation under a slow stream of nitrogen; the residue was then immediately dissolved in 50 μl pentane for subsequent liquid chromatography.

Liquid Chromatography. The cleanup was performed with solid-phase extraction on a Chromabond SiOH column (1 ml). The chromatography was initiated by wetting the adsorbent material with the eluent, in this case pentane; the material was then placed on the column and a gradient elution was applied (Wassgren et al., 1992) by increasing the polarity with 1–9% ethyl acetate in pentane, with 150 μl elution volume in each fraction. Fractions 7 and 8 were mixed in a small conical glass tube with a stopper before evaporation to dryness.

Preparation of Pentafluorobenzoates. The sample was dissolved in 3 μl of a mixture consisting of 2 μl pentafluorobenzoyl chloride in 20 μl pyridine, and the closed reaction tube was kept in a water bath at 60–70°C for 2 min. The solvent and excess reagent were then evaporated to dryness with water suction for 0.5–1 hr. The yellow residue was dissolved with 3 μl methanol, and 3 μl Millipore-redistilled H_2O was added. The pentafluorobenzoate was extracted twice with pentane (2 × 25 μl). The organic phase was washed twice with Millipore-redistilled H_2O, dried with Na_2SO_4, and concentrated to about 20 μl for GC analyses.

GC-ECD. Gas chromatography was performed with a Chrompack model CP-9000 gas chromatograph equipped with an electron capture detector, model 902A, which contains a foil of radioactive ^{63}Ni, a splitless injector, and a HP3396A integrator. The gas chromatograph was fitted with a 1.5-m × 0.32-mm-ID methyl deactivated silica retention gap, connected with a Quick-Seal column connector to fused silica columns coated with Sil-88 (Chrompack, the Netherlands) with the following dimensions: (a) 30 m × 0.15 mm ID, d_f = 0.24 μm; (b) 25 m × 0.25 mm ID, d_f = 0.22 μm; (c) 50 m × 0.25 mm ID, d_f = 0.22 μm. A setup of oxygen trap, molecular sieve, and charcoal filter was placed before the gas inlet. Separations of pentafluorobenzoates of the synthetic enantiomeric mixture of diprionol and the corresponding chiral homologs were carried out by tandem GC. The deactivated retention gap was fitted with a Quick-Seal column connector to the first fused silica capillary column Sil-88 (30 m × 0.15 mm ID). The second fused silica column was also coated with Sil-88 (25 m × 0.25 mm ID). Nitrogen was used as carrier gas at a pressure of 145 kPa, as well as make-up gas and purge gas at flows of 40 ml/min and 35 ml/min, respectively. The oven temperature were 250° and 275°C, respectively. The ECD was working in range 0, and attenuation 0.1.

Individual analyses of *N. sertifer* samples were also performed on Sil-88 columns, namely a 50-m × 0.25-mm column coupled to a 25-m × 0.25-mm column in series. The carrier gas pressure was 90 kPa, and the oven temperature 150°C.

RESULTS AND DISCUSSION

It has always been a special problem to isolate and identify biologically active components in extracts of whole insects. Such extracts, which are complex mixtures of volatile and nonvolatile compounds, usually require cleanup with solid sorbent extraction. This is a rapid isolation technique for compounds such as the alcohols present in pine sawflies. The recovery in this case, when we used the extract of an individual female, was 90%.

In earlier studies we presented the quantification of the amount of diprionol in extracts containing 20 females by using a flame ionization detector. This detector is not sufficiently sensitive to detect diprionol from single individuals or for determining possible homologs. We have therefore improved the method by using a selective electron capture detector (ECD), which is used for very sensitive detection of components with high electron affinity, especially halogenated hydrocarbons. The alcohols were transformed to pentafluorobenzoates with pentafluorobenzoyl chloride, which gives high sensitivity on ECD. The derivatizing procedure is quantitative, and it is therefore possible to perform the whole procedure *in vitro*; in this manner the loss of material is exceedingly small; recovery is 80% after the derivatization procedure. The reproducibility

and accuracy of this approach were determined in an experimental series of standards consisting of diprionol, 10 and 20 ng, respectively, with 50 ng internal standard in each. The pentafluorobenzoyl derivatives of diastereomericdiprionols were separated on commercially available high-polar fused-silica capillary columns, CP Sil-88.

The stationary phase unfortunately is not chemically bonded, since the relatively active cyanate groups (CN^-) do not allow cross-linking without vinyl groups. It is therefore necessary to inject a reference mixture at regular intervals, to check retention time, and carry out coinjection of the material with pure, known stereoisomers. This ensures that the stereoisomers elute at the same time. The uncoated retention gap avoids problems with solvent condensation that occur with splitless injection. One of the main problems we encountered was the occurrence of residual water after extraction, because water both distorts the chromatography and severely reduces the sensitivity of the ECD; anhydrous sodium sulfate can be used to remove residual water. It was also possible to use this gas chromatographic method for the quantification of low concentrations of diprionol its stereoisomers and occurring homologs in both natural and synthetic material (Figure 1). The detection limit is below 1 pg of diprionol homologs and diastereomers. Furthermore, Figure 1 shows the separation of a mixture of known stereochemical composition of isomeric diprionols and homologs. The *erythro*-isomers of the C_{15}- and C_{16}-homologs which have the (S, S, S)-configuration have the same retention time as the (R, R, R)-isomers (Högberg et al., 1990). The *erythro*-isomers (S, S, S) and (R, R, R) were separated as N-isopropylcarbamates. The separations of (2*S*, 3*S*, 7*R*)-diprionol from (2*S*, 3*S*, 7*S*)-diprionol by a 1.5-min retention difference and the resolution between (2*S*, 3*S*, 7*S*)-diprionol and (2*S*, 3*R*, 7*R*)-diprionol are sufficient to identify and

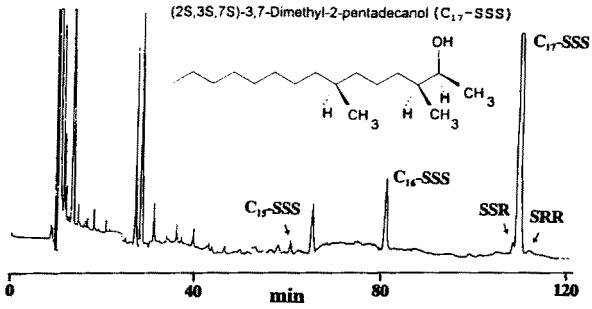
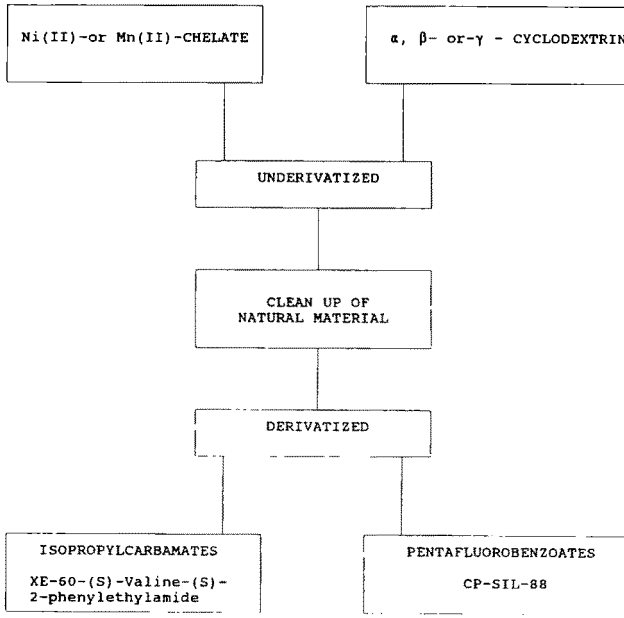


FIG. 1. Enantiomer separation of diprionols as pentafluorobenzoate derivatives, detected by ECD. Columns: fused silica capillary columns, 30 m × 0.15 mm and 25 m × 0.25 mm coated with CP-Sil-88; d_f = 0.22 μm, coupled in series. Oven temperature, 150°C; inlet temperature, 145 kPa nitrogen. C_{15}-*SSS* (2 pg); C_{16}-*SSS* (10 pg); C_{17}-*SSS* (200 pg); C_{17}-*SSR* (4 pg) and C_{17}-*SRR* (2 pg).



SCHEME 1. Summary of procedures used in chiral analyses of sawfly and antlion volatiles.

quantify them. With this derivatization method we can determine the configuration at the 7-position for *erythro* isomers of diprionols. Scheme 1 shows, in summary, the procedure we have used for the analysis of chiral volatiles from sawflies and antlions. We refer here especially to Högberg et al. (1990) and Bergström et al. (1992), respectively.

The analytical results of the quantification of diprionol from single individuals of *N. sertifer* are presented in Figure 2A, which shows a GC-ECD chromatogram of natural material. Two females from the same population were analyzed, and we found 7.5 and 8.2 ng per individual, respectively. A female from another population produced 9.7 ng diprionol. The second chromatogram presents a standard with 10 ng diprionol (Figure 2B). For each quantification, a standard curve was plotted using the peak area of the diprionol per internal standard. This standard curve for diprionol, i.e., the curve obtained by plotting the ratio between the gas chromatographic peak area of the substance being analyzed and the internal standard against the amount of substance determined, was linear. The blank sample, which was prepared equivalently to the other samples, contained extraneous peaks from the reagents and solvents used (Figure 2C).

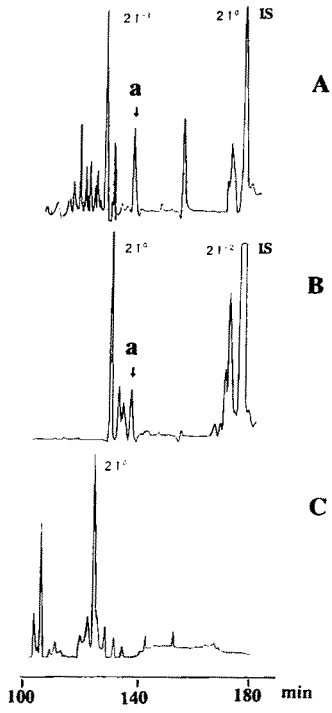


FIG. 2. Quantitative GC-ECD analysis of diprionol in an individual *N. sertifer* female. Column: fused silica capillary columns 50 m × 0.25 mm coupled to 25 m × 0.25 mm coated with Sil-88; d_f = 0.22 μm. Oven temperature, 150°C; carrier gas, nitrogen (90 kPa); injector temperature, 250°C; EC-detector temperature, 275°C. (A) Derivatized LC fractions 7 and 8 from one female: C_{17}-*SSS* (a), internal standard C_{18}-*SSS* (IS), attenuation: $2\uparrow^{-1}$ and $2\uparrow^{0}$. (B) Standard mixture; 10 ng C_{17}-*SSS* (a), C_{18}-*SSS* internal standard (IS), attenuation: $2\uparrow^{0}$ and $2\uparrow^{-2}$. (C) A blank chromatogram with the disturbed peaks from the reagent, attenuation: $2\uparrow^{0}$.

The results demonstrate that complexation gas chromatography can be used as an effective method for stereochemical analyses of molecules that contain suitable functional groups for derivatization. This method may prove generally useful for determination of stereochemical composition.

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ENANTIOMERIC COMPOSITION OF IPSDIENOL: A CHEMOTAXONOMIC CHARACTER FOR NORTH AMERICAN POPULATIONS OF *Ips spp.* IN THE *pini* SUBGENERIC GROUP (COLEOPTERA: SCOLYTIDAE)

STEVEN J. SEYBOLD,[1,2,*] TOSHIKAZU OHTSUKA,[1,3] DAVID L. WOOD,[1] and ISAO KUBO[1]

[1]*Department of Environmental Science, Policy and Management*
Division of Entomology and Plant and Soil Microbiology
University of California at Berkeley
Berkeley, California 94720

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Abstract—Thirty-five populations of *Ips pini* (Say) and one population each of *Ips avulsus* (Eichhoff) and *Ips bonanseai* (Hopkins) were analyzed for the enantiomeric composition of ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol). Populations of *I. pini* occur as at least two distinct regional pheromone variants: New York type [32%-(−) to 56%-(−)-ipsdienol] and California type [94%-(−) to 98%-(−)-ipsdienol]. A third phenotype may occur in southeastern British Columbia, Idaho, and Montana [91%-(−) to 95%-(−)], possibly indicating a zone of hybridization. Populations of the New York type occur in southwestern British Columbia, Alberta, Saskatchewan, Minnesota, and Wisconsin suggesting a continuum through the Canadian provinces and Lake States. The presence of the New York type in western Canada is likely linked to the Quaternary history of the transcontinentally distributed host, *Pinus banksiana* Lamb. Male *I. avulsus* [−25%-(−)] and male *I. bonanseai* [−29%-(−)] both produce ipsdienol, but not ipsenol. Production of ipsdienol by male *I. pini* was evaluated in six different *Pinus* spp. hosts. Following transfer of male *I. pini* to hosts other than the host of origin, the percentage of the (−)-enantiomer of ipsdienol declined when compared to production in the host of origin.

*To whom correspondence should be addressed.
[2]Current mailing address: Dept. of Biochemistry, Mail Stop 330, University of Nevada Reno, Reno, Nevada, 89557.
[3]Current mailing address: Shionogi & Co., Ltd., Aburahi Laboratories, 1405 Gotanda, Koka-cho, Koka-gun, Shiga 520-34, Japan.

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Key Words—Insecta, Coleoptera, Scolytidae, *Ips pini*, *Ips avulsus*, *Ips bonanseai*, ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol, aggregation pheromone, enantiomeric composition.

INTRODUCTION

North American pine engraver beetles in the *pini* subgeneric group include *Ips avulsus* (Eichhoff), *Ips bonanseai* (Hopkins), and *Ips pini* (Say) (S.L. Wood, 1982). Morphological similarities also support the placement of the palearctic species, *Ips mannsfeldi* (Wachtl), in this group (S.L. Wood, 1982). In an exhaustive biosystematic study, Lanier (1972) investigated morphological, karyological, and behavioral (mating and pheromone attraction) characteristics of the North American species. Through a survey of collection records, he also delineated the currently accepted distributions of these species.

I. pini, an economically important pest in certain regions of its extensive range, has been the topic of numerous studies of pheromone biology (Anderson, 1948; Lanier et al., 1972, 1980; Vité et al., 1972; Young et al., 1973; Birch, 1974; Piston and Lanier, 1974; Birch and Wood, 1975; Lanier and Wood, 1975; Stewart, 1975; Plummer et al., 1976; Birch and Light, 1977; Birch et al., 1977, 1980; Angst and Lanier, 1979; Furniss and Livingston, 1979; Mustaparta et al., 1980, 1985; Miller et al., 1989, 1990; Raffa and Klepzig, 1989; Gries et al., 1990; Miller, 1990; Miller and Borden, 1990; Teale, 1990; Herms et al., 1991; Raffa, 1991; Teale et al., 1991, 1994; Teale and Lanier, 1991; Vanderwel, 1991; Seybold, 1992; Seybold et al., 1992; Hager and Teale, 1994). The original impetus for the isolation of the aggregation pheromone of *I. pini* in California was because it colonized the same host niche (i.e., phloem layer of *Pinus ponderosa* Laws.) in sympatry with *Ips paraconfusus* Lanier, but was in a different subgeneric group (Wood, 1970; Birch, 1978). Lanier et al. (1972) first described geographic variation in pheromone production and response for populations of *I. pini* from California, Idaho, and New York. The variation in production was later attributed to the enantiomeric composition of the male-produced terpene alcohol pheromone component, ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Birch et al., 1980; Lanier et al., 1980). Isolation studies and field assays showed that a population in California produced and responded to (4*R*)-(−)-ipsdienol (Young et al., 1973; Stewart, 1975; Birch et al., 1980), while a population in New York produced and optimally responded to a mixture of the two enantiomers (Stewart, 1975; Lanier et al., 1980). In an analysis of hingut extracts from individual male *I. pini*, Miller (1990) characterized the enantiomeric composition of ipsdienol produced by six populations of *I. pini* in British Columbia in addition to the populations from California and New York. Miller et al. (1989) documented substantial intrapopulational variation of the enantiomeric composition of ipsdienol and found that interpopula-

tional variation ranged from 91%-(−) in California and 89%-(−) in southeastern B.C. to 43%-(−) in New York and 34%-(−) in southwestern B.C.

More recently, geographic variation in *I. pini* pheromone production and response has also been associated with the terpenoid-based ketone, lanierone (2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one), a second pheromone component (Teale et al., 1991; Seybold et al., 1992). Lanierone is produced by a New York, but not a California, population of *I. pini*. Teale et al. (1991) demonstrated that lanierone and racemic ipsdienol together elicit a dramatically higher response from New York *I. pini* than either component alone (> 10-fold increase in trap catch). Lanierone significantly increases the response of California *I. pini* when compared to the response to (−)-ipsdienol alone (0.4-fold increase in trap catch), but lanierone is not as strong a synergist with ipsdienol for this population as it is in New York (Seybold et al., 1992).

The pheromone biologies of *I. avulsus*, *I. bonanseai*, and *I. mannsfeldi* have not been investigated to the extent that any geographic variation is evident. By gas chromatographic (GC) analysis of hindgut extracts, Vité et al. (1972) first demonstrated that fed male *I. avulsus* and *I. bonanseai* produced ipsdienol. *I. avulsus* also produced ipsdienol when exposed to myrcene vapors (Hughes, 1974). However, the enantiomeric composition of the ipsdienol produced by either species has not been reported. Nonetheless, in several field behavioral assays in eastern Texas, *I. avulsus* responded to treatments containing racemic ipsdienol (Renwick and Vité, 1972; Hedden et al., 1976). Based on results from assays with 90%-(−)-ipsdienol in Lufkin, Texas, Vité et al. (1978) predicted that (−)-ipsdienol would be produced by *I. avulsus*. In east Texas, Smith et al. (1990) also reported responses of *I. avulsus* to treatments containing the enantiomers of ipsdienol [96%-(−); 94%-(4*S*)-(+)]. Although the trap catches were low in these studies, *I. avulsus* preferred treatments containing racemic and (−)-ipsdienol over those with (+)-ipsdienol.

While assays with isolated semiochemicals have not been conducted with *I. bonanseai*, Lanier (1972) found in the laboratory that the male-produced frass of *I. bonanseai* from the Santa Catalina Mountains in Arizona elicited a walking response from female *I. pini* originating from western North America. This response was lower than the responses to frass produced by male *I. pini* from eastern or western North America. An investigation of hindgut extracts from male *I. mannsfeldi* indicated that 96.5%-(−)-ipsdienol and 99.5%-(4*S*)-(−)-ipsenol (2-methyl-6-methylene-7-octen-4-ol) occurred in an approximate ratio of 1.5 : 1 (Kohnle et al., 1993). Limited field studies conducted 200 km northeast of Madrid, Spain, demonstrated that predominantly male *I. mannsfeldi* responded maximally to the combination of the two racemic compounds, with negligible responses to either compound alone.

I. pini is distributed both boreally and australly, and it has coevolved with a wide variety of *Pinus* spp. These characteristics, when combined with a simple

aggregation pheromone that is easily collected and analyzed, make *I. pini* an ideal model animal to study the evolution of geographic variation in pheromone production. Because the enantiomeric composition of ipsdienol plays a central role in the aggregation behavior and, thus, the mating biology of *I. pini*, we analyzed North American populations of *I. pini* for this character. Single populations of *I. avulsus* and *I. bonanseai* were included in the survey for comparative purposes. The effect of the *Pinus* spp. host on the enantiomeric composition of male-produced ipsdienol was also investigated for populations from British Columbia, California, and New York. We envisioned that the phenotypic trait of ipsdienol enantiomeric composition could be used to organize the variable populations of *I. pini* into geographically coherent pheromone subgroups that might later be recognized as taxonomic units. Through this analysis, we also hoped to identify areas of sympatry where populations of different pheromone subgroups might be in the process of introgression or divergence. In addition, the information provided by this survey would be useful for forest pest managers concerned with trapping or monitoring populations of *I. pini*. Experience with populations of *I. pini* from California and New York has shown that each population generally responds maximally to its locally produced enantiomeric blend of ipsdienol. Similar, and in some cases, identical pheromone extracts will be analyzed for the presence and absence of lanierone and the results will be summarized in a future paper.

METHODS AND MATERIALS

Insects. Ips species were collected in pine logging debris and reared to adults (Browne, 1972). The sexes were separated using morphological secondary sexual characters (Lanier and Cameron, 1969). Adults were held at 5°C on moist filter paper until they were introduced into logs for ipsdienol collection. Collection localities are given in Table 1 and summarized geographically in Figure 1.

Collection of Vapors. Volatiles associated with tunneling adult beetles of each species were collected by artificially colonizing recently cut logs from *Pinus* spp. and trapping the vapors on Porapak Q (Byrne et al., 1975) (50/80 mesh size, Supelco Inc., Bellefonte, Pennyslvania). Since male *Ips* are generally the first sex to colonize the host (see Kirkendall, 1990 for an exception) and are generally thought to be the only sex to produce ipsdienol (see Seybold, 1992, for an exception), we introduced adult males into fresh logs (~28 cm length × 12 cm diameter) by drilling shallow holes through the bark approximately 3 cm apart on the surface. One male was inserted per hole, and then secured with screen. The method was repeated with separate logs for females and, in some cases, for the host alone. Depending on the availability of a given pop-

TABLE 1. COLLECTIONS OF *Ips* SPECIES IN *pini* SUBGENERIC GROUP [*Ips avulsus* (EICHHOFF), *Ips bonanseai* (HOPKINS), AND *Ips pini* (SAY)] FOR NORTH AMERICAN SURVEY OF IPSDIENOL ENANTIOMERIC COMPOSITION[a]

Collection locality	Host[b]	Collection/aeration date	Males/Females (*N*)	Enantiomeric composition ipsdienol [%-(−)]	Quantity of ipsdienol (mg)[c]
Alabama [*Ips avulsus* (Eichhoff)], Lee Co., 15 km southwest of Auburn	PT	I-21-89/I-28-II-4-89[d]	57/49	25.0[e]	0.5
Alberta, 27 km north of Obed, 117°13′W, 53°47′N	PCl	VIII-91/IX-24-X-1-91	107/79	34.5	0.2*
Arizona, Gila Co., ~1.6 km east of Ponderosa Spring, T11 N, R13E, SE 1/4 S36, and T101/2N, R131/2E, NW1/4, S21(Payson)	PP	V-25-93/VIII-1-8-93	75/60	94.7	5.0*
Arizona, Graham Co., Point of Pines, San Carlos Apache Indian Reservation, T1S, R25E, S11	PP	VI-10-93/IX-20-37-93	97/60	95.3	8.6*
Arizona, Yavapai Co., 3.2 km south of Mingus Mountain, Prescott National Forest, T15N, R2E, S22	PP	VII-87/X-27-XI-3-87	123/68	96.9	27.6
British Columbia, Eastgate, Manning Park	PCl	VII-1-88/VIII-20-27-88	107/83	38.6	1.2
British Columbia, Jaffray	PCl	VIII-11-88/IX-18-24-88	89/92	93.1	4.1
California, El Dorado Co., D. L. Bliss Park, T13N, R17E, S9	PJ	XI-4-88/XII-20-29-88	49/0	96.5	11.1
California, El Dorado Co., Fallen Leaf Lake, T12N, R17E, S1	PJ	IX-9-88/X-13-20-88	98/77	98.0[f]	60.6
California, Lassen Co., 5.6 km southwest of Ladder Butte, T34N, R6E, S34 and 35	PJ	V-18-88/VIII-21-28-88	105/0	96.1	20.2
California, Lassen Co., 3.2 km west of Butte Creek Ranch, Hwy 44, T32N, R6E, S9	PCm	V-18-88/XII-1-8-88	25/17	98.5	2.8
California, Modoc Co., Benton Meadow, T44N, R15E, S17 and T44N, R14E, S1	PCm	VIII-4-88/X-22-29-88	59/97	98.3	7.2
California, San Bernardino Co., Polique Canyon Road, 3.2 km NE Fawnskin	PJ	III-12-88/III-25-IV-1-88	68/91	98.3	35.3

TABLE 1. CONTINUED

Collection locality	Host[b]	Collection/aeration date	Males/Females (*N*)	Enantiomeric composition ipsdienol [%-(−)]	Quantity of ipsdienol (mg)[c]
California, San Bernardino Co., Shake Creek, T2N, R2W, S18	PCo	IX-13-88/IX-28-X-5-88	36/64	96.5	12.3
California, Shasta Co., Summit Lake, Hwy 44, 9.7 km east of Hwy 89, T32N, R6E, S6	PP	IV-7-88/IV-18-25-88	76/94	96.5	13.1
California, Tuolomme Co., Long Barn, Stanislaus National Forest, 1615 m.	PJ	X-92/II-24-III-3-93	209/164	96.2*	n.d.
Idaho, Bonner Co., 4.8 km east of Clagstone	PP	VI-91/VII-20-27-91	222/241	94.3	11.6*
Idaho, Latah Co., 11.3 km north of Moscow, T40N, R5W, S22	PP	V-24-89/VII-4-11-89	176/189	92.5	8.8
Minnesota, Sherburne Co., 9.7 km west of Zimmerman, T34N, R27E, S17	PR	VI-11-90/VII-24-31-90	64/49	33.5	1.5*
Montana, Missoula Co., 32.3 km northeast of Missoula	PP	V-29-91/VI-26-VII-2-91	74/89	91.2	8.6*
Montana, Rosebud Co., 9.7 km south of Lame Deer	PP	VII-93/IX-6-13-93	114/312	94.6	3.0*
New Mexico, Otero Co., 15 km west of intersection of Highways 244 and 82, T16S, R14E, S10, 16 km east of Cloudcroft on Hwy 82	PP	VIII-18-90/IX-26-X-3-90	75/89	96.8	0.9*
New Mexico, Otero Co., T16S, R13E, S5	PP	V-14-94/VI-3-10-94	116/71	98.5	38.1*
New Mexico, Otero Co., T16S, R13E, S5	PP	V-14-94/VII-21-28-94	119/0	98.0	8.0*
New York, Cortland Co., Morgan Hill State Forest	PS	VI-4-94/VII-13-20-94	216/225	34.4	36.0*
New York, Essex Co., Newcombe, ex Lab Colony	PR	Fall-84/VI-27-VII-4-88	85/86	52.3	3.8
New York, Onondaga Co., Tully	PR	VIII-3-88/IX-7-14-88	103/70	41.4	1.8
New York, Suffolk Co., Smithtown, Long Island, Bow Drive Greenbelt	PS	VI-8-94/VII-4-11-94	233/204	34.9	25.8*
North Carolina, Clay Co., Nantahala National Forest	PS	X-89/X-26-XI-2-89	121/86	55.9[c]	0.4

North Carolina, Clay Co., Nantahala National Forest	PS	X-89/XII-8-15-89	38/23	52.8	0.1*
Nuevo Leon [*Ips bonanseai* (Hopkins)], Mpio. Galeana, Northeast Slope of Cerro El Potosi, 2800 m.	PH	X-27-93/XI-22-28-93	223/240	28.3	24.5*
Nuevo Leon [*Ips bonanseai* (Hopkins)], Mpio. Galeana, Northeast Slope of Cerro El Potosi, 2800 m.	PH	X-27-93/I-9-16-94	213/0	29.0	4.9*
Oregon, Deschutes Co., 16.1 km southeast of Bend	PP	VII-23-88/VIII-27-IX-3-88	101/98	94.2	6.9
Saskatchewan, 70 km north of Meadow Lake, 108°15′W, 54°45′N	PB	VII-18-90/IX-19-26-90	124/155	39.2	0.2*
Saskatchewan, 50-60 km northeast of Prince Albert, 104°41′W, 53°18′N	PB	VII-18-90/VIII-26-IX-3-90	187/197	41.9	0.2*
South Dakota, Pennington Co., 3 km south of Pactola Reservoir, T1N, R5E, S14	PP	II-22-90/VI-25-VII-3-90	57/105	96.4	0.6*
Washington, Kittitas Co., Reecer Canyon, 19.3 km north of Ellensburg, T20N, R18E, S33	PP	IX-15-89/X-3-10-89	225/232	96.5	65.1
Washington, Okanogan Co., 6.5 km south of Sweat Creek T36N, R31E, S12, Tonasket	PCl	IX-16-89/XI-25-XII-2-89	88/109	95.9	15.4
Washington, Okanogan Co., 19.3 km northwest Winthrop, T36N, R20E, S13	PP	IX-17-89/XI-9-16-89	97/71	96.9	25.0
Wisconsin, Sauk Co., Bakken Pond, Spring Green	PR	IX-87/XII-7-14-88	21/21	32.0[h]	7.4
Wisconsin, Sauk Co., Bakken Pond, Spring Green	PR	IX-87/I-4-11-89	33/0	35.0	5.2

[a] All entries represent collections of *Ips pini* except where *Ips avulsus* or *Ips bonanseai* are indicated in brackets. n.d. = not determined.
[b] PB = *Pinus banksiana* Lamb., PCl = *Pinus contorta latifolia* (Engelm. *ex* Wats.), PCm = *Pinus contorta murrayana* (Balf.), PCo = *Pinus coulteri* D. Don, PH = *Pinus hartwegii* Lindl., PJ = *Pinus jeffreyi* Grev. & Balf., PP = *Pinus ponderosa* Laws., PR = *Pinus resinosa* Ait., PS = *Pinus strobus* L., and PT = *Pinus taeda* L. Volatiles from male and female *I. pini* were collected separately from artificially colonized logs of the local host in which the population was collected.
[c] Asterisk indicates recent sample; see text.
[d] In dates, Roman numeral is the month: I = January, . . . , XII = December.
[e] Determined as 23.7%-(−) by chiral GC.
[f] Determined as 98.0%-(−) by chiral GC.
[g] Determined as 58.2%-(−) by chiral GC.
[h] Determined as 31.8%-(−) by chiral GC.

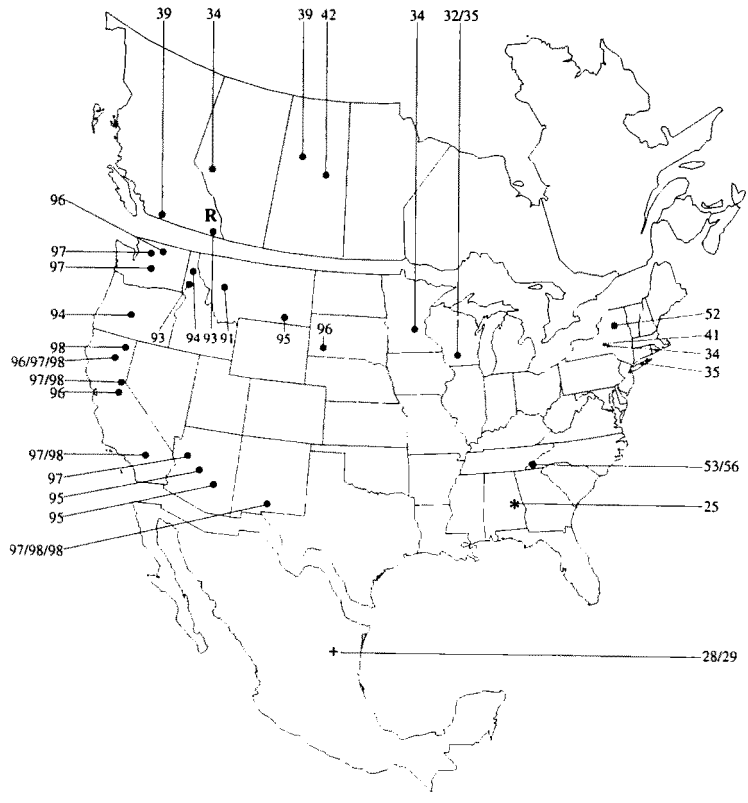

FIG. 1. The enantiomeric composition of ipsdienol [in percentage-(−)] isolated from (*) *Ips avulsus* (Eichhoff), (+) *Ips bonanseai* (Hopkins), and (●) *Ips pini* (Say) from collection sites in North America. R denotes Radium, British Columbia.

ulation, we attempted to use at least 100 insects per aeration. The technique results in an initial colonization density of ~1 insect/4 cm^2.

The artificially colonized logs were incubated at room temperature for 24 hr to allow for frass production and then placed into 19-liter silylated glass carboys (Sylon CT, Supelco Inc., Bellefonte, Pennsylvania) for collection of volatiles. The volatiles were collected for 168 hr by drawing air from the carboys at a rate of 4 liters/min through 15 g Porapak Q. After the collection period, the logs were dissected to determine the number of insects that excavated feeding galleries.

Extraction and Analysis. Compounds were extracted from the Porapak with ~350 ml pentane. Quantitative analysis of ipsdienol present in the extract was performed using one of two methods. In the first samples analyzed, a 10-μl

aliquot of the crude, unconcentrated pentane extract was analyzed for presence and quantity of ipsdienol using silica-phase high-performance liquid chromatography (HPLC). Because of the difficulty in obtaining an internal standard with suitable retention time and UV absorbance, quantities were estimated using a calibration curve derived from the injection of known quantities of authentic ipsdienol (Product #P407, Bedoukian Research Inc., Danbury, Connecticut). Measurement was based on the mean of three sample injections. Recent samples (indicated by an asterisk in Table 1) were analyzed by adding an internal standard of *n*-octanol (Aldrich Chemical Co., No. 36,056-2, Milwaukee, Wisconsin) to the 350-ml crude extract prior to Kuderna-Danish evaporative concentration to 10 ml. A 1-μl aliquot of the concentrated crude extract was analyzed for the presence and quantity of ipsdienol using gas chromatography (GC). In this case, measurement was based on the mean of six sample injections. Enantiomeric analysis that followed both methods of quantitative analysis utilized a fractionated 1-ml aliquot of the 10-ml concentrated crude extract.

The enantiomeric composition of the naturally occurring ipsdienol was determined following isolation by silica-phase preparative HPLC. In the first samples analyzed, isolated ipsdienol was converted to diastereomeric esters and the esters were resolved by silica phase analytical HPLC (Kubo et al., 1986; Seybold, 1992). Integration of peak areas for each diastereomer and calculation of the peak area ratio yielded the enantiomeric composition. The chiral acid used for the esterification of ipsdienol was (1*S*)-camphanic acid [Aldrich Chemical Co., No. 32,822-7, CP = 99%, $[\alpha]_D^{20} = 25°$ (c = 1, dioxane)]. The enantiomers of isolated ipsdienol were thus resolved as the (4*R*)- and (4*S*)-ipsdienyl-(1*S*)-camphanates (Seybold, 1992). In more recently analyzed samples (indicated by an asterisk in Table 1), enantiomeric composition of isolated ipsdienol was determined by chiral GC (Seybold et al., 1992). Selected samples were analyzed by both methods (Table 1) to determine if the methods gave comparable results. Measurement of the enantiomeric ratio for each compound using either technique was based on the mean of five sample injections.

Chromatography. Thin-layer chromatography (TLC) for monitoring reactions utilized silica gel Polygram Sil G/UV254 precoated 4 × 8-cm plastic sheets (0.25 mm, Macherey-Nagel, Duren, Germany) developed with ethyl acetate-hexane (2:1, v/v).

Silica-phase HPLC for quantitative analysis of ipsdienol and resolution of ipsdienol diastereomeric esters employed a Jasco 880-PU pump (Jasco, Tokyo, Japan) equipped with an Eyela UV-9000 Detector (Tokyo Rikakikai Co., Tokyo, Japan); column: Partisil PXS 5/25 (Whatman Inc., Clifton, New Jersey), 25 cm × 5 mm; mobile phase: hexane–acetone (100:2, v/v); flow rate 1.5 ml/min; and UV 225 nm. Silica-phase preparative HPLC for isolation of ipsdienol was conducted with a Japan Analytical Industry model LC-908 instrument equipped with UV and refractive index (RI) detectors. The column and

conditions for isolation of ipsdienol were Nucleosil 50-5 (50 × 10 cm) (Alltech Applied Science, Deerfield, Illinois); mobile phase: hexane–acetone (94:6, v/v); flow rate: 1.6 ml/min; and UV 235 nm.

Gas chromatography (GC) for quantitative analysis of ipsdienol was performed on a Hewlett Packard Series 5890A gas chromatograph. A fused silica capillary (Durabond-5, J&W Scientific, Folsom, California; 30 m × 0.25 mm ID, 0.25-μm film thickness; helium carrier gas 31 cm/sec) was temperature programmed (70°C for 29 min and 70°C to 180°C at 5°C/min) for resolution of ipsdienol from *n*-octanol and a myriad of crude extract components. Injection and detection temperatures were set at 200°C and peak areas were integrated using an HP-3393A integrator. GC conditions for determination of enantiomeric composition of ipsdienol were as previously described (Seybold et al., 1992).

Spectroscopy. High-resolution gas chromatography–mass spectrometry (HRGC-MS) utilized a Hewlett-Packard Series 5790A gas chromatograph interfaced with an HP Series 5970A mass selective detector (MSD). This system was operated under electron impact (70 eV) conditions. A fused silica capillary (Durabond Wax, J&W Scientific; 30 m × 0.25 mm ID, 0.25-μm polyethylene glycol film; helium carrier gas 25 cm/sec) was temperature programmed (75°C to 130°C at 10°C/min, 130°C to 150°C at 2°C/min, and then 150°C to 250°C at 10°C/min) for resolution of ipsdienol. Ipsdienol was unambiguously identified in selected extracts by comparison with the retention time and spectrum from the commercially available authentic standard and by matching retention time of the standard with the retention time of the base peak (ipsdienol m/z = 85) determined by selective ion monitoring of selected extracts.

RESULTS

A total of 35 populations of *I. pini*, one population of *I. avulsus*, and one population of *I. bonanseai* were examined in this survey; in some cases identical populations were subsampled and analyzed twice. The enantiomeric composition of ipsdienol isolated from male *I. pini* in its local host ranged from 32.0%-(−) (Sauk County, Wisconsin) to 98.5%-(−) (Lassen County, California, and Otero County, New Mexico) (Table 1). Male *I. avulsus* (Lee County, Alabama) produced 25.0%-(−)-ipsdienol [23.7%-(−) when measured by chiral GC], while male *I. bonanseai* (Galeana, Nuevo Leon, Mexico) produced 28.3%-(−)- and 29.0%-(−)-ipsdienol in separate analyses of the same population (Table 1). Populations of *I. pini* can be organized into three basic pheromone groups, based on their geographic distribution. Populations from the southwest and the Pacific coast of the United States produced ipsdienol that ranged in enantiomeric composition from 94.2%-(−) (Deschutes County, Oregon) to 98.5%-(−) (Lassen County, California, and Otero County, New Mexico) (Figure 1). Populations

from southeastern British Columbia, Idaho, and Montana produced ipsdienol that varied in enantiomeric composition from 91.2%-(−) (Missoula County, Montana) to 94.6%-(−) (Rosebud County, Montana). Populations from the eastern United States, southwestern British Columbia, Alberta, and Saskatchewan produced ipsdienol with an enantiomeric composition between 32.0%-(−) (Sauk County, Wisconsin) and 55.9%-(−) (Clay County, North Carolina) (Figure 1). An isolated population from the Black Hills of South Dakota (Pennington County) produced 96.4%-(−)-ipsdienol (Figure 1). There was general agreement between results obtained using the HPLC and GC techniques (Table 1). The most pronounced difference was with measurements of ipsdienol isolated from *I. pini* from Clay County, North Carolina: 55.9%-(−) by HPLC; 58.2%-(−) by GC.

When populations of male *I. pini* were transferred to other host pines for ipsdienol production, the percentage of the (−)-enantiomer declined when compared to the percentage produced in the host of origin (Table 2). Host transfers to *P. ponderosa* resulted in absolute reductions of 7.5% (Manning Park, British Columbia from *P. contorta latifolia*); 1.6% (Jaffray, British Columbia from *P. contorta latifolia*); 3.1% (Essex County, New York from *P. resinosa*); and 0.3% (Onondaga County, New York from *P. resinosa*). California populations of *I. pini* collected in *P. jeffreyi* and *P. contorta murrayana* (both Lassen County) showed absolute reductions of 3.4% and 2.0%, respectively, when they were aerated in *P. ponderosa*. A California population of *I. pini* collected in *P. ponderosa* (Shasta County) showed an absolute reduction of 2.5% in the percentage of the (−)-enantiomer when transferred to *P. jeffreyi* and a 0.6% drop when transferred to *P. contorta murrayana* (Table 2). Nonetheless, the ranges for these three California populations in the three hosts were fairly small (*P. jeffreyi*—3.4%; *P. contorta murrayana*—2.5%; and *P. ponderosa*—2.5%).

If we consider the populations from British Columbia, California, and New York as a random sample from the distribution of *I. pini*, then a paired *t* test of the reductions of the (−)-enantiomer can be performed. For biological reasons, we chose to analyze the six cases where *P. ponderosa* was the host of aeration, but not the original host. We also included the single case (Shasta County, California) where the closely related *P. jeffreyi* was the host of aeration and *P. ponderosa* was the original host. The analysis indicated that the 95% confidence interval around the mean reduction in the percentage of the (−)-enantiomer does not include zero, thus the reduction is significant ($\bar{X} = -2.914$; 95% confidence interval = −5.013, −0.816; $N = 7$) (Minitab, Release 8.2, 1991, State College, Pennsylvania).

The structure of ipsdienol was confirmed by mass spectral analysis in samples from the following populations of *I. pini:* Obed, Alberta; Jaffray, British Columbia; El Dorado County, California (Fallen Leaf Lake); Lassen County, California (Ladder Butte); Modoc County, California; San Bernardino County,

TABLE 2. EFFECT OF HOST ON ENANTIOMERIC COMPOSITION OF IPSDIENOL PRODUCED BY *Ips pini* (*Say*) FROM SELECTED NORTH AMERICAN POPULATIONS

Collection locality and host[a]	Collection/aeration date	Males/Females (*N*)	Enantiomeric composition ipsdienol [%-(−)]	Quantity of ipsdienol (mg)
British Columbia, Eastgate, Manning Park				
PCl/PCl	VII-1-88/VIII-20-27-88	107/83	38.6	1.2
PCl/PP	VII-1-88/VIII-27-IX-3-88	119/95	31.1	1.2
British Columbia, Jaffray				
PCl/PCl	VIII-11-88/IX-18-24-88	89/92	93.1	4.1
PCl/PP	VIII-11-88/IX-18-24-88	95/87	91.5	1.3
California, Lassen Co., 5.6 km southwest of Ladder Butte, T34N, R6E, S34 and 35				
PJ/PJ	V-18-88/VIII-21-28-88	105/0	96.1	20.2
PJ/PP	V-18-88/VIII-27-IX-3-88	107/82	92.7	3.3
PJ/PCm	V-18-88/VIII-27-IX-3-88	95/96	96.0	14.7
California, Lassen Co., 3.2 km west of Butte Creek Ranch, Hwy 44, T32N, R6E, S9				
PCm/PCm	V-18-88/XII-1-8-88	25/17	98.5	2.8
PCm/PJ	V-18-88/XI-8-15-88	95/97	96.0	25.9
PCm/PP	V-18-88/XI-12-19-88	65/102	96.5	10.1
California, Shasta Co., Summit Lake, Hwy 44, 9.7 km east of Hwy 89, T32N, R6E, S6				
PP/PP	IV-7-88/IV-18-25-88	76/94	96.5	13.1
PP/PJ	IV-7-88/VII-88	71/99	94.0	21.9
PP/PCm	IV-7-88/VII-88	123/128	95.9	12.3
New York, Essex Co., Newcombe, *ex* Lab Colony				
PR/PR	Fall-84/VI-27-VII-4-88	85/86	52.3	3.8
PR/PP	Fall-84/VII-4-11-88	121/125	49.2	10.4
New York, Onondaga Co., Tully				
PR/PR	VIII-3-88/IX-7-14-88	103/70	41.4	1.8
PR/PP	VIII-3-88/IX-7-14-88	57/53	41.1	0.6

[a] First host entry is host for collection, while second host entry is host for aeration. Hosts include: PCl = *Pinus contorta latifolia* (Engelm. *ex* Wats.), PCm = *Pinus contorta murrayana* (Balf.), PJ = *Pinus jeffreyi* Grev. & Balf., PP = *Pinus ponderosa* Laws., and PR = *Pinus resinosa* Ait. For populations from British Columbia and New York, the alternate host (*P. ponderosa*) originated from Shasta Co., California. For populations from California, the alternate hosts originated from Lassen or Shasta Cos., California.

California (Shake Creek); Latah County, Idaho; Clay County, North Carolina; Deschutes County, Oregon; Meadow Lake, Saskatchewan; Prince Albert, Saskatchewan; Pennington County, South Dakota; Okanogan County, Washington (Winthrop); and Sauk County, Wisconsin.

DISCUSSION

Within the *pini* subgeneric group in North America, all three species produce ipsdienol, but not ipsenol. The palearctic consubgeneric species *I. mannsfeldi* is an exception (Kohnle et al., 1993). The two populations of *I. avulsus* and *I. bonanseai* that we sampled produced surprisingly similar enantiomeric blends of ipsdienol [~25%-(−) and ~29%-(−), respectively] that differed markedly from the majority of *I. pini* populations studied [32%-(−) to 98%-(−)]. Although evaluation of this character alone may suggest similarity of the two southern species, Lanier (1972) concluded that *I. pini* and *I. bonanseai* were more closely related for reasons of morphology, karyology, and interfertility. However, he did not include *I. avulsus* in either interbreeding or pheromone cross-attraction studies, stating that the extreme (morphological?) differences of *I. avulsus* made experiments unnecessary. Lanier (1972) noted that the male genitalia of *I. avulsus* resemble those of *I. bonanseai*, as well as those of species in the *emarginatus* subgeneric group.

It is unclear whether interspecific breeding isolation and competition for host material (Wood, 1970; Lanier and Burkholder, 1974; Lanier and Wood, 1975) or phylogenetic divergence (Lewis and Cane, 1990) better explains current pheromonal specificity among *Ips* spp. However, the similarity in enantiomeric composition of ipsdienol produced by male *I. avulsus* and *I. bonanseai* may not indicate phylogenetic similarity as much as it does similar competitive interactions with *I. pini*. *I. avulsus* and *I. pini* share a zone of sympatry in southern Appalachia (Ciesla and Bell, 1968; Lanier, 1972; S.L. Wood, 1982), while *I. bonanseai* and *I. pini* are sympatric in the northern Mexican state of Chihuahua (Lanier, 1972). Additional populations of *I. pini* may be present in more southerly Mexican states like Durango, providing the possibility of more extensive sympatry for *I. bonanseai* and *I. pini* (J. Flores, personal communication). In these zones, both *I. avulsus* and *I. bonanseai* may have evolved to produce and detect enantiomeric blends of ipsdienol that would minimize the possibility of a wasted mating attempt with *I. pini*. Since the behavioral assays conducted to date in eastern Texas implied a preference by *I. avulsus* for (−)-ipsdienol (Vité et al., 1978; Smith et al., 1990), it is surprising that we found that male *I. avulsus* from Lee County, Alabama produced predominantly (+)-ipsdienol. There could be geographic variation for this character in *I. avulsus* between the disjunct Alabama and Texas populations (Lanier, 1972). Both *I. avulsus* and *I.*

bonanseai may also produce other aggregation pheromone components, possess proximal chemical or auditory cues, or even have different host preferences that provide for interspecific discrimination in the respective zones of sympatry with *I. pini*.

For *I. pini* this study indicated the presence of geographically specific trends in ipsdienol stereochemistry. Birch et al. (1980) and Lanier et al. (1980) showed a clear difference in enantiomeric composition between eastern and western North American populations. Ipsdienol is produced in the east in a wide range of enantiomeric percentages [32%-(−) to 56%-(−)], increasing as populations were sampled from more easterly and southerly localities. In general for eastern populations, the percentage of the (−)-enantiomer tends toward that produced by the consubgeneric species *I. avulsus* and *I. bonanseai*. Anderson (1948) made pioneering observations of the aggregation behavior of *I. pini* on jack pine, *Pinus banksiana* Lamb., in Minnesota. Although the Minnesota population that we analyzed came from red pine, *P. resinosa* Ait., it is likely that Anderson's original observations were made on a population of males that produced ~34%-(−)-ipsdienol and that population was representative of much of eastern North America (see query in D.L. Wood, 1982).

The regional situation in western North America is more complex. Miller et al. (1989) found a particularly interesting regional variant of *I. pini* in *P. contorta latifolia* Engelm. ex Wats. located in southwestern British Columbia near Manning Provincial Park. This population produces an enantiomeric blend of ipsdienol normally associated with *I. pini* in eastern North America (e.g., New York). Surprisingly, this population occurs only 70 km north of a western population [97%-(−)] (Okanogan County, Washington, Winthrop, *P. ponderosa*) and 100 km northwest of a second western population [96%-(−)] (Okanogan County, Washington, Tonasket, *P. contorta latifolia*). This variant population is likely to be contiguous with populations included in this study from Alberta and Saskatchewan (Meadow Lake and Prince Albert), which are also more "New York-like." In addition, populations from southeastern British Columbia, Idaho, and Montana tend to produce an enantiomeric percentage of (−)-ipsdienol that is slightly lower [91%-(−) to 95%-(−)] in our study than that produced by populations from the southwestern and Pacific coast states [94%-(−) to 98%-(−)]. Miller et al. (1989) found a lower population mean for the population from southeastern British Columbia [89%-(−)], while Plummer et al. (1976) reported 100%-(−) for "Idaho" *I. pini*. The latter study was an early attempt at determining the enantiomeric composition of insect pheromone alcohols using NMR chiral shift reagents. This technique may not have been as accurate as currently available chromatographic methods. The existence of a third regional variant in western North America (Idaho/Montana) is generally supported by cuticular hydrocarbon phenotypes (Seybold et al., unpublished)

and by field behavioral responses to ipsidenol and lanierone (Miller et al., unpublished) that differ from those in northern California and New York.

The complexity outlined by this investigation in northwestern North America suggests that further sampling in British Columbia, Alberta, and the central Rocky Mountain region will be important. Perhaps significantly, Swaine (1918) recognized a taxon in southern British Columbia named *Ips interpunctus* Eichhoff [now synonymized with *Ips perturbatus* (Eichhoff) (Wood, 1977; Wood and Bright, 1992)]. Although found most frequently in spruce (*Picea* spp.), *I. interpunctus* was considered closely related to *I. pini* because it was observed in the same galleries with, intergraded morphologically with, and apparently crossed with *Ips oregonis* Eichhoff [a synonym for western populations of *I. pini* (Hopping, 1964; Wood and Bright, 1992)] (Swaine, 1918). The seemingly confusing interspecific taxonomic situation in this area alluded to by early researchers may have foreshadowed the intraspecific complexity presented in the current analysis. Indeed, Lanier and Burkholder (1974) also understood the significance of this region for *I. pini* when they observed that, "The pheromonal differences (between populations of *I. pini*), like the slight morphological differences in both the insect (Hopping, 1964) and its parasitic mites (Lindquist, 1969), probably intergrade in the Rocky Mountains of the northern U.S.A. and Canada." Several possible zones of sympatry between pheromone types of *I. pini* must exist in the northwest, and they would make ideal locations for studies of naturally occurring, interpopulational aggregation and mating behavior. These zones include possible California and New York phenotypes in northern Washington and southwestern British Columbia; Idaho/Montana and possible New York phenotypes in southeastern and southwestern British Columbia; and Idaho/Montana and possible California phenotypes in northern Idaho and northern Washington.

Among the critical questions to be answered by examining the behavior of the populations in these possible zones of sympatry is whether the southwestern British Columbia population has the behavioral and biochemical characteristics of the well-studied New York population. Cross-attraction studies in this area with *I. pini* from California and New York similar to those conducted by Lanier et al. (1972) are needed to address this question. Reciprocal tests in California and New York with southwestern British Columbian *I. pini* would establish whether the latter has New York behavioral traits. Production of lanierone and the dose–response to the highly pure enantiomers of ipsdienol are also key factors to be investigated for this population. However, differences in the cuticular hydrocarbon phenotypes from the New York and southwestern British Columbian populations indicate that these populations are not entirely identical (Seybold et al., unpublished).

If the population in southwestern British Columbia is demonstrated to have

the full complement of New York behavioral attributes, then its origin is of interest: whether through human introduction or natural postglacial migration with *P. banksiana* and *P. contorta* (Critchfield, 1957, 1984, 1985; Mathews, 1979; Seybold et al., 1992). Since male *I. pini* collected from Alberta [34%-(−)]; Meadow Lake, Saskatchewan [39%-(−)]; Prince Albert, Saskatchewan [42%-(−)]; and Minnesota [34%-(−)] all produce an enantiomeric composition of ipsdienol similar to the southwestern British Columbia population [39%-(−)], it is likely that a transcontinental continuum of the New York ipsdienol phenotype exists through the Canadian provinces. Because *P. banksiana* is the only species of pine whose distribution spans the Lake States and most of the Canadian provinces (Critchfield and Little, 1966), the late Quaternary history of *I. pini* in this area is undoubtably linked to the history of this host (Critchfield, 1985). During the Holocene Epoch (the current interglacial episode), *P. banksiana* may have recolonized the Canadian provinces from a principal refugium in southeastern North America or from intraglacial corridors or refugia (Critchfield, 1985). The current distribution of *P. banksiana* ends in a zone of hybridization with *P. contorta latifolia* in northern Alberta, northeastern British Columbia, and southwestern MacKenzie (Critchfield, 1984, 1985). However, throughout the repeated glacial–interglacial sequences of the Pleistocene Epoch, *P. banksiana* and *P. contorta* have experienced increased genetic variation due to multiple alterations in geographic distribution, including periods of genetic contact during earlier interglacials (Critchfield, 1984). For example, there is evidence for introgression of *P. banksiana* into the northern interior range of *P. contorta latifolia*, extending northwest from western Montana to the Yukon (Critchfield, 1984). These genetic traits have been documented to occur to the west of the current zone of hybridization and are separated from this zone by populations of pure *P. contorta latifolia*. Their presence in these locations has been attributed to previous contacts between the species during earlier interglacials (Critchfield, 1984).

Critchfield's (1984) themes of long periods of geographic isolation with possible genetic differentiation, shifting and expansion of the range, and reestablishment of genetic contact with populations from other refugia might apply to historical populations of *I. pini* as well as to its hosts. However, comparable genetic studies of the associated regional populations of *I. pini* that might reveal similar disjunct occurrences of genetic influence have not been conducted. Furthermore, the only currently known, direct fossil evidence related to the Quaternary history of *I. pini* consists of body fragments excavated near Kitchener, Ontario, from peat/marl lacustrine sediments at a depth of 1.75–3.28 m and dated ~9000 BP (Schwert et al., 1985). The sediments indicated that at that time the area was a pine-dominated vegetative zone with floral and faunal conditions similar to those present in southern Ontario today. To our knowledge,

there are no fossil records from the area of interest in the western Canadian provinces.

It is intriguing that while the current distribution of *P. banksiana* ends in a zone of hybridization with *P. contorta latifolia* in northern Alberta, northeastern British Columbia, and southwestern Mackenzie, the distribution of the New York ipsdienol phenotype of *I. pini* continues in *P. contorta latifolia* through southwestern British Columbia to the border of Washington. This asymmetry between the distribution of the New York phenotype of the beetle and the host that it may have followed from the east could be explained by invasion of New York-type *I. pini* into the habitat and host once occupied solely by the western form or by a more rapid postglacial recolonization rate by the eastern host, *P. banksiana.* Miller (1990) studied the enantiomeric composition of ipsdienol produced by males from six populations in British Columbia and the response to the enantiomers in four populations. One population (Radium in southeastern British Columbia, Figure 1) has a distribution of ipsdienol enantiomeric composition that appears to be intermediate between east and west, suggesting possible introgressive hybridization.[4] However, the response of the Radium population of *I. pini* to the enantiomers of ipsdienol appears to be similar to the New York type (Miller, 1990). In our study, the slightly lower enantiomeric compositions of ipsdienol [in percentage-(−)] produced by populations from southeastern British Columbia, Idaho, and Montana may indicate that, similar to the situation with the two hosts, the zone of introgression for the two beetle populations extends south of the international border. Alternatively, the New York type of *I. pini* may have had more rapid entry into northern British Columbia during the present interglacial. Macrofossil data (Critchfield, 1985) and pollen-dating studies (Critchfield, 1985; MacDonald and Cwynar, 1985; Prentice et al., 1991) suggest that *P. banksiana* may have recolonized the area vacated by the receding Laurentide and Cordilleran ice sheets more rapidly than *P. contorta.*

Although the species of pine host used for ipsdienol production did not radically alter the enantiomeric composition of ipsdienol isolated from any *I. pini* population in our study, it is interesting that the enantiomeric composition [in percentage-(−)] was consistently lower when males were placed in any other host compared to their host of origin. These reductions were small when compared to the differences measured from the New York and California phenotypes, but comparable to the differences measured from the Idaho/Montana and California phenotypes. These small reductions might be due to a nutritional effect of feeding in a biochemically different pine species or simply attributable

[4]This area of North America has also been recognized as a site of complex intrapopulational pheromone variation for *Ips tridens* (Mannerheim) (Lanier and Burkholder, 1974).

to variation in our technique. One source of variation is the number of individuals included in the aeration sample. This is related to the inherent variation in the enantiomeric composition between individuals in the population (Miller et al., 1989; Miller, 1989). For example, in a study of gut extracts made from 62 individual male *I. pini* collected in Shasta County, California, Miller et al. (1989) found that 10–20% of the individuals had ipsdienol with enantiomeric compositions lower than 90%-(−) and several individuals had an enantiomeric composition less than 50%-(−). The distribution mean was 91%-(−), which is lower than results obtained by trapping ipsdienol from all individuals simultaneously in the volatile headspace from a similarly-sized population from the same locality (Tables 1 and 2, Figure 1). The slightly lower result noted by Miller et al. (1989) may also be due to host effects. Insects from their study were collected in *P. ponderosa* and analyzed in *P. contorta latifolia*. Real differences associated with their gut extraction technique and our volatile entrapment method may also exist.

Another source of variation may be related to the quantity of ipsdienol produced or collected for analysis. Thus, the number of individuals included in each aeration sample may affect the measurement of the enantiomeric composition. However, we have found that including 20–25 individuals in an aeration sample gives an enantiomeric composition that is repeatable with larger numbers of insects. For example, in four separate aerations of four coastal California populations of male *Ips mexicanus* (Hopkins) (29, 61, 111, and 200 individuals), the enantiomeric compositions of ipsdienol were: 88.9, 90.9, 91.3, and 88.9%-(−), respectively (Seybold, 1992). Similar results were obtained in the present study (see populations of *I. pini* from Shasta and Lassen Counties, California; Table 2).

A third source of variation in our technique involves run-to-run variation in the extraction and chemical analytical procedures. Two similarly-sized samples of male *I. pini* from Sauk County, Wisconsin, of male *I. pini* from Otero County, New Mexico, and of male *I. bonanseai* from Nuevo Leon, Mexico (Table 1) were analyzed. The measurement differed by 3.0% in absolute terms for Wisconsin *I. pini*, yet by only 0.5% for New Mexico *I. pini* and 0.7% for *I. bonanseai*, indicating the possibility for substantial run-to-run variation, although different individuals were also included in each run. Due to these three sources of variation, a larger number of samples will be required to separate the northern Rocky Mountain (i.e., Idaho/Montana) unequivocally from the Pacific coast/southwestern United States variant. Our analysis has been a first-level, bulk aeration assessment. Further sampling in these areas of interest should involve measuremnts of within-population variation by analyzing volatiles trapped from individuals.

Acknowledgments—We dedicate this paper to the memory of Gerald N. Lanier, who recognized both that, "In some instances the entities revealed by pheromonal differences will prove to

be sibling species" and research on the role of optical rotation in pheromone specificity could clarify the role of pheromones in the evolution of the Coleoptera (Lanier and Burkholder, 1974). We wish to thank the following cooperators who assisted us with collections of live populations of *I. pini* and host material: C.W. Berisford, J.H. Borden, B. Borys, J.H. Cane, A. Cognato, R. Dorsett, T. Eager, T. Eiber, J.W. Fox, M.M. Furniss, C. Garza, K. Gibson, P.F. Kolb, D.W. Langor, G.N. Lanier, J. Flores Lara, R.L. Livingston, L.D. Merrill, D.R. Miller, D. Milligan, K.F. Raffa, M.E. Schultz, P.J. Shea, and S.A. Teale. J.L. Tupy, S.T. O'Keefe, M. Kuenzli, D.R. Quilici, and J.A. Tillman provided essential laboratory support for this study. H. Rappaport provided assistance with graphics, and J. Baldwin provided statistical advice. We thank B.B. Kinloch, C.I. Millar, and D. Vanderwel for reviews of the manuscript. This research was supported by the University of California at Berkeley, a National Science Foundation Graduate Fellowship to S.J.S., National Science Foundation Dissertation Improvement Grant BSR-8914625 to S.J.S., and USDA Forest Service Pacific Southwest Research Station Cooperative Research Agreement #PSW-92-0014CA with D.L.W. A substantial portion of this survey was completed by S.J.S. with the support of and at the facilities of the Insect Chemical Ecology Laboratory of the USDA Forest Service, Pacific Southwest Research Station, Albany, California.

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WHY NOT BE A QUEEN? REGIOSELECTIVITY IN MANDIBULAR SECRETIONS OF HONEYBEE CASTES

E. PLETTNER,[1] G.R.J. SUTHERLAND,[1,3] K.N. SLESSOR,[1,*] and M.L. WINSTON[2]

[1]*Department of Chemistry*
[2]*Centre for Pest Management, Department of Biological Sciences*
Simon Fraser University
Burnaby, British Columbia, Canada V5A 1S6

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Abstract—Both female castes of the honeybee (*Apis mellifera* L.) synthesize hydroxylated 2(*E*)-decenoic acids in their mandibular glands. Queens produce 9-hydroxy-2(*E*)-decenoic acid as part of their primer pheromone, while workers produce the regioisomeric 10-hydroxy acid, probably as a larval food source and an antiseptic secretion. Both workers and queens are biosynthetically competent to produce the other caste's dominant hydroxylated compound, as both isomers can be detected in queens and workers. We investigated the source of the caste-determined regioselectivity of hydroxy acid biosynthesis by investigating the production and interconversion of these compounds in isolated worker honeybee mandibular glands with specifically deuterated precursors. Gas chromatographic-mass spectroscopic identification of the labeled product indicates that octadecanoic acid is converted into 10-hydroxy-2(*E*)-decenoic acid with higher efficiency than either hexadecanoic or decanoic acids. 10-Hydroxydecanoic acid is readily converted into 10-hydroxy-2(*E*)-decenoic acid as expected in the β-oxidation process. The saturated and unsaturated 10-hydroxy acids are oxidized to the corresponding ten carbon diacids.

Key Words—Hymenoptera, Apidae, *Apis mellifera* L., mandibular gland secretion, hydroxydecenoic acids, hydroxydecanoic acids, deuterated precursors, gas chromatography-mass spectroscopy, biosynthetic precursors.

INTRODUCTION

There are two female castes in a honeybee (*Apis mellifera* L.) colony: the queen and several thousand workers. The queen's main function is reproduction,

*To whom correspondence should be addressed.
[3]Present Address: Department of Chemistry, University of Utah, Logan, Utah.

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although she also heads the colony by producing primer/releaser pheromones that mediate many aspects of worker behavior and partially inhibit worker reproduction. The workers perform many nonreproductive tasks, such as brood rearing, guarding, and foraging (Winston, 1987), and generally only lay eggs following two to three weeks of queenlessness and broodlessness. Beekeepers and scientists have been fascinated by the differences between queens and workers because fertilized eggs have the potential to follow different developmental pathways leading to worker or queen adults. This process of caste differentiation ultimately results in morphological and physiological differences between adult queens and workers.

One such physiological difference is the composition of the mandibular gland secretion, a blend of several compounds produced in the mandibular glands of female honeybees. Among the many compounds found in their mandibular glands, both queens and workers produce a group of oxygen functionalized 10- and 8-carbon fatty acids. The fatty acids characteristic of queens have the functional group at the penultimate ($\omega - 1$) position in the chain, while the acids typical of workers are functionalized at the last (ω) position. The major component of queen mandibular pheromone (QMP) is 9-keto-2(*E*)-decenoic acid (ODA); the second most abundant is 9-hydroxy-2(*E*)-decenoic acid (9-HDA) (Slessor et al., 1988, 1990). The most abundant component of the worker mandibular blend is 10-hydroxy-2(*E*)-decenoic acid (10-HDA), followed by 10-hydroxydecanoic acid (10-HDAA). The 8-carbon homolog, 8-hydroxyoctanoic acid (8-HOAA), is a minor worker component, as are the diacids corresponding to 10-HDA and 10-HDAA, 2(*E*)-decenedioic acid ($C_{10:1}$ DA) and decanedioic acid ($C_{10:0}$ DA), respectively.

The function of these caste-specific blends also differs. The queen-produced blend is a releaser pheromone that attracts nearby workers to the queen, giving rise to a retinue of workers around the queen (Slessor et al., 1988), among other effects (Winston and Slessor, 1992). The queen pheromone also is a primer pheromone, since it inhibits queen rearing by workers (Winston et al., 1990) and its major component, ODA, inhibits juvenile hormone III biosynthesis in workers (Kaatz et al., 1992). The worker mandibular components are not known to function as semiochemicals, but 10-HDA is found in royal jelly (Weaver et al., 1968), the worker-produced queen food and the 10-carbon diacids, $C_{10:1}$ DA and $C_{10:0}$ DA, are found in honey (Plettner, unpublished observation). Blum et al. (1959) reported antibacterial and antifungal activity for 10-HDA, and Boch et al. (1979) reported the volatile acids present as antibacterials, so this secretion may be involved in food preservation.

Even though the mandibular acids of both castes appear to have important functions in honeybee colonies, their biosynthesis has not been studied. However, the catabolism of ODA in workers was followed with radiolabeled material, in an effort to understand how the workers clear their system of the queen-

produced attractant and primer (Johnston et al., 1965). The results indicated that the ODA was reduced to 9-HDA and to the saturated analogs, 9-ketodecanoic- and 9-hydroxydecanoic acids (ODAA and 9-HDAA). This process appeared to take place mainly in the gut of the workers. The authors speculated that the catabolites of ODA might accumulate in the worker-produced royal jelly and could thus be recycled back to the queen, who would convert the catabolites back to ODA. However, analyses of royal jelly have revealed that it does not contain 9-HDA, 9-HDAA, or ODAA (Weaver et al., 1968), and the biosynthesis of mandibular acids has not been studied since. The objective of our work was to study interconversion among mandibular blend components and to find which precursor is functionalized to give the hydroxy acids, using deuterated potential intermediates and precursors and following their incorporation by gas chromatography–mass spectroscopy. We wished to understand how the caste-specific regioselective functionalization of the hydroxy acids is regulated and chose to initially study workers, since these are more readily available than queens.

The composition of queen and worker mandibular glands had been thought to be mutually exclusive, until it was found that some workers can produce ODA under queenless conditions (Ruttner et al., 1976). Further work led to the realization that this overlap does not occur under special circumstances. Even queenright workers have a small amount of 9-HDA in their mandibular glands, the ratio of 10-carbon hydroxy acids, 10-HDA–10-HDAA–9-HDA being ca. 30:10:1 (Plettner et al., 1993). We have taken advantage of this occurrence of 9-HDA in workers to study its biosynthesis simultaneously with the ω-hydroxy acids. The overlap in gland composition is not restricted to workers; mated, laying queens always have trace amounts of 10-HDAA and variable amounts of 10-HDA, along with ODA and 9-HDA, which are more abundant than the worker acids (Crewe, 1982, Plettner et al., unpublished observation). While workers retain the same ratio of mandibular components throughout their life, queens gradually change from a blend dominated by 10-HDA in newly emerged queens to a blend with mostly ODA and 9-HDA. Both castes experience an increase in the total amount of acids in the mandibular glands as they age (Slessor et al., 1990) (Figure 1). Knowledge of the mandibular gland ontogeny was useful for this project, since young individuals have low levels of endogenous material, which makes labeled products easier to detect.

METHODS AND MATERIALS

Gland Isolation and Treatment

The bees used for the assay were 1–5 days old and were kept caged in an incubator (34°C) with honey and water until required. Both glands were freshly

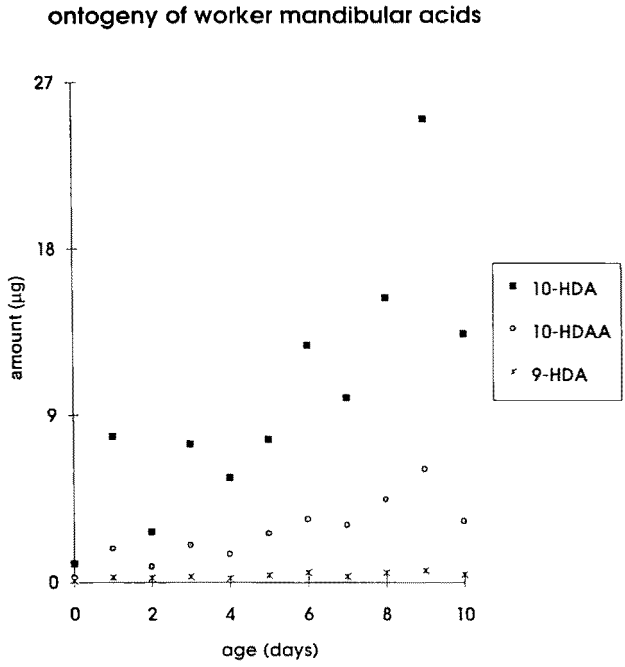

FIG. 1. Ontogeny of three hydroxy acids found in worker mandibular glands: 10-hydroxy-2(*E*)-decenoic acid (10-HDA), 10-hydroxydecanoic acid (10-HDAA), and 9-hydroxy-2(*E*)-decenoic acid (9-HDA). Data represent the total amount of compound found in a pair of mandibular glands, as determined by gas chromatography (Slessor et al. 1990). Each point is an average of three samples.

removed, washed in Tris-buffered physiological saline (pH 6.5–7.5), blotted dry with a piece of tissue, and exposed to a solution of the test substrate in DMSO. The gland was placed in an Eppendorf tube and left to perfuse at room temperature for 5–20 min, washed with physiological saline, blotted dry, and placed in methanol (13 µl) containing 10-undecenoic acid (0.2 µg/µl) as internal standard, crushed, and centrifuged at low speed. The supernatant was removed and the pellet extracted with a further portion of methanol (13 µl) containing internal standard.

Analysis

Two aliquots (2 µl) of mandibular extract were evaporated and trimethylsilyated with bistrimethylsilyltrifluoroacetamide (2 µl of neat reagent, Sigma Chemical Co., St. Louis, Missouri) for at least 40 min at room temperature (Slessor et al., 1990). One sample was diluted with hexane (to 30 µl) and

analyzed by splitless capillary gas chromatography (GC) on a Hewlett-Packard 5890A equipped with a flame ionization detector and a 30 m × 0.25 mm, (film thickness 0.25 μm) DB-1 column (program: 100°C, 1 min; 10°/min to 185°C, 4 min; 3°/min to 200°C, 0 min; 25°/min to 260°C, 20 min), head pressure 120 kPa, flow: 30 ml/min. Response was calibrated with standards of known concentration.

The second sample was diluted such that ca. 10–20 ng of the most abundant component would be injected and analyzed on a Varian 3400 GC interfaced with a Varian Saturn ion trap mass spectrometer (MS), equipped with a 30-m × 0.25-mm, (film thickness 0.25 μm) DB-5 column (program: 100°C, 1 min; 10°/min to 200°C, 3°/min to 220°C, 2.5°/min to 225°C, (15 min), head pressure 105 kPa, flow 20 ml/min. The ionization mode was EI with a filament emission current of 10 μA at 70 eV, a target value of 5500, with the automatic gain control (AGC) on and with 1 scan/sec.

Data Analysis and Statistics

Three evaluations of substrate incorporation were investigated: percentage of label incorporated, quantity of labeled product formed, and rate of incorporation. Small overlapping peaks from artifacts in GC-MS made the latter two evaluations less reliable than percentage label.

The usually dominant $(M\text{-}15)^+$ fragment, arising from loss of a methyl group from the trimethylsilyl derivative of the parent ion, was used to determine the percentage label for each compound of interest. The ion currents for the $(M\text{-}15)^+$ and $(M\text{-}15 + n)^+$ (n = number of D atoms in the compound) were recorded for each scan across the GC peak without prior background subtraction. The percentage of labeled material was obtained from a calibration curve in which the ratio of $\Sigma(M\text{-}15 + n)/\Sigma(M\text{-}15)$ had been plotted against the percentage of label, using prepared mixtures of labeled and unlabeled standard. This correction minimized the effect of overlapping ions, especially $(M\text{-}15 + nD) - n$ signal on the unlabeled (M-15) signal at higher labeling concentrations. With the AGC on, correction parameters varied only slightly with concentration of 0.1–100 ng of injected sample.

The data were analyzed by a one-way ANOVA (SAS statistical package, SAS Institute Inc., Cary, North Carolina) for differences between treatments and blanks (pairwise comparisons). Points were only rejected if a known experimental problem existed with the sample, or if they were strong outliers by the fourth spread criteria (Devore, 1991).

Labeled Substrates

General. TLC was performed on 0.25-mm-thick films of silica gel (Merck, Kieselgel 60G for TLC) on glass plates. Acidic TLC plates were prepared with 2% H_3PO_4 instead of water and were used to monitor reactions and purifications

involving free acids. Silica gel columns were prepared with Merck Kieselgel 60 (230–400 mesh). Acidic gel was prepared by suspending the silica in 2% aqueous H_3PO_4 (silica–aqueous acid 1:2 w/w), leaving the silica to settle, decanting the supernatant, and drying the slurry in an oven at 100°C. Acidic silica gel was used to purify compounds possessing free carboxylic acid functionalities to minimize streaking. Esters dissolved in methanol were hydrolyzed by adding 5% aqueous NaOH and stirring overnight. Hydrolyzates were acidified with 10% HCl and extracted twice with ether, the ether washed with brine, and dried over sodium sulfate. ^{1}H NMR spectra were measured in $CDCl_3$ at 400 MHz on a Bruker WM-400. Both ^{1}H NMR and GC-MS were used to confirm the structure and assess the extent of deuteration in intermediates and products. All deuterated products showed physical properties and retention times identical to the unlabeled compound, except for the differences in the ^{1}H NMR and MS fragmentation expected for the deuterium substitution pattern.

(9,9,10) D_3-10-Hydroxydecanoic Acid (D_3 10-HDAA). 10-Oxodecanoic acid was obtained from 10-undecenoic acid by OsO_4-mediated oxidation with $NaIO_4$ (Graham and Williams, 1966). This material was refluxed 4 hr under Ar at 105°C with D_2O–pyridine (6:7), to give (9,9) D_2-10-oxodecanoic acid (96% D_2). Reduction with $NaBD_4$ in methanol gave (9,9,10) 10-hydroxydecanoic acid (94% yield, 94% labelled).

(4,4) D_2-2(E)-Decenoic Acid [D_2 $C_{10:1}$ 2(E)]. Octanal was deuterated with D_2O–pyridine (1:1, 3 hr, 100°C) to give (2,2) D_2-octanal (93% D_2). Reaction with the ylide of trimethylphosphonoacetate produced by treatment with NaH (Kandil and Slessor, 1985), gave methyl (4,4) D_2-2-decenoate (ca. 86% *E* and 14% *Z*). Chromatography on silica gel (hexane–ether, 5:1) provided an enriched methyl ester fraction (ca. 95% *E*, 30% yield). The methyl ester was base hydrolyzed to yield the free acid (77% yield).

(4,4) D_2-10-Hydroxy-2(E)-Decenoic Acid (D_2 10-HDA). The tetrahydropyranyl derivative of 8-hydroxyoctanal (Chiron, 1982) was deuterated with pyridine–D_2O (1:1, 16 hr, reflux), to give (2,2) D_2-8-THPO-octanal (94% D_2). Condensation with the ylide of trimethylphosphonoacetate gave the THP derivative of methyl (4,4) D_2-10-hydroxy-2-decenoate (ca. 80% *E* and 20% *Z*). Removal of the THP group under acidic conditions (methanol–water–10% HCl, 1:4:10, room temperature for 1.5 hr) and silica gel purification resulted in methyl (4,4) D_2-10-hydroxy-2-decenoate enriched in the *E* isomer (93% *E*, 50% yield, 94% D_2). Base hydrolysis of the methyl ester gave the free acid (58% yield).

(4,4) D_2-2(E)-Decenedioic Acid (D_2 $C_{10:1}$ DA) and (2,2) D_2-Decanedioic Acid (D_2 $C_{10:0}$ DA). Small portions of D_2 10-HDA and D_2-10-oxodecanoic acid were oxidized with pyridinium dichromate (PDC) (Corey and Schmidt, 1979) to the corresponding diacids D_2 $C_{10:1}$ DA and D_2 $C_{10:0}$ DA, respectively.

(12) D_1-Octadecanoic Acid (D_1 $C_{18:0}$). Oxidation of methyl 12-hydroxy-

octadecanoate (Sigma) with pyridinium chlorochromate (PCC) gave the 12-keto compound, which was treated directly with *p*-tolylsulfonylhydrazine (2 hr reflux in MeOH) and reduced with $NaBD_4$ (2 hr 50°C in MeOH) to give methyl 12-D octadecanoate in 5% yield. The methyl ester was hydrolyzed to the free acid (83% D_1).

(7,7) D_2-8-Hydroxyoctanoic Acid (D_2 8-HOAA). Oxidation of monoacetyl 1,8-octanediol (Babler and Coghlan, 1979) with PDC gave 8-acetoxy octanoic acid. The acetate group was removed to give 8-hydroxyoctanoic acid and oxidized with PCC to the 8-oxo acid. The oxo acid was deuterated twice with pyridine–D_2O (1 : 1, 17 hr reflux, 65% D_2) and reduced with $NaBH_4$ in MeOH to give the deuterated ω-hydroxy acid.

(7,7,8,8) D_4-Decanoic Acid (D_4 $C_{10:0}$). 7-Decynoic acid was prepared by the condensation of 6-bromohexanoic acid and 1-butyne in the presence of 2.5 eq. of *n*-butyl lithium, converted to the methyl ester with BF_3–MeOH, and deuterated with deuterium and $RhCl_2[P(phenyl)_3]_2$ as a catalyst (room temperature, 1 atm) to give methyl (7,7,8,8) D_4 decanoate. Hydrolysis gave deuterated decanoic acid.

RESULTS

Treatment with labeled 10-HDAA or 10-HDA led to labeling of 8-HOAA, suggesting that both 10-carbon hydroxy acids can be shortened to 8-HOAA (Table 1). Furthermore, application of labeled 10-HDA resulted in labeling of 10-HDAA and vice versa, indicating that 10-HDA and 10-HDAA can be interconverted. Finally, treatment with labeled 8-HOAA led to no significant labeling

TABLE 1. PERCENTAGE OF LABELED PRODUCT FORMED IN INTERCONVERSION AMONG ω-HYDROXY ACIDS IN WORKER MANDIBULAR GLANDS

Treatment	Incubation time (min)	*N*	Product (means ± SE)[a] 8-HOAA	10-HDAA	10-HDA
D_2 8-HOAA	10	9	**73.4 ± 1.2 a**	1.5 ± 0.1 a	4.0 ± 1.4 a
Blank		10	6.3 ± 1.8 b	3.6 ± 0.2 b	4.1 ± 0.4 a
D_3 10-HDAA	15	9	22.6 ± 11.1 a (N = 8)	**86.0 ± 4.0 a**	15.5 ± 7.0 a
Blank		9	1.3 ± 0.7 b	0.3 ± 0.1 b	0.5 ± 0.1 b
D_2 10-HDA	20	8	48.0 ± 5.0 a	27.0 ± 4.0 a	**89.7 ± 5.1 a**
Blank		9	1.3 ± 0.7 b	0.3 ± 0.1 b	0.5 ± 0.1 b

[a]Treatments were compared to their corresponding blanks. Means within a column followed by the same letter do not differ significantly by Tukey's test ($P < 0.05$).

of the 10-carbon hydroxy acids, which suggests that 8-HOAA was not chain lengthened.

In the D_3 10-HDAA treatment, labeled 8-HOAA accumulated over time. In 1 min, 7.1 ± 3.3 ng of labeled 8-HOAA formed, while in 15 min, 120 ± 62 ng formed. In the same experiment, labeled 10-HDA did not accumulate with time; 12.6 ± 2.2 ng formed in 1 min and 9.2 ± 2.8 ng formed in 15 min. The accumulation of 8-HOAA with time and its failure to convert to other compounds suggests that it may be an end point in the hydroxy acid biosynthetic pathway. By contrast, 10-HDA did not accumulate with time and appears to be converted to other compounds. The percentages of labeled 10-HDA and 10-HDAA formed from 10-HDAA and 10-HDA, respectively, were similar (Table 1), as were the amounts formed during the 15- to 20-min treatments (data not shown). This indicates that in young workers desaturation of 10-HDAA to give 10-HDA occurs more or less at the same rate as the reverse reaction.

Apart from interconversion, the 10-carbon hydroxy acids were oxidized to their respective diacids in both queens and workers. Treatment with D_3 10-HDAA led to significant incorporation in $C_{10:0}$ DA and $C_{10:1}$ DA, while treatment with D_2 10-HDA resulted in significant labelling of $C_{10:1}$ DA, but not of $C_{10:0}$ DA.

No incorporation of label from the ω hydroxy acids into ω-1 hydroxy acids was found. The reverse incorporation of label from a labeled 9-HDA into the 10 hydroxy acids was not observed (data not shown). Both observations suggest that the ω and ω-1 hydroxy acids do not interconvert.

TABLE 2. PERCENTAGE OF LABELED MATERIAL FORMED FROM DECANOIC, 2(*E*)-DECENOIC, HEXADECANOIC, AND OCTADECANOIC ACIDS AS PRECURSORS OF WORKER MANDIBULAR GLAND COMPONENTS

Treatment	*N*	Product (mean ± SE)[a]			
		9-HDA	8-HOAA	10-HDAA	10-HDA
D_4 C10:0	9	1.0 ± 0.5	1.8 ± 1.2	0.3 ± 0.1	**0.8 ± 0.1**
Blank D_4	10	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	**0.2 ± 0.1**
D_2 $C_{10:1}$	6	5.0 ± 0.7	3.0 ± 0.4	2.3 ± 0.3	2.9 ± 0.4
Blank D_2	6	2.4 ± 1.1	3.4 ± 1.8	2.6 ± 0.3	3.9 ± 0.3
D_3 $C_{16:0}$	14	7.5 ± 2.2	**14.2 ± 3.7**	3.3 ± 0.4	3.6 ± 0.6
Blank $D_{2/3}$	10	3.3 ± 0.9	**3.3 ± 1.1**	2.6 ± 0.2	3.0 ± 0.5
D_1 $C_{18:0}$	16	**22.4 ± 2.6**	**16.9 ± 5.0**	**10.0 ± 1.4**	**13.0 ± 3.0**
Blank D_1	10	**1.5 ± 0.7**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**

[a] For entries in bold type, treatments and blanks differed significantly ($P < 0.05$) by Tukey's test.

TABLE 3. INTERCONVERSION AMONG POTENTIAL PRECURSORS TO HYDROXY ACIDS IN WORKER MANDIBULAR GLANDS[a]

Treatment	Product			
	$C_{10:0}$	$C_{10:1}$	$C_{16:0}$	$C_{18:0}$
D_4 $C_{10:0}$		+	n.s.	n.s.[b]
D_2 $C_{10:1}$	n.s.		n.s.	+++
D_3 $C_{16:0}$	++	++		+++
D_1 $C_{18:0}$	n.s.	++	+	

[a]Plus signs indicate significant incorporation of label into the product as compared to the blank ($P < 0.05$, Tukey's). Data corresponding to the compounds used for treatment were left blank. +, up to 20% label; ++, between 20% and 40%; +++, between 40% and 60%; n.s., not significant.
[b]The mass spectra show a small amount of label.

Of the possible precursors to the hydroxy acids tested, D_1 $C_{18:0}$ was incorporated with the greatest efficiency into both ω-1 and ω hydroxy acids (Table 2). This was followed by D_3 $C_{16:0}$, which was incorporated significantly only into 8-HOAA. D_2 $C_{10:1}$ was not incorporated significantly into any of the hydroxy acids, while D_4 $C_{10:0}$ was only weakly incorporated into 10-HDA. Furthermore, application of D_2 $C_{10:1}$ and D_3 $C_{16:0}$ resulted in significant labeling of $C_{18:0}$ (Table 3). Treatment with D_4 $C_{10:0}$ did not lead to significant labeling of $C_{18:0}$, but a small amount of the expected label was seen in the mass spectra. These results suggest that unfunctionalized fatty acids with less than 18 carbons were lengthened to $C_{18:0}$ before functionalization.

DISCUSSION

A study of the possible interconversion among ω-hydroxy 10-carbon acids demonstrated that there was ready interconversion between the saturated and the 2(*E*)-unsaturated ω functionalized species in isolated worker mandibular glands. In addition, the two 10-hydroxy 10-carbon acid substrates were readily chain shortened to the saturated 8-carbon ω acid. Both observations are in accord with the ability of these hydroxylated compounds to enter the β-oxidation pathway. 10-HDAA would first be desaturated to 10-HDA, presumably through activation as its CoA-ester. Our results indicate this to be a reversible process. Hydration of the 2–3 double bond would give a 3,10-dihydroxydecanoate. The final steps are the oxidation of the 3-hydroxy group to a keto moiety, attack of the keto group by free CoA-SH, and displacement of the acetyl-CoA head portion, giving

8-HOAA-CoA. At each step a portion could be hydrolyzed, giving rise to the corresponding free acid. Presumably, a significant proportion of the 10-HDA-CoA gets hydrolyzed, since 10-HDA is the major hydroxy acid component of the worker mandibular gland secretion. Free 3,10-dihydroxydecanoic acid appears to be a minor component of worker mandibular extracts and has been found in royal jelly, along with the other ω-hydroxy acids (Weaver et al., 1968).

In addition to β-oxidation of 8- and 10-carbon hydroxy acids, the glands have the capability to oxidize ω-hydroxy acids to diacids. While 10-HDA was oxidized only to its corresponding diacid ($C_{10:1}$ DA), 10-HDAA was converted to both its corresponding diacid ($C_{10:0}$ DA) and $C_{10:1}$ DA. The latter conversion could have occurred by desaturation of 10-HDAA to 10-HDA, followed by oxidation to the diacid, or vice versa, oxidation to $C_{10:0}$ DA preceding desaturation to $C_{10:1}$ DA. No experiment with labeled $C_{10:0}$ DA was performed to evaluate these two possibilities.

We investigated decanoic acid ($C_{10:0}$) as a potential precursor to find the fatty acid subtrate that is regioselectively oxygenated through caste-determined control. We compared incorporation of labeled straight-chain fatty acids of different chain lengths in the whole gland assay. The results indicated that D_1 $C_{18:0}$, and not D_4 $C_{10:0}$, was incorporated most efficiently into both ω and ω-1 10-carbon components. Furthermore, interconversion among precursors indicated that $C_{10:0}$, $C_{10:1}$, and $C_{16:0}$ were lengthened to $C_{18:0}$, suggesting it to be the entry point to the hydroxy acid pathway. For $C_{10:0}$ to be incorporated into mandibular acids, it must be lengthened to $C_{18:0}$ prior to functionalization, requiring the largest number of steps and experiencing considerable dilution. $C_{18:0}$ would require the smallest number of steps and would experience the smallest isotopic dilution in the final products. The somewhat low incorporation of D_3 $C_{16:0}$ may be due to a primary isotope effect, since one of the deuterium atoms would be removed during ω-functionalization. Our results are consistent with $C_{18:0}$ being the entry point to the hydroxy acid biosynthetic pathway, with either free and/or lipid-bound $C_{18:0}$ constituting a precursor reservoir that ensures continuous hydroxy acid biosynthesis by the mandibular gland.

Two routes are possible to convert $C_{18:0}$ into 10-carbon ω and ω-1 hydroxy acids, which differ only in the order in which functionalization and chain shortening take place. Functionalization may occur at the $C_{18:0}$ (or its CoA ester) stage to give 18-hydroxyoctadecanoic acid (or its CoA ester) followed by chain-shortening by β-oxidation to the 10-carbon length (Figure 2). Alternatively, after chain shortening, a bound $C_{10:0}$ intermediate is functionalized. Both routes have been observed in the biosynthesis of fatty acid-derived semiochemicals. The major component of the sex pheromone of the cabbage looper (*Trichoplusia ni*), (*Z*)-7-dodecenyl acetate, is biosynthesized from $C_{16:0}$-CoA by Δ11 desaturation to give (*Z*)-11-$C_{16:1}$-CoA followed by chain-shortening to (*Z*)-7-$C_{12:1}$-CoA, the immediate precursor (Jurenka and Roelofs, 1993). Chain-shortening

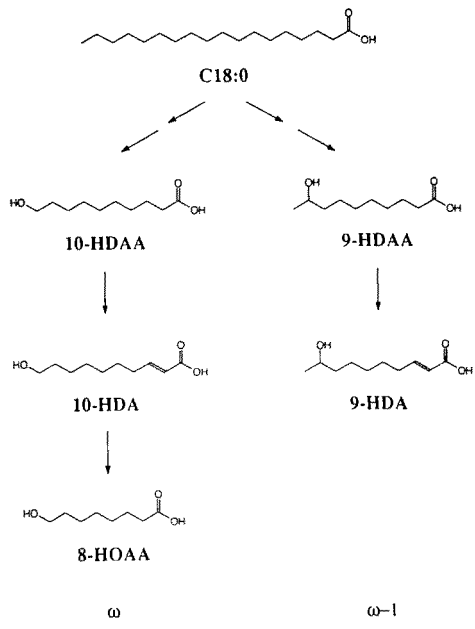

FIG. 2. Formation of ω and ω-1 hydroxy acids from octadecanoic acid ($C_{18:0}$) in worker mandibular glands. The ω hydroxy acids are: 10-hydroxydecanoic acid (10-HDAA), 10-hydroxy-2(*E*)-decenoic acid (10-HDA), and 8-hydroxyoctanoic acid (8-HOAA); the ω-1 hydroxy acids are 9-hydroxydecanoic acid (9-HDAA) and 9-hydroxy-2(*E*)-decenoic acid (9-HDA).

before functionalization is found in the red-banded leafroller moth (*Argyrotaenia velutinana*), where the major sex pheromone component, (*Z*/*E*)-11-tetradecenyl acetate, is made by $C_{18:0}/C_{16:0}$-CoA chain shortening to $C_{14:0}$-CoA, followed by Δ11 desaturation to give (*Z*/*E*)-11-$C_{14:1}$-CoA. (Jurenka and Roelofs, 1993). We are currently investigating which route is responsible for hydroxy acid biosynthesis in honey bee mandibular glands.

One requirement of being a queen is the regioselective functionalization of a fatty acid, or its derivative, at the ω-1 position in the production of the queen's primer pheromone. Our results indicate that the free fatty acid entry point for this caste-determined capability is $C_{18:0}$. As no evidence was found for the interconversion of ω and ω-1 hydroxy acids, two distinct functionalization reactions must be operative: ω for workers and ω-1 for queens. The positional selectivity of this hydroxylation to form hydroxy decenoic acids in the mandibular gland is one of the important developmentally derived characters that differentiate queens from workers. An understanding of the control system reg-

ulating this regioselectivity will provide a better appreciation for the caste differentiation processes in this highly social insect.

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TESTING THE "NEW ASSOCIATIONS" BIOLOGICAL CONTROL CONCEPT WITH A TACHINID PARASITOID (*Euclytia flava*)

J.R. ALDRICH

Insect Chemical Ecology Laboratory, USDA-ARS, Bldg 007
Agricultural Research Center-West
Beltsville, Maryland 20705

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Abstract—Males of the spined soldier bug, *Podisus maculiventris* (Heteroptera: Pentatomidae), produce an attractant pheromone that is exploited as a host-finding kairomone by a complex of parasitic species. The capability to catch hundreds of a generalist tachinid fly parasitoid, *Euclytia flava*, alive in traps baited with the pheromone of *P. maculiventris* provided an opportunity to test the premise of the "new associations" biological control concept. The hypothesis that host species newly associated with a parasitoid are maladapted relative to native-native associations was tested by giving *E. flava* females a choice between native and exotic stink bugs (Heteroptera: Pentatomidae). Wild *E. flava* females preferred to oviposit on exotic pentatomid species rather than indigenous, known host species, both in field traps baited with the pheromone of a native host and in the laboratory. Data presented here demonstrate that an invader may be vulnerable to native parasitoids in one aspect of the parasitism process (acceptance), yet go unrecognized as a potential host.

Key Words—Parasitoid, Tachinidae, Pentatomidae, *Nezara*, *Podisus*, biological control, pheromone, kairomone, exotic host.

INTRODUCTION

Classical biological control has emphasized old associations, seeking natural enemies in the native regions of pests for introduction into areas where target species are out of control (Nechols and Kauffman, 1992). However, coevolution between a parasitoid and its hosts may lead to a physiological equilibrium that could prevent such long-associated parasitoids from being effective biological control agents (Pimentel, 1963). Indeed, the probability of success in biological control is reportedly 75% higher in programs employing new associations than

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those based on long-evolved parasite–host or predator–prey associations (Hokkanen and Pimentel, 1984). These authors proposed that new exploiter–victim associations should be the preferred type of biological control agents in the future. This proposal proved to be controversial among biological control specialists (Greathead, 1986; Hokkanen and Pimentel, 1989; Nechols et al., 1992) but, in their reanalysis of the data on the new association approach for selecting biological control agents, Hokkanen and Pimentel (1989) again concluded that the concept is ecologically and statistically sound. Moreover, they reemphasized that newly associated biological control agents are "virtually the only type of agents for the classical biocontrol of native pests" (Hokkanen and Pimentel, 1989).

The initial controversy over the merits of the new associations approach in biological control has escalated into a lively debate over the wisdom of importing exotic species to control native pests (e.g., Carruthers and Onsager, 1993; Lockwood, 1993a). An overriding concern with the use of exotics to control native species is whether the risk of adverse affects to innocuous and beneficial endemic species is worth the potential benefit of controlling the pest (Howarth, 1991; Pimentel and Hunter, 1992; Lockwood, 1993a,b). Officials in the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) are keenly aware of environmental issues involved in biological control with exotic agents (C.P. Schwalbe, USDA-APHIS, personal communication), and the regulations governing the importation of exotic species are currently under review (M. Firko, USDA-APHIS, personal communication).

Data gleaned from the literature for Tachinidae indicate that biological control efforts involving this group support the new associations concept more clearly than do data for some other groups (Hokkanen et al., 1994). Large numbers of the endemic generalist pentatomid parasitoid, *Euclytia flava* (Diptera: Tachinidae), can be caught alive in traps baited with pheromone for the common North American generalist predator known as the spined soldier bug, *Podisus maculiventris* (Heteroptera: Pentatomidae) (Aldrich et al., 1984). This capability has provided an unprecedented opportunity to test the premise of the "new association" biological control concept. The hypothesis that host species newly associated with a parasitoid are maladapted relative to native–native associations was tested by giving *E. flava* females a choice between native and exotic pentatomids to determine their relative susceptibility to parasitoid oviposition.

METHODS AND MATERIALS

Host Insects. Table 1 lists the pentatomids used for host-choice experiments, the origin of individuals to start colonies, the native ranges for these

TABLE 1. PENTATOMIDS USED FOR HOST-CHOICE TESTS WITH *Euclytia flava* (Diptera: Tachinidae), AND THEIR CLASSIFICATION AS EXOTIC (E) OR NATIVE (N) RELATIVE TO RANGE OF *E. flava*

Species	Class	Collection locality	Native range
Predaceous			
Posisus maculiventris	N	Beltsville, Maryland	North America
P. neglectus	N	Beltsville, Maryland	North America
P. distinctus	E	Brasília DF, Brazil	South America
Phytophagous			
Nezara viridula	E	Stoneville, Mississippi	Africa
Euschistus obscurus	N	Weslaco, Texas	Central America, southern U.S.
E. tristigmus	N	Beltsville, Maryland	North America
E. servus	N	Beltsville, Maryland	North America
Acrosternum hilare	N	Beltsville, Maryland	North America
A. pennsylvanicum	N	Beltsville, Maryland	eastern North America
Thyanta accerra	N	Weslaco, Texas	eastern North America

species (McPherson, 1982; Thomas, 1992), and whether they are exotic or native relative to the range of *E. flava* (British Columbia and California, to Maine, and south to Texas) (Stone et al., 1965). *Nezara viridula*, a native of the Ethiopian region (Hokkanen, 1986; Jones, 1988), now has a cosmopolitan distribution, but Maryland is at least 350 km north of the limit of the range of this species in the eastern United States (Todd, 1989). *Euschistus servus*, *Euschistus tristigmus*, *Acrosternum hilare*, and *Acanthocephala terminalis* (Coreidae) adults used for experiments were collected in May and June, 1993 and 1994, from empress trees (Scrophulariaceae: *Paulownia tomentosa*). *Acrosternum pennsylvanicum* males used in the study were collected in June, 1993, from bracken fern (Filicopsida: Dennstaedtiaceae: *Pteridium aquilinum*) (Aldrich et al., 1989). Adults of other species were from laboratory colonies maintained as previously described (Aldrich et al., 1984, 1987).

Parsitoids. Adult *E. flava* females used for host choice tests were field-collected in traps baited with a simplified formulation (described below) of the synthetic pheromone for *P. maculiventris* (Aldrich et al., 1984). Flies in 1993 were collected from June 3 to 16, and flies in 1994 were collected from April 24 to May 14 in traps deployed at the Research Center.

Pheromone. A three-component blend was used to bait traps to catch *P. maculiventris* and *E. flava*. (*E*)-2-Hexenal (Aldrich Chemicals, Milwaukee, Wisconsin), racemic α-terpineol (Hercules Inc., Wilmington, Delaware), and benzyl alcohol (Aldrich Chem.) were mixed in a molar ratio of 1 : 0.843 : 0.061,

respectively. Batches of 40 ml of pheromone (17.6 ml hexenal, 21.4 ml α-terpineol, 1 ml benzyl alcohol) were normally prepared. A 20% (w/w) formulation of pheromone in plasticized polyvinyl chloride (PVC) was prepared by mixing 107 g of powdered PVC (Tenneco, Piscataway, New Jersey) with 53 ml of dioctyl phthalate (Aldrich), then adding the pheromone (Fitzgerald et al., 1973). The pheromone–PVC mixture was poured into test tubes (to the top), heated at 110°C for 30 min, removed from the tubes, and stored in sealed vials in a freezer until use. Pheromone for *Podisus neglectus* was prepared in a similar manner except linalool (Aldrich Chem.) was substituted for α-terpineol (Aldrich et al., 1986).

Trapping. Traps were made of transparent cylindrical containers having either white or transparent lids (20.2 × 19.7 cm; Tri-State Molded Plastics, Inc., Dixon, Kentucky) by cutting two holes (9 cm diameter) in opposite sides, and covering each hole with an inwardly projecting screen funnel. The containers were turned upside-down, so that the normal top of the container became the trap button, small drainage holes were made in the bottom, and the bottoms were held in place by two elastic cords hooked into each screen funnel. Traps were hung from branches about 1.7 m from the ground, at least 50 m apart, bordering oak-dominated deciduous forest at the Beltsville Agricultural Research Center-East. Each trap was baited with ca. 350 mg of pheromone–PVC material every three to four days. *E. flava* females were removed from traps by first flushing the trap with CO_2 to anesthetize the insects and then placing the flies in cotton-stoppered vials for transport to the laboratory.

Induced Parasitoid Oviposition in the Field. To determine if native tachinids could be induced to oviposit on adults of the southern green stink bug, *N. viridula*, a preliminary experiment was performed as follows in June 1989. A 20 × 20-cm cylinder was made from 1/4-in. mesh screen (large enough for tachinid flies to pass through), and the top and bottom were attached as for the traps described above. Ten laboratory-reared *N. viridula* males were placed inside the cage with a dose of *P. maculiventris* synthetic pheromone, and the cage was hung in the field.

A more extensive experiment was carried out from June 11 to 27, 1993. In this experiment, one or two newly emerged (<48-hr-old) laboratory-reared southern green stink bug males were placed inside traps baited with pheromone for *P. maculiventris*. Traps ($N = 20$) were monitored every one to three days, *Nezara* males exposed to parasitism were removed for examination in the laboratory, and fresh *N. viridula* males were put in the traps. The number and position of tachinid eggs on the *Nezara* males from pheromone-baited traps were counted under a dissecting microscope (tachinids known to use pentatomid pheromones as kairomones lay macro-eggs on their hosts' cuticle). Males were placed in half-pint cartons and held in the insectary for parasitoid emergence.

Host-Choice Bioassays. The same type of plastic containers used to make pheromone traps were used for the host-choice bioassays. Container lids were modified for ventilation by cutting a hole in the top (15-cm diameter) and gluing fine mesh nylon screen over the holes. Vaseline was applied to the upper, inner rim of each bioassay cage to prevent bugs from walking onto the lid, and each cage was provisioned with a water bottle, three green beans propped up against the cage wall, one mealworm pupa per predator if *Podisus* spp. were being tested, and honey was smeared on the screen mesh for the flies to feed upon.

Bioassays involving live bugs were usually performed with three or four wild *E. flava* females collected the same day as the bioassay was initiated, and three males and three females of each of the two heteropteran species were exposed to parasitoids. However, several bioassay experiments deviated from this standard, depending on the number of host specimens and flies available on any given day. In general, lower numbers of bugs and flies were used when the number of individuals for one of the three required species was limiting. The bugs to be tested were added to the bioassay cage, and then *E. flava* females that had been chilled in a refrigerator for 5–10 min were added. Each choice test lasted ca. 24 hr, after which the bugs were removed to count the eggs laid on them and held for parasitoid emergence. Some flies and unparasitized bugs were bioassayed on successive days.

Two choice-tests were performed using dead *Nezara* males treated with cuticular extracts. Six *N. viridula* males (killed by freezing) were mounted on pins in a circle, alternately dipped in extracts (purified through silica gel) of either *E. obscurus* or *N. viridula* males (9 adults/ml pentane/replicate), and placed in cages with six to eight 1-day-old flies.

Data Analysis. Bioassays of live bugs sometimes used flies on successive days and/or fewer than the normal number of bugs; therefore these data were analyzed by chi-square; all extract bioassays used 1-day-old flies and were analyzed by paired Student's *t* test.

RESULTS

Induction of Parasitoid Oviposition in the Field. The preliminary experiment exposing adult *N. viridula* males to parasitoids in the field showed that native tachinids attracted to the vicinity of the cage by *P. maculiventris* pheromone would, in fact, readily oviposit on southern green stink bugs. However, no parasitoids emerged from these *Nezara* males.

In the follow-up experiment in 1993, newly emerged *N. viridula* males with incompletely sclerotized cuticle were used because it seemed possible that the failure of tachinids to develop in the earlier experiment could have been due

TABLE 2. *Euclytia flava* (Tachinidae) OVIPOSITION ON MALE *Nezara viridula* (N.v., Exotic Pentatomid) PLACED IN FIELD TRAPS BAITED WITH *Podisus maculiventris* PHEROMONE[a]

Tachinid eggs/N.v.	$(\bar{X} \pm \text{SEM})^b$
Dorsal	2.17 ± 0.29
Ventral	6.15 ± 0.62
Whole body	8.26 ± 0.79

[a]One or two lab-reared *N. viridula* males (<48 hr old)/trap/day; 12 traps; 12 days, June 11–27, 1993, Beltsville, Maryland.
[b]$N = 72$, range = 1–27.

to an inability of the first-stage larvae to penetrate the host cuticle. Again, southern green stink bugs males received many tachinid eggs (8.26 ± 0.79 eggs/male; $N = 72$), and about three times more eggs were laid ventrally on the bugs than dorsally (Table 2). However, no tachinid larvae emerged from *Nezara* males with tachinid eggs, including several individuals having only one or two eggs.

Host-Choice Bioassays. A total of 317 living adult heteropterans, representing 11 species, were offered to 123 wild *E. flava* females in host-choice bioassays. These *Euclytia* females oviposited 1339 eggs.

Data for host-choice experiments involving live pentatomids are summarized in Figure 1A. Of the 12 choices included in Figure 1A, four combinations offered *E. flava* females a clear-cut choice between an exotic species (black bars) and a native, known host species (white bars): (1) *N. viridula/P. maculiventris*, (2) *P. distinctus/P. maculiventris*, (3) *N. viridula/T. accerra*, and (4) *N. viridula/E. tristigmus*. In all four of these exotic–native host choices, significantly more eggs were laid on the exotic species. In the experiment comparing the acceptability of the *P. distinctus* (Brazilian) versus *P. maculiventris* (No. 2), no eggs were oviposited on *P. maculiventris*. In *N. viridula/P. maculiventris* (No. 1) pairings, well over twice as many eggs were laid on *N. viridula* (508, range 10–57) than *P. maculiventris* (224, range 1–21) ($P < 0.001$). *Nezara viridula* adults were moderately, but significantly, preferred over *T. accerra* ($P < 0.05$) (No. 3), whereas *N. viridula* was greatly preferred over the known host, *E. tristigmus* ($P < 0.001$) (No. 4). Other pairings involving the exotic species, *N. viridula*, and native species not known to be naturally parasitized by *E. flava*, also showed ovipositional patterns heavily skewed toward *N. viridula:* (7) *N. viridula/E. obscurus* ($P < 0.001$), (10) *N. viridula/A. hilare* ($P < 0.001$), and (11) *N. viridula/A. pennsylvanicum* ($P < 0.05$). *Euclytia*

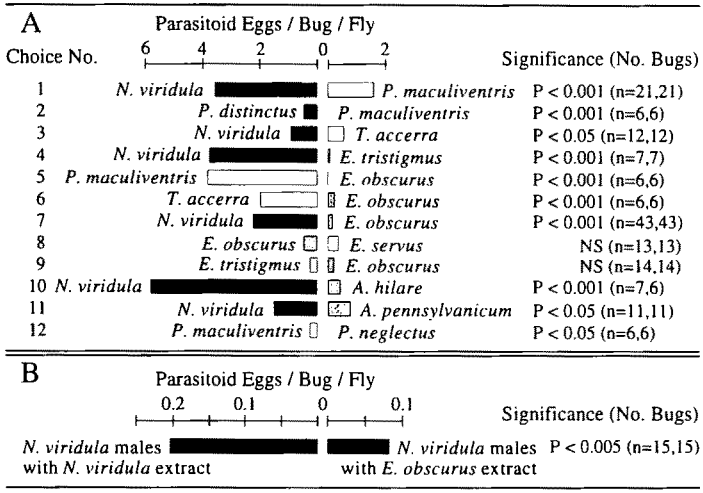

FIG. 1. Oviposition by *Euclytia flava* (Diptera: Tachinidae) given a choice (A) between two species of live Pentatomidae (black bars = exotic species; white = native, known host; gray = native, unknown host status) or (B) dead *Nezara* males treated with cuticular extracts.

flava females did not discriminate among *Euschistus* spp. (Nos. 8 and 9), and laid relatively few eggs on these bugs (Nos. 4 to 9). In the choice-test involving *P. maculiventris* and another native, but comparatively rare, known *Podisus* host, *P. neglectus* (No. 12), no eggs were laid on the latter species. Finally, in the only comparison between a pentatomid and a coreid (not shown in Figure 1), 18 eggs were laid on one *N. viridula* female while no eggs were laid on the *A. terminalis* female.

Euclytia flava females laid significantly more eggs on the ventral versus the dorsal surface of *N. viridula*, *P. maculiventris*, *E. obscurus*, and *A. pennsylvanicum* individuals (Table 3). However, the reverse was true for *T. accerra* individuals; significantly more *E. flava* eggs were laid dorsally. For three species, significantly more eggs were deposited on male bugs than females: *N. viridula*, *P. maculiventris*, and *A. hilare* (Table 3).

For two species, *P. maculiventris* ($P = 0.0082$), and *N. viridula* ($P = 0.0042$) (2×2 chi-square contingency test), there was a significant interaction between the position of *E. flava* eggs on the host body and the sex of the host. For *P. maculiventris*, the distribution of tachinid eggs on the ventral body surface did not differ significantly from the expected for males and females ($P = 0.6529$), whereas the distribution of eggs laid on the dorsal surface was signif-

TABLE 3. DISTRIBUTION OF *Euclytia flava* EGGS OVIPOSITED ON HETEROPTERA.

Species	No. Eggs (*N*)	Position (%)			Sex (%)		
		Dorsal	Ventral	*P*	Male	Female	*P*
N. viridula	965	23.9	76.1	0.001	58.9[a]	41.1[a]	0.0001
P. maculiventris	230	39.6	60.4	0.01	58.4[a]	41.6[a]	0.01
P. distinctus	11	36.4	63.6	NS	36.4	63.6	NS
E. obscurus	61	26.2	73.8	0.001	49.9	50.1	NS
E. tristigmus	16	50.0	50.0	NS	76.6	23.4	NS
E. servus	14	35.7	64.3	NS	50.0	50.0	NS
A. hilare	4	25.0	75.0	NS	62.5	37.5	0.05
A. pennsylvanicum	23	4.3	95.7	0.0001	[b]		
T. accerra	85	65.9	34.1	0.01	58.8	41.2	NS

[a]Normalized for unequal sample sizes.
[b]No female *A. pennsylvanicum* were tested.

icantly greater for males than for females ($P = 0.00018$). For *N. viridula*, significantly more eggs were laid on both the dorsal and ventral surfaces of males versus females than expected ($P < 0.0000001$ and $P < 0.0005$, respectively).

Bioassays using frozen *N. viridula* males treated with cuticular extracts showed that the factor supporting *E. flava* oviposition on *E. obscurus* could be transferred (Figure 1B). Dead *Nezara* males treated with cuticular wash from *E. obscurus* had significantly fewer eggs than did males treated with *N. viridula* extract ($P < 0.005$).

DISCUSSION

Exposing the exotic pest species, *Nezara viridula*, to parasitoids attracted in the field to *P. maculiventris* pheromone resulted in a level of multiple tachinid oviposition beyond that commonly observed for trapped spined soldier bugs. Of the two common tachinid parasitoids attracted to the pheromone of the spined soldier bug (*E. flava* and *Hemyda aurata*), only *E. flava* will enter this type of trap (Aldrich et al., 1984; Aldrich, 1985). Therefore, it was inferred that *N. viridula* males were being attacked by *E. flava* and, in fact, this species was frequently observed in and around the traps. Although no *E. flava* emerged from the southern green stink bug, the surprising degree of oviposition suggested that this exotic species is actually exceptionally attractive at close range to females *E. flava*. In the laboratory, wild *E. flava* females given a choice between *P.*

maculiventris adults and *N. viridula* adults did, indeed, prefer to oviposit on the exotic species rather than the native, known host whose pheromone was used to catch the flies for the experiments.

In the three other offerings where *E. flava* females could choose between an exotic species and a native, known host species, the flies also significantly preferred to oviposit on the exotic species. The *P. maculiventris/P. distinctus* combination is particularly interesting because the South American predator, *P. distinctus*, is congeneric, and males of this species produce a pheromone blend that contains the same compounds as the pheromone of *P. maculiventris* (Aldrich et al., 1991b) (*P. connexivus* redetermined as *P. distinctus* by D. Thomas, unpublished data). In this case, three *E. flava* females were given a choice between three males and three females of each *Podisus* spp.; they laid 11 eggs on *P. distinctus* individuals, and no eggs on *P. maculiventris*. Similarly, *Nezara* and *Acrosternum* are closely related genera whose species produce distinctive blends of the same sesquiterpene hydrocarbon and epoxide isomers (Aldrich et al., 1989). In fact, it is likely that the only Nearctic tachinid (*Trichopoda pennipes*) to naturally adopt *N. viridula* as a host in the 200 years or so since its arrival in the United States did so because it was preadapted to respond to the sesquiterpenes of the native *Acrosternum* spp. (Aldrich et al., 1989). Nonetheless, in close proximity to *N. viridula* and *Acrosternum* spp., *E. flava* females strongly discriminate between these bugs in favor of the exotic species, *N. viridula*.

The distribution data of parasitoid eggs on the bugs used for host-choice experiments give some added insights into the ovipositional behavior of tachinids. In seven of the nine pentatomid species, *E. flava* females laid more eggs ventrally than dorsally, with significant differences in four of these species. However, significantly more eggs were laid dorsally on *T. accerra* than ventrally. *Thyanta accerra* adults were noticeably smaller (ca. 9 mm long; McPherson, 1982) than all of the other pentatomids included in these experiments except for *E. tristigmus*, which showed an equal but nonsignificant positional distribution of eggs. Therefore, small body size may make it more difficult for *E. flava* to oviposit ventrally.

Tabulation of the laboratory ovipositional data by the sex of the host revealed that *E. flava* females laid significantly more eggs on males than females of *P. maculiventris*, *N. viridula*, and *A. hilare*. For the latter species, the sample size was small, and significant at only the 5% level. For *P. maculiventris* and *N. viridula*, large numbers of eggs were laid, and the preferences for males were highly significant. It is not unexpected that the female flies are preferentially stimulated to oviposit on *P. maculiventris* males because *E. flava* uses the male-produced pheromone of the spined soldier bug as a host-finding kairomone. In fact, the significant interaction between egg position and host sex that was detected for *P. maculiventris* is largely accounted for by the significantly greater

than expected number of eggs on the dorsal surface of males, and the pheromone is emitted from dorsal abdominal glands.

It does seem surprising that *E. flava* females prefer males over females for an exotic species. One possible explanation for this behavior is that *Acrosternum* spp. are occasional hosts such that *E. flava*, like *T. pennipes*, is preadapted to recognize the pheromone components of *N. viridula*. This possibility is supported by the finding that male *A. hilare* are preferred over females by ovipositing *E. flava* and that the egg position by sex interaction detected for *N. viridula* is partially accounted for by an excess of eggs on the ventral surface of males. In contrast to *Podisus* spp., the attractant pheromones of *Nezara*, *Acrosternum*, *Euschistus*, *Thyanta*, and most other phytophagous pentatomids are evidently released ventrally from scattered cells in the abdominal epidermis of males (Carayon, 1981; Aldrich, unpublished data).

Euclytia flava has been reared from eight species of pentatomoid bugs, including four species used in the host-choice experiments described here: *P. maculiventris*, *E. servus*, *E. tristigmus*, and *T. accerra* (Aldrich et al., 1991a; Buschman and Whitcomb, 1980; Eger and Ables, 1981; Jones et al., 1994; Jones and McPherson, 1980; Oetting and Yonke, 1971). The female flies exploit the pheromones of their hosts to locate them (Aldrich et al., 1991a; Aldrich, 1995), and these pheromones to some extent stimulate acceptance. However, native hosts appear to have somehow retaliated, perhaps by secreting repellent or masking compounds on their cuticle. This interpretation is substantiated by the demonstration that dead *Nezara* males treated with cuticular wash from *E. obscurus* had significantly fewer eggs than did males treated with *N. viridula* extract.

The two exotic species examined here, *N. viridula* and *P. distinctus*, evidently have no immunity to oviposition by *E. flava*. Most of the *Nezara* individuals that were attacked died, and oviposition by *E. flava* on *N. viridula* is a reproductive waste. By comparison, the *E. flava*–native pentatomid combinations tested here have evolved toward commensalism. Thus, the preference of *E. flava* females for exotic species substantiates the theoretical premise of the new associations hypothesis (Pimentel, 1963; Hokkanen and Pimentel, 1984, 1989).

A multitude of new host–enemy associations are automatically created when a species invades a foreign territory. The data presented here demonstrate that an invader may be maladapted and vulnerable to native parasitoids in one aspect of the parasitism process (acceptance), yet go unrecognized as a potential host. Of the existing community of natural enemies, there likely are some native species capable of becoming successful biological control agents if only they could find the exotics. Perhaps in the future it will be possible to accomplish biological control by "teaching" physiologically competent endemic beneficials to recognize alien hosts. In other words, can artificial selection regimes be

devised, based on appropriate semiochemical information, to speed up the natural process of host shifts?

The upheaval surrounding the use of exotic species for biological control of endemic pests makes managing native beneficials more important than ever (Howarth, 1991). The idea that native natural enemies might be "taught" to accept foreign hosts is a reversal of classical biological control. Today, such an unclassical approach is probably more environmentally and sociologically acceptable than classical biological control.

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SEMIOCHEMICALS FROM BARK BEETLES: NEW RESULTS, REMARKS, AND REFLECTIONS

WITTKO FRANCKE,[1,*] JOCHEN BARTELS,[1] HOLGER MEYER,[1] FRANK SCHRÖDER,[1] ULRICH KOHNLE,[2] ERNST BAADER,[2] and JEAN PIERRE VITÉ[2]

[1]*Institut fur Organische Chemie der Universität Hamburg*
Martin-Luther-King-Platz 6
D-20146 Hamburg, Germany
[2]*Forstzoologisches Institut der Universität Freiburg*
Fohrenbühl 25-27
D-79252 Stegen-Wittental, Germany

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Abstract—A brief survey is given about recent results in the identification of semiochemicals in bark beetles: Males of *Ips sexdentatus* (Boern.), stressed by the attack on resinous trees produce large amounts of (3*S*)-1-methyl-5-(1-hydroxy-1-methylethyl)-cyclohexa-1,3-diene. The compound appears to be derived from Δ^3-carene and acts as a repellent. Males of *Ips typographus* (L.), stressed through the attack on unsuitable host material release 3-methyl-7-methylene-1,3(*E*), 8-nonatriene, which seems to act as a repellent. The odor bouquet of three species of *Pityogenes* is described. The occurrence of (+)-grandisol and other compounds related to weevil pheromones points to a close relation between Scolytidae and Curculionidae. Females of *Dendroctonus simplex* (Le Conte) use (−)-frontalin as the main pheromone. 6-Methyl-6-hepten-2-one, a minor component among the volatile compounds released by the females, is regarded as a possible precursor of frontalin. Similarly, (2*R*,5*S*)-2(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran, pityol, a pheromone of *Pityophthorus* spp., is regarded to at least share a common biogenetic precursor with 6-methyl-5-hepten-2-ol, sulcatol. A new bicylic acetal, 2-ethyl-1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, is described as an aggregation pheromone of the beech bark beetle, *Taphrorychus bicolor* (Herbst). Structural relationships between bark beetle pheromones and plant volatiles are discussed.

Key Words—Bark beetle, identification, pheromone, *Dendroctonus*, *Ips*, *Pityogenes*, *Pityophthorus*, *Taphrorychus*.

*To whom correspondence should be addressed.

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INTRODUCTION

As compared to Lepidoptera, chemical communication in bark beetles appears to be much more complex. While host odors play a major role in host selection, the beetles use so-called aggregation pheromones (Vité and Francke, 1976) to mass attack selected trees. These chemical messages are produced by the habitat-selecting sex (in most polygamous species the males) to attract both males and females. In some species, however, the signals also show certain characters of sex pheromones since the opposite sex is attracted in higher numbers. When females select the host, as in many monogamous species, the arriving males may contribute in a "dialogue" by producing signals that balance the sex ratio or which repel other individuals. Mass attack actually is the outcome of an intriguing system of intra- and intersexual communication, which in many respects appears to be more closely related to communication among social insects than to the sex pheromone systems of moths. The pheromone biology and chemical ecology of bark beetles have been extensively reviewed (D.L. Wood, 1982; Borden, 1989; Byers, 1989; Vité and Baader, 1990); syntheses of pheromones have been carefully compiled by Mori (1992).

While many chemical compounds have been identified from bark beetles, only a few proved to be truely behavior mediating. With the exception of the structures of *Scolytus* pheromones, which seem to be polyketids made up by propanoate units, bark beetle pheromones are represented by either mevalogenins (terpenoid carbon skeletons) (Francke and Vité, 1983) or acetogenins (unbranched carbon skeletons). The structures of 21 known, relevant compounds are depicted in Figure 1.

In the present paper, we present our recent results on the identification of behavior mediating compounds from bark beetles. Some new chemical structures will be presented and some (structural/biogenetic) relations will be discussed.

RESULTS AND DISCUSSION

Ips De Geer

Typical pheromones of many *Ips* species are the male-produced ipsdienol (VIII) and ipsenol (X) (Silverstein et al., 1966). While myrcenol (VII) was found to be a synergistic component of the aggregation pheromone of *Ips duplicatus* (Sahlb.) (Byers et al., 1990b), amitinol (IX), a possible rearrangement product of ipsdienol, appeared to be behavior mediating in *I. sexdentatus* (Boern). (Kohnle et al., 1988). The structures of these compounds suggest a biogenesis from the host terpene, myrcene, as a precursor (Hughes, 1974; Hendry et al., 1980); however, recent results also show that a *de novo* synthesis by the beetles may occur, at least in some species (Ivarsson et al., 1993). While the hemiter-

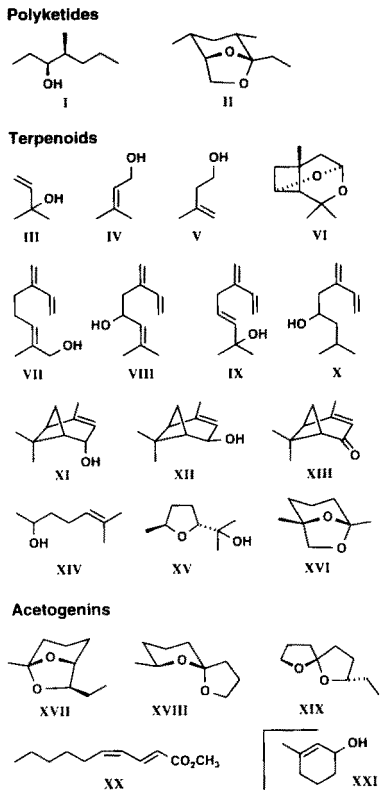

FIG. 1. Chemical structures of known bark beetle pheromones.

pene, 2-methyl-3-buten-2-ol (III), is an important pheromone of *I. typographus* (L.) (Bakke et al., 1977) and *Orthotomicus erosus* (Woll.) (Giesen et al., 1984), its isomers, 4-methyl-3-buten-1-ol (IV) and 3-methyl-3-buten-1-ol (V), are pheromones of the larch bark beetle, *I. cembrae* (Heer) (Stoakley et al., 1978) and *Polygraphus rufipennis* (Kirby), respectively (Bowers et al., 1991). The almost ubiquitous bicyclic terpenol, (*S*)-*cis*-verbenol (XI), is a particularly important compound in *I. typographus*, while its oxidation product, verbenone (XIII), acts as a repellent in many species, including *Dendroctonus*.

Ips sexdentatus (Boern.). Investigations concerning the pheromone system of *Ips sexdentatus* have been carried out earlier (Francke et al., 1986; Kohnle et al., 1988). In continuation of these studies, we analyzed the volatile compounds produced by beetles boring in highly resinous trees. Using a fused silica capillary coated with the polar stationary phase FFAP, pentane extracts of excised

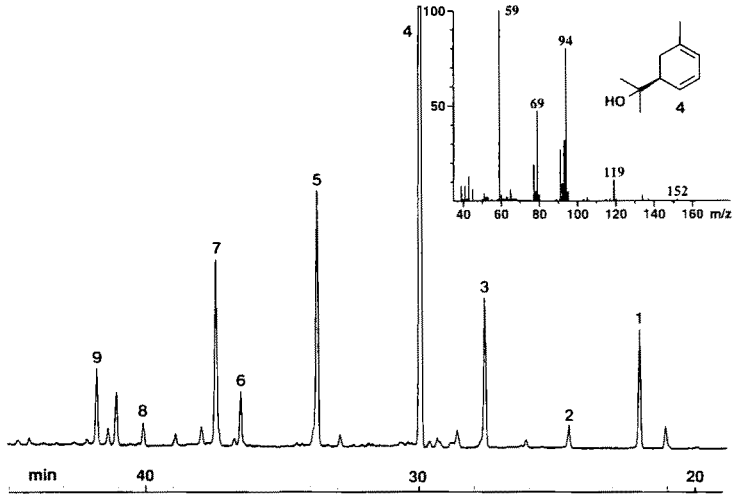

FIG. 2. Gas chromatogram of hindguts of *Ips sexdentatus* males boring in highly resinous pine trees; for conditions see text. Insert: 70-eV EI-MS of (3*S*)-(−)-1-methyl-5-(1-hydroxy-1-methylethyl)-cyclohexa-1,3-diene.

hindguts of males produced the gas chromatogram shown in Figure 2. Structure assignment of peaks was based on GC-MS investigations (a Hewlett-Packard HP 5890 was linked to a double-focusing mass spectrometer, Vacuum Generators VG 70-250SE, 70 eV) and comparison of data obtained with authentic reference samples. A list of identified compounds is shown in Table 1; peak numbers in Figure 2 and numbers in the corresponding table are congruent.

TABLE 1. *Ips sexdentatus*: HINDGUTS OF MALES BORING IN RESINOUS PINE TREE[a]

1. Ipsdienone
2. Myrtenal
3. Ipsdienol (60% ee)
4. (3*S*)-(−)-1-Methyl-5(1-hydroxy-1-methylethyl)-cyclohexa-1,3-diene
5. (+)-Myrtenol (85% ee)
6. *m*-Cymen-8-ol
7. *trans*-Myrtanol
8. β-Phenylethanol
9. 3-Caren-10-ol

[a]Compound numbers refer to peak numbers in Figure 2.

The main compound (peak 4) proved to be 1-methyl-5(1-hydroxy-1-methylethyl)-cyclohexa-1,3-diene, a *m*-mentha-4,6-dien-8-ol, which had been described earlier from *Ips* (Renwick et al., 1976) and *Dendroctonus* (Pierce et al., 1987). The compound shows a mass spectrum (see insert in Figure 2), which is indistinguishable from the corresponding *p*-menthadienol, α-phellandren-8-ol, and which can be separated from the latter only by a rather selective column. Such coincidences may cause misinterpretations concerning *m*- and *p*-menthadienes—including our own report (Francke et al., 1986). Actually, the *m*-menthadienol may be much more widespread than is known today. Optically active samples of the compound were prepared according to Gollnick et al. (1966). Using a 30-m FS capillary coated with a 1:1 mixture of 2,6-di-*O*-methyl-3-*O*-pentyl-β-cyclodextrin as the stationary phase, programmed from 80 to 140°C at a rate of 2°C/min, the stereoisomers of the hydrogenated natural product could be separated and compared with synthetic samples. As a result, the *Ips*-volatile proved to show (5*S*)-configuration with an enantiomeric excess (ee) of ca. 99%.

Although the biosynthesis of the compound has not yet been investigated, it is likely that it is produced from 3-carene, since exposure of the beetles to resin with high amounts of 3-carene resulted in the production of high amounts of the (5*S*)-enantiomer of the *m*-mentha-4,6-dien-8-ol.

During field tests, enantiometrically pure samples showed strong repellent effects, which may indicate a function as a warning signal to avoid the attack of healthy (high resinous) trees with a high content of the (poisonous) monoterpene hydrocarbon, 3-carene.

Apart from the (5*S*)-(−)-*m*-mentha-4,6-dien-8-ol, the first compound of this type to show biological activity, its corresponding dehydrogenation product, the aromatic *m*-cymen-8-ol (6), was also found, which, however, did not exhibit behavior-mediating properties.

In the present samples, ipsdienone (1) and ipsdienol (3), which besides myrtenol (5) usually represent major products in the hindguts of successfully boring beetles, were found to be much less abundant. Chiral gas chromatography, using the cyclodextrin phase mentioned above, proved the myrtenol to show (1*S*)-configuration (85% ee), while the ipsdienol was represented by a 4:1 mixture of (−):(+). The latter result is in contrast to our earlier report (Francke et al., 1986), where we found almost racemic ipsdienol, produced by successfully boring *I. sexdentatus* males.

Ips typographus (L). The chemistry of the pheromone system of *I. typographus* has been described by Bakke et al. (1977). Kohnle et al. (1991) hypothesized that 2-methyl-3-buten-2-ol and (*S*)-*cis*-verbenol play a major role as contact pheromones, while (−)-ipsdienol and (+)-ipsenol, two behavior-mediating components that are produced at a later stage of the attack, are frass pheromones.

We were interested in investigating the pheromone production of male beetles under stress and forced them to bore into rather thin, rapidly drying logs of Norway spruce and collected headspace volatiles on charcoal filters by closed loop stripping (Grob and Zürcher, 1976). As a result, the beetles did not release any volatiles known as attractants. A typical gas chromatogram (50-m fused silica capillary coated with FFAP and programmed from 60 to 200°C at a rate of 3°C/min) is shown in Figure 3. The identified compounds are listed in Table 2; the numbers are congruent. Identifications are based on GC-MS and synthetic reference samples (see above). Besides an array of monoterpene hydrocarbons and a few sesquiterpenes known as host constituents, some oxygenated monoterpenes were found. Upon high-resolution GC-MS, a compound eluting between the monoterpene hydrocarbons and the region of oxygenated monoterpenes proved to be a hydrocarbon, $C_{11}H_{16}$. The compound (mass spectrum shown as an insert in Figure 2) could be hydrogenated to $C_{11}C_{24}$, which suggested an open chain. The base peak *m/z* 81 pointed to the methylene-homologue of *m/z*

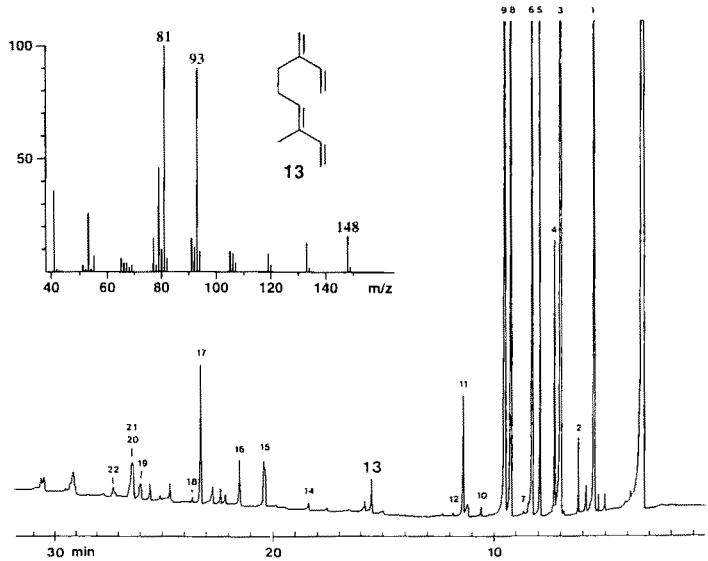

FIG. 3. Gas chromatogram of headspace volatiles collected from *Ips typographus* males boring in Norway spruce; for conditions see text. Insert: 70-eV EI-MS of 3-methyl-7-methylene-1,3(*E*),8-nonatriene. NMR data of this compound obtained with a Bruker AMX 400 are as follows: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.85 (s, 3H); 3.03 (d, J = 7 Hz, 2H); 4.90 (s, 2H); 5.03 (s, 1H); 5.08 (s, 1H), 5.10 (d, J = 10.8 Hz, 1H); 5.24 (d, J = 18 Hz, 1H); 5.72 (td, J = 16 Hz, J = 7 Hz, 1H); 6.20 (d, J = 16 Hz, 1H) 6.43 (dd, J = 18 Hz, J = 10.8 Hz, 1H).

TABLE 2. VOLATILE COMPOUNDS IDENTIFIED FROM HEADSPACE COLLECTIONS OF *Ips typographus* MALES UNDER STRESS CONDITIONS[a]

1. α-Pinene
2. Camphene
3. β-Pinene
4. Verbenene
5. Δ^3-carene
6. Myrcene
7. α-Terpinene
8. Limonene
9. β-Phellandrene
10. γ-Terpinene
11. *p*-Cymene
12. Terpinolene
13. 3-Methyl-7-methylen-1,3(*E*),8-nonatriene
14. *trans*-4-Thujanol
15. Pinocamphone
16. Isopinocamphone
17. Longifolene
18. Bornyl acetate
19. *trans*-Pinocarveol
20. Methylchavicol
21. Humulene
22. α-Terpinyl acetate

[a]Compound numbers refer to peak numbers in Figure 3.

69, which is characteristic for the typical dimethyl allyl terminus of open chain terpenes. In fact, the natural product proved to be 3-methyl-7-methylene-1,3(*E*),8-nonatriene (13), which could be easily synthesized from myrcenal (Büchi and Wuest, 1967) by Wittig reaction with triphenylmethylene phosphorane.

The carbon skeleton of the new homoterpene is the same as in the homoocimene, 3,7-dimethyl-1,3,6-nonatriene, identified from army ants (Keegans et al., 1993), and resembles that of juvenile hormones. A possible biosynthesis suggests homomevalonate to be involved.

Preliminary laboratory bioassays showed a repellent effect of 3-methyl-7-methylene-1,3(*E*),8-nonatriene, indicating a possible function as a warning signal to avoid unsuitable host material.

Pityogenes Bedel/Pityophthorus Eichhoff

Little is known about the pheromone systems in these two genera, since only two species, *Pityogenes chalcographus* (L.) and *Pityophthorus pityographus* (Ratz.) have been investigated so far.

The acetogenin, chalcogran (XIX), has been identified as the principal pheromone component produced by male *Pityogenes chalcographus* (Francke et al., 1977) synergized by methyl (*E*,*Z*)-2,4-decadienoate (XX) (Byers et al., 1990a). The natural spiroacetal shows (2*S*)-configuration (Schurig and Weber, 1984), and the (2*S*,5*S*)-diastereomer proved to be nonattractive (Byers et al., 1989). The structure of chalcogran suggests a formation from five acetate units and loss of one carbon atom: it would not be too surprising if chalcogran and its synergist, the decadienoic acid ester, share a common precursor or if a long-chain unsaturated fatty acid were involved in the biosynthesis. Chalcogran as well as the isomeric spiroacetal, 7-methyl-1,6-dioxaspiro[4.5]decane (XVIII) (see below), are not made up by three propanoate units, as suggested by Vanderwel and Oehlschlager (1987), since such a biosynthesis would yield a compound showing characteristic methyl branching pattern as represented in multistriatin (II).

In a continuation of our earlier investigations, we analyzed the volatiles emitted by males of *P. quadridens* (Hartig), *P. calcaratus* (Eichh.), and *P. bidentatus* (Herbst). Gas chromatograms are shown in Figure 4 and 5A and B; a corresponding list of identified compounds is given in Table 3. Peak attribution was based on GC-MS using synthetic reference samples (see above).

P. quadridens shows an interesting bouquet (Figure 4); while expectedly it contains chalcogran and 1-hexanol, just like *P. chalcographus*, it also exhibits

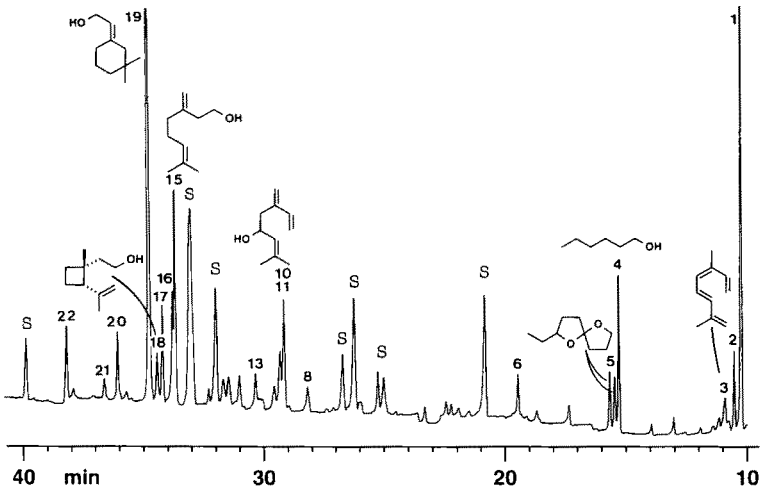

FIG. 4. Gas chromatogram of a pentane extract of *Pityogenes quadridens*; 50-m × 0.25-m-ID glass capillary, coated with WG 11, programmed from 60 to 200°C at a rate of 3°C/min; S = sesquiterpene.

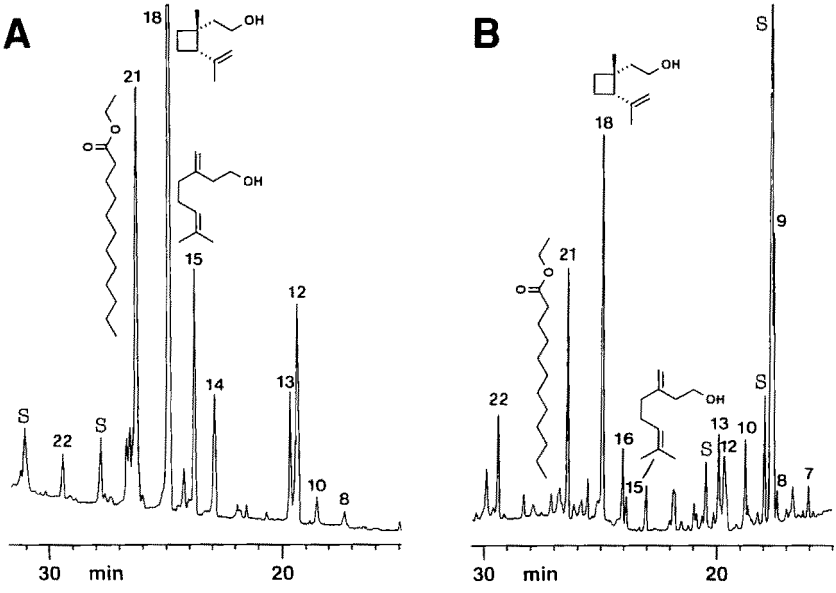

FIG. 5. (A) Gas chromatogram of a pentane extract of *Pityogenes calcaratus*; 50-m × 0.25-mm-ID fused silica column coated with FFAP and programmed from 60 to 200°C at a rate of 3°C/min; S = sesquiterpene. (B) Gas chromatogram of a pentane extract of *Pityogenes bidentatus*; conditions as in Figure 5A; S = sesquiterpene.

typical features of *Ips* pheromones: ipsdienol—absolute configuration unknown—accompanied by traces of 2-methyl-6-methylene-1,3(*E*),7-octatriene (not listed), a possible elimination product formed from either ipsdienol or amitinol. The dehydroocimenes, 2,6-dimethyl-1,3(*E*),5(*Z*),7-octatetraene (3) and traces of its 5(*E*) isomer were also found.

A particularly surprising set of compounds points to close relationships between Scolytidae and Curculionidae: γ-isogeraniol (15), (*E*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol (19) and grandisol (18)—absolute configurations unknown—could be identified. The *cis*-isomer of 19 and grandisol are pheromone compounds of the boll weevil, *Anthonomus grandis* (Boheman) (Tumlinson et al., 1969).

During bioassays with *P. quadridens*, mixtures of chalcogran (15), (+)-grandisol (18), and 19 proved to be attractive in the field (E. Baader, unpublished). The samples obtained from *P. calcaratus* males were dominated by grandisol (Figure 5A). Enantiomeric separation of its trifluoroacetate on 2,3,6-tri-*O*-pentyl-β-cyclodextrin proved the natural product to be the (+)-enantiomer showing ca. 99% ee. It was accompanied by trace amounts of grandisal. Both

TABLE 3. VOLATILES FROM MALES OF *Pityogenes* SPECIES[a]

1. Limonene
2. β-Phellandrene
3. 2,6-Dimethyl-1,3(*E*), 5(*Z*),7-octatetraene
4. 1-Hexanol
5. Chalcogran
6. 1-Octen-3-ol
7. Myrtenal
8. *trans*-Pinocarveol
9. *cis*-Verbenol
10. *trans*-Verbenol
11. Ipsdienol
12. Borneol
13. Verbenone
14. Citronellol
15. γ-Isogeraniol
16. Myrtenol
17. Nerol
18. Grandisol
19. (*E*)-3,3-Dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol
20. Geraniol
21. Ethyl dodecanoate
22. β-Phenylethanol

[a]Compound numbers refer to peak numbers in Figure 4 and 5A and B.

grandisal and grandisol have been described as pheromones of pine weevils, *Pissodes* spp. (Booth et al., 1983). (+)-Grandisol and γ-isogeraniol were also identified in males of *P. bidentatus* (Figure 5B). All three *Pityogenes* species discussed so far contained ethyl dodecanoate (22).

With respect to chemical functionalities, structural relations between γ-isogeraniol and grandisol are similar to those between ipsdienol and lineatin (VI), the aggregation pheromone of the ambrosia beetle, *Trypodendron lineatum* (Oliv.) (Borden et al., 1979). Formation of the cyclobutane ring may follow the same biogenetic mechanism.

While γ-isogeraniol was prepared according to Brown et al. (1963), (*E*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol was obtained by reduction of the corresponding aldehyde (Bedoukian and Wolinsky, 1975). Bioassays with *Pityogenes* species using synthetic compounds are not yet finished.

It is interesting to note that (+)-grandisol was also found in *Pityophthorus pityographus* (Ratz.). In this species, the tetrahydrofuran derivative, pityol (XV), represents the most active pheromone component (Francke et al., 1987). The gas chromatogram (FFAP; standard conditions see above) depicted in Figure 6 shows the relative proportions between pityol, grandisol, and inactive com-

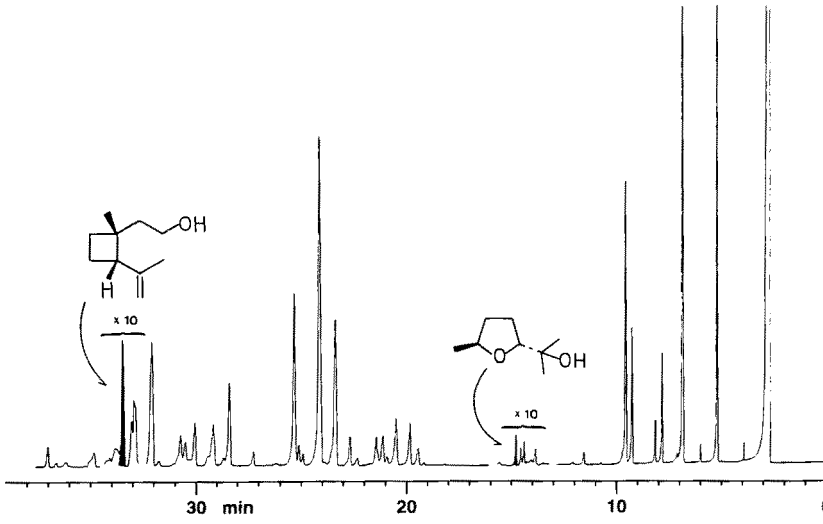

FIG. 6. Gas chromatogram of a pentane extract of *Pityophthorus pityographus*. For conditions see Figure 4.

pounds, which may form a kind of matrix for the active principle. In addition, (2*R*,5*S*)-pityol was identified from some American *Pityophthorus* species and proved to be highly attractive in the field (D. Wood, S. Seybold and W. Francke, unpublished). In pine cone borers, *Conophthorus* spp., the same compound represents a strong female specific attractant (Birgersson et al., 1995).

Although the biosynthesis of pityol is unclear, a degradation of a monoterpene precursor (geraniol or a derivative thereof) is suggested as the initial step, to yield 6-methyl-5-hepten-2-one. Reduction of this ketone would yield sulcatol (XIV), which is known as an aggregation pheromone of the ambrosia beetle, *Gnathotrichus sulcatus* (Le Conte) (Byrne et al., 1974). Enzymatic epoxidation of the double bond of sulcatol by an mfo-system and ring closure would lead to either pityol or vittatol, which had been identified from the elm bark beetle, *Pteleobius vittatus* (F.) (Klimetzek et al., 1989). The stereochemistry of the resulting 2,5-disubstituted tetrahydrofrun would be determined by the stereoselectivity of the reduction step and the stereoselectivity during epoxidation.

The spiroacetal (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane (XVIII), which was first described from common wasps (Francke et al., 1979), could be identified in some *Pityophthorus* spp. (W. Francke, unpublished), in *Conophthorus* spp. (Birgersson et al., 1995), in *Cryphalus piceae* (Ratz.), and in the ash bark beetle, *Leperisinus varius* (F.). In all three species it acts as a male-produced repellent (Birgersson et al., 1995; Kohnle et al., 1992). Using chiral gas chro-

matography (40-m FS capillary coated with 2,3,6-tri-*O*-hexyl-α-cyclodextrin, programmed from 50 to 100°C at a rate of 2°C/min) in some species the natural product could be shown to exhibit (2*S*,5*S*)-configuration, just as in *Paravespula vulgaris* (W. Francke, unpublished). The synthesis of the optically active spiroacetals, used as reference samples and in field tests, followed the procedure described by Jacobsen et al. (1982).

Dendroctonus Erichson

The oxygenated monoterpenes, *trans*-verbenol and verbenone, have been frequently described as behavior-mediating volatiles from *Dendroctonus* spp. The terpenoid, frontalin (XVI) (Kinzer et al., 1969), and the acetogenin, brevicomin (XVII) (Silverstein et al., 1968), play a major role in the communication systems of many species. The compounds are secreted by different sexes and contribute to the above-mentioned dialogue. The isomers of brevicomin are obviously used both with respect to enantiomeric compositions as well as diastereomeric proportions.

In addition to the widespread monoterpenes and bicyclic acetals, 3-methyl-2-cyclohexen-1-one (Kinzer et al., 1971) and its reduction product, 3-methyl-2-cyclohexen-1-ol (XXI) (Vité et al., 1972) as well as 1-methyl-2-cyclohexen-1-ol (Libbey et al., 1983) were specifically found to be pheromones of *D. pseudotsugae* (Hopkins). Although the substituted cyclohexenone might be biosynthesized from a straight-chain precursor (e.g., 2,6-heptanedione or derivative thereof), it appears more likely that it represents a degradation product of a terpene (via a *m*-menthane derivative; see above). The tertiary alcohol may well be a product of an allylic rearrangement of 3-methyl-2-cyclohexan-1-ol, the chemical relations between these two compounds being the same as between ipsdienol and amitinol.

Dendroctonus simplex *(Le Conte)*. Besides the usual array of monoterpene and sesquiterpene hydrocarbons and some oxygenated monoterpenes, four female-specific compounds could be identified in *D. simplex*. The gas chromatogram depicted in Figure 7 and Table 4 show identical numbering; identifications are based on GC-MS using synthetic reference samples (see above). The major sex specific compound was identified to be (−)-frontalin (ca. 95% ee as determined by chiral gas chromatography on the above-mentioned 2,3,6-trihexyl-α-cyclodextrin). The pure enantiomer proved to be highly attractive in the field (J.P. Vité, unpublished). In addition to frontalin, three structurally related terpenoid ketones, 6-methyl-6-hepten-2-one, 6-methyl-5-hepten-2-one, and 6-methyl-3(*E*),5-heptadien-2-one, were found. Close biogenetic relations between these ketones and known bicyclic acetals are suggested.

Bicyclic acetals showing a 6,8-dioxabicyclo[3.2.1]octane system represent

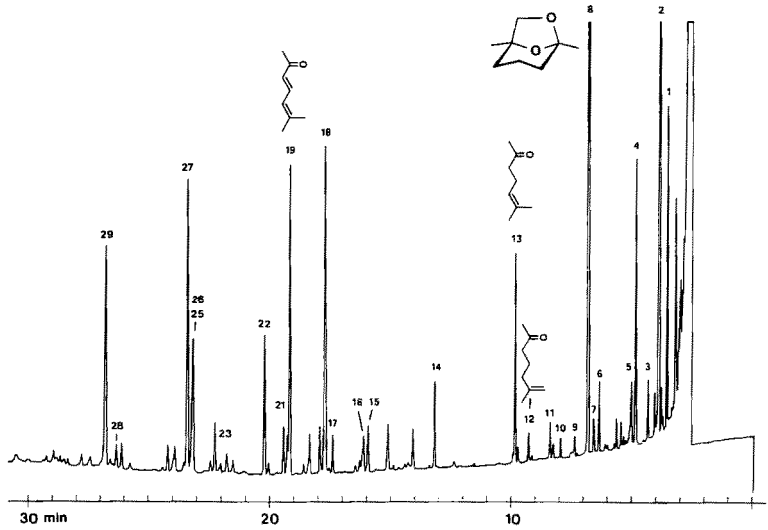

FIG. 7. Gas chromatogram of a pentane extract of *Dendroctonus simplex* males. For conditions see Figure 5.

TABLE 4. VOLATILE COMPOUNDS IDENTIFIED FROM HINDGUTS OF *Dendroctonus simplex* FEMALES[a]

1. Artifact
2. α-Pinene
3. Camphene
4. β-Pinene
5. Sabinene
6. Limonene
7. β-Phellandrene
8. (−)-Frontalin
9. γ-Terpinene
10. *p*-Cymene
11. Acetoin
12. 6-Methyl-6-hepten-2-one
13. 6-Methyl-5-hepten-2-one
14. Artifact
15. Camphor
16. Pentadecane
17. Linalool
18. Longifolene
19. 6-Methyl-3,5-heptadien-2-one
20. 3-Methyl-2-cyclohexenone[b]
21. Terpinen-4-ol
22. Myrtenal
23. Methylchavicol
24. *trans*-Verbenol[b]
25. Borneol
26. α-Terpineol
27. Verbenone
28. Perillaaldehyde
29. Myrtenol

[a]The compound numbers refer to peak numbers in Figure 7.
[b]Not consistently present.

cryptic ketodiols or products of intramolecular cyclizations of epoxy ketones. (Z)-6-Nonen-2-one, which besides *exo*-brevicomin had been identified from the ash bark beetle, *Leperisinus varius* (Francke et al., 1979), was postulated as a precursor to form *exo*-brevicomin upon epoxidation and ring closure (Francke, 1981). Recently, careful investigations by Vanderwel et al. (1992) proved this hypothesis to be true for *D. ponderosae* (Hopkins). Reactions similar to those that lead from 6-nonen-2-one to brevicomin would produce frontalin from 6-methyl-6-hepten-2-one (Figure 8). The stereoselectivity of the epoxidation would determine the stereochemistry of the final product.

It should be noted that 6-methyl-5-hepten-2-one is widespread among bees and ants and that epoxidation and ring closure of this ketone (Figure 8) may similarly be involved in the biosynthesis of 1,3,3-trimethyl-2,7-dioxabicyclo[2.2.1]heptane, which has been found in some ant species (Keegans et al., 1993).

Taphrorychus Eichhoff

Our earlier investigations on the pheromone system of the only species investigated so far revealed the presence of large amounts of acetophenone (Kohnle et al., 1987) along with other beetle-specific volatiles. We now wish to give a more complete list of volatile compounds and report on the identification of the male produced aggregation pheromone.

Taphrorychus bicolor (Herbst). The gas chromatogram depicted in Figure 9 and Table 5 show identical numbering; identifications were based on GC-MS using synthetic reference samples (see above). Pentane extracts of frass produced by boring beetles served as substrate. A most interesting mass spectrum was obtained from the male-specific peak 3, which eluted shortly after nonanal. Its EI-MS is shown as an insert in Figure 9. As determined by high-resolution GC-MS, the molecular formula was found to be $C_{10}H_{18}O_2$. The substance could not

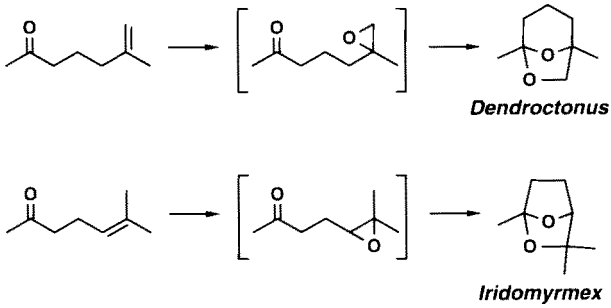

FIG. 8. Possible formation of bicyclic acetals from terpenoid precursors.

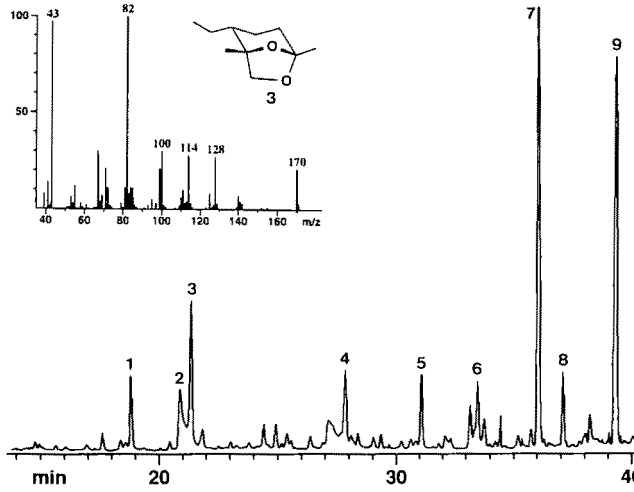

FIG. 9. Gas chromatogram of a pentane extract of frass produced by males of *Taphrorychus bicolor*. For conditions see Figure 5. Insert: 70-eV EI-MS of 2-ethyl-1,5-dimethyl-6,8-dioxaspiro[3.2.1]octane.

be hydrogenated and did not react with silylation reagents. According to known mass spectrometric fragmentation patterns (Gore et al., 1976), the structure of a bicyclic acetal was suggested. Although initially the intense signal of *m/z* 82 (C_6H_{10} as determined by high-resolution MS) could not be satisfactorily interpreted, the characteristic fragments at *m/z* 43 and *m/z* 128 (M-42) pointed to a substituted 5-methyl-6,8-dioxabicyclo[3.2.1]octane. The ^1H NMR spectrum of

TABLE 5. VOLATILE COMPOUNDS IDENTIFIED FROM FRASS PRODUCED BY MALES OF *Taphrorychus bicolor*[a]

1. 1-Hexanol
2. Nonanal
3. (1*S*, 2*R*, 5*R*)-2-Ethyl-1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane
4. Camphor
5. 1-Octanol
6. Terpinen-4-ol
7. Acetophenone
8. Nonanol
9. Verbenone

[a]Compound numbers refer to peak numbers in Figure 9.

a small sample of the natural product obtained by preparative gas chromatography showed two shifted methyl singlets and a methyl triplet as well as two shifted protons in α-positions to oxygen. On the basis of this information, the fragment *m/z* 82 suggested an ethyl group at position 2 and a second methyl group at position 1. Thus, the compound proved to be 2-ethyl-1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, the 2-ethyl bishomolog of frontalin. Using optically active reference samples, chiral gas chromatography (25-m FS capillary coated with 2,6-di-*O*-methyl-3-*O*-pentyl-β-cyclodextrin programmed from 60 to 150°C at a rate of 3°C/min) proved the natural product to show (1*S*,2*R*,3*R*)-configuration.

The compound that we named bicolorin was highly attractive in the field. After brevicomin, frontalin, and multistriatin, which together with (3*S*,4*S*)-4-methyl-3-heptanol (I), plays a major role in pheromone systems of *Scolytus multistriatus* (Marsh.) (Pearce et al., 1975), bicolorin is the fourth substituted 6,8-dioxabicyclo[3.2.1]octane identified as a bark beetle pheromone. Although sharing the same parent system, the four compounds seem to originate from very different precursors. Details on the synthesis of the stereoisomers of bicolorin and results of bioassays will be given in a separate paper.

REMARKS AND REFLECTIONS

Since the identification of the first bark beetle pheromone by Silverstein et al. (1966), a lot has been learned about the chemistry and function of behavior-mediating compounds among the scolytids. Although many of the known intra- and interspecific signals proved to be highly characteristic, a closer look at the chemical structures of the components that make up such chemical messages reveals many compounds that are by no means unique. Only a few of the substances depicted in Figure 1 have not yet been found in a non-bark beetle context. The hemiterpenes III, IV, and V are known from plants and Hymenoptera; and III is the alarm pheromone of European hornets, *Vespa crabro* (Veith et al., 1984). The bicyclic terpenes XI, XII, and XIII are typical constituents of many essential oils and have been known for quite some time. Furthermore, the occurrence of the myrcene derivatives is not restricted to bark beetles. "Ipsdienone," the oxidation product of ipsdienol (VIII) had already been mentioned by Naves (1948; he called it myrcenone) as a constituent of the Verbenacea, *Lippia asperifolia*. Our own analysis of the essential oil of *L. javanica* from South Africa (W. Francke and H. Meyer, unpublished) revealed high proportions of ipsdienone and ocimenone accompanied by small amounts of other monoterpenes including ipsdienol and 6,7-epoxymyrcene. Ipsdienol has also been described as a component of the fragrance of orchids that are pollinated by euglossine bees (Whitten et al., 1988). Amitinol (IX) is a constitutent of the

essential oil of *Ledum palustre* (Schantz et al., 1973). Sulcatol (XIV) and the corresponding ketone are particularly widespread among the secretions of animals (including mammals); they are produced by microorganisms as well as by plants. The orchid *Aerides lawrenceae* produces a nice "bark beetle bouquet" by emitting both sulcatol and 2-methyl-3-buten-2-ol (III) (Kaiser, 1993). Although grandisol has not yet been reported from sources other than beetles, its *trans* isomer, fragranol, has been identified from *Artemisia fragrans* (Bohlmann et al., 1973). Methyl (*E*,*Z*)-2,4-decadienoate (XX), which along with the corresponding ethylester ("pear ester") is known to account for the typical aroma of Bartlett pears, was also found in orchids (Kaiser, 1993). Even the famous bicyclic acetals XVI, XVII, and XIX do not represent "unique beetle chemistry." We found *endo*-brevicomin in the scent of the orchid *Ophrys speculum* (W. Francke and D. Erdmann, unpublished), while 3,4-dehydrobrevicomin proved to be a pheromone of mice (Novotny et al., 1984). Chalcogran (XIX) was identified in flowers of orchids, *Liparis viridifoli*, *Eria hyacinthoides*, and *Oncidium ornithorhynchum*. Along with the isomeric spiroacetal (XVIII) it also occurs in *Dracaena fragrans* (Kaiser, 1993). The latter compound is also widespread among common wasps and was detected both in cork oak and spruce (W. Francke, unpublished). What do *Dendroctonus* and *Elephas* have in common? The "typical" bark beetle pheromone, (1*S*)-frontalin is a constituent of the temporal gland secretion exuded by male Asian elephants, during their musth cycles (Rasmussen et al., 1994). The list of parallels is long and certainly does not exclude polyketides such as the "*Scolytus* pheromones," 4-methyl-3-heptanol (I), which is known from ants, too.

Degradation and elimination of the signal with time and space is a principal feature of pheromones, and one may speculate about the environmental impact on the fate of the bark beetle pheromones once they are released. Oxygenated monoterpenes may undergo acid-catalyzed or UV-induced rearrangements, and in addition their structures may be changed by elimination of water or further oxidation. Verbenone is easily rearranged to chrysantheone and 2,4,4-trimethylbicyclo[3.3.1]hept-2-en-6-one (Hurst and Whitham, 1960). In our own investigations we found that this reaction may take place under the conditions of a sunny warm day. During our irradiation experiments (400 W, Heraeus), isopiperitone as well as (*E*)- and (*Z*)-ocimenone were produced. Formation of the acyclic ketones is similar to the conversion of α-pinene to ocimenes as described by Kropp (1969). Although verbenols show a less suitable chromophore, allylic rearrangements to the corresponding bicyclic alcohols and ring-opening to *cis*-isopiperitenol and *cis*-3-methyl-6-(1-methylethenyl)-3-cyclohexen-1-ol follows the same mechanisms as in the case of verbenone. Irradiation of ipsenol and ipsdienol affects the butadiene moiety, yielding 1-substituted cyclobutenes. While 1-(2-hydroxy-4-methyl)-butylcyclobutene is formed from ipsenol, ipsdienol leads to 1-(2-hydroxy-4-methyl)-but-3-enylcyclobutene and

bicyclic alcohols. The composition of the mixture of alcohols formed from these alcohols corresponds to the mixture of hydrocarbons obtained from irradiation of myrcene (Liu and Hammond, 1967 and references cited therein). Interestingly, we identified 1-(2-hydroxy-4-methyl)-but-3-enylcyclobutene as a new natural product in some bark beetle species; however, no bioassays have yet been carried out. It would be interesting to know more about degradation products form (bark beetle) pheromones and to learn something about their (possible) bioactivities.

Some of the known systems of bark beetle pheromones look rather primitive, being made up of simple oxygenated monoterpenes that are indistinguishable from products of autoxidation processes, while other species developed elaborate biosynthetic pathways to produce and use highly sophisticated compounds. This may suggest at least a certain trend in the evolution of bark beetle pheromones. However, with respect to production and use of relevant compounds, there is no correlation between chemical structures of bark beetle pheromones and the position of the corresponding species within the phylogenetic tree, described by Steven Wood (1982). Chemical communication seems to follow higher principles, as is thought today. It makes use of a wealth of chemical compounds that seem to develop specificity through the formation of unique blends that are used in a specific biological context. With respect to bark beetles, we are just starting to learn how chemical ecology works.

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CODLING MOTH, *Cydia pomonella*, (LEPIDOPTERA: TORTRICIDAE): IS ITS SEX PHEROMONE MULTICOMPONENT?

L.M. McDONOUGH,* H.G. DAVIS, P.S. CHAPMAN, and C.L. SMITHHISLER

Agricultural Research Services, USDA
3706 W. Nob Hill Blvd.
Yakima, Washington 98902

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Abstract—When the nine identified components in the effluvium of calling female codling moths were compared to pure synthetic (*E,E*)-8,10-dodecadien-1-ol in flight-tunnel tests, equal responses were obtained over a concentration range of 300-fold. When synthetic (*E,E*)-8,10-dodecadien-1-ol was compared to extract of female sex pheromone glands by a male wing-flutter bioassay, or in flight-tunnel tests, equal responses were obtained over a concentration range of 1000-fold. The sum total of these and previous studies indicate that the codling moth sex pheromone consists of only one component.

Key Words—Codling moth, *Cydia pomonella*, sex pheromone, (*E,E*)-8,10-dodecadien-1-ol.

INTRODUCTION

Sex pheromones of insects are generally multicomponent (Wood et al., 1967; Roelofs, 1977; Silverstein, 1977). The codling moth sex pheromone was originally identified as the single component, (*E,E*)-8,10-dodecadien-1-ol by Roelofs et al. (1971). Subsequently, data from flight-tunnel tests and male wing-flutter bioassays indicated that dodecan-1-ol and tetradecan-1-ol were synergistic components of the sex pheromone (Einhorn et al., 1984, 1986; Arn et al., 1985; Bartell et al., 1988), but in later flight tunnel tests the activity of these compounds could not be substantiated (McDonough et al., 1993).

The lack of substantiation of pheromonal activity for dodecan-1-ol and

*To whom correspondence should be addressed.

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tetradecan-1-ol did not rule out the possibility that other undiscovered active components existed. Arn et al. (1985) identified nine components in the effluvium of calling females and many of these components had not been tested. Using a male wing-flutter bioassay, Bartell and Bellas (1981) reported that extract of female sex pheromone glands produced a stronger response than synthetic (*E,E*)-8,10-dodecadien-1-ol.

To define the sex pheromone of the codling moth more accurately, we conducted tests in a flight tunnel comparing the natural ratio of known components of the effluvium of calling females with synthetic (*E,E*)-8,10-dodecadien-1-ol and a comparison of sex pheromone gland extract to synthetic (*E,E*)-8,10-dodecadien-1-ol by wing-flutter and by flight-tunnel bioassays. None of these tests indicated the presence of sex pheromone components other than (*E,E*)-8,10-dodecadien-1-ol.

METHODS AND MATERIALS

Insects. Pupae were obtained from a laboratory colony reared on artificial diet (Howell, 1972; Toba and Howell, 1991). Individual pupae were placed in 15 × 50-mm vials and maintained at 20°C and 65% relative humidity under constant light. Males for wing-flutter or flight-tunnel bioassays were used three days after eclosion and 0.25–2.5 hr after darkening the room.

Extracts of Sex Pheromone Glands. Female moths (2–3 days old) in individual vials were collected 0.5 hr after the beginning of darkness and placed in a refrigerator to inactivate them. They were removed individually, and their terminal abdominal segments containing the sex pheromone gland were severed and steeped for 15–60 min in ca. 200 μl dichloromethane. The dichloromethane extract was removed with a syringe and kept in a freezer until needed.

Chemicals. (*E,E*)-8,10-Dodecadien-1-ol, decan-1-ol, dodecan-1-ol, tetradecan-1-ol, hexadecan-1-ol, and octadecan-1-ol were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). (*E,E*)-8-10-Dodecadien-1-ol was purified by three low-temperature recrystallizations from pentane, and the isomeric purity was 99.7% (ca. 0.1% *ZE* and 0.2% *EZ*) by gas chromatographic analysis. (*E*)-9-Dodecen-1-ol was purchased from the now-defunct Farchan Division of Story Chemical Corp. (Willoughby, Ohio). All of these chemicals were at least 98% pure by GC analysis, and (*E*)-9-dodecen-1-ol contained less than 1% of the *Z* isomer. (*E,Z*)-8,10-dodecadien-1-ol was synthesized by the method of McDonough and Smithhisler (1989) for the synthesis of (*E,Z*)-10,12-tetradecadien-1-ol except that 1,8-octandiol was used in place of 1,10-decandiol. The isomeric purity was 96% (ca. 1% *ZE* and 3% *ZZ*).

Pheromone Dispensers. In flight tunnel tests, two types of dispensers were used. When (*E,E*)-8,10-dodecadien-1-ol was compared to extract of sex pher-

omone gland, a piece of cotton dental roll (6 mm thick × 6 mm diameter) was used. The test materials were applied as dichloromethane solutions in doses of 0.3, 1.0, 3.0, 10.0, 30.0, 100.0, and 300 ng of (*E,E*)-8,10-dodecadien-1-ol. The amount of (*E,E*)-8,10-dodecadien-1-ol in the extract was determined by gas chromatographic analysis and was usually about 3 ng/female. When (*E,E*)-8,10-dodecadien-1-ol was compared to the nine known components of the effluvium of calling females (Arn et al., 1985), gray elastomer septa (Brown and McDonough, 1986) were used. The test compositions were applied as dichloromethane solutions, and for the nine component mixtures, in ratios that would reproduce the vapor ratios reported by Arn et al. (1985). The ratios applied were calculated in the manner previously reported (McDonough et al., 1993) from known relative half-lives (McDonough, 1991). The applied ratios were: (*E,E*)-8,10-dodecadien-1-ol, 100; (*Z,E*)-8,10-dodecadien-1-ol, 0.3; (*E,Z*)-8,10-dodecadien-1-ol, 5.6; (*E*)-9-dodecen-1-ol, 9.08; decan-1-ol, 0.25; dodecan-1-ol, 43.9; tetradecan-1-ol, 46.9; hexadecan-1-ol, 704; octadecan-1-ol, 6,340. The doses applied of (*E,E*)-8,10-dodecadien-1-ol were: 0.1, 0.3, 1.0, 3.0, 10.0, 30.0 μg.

Wing-Flutter Bioassay. The 10 males for testing were placed in a pint fruit jar. The metal jar tops were pierced with a small hole that just allowed the insertion of a 3-mm-diameter glass rod. Between tests, a piece of tape over the hole prevented odors from entering the jar. The test room was dimly lighted by red lights. A 3-mm-diameter glass rod was inserted to a depth of 6 mm into the test solution. As soon as the solvent evaporated from the rod, it was inserted into the jar. The number of males making a circling motion while wing fanning were counted. Each concentration was tested three times. The concentrations tested in female sex pheromone gland equivalents per milliliter and in grams of codlemone per milliliter were 0.01 and 3×10^{-11}; 0.1 and 3×10^{-10}; 1.0 and 3×10^{-9}; and 10 and 3×10^{-8}, respectively.

Flight Tunnel. A flight tunnel constructed as described by Miller and Roelofs (1978) was used. The flight compartment was 210 cm in length. The air speed directly downwind of the dispenser was 12 cm/sec, and the temperature was maintained at 23°C. The tunnel was dimly lighted overhead by red lights controlled by a rheostat. The effluvium from the flight tunnel was evacuated directly into a fume hood.

Flight Tunnel Bioassay. There were two observers, one at each end of the flight tunnel. Test mixtures in their dispensers were held in place at the upwind end of the flight tunnel by a bent wire. At the downwind end of the tunnel, five male codling moths in a small covered dish were placed on a platform in the middle of the airstream. After 1 min, the cover was removed and behavior was observed. If a male did not flutter its wings, it was considered nonviable. The number of males tested for each data point was 25. When sex pheromone gland extract was tested, a fresh extract was used for each test of five males. Upwind

flights culminating in contact with the dispenser were recorded. Once a male contacted the dispenser, it was removed from the flight tunnel via a tube connected to a vacuum.

RESULTS

Flight Tunnel Tests of Components of Effluvium of Calling Female Codling Moths. Figure 1 shows a graph of log dose versus male response to synthetic (*E,E*)-8,10-dodecadien-1-ol and to the nine components of the effluvium of calling females over a dosage range of 0.1–30.0 μg of (*E,E*)-8,10-dodecadien-1-ol. The nine components were formulated in gray elastomer septa in ratios that would reproduce ratios in the vapor equal to those Arn et al. (1985) found in the effluvium of calling females. The two curves are superimposable and indicate that none of the known components of the effluvium of calling females other than (*E,E*)-8,10-dodecadien-1-ol elicited behavioral activity in these tests.

Wing-Flutter Bioassay. The results of the wing-flutter bioassay are shown in Table 1. The response of males to synthetic (*E,E*)-8,10-dodecadien-1-ol varied from zero for the blanks to almost 100% for a concentration of 3×10^{-8} g/ml. The responses of males to the sex pheromone gland extract were quite similar to synthetic (*E,E*)-8,10-dodecadien-1-ol and well within the standard errors of the means. Thus, in these tests, there is no indication of active components in the extracts other than (*E,E*)-8,10-dodecadien-1-ol.

Flight Tunnel Tests of Extract of Female Sex Pheromone Glands. Male response in flight tunnel tests to synthetic (*E,E*)-8,10-dodecadien-1-ol and to

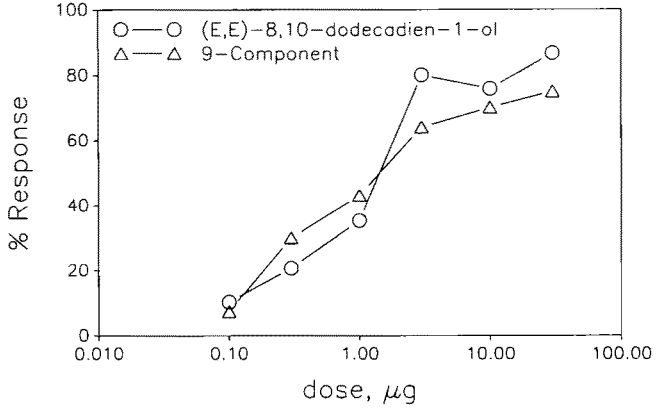

FIG. 1. Flight-tunnel bioassay comparing synthetic (*E,E*)-8,10-dodecadien-1-ol with the blend of the nine components emitted by calling female codling moths.

TABLE 1. COMPARISON OF EXTRACT OF FEMALE CODLING MOTH SEX PHEROMONE GLANDS WITH SYNTHETIC (*E,E*)-8,10-DODECADIEN-1-OL BY WING-FLUTTER BIOASSAY (10 FEMALES PER TEST REPLICATE)

Concentration		% Response ± SE (N = 3)	
Female eq/ml	g/ml	SPG extract	Synthetic
10	3×10^{-8}	93.0 ± 12.1	96.5 ± 4.9
1	3×10^{-9}	71.5 ± 6.4	66.5 ± 9.2
0.1	3×10^{-10}	38.6 ± 5.0	43.5 ± 24.7
0.01	3×10^{-11}	15.3 ± 16.6	20.5 ± 20.5
0.0	0.0	3.9 ± 3.4	0.0

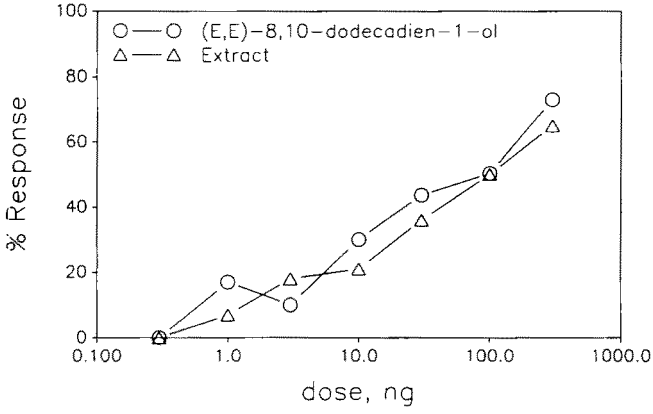

FIG. 2. Flight-tunnel bioassay comparing (*E,E*)-8,10-dodecadien-1-ol with extract of female codling moth sex pheromone glands.

extract of female sex pheromone glands as a function of log dose is shown in Figure 2. Over the dosage range of 0.3–300 ng of (*E,E*)-8,10-dodecadien-1-ol on a cotton dental roll dispenser, the response curves were superimposable. Thus, in these tests also there is no indication of active components in the extracts other than (*E,E*)-8,10-dodecadien-1-ol.

DISCUSSION

Generally, it is expected that insect sex pheromones will consist of two or more components (Silverstein et al., 1966; Wood et al., 1967), but although

the evidence is not rigorous, there is little doubt that the sex pheromone of the codling moth consists of only one component. None of the identified components in the effluvium of calling females, when released in the natural ratio to (*E,E*)-8,10-dodecadien-1-ol, elicited greater responses in flight tunnel tests than (*E,E*)-8,10-dodecadien-1-ol alone, either in this study or in our previous study (McDonough et al., 1993). Furthermore, in both the wing-flutter and the flight-tunnel bioassays, synthetic (*E,E*)-8,10-dodecadien-1-ol elicited responses identical to extracts of female sex pheromone glands containing an equivalent amount of (*E,E*)-8,10-dodecadien-1-ol. In flight tunnel tests a higher response was elicited by synthetic (*E,E*)-8,10-dodecadien-1-ol than by five calling females, and male responses to synthetic (*E,E*)-8,10-dodecadien-1-ol emitted at the low rate of about 3–5 ng/hr were equivalent to the responses to the five caged females (McDonough et al., 1993). Several authors have suggested that the codling moth sex pheromone is inadequate as a mating disruptant because the pheromone was incomplete. A more likely reason for the unsatisfactory results is the ease of oxidative degradation (McDonough et al., 1992), which greatly reduces the amount of (*E,E*)-8,10-dodecadien-1-ol available to disrupt mating. Based on the evidence now available, male codling moths are highly sensitive to their single component sex pheromone.

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BIOASSAY APPROACHES TO ASSESSING BEHAVIORAL RESPONSES OF PLUM CURCULIO ADULTS (COLEOPTERA: CURCULIONIDAE) TO HOST FRUIT ODOR

RONALD J. PROKOPY,[1,*] SYLVIA S. COOLEY,[1] and P. LARRY PHELAN[2]

[1]*Department of Entomology*
University of Massachusetts
Amherst, Massachusetts 01003

[2]*Department of Entomology*
Ohio Agricultural Research and Development Center
Wooster, Ohio 44691

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Abstract—We evaluated several approaches to developing a simple, sensitive, and reliable laboratory bioassay of responses of overwintered adult plum curculios (PCs), *Conotrachelus nenuphar* (Herbst), to host fruit odor or its attractive components. A high proportion of assayed PCs responded positively to odor of wild plums under no-choice, moving-air conditions in a wind tunnel and under dual-choice, still-air conditions in enclosed Petri dishes. Positive response to controls lacking host odor, however, was much greater in the wind tunnel, arguing in favor of bioassays under dual-choice conditions in still air to provide greater PC discrimination. Response to host odor (from wild plums or hexane extract of wild plums or Liberty apples) in Petri dish bioassay chambers proved greatest: (1) during the scotophase of PCs under total dark or dim red light conditions, (2) when Petri dishes were completely enclosed, (3) when PCs were starved for 24 or 48 hr, and (4) when PCs were tested within seven weeks after apple tree petal fall. Neither the sex of a PC nor the direction in which a PC was obliged to move to find the source of host odor (upward through a port in the Petri dish lid or downward through a port in the base) had a substantial effect on level of response to host odor or discrimination of host odor from a nonodorous control. We conclude that an enclosed Petri dish bioassay chamber of the type described here should be a valuable asset in the process of chemically identifying components of host fruit odor attractive to PCs.

Key Words—Fruit odor, bioassay methods, *Conotrachelus nenuphar*.

*To whom correspondence should be addressed.

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INTRODUCTION

The plum curculio (PC), *Conotrachelus nenuphar* (Herbst), is among the most damaging of all insect pests of stone and pome fruit in eastern and central North America (Racette et al., 1991). Despite this economic importance, currently there exists no effective trap for monitoring the appearance, abundance, or disappearance of PC adults in commercial orchards (Racette et al., 1992; Prokopy et al., 1993).

In contrast, effective monitoring traps are available for essentially all other key frugivorous insect pests of North American stone and pome fruit orchards (Prokopy and Croft, 1994). They also are available for several other economically important Curculionidae. For example, traps baited with pheromone in combination with host plant volatiles are used to monitor the cotton boll weevil, *Anthonomus grandis* Boheman (Dickens, 1989), the palm weevil, *Rhynchophorus palmarum* (L.) (Jaffé et al., 1993; Oehlschlager et al., 1993), the palmetto weevil, *Rhynchophorus cruentatus* (Fabricius) (Giblin-Davis et al., 1994), and the rice weevil, *Sitophilus oryzae* (L.) (Phillips et al., 1993). Traps baited with pheromone alone are employed in monitoring the sweetpotato weevil, *Cylas formicarius* (Fabricius) (Jansson et al., 1992), legume-infesting *Sitona weevils* (Neilsen and Jensen, 1993) and the pepper weevil, *Anthonomus eugenii* Cano (Eller et al., 1994), while traps baited with host plant volatiles alone have been used to monitor *Hylobius* and *Pachylobius* weevils in pine forests (Hunt and Raffa, 1989).

The principal native host of PC is suspected to be wild plum, *Prunus americana* (Maier, 1990). Within the past 150 years, however, PC has expanded its host range onto introduced commercial apple, pear, plum, peach, and cherry trees (Maier, 1990). Following eclosion in mid-summer, adults feed on host fruit somewhat briefly and then migrate to hibernation sites in nearby woods or hedgerows (Lafleur et al., 1987). In spring, overwintered adults move toward host trees, beginning when apple buds are in the tight cluster stage of development and peaking several weeks later at fruit set (Lafleur and Hill, 1987). Until petal fall, activity within host trees is largely nocturnal, with adults spending a majority of daylight hours in the ground cover beneath trees (Racette et al., 1991). After petal fall, activities of feeding and egg-laying on host fruit occur both diurnally and nocturnally but tend to be concentrated from mid- or late afternoon until dawn the next day (Racette et al., 1991).

Our ultimate goal is to develop an effective monitoring trap for PC adults that incorporates both attractive odor and attractive visual stimuli. Toward this end, we have found that overwintered PC adults respond positively to the odor of intact developing host fruit under both field (Butkewich and Prokopy, unpublished data) and laboratory (Butkewich and Prokopy, 1993) conditions and that adults respond positively to odor of the same or opposite sex (Prokopy and

Cooley, unpublished data). Identification of host fruit or pheromonal chemicals eliciting attraction cannot proceed efficiently, however, without a simple, sensitive, and reliable laboratory bioassay procedure. The laboratory assay approach we had employed until now involved hanging candidate fruit or cotton wicks containing fruit extracts from ends of wooden crosspieces enclosed in clear plastic cylinders, allowing an introduced adult to climb the central stem supporting the crosspiece, and continuously observing the response pattern of the adult thereafter (Butkewich and Prokopy, 1993). Although reasonably sensitive and reliable, this bioassay procedure proved too cumbersome for use on a large scale.

Our objective here was to develop an efficient approach to bioassaying response of PC adults to attractive host fruit odor. To this end, we assessed PC responses to fruit odor under wind-tunnel versus still-air conditions, under photophase versus scotophase conditions, and under various modified still-air conditions. We also assayed responses of adults of varying physiological states with respect to hunger and age and responses of females versus males. The result has been development of a still-air, choice-test bioassay approach that is simple, sensitive, and reliable.

METHODS AND MATERIALS

PCs used in the assays were obtained as adults from unmanaged apple trees from mid-May (petal fall) to early June by tapping tree limbs and collecting fallen adults on sheets of cloth placed beneath the trees. Collected and unsexed PCs were placed in groups of 20–30 in waxed, lid-covered paper cups (500 cc), maintained in the laboratory at 26°C and 80% relative humidity, and provided with several fresh-picked wild plum or apple fruit that were renewed every other day. Unless stated otherwise, the scotophase in the maintenance room extended from 10:00 to 18:00 hr, and fruit were removed from the cups 24 hr before testing. Owing to the limited number of available PCs, the same PCs were used in more than one experiment, but never in more than one experiment per week. Individuals used in experimental treatments were chosen at random from the available pool of PCs.

Intact fruit used in assays were wild plums, picked fresh (when 11–13 mm diam.) about two weeks after petal fall. For several assays, we used a hexane extract of unsprayed domestic plums cv. Fellenberg or domestic apples cv. Liberty. For extracts, fruit were picked fresh about one week after petal fall (when 0.7 g in weight) and were soaked in hexane (1 g fruit/1 ml hexane) for 24 hr at 25°C. In tests, 50 μl of raw extract was applied using a micropipet to a 1-cm^3 piece of cotton dental wick (Absorbal, Wheat Ridge, Colorado) and allowed to evaporate for 4 min before use. As a control treatment, we used

either 12-mm-diam wooden beads painted with green artist pigment to approximate the color of wild plums or 1-cm^3 pieces of cotton dental wick, each treated with 50 μl of hexane and allowed to dry for 4 min before use.

The wind tunnel (Phelan, unpublished) consisted of a 78-cm-long × 34-cm-wide × 10-cm-high Plexiglas box that was open at both ends and divided into four 7-cm lanes by Plexiglas dividers. Air, purified by a charcoal filter, was blown through the lanes at 0.4 m/sec by a fan attached to the filter by a plastic bag. The tunnel was illuminated by fluorescent white light (130 lux) or by an incandescent red lamp (1 lux). Assays were conducted under no-choice conditions. Five minutes before a trial, a treatment or control substance was placed 3 cm downwind of the filter at the upwind end of a lane, separated from the lane by wire mesh. Within 30 sec of the start of a trial, a single PC was introduced into the midway point of each lane, immediately after which the downwind end was closed with wire screen. Each PC was observed continuously until it reached the upwind screen (recorded as a positive response) or until 5 min elapsed without reaching the upwind screen (trial terminated).

Still-air test chambers consisted of plastic Petri dishes (90 mm diam × 15 mm high) modified in the following way (Figure 1). Two holes, 10 mm diam. and 40 mm apart, were bored through a Petri dish lid using a heated cork borer. A tapered disposable polyethylene micropipet tip, 10 mm diam. at the base, 30 mm tall, and 7 mm diam at the tip (after cutting) was fitted snugly into each

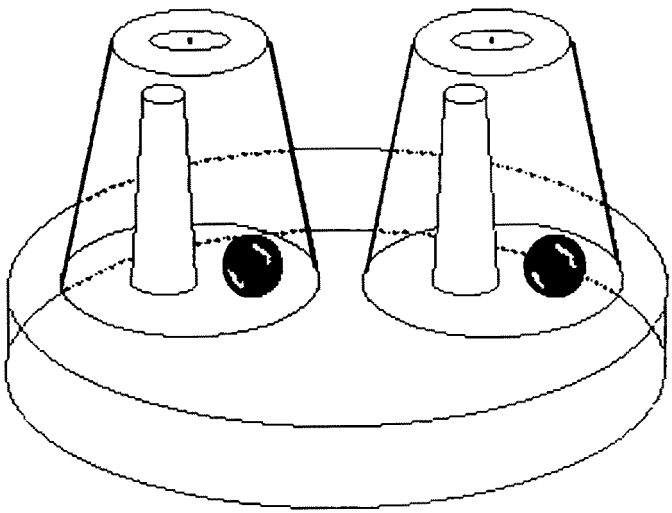

FIG. 1. An enclosed dual-choice Petri dish bioassay apparatus in standard orientation. The dark spheres represent a wild plum (left) and a wooden model of a plum (right).

hole so that the base was flush with the lid of the dish and the tip protruded above the lid. A 35-ml transparent polystyrene cup was centered over each tube, enclosing it. One of the cups contained a treatment inserted within 5 min of the start of a trial. The other cup contained an appropriate control. Within 2 min of the start of a trial, a single PC was placed in the bottom of the dish. It could choose to remain in the dish or to crawl up the inner surface of one of the two tubes and enter a cup containing a treatment or control. In initial tests, the location of each PC was observed every 15 min up to 4 hr. Thereafter, it was observed only at 3 hr.

Data were analyzed according to a G test ($P < 0.05$) (Sokal and Rohlf, 1981) in which the number of PCs that entered a treatment cup was compared with the number that entered a control cup. In addition, to provide a common criterion for judging the power of a treatment to attract PCs under various Petri dish assay conditions, we adopted a response index (RI) used by Phillips et al. (1993) for assessing laboratory attraction of rice weevils to odors of grain. $RI = [(T - C)/\text{total}] \times 100$, where T is the number of PCs responding to a treatment, C is the number of PCs responding to the control, and total is the number of PCs evaluated in the test.

RESULTS

Wind-Tunnel versus Still-Air Conditions. In wind-tunnel assays conducted under white light 8–12 hr after the onset of the photophase of PCs (4–8 hr before the scotophase), 3% of PCs responded positively to a wild plum and 3% to a wooden plum model within 1 min (Table 1, experiment 1). Response levels at 5 min (the end of a trial) were 27 and 20%, respectively. Even though substantial numbers of PCs in nature have been observed to feed and oviposit during this part of the photophase (Owens et al., 1982; Racette et al., 1991), we speculated that response to host fruit might be greater during the scotophase. Indeed, this proved to be so. In assays carried out under dim red light 0–4 hr after the onset of the scotophase, 40% of PCs responded positively to a wild plum and 13% to a wooden plum model within 1 min (Table 1, experiment 2). Response levels at 4 min were identical to those at 5 min: 67% to a wild plum and 30% to a wooden plum model. Response to hexane extract of plums compared with hexane alone under the same conditions as experiment 2 yielded results very similar to those of experiment 2 (Table 1, experiment 3).

Despite the significantly greater proportion of PCs responding to intact plums or plum extract compared with controls during scotophase under wind-tunnel conditions, we believed that this bioassay lacked sufficient discrimination and was too time-consuming to be useful when conducting large numbers of assays associated with identification of chemical components of fruit or sex odor.

TABLE 1. PC RESPONSE TO WILD PLUM (WOODEN MODEL AS CONTROL) OR HEXANE EXTRACT OF PLUMS (HEXANE AS CONTROL) UNDER WIND-TUNNEL OR STILL-AIR (PETRI DISH) TEST CONDITIONS DURING PHOTOPHASE OF PC (WHITE LIGHT) OR SCOTOPHASE OF PC (RED LIGHT)[a]

	Assay condition					PC responding (%)	
Experiment	Air	PC phase	Light	Treatment stimulus	Response at (min)	Treatment	Control
1	Wind	Photo	White	Plum	1	3	3
					5	27	20
2	Wind	Scoto	Red	Plum	1	40[b]	13
					5	67[b]	30
3	Wind	Scoto	Red	Extract	1	37[b]	10
					5	63[b]	37
4	Still	Photo	White	Plum	240	47[b]	17
5	Still	Scoto	Red	Plum	30	37[b]	7
					60	60[b]	10
					120	70[b]	10
					180	73[b]	10
					240	73[b]	10
6	Still	Scoto	Red	Extract	30	30[b]	3
					60	43[b]	3
					120	57[b]	3
					180	63[b]	3
					240	63[b]	3

[a]Thirty PCs were tested per experiment.
[b]Treatments are significantly different from control at $P < 0.05$.

In particular, PCs were prone to move upwind even in clean air. We reasoned that a choice test under still-air conditions might prove more rewarding.

Using still-air Petri dish test chambers, we repeated the treatments of the three wind-tunnel experiments. In each case, PC discrimination between treatment and control exceeded that in the comparable wind-tunnel experiment. Thus, when tested during the photophase, 47 vs. 17% of PCs had responded positively to a wild plum compared with a wooden plum model by 4 hr (Table 1, experiment 4). In a similar test during the scotophase, response levels at 4 hr were 73 vs. 10%, respectively (Table 1, experiment 5). When hexane extract of plums was compared with hexane alone during the scotophase, response levels at 4 hr were 63 vs. 3%, respectively (Table 1, experiment 6). For experiments 5 and 6, we provide response levels at 30, 60, 120, 180, and 240 min. In each experiment, response to treatment as well as control reached a maximum at 3

hr. In each experiment, 3% of the PCs assayed changed location from a treatment cup to a control cup or vice versa between observation periods.

We concluded from this set of six experiments that assays conducted in still air in Petri dishes under dim red light early in the scotophase of PC permitted a substantially greater level of PC discrimination than assays carried out in a wind tunnel or during the photophase of PC. Unless stated otherwise, all remaining assays were conducted in still air in Petri dishes.

Photophase versus Scotophase Conditions. To confirm and expand our conclusions with respect to effects of photophase versus scotophase on PC response levels, we conducted two additional experiments in which PCs were evaluated under photophase or scotophase conditions using plum extract as the treatment substance. When assayed early in the scotophase, PCs responded identically under conditions of dim red light or total darkness: 61% to plum extract versus 3% to control, respectively (Table 2, experiment 1). For PCs entrained for one week to a photophase from 18:00 to 10:00 hr the next day, response levels to plum extract versus control were 60 vs 4% when the assay was initiated at 10:00 hr under red light but 25 vs. 2% when initiated at 10:00 hr under white light (Table 2, experiment 2). For PCs entrained for one week to a photophase from 05:00 to 21:00 hr, response levels to plum extract versus control were 23 vs. 6% when the assay was initiated at 10:00 hr under red light and 27 vs. 2% when initiated at 10:00 hr under white light (Table 2, experiment 2). These results show that PC response to host odor under dim red light does not differ from that in total darkness and confirm that response to host odor is substantially greater when assays are initiated at the beginning of scotophase under dim red light (or total darkness) than otherwise. All remaining assays were conducted under this condition.

Closed versus Open-to-Air Petri Dishes. We hypothesized that the level of PC response to host odor in Petri dish test chambers might be even greater if air were allowed to pass into the chambers rather than maintaining closed air conditions. We reasoned that PCs might be less likely to adapt or habituate to host odor in dishes that permitted exchange of interior with exterior air. Results showed, however, that discrimination between plum extract and control was slightly greater in Petri dishes that were closed (58 vs. 8%) than in Petri dishes having an entirely screened bottom that permitted entry of air from below when dishes were raised 2 cm above floor level (56 vs. 19%) (Table 2, experiment 3).

Orientation of Petri Dishes. We asked whether PC response to host odor differed according to whether PCs in closed Petri dishes were obliged to crawl upward through tubes to reach sources of host odor in cups resting upon lids of dishes (our standard orientation) or were obliged to crawl downward through tubes to reach sources of host odor in cups when dishes, tubes, and cups were flipped 180 degrees so that dishes rested on cups beneath. Results revealed little

TABLE 2. PC RESPONSE TO WILD PLUM (WOODEN MODEL AS CONTROL) IN EXPERIMENTS 6 AND 7 TO HEXANE EXTRACT OF PLUMS (HEXANE AS CONTROL) IN EXPERIMENTS 1, 2, 3, AND 4 OR TO HEXANE EXTRACT OF APPLES (HEXANE AS CONTROL) IN EXPERIMENTS 5 AND 8 UNDER TREATMENT MODIFICATIONS OF STANDARD STILL-AIR PETRI DISH (PD) ASSAY CONDITION[a]

Experiment	Assay condition	PC assayed (*N*)	PC responding (%)		RI[c]
			Treatment	Control	
1	Scotophase, red light	36	61[b]	3	58
	Scotophase, darkness	36	61[b]	3	58
2	Scotophase, red light	48	60[b]	4	56
	Scotophase, white light	48	25[b]	2	23
	Photophase, red light	48	23[b]	6	17
	Photophase, white light	48	27[b]	2	25
3	PD enclosed	36	58[b]	8	50
	PD screened bottom	36	56[b]	19	36
4	Odor above PD	50	62[b]	8	54
	Odor below PD	50	64[b]	14	50
5	PD tubes without Fluon	56	68[b]	9	59
	PD tubes with Fluon	56	38[b]	13	25
6	PC deprived 0 h	40	38	40	−3
	PC deprived 24 h	40	73[b]	13	60
	PC deprived 48 h	40	75[b]	8	68
	PC deprived 72 h	40	35[b]	0	35
7	PC tested May 31	36	69[b]	11	58
	PC tested July 5	36	64[b]	6	58
	PC tested July 19	36	47[b]	14	33
8	Female PC	69	70[b]	10	59
	Male PC	31	68[b]	10	58

[a] Standard assays employed mixed sexes of PCs starved for 24 hr and were conducted early in the scotophase of PC under dim red light conditions in enclosed Petri dishes in which fruit-odor-containing and control cups were placed on lids of dishes and no Fluon was used to coat the exterior of micropippet tubes. All assays except experiment 7 were conducted between June 1 and June 30.

[b] Treatments are significantly different from control at $P < 0.05$.

[c] Response index; see text for definition.

response difference between these two assay conditions: 62% to plum extract versus 8% to control under the standard orientation compared with 64 and 14%, respectively, under the reverse orientation (Table 2, experiment 4). Because it was substantially less effort to set up Petri dishes for assays under the standard dish orientation, we adopted this position rather than the reverse orientation for all tests.

Clean versus Fluon-Coated Micropipet Tubes in Petri Dishes. Even though

only 3% of assayed PCs were observed to change cups during the course of experiments 5 and 6 of Table 1, we speculated that coating the outside of each micropippet tube with Fluon (polytetrafluoroethylene), a substance to which insect tarsi cannot adhere, might prevent any PCs whatsoever from changing location. Unexpectedly, results showed that PC response was only 38% to apple extract (versus 13% to control) under such assay conditions compared with 68% to apple extract (versus 9% to control) under standard conditions with clean micropipette tubes (Table 2, experiment 5). Observations indicated that oftentimes PCs that had climbed up inside surfaces of tubes to reach open tips were reluctant to crawl down Fluon-coated outside surfaces to reach sources of odor.

Physiological State of PCs. Under standard Petri dish assay conditions, we compared response patterns of PCs in varying physiological states with respect to hunger or age. For PCs deprived of all food for 0, 24, 48, or 72 hr before testing, proportions responding to an intact plum versus a wooden plum model were 38 vs. 40%, 73 vs. 13%, 75 vs. 8% and 35 vs. 0%, respectively (Table 2, experiment 6). For PCs collected in late May (within two weeks of apple tree petal fall), proportions responding to apple extract versus control were 69 vs. 11% on May 31, 64 vs. 6% on July 5 and 47 vs. 14% on July 19 (Table 2, experiment 7). We conclude from these tests that nonstarved PCs do not discriminate between fruit odor and control, that PCs starved for 72 hr are perhaps too weak to respond strongly to fruit odor, and that response to fruit odor begins to fade by two months after apple petal fall, when collected overwintering PCs held under laboratory conitions begin to die in substantial numbers. We further conclude that deprivation of food for 24 hr rather than 48 hr is likely to pose less threat to the long-term survival of collected PCs and is the deprivation period of choice where the intent is maximizing PC response to host odor while preserving the health of PCs for future testing.

Sex of PCs. Using Thomson's (1932) criterion for determining the sex of PCs, we attempted to segregate females from males before testing but were unable to do so effectively without injuring some PCs. We did kill and sex 100 assayed PCs after termination of a test of response to apple extract and found 69 to be females. Among the 69 females, response to apple extract versus control was 70 vs. 10%. Among the 31 males, response was 68 vs. 10%, respectively (Table 2, experiment 8). Thus, females and males appear to respond similarly to host odor.

DISCUSSION

Our findings indicated that about 65% of PCs assayed in a wind tunnel early in the scotophase moved upwind in an airstream flowing at 0.4 m/sec in response to odor of wild plums or hexane extract of wild plums before the

proportion responding reached a plateau. Similarly, about 68% of PCs assayed in still-air Petri dishes early in the scotophase responded positively to such host odor before reaching a plateau. The principal difference between the results of these two bioassay approaches was the degree of PC response to controls, which averaged 34% in the wind tunnel but only 7% in Petri dishes. It should not be surprising that PCs responded to a similar extent to host fruit odor under wind-tunnel and still-air conditions given that PCs appear to use host odor as an informational cue both when locating host trees under windy field conditions (Butkewich and Prokopy, unpublished data) and when locating individual host fruit within a tree under calm conditions (Butkewich and Prokopy, 1993). The lower level of response of PCs to controls in closed Petri dish bioassays than in wind-tunnel bioassays argues in favor of conducting bioassays in still air to maximize PC discrimination.

Petri dishes proved to be convenient still-air test chambers. We estimate it requires about 70 min for one person to set up 100 Petri dishes for assay (each dish containing a test substance, a control substance, and a single PC) and about 40 min to record the data 3 hr later and disassemble the dishes. We do not recommend introducing more than one PC per Petri dish owing to the array of sounds emitted by PCs that could attract, repel, or otherwise affect PCs (Webb et al., 1980).

Use of response indices (Table 2) facilitates comparison among experiments. Response indices were greater for PCs assayed at the beginning of scotophase under dim red light than during photophase or under white light, were no different for assays under dim red light or total darkness, were greater for Petri dishes that were enclosed than for Petri dishes whose bottom was screened and open to incoming air, were no different for Petri dishes in which PCs crawled upward to reach cups than for Petri dishes in which PCs crawled downward to reach cups, were greater for bioassays without Fluon compared to bioassays where Fluon was used, were greater for PCs starved for 24 or 48 hr before testing than for unstarved PCs or PCs starved for 72 hr, were greater for overwintered PCs tested two or seven weeks after petal fall than for PCs tested nine weeks after petal fall, and were no different for females and males.

Laboratory procedures reported recently for bioassaying responses of curculionid adults to host odor or pheromonal stimuli include use of an olfactometer under moving air for assaying palm weevils during photophase (Jaffé et al., 1993) and a variety of olfactometers under still-air, two-choice conditions for assaying palm weevils (Rochat et al., 1991), rice weevils (Phillips et al., 1993), and banana weevils, *Cosmopolites sordidus* (Germar), (Budenberg et al., 1993) during scotophase. Our results support the latter approach as being the most effective for PCs.

Currently, we are attempting to identify the compound(s) in host fruit odor attractive to PCs. Such a compound(s) alone, or in combination with pheromonal

components, might serve as an effective odor stimulus for use in traps for monitoring or possibly even directly controlling PCs.

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ALLELOPATHIC POTENTIAL OF *Ipomoea tricolor* (CONVOLVULACEAE) IN A GREENHOUSE EXPERIMENT

A.L. ANAYA,[1,*] D.J. SABOURIN,[2] B.E. HERNANDEZ-BAUTISTA,[1] and I. MENDEZ[3]

[1]*Instituto de Fisiología Celular, UNAM*
Apdo. Postal 70-243, México 04510, D.F.

[2]*The University of Michigan, Flint*
264 MSB Biology Department
303 East Kearsley Street
Flint, Michigan 48502-2186

[3]*Instituto de Investigación en Matemáticas Aplicadas y Sistemas, UNAM*
Ciudad Universitaria
México 04510, D.F.

Abstract—The allelopathic potential of *Ipomoea tricolor*, a plant used in Mexican agriculture to control weeds, and tricolorin A, the major phytogrowth inhibitor present in the so-called "resin glycosides" of this plant, have been evaluated by testing leachates of the plant and the compound on the germination and radicle growth of *Amaranthus hypochondriacus*, *Echinochloa crus-galli*, *Senna uniflora*, *I. tricolor*, and *I. purpurea*. The allelopathic potential of *I. tricolor* was evaluated in a greenhouse experiment with dry *I. tricolor* mixed with sterile and nonsterile soil in pots. *A. hypochondriacus* was sown in pots containing *I. tricolor*, 2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5 triazine (Gesaprim) or 1-glyphosphate, and the glyphosphate salt of isopropylamine (Faena), two different commercial herbicides used as a comparison to *I. tricolor*. Number and dry weights of different monocotyledonous and dicotyledonous weeds and *A. hypochondriacus* growing in the different treatments were measured. *Ipomoea* and Faena herbicide had a similar inhibitory effect on monocots.

Key Words—Allelopathy, *Ipomoea tricolor*, Convolvulaceae, tricolorin A, resin glycosides, herbicides.

INTRODUCTION

The phytotoxic effect of plant residues on the growth of crops and weeds is an aspect that has been extensively studied in allelopathy. Extracts of decomposing

*To whom correspondence should be addressed.

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field residues of barley, rye, broccoli, broadbean, wheat, rice, vetch, and sudangrass, among other crops and weeds, were found to be toxic to the growth of different test species. The challenge is to find plants and residues that are selectively phytotoxic to weeds and with an adequate safety margin on crops (Chou et al., 1977; Altieri and Doll, 1978; Anaya and del Amo, 1978; Ramos et al., 1983; Anaya et al., 1987, 1988; Einhellig and Leather, 1988).

Ipomoea tricolor Cav. (Convolvulaceae) is extensively used in Mexico in folk medicine and agriculture for many purposes. The seeds, with a high content of ergot-type alkaloids, have been used as a hallucinogenic agent in ritual ceremonies by members of the Zapotec and Chatin Indians in Mexico. Other species of the morning glory family are used traditionally all over the world as powerful cathartics. This purgative action is a consequence of the presence of glycosidic resins in foliar tissues, especially in roots and rhizomes of the species belonging to the Convolvulaceae family (Wagner, 1973). In the tropical zones of Mexico, farmers promote the growth of *I. tricolor*, other species of this genus, and some semidomesticated legumes, e.g., *Stizolobium* spp., to protect the soil and to control the growth of weeds. In the sugar cane fields of the state of Morelos, México, *I. tricolor* is grown extensively as a cover crop, especially from August to October. After this time, the whole plant is cut and incorporated into the soil as a green manure. Bioactivity-directed fractionation of organic extracts of the plant led to the isolation of a mixture of resin glycosides with high phytotoxic effects (Anaya et al., 1990). Tricolorin A, the major phytogrowth inhibitor present in the active fraction of the $CHCl_3$ extract (Figure 1) inhibited the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa*

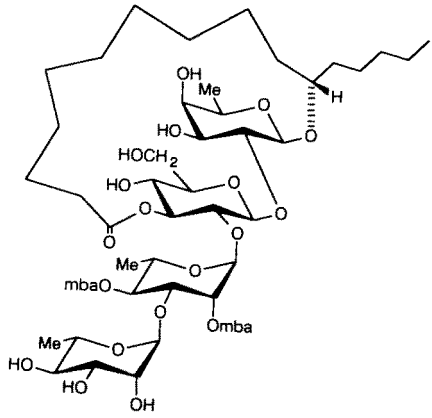

FIG. 1. Structure of tricolorin A (mba, 2-methylbutyroyl).

crusgalli with IC_{50} values ranging from 12 to 37 μM. This bioactive glycolipid displayed a broad range of activities, including antimicrobial and cytotoxic effects and inhibition of protein kinase C activity (Pereda-Miranda et al., 1993).

An objective of the present study was to compare the phytotoxicity of *I. tricolor* leachates and tricolorin A in vitro with that of two commercial herbicides on the germination and growth of *A. hypochondriacus* (Amaranthaceae) and *E. crusgalli* (Poaceae). A second objective was to compare the effect of dry *I. tricolor* incorporated into the soil (sterile and nonsterile) in pots with that of two commercial herbicides on the number and growth of weeds, and the growth of *A. hypochondriacus* (Amaranthaceae).

METHODS AND MATERIALS

Whole plants of *I. tricolor* were collected in Cañón de Lobos, Morelos, México, in March 1993. The plant material was air dried and crushed by hand.

Comparative Bioassays for Phytotoxicity. A bioassay was performed to determine the phytogrowth inhibitory effect of the leachate (2%) of *I. tricolor* mixed (50:50) with 1.5% pure agar. Osmotic pressure was checked to prevent the use of a highly concentrated leachate. Tricolorin A, and two commercial herbicides, 2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5 triazine (Gesaprim, Ciba-Geigy), and 1-glyphosate and the glyphosate salt of isopropylamine (Faena, Monsanto) in concentrations of 10, 50, 100, 150, and 200 μg/ml were tested. Phytogrowth inhibitory activity was determined by seed germination and radicle growth tests on *A. hypochondriacus*, *E. crusgalli* (Poaceae), *Senna uniflora* (Fabaceae), *I. tricolor*, and *I. purpurea*. Distilled water plus methanol (9.9:0.1) mixed with 1.5% agar (50:50) was used as control.

The bioassay was performed in Petri dishes (10 seeds per dish for *A. hypochondriacus*, *E. crusgalli*, *I. tricolor*, and *I purpurea*, and eight seeds for *Senna uniflora*) with four repetitions per treatment in a complete block design. Petri dishes were incubated in darkness at 27°C. Percent germination and radicle growth were obtained after 24 hr for *A. hypochondriacus* and 48 hr for the rest of the test species. Data were analyzed by an ANOVA and Tukey's test.

Greenhouse Experiment. Soil for pots was collected in a crop field recently tilled from a farming community where *I. tricolor* is used as a cover crop for weed control, in the locality of Yautepec, near Cañón Lobos, Morelos, in July 1993. The soil was passed through a 2-mm sieve and divided in two parts. One part was sterilized before the addition of treatments. The experiment was performed in a complete random block design with four blocks. Each block contained 16 representative pots for a total of 64 pots. The pots contained 3 kg of soil and were rotated every three weeks to ensure uniform sunlight. The following treatments were used.

Abbreviations	Treatments
1VS	Sterile soil + 2.5% of vermiculite (control 1)
2VS	Sterile soil + 5% of vermiculite (control 2)
1VNS	Nonsterile soil + 2.5% of vermiculite (control 3)
2VNS	Nonsterile soil + 5% of vermiculite (control 4)
1IS	Sterile soil + 2.5% of *I. tricolor* (dried and milled)
2IS	Sterile soil + 5% of *I. tricolor* (dried and milled)
1INS	Nonsterile soil + 2.5% of *I. tricolor* (dried and milled)
2INS	Nonsterile soil + 5% of *I. tricolor* (dried and milled)
1GS	Sterile soil + 2.5% of vermiculite + 0.4% of Gesaprim
2GS	Sterile soil + 5% of vermiculite + 0.8% of Gesaprim
1GNS	Nonsterile soil + 2.5% of vermiculite + 0.4% of Gesaprim
2GNS	Nonsterile soil + 5% of vermiculite + 0.8% of Gesaprim
1FS	Sterile soil + 2.5% of vermiculite + 0.4% of Faena
2FS	Sterile soil + 5% of vermiculite + 0.8% of Faena
1FNS	Nonsterile soil + 2.5% vermiculite + 0.4% of Faena
2FNS	Nonsterile soil + 5% of vermiculite + 0.8% of Faena

Gesaprim is a 1,3,5-triazine herbicide with a selective pre- and postemergent action used to control annual weeds. Faena is a phosphonic acid product, a glyphosate that acts as a nonselective, nonresidual postemergent herbicide (Worthing, 1991; Draber and Fujita, 1992). The tested concentrations of herbicides were designed to be similar and higher than the mean content of Tricolorin A in *Ipomoea tricolor* (Pereda-Miranda et al., 1993).

Five seeds of *A. hypochondriacus* were sown in each pot. After two weeks, pots were thinned to one seedling of *A. hypochondriacus*. Five seeds of *E. crusgalli* and *S. uniflora* were sown in each pot that contained sterile soil. Number of monocotyledonous and dicotyledonous weeds in the nonsterile soil treatments was recorded five times during the experiment. Numbers of *E. crusgalli* and *S. uniflora* were recorded in the sterile soil treatments. The number of *I. tricolor* seedlings in the four treatments that contained this plant incorporated into the soil (sterile and nonsterile) was recorded four times during the experiment. Pots were watered when necessary. Basins were placed underneath each pot in order to collect drainage water, which was refrigerated for further bioassays and chromatographic analysis. After 45 days the experiment was completed. Plants were removed and separated into monocot and dicot weeds and *A. hypochondriacus*. Plants were dried and weighed. Data were analyzed by an ANOVA, contrasts, and Tukey studentized range tests (Méndez et al., 1992).

Bioassays with Drainage Water from Pots. A bioassay was performed to determine the effects of the drainage water from pots containing each of the treatments in the greenhouse experiment. In this bioassay, the drainage water

of the pot with the 2.5% proportion was mixed with that of the pot with 5% proportion of each treatment to make only eight treatments. These drainage waters were tested on germination and radicle growth of *A. hypochondriacus* and *E. crusgalli*. Bioassay was performed in a complete block design with four repetitions in Petri dishes with Whatman filter paper (No. 42), 10 seeds per treatment. Petri dishes were placed under the same conditions as the comparative phytotoxicity bioassay. Data were analyzed by ANOVA and Tukey's test.

Soil Analysis. Soil samples before and after the greenhouse experiment were analyzed at the Center of Ecology at UNAM in order to measure physical and chemical properties.

RESULTS AND DISCUSSION

Comparative Bioassays for Phytotoxicity

Results of the bioassays in Petri dishes testing aqueous leachates of *I. tricolor*, tricolorin A, and the two herbicides, Gesaprim and Faena, on the germination of *A. hypochondriacus*, *E. crusgalli*, *Senna uniflora*, *Ipomoea tricolor*, and *I. purpurea* are shown in Table 1. Aqueous leachates of the plant significantly inhibited germination of *E. crusgalli* and *I. tricolor*. Tricolorin A was the only treatment that strongly inhibited germination of *A. hypochondriacus*, and to a lesser extent, *E. crusgalli*. Tricolorin A slightly inhibited *I. tricolor* and *I. purpurea* germination, as all other treatments had no effect, on *S. uniflora*, a species that coexists with *I. tricolor* in the same habitat. Both herbicides slightly inhibited germination of *A. hypochondriacus* and *E. crusgalli*. Germination of *I. tricolor* was significantly inhibited by tricolorin A (100–200 μg/ml) and Faena herbicide. Germination of *I. purpurea* was slightly affected by tricolorin A and Faena.

Results of the bioassays in Petri dishes, which tested the same treatments on the radicle growth of test species, are shown in Table 2. Leachate of *I. tricolor* strongly inhibited the radicle growth of *A. hypochondriacus* and had a slight effect on *E. crusgalli* and *S. uniflora*, but it showed no effect on the radicle growth of the two *Ipomoea* species tested. Tricolorin A displayed a strong inhibitory effect on radicle growth of *A. hypochondriacus* and *E. crusgalli*. However, this bioactive compound had no effect on *S. uniflora*. Tricolorin A significantly inhibited radicle growth of *I. tricolor* at 150 and 200 μg/ml. This inhibitory effect was stronger on *I. purpurea*. The effect of the two herbicides tested in Petri dishes was different to that in the soil. Gesaprim significantly inhibited (34%) the radicle growth of *A. hypochondriacus* at 150 and 200 μg/ml. It stimulated *A. hypochondriacus* at 10 μg/ml, and had no effect on *E. crusgalli* and *S. uniflora*. Gesaprim caused a significant stimulation of radicle

TABLE 1. EFFECTS OF AQUEOUS LEACHATE AND TRICOLORIN A OF *Ipomoea tricolor* AND GESAPRIM AND FAENA HERBICIDES ON GERMINATION OF FIVE SPECIES

Treatments	Germination (%)				
	Amaranthus hypochondriacus	*Echinochloa crusgalli*	*Senna uniflora*	*Ipomoea tricolor*	*Ipomoea purpurea*
Control	95.0	90	80	65	60
Aqueous leachate (1%)	95.0	67.5[a]	100	55[a]	60
Tricolorin A (μg/ml)					
10	70.0[a]	92.5	80	65	45[a]
50	57.7[a]	70[a]	80	62.5	67.5
100	0	35[a]	80	62.5	50[a]
150	0	35[a]	80	55[a]	50[a]
200	0	27.5[a]	75	25[a]	57.5
Gesaprim (μg/ml)					
10	87.5	90	80	90	72[a]
50	92.5	90	80	70	52
100	97.5	90	72.5	76	56
150	85[a]	92.5	80	88	52
200	80[a]	82.5	80	76	52
Faena (μg/ml)					
10	100	77.5[a]	80	47.5[a]	57.5
50	92.5	75[a]	77.5	42.5[a]	55
100	85[a]	90	80	55[a]	50[a]
150	87.5	75.0[a]	80	42.5[a]	47.5[a]
200	85[a]	77.5[a]	80	30[a]	57.5

[a]Differences statistically significant ($P < 0.05$) with respect to control using a HSD Tukey's test.

growth of the two *Ipomoea* species tested. Faena had an inhibitory effect on radicle growth of *E. crusgalli*, *S. uniflora*, *I. tricolor*, and *I. purpurea* starting at 10–50 μg/ml. This herbicide did not affect the growth of *A. hypochondriacus*.

Greenhouse Experiment

Number of Seedlings of I. tricolor in Pots. The dry *I. tricolor* that was mixed with sterile and nonsterile soil (1IS, 2IS, 1INS, 2INS), contained some seeds of the species that went unnoticed until they germinated in the pots. The number of seedlings that emerged in these four treatments was recorded four times during the experiment and then was completely eliminated to avoid its competitive effect on the other plants. The split-plot ANOVA made with the logarithms of the number of these seedlings in the four dates recorded showed a significant effect for the interaction date–treatment.

TABLE 2. EFFECTS OF AQUEOUS LEACHATE AND TRICOLORIN A OF *Ipomoea tricolor* AND GESAPRIM AND FAENA HERBICIDES ON RADICLE GROWTH OF FIVE SPECIES

Treatments	Radicle growth inhibition (%)				
	Amaranthus hypochondriacus	*Echinochloa crusgalli*	*Senna uniflora*	*Ipomoea tricolor*	*Ipomoea purpurea*
Control	0.0	0.0	0.0	0.0	0.0
Aqueous leachate (1%)	60.0[a]	26.4[a]	20.8[a]	11.6	7.0
Tricolorin A (μg/ml)					
10	39.7[a]	52.2[a]	(13.1)[b]	(5.4)	7.2
50	55.0[a]	72.1[a]	9.3	17.3	36.7[a]
100	100[a]	78.2	7.7	17.1	32.6[a]
150	100[a]	78.0[a]	8.3	32.4[a]	23.0[a]
200	100[a]	77.5[a]	4.8	40.7[a]	43.0[a]
Gesaprim (μg/ml)					
10	(24.0)[a]	(14.6)	(13.1)	(7.2)	(55.0)[a]
50	(8.0)	(12.8)	5.1	(18.4)	(10.0)
100	19.6	3.7	10.0	(27.0)[a]	(55.8)[a]
150	20.0[a]	11.7	(6.2)	(31.0)[a]	(28.0)[a]
200	34.4[a]	(15.0)	8.2	(24.3)[a]	(70.5)[a]
Faena (μg/ml)					
10	(1.8)	25.0[a]	(15.9)	41.8[a]	46.0[a]
50	(6.0)	41.2[a]	40.9[a]	46.4[a]	55.4
100	(7.5)	55.5[a]	42.9[a]	53.4	59.7[a]
150	(1.8)	69.1[a]	52.3[a]	66.5[a]	58.5[a]
200	18.6	75.0[a]	52.3[a]	61.4[a]	66.0[a]

[a]Differences statistically significant ($P < 0.05$) with respect to control using a HSD Tukey's test.
[b]Number in parentheses indicates stimulation.

The analysis of interaction of treatments by dates on number of seedlings of *Ipomoea* is shown in Figure 2. There was a significant difference in the number of seedlings of *I. tricolor* in sterile (1IS and 2IS) and nonsterile (1INS and 2INS) treatments, particularly in the first date. There was a significant difference between 1INS treatment (prepared with the low proportion of *I. tricolor* added to soil) and the other three treatments in the second, third, and fourth measurements. Probably, in nonsterile soil, microorganisms quickly attack the seed population of *I. tricolor*, diminishing the possibility of germination and survival. Absence of microorganisms in sterile soil may have favored the integrity of *Ipomoea* seeds and their probability of survival and germination. With time, conditions of soil sterility were lost, and the number of germinated seeds came to be similar in all treatments, except in the 1INS. It is possible that in natural conditions, *I. tricolor* inhibits germination and growth of its own seeds.

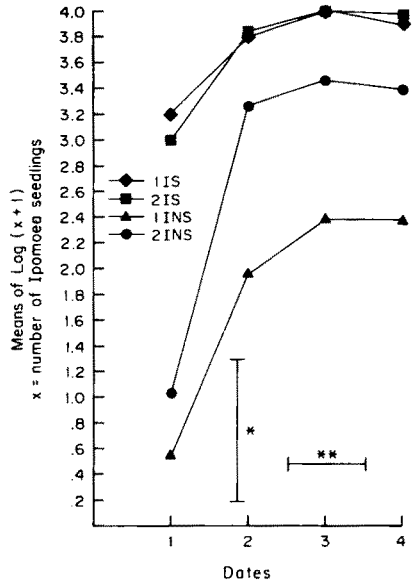

FIG. 2 Study of the interaction treatments by dates on number of seedlings of *Ipomoea* (all experimental data). *HSD for comparing treatments; **HSD for comparing dates.

The phytotoxic activity of the leachates and/or residues could be an autocontrol mechanism of the population. These seeds are rich in alkaloids and other important secondary metabolites, including resin glycosides, so it is also important to explore the ecological role that these compounds play in the establishment of biotic interactions, especially with predators and microorganisms.

Effects of Treatments on Number of Echinochloa *(Monocot) and* Senna *(Dicot) Plants in Sterile Soil.* The number of plants for each pot was recorded five times during the experiment. ANOVA showed that there was a significant difference among treatments. For both plant types, it is also possible to observe a significant effect for the interaction treatments–dates.

Tukey's studentized range test for the means of the logarithm of number of *Echinochloa* (monocot) plants in sterile soil (Table 3) shows that the number of plants is higher in control 2VS compared with the other treatments. There was no difference among IVS, 1IS, 2IS, and 2FS. These four treatments were different from 1FS, 1GS, and 2GS. These last two treatments were statistically different from each other and from 1IFS. The number of *Echinochloa* was significantly diminished by both Gesaprim treatments and treatment with Faena in low concentration (1FS).

TABLE 3. TUKEY'S STUDENTIZED RANGE TEST FOR LOGARITHM OF NUMBER OF *Echinochloa* (MONOCOT) AND *Senna* (DICOT) PLANTS IN STERILE SOIL[a]

Echinochloa (monocot)		*Senna* (dicot)	
Treatment	Mean	Treatment	Mean
2VS	1.40 ± 0.61 a	2VS	1.56 ± 0.38 A
2FS	1.30 ± 0.75 ab	2FS	1.40 ± 0.34 A
1VS	1.28 ± 0.73 ab	2IS	1.35 ± 0.48 AB
2IS	1.25 ± 0.72 ab	1VS	1.33 ± 0.75 AB
1IS	1.21 ± 0.71 ab	1IS	1.31 ± 0.19 AB
1FS	1.08 ± 0.73 bc	1FS	1.02 ± 0.32 BC
2GS	0.87 ± 0.74 cd	2GS	0.70 ± 0.78 DC
1GS	0.66 ± 0.64 d	1GS	0.35 ± 0.40 D

[a]Numbers with different letters are significantly different ($p < 0.05$).

The number of *Senna* (dicot) (Table 3) was the same in the control 2VS and Faena in high concentration (2FS). The control 1VS and the treatments 1IS and 2IS are not different from each other. The other treatments (1FS, 2GS, and 1GS) were significantly different. In fact, the number of *Senna* plants was significantly diminished by the two treatments with Gesaprim and Faena in low concentration. *Ipomoea* treatments affected the number of *Senna* only when compared with 2VS control and Faena in high concentration. The effect of Faena was unexpected when the results of the low and high concentrations were compared. The number of *Senna* and *Echinochloa* seedlings was affected only by the low concentration of Faena.

The analysis of interaction of treatments by dates on number of *Echinochloa* (monocot) plants in sterile soil is shown in Figure 3. Faena at low concentration (1FS) had the lowest number of seedlings at the second and third dates. There was a significant difference between both Gesaprim treatments and the other treatments at the fourth and fifth dates.

The analysis of interaction of treatments by dates on number of *Senna* (dicot) plants in sterile soil is shown in Figure 4. Regarding differences among treatments, Gesaprim and Faena at low concentration had low numbers of *Senna* plants at the third and fourth dates. At these same dates, control 2VS had the higher number of plants. Both concentrations of Gesaprim (1GS and 2GS) were significantly different from the other treatments in the fourth and fifth dates. Gesaprim and Faena at low concentration (1GS and 1FS) had a stronger effect on the number of *Echinochloa* and *Senna* plants compared with the high concentration (2GS and 2FS).

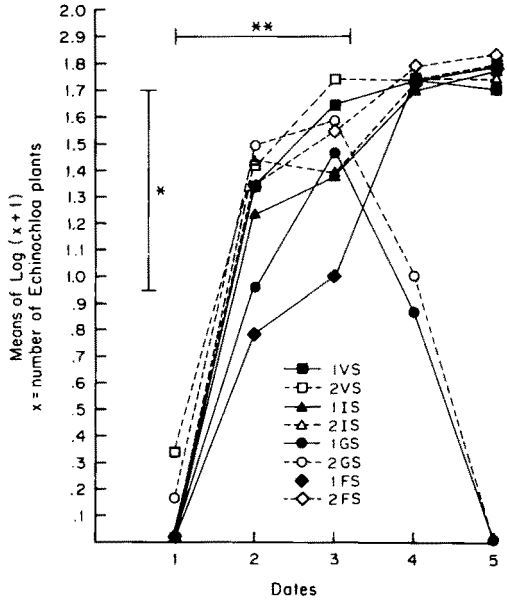

FIG. 3 Study of the interaction treatments by dates on number of *Echnochloa* plants (only data for sterile soil). *HSD for comparing treatments; **HSD for comparing dates.

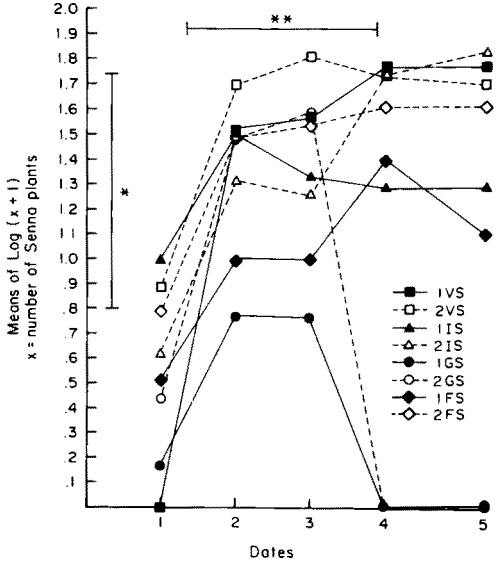

FIG. 4 Study of the interaction treatments by dates on number of *Senna* plants (only data for sterile soil). *HSD for comparing treatments; **HSD for comparing dates.

TABLE 4. TUKEY'S STUDENTIZED RANGE TEST FOR LOGARITHM OF NUMBER OF MONOCOTYLEDONOUS AND DICOTYLEDONOUS PLANTS IN NONSTERILE SOIL[a]

Monocotyledonous plants		Dicotyledonous plants	
Treatment	Mean	Treatment	Mean
2VNS	1.09 ± 0.42 a	1VNS	2.26 ± 0.23 A
1VNS	1.09 ± 0.40 a	2VNS	2.24 ± 0.12 A
1FNS	0.97 ± 0.64 a	1FNS	1.53 ± 0.33 B
1INS	0.73 ± 0.49 ab	1INS	1.41 ± 0.27 B
2INS	0.52 ± 0.41 bc	2INS	1.24 ± 0.34 B
2FNS	0.37 ± 0.23 bcd	2FNS	0.81 ± 0.26 C
2GNS	0.10 ± 0.23 cd	1GNS	0.55 ± 0.69 C
1GNS	0.07 ± 0.15 d	2GNS	0.54 ± 0.68 C

[a]Numbers with different letters are significantly different ($p < 0.05$).

Effects of Treatments on Number of Monocotyledonous and Dicotyledonous Plants in Nonsterile Soils. The split-plot ANOVA with the logarithms of the number of monocotyledonous and dicotyledonous plants in nonsterile soils showed that there was a significant difference between treatments. In the case of the number of dicotyledonous plants, there was a significant effect for the interaction treatments–dates. Tukey's studentized range test for the logarithm of the number of monocots in nonsterile soil (Table 4) showed that the two controls (1VNS, 2VNS) and 1FNS are not different. The rest of the treatments were different from each other. The number of monocots was significantly diminished by *Ipomoea* and Faena in high concentration; this effect was stronger with Gesaprim in both concentrations. In relation to the number of dicots, it was observed that treatments 1FNS, 1INS, and 2INS were significantly different from the controls. Treatments 2FNS, 1GNS, and 2GNS had a strong phytotoxicity on the number of dicots.

The analysis of interaction of treatments by dates on the number of monocotyledonous plants in nonsterile soil is shown in Figure 5. The two controls maintained the higher number of monocots from the beginning of the experiment. At the fourth and fifth dates, this is true also for Faena at low concentration. The two treatments with *Ipomoea* had a low number of monocots from the first to the fourth dates. Both treatments with Gesaprim differed from the other treatments from the third date to the final date. Faena at high concentration was significantly different from Faena at low concentration. It is possible to see that the effect of *Ipomoea* treatments is stronger compared with Faena at low concentration.

The analysis of interaction of treatments by dates on the number of dico-

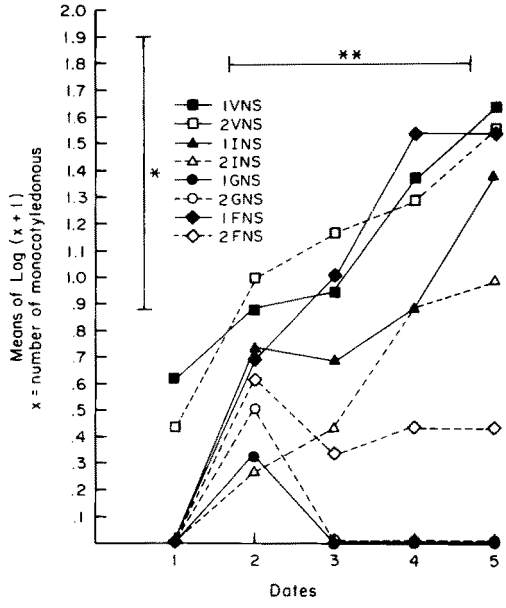

FIG. 5 Study of the interaction treatments by dates on number of monocotyledonous (only data for non-sterile soil). *HSD for comparing treatments; **HSD for comparing dates.

tyledonous plants in nonsterile soil is shown in Figure 6. Both controls had the higher number of dicots at the five dates recorded. At the first date both *Ipomoea* treatments and Gesaprim at the low concentration had the lower numbers of dicots. *Ipomoea* treatments and Faena at low concentration showed a similar effect on the number of dicots from the second date to the fifth date. From the third date to the end of the experiment, 2FNS and both Gesaprim concentrations had a strong phytotoxic effect on dicots.

Gesaprim herbicide eliminated all plants in the pots at the two concentrations used at the end of the experiment. The effect of Gesaprim on growth of plants in sterile and nonsterile soil was opposite to its effect in Petri dishes. In general, Gesaprim did not affect germination and growth of the test species in Petri dishes bioassays. Undoubtedly, the mediator role of the soil is a fundamental factor for the phytotoxic expression of this herbicide. On the other hand, Faena did not affect germination and growth of *Amaranthus* in vitro, nor the germination of *Senna*, but it inhibited significantly the radicle growth of *Echinochloa*, *Senna*, *Ipomoea tricolor*, and *I. purpurea* seedlings in these Petri dish bioassays.

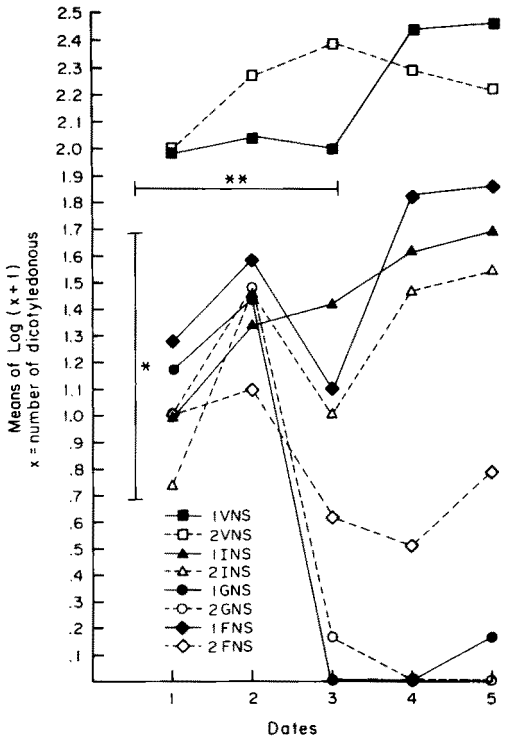

FIG. 6 Study of the interaction treatments by dates on number of dicotyledonous (only data for non-sterile soil). *HSD for comparing treatments; **HSD for comparing dates.

Effects on Echinochloa *and* Senna *Biomass in Sterile Soils and Monocot and Dicot in Nonsterile Soils.* Biomass, expressed as dry weight, was evaluated at the end of the experiment. Mean weights of *Echinochloa* (monocot) and *Senna* (dicot) in sterile soil, and monocots and dicots in nonsterile soil are shown in Table 5. Gesaprim eliminated all the plants at the two concentrations tested in sterile and nonsterile soils. In relation with sterile soil, the significant difference between treatments is focused on the effect of *Ipomoea* mixed in the soil and on biomass of *Echinochloa*. In relation with nonsterile soils, there was no significant difference in the biomass of monocots and dicots in the control, *Ipomoea*, and Faena treatments. On the other hand, there were no significant differences in the biomass of *Aviaranthus hypochondriacus* among all the treatments (data not shown).

TABLE 5. EFFECTS OF EIGHT TREATMENTS ON BIOMASS OF *Echinochloa* AND *Senna* PLANTS IN STERILE SOILS AND BIOMASS OF MONOCOTS AND DICOTS IN NONSTERILE SOILS

Treatment	Mean weight (g) *Echinochloa*	Mean weight (g) *Senna*	Total
Sterile soil			
1VS	2.2	0.15	2.35
2VS	2.78	0.15	2.93
1IS	1.6[a]	0.12	1.72
2IS	1.25[a]	0.11	1.36
1GS	0	0	0
2GS	0	0	0
1FS	2.02	0.11	2.13
2FS	2.12	0.10	2.22
Total	11.97	0.74	12.71
	Mean weight (g) of monocot	Mean weight (g) of dicot	
Nonsterile soil			
1VNS	0.03	0.08	0.11
2VNS	0.007	0.09	0.097
1INS	0.09	0.16	0.25
2INS	0.013	0.13	0.143
1GNS	0	0	0
2GNS	0	0	0
1FNS	0.003	0.04	0.043
2FNS	0.005	0.0017	0.0067
Total	0.148	0.50	0.6497

[a]Differences statistically significant ($p < 0.01$) with respect to control using a HSD Tukey's test.

Results of Bioassay with Drainage Water from Pots

The results from this bioassay are shown in Table 6. Effects of the drainage water from sterile and nonsterile soil treatments on radicle growth of *A. hypochondriacus* and *E. crusgalli* were different. It is possible to see that there was a specific response of each species to the treatments. ANOVA and Tukey's test showed that significant effects, particularly stimulations, were produced only by drainage water from nonsterile soils. The water from pots with *I. tricolor* produced a significant stimulation of radicle growth of *A. hypochondriacus*. Drainage water from Faena treatments significantly inhibited the growth of this species.

TABLE 6. EFFECTS OF THE LEACHATES FROM THE POTS ON THE RADICLE GROWTH OF *Amaranthus hypochondriacus* AND *Echinochloa crusgalli*.

Treatment	Inhibition (%)	
	Amaranthus hypochondriacus	*Echinochloa crusgalli*
Sterile soil		
VS	0	0
IS	(8)[a]	(2)
GS	1	(3)
FS	3	(4)
Nonsterile soil		
VNS	0	0
INS	(24)[a]	(54)[a]
GNS	9	(47)[a]
FNS	44[a]	(36)

[a]Differences statistically significant ($P < 0.05$) with respect to control using a HSD Tukey's test. Numbers in parentheses are stimulations.

On the other hand, *E. crusgalli* was significantly stimulated by water from pots with *I. tricolor*, Gesaprim, and Faena. Neither of the phytotoxic agents from the different treatments, except that from Faena, was leached out by the drainage waters. If we compare the results of the bioassays in Petri dishes (Table 2) with these of the drainage waters bioassays (Table 6), we can observe the essential effect of the soil on the expression of phytotoxicity and allelopathy. In relation to this, the effectiveness of a soil-applied herbicide depends on its adsorption in the soil. Organic matter is the predominant adsorbing component in it, so the amount of organic matter in the soil is a determining factor (Johnston, 1986; Rowell, 1994). The selectivity of triazines, for instance, results from their low solubility in water and their high colloidal absorption grade in the soil, so they have relatively long-term activity in the soil. This could explain the strong phytotoxicity of Gesaprim in the soil of the experiment that was rich in organic matter (Gerber et al., 1970; Addiscot and Wagenet, 1985).

Table 7 shows some physical and chemical properties of soil before and after the experiment. In general, conditions of the soil before and after the experiment change slightly. Before the experiment, sterilization of the soil increased total nitrogen and total phosphorus. After the experiment, the pH tended to be neutral. The soil was not saline, but *Ipomoea* and Gesaprim caused a small increase in the electrical conductivity of it. Organic matter is higher in sterile and nonsterile soil with *Ipomoea* and in soil of the nonsterile control and

TABLE 7. PHYSICAL AND CHEMICAL CHARACTERISTICS OF SOIL BEFORE AND AFTER GREENHOUSE EXPERIMENT[a]

Soil	pH 1:2.5	EC (μohm)	MO (%)	K (meq/100 g)	Ca (meq/100 g)	Mg (meq/100 g)	CEC	TN (ppm)	TP (ppm)	Clay (%)	Silt (%)	Sand (%)	Texture
SSBE	6.7	112	3.45	4.15	8.63	5.9	30.46	1630	1100	20	30	50	Loam
NSSBE	6.2	143	3.90	4.42	7.95	8.85	31.62	1195	450	24	30	46	Loam
VS	7.0	113	3.68	4.37	8.40	9.31	31.40	1175	3250	26	30	44	Loam
VNS	7.0	95	4.21	3.84	7.72	8.85	23.32	1200	1350	26	28	46	Loam
IS	6.7	154	4.79	5.93	9.31	6.81	40.98	1605	1400	30	28	42	Clay loam
INS	7.4	209	4.23	6.11	8.17	6.58	27.93	1680	950	24	30	46	Loam
GS	6.7	239	3.81	4.43	9.08	7.26	32.64	1340	1250	26	28	46	Loam
GNS	7.0	154	3.49	7.74	9.31	6.58	27.91	1160	950	26	28	46	Loam
FS	7.3	125	3.64	4.20	9.53	8.63	32.90	1055	700	22	28	50	Loam
FNS	7.0	128	4.70	3.86	7.49	8.40	27.37	1145	1050	24	28	48	Loam

[a]Before the experiment: SSBE, sterile soil; NSSBE, nonsterile soil. After the experiment: VS, soil of the sterile control; VNS, soil of the nonsterile control; IS, sterile soil with *Ipomoea*; INS, nonsterile soil with *Ipomoea*; GS, sterile soil with Gesaprim; GNS, nonsterile soil with Gesaprim; FS, sterile soil with Faena; FNS, nonsterile soil with Faena.

nonsterile soil with Faena. Potassium was increased in *Ipomoea* treatments and in nonsterile soil with Gesaprim. Nitrogen was increased in sterile soil before the experiment and in both treatments with *Ipomoea*. Phosphorus is higher in almost all the sterile soils compared with the nonsterile ones.

In the greenhouse experiment, the biomass of *Echinochloa crusgalli* (sterile soil) was significantly diminished by the incorporation of *I. tricolor* into the soil, and also, the number of monocots and dicots in nonsterile soil. In fact, the number of monocots and dicots tended to diminish starting from the third date recorded in the greenhouse experiment in both *Ipomoea* treatments. It is possible to see that this tendency is stronger comparing these two treatments with Faena at low concentration. In the field, it is possible that the weed control effect of *Ipomoea tricolor* takes place in two stages, first, by the combination of the allelopathic potential of the living plant expressed through rain leachate and competition (interference); and second, when the plant is cut and incorporated into the soil. Decomposition of its residues acts as the second weed control factor before the sowing of sugar cane, corn, or other crops.

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VARIATION IN CONDENSED TANNIN CONCENTRATION OF A TEMPERATE GRASS (*Holcus lanatus*) IN RELATION TO SEASON AND REPRODUCTIVE DEVELOPMENT

G.R. IASON,[1,2,*] J. HODGSON,[2] and T.N. BARRY[1]

[1]*Department of Animal Science*
[2]*Department of Plant Science*
Massey University
Palmerston North, New Zealand

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Abstract—*Holcus lanatus*, a temperate grass, was found to contain low but measurable concentrations of condensed tannins (CT), according to the vanillin-HCl and the butanol-HCl methods. The variation in CT concentrations between different types of tissue including leaf age and reproductive versus nonreproductive tissue was examined. There was no evidence that floral tissue contained more CT than nonreproductive tissue. A sequential extraction and analysis procedure showed a decreasing proportion of free-unbound CT and greater preponderance of protein-bound and fiber-bound CT with increasing leaf age and in dead matter. Samples collected from an agricultural pasture on two sampling dates, January 24 and March 5, 1993, showed short-term temporal variation in free-unbound CT.

Key Words—Condensed tannin, *Holcus lanatus*, flowering, vegetative, seasonal variation.

INTRODUCTION

Despite having no known role in primary metabolism, evidence has accumulated that plant secondary metabolites may have adaptive value in the ecological interactions of the plant, often providing effective defense against herbivores or pathogens (Zucker, 1983; Rhoades and Cates, 1976). Current theories of plant

*To whom correspondence should be addressed at current address: Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB9 2QJ, UK.

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chemical defense predict that tissues of greatest value should be most heavily defended (Rhoades, 1979). In fitness terms, the influorescences are among the most valuable of plant tissues, representing a large investment of resources in reproduction. Flower pigments of plants, whose role is usually considered to be attraction of pollinators, often contain phenolic compounds (Harborne, 1988). This factor can confound comparisons of the extent to which flowering tissue versus vegetative tissue is chemically defended by phenolics. Although condensed tannins (CT) are generally considered to be absent from temperate or high-latitude grasses (Jung et al., 1979; Bernays et al., 1989), Yorkshire fog (*Holcus lanatus* L.) is reported to contain small but measurable amounts (Terrill et al., 1992; Douglas et al., 1994). Since grasses are wind pollinated, this permits comparison of concentration of CT in flowering and vegetative tissue, without confounding the possible defensive functions of phenolics in influorescences with the insect attractant function.

Although there is much information on the variation in concentration of phenolic compound in woody plants, both temporally and between plant parts (Feeny, 1970; Palo et al., 1985; Iason et al., 1993), little is known about the distribution of tannins in grasses. The development of techniques to quantify the amounts of free-unbound, versus protein- and fiber-bound CT in plants (Terrill et al., 1992) permits examination of these different forms of CT and enhanced interpretation of their ecological roles. In this study, wild *H. lanatus* and a cultivar, *H. lanatus* (cv. Massey Basyn), were used to address the following specific aims: (1) to investigate variation in the concentrations of free-unbound, protein-bound, and fiber-bound CT between different leaf ages and between vegetative and reproductive parts of *H. lanatus*; and (2) to examine the variation in CT concentration of vegetative shoots of *Holcus lanatus* cv. Massey Basyn, between two different sampling dates in the growing season, independently of flowering.

METHODS AND MATERIALS

Vegetation Sampling. Differences in chemical composition between vegetative and reproductive tissues of *H. lanatus* were compared using samples collected on February 28, 1993, from separate sites, 40 m apart along an unmown roadside verge at Aokautere, 10 km NW of Massey University, Palmerston North, New Zealand. Each sampling site was at least 2 m from the road edge, well drained, and consisted of at least 70% ground cover of *H. lanatus*. The *H. lanatus* plants were dissected into flowering stems, inflorescences, live vegetative stems, dead leaf, and live leaves 1, 2, and 3. Leaf 1 was defined as the youngest leaf that had emerged from the sheath by at least 1 cm. Leaves 2 and 3 were the two next oldest leaves, respectively.

In order to examine temporal variation in the tannin concentration of the vegetative tissue of *H. lanatus*, two sets of herbage samples were collected from a quadrat placed at random in each of four 0.2-ha plots of *H. Lanatus* cv. Massey Basyn, on January 24, 1993 and six weeks later on March 5, 1993. The plots were at Massey University, New Zealand, and were each grazed continuously by weaned lambs to a target sward height of 5 cm from December 1992 until after the second sampling date, as part of a larger study of the nutritive value of *H. lanatus* for sheep. These samples were harvested at ground level and aerial tissue above the highest node with adventitious roots was separated into live leaf, dead leaf, and live stem.

Material was stored in sealed bags at 4°C for a maximum of three days after harvesting while being dissected. Only about 30 g was dissected at any time to prevent air drying during this process. All sorted samples were stored at −20°C prior to freeze drying and subsequent milling to pass a 1 mm sieve.

Chemical Analyses. CT were measured using the sequential extraction procedure described by Terrill et al. (1992) in which the CT present in each of the free-unbound, protein-bound, and fiber-bound forms were determined using the butanol–HCl procedure (the proanthocyanidin assay) of Porter et al. (1986). Free-unbound CT was extracted with aqueous acetone and diethyl ether and partitioned into the aqueous phase to exclude chlorophyll. Subsequent extraction with boiling sodium dodecyl sulfate (SDS)/mercaptoethanol solution yielded protein-bound tannin. The residue from this extraction was boiled with SDS and butanol–HCl reagent to yield fiber-bound CT. Additionally, the free-unbound tannin fraction was analyzed for CT by the vanillin–HCl assay (Broadhurst and Jones, 1978). Vanillin reacts with end-flavanoid units only, whereas in the butanol–HCl procedure CT is depolymerized and anthocyanidin color pigment can be formed from any flavonoid unit in the CT (Hagerman and Butler, 1989). Results are expressed as proportions of dry matter relative to a standard of purified CT, extracted from *Lotus pedunculatus*, which was made up in either water (free-unbound CT) or SDS solution (protein- and fiber-bound CT).

Holcus lanatus is known to contain low concentrations of anthocyanin pigments (Beddows, 1961), which can cause some color development in assays for CT (Watterson and Butler, 1983). Consequently, for each fraction of each sample, a sample blank was prepared by either replacing the HCl in the reagent with the same volume of water (butanol–HCl assay), or replacing the vanillin/methanol with methanol only (vanillin assay). These blanks were otherwise treated in exactly the same way as the sample. Any color development was subtracted from that of the normally assayed sample.

Samples were also analyzed for total nitrogen, using a Kjeldahl technique. Due to a shortage of material, samples of each type of tissue were pooled across plots or sites prior to analysis for neutral detergent fiber (NDF) according to the system of Van Soest and Wine (1967).

Statistical Analyses. Statistical comparisons of chemical composition were made using Genstat 5 (1987). One-way analysis of variance was used to compare between plant tissues or leaf ages, and when data were available on different harvest dates, a two-way analysis of variance was used entering plant tissue and date as factors. Data were blocked within plots for *H. lanatus* on the grazed pasture or within sampling sites for wild *H. lanatus*. The protected least significant difference (LSD) method was used to make comparisons between individual classes where overall F tests showed significant heterogeneity (Snedecor and Cochran, 1980).

RESULTS

Changes in Composition with Age of Leaf. There were significant differences in concentration of free-unbound, protein-bound, fiber-found, and total CT between different types of shoot tissue as measured by the butanol–HCl assay ($P < 0.01$, Table 1). Although leaves 1–3 did not contain significantly different concentrations of total CT, there was a significantly higher concentration of CT in dead versus living leaf. There was a gradual decrease of free-unbound CT with leaf age, as shown by both the vanillin–HCl and the butanol–HCl assays (Table 1). Free-unbound CT formed 55% of total CT in leaf 1 but only 9% of total CT in dead leaf tissue. Although there were no significant differences, both protein-bound and fiber-bound CT tended to increase with leaf age. Protein-bound and fiber-bound CT were significantly ($P < 0.05$) higher in dead than living leaf material (Table 1). As a percentage of total CT, protein-bound CT represented 45% in leaf 1, 54% in leaf 2, 62% in leaf 3, and 69% in dead leaf. The corresponding percentages of the total CT formed by fiber-bound CT were 0%, 3%, 6%, and 22%. Total nitrogen concentration declined with leaf age. It was significantly lower in leaf 3 than leaves 1 and 2 ($P < 0.05$) and declined markedly in the dead leaf matter (Table 1). Although no statistical comparisons were possible, there was no indication that cell wall content (NDF) changed progressively with leaf age, although dead leaf material contained a higher concentration than living leaf (Table 1).

Analysis of the samples collected on different dates from the grazed pasture confirmed the overall pattern of decrease in total nitrogen and increase in CT and NDF as a proportion of dry matter with leaf senescence. The shift in the form of the CT from free-unbound to the protein- and fiber-bound forms with leaf senescence was also confirmed (Table 2).

Reproductive versus Nonreproductive Tissue. The concentration of fiber-bound CT was higher in flowers than in leaf 1, and the concentration of free-unbound CT was lower in flowers than leaves 1 or 2 according to the butanol–HCl assay ($P < 0.05$, Table 1) and lower than leaf 1 according to the vanillin assay. There was no significant difference in the concentration of total CT

TABLE 1. CONCENTRATION OF CONDENSED TANNIN (CT) AND TOTAL NITROGEN ($N = 4$), AND NDF (SINGLE POOLED SAMPLE) IN VEGETATIVE AND REPRODUCTIVE TISSUE OF WILD *Holcus lanatus*[a]

	Vegetative tissue					Reproductive tissue			
Concentration (g/kg dry matter)	Leaf 1	Leaf 2	Leaf 3	Dead leaf	Vegetative stem	Reproductive stem	Flowers	SED	F probability
CT (butanol–HCl)									
Free-unbound	0.72a	0.52ab	0.41c	0.35c	0.30c	0.21c	0.26c	0.111	<0.01
Protein-bound	0.59ac	0.64ac	0.78a	1.90b	0.47cd	0.25d	0.69ac	0.118	<0.001
Fiber-bound	NDa	0.03ac	0.07a	0.61b	NDa	NDa	0.14c	0.061	<0.001
Total	1.30a	1.19c	1.26a	2.76b	0.81c	0.47d	1.08ac	0.130	<0.001
CT (vanillin–HCl)									
Free-unbound	3.51a	2.85abc	2.44abc	1.64bc	2.96ac	1.74bc	1.60b	0.629	<0.05
Total nitrogen	32.3a	32.0a	27.9b	9.3c	11.0d	10.5cd	19.8e	0.07	<0.001
NDF	533	549	545	733	559	672	654	—	—

[a]Means followed by unlike superscripts are significantly different ($P < 0.05$). ND: not detectable.

TABLE 2. CONCENTRATION OF CONDENSED TANNIN (CT) AND TOTAL NITROGEN ($N = 4$) AND NDF (SINGLE-POOLED SAMPLE) IN LIVE LEAF, LIVE STEM, AND DEAD MATTER TISSUE OF *Holcus lanatus* CV. MASSEY BASYN SAMPLED FROM SAME FOUR PLOTS ON JANUARY 24 AND MARCH 05 1993[a]

Concentration (g/kg dry matter)	Sampling date	Live leaf	Live stem	Dead matter	SED	F probability		
						Date	Tissue	D × T
CT (butanol-HCl)								
Free-unbound	Jan 24	0.90	0.45	0.39	0.115	<0.01	<0.001	NS
	Mar 5	0.46	0.25	0.27				
Protein-bound	Jan 24	0.60	0.30	1.39	0.115	NS	<0.001	NS
	Mar 5	0.67	0.24	1.67				
Fiber-bound	Jan 24	0.07	0.03	0.67	0.119	NS	<0.001	NS
	Mar 5	ND	ND	0.45				
Total	Jan 24	1.56	0.78	2.44	0.261	NS	<0.001	NS
	Mar 5	1.13	0.50	2.39				
CT (vanillin-HCl)								
Free-unbound	Jan 24	1.83	2.18	1.95	0.26	NS	NS	NS
	Mar 5	1.96	2.19	2.19				
Total nitrogen	Jan 24	35.3	23.1	15.4	2.13	<0.05	<0.001	<0.05
	Mar 5	43.9	23.1	15.7				
NDF	Jan 24	498	560	709	—	—	—	—
	Mar 5	486	552	695				

[a]ND: Not detectable. SED: SED of interaction.

between flowers and live leaf tissue (Table 1). In general, stem tissue contained lower levels of total CT than other types of tissue (Table 1), and the reproductive stem contained lower concentrations of total CT measured by butanol–HCl than vegetative stem.

Total nitrogen concentrations in the samples of flowers were significantly lower than in leaves ($P < 0.05$) and NDF was greater in flowers and reproductive stems than in vegetative parts (Table 1).

Chronological Differences. Total CT concentration did not differ between sampling dates (Table 2), although free-unbound CT was lower in March than in January ($P < 0.01$) and the ratio of vanillin–HCl/butanol–HCl for free-unbound CT was higher in March ($P < 0.05$). Total nitrogen was lower on the earlier sampling date ($P < 0.05$) but only in the live leaf tissue (Table 2).

DISCUSSION

Although other phenolics are present (Morrison, 1974; Mole and Joern, 1993), grasses are generally reported to contain no CT (Rhoades and Cates,

1976; Jung et al., 1979). Rather than defending themselves against herbivory with carbon-based secondary metabolites, they usually respond to defoliation by herbivores with rapid regrowth (Bryant et al., 1983; McNaughton, 1983). Wild *H. lanatus* grows under a wide range of nutrient and ecological conditions (Watt, 1978), and in agricultural pastures it has the potential for rapid regrowth after grazing that is typical of grasses (Haggar, 1976). However, low concentrations of CT in *H. lanatus* has been confirmed in the present study, using two analytical methods working on different principles (vanillin–HCl and butanol–HCl).

The overall higher concentrations of total CT with leaf age, particularly in senescent leaves, are due partly to a concentrating effect as soluble and mobile constituents are withdrawn from the leaf prior to abcission. Insoluble, nonmobile constituents such as CT (Rhoades and Cates, 1976) and cell-wall polymers, measured here as NDF, are hence increased relative to dry matter. The pattern of change of free-unbound, protein-bound, and fiber-bound tannin with leaf age suggests that the greater proportion of fiber-bound CT, and to a lesser extent of protein-bound CT in older and dead leaf material, is due to progressive complexation of free-unbound CT. This greater binding of CT to cell wall in senescing leaves may inhibit microbial degradation (Swain, 1979), leading to slow rates of decomposition and the accumulation of litter in *Holcus*-dominated grasslands (Watt, 1976).

Condensed tannin concentrations may increase (Feeney, 1970; Mauffette and Oechel, 1989) or decrease (Baldwin et al., 1987) with age of leaf in trees. Most studies only measure free-unbound tannin, and in *H. lanatus* this form of tannin declined with leaf age, although total CT did not change consistently. As well as reflecting greater binding of tannins with increasing leaf age, the decrease in free-unbound CT is probably also associated with a decrease in the production of CT in the older leaves. These leaves are lower in the canopy and receive less incident light, an environmental factor that has been shown consistently to lead to strong reductions in phenolic levels (Waterman and Mole, 1989; Nichols-Orians, 1991; Iason and Hester, 1993). Because of the rapid leaf turnover in grasses throughout the season of growth, a sustained trend of increasing or decreasing concentration of CT in the live tissue would not be expected except as a result of changes in the proportions of young leaf. Indeed, differences in free-unbound CT between the two dates sampled in the grazed pasture are low in comparison to the differences between tissues and leaf ages. Temporal effects may also result from variation in climatic conditions causing short-term changes in growth or photosynthetic rate. The total CT concentration in flowering tissue did not differ significantly from that in live leaf tissue of any age (Table 1), and the observed differences in the form of the CT are consistent with the greater maturity of the inflorescences relative to the young leaf ages.

In those environments where tannin levels are high and plant nutrients and protein levels are low, tannins may cause avoidance by herbivores (Mole and

Waterman, 1987) and are considered to inhibit forage digestion by complexing with plant proteins and digestive enzymes (Robbins et al., 1987). In contrast, nutrient levels and net primary production of plants in some natural communities and most intensively managed agricultural systems are higher than in less intensively managed communities. In these nutrient-rich grazing systems, low levels of CT in forages are thought to be of benefit to grazing ruminants, enhancing animal performance by complexation with protein and protecting it from wasteful microbial degradation in the rumen (Barry, 1989). The low levels of CT in *H. lanatus* are within the range that results in a positive effect on performance of ruminant herbivores (<20 g/kg dry matter; Barry, 1989). Furthermore, CT have no effect as digestion inhibitors in insects (Bernays et al., 1989; Feeney, 1992). In summary, it is unlikely that the low levels of CT in *H. lanatus* have a digestion-inhibiting effect on herbivores, but the possibility that they act as a defense against pathogenic attack from fungi and bacteria (Rhoades, 1979) cannot be dismissed.

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COMPARISON OF ANTIALGAL ACTIVITY OF BROWN-ROTTED AND WHITE-ROTTED WOOD AND *IN SITU* ANALYSIS OF LIGNIN

J.M. PILLINGER,[1,2,*] I. GILMOUR,[2] and I. RIDGE[1]

[1]*Department of Biology*
[2]*Department of Earth Sciences*
Open University, Walton Hall
Milton Keynes, MK7 6AA, U.K.

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Abstract—Brown-rotted wood has been used as a source of lignin to investigate further the antialgal effects of lignocellulosic materials such as decomposing barley straw. The antialgal activity of brown-rotted and white-rotted wood has been determined in a laboratory bioassay. Using pyrolysis gas chromatography–mass spectrometry, the lignin of the rotted wood samples has been compared and the significance of the structure of the lignin in antialgal activity is discussed.

Key Words—Antialgal compounds, brown-rotted wood, white-rotted wood, lignin, macromolecular analysis, pyrolysis GC-MS, *Chlorella*, *Microcystis*.

INTRODUCTION

Barley straw rotting, submerged and well-aerated, in water has been shown to release substances that inhibit the growth of a range of algae and cyanobacteria (Gibson et al., 1990; Newman and Barrett, 1993). Indeed, this effect is being exploited widely for the control of nuisance algae in waterbodies. We have suggested that the antialgal factor(s) is derived directly from the straw per se (Pillinger et al., 1992) and may be phenolic, particularly polyphenolic, in origin (Pillinger et al., 1994). Straw contains some 15% by weight of the polyphenolic structural material lignin. We report the effect of lignin on the growth of an alga and a cyanobacterium in a laboratory bioassay.

Unfortunately, no method is available that permits the extraction of total

*To whom correspondence should be addressed.

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lignin (Lewis and Yamamoto, 1990); different forms of lignin reflect the method by which they have been isolated. The lignin extract that best reflects unchanged native lignocellulose is "enzymatically liberated lignin" (Ander and Eriksson, 1975), formed by the action of brown-rot fungi on lignocellulose, whereby cellulose is preferentially utilized leaving a brown powder highly enriched in lignin (Kirk and Adler, 1970). Brown-rotted wood is readily available in nature, and for this reason we have investigated initially the effect of lignin derived from wood rather than from straw. Although brown-rot lignin is representative of the original lignin of the wood, minor chemical changes cannot be ruled out (Kirk and Adler, 1970). On the other hand, a group of specialized fungi that are able to effect white-rotting of lignocellulose substrates do so by metabolizing lignin. The action of fungi on wood has been reviewed by Levy (1987). Mulder et al. (1991) demonstrated that a thoroughly white-rotted wood did not contain lignin moieties, only those of cellulose. We thus chose to use white-rotted wood in antialgal tests as a control to the lignin-enriched brown-rotted wood.

We have used on-line pyrolysis gas chromatography–mass spectrometry (Py-GC-MS) to compare the lignin in each of the rotted wood samples. This technique increasingly is being employed to study lignocellulose (e.g., Scheijen and Boon, 1991; Galletti and Reeves, 1991) and was recently summarized by Boon (1989). Macromolecular lignin-rich material can be analyzed directly avoiding selective (Lapierre et al., 1989) and time-consuming chemical extractions. The solid sample is thermally disrupted in the absence of air, the subcomponents separated by conventional gas chromatography, and identified by their characteristic mass spectra.

METHODS AND MATERIALS

Algal Toxicity Experiments. A unicellular green alga, *Chlorella vulgaris* Beijerinck (CCAP 211/12), and the cyanobacterium (blue–green alga) *Microcystis aeruginosa* Kutz, emend Elenkin 1924 (CCAP 1450/6), were maintained in liquid culture as below with weekly subculturing. The growth of either organism in the absence (control) and presence of rotted wood (see Table 1 below for details) in algal culture medium was assessed using a laboratory bioassay (Pillinger, 1993). Briefly, an aliquot (1 ml) of stock culture in the exponential phase of growth was added to 50 ml modified Jaworski's medium, buffered with 20 mM HEPES at pH 8.2, in a conical flask and incubated at random in continuous light at 20°C with daily shaking to maintain aeration. All controls and treatments were replicated five times. A comparison of the growth of the organisms between the controls and treatments was obtained by quantifying the chlorophyll *a* content after three to four days of incubation. The absorbance due to chlorophyll *a* of a methanolic extract (10 ml) of the dry biomass retained on

a glass-fiber filter (pore size 1.2 μm) was measured at 665 nm (less a background reading taken at 750 nm). In some experiments the growth of *Chlorella* was measured by counting algal cells using a hemacytometer. Preliminary data indicated a linear correlation between the two methods. All data are reported as percentage of the control growth, allowing comparison between experiments conducted under slightly varying conditions, and, where quoted, standard errors of the mean of the normalized values. The significance of the treatment value ($N = 5$) as compared to the respective control ($N = 5$) was determined by an unpaired t test.

Analytical Pyrolysis Gas Chromatography–Mass Spectrometry. A sample of dried, pulverized wood of between 1 and 2 mg was weighed accurately into a Pelletiser (Scientific Glass Engineering, SGE, Milton Keynes, U.K.) and gently compressed. The pellet was introduced into the quartz liner of the furnace (SGE Pyrolyser) maintained at a preset temperature (500°C). A slight positive pressure of helium in the furnace keeps pyrolysis time constant and reduces secondary pyrolysis; pyrolysis products were thus swept onto the column of the gas chromatograph (GC). The GC (Hewlett Packard HP5890 Series II) was fitted with a WCOT 50-m × 0.32-mm capillary column column coated with 0.5 μm film of 5% phenyl methyl silicone. The initial oven temperature was set at 50°C and programmed to rise at 10°C/min to 100°C after a 1-min hold, then at 5°C/min to 300°C, after which it was isothermal; the complete run lasted for 60 min. Pyrolysis subcomponents were detected using a mass selective detector (HP 5971 series MSD); electron impact ionizing voltage was 70eV with continuous scanning from 50 to 500 mass units. Identification was based on computer matching of mass spectra.

The experimental conditions, including the temperature of pyrolysis, gave an acceptable separation of the subcomponents. Blank runs confirmed that memory effects of the pyrolyzer system were negligible. Preliminary analyses indicated a good measure of reproducibility with a kraft lignin standard, Indulin AT (Sigma), and replicate analyses of a white-rotted wood sample (Pillinger, 1993).

Pyrolysis is not a highly quantitative method (Faix et al., 1987). However, where similar weights of comparable samples are pyrolyzed under identical conditions within a short time period to reduce effects of instrumental variability, then a quantitative estimate of the various pyrolysis fragments can be made. This was achieved by determining the computer-integrated peak areas of interest as a ratio of a chosen peak that occurred in each sample.

RESULTS

Antialgal Effect of Brown- and White-Rotted Wood. Both brown-rotted (BRW) and white-rotted (WRW) sycamore wood were significantly (t test, P

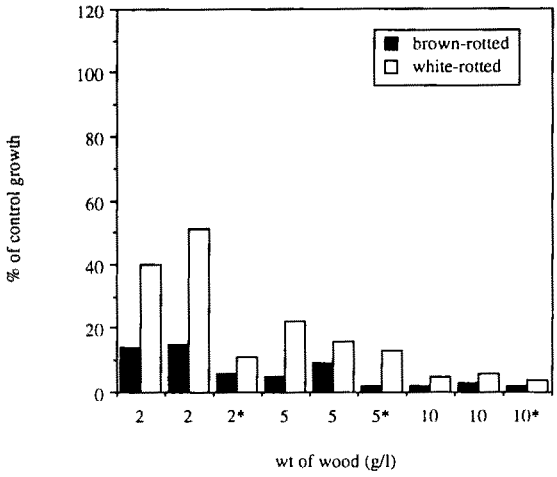

FIG. 1. Effect of brown- and white-rotted sycamore wood on growth of *Chlorella* in vitro. Algal growth quantified by cell counts. The asterisk denotes that the wood was autoclaved in the algal medium prior to the bioassay, otherwise wood was tested without autoclaving. All treatments ($N = 2$) significantly (t test, $P < 0.001$) inhibitory compared to controls ($N = 2$).

< 0.001) inhibitory to the growth of *Chlorella*, although it can be seen from Figure 1 that BRW was consistently more inhibitory than WRW. The results shown in Figure 1 represent the data from duplicate experiments in which wood was included in the algal bioassay at a dose equivalent to 2, 5, and 10 g/liter (g/dm^3) respectively. In a third experiment (denoted by an asterisk in Figure 1), similar dose rates of wood were used, but the wood was autoclaved in the algal bioassay medium prior to the experiment. Autoclaving increased the antialgal activity of both types of wood, discernible only at the lower dose rates. Microscopic examination of the WRW (sycamore) indicated that the sample was not homogeneously rotted.

When compared to the data for sycamore, a greater antialgal effect of brown- and white-rotted elm was recorded and thus the elm samples were selected for chemical analysis. The latter sample was well rotted and homogeneous on microscopic examination. WRW (elm) was not significantly inhibitory compared to control growth at the three dose rates tested (1, 2, and 4 g/liter whereas BRW (elm) was highly inhibitory to *Chlorella* (Figure 2). In all cases, the wood was autoclaved in the medium previously shown (Figure 1) to result in greater algal toxicity than with wood not exposed to the period of hot aqueous extraction afforded by autoclaving.

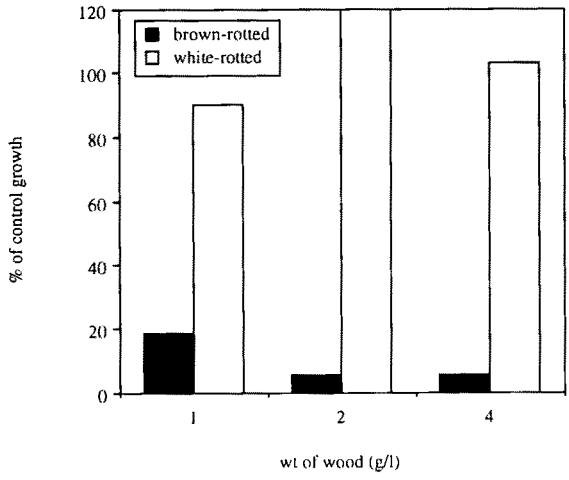

FIG. 2. Effect of brown- and white-rotted elm wood on growth of *Chlorella* in vitro. Algal growth quantified by chlorophyll *a* estimation.

A heartwood sample from the white-rotted elm trunk (Table 1) was quite brown in color, although the wood had the texture of WRW. The lack of antialgal activity of the elm heartwood sample was similar to that of the white-rotted fraction, namely 1 g/liter wood resulted in 140% (SE $\pm$14.7) of the control growth; 2 g/liter in 130% (12.6); and 4 g/liter in 98% (6.0). On autoclaving the elm heartwood sample in algal medium prior to the bioassay, a brown coloration was imparted to the medium as occurred also with BRW. The resulting inactivity of the white-rotted, but brown-colored, elm wood towards *Chlorella* indicated that reduced light penetration was not responsible for the algal toxicity observed with BRW.

Two further white-rotted wood samples, birch and hazel, were not inhibitory to *Chlorella* (Table 2). However, both samples were inhibitory to the growth of the cyanobacterium *Microcystis* (Table 2). The data in Table 2 also show the results of the bioassay of WRW (elm) against *Microcystis*; it can be seen that this white-rotted sample, which was not toxic towards *Chlorella* (Figure 2), significantly inhibited the growth of *Micocystis* at the same three dose rates.

Pyrolysis GC-MS of Wood. Quantitative and qualitative differences in the lignin of brown- and white-rotted woods were determined using pyrolysis GC-MS. Figure 3 shows the total ion current (TIC) chromatogram, or pyrogram, generated by the mass spectrometer in response to the intensity of ions of the

TABLE 1. PROVENANCE AND DESCRIPTION OF WOOD SAMPLES[a]

Wood sample	Location, UK	Type of rot
Sycamore, *Acer pseudoplatamus* L.	old woodland, Lancashire	brown
	old woodland, Lancashire	white (partial)
Elm, *Ulmus minor* Miller	old woodland, Cambridgeshire	brown
	old farmland hedge, Cambridgeshire	white (well-rotted)
	old farmland hedge, Cambridgeshire	heartwood (brown) from white (well-rotted) log
Birch, *Betula* sp.	parkland, Berkshire	white (well-rotted), *Coriolus* (*Trametes*) *versicolor* fruiting on surface
Hazel, *Corylus avellana* L.	twig from parkland, Berkshire	white (well-rotted) under bark, *Merulius corium* fruiting on surface

[a]All samples were air dried, homogenized in a laboratory blender, and sieved to <2 mm. Samples for pyrolysis GC-MS were further sieved to <125 μm and stored in glass vials in a desiccator.

TABLE 2. EFFECT OF WHITE-ROTTED WOOD ON GROWTH OF *Chlorella* AND *Microcystis*

Wood	Weight of wood (g/liter) in algal medium	% of control growth (±SE)[a]			
		Chlorella		**Microcystis**	
Birch	1	102	(8.2)	14	(3.7)
	2	109	(3.9)	8	(1.5)
	4	101	(9.5)	8	(2.4)
Hazel	1	174	(12.3)	9	(3.2)
	2	nd		5	(1.4)
	4	nd		1	(0.7)
Elm	1	90[b]	(3.4)	60	(4.8)
	2	>120[b]	(5.8)	10	(3.8)
	4	103[b]	(6.6)	2	(0.2)
Elm (brown-colored heartwood of sample above)	1	140	(14.7)	nd[c]	
	2	130	(12.6)	nd	
	4	98	(6.0)	nd	

[a]Algal growth quantified by chlorophyll *a* estimation. *Chlorella*, no significant inhibition ($P > 0.05$); *Microcystis*, all data are significantly different from controls ($P < 0.001$).
[b]Data shown in Figure 2.
[c]nd = not determined.

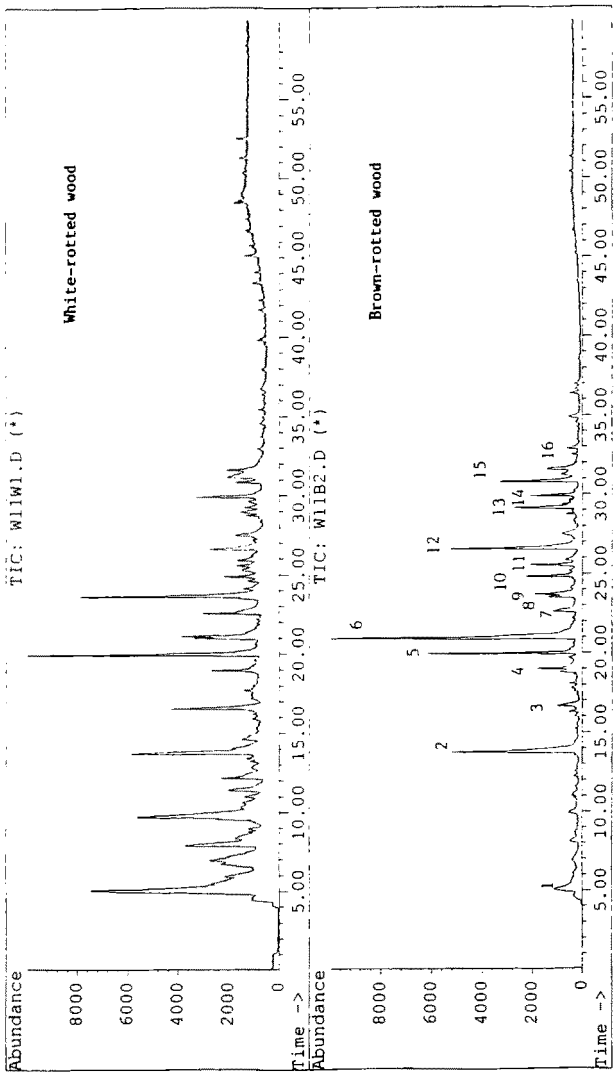

FIG. 3. Comparison of pyrograms of brown-rotted and white-rotted elm wood. Weight of WRW pyrolyzed = 1.52 mg, BRW pyrolyzed = 1.20 mg. Peaks 1–16 on the BRW trace refer to Figure 4 for quantitative comparison and Table 3 for identification.

various pyrolysis subcomponents of the white-rotted and brown-rotted elm samples. The trace for the WRW (elm) shows pyrolytically produced subcomponents of short retention time, which, together with their mass spectra, indicates they were derived from polysaccharide (Boon, 1989; Kivaisi et al., 1990). Such peaks were typically lacking from the BRW (elm) pyrogram. In BRW (elm) and WRW (elm), the area of the peak due to the simplest monomethoxy fragment, methoxy phenol or guaiacol, was remarkably similar (peak number 2 on Figure 3). Thus the integrated areas of other peaks for which spectra were obtained were normalized to that of methoxy phenol.

Figure 4 graphically shows the normalized distribution of the 16 major peaks in the BRW (elm) sample compared to the normalized values of the peaks of the same (as determined by their mass spectra) fragments where present in WRW (elm). The tentative identification of the peaks is given in Table 3. The convention of describing lignin subcomponents as either G (guaiacyl unit, with one methoxy group) or S (syringyl unit, dimethoxylated) has been adopted.

It can be seen from Figure 4 that peak 6 (2,6-dimethoxyphenol) was some six times more abundant in BRW than WRW. A greater abundance of dimethoxy to monomethoxy substitution was reflected in the overall pattern of the BRW compared to WRW. The ratio of S/G lignin subcomponents in BRW (elm) was

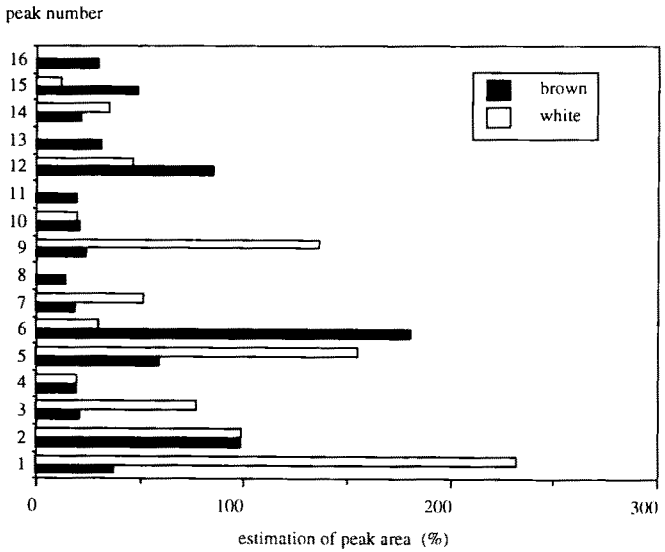

FIG. 4. Comparison of peak areas obtained by pyrolysis GC-MS of brown-rotted and white-rotted elm wood. Computer-integrated peak areas are normalized to the area of peak number 2, identified as methoxyphenol, arbitrarily set at 100%. Peaks 1–16 refer to the pyrograms shown in Figure 3 and identification is shown in Table 3.

TABLE 3. IDENTIFICATION OF PEAKS IN PYROGRAM OF BRW ELM

Peak number	Retention time (min)	M+[a]	Base peak[b]	Other peak	G or S[c]	Identification or partial identification[d]
1	5.08	60				unidentified
2	13.76	124	109		G	methoxyphenol
3	16.67	138	123	95	G	2-methoxy-4-methyl phenol
4	19.02	152	137		G	G + 2 m or G + e
5	20.00	150	150	135	G	G + v
6	20.85	154	154	139	S	2,6-dimethoxyphenol
7	22.63	152	152	151	G	G + 2 m or G + e
8	23.52	168	168	153	S	S + m[e] or 3 OMe
9	23.70	164	164	149	G	2-methoxy-4-(1-propenyl) phenol
10	24.83	166	166	151	G	G + (2 m + e) or G + 3 m
11	25.57	182	182	167	S	4-hydroxy-3,5-dimethoxy benzaldehyde
12	26.60	180	180	165	S	S + v
13	29.18	182	182		S	4-hydroxy-3,5-dimethoxy benzaldehyde
14	29.92	194	194		S	2,6-dimethoxy-4-(2-propenyl) phenol
15	30.83	196	181		S	1-(4-hydroxy-3,5-dimethoxyphenyl) ethanone
16	31.62	212	167	181	S	unidentified

[a]M+ = molecular ion, equivalent to molecular weight
[b]Base peak = the peak of greatest intensity in the mass spectrum, other significant peaks aid interpretation
[c]G, guaiacyl; S, syringyl.
[d]m = methyl (adds 14 mass units to G or S), e = ethyl (adds 28 mass units to G or S), h = hydroxyl (adds 16 mass units to G or S), v = vinyl (adds 26 mass units to G or S), p = propenyl (adds 40 mass units to G or S).
[e]Computer matched as 1,2,3-trimethoxy benzene (93% certainty).

calculated as 1.8, whereas in the WRW (elm) it was 0.3. Both hazel and birch WRW samples had S/G ratios of less than 0.5. The heartwood sample of white-rotted elm, which was ineffective against *Chlorella*, had a pyrogram typical of the WRW (elm). The S/G ratio of the elm heartwood sample was calculated to be 0.1, consistent with depletion of syringyl lignin following white-rotting, irrespective of the brown coloration typical of BRW.

DISCUSSION

Brown-rotted wood, elm or sycamore, was consistently more inhibitory to the growth of *Chlorella* than was white-rotted wood. The partial inhibitory

effects of sycamore WRW may be explained by the poor degree of homogeneity of the white-rotting as noted on microscopic examination. Extensively white-rotted wood (three species) did not prevent the growth of *Chlorella*. Based solely on the response of *Chlorella*, it is tempting to conclude that the lignin enrichment of BRW is responsible for antialgal activity. These data support our hypothesis that, of the lignocellulose content of straw, it is indeed the lignin portion that has antialgal potential.

White-rotted wood (three species), however, significantly prevented the growth of *Microcystis*. Cyanobacteria are reportedly more sensitive than green algae to barley straw (Newman and Barrett, 1993), although the possibility that the prokaryote and eukaryote are adversely affected by different factors cannot be ignored.

Pyrolysis GC-MS has allowed us to compare the lignin from a range of rotted wood samples. In general, components with short retention times and mass spectral identification indicating a polysaccharide origin were recorded only in the WRW samples, consistent with the typical action of white-rot fungi, which deplete lignin while preserving holocellulose. Lignin-derived subcomponents were observed in both BRW and WRW; the latter results are in contradiction to those reported by Mulder et al. (1991). A measure of the extent of white-rotting is obviously critical for the interpretation of analytical data relating to the persistence of ligninaceous material.

Analysis by pyrolysis GC-MS does not allow us to quantify the total phenolic macromolecular material remaining in the two types of rotted wood, so we cannot discount the possibility that antialgal activity is dependent only on the amount of total lignin, the more resistant *Chlorella* being unaffected by lower levels of lignin in WRW. However, it is also possible that the structural difference in lignin as identified by Py GC-MS is a significant factor in determining antialgal activity. The pyrograms of the elm wood, rotted in the two distinct fashions, allows a comparison of the nature of the lignin in each. BRW [regarded as closely repesentative of the native (unrotted) wood lignin] shows a high proportion of syringyl to guaiacyl lignin subcomponents, presumably reflecting the distribution of S to G lignin prior to pyrolysis. The action of brown-rot fungi in modifying the native lignin (Mulder et al., 1991; Kirk and Alder, 1970) may account partially for an increase in S units reflected in the S/G ratio of BRW. The pyrogram of WRW, on the other hand, indicates the presence of G units of lignin. Overall the S/G ratio of the two elm samples, supported by the data from other WRW samples, reflects the structurally different lignin resulting from brown- or white-rot processes.

The preferential utilization of syringyl lignin during white-rotting, which is limited to specialized fungi, is well documented (Dare et al., 1988). Niemann et al. (1991), however, have suggested that overall reduction in S lignin in some cases may result from the fungal preference for secondary cell walls, which are

higher in S lignin than primary cell walls, rather than specific action on S lignin. Likewise chemical extraction is selective; Scheijen and Boon (1991) cautioned that the Bjorkman method preferentially extracts secondary cell walls high in S lignin units. Such reports underline the value of the in toto analysis afforded by Py-GC-MS.

Under alkaline and oxygenated conditions, precisely those that exist in both field sites and laboratory bioassays where the antialgal effect of barley straw has been demonstrated, phenolic groups would readily autoxidize to quinones that are antialgal (Pillinger et al., 1994). We have proposed (Pillinger et al., 1994) that the process of oxidation of phenolic hydroxy groups increases the antialgal activity of phenolic or polyphenolic substrates. Detailed studies of chemical processes associated with wood pulping have shown that syringyl lignin components have a greater tendency to undergo abiotic oxidation than do guaiacyl units (Kempf and Dence, 1975). Furthermore, the toxicity of a range of phenylpropanoid compounds towards a green alga, *Selenastrum capricornutum*, increased with increasing methoxy substitution, and the position of the methoxy groups also influenced sensitivity (Della Greca et al., 1992). The digestibility of forage crops containing similar amounts of lignin was found to be greater in those feeds with lower syringyl lignin (Jung and Himmelbach, 1989 and references therein), suggesting that microbial action is reduced by the dimethoxy substitution of the lignin. It is conceivable, therefore, that lignin with increased dimethoxy groups as compared to a high proportion of monomethoxy substitution could be a significant factor in the efficacy of lignin to reduce the growth rate of algae and cyanobacteria. The greater antialgal activity shown by brown-rotted wood over white-rotted wood may be accounted for, at least partially, not only by the total amount of lignin present, but also by the increased potential for oxidation offered by syringyl lignin compared to guaiacyl lignin. Softwood lignin is typically of the guaiacyl type, with negligible dimethoxy substitution (Sarkanen and Ludwig, 1971); preliminary studies in our laboratory suggest that brown-rotted conifer wood is indeed less toxic towards *Chlorella* than that of hardwoods.

CONCLUSIONS

Brown-rotted wood inhibits the growth of two freshwater microorganisms, a green alga, *Chlorella*, and a cyanobacterium, *Microcystis*. White-rotted wood, however, was not inhibitory to the green alga but significantly inhibited growth of the cyanobacterium. Lignin was still present in the white-rotted samples, detected by on-line pyrolysis followed by combined gas chromatography–mass spectrometry. Quantification of data from pyrolysis studies must be treated with caution, but nevertheless the method provided a valuable comparison of the type

of lignin in similar samples. The lignin remaining in the white-rotted samples had a lower proportion of syringyl (dimethoxy substituted) to guaiacyl lignin than in the brown-rotted wood. Apparently, lignin rich in syringyl units shows greater antialgal activity, possibly as a result of the greater case of phenolic oxidation afforded by the presence of dimethoxy groups compared to monomethoxy ones.

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MECHANISM UNDERLYING CUTICULAR HYDROCARBON HOMOGENEITY IN THE ANT *Camponotus vagus* (SCOP.) (HYMENOPTERA: FORMICIDAE): ROLE OF POSTPHARYNGEAL GLANDS

MOHAMED MESKALI,* ANNIE BONAVITA-COUGOURDAN, ERIC PROVOST, ANNE-GENEVIEVE BAGNÈRES, GEORGES DUSTICIER, and JEAN-LUC CLÉMENT

Lab. Neurobiologie UPR 9024, CNRS
Université de Provence
31, CH. J. Aiguier, 13402 Marseille, France

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Abstract—The aim of the present study was to investigate the regulation and the homogeneity of the chemical signature between members of *Camponotus vagus* after experimentally changing the cuticular chemical signature by topically applying hydrocarbons. Topical application of pentane (solvent) to the cuticle of isolated workers led to a significant decrease in the quantities of the cuticular hydrocarbons measured within 3 hr, followed by an increase within the following 3 hr and a period of relative stability from 9 hr to 14 days. On the other hand, after topical application to isolated workers of *n*-tetracosane, a hydrocarbon existing only in trace quantity in this species, the quantity of this hydrocarbon measured over time in the epicuticular wax tended to level out at about 14 days after treatment. In contrast, topically applied (Z)-9-tricosene, an unsaturated hydrocarbon not normally synthesized by this species, decreased dramatically within a few hours and had completely disappeared within 14 days. (Z)-9-Tricosene applied to one member of a group was present in the postpharyngeal glands of the other members from 30 min to seven days of cohabitation. The highest levels were recorded in all six workers in each group after one day. GC-MS analyses showed that (Z)-9-tricosene was present in the cuticles of some untreated workers only after four and seven days of cohabitation with a treated worker. These data suggest: (1) that the deposited (Z)-9-tricosene decreased very quickly on the cuticle of the treated worker, although the total amount was spread over the cuticle and postpharyngeal gland and (2) that it was absorbed by the nontreated workers via the postpharyngeal glands during licking or grooming activities and reincorporated into the cuticle at four and seven days. When the treated worker

*To whom correspondence should be addressed.

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was separated from the other ants by a wire mesh, (Z)-9-tricosene was detected neither in the cuticle nor in the postpharyngeal gland of nontreated workers.

Key Words—Cuticular hydrocarbons, chemical signature, postpharyngeal glands, nestmate recognition, Hymenoptera, ant, *Camponotus vagus*.

INTRODUCTION

It was suggested long ago by Fielde (1904) that a colony odor might exist among ants. Hölldobler and Michener (1980) more recently suggested that individuals might produce signals or discriminators that are learned by the members of a colony and that subserve the recognition and discrimination processes at work in the colony. Several studies subsequently focused on the nature and the source of these colony signatures. It has been established that qualitative and quantitative differences in the cuticular hydrocarbon mixtures are used for interspecies recognition purposes by the termites *Reticulitermes* (Howard et al., 1982b; Bagnères et al., 1988, 1991a) and *Zootermopsis* (Haverty et al., 1988). The cuticular hydrocarbons play a role in recognition among colony members in the ants *Camponotus vagus* (Bonavita-Cougourdan and Clément, 1986; Bonavita-Cougourdan et al., 1987; Clément et al., 1987), *C. floridanus* (Morel and Vander Meer, 1988), and *Cataglyphis cursor* (Nowbahari et al., 1990) and possibly also in social bees (Page et al., 1991; Getz and Page, 1991; Breed and Stiller, 1992) and social wasps (Espelie et al., 1990; Bonavita-Cougourdan et al., 1991).

Colony odor may be important to the mechanisms whereby termitophiles and myrmecophiles are integrated into colonies of termites and ants, respectively. Howard et al. (1980, 1982a) established that the termitophiles, *Tricopsenius frosti*, *T. depressus*, and *Philotermes howardi*, chemically mimic the cuticular profiles of their respective termite hosts (*Reticulitermes flavipes* in the case of *Tricopsenius frosti* and *R. virginicus* in that of the other two termitophiles). A similar mimetic chemical process occurs in the parasitic dipteran *Microdon piperi*, which facilitates its integration into colonies of *Camponotus modoc* ants (Howard et al., 1990a). Although *de novo* biosynthesis of hydrocarbons occurs among *Microdon albicomatus* parasites living in *Myrmica incompleta* ant colonies (Howard et al., 1990b), Vander Meer and Wojcik (1982) reported that the myrmecophile beetle *Martinezia duterteri* (=*Myrmecaphodius excavaticollis*) initially has its own cuticular profile, but acquires the cuticular hydrocarbons characteristic of the host ant species, *Solenopsis richteri* or *Solenopsis invicta*. Vander Meer et al. (1989a) observed that the larvae of the eucharitid parasitoid, *Orasema* spp., which becomes closely integrated into colonies of the fire ant, *solenopsis* spp., has a cuticular hydrocarbon signature that is qualitatively identical and quantitatively very similar to that of its host.

A hybrid cuticular signature was developed by workers of the species *Man-*

ica rubida and *Formica selysi* that were placed together at emergence and formed a heterospecific colony (Errard and Jallon, 1987; Errard et al., 1989; Errard and Jaisson, 1991; Bagnères et al., 1991b). The hybrid signature differed both qualitatively and quantitatively from the original species' profiles. *Camponotus vagus* ant larvae (Bonavita-Cougourdan et al., 1989) that were adopted and tended by workers in an unknown colony showed changes in proportions of cuticular hydrocarbons as the result of the change of social environment.

Three hypotheses have been put forward to account for the mechanisms whereby hydrocarbon components may be acquired and/or chemical signatures modified in this way (Bonavita-Cougourdan et al., 1989); (1) larvae may be able to synthesize hydrocarbons of their brood-tenders that they have perceived via the olfactory pathway, (2) larvae may synthesize hydrocarbons constituting the colony signature from precursors provided by workers in variable quantities from one colony to another, or (3) hydrocarbons synthesized by brood-tenders may be transmitted orally to a larva, and these may be transferred to the latter's cuticle.

Some investigations on ants have shown that the postpharyngeal glands are involved in the absorption of ingested alimentary lipids (Peregrine et al., 1972, 1973; Peregrine and Mudd, 1974; Delage-Darchen, 1976). Others have established that the main components of the postpharyngeal glands are hydrocarbons (for example: Thompson et al., 1981; Attygalle et al., 1985). Hydrocarbons present on the cuticle and in the gland are qualitatively identical and quantitatively similar, at least in the case of some species (Bonavita-Cougourdan et al., 1987; Clément et al., 1987; Bagnères and Morgan, 1991). Hefetz et al. (1992) reported that the postpharyngeal glands of *Manica rubida* living in mixed associations with *Formica selysi* contained some of the latter's specific cuticular hydrocarbons in addition to their own.

The present paper reports studies of chemical interactions occurring in a social group and analyzes the mechanisms possibly regulating the homogeneity of chemical signals. First, we reinvestigated the nature of the cuticular hydrocarbons carried by the ant *Camponotus vagus* (Scop.). Secondly, we studied the variations with time of (*Z*)-9-tricosene, an exogenously added hydrocarbon not normally synthesized by this species, and an endogenous hydrocarbon, *n*-tetracosane, existing only in trace amounts in this species. The third aspect studied was the chemical composition of the cuticles and postpharyngeal glands to determine whether transfer of (*Z*)-9-tricosene occurred over time within small groups containing one worker topically treated with (*Z*)-9-tricosene and five untreated workers. We chose (*Z*)-9-tricosene because it is an unsaturated hydrocarbon known to exist in other insects, but not in the ants with which we worked. It was described as the sex pheromone of the common housefly (Carlson et al., 1971) and as one of the cuticular hydrocarbons carried by termites of the genus *Reticulitermes* and by ants of the genus *Formica* (Bagnères, 1989).

METHODS AND MATERIALS

Insects. We used colonies of the carpenter ant *Camponotus vagus* (Scop.), a monogynous species living in stumps and fallen trees. All the colonies were collected in the same biotope, at Nans les Pins, 45 km from Marseille, France. At the laboratory, the colonies were reared in Plexiglas boxes connected to two well-aired compartments, where food consisting of honey and crickets was provided. The ants were reared and maintained with a 12L:12D photoperiod at approximately 22°C and 70% relative humidity.

Experiment 1 (Control). First we studied the ants' cuticular hydrocarbons and their variations with time in response to topical application of solvent (pentane). Nine sets of five workers each were used for this purpose. With a 1-μl Hamilton syringe, 1 μl of pentane was deposited on the thorax of these separately housed sets of workers. Cuticular extracts of the five workers in each set were analyzed with gas chromatographic technique (GC) at 0, 1, 3, 6, 9, 24, 48, 168, and 336 hr after application of pentane. We recorded as a function of time the amounts (in nanograms) of the main hydrocarbon families present (*n*-alkanes, monomethylalkanes, and dimethylalkanes) in the workers' cuticular profiles.

Experiment 2. We studied as a function of time the variations in added quantities of an endogenous cuticle component, *n*-tetracosane (*n*-C_{24}), and an exogenous compound, (*Z*)-9-tricosene ($C_{23:1}$), applied to the thorax of workers. One microliter (0.5 μg/μl) of a solution of (*Z*)-9-tricosene was applied to the cuticle of a single individual in each set of workers. The cuticular extract of each individual was then analyzed by GC at 0 ($N = 2$), 1 ($N = 10$), 3 ($N = 11$), 6 ($N = 10$), 9 ($N = 9$), 24 ($N = 10$), 48 ($N = 9$), 168 ($N = 11$), and 336 ($N = 10$) hr after application of (*Z*)-9-tricosene.

In a separate series, 1 μl of *n*-tetracosane solution (0.8 μg/μl in pentane) was applied to the cuticle of a single individual in each set of workers ($N = 5$ individuals per set), and the cuticular extract of each individual was then analyzed by GC at the same time intervals as in the (*Z*)-9-tricosene experiment above. The solutions of (*Z*)-9-tricosene were prepared from a 97% solution of (*Z*)-9-tricosene (Aldrich Inc., St Quentin Fallavier, France) and pentane (Atrasol 99%), and the *n*-tetracosane solution was prepared from a pure *n*-tetracosane solution (PolyScience Corp., Niles, USA).

Experiment 3. One worker was topically treated with 1 μl of the solution of (*Z*)-9-tricosene (1 μg/μl in pentane), marked with a metal belt (diameter 0.06 mm) placed around its petiole (Provost, 1983), immediately isolated for 1 hr, and then placed with five nontreated workers in a transparent plastic box (12 × 8 × 2 cm). The cuticular profile of each of the six individuals in the group was analyzed after 0, 0.5, 1.5, 9, 24, 48, 96, 168, 264, and 336 hr of cohabitation. In this experiment, we also analyzed by GC-MS a blend of eight cuticular extracts from the eight untreated individuals taken at a cohabitation time of 96 hr.

Experiment 4. The purpose of this experimental series was to evaluate the amounts of (Z)-9-tricosene present over time in the postpharyngeal glands of a worker topically treated with (Z)-9-tricosene, and in those of the five nontreated workers with which it cohabited. The same experimental procedure was used as in experiment 3, except that the postpharyngeal glands were dissected from each worker in distilled water, extracted with pentane, and then analyzed by GC. The same periods of cohabitation were used as above with five groups for each time period with the exceptions of six groups for 9 hr, nine groups for 48 hr, and four groups for 264 hr and 336 hr. In experiment 4, we analyzed by GC-MS a blend of two cuticular extracts from two treated individuals and blends of postpharyngeal gland extracts from treated and untreated individuals at each cohabitation time, except at 48, 264, and 336 hr.

Experiment 5. A worker treated with 1 $\mu g/\mu l$ of (Z)-9-tricosene as above was separated by a double layer of wire mesh for 24 hr from five nontreated workers. This experiment was repeated five times with different workers. The postpharyngeal glands were then dissected from all individuals, each extracted separately (pentane), and analyzed by GC.

Chemical Analysis. Prior to dissection and/or extraction, ants were killed by freezing. Either the dissected postpharyngeal glands or whole ants were immersed for 5 min in 1 ml pentane in a Weaton tube. An internal standard of 200 ng *n*-docosane was then added. Each extract was completely evaporated under a stream of nitrogen, and 40 μl of pentane added to extracts of postpharyngeal glands and 30 μl to cuticular extracts. Samples of 2 μl were analyzed by GC on a Delsi 200 DN GC coupled to an Enica 31 integrator and equipped with a flame ionization detector (FID) and a Chrompack CPSil5 WCOT column (25 m $\times$ 0.25 mm). The injector was of the split-splitless type, and the injection was performed within 15 sec in the splitless mode. The temperature program was from 180°C to 320°C at 5°C/min. The carrier gas was helium at a pressure of 1 bar.

A silver nitrate silica microcolumn (20% silver nitrate on silica, 6 cm $\times$ 6 mm) was used to separate (Nelson et al., 1981) (Z)-9-tricosene from the cuticular hydrocarbons synthesized by *Camponotus vagus* after a period of 168 hr. Saturated hydrocarbons were eluted from the column with 30 ml of hexane, and then 10 ml (in 2-ml portions) of ether–hexane (1 : 20) was added to elute (Z)-9-tricosene. The eluates obtained were analyzed by GC to determine the purity and the efficiency of the separation technique. In our experiments, 10 ml of ether–hexane sufficed to collect (Z)-9-tricosene, and no further (Z)-9-tricosene was detected by GC after further washing of the microcolumn.

The gas chromatography–mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 5890 GC coupled to a 5989A MS. The whole system was controlled by a HPUX MS Chemstation. The analyses were first performed in an electron impact mode (EI; 70 eV) over the mass range *m/z* 30 to *m/z* 600 amu (atomic mass units), with a scan time of 1 sec. Positive chemical

ionization (CI; 150 eV/CH_4^+) was then used over the mass range *m/z* 100 to *m/z* 600 amu at a rate of approximately 1 scan/sec. The GC contained an HP5 column (30 m × 0.25 mm). The injection was made in the splitless mode (1 min). Helium was used as the carrier gas at a pressure of 2 bars. During chemical ionization, the methane was at a pressure of 1.5 bars. For hydrocarbon identification, the GC oven was programmed from 70°C to 180°C at 30°C/min and then to 320°C at 3°C/min with a 5-min isotherm at 320°C. For (*Z*)-9-tricosene determinations, the oven was programmed from 70°C to 200°C at 30°C/min and then to 240°C at 5°C/min, and again to 320° at 10°C/min with a 10-min isotherm at 320°C.

Confirmation of Typical Hydrocarbon Pattern of C. vagus. Some of the cuticular hydrocarbons of *C. vagus* have been previously identified by GC-MS (Bonavita-Cougourdan et al., 1987). For confirmation and reference, we prepared a cuticular extract by immersing 80 workers in pentane for 5 min. The apolar fraction was separated from the polar fraction with a silica microcolumn (6 cm × 6 mm), and the apolar fraction was analyzed by GC-MS. This enabled us to confirm that no (*Z*)-9-tricosene was present among the endogenous substances, to check which substances (such as *n*-tetracosane) were present only in trace amounts, and to look more closely at some of the cuticular hydrocarbons previously found to be present, but whose identity was doubtful. Our identifications were compared with the previously published results (Bonavita-Cougourdan et al., 1987; Lange et al., 1989; Bagnères and Morgan, 1991).

Detector response to the hydrocarbon series was corrected with a response coefficient defined for the flame ionization detector used (Bagnères, 1989). A coefficient of 0.9 was used for (*Z*)-9-tricosene.

Statistical comparisons were made by using the Mann-Whitney two-tailed *U* test and the Jonckeere test (Jonckeere, 1954).

RESULTS

GC-MS Identification of Cuticular Hydrocarbons

Chemical analyses based on the GC-MS data confirmed that the extracts from *C. vagus* (Table 1 and Figure 1) mainly consisted of saturated hydrocarbons with 21–39 carbon atoms (59 peaks), but two unsaturated compounds represented by peaks 19 and 24 were found. The saturated hydrocarbons were *n*-alkanes, monomethylalkanes, dimethylalkanes, and trimethylalkanes. Some further hydrocarbons were identified in addition to those previously described (Bonavita-Cougourdan et al., 1987; Lange et al., 1989). These included monomethylalkanes, dimethylalkanes, and trimethylalkanes. The latter is a new hydrocarbon class that has not been detected previously in *C. vagus*. Hydrocarbons with 21–24 carbon atoms (peaks 0–5) were present only in trace amounts (Table 1) and were not included in Figure 1.

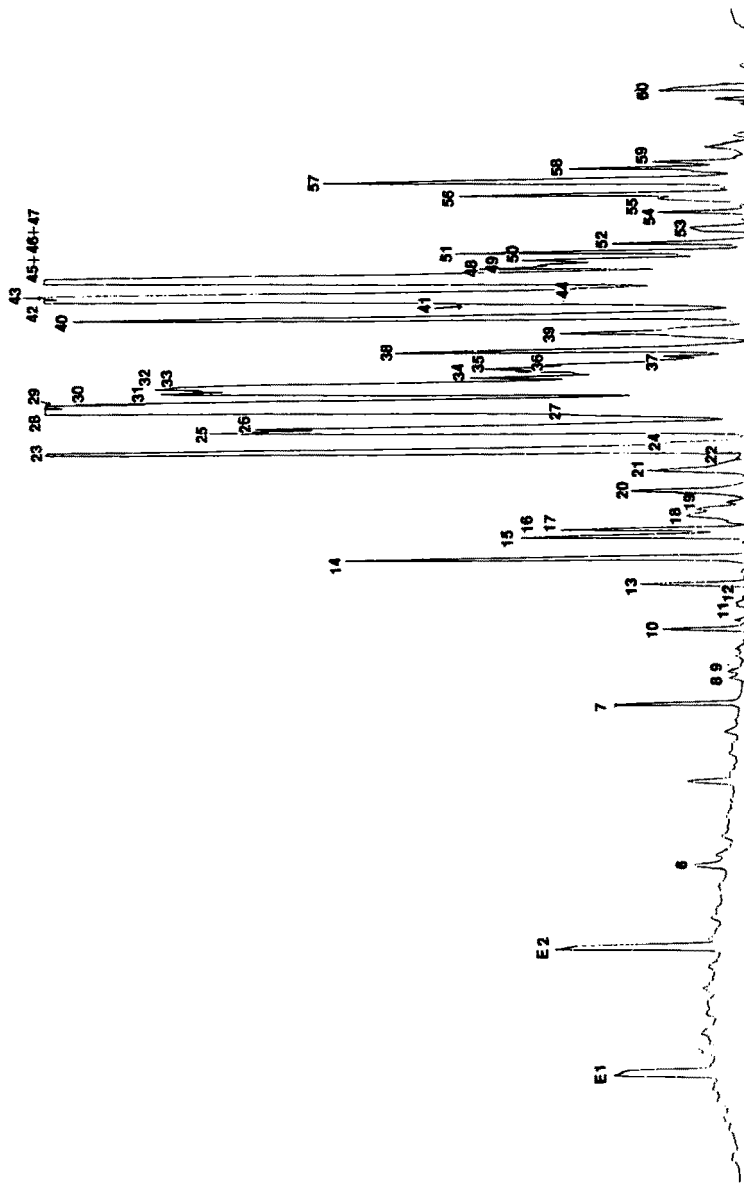


FIG. 1. Cuticular hydrocarbon profile of a worker ant of *C. vagus*. E1 (*n*-docosane) and E2 (*n*-tetracosane) were internal standards. Analyses were conducted on a fused silica capillary column with a temperature program from 180°C to 320°C programmed to rise at 5°/min. The carrier gas was helium and the injection mode was splitless (15 sec).

TABLE 1. DETERMINATION BY GC-MS OF CUTICULAR HYDROCARBONS OF *C. vagus*[a]

Peak	ECL	Hydrocarbon	CN	Diagnostic ions MS/EI	Cl(CH_4^+)
0	21.00	*n*-Heneciosane	21	296	295
1	22.00	*n*-Docosane	22	310	309
2	22.39	10-Methyldocosane	23	154/5,196/7,309	323
3	22.75	3-Methyldocosane	23	56/7,294/5,309	323
4	23.00	*n*-Tricosane	23	324	323
5	24.00	*n*-Tetracosane	24	338	337
6	25.00	*n*-Pentacosane	25	352	351
7	27.00	*n*-Heptacosane	27	380	379
8	27.33	11 − + 13-Methylheptacosane	28	168/9,196/7,224/5,252/3,379	393
9	27.42	7-Methylheptacosane	28	112/3,308/9,379	393
10	28.00	*n*-Octacosane	28	394	393
11	28.37	10 − + 11 − + 12-Methyloctacosane	29	154/5,280/1,168/9,266/7,210/1, 224/5,393	407
12	28.42	8-Methyloctacosane	29	126/7,308/9,393	407
13	28.64	4/2-Methyloctacosane	29	364/5,393	407
14	29.00	*n*-Nonacosane	29	408	407
15	29.33	11 − + 13 − + 15-Methylnonacosane	30	168/9,196/7,252/3,280/1,407	421
16	29.35	9-Methylnonacosane	30	140/1,308/9,407	421
17	29.43	7-Methylnonacosane	30	112/3,336/7,407	421
18	29.65	9,13-Dimethylnonacosane	31	140/1,210/1,252/3,322/3,421	435
19	29.70	*n*-Triacontene	30	420	419
20	30.00	*n*-Triacontane	30	422	421
21	30.30	11 − + 12 − + 13 − + 14 − + 15-Methyltriacontane	31	168/9,182/3,196/7,210/1,224/5, 238/9,252/3,266/7,280/1,294/5,421	435
22	30.43	7-Methyltriacontane	31	112/3,350/1,421	435
23	30.65	4/2-Methyltriacontane	31	392/3,421	435
24	30.81	Hentriacontene	31	434	433
25	30.93	Dimethyltriacontane	32	435	449

26	31.00	*n*-Hentriacontane	31	436	435
27	31.19	Trimethyltriacontane	33	449	463
28	31.29	15 − + 13 − + 11-Methylhentriacontane	32	168/9,196/7,224/5,252/3,280/1, 308/9,435	449
29	31.35	9-Methylhentriacontane	32	140/1,336/7,435	449
30	31.40	7-Methylhentriacontane	32	112/3,364/5,435	449
31	31.54	13,17 − + 13,19-Dimethylhentriacontane	33	196/7,224/5,238/9,252/3,266/7, 294/5,449	463
32	31.61	11,15 − + 11,17 − + 11,19 − + 11,21-Dimethylhentriacontane	33	168/9,196/7,224/5,238/9,252/3, 266/7,294/5,322/3,449	463
33	31.68	9,13 − + 9, 15 − + 9,17 − + 9,19 − + 9,21 − + 9,23-Dimethylhentriacontane	33	140/1,168/9,210/1,224/5,238/9, 252/3,266/7,280/1,294/5,322/3, 350/1,449	463
34	34.73	3-Methylhentriacontane + 7,11 − + 7, 13 − + 7,15 − + 7,17 − + 7,19 − + 7,21 − + 7,23 − + 7,25-Dimethylhentriacontane	32	56/7,420/1	449
			33	112/3,140/1,168/9,182/3,210/1, 224/5,238/9,252/3,266/7,280/1, 294/5,322/3,350/1,378/9,449	463
35	34.77	5,9 − + 5,11 − + 5,13 − 5,15 − + 5,17 − + 5,19 − + 5,21 − + 5,23 − + 5,25 − + 5,27-Dimethylhentriacontane	33	84/5,112/3,140/1,154/5,168/9, 182/3,210/1,224/5,238/9,252/3, 266/7,280/1,294/5,322/3,336/7, 350/1,378/9,406/7,449	463
36	32.00	*n*-Docotriacontane	32	450	449
37	32.10	Trimethylhentriacontane	34	478	477
38	32.33	15 − + 13 − + 11-Methyldocotriacontane	33	168/9,196/7,225/6,266/7,294/5, 322/3,449	463
39	32.65	4/2-Methyldocotriacontane (+ Dimethyldocotriacontane)	33	420/1,449	463
			34		477
40	33.00	*n*-Tritriacontane (+ Dimethyldocotriacontane)	33	464	463
			34		477
41	33.05	Trimethyldocotriacontane	35		491
42	33.25	15 − + 17-Methyltritriacontane	34	252/3,224/5,280/1,463	477
43	33.35	11 − + 13 − + 9-Methyltritriacontane	34	140/1,168/9,196/7,308/9,336/7, 366/7,463	477

TABLE 1. Continued

Peak	ECL	Hydrocarbon	CN	Diagnostic ions MS/EI	CI(CH_4^+)
44	33.40	7-Methyltritriacontane	34	112/3,392/3,463	477
45	33.60	13,17 − + 13,19 − + 13,21-Dimethyltritriacontane	35	196/7,224/5,252/3,266/7,294/5, 322/3,477	491
46	33.67	11,17 − + 11,19 − + 11,21-Dimethyltritriacontane	35	168/9,196/7,224/5,252/3,266/7, 294/5,322/3,350/1,477	491
47	33.70	9,17 + 9,19 − + 9,21 − + 9,23-Dimethyltritriacontane	35	140/1,168/9,196/7,224/5,252/3, 266/7,294/5,322/3,350/1,378/9,477	491
48	33.75	11,15,21-Trimethyltritriacontane	36	168/9,196/7,238/9,296/7,336/7, 364/5,491	505
49	33.80	Dimethyltritriacontane	35		491
50	33.86	9,13,23 − + 9,15,23-Trimethyltritriacontane	36	140/1,168/9,210/1,238/9,296/7, 322/3,364/5,392/3,491	505
51	33.96	7,11,21 − + 7,13,21 − + 7,15,21-Trimethyltritriacontane	36	112/3,182/3,196/7,210/11,238/9, 296/7,322/3,336/7,350/1,491	505
52	34.33	12 − + 14-Methyltetratriacontane	35	182/3,210/1,308/9,336/9,477	491
53	34.45	9,19-Dimethyltetratriacontane	36	196/7,238/9,294/5,336/7,491	505
54	34.91	Dimethyltetratriacontane	36		505
55	35.25	Trimethyltetratriacontane	37		519
56	35.33	9 − + 11 − + 13 − + 15 − + 17-Methylpentatriacontane	36	140/1,168/9,196/7,224/5,252/3, 280/1,308/9,336/7,364/5,392/3,491	505
57	35.62	11,21 − + 13,21 − + 15,19 − + 15,21-Dimethylpentatriacontane	37	168/9,196/7,224/5,252/3,296/7, 322/3,350/1,378/9,505	519
58	35.75	Dimethylpentatriacontane +	37		519
		Trimethylpentatriacontane	38		533
59	35.95	Trimethylpentatriacontane	38		533
60	37.60	Dimethylheptatriacontane	39		547

[a]CN represents the number of carbon atoms and ECL the equivalent chain length.

Variations in Cuticular Components as a Function of Time and Treatment

Experiment 1. To determine the effect of applying the solvent (pentane) alone to the workers' thorax on the cuticular hydrocarbon profiles, we quantitatively analyzed peaks 23, 26, 28, and 42, and the incompletely separated compounds represented by peaks 45–47 as a function of the time elapsed after the application of solvent. At t_{0+3} hr (Figure 2), the quantities decreased 3.1, 3.3, 3.8, 4.3, and 4.7 times for peaks 23, 26, 28, 42, and 45 + 46 + 47, respectively. Comparisons between the values recorded at t_{0+3} hr and those recorded at t_{0+1} hr and t_0 hr using Jonckeere's test (Jonckeere, 1954) showed that the quantities of all tested compounds decreased significantly ($P < 0.05$). The statistical test was carried out separately on each peak with the five values recorded at each of the times considered. At t_{0+6} hr, a significant increase (6.3, 5.8, 9.5, 7.1 and 8.6 times in the case of peaks 23, 26, 28, 42, and 45 + 46

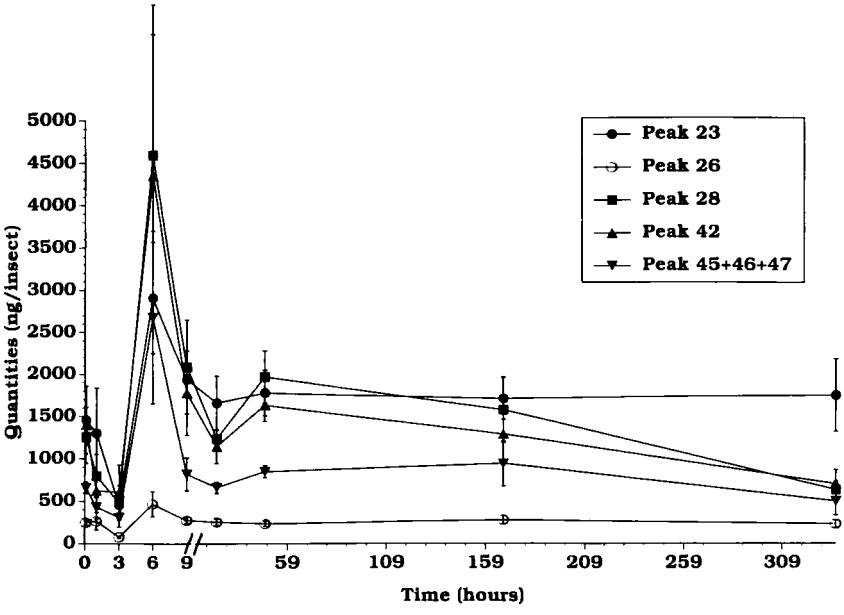


FIG. 2. GC analysis of cuticular hydrocarbons in workers topically treated ($N = 5$) with 1 μl of pentane. Points represent means and bars the standard error (SE). For each time period, five workers were analyzed. Peak 23 = 4/2-me-C_{30}; peak 26 = n-C_{31}; peak 28 = 15-me-C_{31} + 13-me-C_{31} + 11-me-C_{31}; peak 42 = 15-me-C_{33} + 17-me-C_{33}; and peaks 45 + 46 + 47 = 1 mixture of 13,17-dime-C_{33} + 13,19-dime-C_{33} + 13,21-dime-C_{33}, 11,17-dime-C_{33} + 11,19-dime-C_{33} + 11,21-dime-C_{33} and 9,17-dime-C_{33} + 9,19-dime-C_{33} + 9,21-dime-C_{33} + 9,23-dime-C_{33}.

+ 47, respectively) in the quantities of all five peaks occurred compared with the values at t_{0+3} hr ($P < 0.05$; Mann-Whitney two-tailed U test). The quantities recorded at t_{0+6} hr were conspicuously higher than at t_0. The differences were significant in the following cases: peak 23 (twofold increase); peak 28 (3.6-fold increase), and peak 45 + 46 + 47 (4.1-fold increase) ($P < 0.05$; Mann-Whitney two tailed U test). The quantities of the methylated products had increased at least two fold at t_{0+6} hr. From t_{0+9} hr onwards, values tended to return to the initial levels; they no longer differed significantly from values recorded at t_0.

The mean weight of the hydrocarbons present in the cuticular mixture at t_0 was 11.4 μg/individual; assuming that all the cuticular products behaved in the same way as the five peaks investigated here. Based on the same assumptions, the total hydrocarbons present at t_{0+3} hr were estimated to be 4.4 μg/individual (which is 2.6 times less than the level measured at t_0). At t_{0+6} hr after the solvent was applied, it was estimated that there would be 34 mg/individual, or more than three times the amount measured at t_0.

The data show that in response to the application of pentane alone, the quantity of hydrocarbons decreased about 2.6-fold during the first 3 hr after the application of pentane, then increased to about three times their initial level during the following 3 hr, and finally returned to normal at about t_{0+9} hr.

Experiment 2. The changes with time in the percentages (Figure 3) of *n*-tetracosane and (*Z*)-9-tricosene measured on the cuticles with respect to the

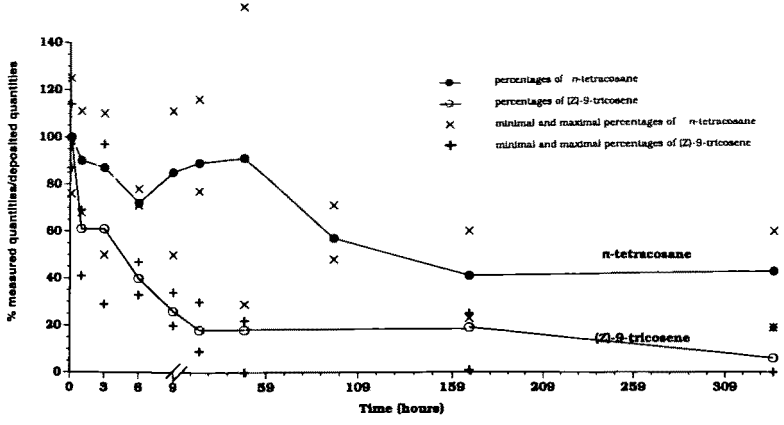


FIG. 3. Percentages of *n*-tetracosane and (*Z*)-9-tricosene measured in topically treated workers isolated at different time periods and cuticular extracts analysed by GC. The *n*-tetracosane was deposited at 800 ng in 1 μl of pentane and the (*Z*)-9-tricosene at 500 ng in 1 μl of pentane. The percentages were calculated from quantities initially deposited at t_0.

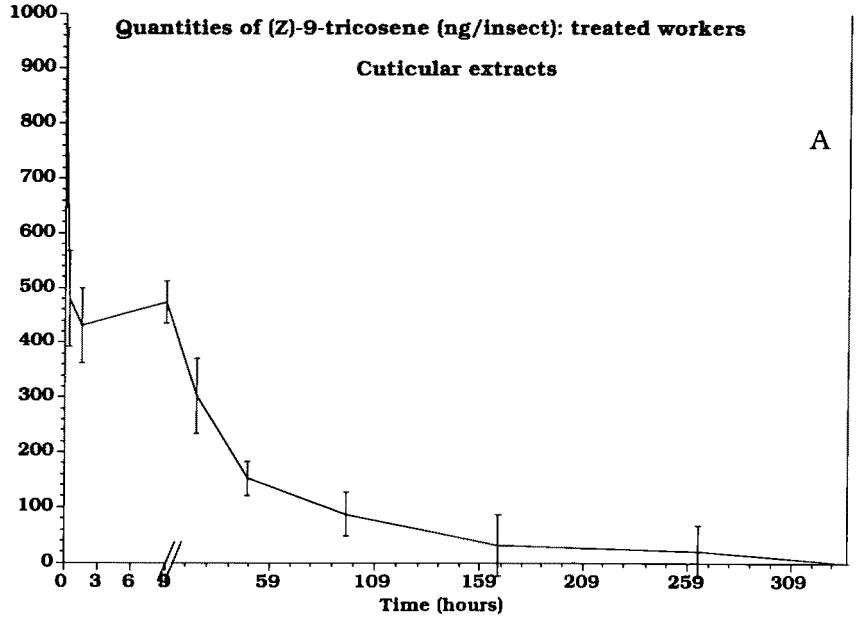
amounts initially deposited at t_0 showed the occurrence of a slower decrease of *n*-tetracosane (half of the amount deposited remained between t_{0+96} and t_{0+168} hr), and a faster decrease of (*Z*)-9-tricosene (half of the amount deposited remained between t_{0+3} and t_{0+6} hr). From t_{0+48} to t_{0+336} hr, the percentage levels of *n*-tetracosane stabilized, whereas those of (*Z*)-9-tricosene tended to decrease.

Changes with Time in Quantities of Added (Z)-9-*tricosene on Cuticles and in Postpharyngeal Glands of a Group Consisting of One Treated and Five Untreated Workers*

Experiment 3. The mean quantity of (*Z*)-9-tricosene applied in this test was 890 ng (SD = 89). The mean quantity of (*Z*)-9-tricosene measured on the cuticle at time t_0 (at the beginning of cohabitation, 1 hr after application) was 810 ng/insect (SD = 168) (Figure 4A). The quantity decreased to 474 ng/insect (SD = 40), about half the initial level, after cohabitation period of 9 hr, and then continued to decrease with time. At 336 hr, the amount of (*Z*)-9-tricosene remaining on the cuticle was practically zero.

(*Z*)-9-Tricosene was detected on the cuticles of the nontreated workers (Figure 4B) only after cohabitation periods of 96 and 168 hr. After 96 hr, the cuticular extracts of 32% of the nontreated workers (eight of the 25 nontreated workers in the five groups studied) contained on average 30 ng/insect (SD = 11); the mean level measured on all 25 nontreated workers was 10 ng (SD = 15). The total amount of (*Z*)-9-tricosene (243 ng) present in the nontreated workers was confirmed by the GC-MS data. After 168 hr of cohabitation, the cuticles of 24% of the nontreated workers (six of 25) contained on average 40 ng/insect (SD = 15), and the mean calculated for all 25 workers studied was 10 ng (SD = 18). At t_{0+168} hr, the total amount of (*Z*)-9-tricosene recorded, 241 ng, was confirmed by the data obtained using the silica–silver nitrate microcolumn followed by the GC and GC-MS. The (*Z*)-9-tricosene subsequently disappeared completely from the cuticles of nontreated workers (cohabitation times of 264 and 336 hr).

Experiment 4. The mean quantity of (*Z*)-9-tricosene deposited on the thorax of the treated workers was 867 ng (SD = 140). The results (Figure 4C) show that the mean quantities of (*Z*)-9-tricosene present in the postpharyngeal glands of some of the treated and nontreated workers amounted to 69 ng (SD = 66), i.e., to 9% of the total amount deposited; whereas in other cases mean values of 43 ng (SD = 28) equal to 5%, and 53 ng (SD = 36) equal to 7%, of that initially deposited were recorded after periods of cohabitation of 30 min (10 workers from five groups), 90 min (five workers from five groups) and 9 hr (10 workers from six groups). The highest levels recorded were those obtained on the whole set of treated and nontreated individuals belonging to the five groups under investigation at a cohabitation time of 24 hr. Low levels were observed



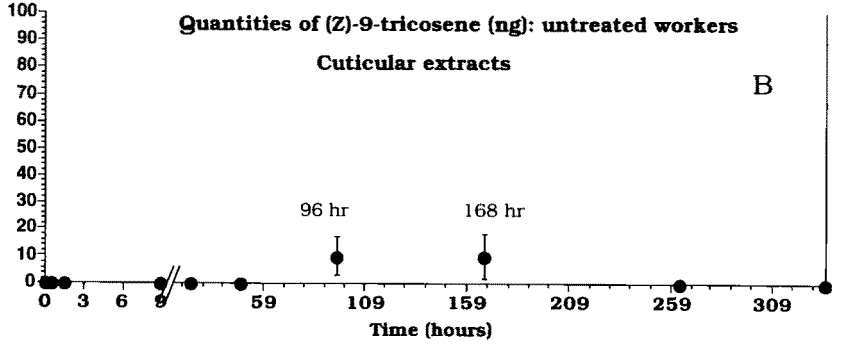


FIG. 4. Mean quantities of (Z)-9-tricosene measured with time in groups of workers. A worker was topically treated with 1 μl of the (Z)-9-tricosene solution at 1000 ng/μl pentane approximately, isolated 1 hr, and then placed with five untreated workers to form a group. Cuticular extracts were analyzed after various time periods, and (Z)-9-tricosene was quantified in treated workers (A) and in untreated workers (B). Extracts from postpharyngeal glands were analyzed from treated and untreated workers (C) for the same time intervals. Bars represent the 95% confidence intervals.

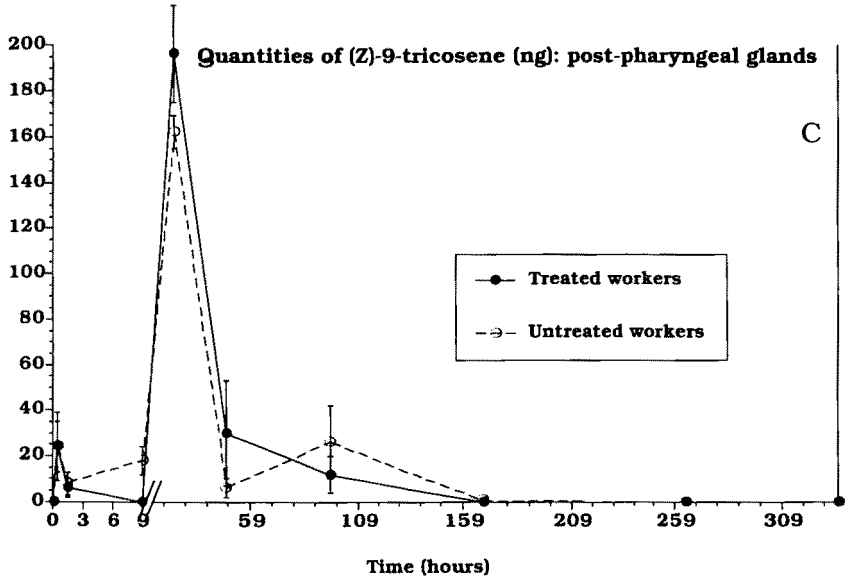


FIG. 4. Continued.

at 48 and 96 hr of cohabitation (five of 54 workers and 12 of 30 workers, respectively), and the levels tended towards zero at cohabitation periods of 168, 264, and 336 hr (Figure 4C). The presence of (Z)-9-tricosene was confirmed by the GC-MS data obtained on a mixture of two cuticular extracts from two individuals chosen at random among the set of treated workers.

In order to determine the combined amounts of (Z)-9-tricosene present over time in a small group of individuals, the mean totals present on the cuticles and in the postpharyngeal glands of treated and nontreated workers were measured (Figure 5). Three phases were distinguished in this way: first, it was observed that at cohabitation times of 30 and 90 min and 9 hr, the mean total quantities of (Z)-9-tricosene measured both on the cuticles and in the postpharyngeal glands of the workers were lower than those measured at t_0 (at the beginning of cohabitation) (Figure 5); at the same time, the quantities measured in the postpharyngeal glands were low and amounted to only 18%, 6% and 12%, respectively, of the levels measured at the beginning of cohabitation. An overall loss of (Z)-9-tricosene therefore occurred during cohabitation, amounting to the difference between the total amounts measured at both loci and those measured at the beginning of cohabitation. This loss amounted to around 23%, 41%, and 30% at the above respective cohabitation times.

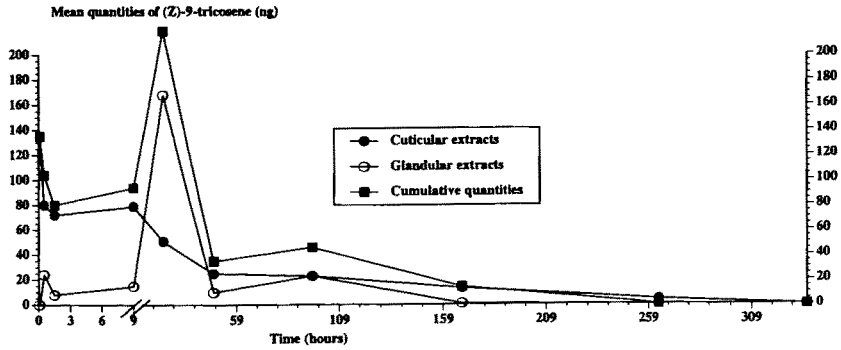


FIG. 5. Mean of the total quantity of (Z)-9-tricosene measured with time in cuticular extracts (experiment 3) and postpharyngeal glands extracts (experiment 4) in groups of workers. A worker was topically treated with 1 μl of the (Z)-9-tricosene solution at 1000 ng/μl pentane approximately, isolated 1 hr, and then placed with five untreated workers to form a group. Cuticular and glandular extracts were analyzed after several time periods, and the (Z)-9-tricosene was quantified in treated and untreated workers. The total quantity was calculated by group, and the mean calculated on all groups for each time period.

During the second phase, which corresponded to 24 hr of cohabitation (Figure 5), the mean total amount of (Z)-9-tricosene recorded per individual was similar to that observed at t_0. During the same period, the total amounts of (Z)-9-tricosene recorded reached a maximum in the postpharyngeal glands.

From cohabitation periods of 48–336 hr (third phase) (Figure 5), the mean combined quantities of (Z)-9-tricosene per individual decreased to zero, and the differences in comparison with the amounts initially deposited gradually increased.

Experiment 5. In the experiments where the treated worker was separated from the nontreated workers in the group by a double layer of wire mesh, the mean amount of (Z)-9-tricosene present in the cuticular extracts of the treated workers (in the case of the five groups studied for 24 hr) was 264 ng (SD = 82). (Z)-9-Tricosene was detected in the postpharyngeal glands of two of the five treated workers (10 ng and 71 ng). Among the nontreated workers, which were separated from the treated member of the group by the wire mesh, not even a trace of (Z)-9-tricosene was detected either in the cuticular extracts or in the extracts obtained from the postpharyngeal glands. When no physical contact was possible between the treated and nontreated individuals, (Z)-9-tricosene was therefore neither transferred nor synthesized.

DISCUSSION

The application of an apolar solvent (pentane) to the cuticle of a *Camponotus vagus* worker led to a significant decrease (2.6-fold) within 3 hr in the amounts of the cuticular products investigated. A threefold increase in the levels of these substances then occurred with respect to the initial levels (t_0) during the t_{0+3} to t_{0+6} hr period, followed by a return to the initial values and a period of relative stability that persisted for up to two weeks. The rebound and increase in cuticular hydrocarbons between 3 and 6 hr after treatment may be due to enhancement of biosynthesis. In ants, the site of hydrocarbon biosynthesis is probably located in the oenocytes, as hypothesized in the case of *Apis mellifera* (Piek, 1964) and other insects (Lockey, 1988).

The data showed that a very fast decrease occurred in applied (Z)-9-tricosene, which is not normally synthesized (according to the GC-MS identification of the extracts analyzed with a threshold of sensitivity of 1 pg/ant). The (Z)-9-tricosene curve was that of an exponential function in the case of single treated individuals during the experiment as well as in that of mixed groups. Half of the amount deposited had disappeared (ie., "half-life" was reached) in about 6 hr after application. It was no longer detectable after a period of 14 days. In contrast, a substance synthesized by the species in small quantities, *n*-tetracosane, stabilized at lower levels than that deposited on the cuticle, but at much higher levels than occurred naturally in cuticular wax. Half of the quantity of *n*-tetracosane deposited disappeared within 140 hr [as compared with 6 hr in the case of (Z)-9-tricosene]. These data show that differences existed depending on the nature of the cuticular hydrocarbon. When the component was normally present among the cuticular hydrocarbons, its quantity decreased but became stable at a particular level; when the component was not normally present, it disappeared quickly.

Added (Z)-9-tricosene may have been mostly metabolized. It probably cannot be resynthesized by *C. vagus*, which seems unlikely to possess the appropriate enzymatic system for synthesizing this substance. Blomquist et al. (1984) suggested that when (Z)-9-tricosene, the sexual pheromone of the female housefly, is deposited on the cuticle of the male, which does not normally synthesize it, the pheromone may be internalized and metabolized into epoxide and ketone products that are then incorporated into the cuticle. The eight-hour "half-life" of added (Z)-9-tricosene recorded in their study of male houseflies is similar to what we have found when it is added to the cuticle of *C. vagus*. Blomquist and Jackson (1973) reported that in the grasshopper, *Melanoplus sanguinipes*, *n*-alkanes with 21 to 29 carbon atoms administered to the cuticle surface were quickly metabolized; the lighter ones were more readily metabolized than the heavier ones, and around 30% of the *n*-tricosane, for example,

was metabolized into secondary alcohols within 18 hr. In our study with *C. vagus*, *n*-tetracosane with a "half-life" of about 140 hr may possibly be resynthesized.

All the treated and nontreated workers at t_{0+24} hr had (*Z*)-9-tricosene in their postpharyngeal glands, and the amounts detected were related to that deposited on the treated worker in contact with a group of nontreated ones. Between 24 and 96 hr, few workers had (*Z*)-9-tricosene in their postpharyngeal glands, and none carried this substance on their cuticle. Only after 96 and 168 hr did we detect small quantities of (*Z*)-9-tricosene on the cuticles (around 1% on average of the amount initially administered). These results suggest that in a treated worker (*Z*)-9-tricosene applied to the cuticle may have been transported to the postpharyngeal glands via the hemolymph. The presence of (*Z*)-9-tricosene in the postpharyngeal glands of nontreated workers after 24 hr of cohabitation with the treated one suggests that (*Z*)-9-tricosene must have been transferred from the one treated individual to the others. The transfer probably occurred during interactions such as trophallactic exchanges and licking activities. Separation of the treated individual from nearby workers by the presence of a double wire mesh prevented these exchanges from occurring. During trophallactic exchanges, (*Z*)-9-tricosene must have been transferred from the postpharyngeal glands of the treated worker to those of the nontreated workers, and during licking activities, this substance must have been transferred from the cuticle of the treated worker to the postpharyngeal glands of the nontreated workers. The fact that no (*Z*)-9-tricosene was detected during the first few hours of cohabitation on the nontreated workers' cuticles shows that the transfer was never effected simply by a cuticle-to-cuticle contact between treated and untreated workers. Large quantities of (*Z*)-9-tricosene were detected in postpharyngeal glands of nontreated workers at a cohabitation time of 24 hr, followed by small quantities only at later times. No traces of (*Z*)-9-tricosene were detected on the cuticles of the ants. It seems reasonable to hypothesize that (*Z*)-9-tricosene may have traveled from the postpharyngeal glands to the cuticle via an internal pathway. Vinson et al. (1980) established that triolein, a triglyceride, was transferred by the hemolymph partly via the postpharyngeal glands of the *Solenopsis invicta* queen. In a recent study, Soroker and Hefetz (poster at the congress held in Tampa, Florida, in 1993) established that the postpharyngeal glands of *Cataglyphis niger* stored hydrocarbons that were synthesized at the cuticular level and not by the postpharyngeal glands themselves. Zylberberg et al. (1974) suggested that the postpharyngeal glands may be involved in selectively absorbing ingested lipids present in nutrients, and thus may play an alimentary role.

It has been established that recognition signals of genetic origin, or discriminators as they have been called, can be produced either mainly by the workers (Morel et al., 1990) or mainly by the queens (Carlin and Hölldobler, 1986). The genetically determined hydrocarbons may be stored in the post-

pharyngeal glands and transferred between the members of a social group during trophallaxis, licking, and grooming activities, according to a gestalt model (Crozier and Dix, 1979) where individuals may consistently regulate and adjust their chemical signals so as to homogenize the substances involved in the recognition processes. Regulatory processes of this kind may constitute one of the mechanisms whereby social cohesion is maintained. They may help to give us a better understanding of the mechanisms involved both in the cohabitation that occurs in artificially mixed colonies of *Manica rubida* and *Formica selysi* (Bagnères et al., 1991b), in the changes observed with time in some of the hydrocarbons carried by *Solenopsis invicta* (Vander Meer et al., 1989b), and in the synchronous patterns of change occurring with time in the cuticular hydrocarbon mixture of *Leptothorax lichtensteini* (Provost et al., 1993).

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INDOLE AS AN OLFACTORY SYNERGIST FOR VOLATILE KAIROMONES FOR DIABROTICITE BEETLES

ROBERT L. METCALF,* RICHARD L. LAMPMAN, and LESLEY DEEM-DICKSON

Department of Entomology, University of Illinois Urbana-Champaign, Illinois 61801

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Abstract—Olfactory synergism, where combinations of plant volatile kairomones are quantitatively more attractive to insects than the sum of attraction of the individual components, is an important but little-studied phenomenon in host plant selection and feeding and in pollination ecology. Diabroticite beetles (Coleoptera: Chrysomelidae) are strongly attracted to *Cucurbita* blossoms, and 2- to 3-fold olfactory synergism has been demonstrated in four species by combinations of the key blossom volatiles, 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde. This TIC mixture represents an optimized *Curcurbita* blossom volatile kairomone mixture useful in monitoring Diabroticite populations and in studying their behavior and ecology. Indole, which exhibits a spectrum of attraction to these beetles ranging from moderate for *Diabrotica virgifera virgifera* and *Acalymma vittatum* to very weak for *D. barberi*, is the primary synergistic component. Indole combined with 4-methoxycinnamaldehyde was significantly synergistic to *D. v. virgifera* at a ratio of 1:300 and produced 4-fold synergism at a ratio of 1:1. Indole combined with 4-methoxyphenethanol was less synergistic to *D. barberi* with 1.5- to 2-fold synergism at a 1:1 ratio. These consistent variations in diabroticite beetle olfactory responses presumably indicate evolutionary divergences in the numbers of relict indole antennal receptors.

Key Words—Kairomonal attractants, Diabroticite beetles, Coleoptera, Chrysomelidae, olfactory synergism, trimethoxybenzene, indole, cinnamaldehyde, 4-methoxycinnamaldehyde, 4-methoxyphenethanol.

INTRODUCTION

Abundant evidence from chemical ecology supports the viewpoint that diabroticites (Coleoptera: Chrysomelidae: Luperini) coevolved with plants of the fam-

*To whom correspondence should be addressed.

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ily Cucurbitaceae (Metcalf, 1986, 1994; Metcalf and Lampman, 1989c). The preponderance of species for which host records are available were collected from foliage, blossoms, and fruits of this family. This, combined with the fact that the tetracyclic triterpenoid cucurbitacins, characteristic of the Cucurbitaceae, are arrestants and phagostimulants for *Diabrotica* spp. in quantities as small as 5×10^{-12} to 1×10^{-9} g (Metcalf et al., 1980; Peterson and Schalk, 1985), indicates a coevolutionary relationship. The aggregations of diabroticite beetles commonly found in *Cucurbita* blossoms, where they feed avidly on pollen, are the result of concerted attraction from an array of blossom volatiles that increases the arrival rate acting together with the arrestant properties of the cucurbitacins that delay departure (Andersen and Metcalf, 1987; Metcalf and Lampman, 1989c, 1991; Lewis et al., 1990). Coleoptera are considered to be the most primitive pollinators of the angiosperms and were probably associated with open bowl-shaped flowers, where the beetles fed on floral secretions, nectar, and pollen (Kevan and Baker, 1983). *Diabrotica* spp. are among the most important pollinators of *Cucurbita* spp. (Avila et al., 1989).

The blossoms of *Cucurbita maxima* Duchesne produce an array of more than 40 volatile chemicals (Andersen and Metcalf, 1986, 1987), of which 22 have been identified by GC-MS (Andersen, 1987). Extensive field evaluations have shown that at least 10 of these volatile compounds are kairomone attractants for both sexes of diabroticites (Lampman et al., 1987; Metcalf, 1994; Metcalf and Lampman, 1989a–c). Most of these volatiles are phenyl propanoids formed through the shikimic acid pathway from phenyl alanine to cinnamic acid, which is produced in relatively large amounts during pollen maturation in blossoms (Wierman, 1981).

All of the four diabroticites examined in field studies are attracted to chemically related phenyl propanoids; however, each species has an intrinsic and characteristic pattern of response when exposed to an array of *Cucurbita* blossom volatiles (Lampman and Metcalf, 1988; Metcalf and Lampman, 1991; Metcalf, 1994). Indole is a condensed phenyl propanoid that is a major component of the rich, heavy aroma of squash blossoms and is attractive to the western corn rootworm, *D. virgifera virgifera* LeConte, and to the striped cucumber beetle, *Acalymma vittatum* (F.). However, indole alone is only slightly attractive to the southern corn rootworm *D. undecimpunctata howardi* Barber and is unattractive to the northern corn rootworm *D. barberi* (Smith and Lawrence) (Andersen and Metcalf, 1986; Lewis et al., 1990; Metcalf, 1994). During the summers of 1986–1994, we evaluated more than 30 combinations of volatile components identified from the blossom aroma of *C. maxima* (Andersen and Metcalf, 1987; Andersen, 1987) as attractants for adult diabroticites and found indole to be a key component of blossom volatile mixtures that were strongly attractive to all four species investigated (Metcalf et al., 1993) (Figure 1). Thus the role of indole as a diabroticite host plant kairomone is complex.

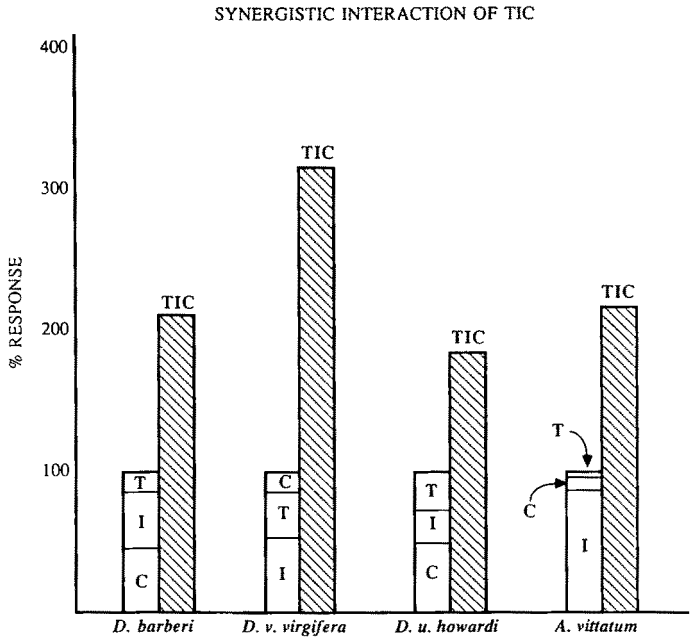

FIG. 1. Synergistic interactions of 1,2,4-trimethoxybenzene (T), indole (I), and (*E*)-cinnamaldehyde (C) to four species of diabroticites. Each compound at 20 mg/trap, separately (open), combined (shaded).

A mixture of equal parts of veratrole, indole, phenyl acetaldehyde, anethole, and eugenol (VIPAE) applied at 100 mg/sticky trap, showed a mean trap catch of *D. u. howardi* adults of 28.0 ± 1.8 times that of the unbaited controls ($N = 4$) (Lampman and Metcalf, 1987). The relationship between log dosage of VIPAE and mean beetle catch was linear over a range of 1–300 mg of lure per trap ($r^2 = 0.98$, $P < 0.001$). Further evaluation of the role of each of the components led to simplification of the lure mixture to equal parts of veratrole, indole, and phenyl acetaldehyde (VIP), which was approximately as attractive to *D. u. howardi* as the VIPAE mixture. However, the catch of *D. v. virgifera* adults by the VIP mixture was not greater than that of indole alone (Lampman and Metcalf, 1987). Further elaboration of the volatile components of *C. maxima* blossoms suggested the substitution in the VIP mixture of 1,2,4-trimethoxybenzene (T) for veratrole, and (*E*)-cinnamaldehyde (C) for phenyl acetaldehyde. These, combined in equal proportions with indole (I), formed the TIC mixture that was as attractive to *D. u. howardi* adults as the VIP mixture and was equally attractive to *D. v. virgifera* adults (Lampman and Metcalf, 1987). Used at 60

mg/trap in experiments conducted during 1986–1989, the TIC mixture showed a mean trap catch of *D. u. howardi* of 35.9 ± 54.1 times that of the unbaited controls ($N = 5$) and for *D. v. virgifera* of 43.7 ± 66.3 times that of the unbaited controls ($N = 8$) (unpublished data). The TIC mixture was also strongly attractive to *Acalymma vittatum* adults and, when applied at 60 mg/trap, showed a mean catch of 14.3 ± 10.9 times that of the unbaited controls ($N = 8$) (Lewis et al., 1990).

From these investigations, we concluded that the TIC mixture represents an optimized *Cucurbita* blossom volatile kairomone that is highly attractive to diabroticite species. In this report we further characterize the role of indole as an olfactory synergist for kairomone lures to various species of Diabroticites.

METHODS AND MATERIALS

Attractants and Field Trapping. The chemicals evaluated as lures were all 98% pure by GC and were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin; except for 4-methoxycinnamaldehyde, which was obtained from Chemical Dynamics, Plainfield, New Jersey. The multicomponent TIC lure was a 1 : 1 : 1 mixture of 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde. Solid lures such as indole and 4-methoxycinnamaldehyde were diluted with reagent-grade acetone before application.

The relative effectiveness of the various lures was determined by baiting white, 1-liter cylindrical paper cartons coated with TangleTrap insect adhesive (Tanglefoot Company, Grand Rapids, Michigan). These traps were inverted over 1-m lath stakes placed approximately 10 m apart in a linear randomized complete block design at the edge of hybrid corn fields infested with adult *Diabrotica* corn rootworms. The lures were dispensed quantitatively from capillary micropipets onto cotton dental wicks (Table 1) or onto 5 × 5-cm squares of blotting paper (Tables 2 and 3), affixed to the top center of the sticky trap. The individual lure components were applied to separate sections of wick or blotting paper, to obviate interference with volatility. The three components of TIC were thoroughly mixed and applied as a mixture. Under each experimental date, four replicate traps were run at each dose together with four untreated control traps. The adult beetle catch was counted at either 4 or 24 h after treatment, depending upon the densities of the beetle populations.

Statistical Analyses. The significance of the treatments in the experiments reported was determined by two-way ANOVA on transformed data [log (x + 1)], and where appropriate, individual means were separated by Fisher's protected least significant difference test (PLSD) (Statview, 1992; Norusis, 1988). For evaluation of dose–response data, linear regression analysis of the mean number of beetles per trap as a function of logarithm of dose was computed (Statview, 1992; Sokal and Rohlf, 1973).

TABLE 1. INDOLE (I) AS OLFACTORY SYNERGIST FOR CINNAMALDEHYDE (C) AND TRIMETHOXYBENZENE (T) TO *Diabrotica v. virgifera*

Date	Mean trap catch ($N = 4$)[a]								SR[b]
	Control	I	C	T	TI	CI	TC	TIC	
Aug. 5, 1986[c]	18.5 ± 3.2a	68.2 ± 17.7b		17.7 ± 2.4a	172.2 ± 62.2c				3.0
Aug. 7, 1986	7.0 ± 2.2a	27.5 ± 10.2b		9.7 ± 6.8a	73.7 ± 33.7c				2.9
July 22, 1988[d]	2.0 ± 2.8a	10.0 ± 5.7b	4.7 ± 2.1b			36.2 ± 18.9c			3.2
	2.0 ± 2.8a	10.0 ± 5.7b	4.7 ± 2.1b	5.2 ± 3.0b				46.0 ± 12.4c	3.2
Sep. 21, 1989[d]	1.7 ± .05a	50.0 ± 31.6b		8.2 ± 2.6c	246.0 ± 109.6d				4.5
	1.7 ± 0.5a	50.0 ± 31.6b	50.0 ± 29.6b			226.2 ± 34.6d			3.3
	1.7 ± 0.5a		50.0 ± 29.6b	8.2 ± 2.6c			48.0 ± 20.4b		1.0
	1.7 ± 0.5a	50.0 ± 31.6b	50.0 ± 29.6b	8.2 ± 2.6c				357.7 ± 185.5d	3.3

[a] Each attractant chemical at 20 mg per trap for a total dose of 20, 40, or 60 mg.
[b] Synergistic ratio.
[c] Means for each test date followed by different letters are significantly different ($P < 0.05$) by Fisher's PLSD test.
[d] Subdivided to show differences in synergistic ratios (SR).

TABLE 2. OLFACTORY SYNERGISM OF INDOLE (I) WITH METHOXYCINNAMALDEHYDE (MCA) TO *Diabrotica v. virgifera*

Date	Mean trap catch (N = 4)				
	Control	MCA (20 mg)	Indole (20 mg)	MCA-Indole (mg ratio)	SR[a]
Aug. 6, 1993[b]	1.5 ± 1.7a	36.2 ± 12.3b	9.2 ± 8.2a	243.2 ± 41.3c (20:20)	5.7
Aug. 7, 1993	3.2 ± 1.0a	34.2 ± 23.0b	4.7 ± 2.9a	169.5 ± 23.8c (20:20)	5.0
Aug. 13, 1993	1.7 ± 1.0a	55.7 ± 30.0b	7.2 ± 3.0a	179.2 ± 31.5cd (20:20)	3.0
				197.0 ± 30.3c (20:7)	3.2
				147.5 ± 32.1d (20:2)	2.4
Aug. 16, 1993	1.0 ± 1.4a	20.2 ± 13.7a	10.7 ± 10.3ab	92.5 ± 20.5c (20:2)	3.2
				73.0 ± 26.1cd (20:0.7)	2.5
				47.5 ± 33.1bd (20:0.2)	1.6
Aug. 17, 1993	1.2 ± 1.0a	34.5 ± 13.2b	7.5 ± 4.4a	70.0 ± 22.3c (20:0.2)	1.7
				69.0 ± 11.1c (20:0.07)	1.7
				38.2 ± 20.9b (20:0.02)	1.0
July 18, 1994	2.0 ± 2.0a	34.5 ± 7.9a	9.7 ± 5.2a	123.2 ± 13.8b (20:20)	3.0
				121.2 ± 58.2b (20:2)	3.0
July 22, 1994	0.5 ± 0.5a	10.2 ± 7.9a	0.2 ± 0.5a	31.5 ± 14.7b (20:20)	3.3
				21.5 ± 12.9b (20:2)	2.2
				21.5 ± 5.5b (20:0.2)	2.2

[a] Synergistic ratio.
[b] Means for each test date followed by different letters are significantly different ($P < 0.05$) by Fisher's PLSD test.

Olfactory synergism is defined as the more than additive response of diabroticite beetles (trap catch) to a mixture as compared with the summed responses to its component parts and is illustrated in Figure 1. The synergistic ratio (SR) is the ratio of the mean trap catch of the mixture (control subtracted) to the summation of the mean trap catches of the components (controls subtracted).

RESULTS AND DISCUSSION

Olfactory Synergism of Indole with Cinnamaldehyde and Trimethoxybenzene. Several years of extensive investigations (Metcalf and Lampman, 1989c; Metcalf, 1994) have demonstrated that indole is the key component of *Cucurbita* blossom volatiles in promoting olfactory synergism among the several species of Diabroticites. The experiments summarized in Table 1 show the synergistic ratio (SR) for catches of *D. v. virgifera* on cylindrical sticky traps baited with 20 mg of the several components of the TIC mixture. As shown in Table 1, indole together with trimethoxybenzene (TI) was strongly synergistic with a

TABLE 3. OLFACTORY SYNERGISM OF INDOLE WITH METHOXYPHENETHANOL (MPE) TO *Diabrotica barberi*

Date	Mean trap catch (N = 4)				
	Control	MPE (20 mg)	Indole (20 mg)	MPE–Indole (mg ratio)	SR[a]
Aug. 3, 1993[b]	1.0 ± 1.1a	26.7 ± 6.3a	2.0 ± 2.2a	50.7 ± 41.0b (20:20)	2.0
Aug. 13, 1993	0a	17.0 ± 5.0b	1.0 ± 0.8a	32.0 ± 18.4c (20:20)	2.0
				36.0 ± 9.3c (20:7)	1.9
				36.5 ± 6.8c (20:2)	1.9
Aug. 18, 1993	0.5 ± 0.6a	20.0 ± 10.4b	3.5 ± 5.0a	21.2 ± 3.6b (20:20)	1.0
				13.2 ± 2.5b (20:7)	1.0
				23.7 ± 13.4b (20:2)	1.0
Aug. 18, 1993	4.2 ± 1.8a	31.0 ± 9.3b	5.2 ± 7.9a	45.2 ± 18.6b (20:20)	1.5
				32.0 ± 3.7b (20:7)	1.0
				52.0 ± 10.4b (20:2)	1.7
July 12, 1994	5.7 ± 2.2a	129.7 ± 39.5b	6.5 ± 5.7a	147.0 ± 36.6b (20:20)	1.1

[a]Synergistic ratio.
[b]Means for each test date followed by different letters are significantly different ($P < 0.05$) by Fisher's PLSD test.

mean SR for three investigations of 3.5 ± 2.7. Indole together with cinnamaldehyde (CI) was also synergistic, with a mean SR for four evaluations of 1.9 ± 0.9. However, in contrast cinnamaldehyde together with trimethoxybenzene (TC) showed no evidence of olfactory synergism.

The three components—indole, cinnamaldehyde, and trimethoxybenzene—used together (TIC) were strongly synergistic, with a mean SR for four evaluations of 2.3 ± 0.9. It should be evident that synergism between an active component and an inactive component may produce higher synergistic ratios than that between two active components, e.g., indole and cinnamaldehyde.

Olfactory Synergism to Several Diabroticite Species. Indole is the key *Cucurbita* blossom volatile, producing olfactory synergism not only to *D. v. virgifera*, as demonstrated in Table 1, but also to other related species, all of which feed on pollen in *Cucurbita* blossoms. The dose–responses of the several species to indole alone in field experiments with cylindrical sticky traps ranges from a limit of response (LR) of approximately 1 mg per trap for *D. v. virgifera* and *Acalymma vittatum* to 20 mg for *D. u. howardi* and to 200 mg for *D. barberi* (Andersen and Metcalf, 1986; Lewis et al., 1990; Metcalf, 1994). However, the TIC mixture is synergistic to all four species, as shown in Figure 1 from data of typical field experiments (Lewis et al., 1990, unpublished data) where the sum of the trap catches for the individual TIC compounds, at 20 mg

each was normalized to 100 and compared to the normalized catches of the TIC mixture at 60 mg/trap. Figure 1 portrays synergistic ratios of 2.1 for *D. barberi* (July 7, 1988), 3.2 for *D. v. virgifera* (July 2, 1988), 1.8 for *D. u. howardi* (Sept. 13, 1986), and 2.1 for *A. vittatum* (Aug. 2, 1988) (Lewis et al., 1990).

Olfactory Synergism of Indole with Methoxycinnamaldehyde. Methoxycinnamaldehyde (MCA) is the most effective single component kairomone-type lure yet found for adult *D. v. virgifera* (Metcalf and Lampman, 1989a). It is an effective lure for both sexes and the male/female trap catch was 108/356 = 0.30 (Aug. 8, 1987) and was 62/37 = 1.67 (Aug. 25, 1993). In 23 comparisons ($N = 4$ traps each) in hybrid corn conducted during July, August, and September of 1987–1993, the one-day mean capture of *D. v. virgifera* beetles on 1-liter cylindrical sticky traps baited with 100 mg of MCA was 182.5 ± 82.0 as compared with a mean of 7.1 ± 12.0 beetles captured on an equal number of unbaited control traps. Therefore the MCA-baited traps captured a mean of 25.7 times more *D. v. virgifera* adults than the controls. MCA is an almost specific lure for *D. v. virgifera* adults, and in 18 comparisons over the same years, the MCA-baited traps captured a mean of only 2.5 ± 3.4 *D. barberi* beetles as compared with a mean of 2.7 ± 3.9 for unbaited controls. In 16 comparisons, the MCA-baited traps captured a mean of only 0.9 ± 2.0 *D. u. howardi* beetles as compared with a mean of 0.7 ± 1.2 for unbaited controls.

The limit of response for *D. v. virgifera* to MCA is approximately 0.03 mg/trap as compared to 1.0 mg for indole (Andersen and Metcalf, 1986; Metcalf and Lampman, 1991). Although MCA has not been isolated from *Cucurbita* blossoms, it is a widely distributed natural product (Metcalf and Lampman, 1991) and is likely to be present in *Cucurbita* blossom aroma because of the presence there of relatively large amounts of cinnamaldehyde, di- and trimethoxybenzene, and 4-methoxybenzaldehyde (Andersen, 1987).

Methoxycinnamaldehyde has a relatively low volatility and has a release rate of only about 0.012 that of indole (Metcalf and Lampman, 1991). Despite this nearly 100-fold disparity in release rates, when MCA and indole were compared at 20 mg/cylindrical sticky trap as lures for *D. v. virgifera* beetles, the mean trap catches from four experiments averaged 10.0 ± 6.7-fold greater for MCA than for indole ($N = 4$) as shown in Table 2. Similar experiments comparing MCA at 20 mg with TIC at 20 mg (ca. 7 mg indole) showed that the mean trap catches of *D. v. virgifera* were almost equal, with an average MCA/TIC ratio of 0.98 ± 0.22 for four experiments ($N = 4$ traps each). This information provides further demonstration of the remarkable olfactory synergism of the TIC mixture.

The ranges of olfactory synergism of mixtures of MCA and indole to *D. v. virgifera* beetles were compared using 20 mg of MCA and a range of doses of indole, from 0.02 to 20 mg, applied to separate sections of blotting paper squares in simultaneous experiments with cylindrical sticky traps (Table 2).

When applied in equivalent doses of 20 mg in four experiments, the mean trap catches for the mixture as compared to the additive catches of the separate components (controls subtracted) showed a mean synergistic ratio (SR) of 4.0 ± 1.3. Further comparisons (Table 2) using MCA at 20 mg and decreasing the amounts of indole, showed only a gradual reduction in SR, which averaged 2.7 at 20 mg:2 mg, 1.6 at 20 mg:0.2 mg, and 1.0 at 20 mg:0.02 mg. Linear regression of the synergistic ratio versus the log dose of indole in the MCA/indole mixtures evaluated in a single field over a short period of time (August 6–17, 1993), shows a highly significant relationship (Figure 2).

The appreciable olfactory synergism (Table 2, Figure 2) at a trap dose of 100 parts of MCA to 1 part of indole appears to result from the approximately 100-fold difference in the release rates of the two lures, so that approximately equal concentrations of MCA and indole were present throughout the "active space" downwind from the baited traps. However, since even at equivalent trap doses, MCA was approximately 10 times more attractive than indole as a lure for *D. v. virgifera* (Table 2), concentrations of indole throughout the MCA active space ranging from 1- to 100-fold greater than those of MCA must have an indirect effect in modifying the *D. v. virgifera* behavior, presumably by central nervous system interactions (Mustaparta, 1990).

Olfactory Synergism of Indole with Methoxyphenethanol. Methoxyphenethanol (MPE) is an effective single-component kairomone lure for adult *Diabrotica barberi*. It is a lure for both sexes and the male/female trap catch was

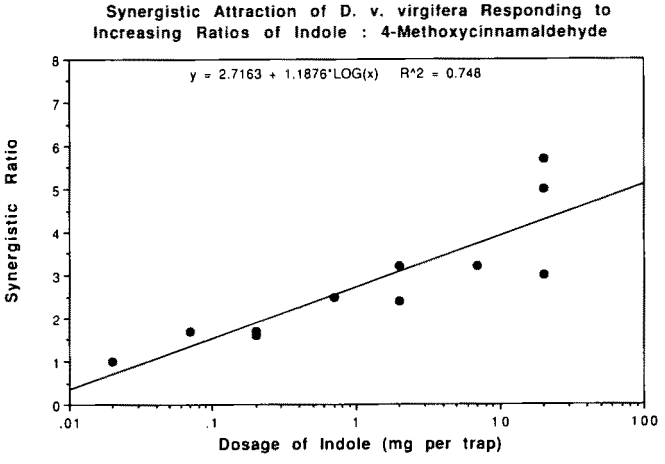

FIG. 2. Relationship between synergistic ratio and dose of indole together with 20 mg 4-methoxycinnamaldehyde per sticky trap ($N = 4$) to *Diabrotica v. virgifera* (1993 data from Table 2). Regression line is significant at $P = 0.01$.

7/45 = 0.16 (Aug. 16, 1989) and 29/135 = 0.4 (Sept. 9, 1993). In 27 comparisons (N = 4 traps each) conducted during July, August, and September of 1989–1993, the one-day captures of *D. barberi* beetles on cylindrical sticky traps baited with 100 mg of MPE had a mean value of 117.8 ± 156.7 as compared with 5.6 ± 8.1 for an equivalent number of unbaited control traps. Therefore, the MPE-baited traps captured a mean of 21.0 times more *D. barberi* adults than the unbaited control traps. MPE is a highly specific lure for *D. barberi* and in 17 identical comparisons over the same four-year period, the MPE baited traps captured a mean of 3.3 ± 4.4 *D. v. virgifera* adults as compared to a mean of 3.5 ± 4.7 for unbaited control traps. No significant numbers of *D. u. howardi* were captured by the MPE baited traps.

The limit of response of *D. barberi* beetles to traps baited with MPE was approximately 0.1 mg (Metcalf and Lampman, 1991). In contrast, indole is not appreciably attractive to *D. barberi* adults at trap doses as high as 200 mg (Lampman and Metcalf, 1987). When MPE and indole were compared at equivalent 20 mg doses (Table 3), mean trap catches of *D. barberi* averaged 46.0 ± 55.0-fold greater for MPE than for indole (N = 5). The mean trap catches of *D. barberi* with a 1:1 mixture of indole and MPE showed some evidence of olfactory synergism (Table 3); however, the SR values were substantially lower than those recorded for corresponding mixtures of MCA and indole to *D. v. virgifera*. This is to be expected, as indole has consistently been less effective in producing an olfactory response in *D. barberi* either alone or in the TIC mixture.

Atmospheric Behavior of Synergistic Mixtures. The TIC attractant mixture diffuses downwind and produces directed anemotactic flight behavior in diabroticite species that is superficially indistinguishable from that of single-component kairomone odorants. There is, for example, a highly precise relationship between mean beetle trap catch and log dose TIC for *D. v. virgifera* and for *D. u. howardi* (Figure 3), using data from Lampman and Metcalf (1987). These responses of the two *Diabrotica* spp. are virtually identical. A similar relationship was shown for *A. vittatum* (Lewis et al., 1990). Such log dose–odorant response curves are also observed with single odorants such as 4-methoxycinnamaldehyde to *D. v. virgifera* (r^2 = 0.984, P = 0.01) and 4-methoxyphenethanol for *D. barberi* (r^2 = 0.937, P = ca. 0.05) (calculated from Metcalf and Lampman, 1991). These plots are useful in determining the approximate limit of response (LR), which for TIC is about 0.5 mg/trap for *D. v. virgifera* and about 1 mg/trap for *D. u. howardi* (Figure 3). These values compare with that of MCA to *D. v. virgifera* of 0.03 mg/trap and for MPE to *D. barberi* of about 0.1 mg/trap (Metcalf and Lampman, 1991).

Experiments with sticky traps baited with 100 mg of TIC or MCA and placed in newly mowed alfalfa stubble devoid of western corn rootworm beetles and adjacent to corn infested with *D. v. virgifera*, have demonstrated directed

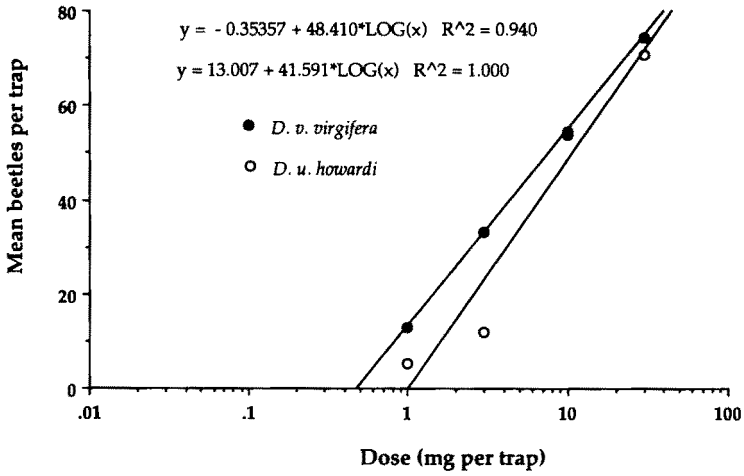

FIG. 3. Relationship of mean trap catch (N = 4) to trap dose for TIC attractant, for *Diabrotica v. virgifera* (P = 0.0001) and *Diabrotica u. howardi* (P = 0.03).

anemotactic searching over distances of at least 100 m in winds of 2–6 m/sec. The mean capture rates for attractant baited/unbaited traps (N = 4, at each distance) were: 10 m TIC 49×, MCA 59×; 30 m TIC 14×, MCA 30×; and 100 m TIC 16×, MCA 20×. The differences in mean capture rates for each lure over the three distances were significant at P = 0.001 (Metcalf and Metcalf, 1992).

The responses of insects to semiochemical odorants are a function of the Q/K ratio, where Q is the release rate of odorant in molecules per second and K is the insect behavioral threshold in molecules per cubic centimeter (Bossert and Wilson, 1963; Metcalf and Metcalf, 1992). Release rates from 20-mg films of the several lures have been measured at 22–24°C and an airflow of 75 cm/sec as: trimethoxybenzene 1.9×10^{14}, indole and cinnamaldehyde both as 1.4×10^{15}, 4-methoxycinnamaldehyde (MCA) as 1.6×10^{13}, and 4-methoxyphenethanol (MPE) as 1.2×10^{14} molecules/sec (Metcalf and Metcalf, 1992). Thus the release rates of indole and cinnamaldehyde, the most volatile of the components of the TIC mixture are approximately 80-fold greater than that of MCA. From this disparity and the 33-fold greater sensitivity of *D. v. virgifera* to MCA (LR 0.03) as compared to indole (LR 1.0) it appears that MCA has a chemosensory activity of about 2750-fold greater than indole (Metcalf and Lampman, 1991).

From these facets of evidence, how can we account for the impressive long-range attractivity of the TIC mixture and the demonstrated synergistic

response of *D. v. virgifera* to indole–MCA mixtures at ratios as low as 0.003? Calculations using the point source atmospheric diffusion model of Bossert and Wilson (1963), where $Q/K = 0.125\ uX_{max}^{7/4}$, indicate that under the conditions noted above, the maximum downwind "active space" (X_{max}) is almost congruent at a ratio of indole/MCA = 0.01. For example, with K assumed as 1×10^5 (Metcalf and Metcalf, 1992) and a wind (u) of 100 cm/sec, the calculated X_{max} for 20 mg of MCA is 32.5 m and for 0.2 mg of indole is 30.0 m (unpublished). Therefore, we suggest that exposure of diabroticites to low levels of indole may stimulate the anemotactic response to search upwind, thus leading them into the odor corridor of the primary kairomone attractant.

The observations discussed above emphasize the complex role of indole as a kairomone for various diabroticites. Indole is a component in *Cucurbita* blossom aroma (Andersen and Metcalf, 1986). This association is a very primitive one, and antennal kairomone receptors to indole must have developed very early in the coevolutionary association of diabroticites and Cucurbitaceae. The presence of indole along with a few other prominent odorants from *Cucurbita* blossoms provided a chemical fingerprint leading diabroticites toward a readily accessible source of nectar and pollen. The indole receptor appears to have been conserved for over 30 million years of diabroticite evolution (Krysan et al., 1989), and remains functional in representatives of both the nondiapausing *fucata* group and in the diapausing *virgifera* group of species. However, evolutionary processes have substantially tempered the nature of the behavioral patterns elicited by depolarization of the indole receptors, probably by reducing the relative numbers of responsive sensilla. In species as diverse as *D. v. virgifera* and *A. vittatum*, there is a clearly defined behavior response to indole, which is much less evident in the sibling species of *D. v. virgifera*, i.e., *D. barberi* and *D. cristata*. In *D. u. howardi*, which probably most clearly resembles the *fucata* group ancestor (Krysan et al., 1989), there is a strong EAG response to the presence of indole but the corresponding behavioral response is suppressed (Andersen and Metcalf, 1986; Lampman et al., 1987).

These interesting evolutionary anomalies can be assessed in a preliminary way by considering the two basic components of the insect nervous system involved in semiochemical communication. The antennal receptors and their associated peripheral nervous system represent the detection system that feeds information into association centers of the CNS where directed behavioral responses are generated (Mustaparta, 1990). As the conservation of the indole receptors has been inferred in the range of species discussed, it appears that evolutionary modifications during speciation have preferentially modified the first integration process in the antennal lobes of the CNS. The extent and magnitude of the synergistic effect resulting from kairomone mixtures containing indole suggest that exposure to very low levels of this kairomone may stimulate

the anemotactic response resulting in upwind searching behavior. It is obvious that the phenomenon of olfactory synergism needs further intensive study.

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DISTRIBUTION OF AUTOGENOUS AND HOST-DERIVED CHEMICAL DEFENSES IN *Oreina* LEAF BEETLES (COLEOPTERA: CHRYSOMELIDAE)

JACQUES M. PASTEELS,[1,*] SUSANNE DOBLER,[2] MARTINE ROWELL-RAHIER,[2] ADELHEID EHMKE,[3] and THOMAS HARTMANN[3]

[1]*Laboratoire de Biologie animale et cellulaire, C.P. 160/12*
Université libre de Bruxelles
50 av. F.D. Roosevelt, B-1050 Brussels, Belgium

[2]*Zoologisches Institut der Universität Basel*
Rheinsprung 9, 4051 Basel, Switzerland

[3]*Institut für Pharmazeutische Biologie der Technischen Universität*
D-38106 Braunschweig, Germany

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Abstract—The pronotal and elytral defensive secretions of 10 *Oreina* species were analyzed. Species feeding on Apiaceae, i.e., *O. frigida* and *O. viridis*, or on Cardueae (Asteraceae), i.e., *O. bidentata*, *O. coerulea*, and *O. virgulata*, produce species-specific complex mixtures of autogenous cardenolides. *O. melanocephala*, which feeds on *Doronicum clusii* (Senecioneae, Asteraceae), devoid of pyrrolizidine alkaloids (PAs) in its leaves, secretes, at best, traces of cardenolides. Sequestration of host-plant PAs was observed in all the other species when feeding on Senecioneae containing these alkaloids in their leaves. *O. cacaliae* is the only species that secretes host-derived PA N-oxides and no autogenous cardenolides. Differences were observed in the secretions of specimens collected in various localities, because of local differences in the vegetation. The other species, such as *O. elongata*, *O. intricata*, and *O. speciosissima*, have a mixed defensive strategy and are able both to synthesize de novo cardenolides and to sequester plant PA N-oxides. This allows a great flexibility in defense, especially in *O. elongata* and *O. speciosissima*, which feed on both PA and non-PA plants. Populations of these species were found exclusively producing cardenolides, or exclusively sequestering PA N-oxides, or still doing both, depending on the local availability of food-plants. Differences were observed between species in their ability to sequester different plant PA N-oxides and to transform them. Therefore sympatric species demonstrate differences in the composition of their host-derived

*To whom correspondence should be addressed.

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secretions, also resulting from differences in host-plant preference. Finally, within-population individual differences were observed because of local plant heterogeneity in PAs. To some extent these intrapopulation variations in chemical defense are tempered by mixing diet and by the long-term storage of PA N-oxides in the insect body that are used to refill the defensive glands.

Key Words—*Oreina* spp., Coleoptera, Chrysomelidae, Asteraceae, Senecioneae, Cardueae, Apiaceae, chemical defense, cardenolides, pyrrolizidine alkaloids, sequestration.

INTRODUCTION

Adult *Oreina* leaf beetles are chemically protected by a secretion oozing from elytral and pronotal glands. Such glands are characteristic of leaf beetles belonging to the Chrysomelinae and some other leaf beetle taxa (reviewed in Pasteels et al., 1988a, 1994). Although only six *Oreina* species have been previously studied, these can be classified into three categories according to their defensive chemistry, exemplifying different defensive strategies.

First *O. gloriosa*, feeding exclusively on *Peucedanum ostruthium* (Apiaceae), relies for its defense on de novo synthesis of cardenolides from a sterol precursor (Van Oycke et al., 1988; Eggenberger et al., 1992). The mixture of cardenolides produced is complex and shows high interindividual variability. Individual variation is in part correlated with physiological factors such as age and mating status (Eggenberger and Rowell-Rahier, 1993), but a strong heritability of the proportions of compounds was demonstrated by full-sib and mother–offspring correlations (Eggenberger and Rowell-Rahier, 1992). Significant differences in the proportions of the cardenolides making up the defensive mixtures also were observed between different alpine populations. These differences were correlated with the genetic distances (estimated from allozyme data) and the geographic distances separating the populations (Eggenberger and Rowell-Rahier, 1991). Three other species, *O. bifrons*, *O. speciosa*, and *O. variabilis*, feeding on Apiaceae, similarly secrete mixtures of cardenolides. The average mixtures are species specific, and their level of similarity correlates with the genetic distances between the species (allozyme electrophoresis) (Rowell-Rahier and Pasteels, 1994). Thus, chemical defense is able to evolve rapidly, possibly in part by genetic drift. In this context, it should be noted that *Oreina* species apparently form metapopulations, the genetic structure of which has been analyzed in two species, *O. cacaliae* and *O. speciosissima* (Rowell-Rahier, 1992). The secretion of cardenolides is considered to be plesiomorphic in the genus, since it is frequently observed in other genera belonging to the same subtribe Chrysolinina (i.e., in the genera *Chrysolina* and *Ambrostoma*) or to a related subtribe, the Doryphorina (genera *Calligrapha* and *Zygogramma*) (Pasteels et al., 1994).

Secondly, *O. cacaliae*, feeding exclusively on members of the Senecioneae (Asteraceae), secretes sequestered plant pyrrolizidine alkaloid N-oxides (PA N-oxides) for defense (Pasteels et al., 1988b; Ehmke et al., 1991). In the beetles studied from the Vosges (France) and the Black Forest (Germany), only PA N-oxides from *Adenostyles alliariae* were sequestered in the defensive secretion, but not PAs from *Senecio fuchsii*, another food plant in the field. PAs from both plants, however, were sequestered in the body (Rowell-Rahier et al., 1991).

Finally, *O. speciosissima*, also feeding on Senecioneae, has a mixed defensive strategy, being able to synthesize cardenolides and to sequester PAs. Interestingly, although the beetles readily sequestered PAs when fed with *A. alliariae* in the laboratory, pooled secretions of beetles collected from two different localities contained, at best, trace amounts of PAs (Rowell-Rahier et al., 1991). In the field, these beetles fed mainly on *Petasites albus*, which is devoid of PAs in its leaves, although *A. alliariae* was equally frequent in one locality. The mixed strategy possibly illustrates an intermediate evolutionary stage, but the flexibility it allows could be selectively advantageous in its own right.

Our ultimate goal is to understand the evolution of chemical defense in the genus. Independent phylogenies based on molecular data will be presented elsewhere. Here, we will focus on the distribution of the three defensive strategies within the genus (i.e., de novo synthesis, sequestration, and mixed strategy) and limit ourselves to adult defense. Larval defense is described by Dobler and Rowell-Rahier (1994a) and Ehmke et al. (in preparation). In the present paper, 10 species will be examined (*O. cacaliae*, *O. speciosissima*, and eight previously unstudied species), bringing the total of species studied to 14, i.e., the majority of species occurring in the Swiss and French Alps. Two main questions will be addressed: How are the different strategies linked to host-plant affiliation, i.e., Apiaceae or Asteraceae, and among the latter, Senecioneae or Cardueae? In sequestering species, how is the defensive chemistry influenced by the local host-plant community? The emphasis will be on the defensive chemistry of beetles collected in the field. Laboratory feeding experiments with a specific food plant will be described only when necessary to confirm the beetles' ability for sequestration.

METHODS AND MATERIALS

Collecting Sites and Host Plants

Unless specified otherwise host-plant affiliations are based on direct observations of feeding beetles. Beetle nomenclature follows Bourdonné and Doguet (1991), and plant nomenclature, Flora Europaea. The species with their host plants are listed in Table 1.

TABLE 1. DISTRIBUTION OF DE NOVO SYNTHESIZED CARDENOLIDES AND SEQUESTERED PAs IN DEFENSIVE SECRETIONS OF *Oreina* spp.

Species	Defensive compound	Host plants	Localities
O. viridis	cardenolides	*Meum athamanticum* (Apiaceae)	Tschiertschen
O. frigida	cardenolides	*Meum athamanticum* (Apiaceae)	Hörnlihütte, Engstligenalb
O. bidentata	cardenolides	*Leuzea rhapontica* (Asteraceae-Cardueae)	Bosco Gurin
O. coerulea	cardenolides	*Centaurea nemoralis* (Asteraceae-Cardueae)	Calmbach, Bad Liebenzell
O. virgulata	cardenolides	*Cirsium spinosissimum* (Asteraceae-Cardueae)	Engstilgenalb, Hörnlihütte
O. melanocephala	cardenolides ?[a]	*Doronicum clusii* (Asteraceae-Senecioneae)	Hörnlihütte
O. cacaliae	PAs	*Adenostyles alliariae*, *Senecio nemorensis* (Asteraceae-Senecioneae)	Tschiertschen
O. intricata	cardenolides PAs	*Adenostyles alliariae*, *Senecio nemorensis*, (Asteraceae-Senecionae)	Tschiertschen
O. speciosissima	cardenolides PAs	*Petasites albus*, *Senecio nemorensis* (Asteraceae-Senecioneae)	Tschiertschen
	PAs	*Adenostyles alliariae* (Asteraceae-Senecioneae)	Zinal
O. elongata	cardenolides PAs	*Adenostyles leucophylla*, *Cirsium spinosissimum* (Asteraceae-Senecionae, Cardueae)	Vallée des Merveilles
	PAs	*Adenostyles alliariae*, *A. alpina* (Asteraceae-Senecioneae)	Col du Lautaret
	cardenolides	*Cirsium spinosissimum* (Asteraceae-Carduae)	Mattmark dam

[a] At best traces; needs confirmation.

Species Feeding on Apiaceae. O. frigida (Weise) was collected at Hörnlihütte above Arosa (Graubünden, Switzerland, 2600 m) and Engstligenalb (Berner Oberland, Switzerland, 2000 m). This species was found under stones at the first location and on *Cirsium spinosissimum* (L.) Scop. (Asteraceae, Cardueae) at the second. In the laboratory, it fed on *Meum athamanticum* Jacq.

(Apiaceae), but also on *Adenostyles alliariae* (Gouan) Kern. (Asteraceae, Senecioneae) and not on *C. spinosissimum*, *Doronicum clusli* (All.) Tausch, or *Petasites albus* (L.) Gaertn. (both Asteraceae, Senecioneae). The food plant of this species was not yet reported.

O. viridis (Duftschmid) was collected at Tschiertschen (Graubünden, Switzerland, above the tree line, 2000 m) under stones. The host plant reported in the literature is *M. athamanticum* (Bourdonné and Doguet, 1991), which is accepted in the laboratory as are other Apiaceae, but no Asteraceae.

Species Feeding on Asteraceae. O. bidentata Bontems was collected at Bosco Gurin (Tessin, Switzerland, 1600 m) on *Leuzea* (=*Centaurea*) *rhapontica* (L.) J. Holub (Asteraceae, Cardueae).

O. cacaliae (Schrank) was collected at Tschiertschen on *A. alliariae* and *Senecio nemorensis* L. (s.str.) (Asteraceae, Senecioneae).

O. coerulea (Olivier) was collected at Calmbach (Black Forest, Germany, 550 m) and Bad Liebenzell (Black Forest, Germany, 550 m) both on *Centaurea nemoralis* Jordan (Asteraceae, Cardueae).

O. elongata (Suffrian) was collected at Col du Lautaret and Col du Petit St. Bernard. (near Briançon, France, 2000 m) on *A. alliariae* and *Adenostyles alpina* (=*glabra*) (L.) Bluff & Fingerh.; Mattmark dam (Valais, Switzerland, 2400 m) on *C. spinosissimum*; and Vallée des Merveilles (Parc National du Mercantour, France, 2500 m) on *Adenostyles leucophylla* (Wild.) Reichenb. and *C. spinossissimum*.

O. intricata (Germar) was collected at Tschiertschen on *S. nemorensis*.

O. melanocephala (Duftschmid) was collected at Hörnlihütte. This species was found under stones, close to *D. clusii*. The beetles fed on *D. clussi*, but not on other Asteraceae or Apiaceae present in the same location. *Doronicum* spp. are the host plants reported in the literature for this *Oreina* by Bourdonné and Doguet (1991).

O. speciosissima (Scopoli) was collected at Tschiertschen mostly on *P. albus*; and occasionally on *S. nemorensis* and *A. alliariae*, and at Zinal (Valais, Switzerland, 1800 m) on *A. alliariae*.

O. virgulata (Germar) was collected at Engstligenalb and Hörnlihütte on *C. spinosissimum*.

Detection and Quantification of Cardenolides in Secretion

For most species, secretions of five or more beetles were collected on pieces of filter paper or in glass capillaries and pooled in methanol. Only two specimens of *O. frigida* and three of *O. melanocephala* were available for study. The methanol extract of pooled secretions was evaporated under vacuum, the residue redissolved in acetonitrile–water 1 : 10, and analyzed after filtration by reverse-phase HPLC [two pump system (Waters 510); detector: photodiode array (Waters

994), 220 nm; column: Macherey-Nagel cartridge, C18, 3 mm, 4 × 130 mm; eluent: chromatography grade acetonitrile (Baker) and water (Merck), 15–42% acetonitrile linear in 36 min, 0.45 ml/min; data analysis system: Maxima 820 data station]. Cardenolides were recognized by their UV spectra. For quantitative analyses, known volumes of secretion were processed and 2 mg ouabain was used as internal standard during HPLC analysis. Quantification of cardenolides was based on peak area measurements and expressed in milligram ouabain equivalents.

Alkaloid Extraction

Individual beetles previously kept frozen at −20°C were ground with quartz sand in 4 ml acidic MeOH (1% HCl) in a mortar for 10 min. After centrifugation the supernatant was divided into two aliquots, which were evaporated under reduced pressure. One aliquot was redissolved in 2 ml of dilute NH_4OH and directly applied to an Extrelut column (3 ml, Merck). PAs were eluted with 20 ml of CH_2Cl_2. After evaporation, the residue was redissolved in 0.1–0.5 ml MeOH. This extract provided the fraction occurring as tertiary PAs in the beetle. To obtain the fraction of total PAs (tertiary PAs and PA N-oxides), the remaining aliquot was redissolved in 2 ml of 0.1 M H_2SO_4 and mixed with Zn dust in excess. The mixture was stirred at room temperature for 4 hr, then the solution was made alkaline and treated as described above. Methanolic extracts of secretions were evaporated to dryness and the residue redissolved in 5 ml of 1 M HCl. Aliquots of 2 ml each were treated to reduce PA N-oxides in tertiary PAs as described above.

Plant leaves were preserved by freezing (−18°C) as soon as possible after harvesting in the field. Following freeze-drying, 0.5–1.0 g of the powdered material was extracted in 25 ml of 0.05 M H_2SO_4 for 3–4 min (Ultra-turrax) and left to stand for 30 min. After centrifugation, one part of the supernatant was adjusted to pH 11 with NH_4OH and applied to an Extrelut column as above. For total PA analysis, part of the acidic supernatant was adjusted to 0.25 M H_2SO_4, mixed with Zn dust, and further processed as described above.

Alkaloid Analysis

PAs were separated and analyzed quantitatively by capillary GC on quartz columns (WCOT, 15 × 0.25 mm DB-1, J & W Scientific) using a Hewlett Packard 5890 series II. Conditions: injector, 250°C; temperature program, 150–300°C at 6°C/min; split ratio, 1:20; injection vol., 1–2 μl; carrier gas, He 0.75 bar; detection, flame ionization and nitrogen detectors. Atropine was used as internal standard. The GC response factors of the major PAs analyzed in these experiments were shown to be almost identical. Retention indices were calculated from cochromatographed hydrocarbon standards.

GC-MS. A Carlo Erba Mega 5160 gas chromatograph, equipped with a quartz column (30 m × 0.32 mm) as specified above, was directly coupled to a quadripole mass spectrometer Finnigan MAT 4515. GC conditions were as specified above (Witte et al., 1993).

RESULTS

Species Secreting Cardenolides and No PAs. All species feeding on Apiaceae as well as those feeding exclusively on Cardueae secrete complex mixtures of cardenolides (Table 1). At least 14 cardenolides were observed in the secretion of *O. bidentata*, 15 in that of *O. coerulea*, 5 in *O. frigida*, 15 in *O. viridis*, and 19 in *O. virgulata*. The secretions of *O. coerulea* from both sites in the Black Forest are very similar, not only in the different compounds observed, but also in their proportions. By contrast, the secretion of *O. bidentata* differs markedly from that *O. coerulea* in the proportions of the constituents. The two species are morphologically very similar (Kühnelt, 1984), but the difference in the composition of the secretions confirms the separation into different species, as in general our analyses show that cardenolide mixtures offer discriminating taxonomic characters (Rowell-Rahier and Pasteels, 1994).

Interestingly, no PAs were observed in the secretion of *O. frigida*, although the beetles accepted *A. alliariae* in the laboratory. Either the beetle is unable to sequester PAs or it mainly fed on *M. athamanticum* in the field. Only a few beetles were available for study, and more experiments are needed to answer this question.

Similarly, PAs were not observed in the secretion of *O. melanocephala* feeding on *D. clusii*. Although PAs were observed in some *Doronicum* (e.g., *D. macrophyllum* and *D. pardalianches*) (Hartmann and Witte, 1994), no PAs were found in *D. clusii* (by TLC analysis of 2 g of dried leaves material). Intriguingly, no or only traces of cardenolides were observed in the few available secretions. More secretion of this rare species is needed for determining what it actually secretes.

Besides cardenolides, all these species may secrete ethanolamine and tyrosine betaine, as do the other previously studied *Oreina* (Pasteels et al., 1994). No attempt was made to identify unambiguously these constituents of unknown function, or to identify the structure of the different cardenolides detected in the secretions.

Species Secreting Sequestered PA N-Oxides and No Cardenolides. O. cacaliae is the only species that secretes host-plant-derived PAs but no autogenous cardenolides. Only PA N-oxides were observed in the beetles' secretions and stored in their bodies, confirming our previous observations (see above). For simplicity, PAs will be designated by their names, omitting the fact that they are in the form of N-oxides.

In Tschiertschen, the beetles were found on both *A. alliariae* and *S. nemorensis*, which grew in close proximity. The glands of wild caught specimens were experimentally emptied, and then the animals were fed for five days on one or the other host plant. After this feeding period, their secretions were collected and both the secretions and the beetles were analyzed for PAs (Table 2). The chemical defense of the beetles depended on the plant on which they fed, but did not exactly mirror food-plant chemistry. The beetles that fed on *A. alliariae* secreted and had in their bodies seneciphylline and senecionine, as did the beetles from the Vosges studied previously (Rowell-Rahier et al., 1991). However, contrary to the latter, they contained traces of other PAs, e.g., doronenine and bulgarsenine, which must have been acquired from *S. nemorensis* in the field. By contrast, beetles fed in the laboratory with *S. nemorensis* secreted

TABLE 2. RELATIVE ABUNDANCES OF PAS AND CONTENTS IN *O. cacaliae* AND ITS HOST PLANTS FROM TSCHIERTSCHEN

	Abundance (%)					
	O. cacaliae[a]					
	Fed on *A. alliariae*		Fed on *S. nemorensis*		Host plants	
	Secretion (N = 8)	Body (N = 3)	Secretion (N = 7)	Body (N = 3)	*A. alliariae*	*S. nemorensis*
Nemorensine						trace
Retroisosenine[b]	trace			trace		trace
Senecionine	20.9	10.7	trace	trace	3.4	
Seneciphylline	79.1	89.3	31.6	22.9	93.0	trace
Nemorensine isomer[b]						0.6
Doronenine	trace	trace	68.4	61.5		trace
Spartioidine + platyphylline[c]	trace	trace		trace	3.6	
Bulgarsenine	trace		trace	15.6		92.4
Intergerrimine					trace	
Neoplatyphylline				trace	trace	6.9
Total PAs μg/beetle; mg/g dry wt leaves	4.2	102.0	4.5	74.3	66.0	8.0

[a] The glands were emptied and the beetles fed for 5 days before the secretions were collected. N: number of pooled secretions or specimens.
[b] Tentative identification.
[c] Not separated in the analysis.

doronenine as the major constituent and also contained in their body significant amounts of bulgarsenine, the major constituent of *S. nemorensis*. Only traces of bulgarsenine were found in the secretions, however, and significant amounts of seneciphylline were observed in both bodies and secretions, possibly sequestered from *A. alliariae* in the field. These data demonstrate that the beetles selectively sequester (and probably metabolize to some extent) certain PAs more than others (Hartmann et al., in preparation). Heterogeneity in the plant community causes heterogeneity in beetle defense. It should be emphasized, however, that the analyses were performed on pooled secretions and the beetles fed for some time in the laboratory. Individual secretions and beetles from the field must be analyzed in order to perceive fully the variation in beetle defense due to plant diversity and heterogeneity.

Species Secreting Both De Novo Synthesized Cardenolides and Host-Derived PA N-Oxides. The mixed defensive strategy was observed in *O. speciosissima* as already reported (Rowell-Rahier et al., 1991), and also in *O. intricata* and *O. elongata* (Table 1). At least 13 cardenolides were observed in the secretion of *O. intricata*, but, as for all species secreting PAs, the presence of PA N-oxides interferes with the detection of cardenolides in HPLC. In Tschiertschen, the beetles fed mainly on *S. nemorensis*, and doronenine was found both in their secretion and their bodies (Table 3), as observed in *O. cacaliae* fed on *S. nemorensis*. However, when this beetle was observed on *A. alliariae* in the field, seneciphylline was sequestered in secretion and body. Both seneciphylline and doronenine were found in the body of one beetle. This beetle presumably had a mixed diet in the field. The body of another beetle contained 255.3 μg/g of seneciphylline and only traces of doronenine, showing that the PA composition may greatly depend on the diet in the field.

The mixed defensive strategy is best illustrated in *O. speciosissima* and *O. elongata*. *O. speciosissima* was observed in Tschiertschen mostly on *P. albus*, which is devoid of PAs in its leaves, but also to a lesser extent on *S. nemorensis* and *A. alliariae*. The beetles sequestered PAs from both *Adenostyles* and *Senecio*, suggesting that they had a mixed diet in the field, like *O. intricata* and probably *O. cacaliae*. In Zinal, *O. speciosissima* fed exclusively on *A. alliariae*. Preliminary analysis by HPLC of secretion from this location indicates that it contains, at best, traces of cardenolides, but the presence of sequestered PAs (characteristic of *A. alliariae*) was obvious.

Striking differences in chemical defense between populations as the result of local differences in the distribution of food plants were also observed in *O. elongata*. Beetles from Mattmark dam fed exclusively on *C. spinosissimum*; no other potential food plants were observed in the vicinity. As expected, they secreted only cardenolides (more than 15 compounds), as no PAs are present in *C. spinosissimum*. This patch of beetles probably has been isolated on *Cirsium* since the building of the dam in 1970. The beetles, however, have kept their

TABLE 3. RELATIVE ABUNDANCE OF PAs AND CONTENTS IN SECRETIONS AND BODIES OF *O. intricata* AND *O. speciosissima* FROM TSCHIERTSCHEN

	Abundance (%)[a]			
	O. intricata		*O. speciosissima*	
	Secretion (N = 8)	Body (N = 1)	Secretion (N = 12)	Body (N = 5)
Retroisosenine isomer[b]			trace	
Retroisosenine[b]		trace	trace	trace
Senecionine		trace	trace	
Seneciphylline	83.8	56.3	59.0	trace
Nemorensine isomer[b]		trace		
Doronenine	16.2	43.7	41.0	99.0
Spartioidine + platyphylline[c]	trace	trace	trace	trace
Bulgarsenine		trace	trace	
Neoplatyphylline		trace	trace	trace
Total content (μg/beetle)	7.4	25.0	8.3	25.3

[a] N: number of pooled secretions or specimens.
[b] Tentative identification.
[c] Not separated in the analysis.

ability to sequester PAs, as proved by feeding them on *A. alliariae* in the laboratory. In contrast, no cardenolides could be detected in the secretion of specimens from the Col du Lautaret, which fed on *A. alliariae*. The secretion contained, however, significant amounts of PAs (Table 4). When the beetles were fed for two weeks on *C. spinosissimum*, after their glands had been emptied before the experiment and again after one week, the renewed secretion at the end of the two weeks contained only PAs obviously mobilized from their body reservoir (Pasteels et al., 1992). Nevertheless, *O. elongata* from the Col du Lautaret do have the capacity to synthesize cardenolides, since cardenolides were found in their eggs and larvae (Dobler and Rowell-Rahier, 1994a). Finally, *O. elongata* collected in the Vallée des Merveilles on either *A. alliariae* or *C. spinosissimum* secreted both cardenolides and PAs. The beetles collected on *Cirsium* contained nearly as much PAs in their secretion and body as those collected on *Adenostyles*, demonstrating that they had also fed on *Adenostyles* (Table 4). A mixed diet in these beetles is not surprising, both plants are growing intimately intermixed in the field.

Interestingly, although the food plants of *O. elongata* contained seneciphylline as a major constituent (Table 5), the secretion and body of the beetles

TABLE 4. PA COMPOSITION AND CONTENT IN *O. elongata* AND IN ITS HOST PLANTS

	PA composition (%)[a]					
			Vallée des Merveilles			
	Col du Lautaret on *A. alliariae* and *A. alpina*, secretion (N = 19)	Petit St Bernard on *A. alliariae*, secretion (N = 8)	On *A. leucophylla*		On *C. spinosissimum*	
			secretion (N = 5)	body (N = 5)	secretion (N = 5)	body (N = 5)
Senecionine	9.8	43.6	56.0	11.2	66.8	11.9
Seneciphylline	58.8	34.5	14.5	41.1	13.3	43.3
Spartioidine + platyphilline[b]		9.4	trace	1.9	trace	trace
Intergerrimine		trace	trace	trace	trace	trace
Oreine	31.4	12.5	29.4	45.8	19.9	44.8
Total (μg/individual)	6.4	N.D.[c]	7.03	247.3	4.5	130.1
Concentration (M)	1.9×10^{-1}		1.4×10^{-1}		1.0×10^{-1}	

[a] N: number of pooled secretions or specimens.
[b] Not separated in the analysis.
[c] Not determined because of leachage during transport.

TABLE 5. PA COMPOSITION AND CONTENT IN LEAVES OF HOST PLANTS OF *O. elongata*

	PA composition (%)		
	A. alliariae	*A. glabra*	*A. leucophylla*
Senecionine	trace	trace	trace
Seneciphylline	92.0	94.8	96.5
Spartioidine + platyphylline[a]	8.0	5.2	3.5
Neoplatyphylline	trace		
Total (mg/g dry wt)	30.9	23.4	18.9

[a]Not separated in the analysis.

also contained significant amounts of senecionine and a new PA derivative, oreine (Table 4) (Hartmann et al., in preparation). A similar pattern of PAs was found in the secretion of beetles from a third population, collected in the Col du Petit St Bernard. This contrasts with the PA composition of secretion of *O. cacaliae* fed on the same host plant, where no oreine and less senecionine compared to seneciphylline was found. The amounts of PAs sequestered in the body of *O. elongata* are much larger than the amounts sequestered by the other *Oreina* species (compare data in Table 4 with those in Tables 2 and 3).

O. elongata collected in the Vallée des Merveilles on *Adenostyles* had an average of 3.5 μg (ouabain equivalent) of cardenolides/secretion ($N = 15$, pooled secretions); those collected on *Cirsium*, 2.7 μg ($N = 5$). This is somewhat less, but within the same order of magnitude, as the amount of PAs sequestered in the secretion (Table 4). However, sequestration of PAs in the glands seems to occur much faster than synthesis of cardenolides, as proved by the following experiment. The glands of beetles ($N = 10$) collected on *Adenostyles* were emptied. The beetles were then fed during nine days in the laboratory on *Cirsium* and their secretion collected again. The mean amount of cardenolides was only 0.04 μg (ouabain equivalent)/secretion, but that of PAs was 16.3 μg/secretion, although *Cirsium* does not contain PAs in its leaves. Clearly, PAs stored in the body can be mobilized in the glands before significant amounts of cardenolides could be synthesized.

DISCUSSION

Relation Between Host-Plant Use and Defensive Chemistry. All species studied here, except *O. cacaliae* and possibly *O. melanocephala*, secrete cardenolides. As in the other leaf beetles studied to date, complex mixtures of cardenolides are produced, the mixture being species specific.

PAs are sequestered from species of the tribe Senecioneae, and species feeding exclusively on Apiaceae or Cardueae only secrete autogenous cardenolides in addition to ethanolamine and tyrosine betaine. All species feeding on Senecioneae containing PAs seem able to sequester some plant PAs, sometimes after transformation. Only PA N-oxides are observed in beetles' secretions and stored in their bodies, confirming our previous observations (Ehmke et al., 1991).

Although ethanolamine and tyrosine betaine were not systematically searched for, so far tyrosine betaine was found in all species producing cardenolides with or without PAs, but is clearly absent in *O. cacaliae*, which does not secrete cardenolides. Ethanolamine was detected in all secretions in which it was looked for, not only in the genus *Oreina* (i.e., *O. cacaliae*, *O. gloriosa*, *O. speciosissima*), but also in the Chrysolinina and Doryphorina (Pasteels et al., 1988a, 1994).

Additional experiments are needed to determine if *O. frigida* and *O. melanocephala* conform to these general rules. First, *O. frigida* collected in the field did not secrete PAs, although it is able to feed on *Adenostyles* in the laboratory. The absence of PAs in this species could simply result from the fact that the few beetles collected did not feed on *Adenostyles* in the field or could be due to an inability to sequester. Second *O. melanocephala*, which fed on *Doronicum* devoid of PAs, did not secrete cardenolides or secreted only in trace amounts. More material of this rare species is needed for a better understanding of its secretion and for deciding with confidence if it has lost the ability to synthesize cardenolides, as did *O. cacaliae*.

Sources of Variation in Defensive Chemistry. As expected, important within-species differences are observed in those beetles that sequester PAs, because of local differences in the vegetation. The secretion of *O. cacaliae* from Tschiertschen contained a spectrum of PAs including compounds characteristic of both *A. alliariae* and *S. nemorensis*, whereas the secretion of specimens collected in the Vosges or the Black Forest contained only PAs from *A. alliariae* (Rowell-Rahier et al., 1991). In these localities, *S. nemorensis* is substituted by *S. fuchsii*, which, instead of macrocyclic PAs, contains open-chain mono- and diesters. The beetles from Tschiertschen sequestered PAs from *S. nemorensis* in both their bodies and secretions, whereas the beetles from the Vosges only sequestered PAs from *S. fuchsii* in their bodies, but not in their secretion. PAs from *A. alliariae* were sequestered in both the bodies and secretion of beetles from both localities.

Individual variation in sequestered defense within a population is also to be expected, since the various host plants with different secondary chemistry are distributed in patches of various sizes, and since beetles have low vagility for long periods of time (S. Knoll, unpublished observation). Our results indicate, however, that mixed diets occur in the field. Sequestration and storage of

large quantities of PAs for long periods occur in the body from which refilling the glands is possible. This could temper individual variation in host-derived defense (Pasteels et al., 1992).

Interpopulation variation in chemical defense is even more obvious in the beetles with the mixed defensive strategy, illustrating the flexibility of this mode of defense. In both *O. speciosissima* and *O. elongata*, populations were observed that practically only sequestered PAs or that secreted only autogenous cardenolides or that did both. Of course, this reflects local variation in host-plant distribution, but intrinsic genetic differences between beetles from distant populations adapted to local vegetation cannot be excluded, and has been suggested in *O. elongata* populations (Dobler and Rowell-Rahier, 1994b). As already stated, *Oreina* species form metapopulations and morphological differences between populations have been reported that justify for some taxonomists the subdivision of species into subspecies or even different species. For example, *O. speciosissima* from Zinal and other alpine populations differ from beetles from lower altitudes in details in their morphology to the point that they were considered as a distinct species: *O. perenei* (e.g., Freude et al., 1966). However, this distinction does not appear to be valid (for detailed taxonomic discussion, see Kühnelt, 1984). Morphological differences also exist between *O. elongata* from the Col du Lautaret, the Vallée des Merveilles, and Mattmark dam, possibly reaching subspecies level. The two first populations belong to *O. elongata occidentalis* Ruffo and the latter to *O. elongata ruffoi* Franz (e.g., Kühnelt, 1984).

The same exocrine glands that store and possibly synthesize autogenous cardenolides also store and possibly transform sequestered PAs. We do not know how these two processes interfere with each other, but it is tempting to speculate that sequestration competes with the secretion of cardenolides. At least in *O. elongata*, our data indicate that the refilling of the glands with sequestered PAs is much faster than with autogenous cardenolides. If sequestration competes with de novo synthesis, it could explain why beetles feeding exclusively for many generations on *Adenostyles* have their production of cardenolides suppressed. If this suppression becomes constitutive, a situation similar to that observed in *O. cacaliae* would be reached. This could occur by genetic drift the more easily as no selective force can act on the synthesis of cardenolides, which is not expressed anymore. Furthermore, experiments demonstrated that naïve red-winged blackbirds more often rejected *O. cacaliae*, which only sequesters PAs, than *O. gloriosa*, a species which is very similar in both size and color, but only produces cardenolides (Rowell-Rahier et al., 1995).

It would be too simplistic, however, to consider the production of both cardenolides and PAs as a transitory evolutionary step towards exclusive plant-derived defense. The advantages of one or the other defense is difficult to assess and depends not only on the distribution of natural enemies and their answer to

both kinds of compounds, but also on the distribution of plants and of their nutritional value. The mixed strategy is observed in three species, but only a single species relies solely on sequestered PAs.

Interspecific differences in host-derived defenses is, of course, even greater that intraspecific variation in both allopatric and sympatric situations. Not only do the different *Oreina* exhibit differences in their host-plant preferences, but they handle PAs from the same plant species in different ways. For example, *O. elongata* feeding on *A. alliariae* sequester higher proportions of senecionine compared to seneciphylline, than *O. cacaliae* or *O. speciosissima* feeding on the same plant, and above all produce significant quantities of oreine, which is not detected or is detected only as traces in the other species. Oreine is not present in *A. alliariae*, and it must be derived from seneciphylline (Hartmann et al., in preparation). Similarly, among PAs belonging to the same structural group (see Hartmann and Witte, 1994, for a classification of PAs) doronenine is the major PA found in *O. cacaliae*, *O. speciosissima*, and *O. intricata*, feeding on *S. nemorensis*. In *S. nemorensis*, the only possible source of such PAs, doronenine is not very abundant and bulgarsenine is far more abundant. Either these beetles are very selective in their sequestration, or they transform bulgarsenine into doronenine (Hartmann et al., in preparation).

Strong inter- and intraspecific variations in host-derived defense resulting from local heterogeneity in phytochemistry, differences in plant communities, and differences in processing plant compounds by insects are of course not surprising; they are already well documented in other insects, predominantly in butterflies and moths (Brower, 1970; Bowers, 1992; Rowell-Rahier and Pasteels, 1992; Hartmann and Witte, 1994). In *Oreina* species, the full description of allopatric and sympatric variations in host-derived defense requires further analysis of individual beetles freshly collected in the field. The real challenge, however, will be to evaluate the consequences for beetle fitness of this diversity and flexibility of defense.

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IDENTIFICATION OF FEMALE SEX PHEROMONE COMPONENTS OF RICE LOOPER, *Plusia festucae* (L.), (LEPIDOPTERA: NOCTUIDAE)

TETSU ANDO,[1,*] RYUTA OHNO,[1] TOMOYUKI KOKURYU,[1] ATSUNORI FUNAYOSHI,[1] and MASASHI NOMURA[2]

[1]*Department of Applied Biological Science, Faculty of Agriculture*
Tokyo University of Agriculture and Technology
Fuchu, Tokyo 183, Japan

[2]*Department of Bioproduction Science, Faculty of Horticulture*
Chiba University
Matsudo, Chiba 271, Japan

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Abstract—The rice looper, *Plusia festucae*, is a defoliator of the rice plant. Chromatographic behavior, chemical reactions, and GC-MS analyses of the female sex pheromone revealed that the main component was (*Z*)-5-dodecenyl acetate (Z5-12:OAc, component I). The GC-MS analysis also indicated that the pheromone gland extract included another three monounsaturated components, (*Z*)-5-dodecen-1-ol (Z5-12:OH, component II), (*Z*)-7-tetradecenyl acetate (Z7-14:OAc, component III), and (*Z*)-7-tetradecen-1-ol (Z7-14:OH, component IV) in the following ratio: I:II:III:IV = 100:6:15:1. In a paddy field, the mixture of synthetic I, II, and III in a ratio of 100:6:15 showed stronger attractancy than the virgin female, while the role of IV was unknown.

Key Words—Sex pheromone, lepidopterous pheromone, rice looper, *Plusia festucae*, (*Z*)-5-dodecenyl acetate, (*Z*)-5-dodecen-1-ol, (*Z*)-7-tetradecenyl acetate

INTRODUCTION

The rice looper, *Plusia festucae* (L.) (Lepidoptera: Noctuidae: Plusiinae), is a defoliator of rice plants. The larvae of the first generation appear in paddy fields in the Tokyo area from late May to mid-June, and the adult moths emerge from late June to July. Although traps baited with virgin females that were reared in

*To whom correspondence should be addressed.

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our laboratory attracted male moths in the paddy field in September, the larvae of the second generation could not be found on the leaves of rice plants in August (Kokuryu et al., unpublished). In order to reveal ecological aspects of this species, we have defined the female sex pheromone, which is expected to be a useful tool for monitoring the population of adult males.

METHODS AND MATERIALS

Insects. Larvae of *P. festucae* were collected in the experimental paddy field of Tokyo University of Agriculture and Technology (Fuchu-shi, Tokyo) during June 1993. Three generations were reared on the artificial diet prepared mainly from kidney bean powder for *Acanthoplusia agnata* (Kawasaki et al., 1987) at 25°C and on a 16:8-hr light–dark cycle. Abdominal tips containing the pheromone glands were removed from about 500 female moths (2–3 days old) 4 hr after the beginning of scotophase, and these were soaked in *n*-hexane for 30 min to extract the pheromone. In order to examine how the attractancy of the virgin female compares with the synthetic pheromone, the larvae were collected again in 1994 and reared on the same diet.

Electroantennogram (EAG) Method. EAG activities of the natural pheromone and synthetic compounds were measured by modifying the method described for the pheromone study of the rice green caterpillar (Ando et al., 1980; Roelofs, 1984). The head of a *P. festucae* male moth was also removed, and the responses produced on one of the two antennae were recorded with a handy recorder (model 8K30; Nippondenki-Sanei, Co., Tokyo, Japan) instead of a cathode ray oscilloscope.

Chemical Reactions with Pheromone Extracts. Crude extract from 50 virgin females was treated with $NaBH_4$ (5 mg) in 2-propanol (0.2 ml) for 3 hr at room temperature. After mixing with water (2 ml), crude products were extracted with *n*-hexane and the EAG activity was measured. Catalytic hydrogenation of the pheromone extract (50 females) was carried out with 10% Pd-C (2 mg) in *n*-hexane (0.5 ml) for 1 hr at room temperature. After filtration of the catalyst and evaporation of the solvent, the hydrogenated products were used for the EAG assay. Another pheromone extract from 100 females 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. Half of this extract was offered in the EAG assay, and the remainder was treated with pyridine (0.1 ml) and acetic anhydride (0.1 ml), after removing the *n*-hexane. This mixture was added to water (1 ml), and reacetylated product, which was extracted with *n*-hexane, was offered in the EAG assay. To determine the double bond position of the pheromone, ozone was added to a *n*-hexane solution (50 μl) of the crude extract (100 females), which was cooled in a dry ice–acetone bath. An aliquot of this solution was analyzed by GC-MS to determine

the thermal decomposition products from the ozonide of the pheromone components.

Chromatography. EAG activities of the fractions obtained by TLC of 50 female extracts on a normal-phase plate (silica gel, Merck GF_{254}, 0.25 mm) using *n*-hexane–benzene (1 : 10) were measured. Under these conditions, R_f values of authentic compounds, (*Z*)-5-dodecenyl acetate (Z5-12 : OAc)** and the parent alcohol, were 0.67 and 0.16, respectively. Another 50 female extracts were injected into a reverse-phase HPLC column (Deverosil ODS-5, 8 mm ID × 15 cm, Nomura Chem. Co., Seto-shi, Japan), using an acetonitrile–water (97 : 3) solvent system at a flow rate of 1.5 ml/min, and the elutions were collected every minute to measure the EAG activity. Under these HPLC conditions, authentic compounds showed the following R_t: Z5–10 : OAc 6.0 min; Z5–12 : OAc 7.8 min; Z7–14 : OAc 14.1 min; Z11–16 : OAc 21.3 min.

GC-MS Analyses. Electron impact (EI) GC-MS was achieved using a JEOL JMS-SX102A mass spectrometer with DB-23 and DB-1 capillary columns (0.25 mm ID × 30 m, J & W Scientific). The former column was used for the natural pheromone and synthetic standards, and the column temperature program was 80°C for 1 min and 8°C/min to 210°C. The latter column was used for the ozonolysis products, and the temperature program was 40°C for 1 min, 50°C/min to 150°C, and finally 8°C/min to 300°C. The ionization voltage of every measurement was 70 eV. The ion source temperature was 240°C. The spectra (*m/z* 50–800) were measured twice per minute in a cyclic scan mode lasting from 5 to 20 min after the sample injection.

Field Tests. Sticky-type traps (30 × 27 cm bottom plate with a roof, Takeda Chemical Ind., Ltd., Osaka, Japan) were set at a height of 0.4 m in the paddy field where the larvae were collected. Synthetic chemicals (>97% purity) were applied to natural rubber septa [8 mm OD, white rubber (Aldrich)] as dispensers, which were set at the center of the traps and used without renewal during the experiments. Attractancy of the female for the male moths was examined using the trap baited with two virgin females in a plastic cup with wire-screen windows (3 × 5 cm). The females were replaced every day.

RESULTS

EAG Analyses. The chemical and chromatographic characteristics of the *P. festucae* pheromone were examined using an EAG technique as the bioassay. Reduction with $NaBH_4$ had little effect on the EAG activity of the crude extract. On the other hand, the activity of the crude extract was completely lost by catalytic hydrogenation, but not completely lost by treatment with methanolic potassium hydroxide. Acetylation of the crude alkaline hydrolyzed mixture with

**Other monoene compounds are abbreviated in the same manner (Arn et al., 1992).

acetic anhydride–pyridine restored the original activity. Furthermore, on preparative TLC with a normal-phase plate, an acetate fraction (R_f 0.6–0.8) showed the strongest EAG activity. The activity of an alcohol fraction (R_f 0.1–0.2) was weak but not negligible, and no other fractions showed remarkable activities. It has been reported that reverse-phase chromatography is available to examine the carbon length of straight-chain compounds (Ando et al., 1986). Strong activity was observed in a fraction (R_t 7–9 min), from which C_{12} monoene acetates were recovered. Figure 1 shows the EAG profiles of a series of C_{12} monoene acetates. Dodecenyl acetate with a (*Z*)-double bond at the 5 position produced the strongest response of the tested compounds. These experiments indicate that the main component of the female pheromone is Z5–12:OAc.

Identification by GC-MS. A GC-MS analysis of one female sex pheromone extract from *P. festucae* on a polar capillary column (DB-23) produced the total ion chromatogram (TIC) shown in Figure 2A. This analysis covers the volatility range of C_{10}–C_{16} alcohols and their derivatives (acetate and aldehyde). Monoene acetates and alcohols with straight chains of the same length showed characteristic fragment ions ($[M-AcOH]^+$ and $[M-H_2O]^+$]) with the same mass number after the elimination of the acetic acid and water, respectively, from molecular ions. Figures 2B–E are mass chromatograms to detect the monoene compounds that monitor fragment ions at *m/z* 222 for C_{16}, *m/z* 194 for C_{14}, *m/z* 166 for C_{12}, and *m/z* 138 for C_{10} chain compounds. Mass chromatogram monitoring of an ion at *m/z* 61 (Figure 2F) was performed to detect acetates. As shown in Figure 2 the pheromone extract included four monoene compounds, C_{12} acetate (component I, R_t 10.91 min), C_{12} alcohol (component II, R_t 11.19 min), C_{14} acetate (component III, R_t 13.10 min), and C_{14} alcohol (component IV, R_t 13.76 min), in a ratio of 100:6:15:1. GC-MS analysis of a mixture of the natural pheromone and synthetic standards showed that the most abundant component (I) had the same R_t and mass spectrum as Z5–12:OAc, as shown in Figure 3.

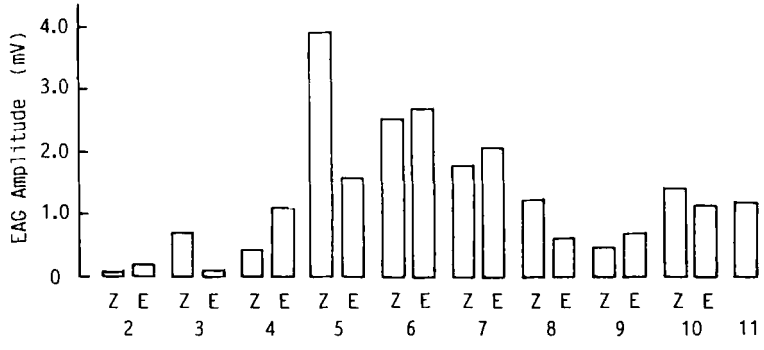

FIG. 1. EAG responses of male *P. festucae* to 1 μg of C_{12} monoene acetates.

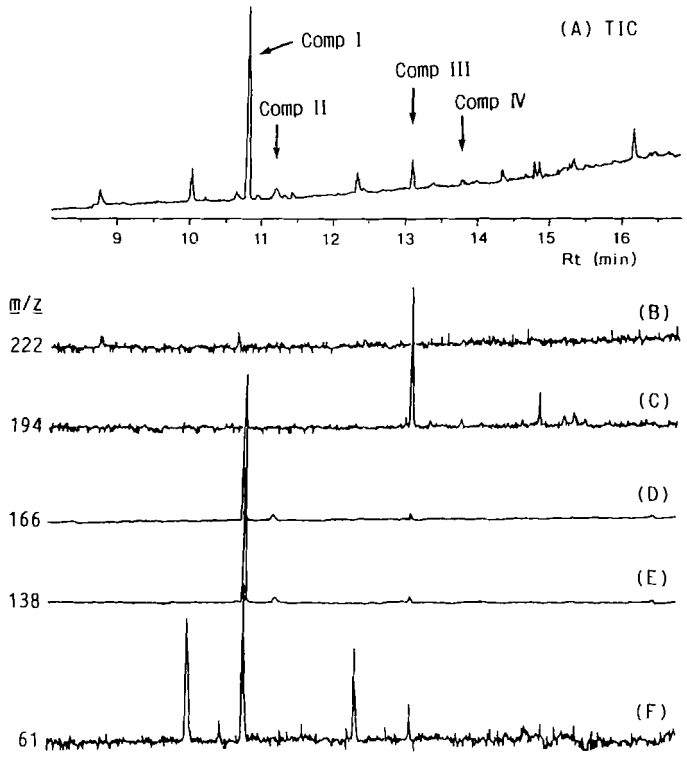

FIG. 2. GC-MS analysis of the pheromone gland extract (one female equivalent) of *P. festucae*. (A) Total ion chromatogram (TIC); (B–F) mass chromatograms monitoring the fragment ions at *m/z* 222, 194, 166, 138, and 61.

The peak height of component I in the TIC (Fig. 3A) indicates that one female possessed ca. 20 ng of Z5–12:OAc. The double-bond positional isomer Z7–12:OAc, a common pheromonal component of Plusiinae pheromones (Arn et al., 1992), had a different R_t (11.06 min) and spectrum and was not detected in the *P. festucae* pheromone extract. Further cochromatographic experiments revealed that the synthetic Z5–12:OH, Z7–14:OAc, and Z7–14:OH had the same R_t as components II, III, and IV, respectively. No other related components were observed in the extract, while the GC-MS data were examined in detail by mass chromatogram, which monitored $[M\text{-AcOH}]^+$ for saturated acetates; $[M-H_2O]^+$ for saturated alcohols, saturated aldehydes, and monoene aldehydes; and molecular ions $[M]^+$ for diene compounds.

The double bond position of component I was furthermore confirmed by an ozonolysis experiment with the pheromone extract. GC-MS analysis of the

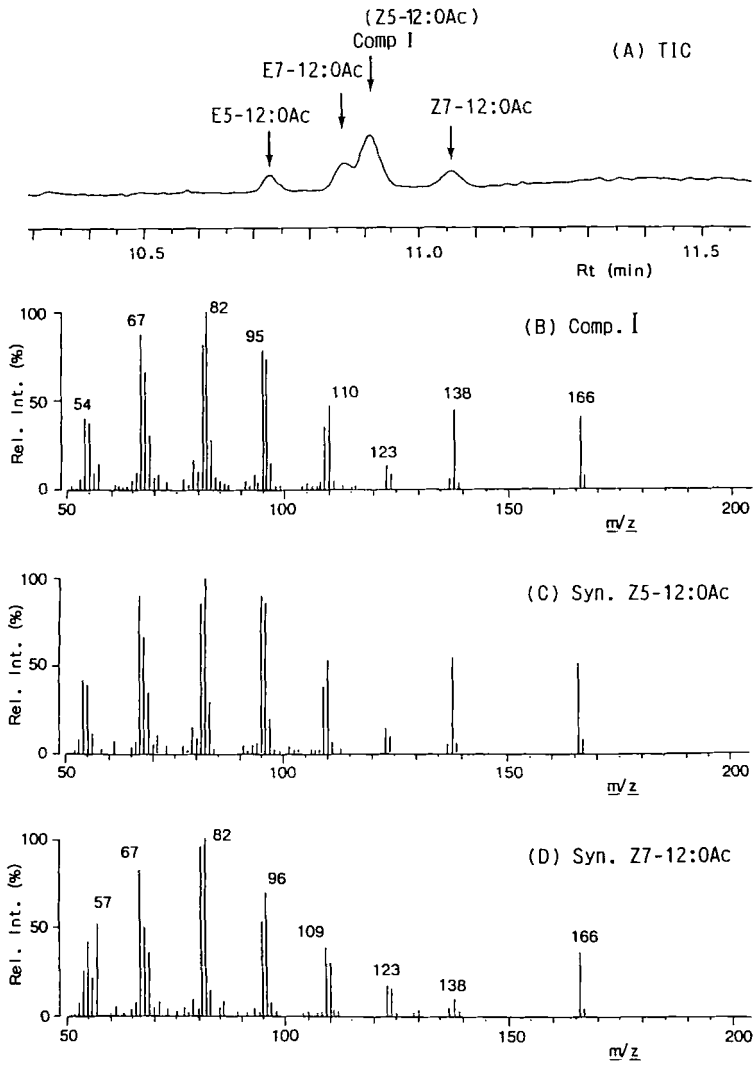

FIG. 3. GC-MS analysis of *P. festucae* pheromone and synthetic standards. (A) TIC of pheromone gland extract (one female equivalent) mixed with some synthetic monoene acetates (10 ng each); (B) mass spectrum of I; (C) mass spectrum of synthetic Z5-12:OAc; (D) mass spectrum of synthetic Z7-12:OAc.

crude products showed 5-acetoxypentanal (R_t 3.54 min) with diagnostic fragment ions at *m/z* 101, 87, and 73. No other acetoxyaldehydes were detected in the ozonolysis products.

Average Fitness Indexes. Kuwahara et al. (1986) proposed an identification method for a double-bond position using the newly devised average fitness index, which was computed with the mass spectral data of a series of synthetic isomers. The fitness index was calculated by dividing the absolute difference between the intensity ratio of the fragment ion-pair of a synthetic isomer and the corresponding ratio of the natural pheromone by the latter. All the index values were summed from each isomer and averaged to determine the similarity to the pheromone. The smallest average among the isomers indicates the best fit to a natural pheromone. Based on the published data (Horiike et al., 1981; Horiike and Hirano, 1982, 1984), the smallest values of the indexes for components I, II, and III were obtained with Z5–12 : OAc (19.6), Z5–12 : OH (76.3), and Z7–14 : OAc (41.6), respectively (Table 1). Since the published data were

TABLE 1. AVERAGE FITNESS INDEXES OF NATURAL PHEROMONE COMPONENTS (I–III) TO SYNTHETIC MONOENES[a]

Pheromone component	Position of (Z)-double bond						
	5-ene	6-ene	7-ene	8-ene	9-ene	10-ene	11-ene
I (C_{12} acetate)[b]	19.6 (5.0[f])[e]	36.6 (41.1)[e]	62.7 (38.9)[e]	38.3	53.3	73.8	57.1
II (C_{12} alcohol)[c]	76.3 (13.1)[e]	79.6 (14.1)[e]	83.1	80.3	101.5	123.6	96.8
III (C_{14} acetate)[d]	44.1	56.1	41.6 (10.3[g])[e]	62.9	59.9	41.8 (34.2)[e]	81.0

[a] Fitness index (%) = |(ratio of an ion-pair/corresponding ratio of the pheromone) − 1| × 100 (Kuwahara et al., 1986). Mass spectra of the natural pheromone components were measured by a JEOL JMS-SX102A mass spectrometer operated at 70 eV ionization voltage.

[b] Calculated using the published ratios (Horiike and Hirano, 1982) of the following seven ion pairs [(*m/z*)/(*m/z*)] by a Shimadzu-LKB 9000 spectrometer at 70 eV; 54/55, 55/68, 67/68, 68/95, 81/82, 81/95, and 95/96.

[c] Calculated using the published ratios (Horiike and Hirano, 1984) of the following eight ion pairs [(*m/z*)/(*m/z*)] by a Shimadzu-LKB 9000 spectrometer at 70 eV; 54/69, 55/67, 56/83, 68/81, 68/82, 69/81, 81/82, and 67/68.

[d] Calculated using the published ratios (Horiike et al., 1981) of the following six ion pairs [(*m/z*)/(*m/z*)] by a Shimadzu-LKB 9000 spectrometer at 50 eV; 67/68, 68/96, 95/96, 67/82, 55/82, and 109/110.

[e] Calculated using the spectral data of the natural and synthetic compounds measured by the same instrumental condition.

[f] Significant at the 1% level by the *t* test.

[g] Significant at the 5% level by the *t* test.

measured with different instrumental conditions from those for the natural pheromone, these smallest indexes are still rather large. The values in parentheses in Table 1, which were calculated with data measured by the same instrument, were much smaller than those calculated with the published data. Because of the spectral similarity of Z5-12:OH and Z6-12:OH, determination of the double-bond position of component II was difficult even though the indexes in parentheses (13.1 and 14.1) were compared each other. The R_t of Z6-12:OH (11.28 min) disqualified this candidate. With component III, the index of Z7-14:OAc (41.6) was almost the same as that of Z10-14:OAc (41.8), but the difference between their indexes increased until they were large enough to be distinguished from each other (10.3 and 34.2), when the calculation used data that were measured with the same instrument. The amount of component IV was so small in the pheromone extract that the mass spectrum was not available for calculating the indexes.

Field Attraction. In order to examine whether the four compounds are real pheromone components, their attractive activities were evaluated in a paddy field. Table 2 shows results of the field tests with the synthetic pheromones carried out in the suburbs of Tokyo from June to July 1994. A rubber septum containing a mixture of Z5-12:OAc (I), Z5-12:OH (II), and Z7-14:OAc (III) in a ratio of 100:6:15 attracted male moths of *P. festucae* more strongly than two virgin females. The trap baited only with Z5-12:OAc, a major component of the natural pheromone, caught no males, and Z7-14:OAc, one of the two

TABLE 2. CAPTURE OF MALE *P. festucae* WITH VARIOUS COMBINATIONS OF PHEROMONE GLAND COMPONENTS (I-IV)[a] IN 1994

Lure components I + II + III + IV (μg)	Cumulative captures[b] and significance[c]	
	June 25-29[d]	July 2-15
1000 + 0 + 0 + 0	0 c	0 c
1000 + 60 + 0 + 0	0 c	0 c
1000 + 0 + 150 + 0	8 b	39 b
1000 + 60 + 150 + 0	19 a	108 a
1000 + 60 + 150 + 10	17 a	99 a
0 + 0 + 0 + 0	0 c	0 c

[a] I = Z5-12:OAc; II = Z5-12:OH; III = Z7-14:OAc; IV = Z7-14:OH.
[b] Two traps for each combination.
[c] Numbers within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple range test.
[d] A trap baited with two virgin females captured five males in this period.

minor components, was shown to be indispensable for the attraction. Z5-12:OH, another minor component, had an important role in strongly attracting the males. Z7-14:OH (IV) is not necessary for attraction.

DISCUSSION

Chromatographic behavior, chemical reactions, and GC-MS analyses of the pheromone gland extract conclusively show that the *P. festucae* female pheromone is composed of Z5-12:OAc, Z5-12:OH and Z7-14:OAc. This conclusion was confirmed by a field test in a paddy field. The attractancy of the mixture in a ratio found in the extract (100:6:15) was stronger than that of virgin females. The role of Z7-14:OH, the most minor component identified so far, was unknown because the activity of three-component lure did not increase after the addition of this forth component.

To date, sex pheromones of 13 species in the subfamily Plusiinae have been identified (Arn et al., 1992). Nine species among the above insects secrete Z7-12:OAc or Z7-12:OH as a major component of their sex pheromones, and only one species, *Autographa nigrisigna* Walker, utilizes Z5-12:OAc. *P. festucae* is the second species in this subfamily, the pheromone of which is constituted mainly of Z5-12:OAc. Considering that *A. nigrisigna* males were attracted to the lure baited with Z5-12:OAc, Z7-12:OH, Z7-12:OAc, and Z7-14:OAc in a ratio of 100:62:2:4 (Sugie et al., 1991), Z7-14:OAc seems to be another key component of the Plusiinae pheromones.

Chemical analysis of the double-bond position of a minor component is difficult. The positional isomers are, however, distinguishable by carefully examining the relative intensities of some fragment ions in their EI-MS without any chemical modifications. When a mass spectrum of the minor component is recorded with no confusing background, the identification is easily accomplished based on the chromatographic and spectral data measured with a capillary GC-MS and comparing them with those of a series of authentic compounds. Even if the authentic standards are not available, the double-bond positions can be estimated using the average fitness indexes calculated with the published spectral data. In these experiments, structures of II and III were focused in an interesting way by the data measured with a different mass spectrometer.

We are now investigating *P. festucae* using the pheromone traps, and the results will be reported elsewhere.

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MAMMALIAN EXOCRINE SECRETIONS[1]: IX. CONSTITUENTS OF PREORBITAL SECRETION OF ORIBI, *Ourebia, ourebi*

W.-P. MO, B.V. BURGER,* M. LeROUX, and H.S.C. SPIES

Laboratory for Ecological Chemistry
Department of Chemistry
University of Stellenbosch
Stellenbosch 7600, South Africa

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Abstract—Using gas chromatography–mass spectrometry in conjunction with ancillary techniques such as chemical ionization with different reactant gases, determination of the position of double bonds by means of dimethyl disulfide derivatization, and finally gas chromatographic and mass spectrometric comparison with authentic synthetic material, 75 constituents were identified in the preorbital secretion of the male oribi, *Ourebia ourebi*. The secretion contains compounds with long-chain, unbranched structures similar to those found in many other preorbital secretions but with a finite volatility range, in contrast to the seemingly endlessly increasing chain lengths typical of other preorbital secretions.

Key Words—*Ourebia ourebi*, Bovidae, mammalian semiochemicals, mammalian pheromones, exocrine secretion, preorbital secretion, dimethyl disulfide derivatization, skipped dienes, chemical-ionization mass spectrometry, NMR.

INTRODUCTION

Cutaneous glands producing odoriferous substances that act as semiochemicals occur in most mammalian species. They are common in carnivores, rodents, and in most ungulate groups, but are also found in many other orders (Schaffer, 1940). The glands may be situated in many different regions of the body, and they may have different functions. For example, the scent glands described in

*To whom correspondence should be addressed.

[1] The heading of the series has been changed as the semiochemical activity of these secretions cannot always be demonstrated.

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the black-tailed deer, *Odocoileus hemionus columbianus*, include preorbital (Albone, 1984), interdigital, metatarsal (Müller-Schwarze, 1971), and tarsal glands (Müller-Schwarze, 1971; Müller-Schwarze and Müller-Schwarze, 1975).

Volatile substances may be released into the air by diffusion from the gland, but in most cases described, objects are marked with secretions from these glands. Scent marking with glandular exudate is often associated with territorial behavior (Eisenberg and Kleiman, 1972) and many bovid species utilize secretions from their preorbital glands for this purpose.

The exocrine secretions of several South African antelope species have been studied in considerable detail in the Laboratory for Ecological Chemistry at the University of Stellenbosch. Some of them, such as the grysbok and the grey duiker cause considerable damage in vineyards, orchards, and pine plantations in parts of South Africa (Bigalke, 1974). A research program was therefore initiated to identify the semiochemicals produced in these preorbital glands and to investigate the feasibility of reducing damage by the use of synthetic pheromones. The first stage of the program was the chemical characterization of the preorbital gland secretion of the grysbok. Although the ritual involved in marking objects with preorbital gland secretions undoubtedly plays an important part in the behavior of male grysbok, no experimental evidence could be found that the olfactory stimuli released from the deposited material serve as deterrents signalling territorial occupancy to other males (Novellie et al., 1984).

Preliminary behavioral studies with other antelope species indicated that the response-guided strategy (Albone, 1984) was unlikely to lead to a complete understanding of the function of the various constituents of the preorbital secretions of these animals in their territorial and social behavior, and it became clear that more background information would be needed on the preorbital secretions from individual conspecific animals, as well as from related and unrelated species.

One of the species selected for this purpose is the oribi, *Ourebia ourebi*, which belongs to the tribe Raphicerini. Other members of this tribe are the grysbok, *Raphicerus melanotis*, and the steenbok, *R. campestris*. The oribi normally inhabits grasslands, open plains, and thickly bushed country (Kenmuir and Williams, 1975) and is found in the eastern Cape, Natal, southeastern Transvaal, Mozambique, and southeastern Zimbabwe (Walker, 1982). They are normally seen in pairs or in small groups (Kenmuir and Williams, 1975).

There are six scent glands on the oribi's body, namely preorbital glands, tracts of glandular skin on the forelegs covered with long hair and on the hindlegs covered with shorter brushes, inguinal glands, pedal glands, as well as ear glands (Kingdon, 1982). In the oribi, marking with the preorbital glands is associated mainly with territorial and sexual activity (Kingdon, 1982). Only males mark with their well-developed preorbital glands (Smithers, 1986), while females induce the male's marking behavior (Kingdon, 1982). The male oribi's preor-

bital secretion consists of a mucoid emulsion containing black insoluble material and small quantities of volatile organic compounds.

In this paper, the identification of the volatile organic constituents of the preorbital gland secretion of the oribi, and the synthesis of some of them, are reported.

METHODS AND MATERIALS

General. All Pyrex glassware used in the handling of biological material and extracts, as well as in the preparation of reference compounds, was heated to 500°C in an annealing oven to remove any traces of organic material. Dichloromethane (Merck, Residue Analysis Grade) was analyzed gas chromatographically and found to be pure enough for extraction purposes when used in small quantities. Syringes, stainless-steel needles, etc., were cleaned by rinsing with this solvent.

Analytical Methods. Gas chromatographic (GC) analyses were carried out with Carlo Erba 4200 and 5300 gas chromatographs equipped with flame ionization detectors, Grob split-splitless injectors, and glass columns coated by the Laboratory for Ecological Chemistry with a 0.25-μm film of the apolar stationary phase PS-089, a silanol-terminated 95% dimethyl-5% diphenylsiloxane copolymer. Helium was used as carrier gas at a linear velocity of 28.6 cm/sec at 40°C. The flame ionization detector was operated at 280°C and the injector was normally used at 220°C. Samples were injected in the split mode, and the volatiles entering the column were thermally focused at ca. 30°C and subsequently analyzed using a temperature program of 2°C/min. For the analysis of synthetic products and intermediates, a 4°C/min programming rate was used. Methyl heptanoate was used as an internal standard in a quantitative analysis of the preorbital secretion.

Electron impact (EI) mass spectra were recorded at 70 eV on a Carlo Erba QMD 1000 GC-MS instrument, using the column and conditions described above. Chemical ionization (CI) mass spectra were obtained with methane and nitric oxide as reactant gases.

^{1}H and ^{13}C NMR spectra of synthesized compounds were obtained on a Varian VXR-300 spectrometer operating at 299.9 MHz for the observation of ^{1}H and at 75.42 MHz for the observation of ^{13}C, respectively. All spectra were recorded at 25°C. ^{13}C Multiplicities were determined by means of the APT technique. Two-dimensional ^{1}H–^{1}H correlation spectra (COSY) were obtained using the pulse sequence: D1-90°-(t1 + D3)-60°-AQ (Bax et al., 1981), with D3 = 0.06 sec to enhance long-range coupling effects. Two-dimensional ^{1}H–^{13}C correlation spectra (HETCOR) were obtained by means of the pulse sequence as described previously (Wilde and Bolton, 1984). Where necessary

^{13}C assignments were verified by means of a long-range ^{1}H–^{13}C heteronuclear correlation experiment (Reynolds et al., 1989). The ^{13}C spectrum of the whole, unprocessed secretion was obtained using a 67° pulse angle and a pulse repetition time of 0.8 sec. A total of 29,600 transients were accumulated. The ^{13}C chemical shifts were measured in benzene-d_6 as lock solvent relative to tetramethylsilane as internal reference.

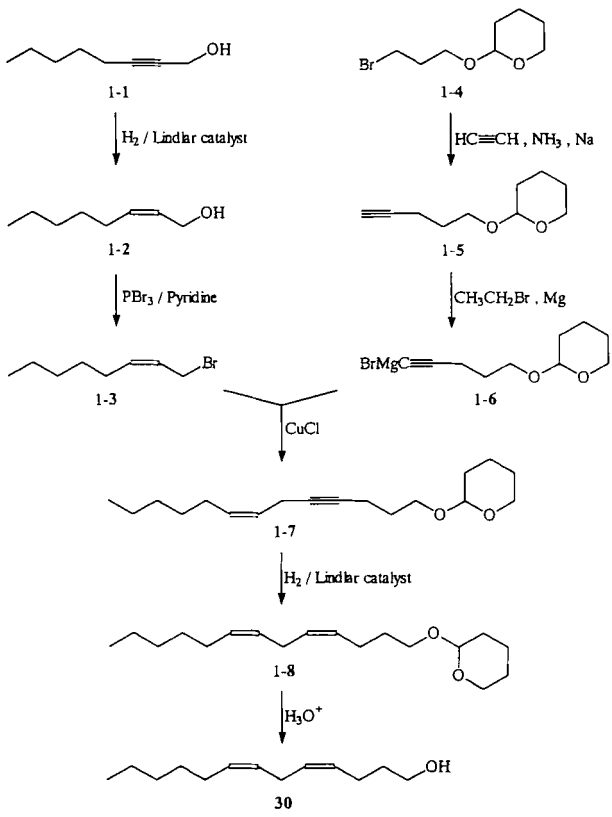
Infrared spectra were recorded on a Mattson Galaxy Series FT-IR 3000 instrument, equipped with a Roland DXY-1100 plotter.

Collection and Sample Preparation. Preorbital gland secretion was collected from a netted, sexually mature male animal by scooping the exudate from the preorbital cavity with a tubular PTFE scoop fitted with a PTFE plunger with which the material was ejected from the scoop into a Reacti-Vial. The organic volatiles were extracted by stirring each collected sample with dichloromethane (ca. 100 μl), using a thin (0.5 mm) glass rod to produce a homogeneous suspension, centrifuging the suspension at 2000–3000 rpm for ca. 15 min, removing the dichloromethane extract from underneath the supernatant water and mucus layer with a 100-μl syringe, and transferring it to a clean Reacti-Vial. Most extracts could be used without subsequent concentration. When more concentrated solutions were required, the solvent was removed in a slow stream of purified (activated charcoal) nitrogen.

Dimethyl Disulfide Derivatization. The derivatization was carried out using two methods. Following the method described by Buser et al. (1983), an aliquot (10 μl) of a dichloromethane extract of the preorbital secretion was concentrated in a 1-ml Reacti-Vial using a slow stream of purified nitrogen. The residual material was dissolved in hexane (50 μl) and treated with 5 μl of iodine solution (60 mg/ml of diethyl ether) and 50 μl of dimethyl disulfide (DMDS). The Reacti-Vial was sealed using a PTFE-faced rubber septum and the reaction mixture left at 40°C for 15 hr in the oven of a gas chromatograph. The reaction was quenched with an aqueous solution of sodium thiosulfate (5%). The organic and aqueous layers were separated by centrifuging for a few minutes at 2000 rpm, after which the organic layer was transferred to a clean Reacti-Vial. The solution was concentrated to 5 μl for GC-MS analysis. In the alternative method, described by Vincenti et al. (1987), a similar procedure is employed except for the substitution of hexane with carbon disulfide as solvent, a higher reaction temperature (60°C), and a longer reaction time (40 hr).

Reference Compounds. Compounds required for comparison with constituents of the preorbital secretion that are not commercially available were synthesized from authentic starting materials.

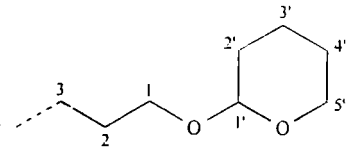
(4*Z*,7*Z*)-4,7-Tridecadien-1-ol, **30,** was synthesized according to Scheme 1. Partial hydrogenation of 2-octyn-1-ol 1-**1** (Taylor and Strong, 1950) with Lindlar catalyst (Augustine, 1965) gave pure (*Z*)-2-octen-1-ol 1-**2** in 80% yield; bp 97–



SCHEME 1. Synthesis of (4Z,7Z)-4,7-tridecadien-1-ol.

98°C (14 mm Hg); ^{13}C NMR ($CDCl_3$): Table 1. Bromination of the octenol 1-**2** with phosphorus tribromide in the presence of pyridine (Taylor and Strong, 1950) gave (Z)-1-bromo-2-octene 1-**3**, containing 9.8% of the *E* isomer (GC-MS), in 81% yield; bp 89–90°C (16 mm Hg). 5-Tetrahydropyranyloxy-1-pentyne 1-**5** was synthesized from 1-bromo-3-tetrahydropyranyloxypropane 1-**4** and sodium acetylide according to the method of Vaughn et al. (1937) in 60% yield; bp 116–118°C (28–30 mm Hg); ^{13}C NMR ($CDCl_3$): Table 1. Coupling of the bromooctene 1-**3** with the acetylenic Grignard reagent 1-**6** in the presence of CuCl, according to the method described by Osbond et al. (1961), gave 1-tetrahydropyranyloxy-7-tridecen-4-yne 1-**7** in 72% yield; ^{13}C NMR ($CDCl_3$):

TABLE 1. ^{13}C NMR SPECTRAL ASSIGNMENT FOR SYNTHETIC INTERMEDIATES IN SCHEME 1



Position	Compounds		
	1-2	1-5	1-7
1	58.55(t)	65.78(t)[a]	65.78(t)
2	133.10(d)i[b]	28.75(t)[a]	28.76(t)
3	128.46(d)i[b]	15.36(t)[a]	15.37(t)
4	27.43(t)	83.95(s)	83.93(s)
5	29.32(t)	68.49(d)	79.22(s)
6	31.45(t)		17.19(t)
7	22.54(t)		124.98(d)
8	14.04(q)		131.31(d)
9			27.10(t)
10			29.23(t)
11			31.49(t)
12			22.57(t)
13			14.06(q)
1'		98.79(d)	98.78(d)
2'		30.69(t)[a]	30.69(t)
3'		19.53(t)[a]	19.52(t)
4'		25.53(t)	25.53(t)
5'		62.15(t)[a]	62.14(t)

[a] According to HETCOR.
[b] i denotes interchangeable assignments.

Table 1. Partial hydrogenation of 1-tetrahydropyranyloxy-7-tridecen-4-yne with Lindlar catalyst followed by deprotection of the resulting skipped diene derivative with hydrochloric acid (Le Roux, 1980), gave 5.84 g (38% overall yield from 1-**1**) of a mixture of 4,7-tridecadien-1-ol isomers containing 32% of the required Z,Z isomer **30**. Chromatography on 7% $AgNO_3$-coated silica gel gave pure (GC) (4Z,7Z)-4,7-tridecadien-1-ol **30**: ^{13}C NMR ($CDCl_3$): Table 2.

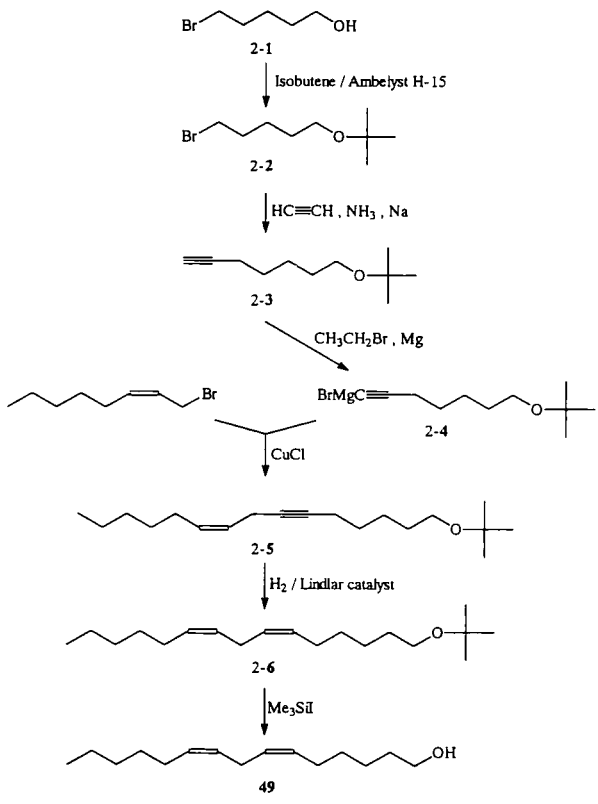
(6Z,9Z)-6,9-Pentadecadien-1-ol, **49**, was prepared according to Scheme 2. Treatment of 5-bromo-1-pentanol 2-**1** with isobutene and Amberlyst H-15 as catalyst (Alexakis et al., 1988) gave 5-bromo-1-*tert*-butoxypentane 2-**2** in quan-

TABLE 2. ^{13}C NMR SPECTRAL ASSIGNMENT FOR SYNTHETIC ALKADIENOLS

Position	Compounds		
	30	**49**	(**9Z**, **12Z**)-9,12-Octadecadien-1-ol
1	62.55(t)	62.98(t)	63.06(t)
2	32.55(t)	32.70(t)	32.83(t)
3	23.60(t)	25.41(t)	25.77(t)
4	129.17(d)i[a]	29.45(t)	29.53(t)i
5	127.70(d)i	27.18(t)i	29.43(t)i
6	25.62(t)	129.80(d)ii	29.27(t)i
7	128.92(d)i	127.80(d)iii	29.68(t)ii
8	130.44(d)	25.66(t)	27.25(t)
9	27.24(t)	128.20(d)iii	130.13(d)iii[b]
10	29.34(t)	130.25(d)ii	127.96(d)iv[b]
11	31.54(t)	27.24(t)i	25.67(t)[b]
12	22.59(t)	29.35(t)	128.03(d)iv[b]
13	14.07(q)	31.54(t)	130.22(d)iii[b]
14		22.58(t)	27.25(t)
15		14.06(q)	29.38(t)ii
16			31.56(t)
17			22.60(t)
18			14.08(q)

[a]i–iv denote interchangeable assignments.
[b]According to HETCOR.

titative yield; ^{13}C NMR ($CDCl_3$): Table 3. 1-*tert*-Butoxy-6-heptyne 2-**3** was synthesized from 2-**2** in a manner analogous to the preparation of 1-**5** in 97% yield; ^{13}C NMR ($CDCl_3$): Table 3. 1-*tert*-Butoxy-(*Z*)-9-pentadecen-6-yne 2-**5** was prepared from the acetylenic Grignard reagent 2-**4** and 1-bromo-2-octene by the procedure used for the preparation of 1-**7** in 87% yield; ^{13}C NMR ($CDCl_3$): Table 3. Partial hydrogenation of 2-**5** with Lindlar catalyst gave 1-*tert*-butoxy-6,9-pentadecadiene as a mixture of *Z,Z*, *Z,E*, *E,Z*, and *E,E* isomers containing 62% of the *Z,Z* isomer 2-**6**; ^{13}C NMR ($CDCl_3$): Table 3. Deprotection of the mixture of 1-*tert*-butoxy-6,9-pentadecadiene isomers with Me_3SiI according to the method of Jung and Lyster (1977), proceeded quantitatively to give 3.40 g of the isomeric 6,9-pentadecadienols in an overall yield of 68% from the starting compound 5-bromo-1-pentanol 2-**1**. Column chromatography on 7% $AgNO_3$-coated silica gel gave the pure (6*Z*,9*Z*)-6,9-pentadecadien-1-ol **49**; ^{13}C NMR ($CDCl_3$): Table 2.

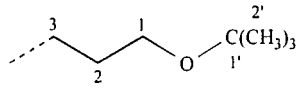


SCHEME 2. Synthesis of (6Z,9Z)-6,9-pentadecadien-1-ol.

(9Z,12Z)-9,12-Octadecadien-1-yl formate, **74,** was synthesized in 92% yield by the reduction of linoleic acid methyl ester with $LiAlH_4$ followed by the esterification of the resulting (9Z,12Z)-9,12-octadecadien-1-ol with formic acid at room temperature for 5 hr. The reaction product was purified by bulb-to-bulb distillation to give (9Z,12Z)-9,12-octadecadien-1-yl formate **74** in 95% yield; bp 165°C (0.015 mm Hg). The ^{13}C NMR data for this and other formates synthesized according to this method, are given in Table 4.

(9Z,12Z)-9,12-Octadecadien-1-yl acetate, **77**, was prepared by the esterification of (9Z,12Z)-9,12-octadecadien-1-ol with acetyl chloride in the presence of pyridine in 99% yield; bp 159°C (0.007 mm Hg). The ^{13}C NMR data for

TABLE 3. ^{13}C NMR SPECTRAL ASSIGNMENT FOR SYNTHETIC INTERMEDIATES IN SCHEME 2



Position	Compounds				
	2-**1**	2-**2**	2-**3**	2-**5**	2-**6**
1	62.44(t)	61.22(t)	61.38(t)	61.48(t)	61.60(t)
2	31.59(t)	29.81(t)	30.20(t)	30.27(t)	30.65(t)
3	24.44(t)	25.06(t)	25.51(t)	25.61(t)	26.00(t)
4	32.50(t)	32.75(t)	28.46(t)	29.01(t)i[a]	29.60(t)
5	33.76(t)	33.80(t)	18.42(t)	18.82(t)[b]	27.23(t)i
6			84.63(s)	79.92(s)ii	130.04(d)ii
7			68.14(d)	78.50(s)ii	127.95(d)iii
8				17.19(t)[b]	25.65(t)
9				125.02(d)[b]	128.09(d)iii
10				131.33(d)[b]	130.22(d)ii
11				27.09(t)	27.26(t)i
12				29.09(t)i	29.37(t)
13				31.49(t)	31.55(t)
14				22.56(t)	22.59(t)
15				14.05(q)	14.08(q)
1'		72.52(s)	72.46(s)	72.44(s)	72.42(s)
2'		27.58(q)	27.59(q)	27.60(q)	27.60(q)

[a] i–iii denote interchangeable assignments.
[b] According to HETCOR.

this and other acetates synthesized according to this method are listed in Table 5.

(4Z,7Z)-4,7-Tridecadienal, **22,** was obtained by the oxidation of the dienol **30** on pyridinium chromate-silica gel in 57% yield according to the procedure described by Singh et al. (1979). The ^{13}C NMR data for the aldehydes synthesized for comparison by this method, are given in Table 6.

RESULTS AND DISCUSSION

Although the oribi enjoys a widespread distribution in the eastern parts of sub-Saharan Africa, its distribution is patchy, and it is therefore a relatively rare

TABLE 4. ^{13}C NMR SPECTRAL ASSIGNMENT FOR SYNTHETIC ALKADIENYL FORMATES

	Compounds		
Position	35	53	74
1	63.43(t)	64.00(t)	64.10(t)
2	28.44(t)	28.47(t)	28.53(t)
3	23.50(t)	25.50(t)	25.83(t)
4	129.52(d)i[a]	29.21(t)	29.39(t)i
5	127.50(d)ii	27.06(t)	29.20(t)i
6	25.60(t)	129.56(d)i	29.17(t)i
7	128.14(d)ii	127.80(d)ii	29.63(t)ii
8	130.52(d)i	25.66(t)	27.22(t)
9	27.24(t)	128.45(d)ii	130.07(d)iii
10	29.33(t)	130.30(d)i	127.93(d)iv
11	31.54(t)	27.24(t)	25.65(t)
12	22.59(t)	29.36(t)	128.06(d)iv
13	14.07(q)	31.55(t)	130.22(d)iii
14		22.59(t)	27.22(t)
15		14.07(q)	29.36(t)ii
16			31.55(t)
17			22.58(t)
18			14.07(q)
1′	161.07(d)	161.11(d)	161.16(d)

[a]i–iv denote interchangeable assignments.

TABLE 5. ^{13}C NMR SPECTRAL ASSIGNMENT FOR SYNTHETIC ALKADIENYL ACETATES

	Compounds		
Position	**42**	**58**	**77**
1	63.96(t)	64.56(t)	64.64(t)
2	28.54(t)	28.56(t)	28.64(t)
3	23.60(t)	25.61(t)	25.93(t)
4	129.28(d)i[a]	29.29(t)	29.43(t)i
5	127.59(d)ii	27.09(t)	29.25(t)i
6	25.59(t)	129.66(d)i	29.23(t)i
7	128.42(d)ii	127.83(d)ii	29.66(t)ii
8	130.45(d)i	25.66(t)	27.23(t)
9	27.23(t)	128.37(d)ii	130.09(d)iii
10	29.34(t)	130.28(d)i	127.94(d)iv

TABLE 5. Continued

Position	Compounds		
	42	**58**	**77**
11	31.54(t)	27.23(t)	25.66(t)
12	22.59(t)	29.36(t)	128.05(d)iv
13	14.06(q)	31.55(t)	130.22(d)iii
14		22.59(t)	27.23(t)
15		14.07(q)	29.37(t)ii
16			31.56(t)
17			22.59(t)
18			14.08(q)
1′	171.11(s)	171.14(s)	171.19(s)
2′	20.97(q)	20.98(q)	21.01(q)

[a]i–iv denote interchangeable assignments.

TABLE 6. ^{13}C NMR SPECTRAL ASSIGNMENT FOR SYNTHETIC ALKENALS AND ALKADIENALS

Position	Compounds				
	10	**23**	**34**	**22**	**43**
1	202.63(d)	202.78(d)	202.84(d)	202.00(d)	202.55(d)
2	43.31(t)	43.89(t)	43.91(t)	43.74(t)	43.79(t)
3	22.10(t)	22.02(t)	22.09(t)	20.10(t)	21.72(t)
4	26.48(t)	28.80(t)	28.99(t)i	129.86(d)i	29.14(t)
5	128.19(d)	29.45(t)i	29.08(t)i	127.28(d)ii	26.93(t)
6	131.43(d)	26.96(t)	29.52(t)ii	25.62(t)	129.21(d)i
7	27.22(t)	129.35(d)	27.11(t)	127.38(d)ii	127.71(d)ii
8	29.35(t)	130.32(d)	129.58(d)	130.68(d)i	25.66(t)
9	31.51(t)	27.20(t)	130.17(d)	27.25(t)	128.71(d)ii
10	22.56(t)	29.42(t)i	27.21(t)	29.31(t)	130.36(d)i
11	14.05(q)	31.53(t)	29.45(t)ii	31.53(t)	27.24(t)
12		22.58(t)	31.55(t)	22.58(t)	29.35(t)
13		14.06(q)	22.59(t)	14.07(q)	31.54(t)
14			14.08(q)		22.59(t)
15					14.08(q)

[a]i and ii denote interchangeable assignments.

to $(M+30)^+$ in the CI(NO) mass spectra of the saturated formates was used to determine the molecular mass of the formates. This ion is formed by addition of a nitrosonium ion to the formate. The M^+ and $(M-46)^+$ ions are the base peaks in the mass spectra of the di- and monounsaturated formates, respectively. Formates were distinguished from other long-chain constituents by the presence in their CI(CH_4) mass spectra of a $(M+1-HCOOH)^+$ ion, which forms the base peak.

The acetates present in the secretion were characterized by a characteristic ion at *m/z* 61 (Sharkey et al., 1959; Beynon et al., 1961). In addition, the saturated acetates also have an ion at *m/z* 116, formed by cleavage of the bond between C-3 and C-4 with rearrangement of one hydrogen atom. The presence of an acetate moiety was confirmed by the presence of $(M+1-CH_3COOH)^+$ ions in the CI(CH_4) mass spectra of these compounds. The $(M+1)^+$ and $(M+NO)^+$ ions in their CI(CH_4) and CI(NO) spectra, respectively, were used to determine the molecular mass of these constituents.

The aldehydes present in the secretion were distinguished by an ion at *m/z* 44 accompanied by $(M-CH_2{=}CH_2)^+$ and $(M-CH_2{=}CHOH)^+$ ions in the high mass range of their EI mass spectra. The $(M-1)^+$ ion constitutes the base peak in these CI(NO) mass spectra, which also contain $(M+NO)^+$ and $(M-H_2O)^+$ ions, whereas the ion at *m/z* 44 has a very low abundance (<ca. 1%) in the spectra of the mono- and diunsaturated aldehydes.

The position of the double bonds in the monounsaturated alcohols, aldehydes, formates, and acetates present in the secretion was determined by GC-MS analysis of an extract of the secretion that had been subjected to the dimethyl disulfide (DMDS) derivatization procedure described by Buser et al. (1983). A total ion current chromatogram of organic material extracted from the secretion and subjected to DMDS derivatization is shown in Figure 2. The EI mass spectra of the bis(methylthioether) derivatives exhibited easily recognizable molecular and less abundant $(M-CH_3S)^+$ ions. The position of the double bonds was derived using the diagnostic ions A^+, B^+, and C^+, as shown in Table 7.

The configuration of the double bonds was established by coinjection of an extract of the secretion with a number of synthetic reference compounds after it had been confirmed that the *E* and *Z* isomers of these compounds could be separated on the available capillary column. Although the concentration of many of the unsaturated compounds in the preorbital secretion is below the detection limit of the NMR instrument, it was possible to confirm the presence of a double bond with *Z* configuration in the major constituents because of a resonance at δ 27.2 in the ^{13}C NMR spectrum of the whole secretion and the total absence of a resonance at δ ca. 33. It is known that the carbon atoms of the allylic methylene group in long-chain unbranched compounds resonate at δ ca. 27 in the *Z* isomers and at δ ca. 33 in the *E* compounds (Breitmaier et al., 1979; Wenkert et al., 1976).

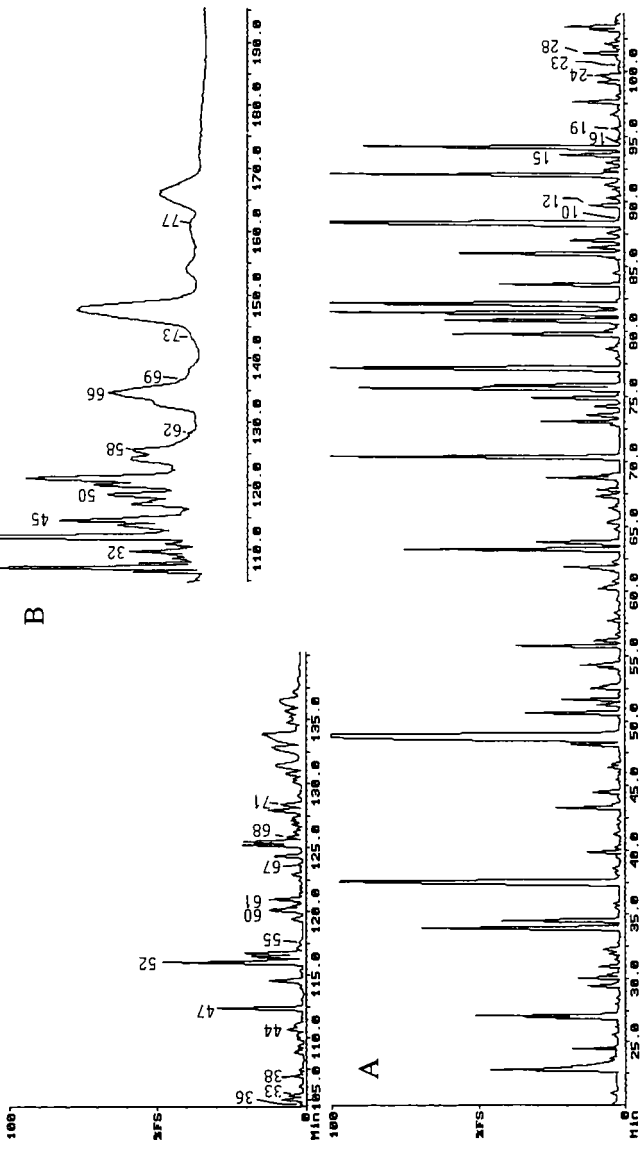
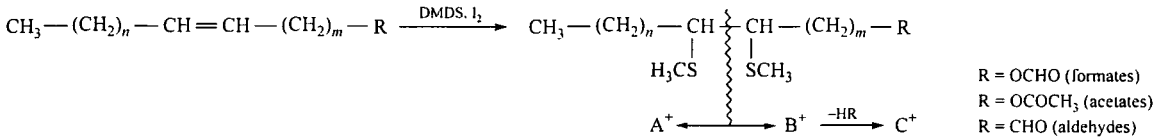


FIG. 2. Total ion chromatogram of an extract of the preorbital secretion of the oribi subjected to dimethyl disulfide derivatization. (A) at 40°C, (B) at 60°C.

TABLE 7. DIAGNOSTIC IONS IN EI MASS SPECTRA OF DMDS DERIVATIVES OF MONOUNSATURATED COMPOUNDS PRESENT IN PREORBITAL SECRETION OF ORIBI



Compound	Compound No. in Figures 1 and 2	Diagnostic ion (%, normalized abundance)				
		M^+	$(M-47)^+$	A^+	B^+	C^+
(Z)-4-Decen-1-yl formate	12	278(9)	231(2)	131(100)	147(3)	101(38)
(Z)-5-Undecen-1-yl formate	19	292(10)	245(2)	131(100)	161(10)	115(26)
(Z)-6-Dodecen-1-yl formate	28	306(10)	259(2)	131(100)	175(14)	129(16)
(Z)-5-Tridecen-1-yl formate	36	320(16)	273(–)	159(100)	161(17)	115(43)
(Z)-7-Tridecen-1-yl formate	38	320(9)	273(3)	131(100)	189(37)	143(11)
(Z)-8-Tetradecen-1-yl formate	47	334(10)	287(3)	131(100)	203(68)	157(11)
(Z)-9-Pentadecen-1-yl formate	55	348(12)	301(3)	131(100)	217(96)	171(6)
13-Methyl-(Z)-8-pentadecen-1-yl formate	60	362(10)	315(3)	159(97)	203(78)	157(7)
(Z)-4-Decen-1-yl acetate	15	292(13)	245(1)	131(100)	161(3)	101(85)
(Z)-5-Undecen-1-yl acetate	24	306(4)	259(1)	131(44)	175(5)	115(25)
(Z)-6-Dodecen-1-yl acetate	33	320(10)	273(1)	131(100)	189(12)	129(49)
(Z)-7-Tridecen-1-yl acetate	44	334(11)	287(2)	131(100)	203(30)	143(36)
(Z)-8-Tetradecen-1-yl acetate	52	348(10)	301(2)	131(100)	217(61)	157(31)
(Z)-9-Pentadecen-1-yl acetate	61	362(9)	315(1)	131(100)	231(76)	171(22)
(Z)-8-Hexadecen-1-yl acetate	67	376(9)	329(2)	159(94)	217(70)	157(34)
(Z)-10-Hexadecen-1-yl acetate	68	376(7)	329(1)	131(97)	245(73)	185(23)
(Z)-9-Heptadecen-1-yl acetate	71	390(4)	343(–)	159(41)	231(39)	171(12)
(Z)-5-Undecenal	10	262(6)	215(–)	131(46)	131(46)	
(Z)-6-Dodecenal	16	276(10)	229(2)	131(100)	145(18)	
(Z)-7-Tridecenal	23	290(9)	243(3)	131(92)	159(9)	

Attempts to determine the position of the double bond in the unsaturated alcohols were unsuccessful as their DMDS derivatives could not be found in the total ion chromatogram of the derivatized secretion. This was probably due to the low concentration of these alcohols in the secretion. The assumption, based on the structures of the other constituents of the secretion, that the double bond in these compounds is situated between C-6 and C-7 from the methyl end of the carbon chain, was confirmed by retention time comparison of the natural constituents with the authentic synthetic alcohols.

In the EI spectrum of component **60,** the $(M-HCOOH)^+$ ion at *m/z* 222 is weaker than the molecular ion at *m/z* 268, and the base peak appears at *m/z* 70. These two phenomena distinguish this monounsaturated formate from other alkenyl formates present in the secretion. The $(M+1)^+$ and $(M+1-HCOOH)^+$ ions at *m/z* 269 and 223 in its CI(NO) spectrum as well as the $(M+NO)^+$, M^+, and $(M-HCOOH)^+$ ions at *m/z* 298, 268, and 222, respectively, in its CI(NO) spectrum confirm that component **60** is a monounsaturated hexadecen-1-yl formate. In the EI spectrum of its DMDS derivative, the presence of the diagnostic ions at *m/z* 159, 203, and 157 suggested the presence of a C-8–C-9 double bond. Synthetic (*Z*)-8-hexadecen-1-yl formate was coinjected with the oribi secretion, and the retention time of component **60** was found to be much shorter than that of the reference compound. On account of this difference in retention time, component **60** was assumed to be a branched monounsaturated formate. According to Brown et al. (1954), Friedel et al. (1956), Dolejs et al. (1968), and Bowen and Maccoll (1984), the base peak in the EI mass spectra of anteisoalcohols appears at *m/z* 70. Component **60** was therefore assumed to be an anteisoformate and, by combining all the relevant information, was identified as 13-methyl-(*Z*)-8-pentadecen-1-yl formate. This was not, however, confirmed by comparison with synthetic material.

According to the EI, CI(CH_4), and CI(NO) mass spectra, component **59** was assumed to be a pentadecen-1-yl acetate. The DMDS derivative of 7-pentadecen-1-yl acetate was found in the EI total ion chromatogram of the derivatized oribi preorbital secretion. In addition, the retention time of component **59** is shorter than the retention time of component **61,** (*Z*)-9-pentadecen-1-yl acetate, the difference between the retention times of these two components being only slightly longer than the difference between the retention times of (*Z*)-8- and (*Z*)-10-hexadecen-1-yl acetate, components **67** and **68**, respectively. It was therefore reasonable to assume that component **59** could be 7-pentadecen-1-yl acetate. However, the retention time of the (*Z*)-7-pentadecen-1-yl acetate reference, which was coinjected with oribi secretion, was longer than that of component **59.** The question arises as to why the DMDS derivative of 7-pentadecen-1-yl acetate was unequivocally identified in the EI total ion chromatogram of the DMDS-derivatized secretion but could not be detected in the EI total ion chromatogram of the secretion itself. The reason may possibly be that

7-pentadecen-1-yl acetate is actually present in the secretion, but in a low concentration and that it is obscured in the EI total ion chromatogram by other components. However, component **59** is not 7-pentadecen-1-yl acetate and could therefore only be characterized as an unidentified pentadecenyl acetate.

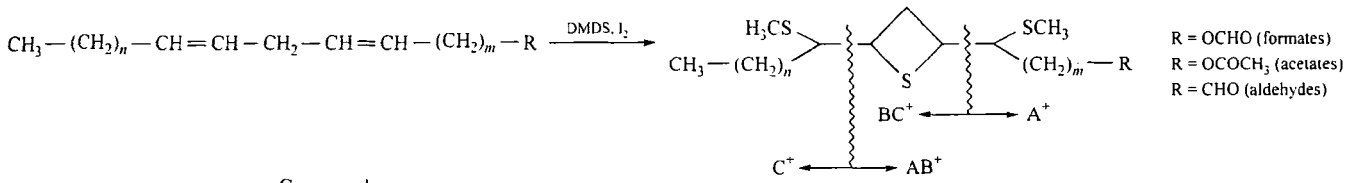
The molecular mass and functionality of the diunsaturated compounds present in the secretion were determined from EI, CI(CH_4), and CI(NO) mass spectral data.

The DMDS derivatization method for the determination of the position of double bonds as described by Buser et al. (1983) produced only two mono-DMDS derivatives of a single diunsaturated acetate, component **50.** Their EI spectra both have a molecular ion at *m/z* 346. Typical diagnostic ions appear at *m/z* 171 (A^+), 175 (B^+), 115 ($B^+ - CH_3COOH$) in one of the spectra, suggesting that one double bond in component **50** is in the 5 position. The diagnostic ions appear at *m/z* 131 (A^+), 215 (B^+), and 155 ($B^+ - CH_3COOH$) in the spectrum of the second DMDS derivative, indicating that the other double bond in component **50** is in the 8 position. Component **50** was therefore concluded to be 5,8-tetradecadien-1-yl acetate. The formation of the mono-DMDS derivative of a diunsaturated acetate has been described by Tonini et al. (1986) in a paper on the sex pheromone of the leopard moth. The DMDS derivatization procedure described by Vincenti et al. (1987), in which a higher reaction temperature and a longer reaction time are used, was therefore employed as an alternative method. According to Vincenti et al. (1987) and Carlson et al. (1989), the structures of the DMDS derivatives of compounds containing a diene system depend on the number of carbon atoms between the two double bonds. Information furnished by the partial derivatization of the skipped diene system of component **50,** 5,8-tetradecadien-1-yl acetate, according to Buser et al. (1983), proved useful in the interpretation of the more complex results produced by the complete derivatization according to Vincenti et al. (1987).

The reaction of a skipped diene with two molecules of DMDS gives four-membered cyclic thioethers substituted with two alkyl chains, each containing a methylthio group α to the ring. The mass spectrometric fragmentation of the DMDS derivatives of constituents containing a skipped diene system is shown in Table 8.

Information on the molecular mass and functionality of a number of alkadienals was supplied by the presence of $(M+1)^+$ and $(M+1-H_2O)^+$ ions in their CI(CH_4) mass spectra. However, only one DMDS derivative of an alkadienal, that of 5,8-tetradecadienal **32,** could be detected in the total ion current chromatogram of the derivatized secretion. The molecular ion, which is absent from the mass spectra of the derivatized alkadienyl acetates and formates, appears at *m/z* 334 in the mass spectrum of the derivatized dienal **32.** The ion at *m/z* 287 is formed by the loss of a methylthio radical from the molecular ion. The diagnostic ions at *m/z* 203 [AB^+ and BC^+], 155 [$(AB-CH_3SH)^+$ and

TABLE 8. DIAGNOSTIC IONS IN EI MASS SPECTRA OF DMDS DERIVATIVES OF DIUNSATURATED COMPOUNDS PRESENT IN PREORBITAL SECRETION OF ORIBI



Compound	Compound No. in Figures 1 and 2	Diagnostic ion (%, normalized abundance)											
		M$^+$	(M-47)$^+$	(M-48)$^+$	(M-95)$^+$	AB$^+$	(AB-48)$^+$	(AB-RH)$^+$	(AB-48-RH)$^+$	BC$^+$	(BC-48)$^+$	A$^+$	C$^+$
(5Z,8Z)-5,8-Tetradecadien-1-yl acetate	50	378(−)	331(7)	330(17)	283(8)	247(−)	199(—)	187(−)	139(84)	203(7)	155(51)	175(1)	131(22)
(6Z,9Z)-6,9-Pentadecadien-1-yl acetate	58	392(−)	345(15)	344(36)	297(20)	261(−)	213(3)	201(−)	153(60)	203(16)	155(100)	189(−)	131(31)
(7Z,10Z)-7,10-Hexadecadien-1-yl actate	66	406(−)	359(6)	358(14)	311(13)	275(−)	227(4)	215(1)	167(30)	203(7)	155(39)	203(7)	131(16)
(8Z,11Z)-Heptadecadien-1-yl acetate	73	420(−)	373(4)	372(6)	325(6)	289(2)	241(4)	229(3)	181(10)	203(5)	155(35)	217(5)	131(7)
(9Z,12Z)-9,12-Octadecadien-1-yl acetate	77	434(−)	387(3)	386(4)	339(5)	303(−)	255(−)	243(14)	195(5)	203(4)	155(22)	231(6)	131(−)
(5Z,8Z)-5,8-Tetradecadien-1-yl formate	45	364(−)	317(10)	316(27)	269(16)	233(1)	185(5)	187(−)	139(36)	203(11)	155(73)	161(5)	131(18)
(7Z,10Z)-7,10-Hexadecadien-1-yl formate	62	392(−)	345(3)	344(7)	297(6)	261(−)	213(2)	215(−)	167(4)	203(2)	155(18)	189(2)	131(11)
(8Z,11Z)-8,11-Heptadecadien-1-yl formate	69	406(−)	359(6)	358(25)	311(18)	275(−)	227(13)	229(−)	181(27)	203(2)	155(28)	203(2)	131(16)
(5Z,8Z)-5,8-Tetradecadienal	32	334(32)	287(4)	286(−)	239(−)	203(29)	155(50)			203(29)	155(50)	131(100)	131(100)

$(BC-CH_3SH)^+$], and 131 [A^+ and C^+] provide evidence that the double bonds are in the 5 and 8 positions. Component **32** was therefore identified as a 5,8-tetradecadienal. All of the alkadienals were found to contain a skipped diene system. Their structures and the configuration of the double bonds were confirmed by GC retention time comparison with synthetic compounds, employing coinjection techniques.

The EI mass spectra of the four components, **30, 40, 49,** and **57,** are similar to those of the alkadienals except for the presence in their spectra of the ion at *m/z* 31, which is typical for alcohols and formates. The prominent $(M+1)^+$ and $(M+1-H_2O)^+$ ions in their CI(CH_4) spectra indicated that they could be alkadienols. Although the $(M-3)^+$ ion, normally present in the CI(NO) spectra of primary alcohols, does not appear in an appreciable abundance in their CI(NO) mass spectra, the presence of a primary hydroxyl function in these compounds is confirmed by the presence of $(M-1)^+$ and $(M-2+NO)^+$ ions and the absence of a $(M-OH)^+$ ion. No DMDS derivative of an alkadienol could, however, be located in the total ion chromatogram shown in Figure 2 and, as a working hypothesis, these compounds were assumed to contain skipped diene systems similar to those found in the formates and acetates. This assumption was confirmed by coinjection of the secretion with the synthetic compounds.

In contrast to the other compound types, only saturated carboxylic acids are present in the secretion. The compounds identified in the preorbital secretion of the oribi are listed in Table 9 together with quantitative data and information on the methods used in their identification.

(4*Z*,7*Z*)-4,7-Tridecadien-1-ol, **30,** was synthesized using published procedures as outlined in Scheme 1. The bromination of the alkenol 1-**2** with phosphorus tribromide and pyridine gave (*Z*)-1-bromo-2-octene 1-**3** containing 10% of the *E* isomer. Condensation of this bromide with an excess of the acetylenic Grignard reagent 1-**6** in the presence of CuCl under reflux conditions, followed by partial hydrogenation and acid catalyzed removal of the protective groups, gave a mixture of 4,7-tridecadien-1-ol isomers. Column chromatography on 7% $AgNO_3$-coated silica gel was used to separate the product isomers and to isolate (4*Z*,7*Z*)-4,7-tridecadien-1-ol, **30.**

(6*Z*,9*Z*)-6,9-Pentadecadien-1-ol, **49,** was synthesized according to Scheme 2. In this synthesis the alcohol function was protected as its *tert*-butyl ether (Alexakis et al., 1988). The use of Ac_2O and $FeCl_3$ for the conversion of *tert*-butyl ethers to the corresponding acetates has been reported (Alexakis et al., 1988; Ganem and Small, 1974; Gross and Watt, 1987; Alexakis and Duffault, 1988). However, this method proved unsuccessful in this synthesis. Me_3SiI as deprotection reagent (Jung and Lyster, 1977), chosen for its reported high yield and limited reaction time, afforded complete cleavage of the ether in 6 min at room temperature. Column chromatography on 7% $AgNO_3$-coated silica gel was used to isolate pure (6*Z*,9*Z*)-6,9-pentadecadien-1-ol, **49.**

TABLE 9. COMPOUNDS IDENTIFIED IN PREORBITAL SECRETION OF ORIBI

No. in Figure 1	Compounds	Analytical methods	Quantity (ng/animal)
3	1-Nonanol	a,b,c,f[a]	0.16
9	1-Decanol	a,b,c,f	0.10
2	1-Octyl formate	a,b,f	0.03
7	1-Nonyl formate	a,b,c,f	0.33
14	1-Decyl formate	a,b,c,f	0.58
20	1-Undecyl formate	a,b,c,f	1.79
29	1-Dodecyl formate	a,b,c,f	4.21
39	1-Tridecyl formate	a,b,c,f	7.64
48	1-Tetradecyl formate	a,b,c,f	9.56
56	1-Pentadecyl formate	a,b,c	2.51
64	1-Hexadecyl formate	a,b,c,f	0.66
6	1-Octyl acetate	a,b,f	[b]
13	1-Nonyl acetate	a,b,c,f	0.19
18	1-Decyl acetate	a,b,c,f	0.55
27	1-Undecyl acetate	a,b,c,f	0.51
37	1-Dodecyl acetate	a,b,c,f	1.47
46	1-Tridecyl acetate	a,b,c	5.88
54	1-Tetradecyl acetate	a,b,c,f	14.87
63	1-Pentadecyl acetate	a,b,c	18.60
70	1-Hexadecyl acetate	a,b,c,f	5.21
75	1-Heptadecyl acetate	a,b,c	0.50
1	Nonanal	a,b,c,f	0.02
4	Decanal	a,b,c,f	0.02
11	Undecanal	a,b,c,f	0.06
17	Dodecanal	a,b,c,f	0.30
26	Tridecanal	a,b,c	0.33
65	Hexadecanoic acid	a,b,c,f	0.94
72	Heptadecanoic acid	a,b,c,f	0.57
76	Octadecanoic acid	a,b,f	0.31
8	(Z)-4-Decen-1-ol	a,b,c	0.20
21	(Z)-6-Dodecen-1-ol	a,b,c,f	0.27
31	(Z)-7-Tridecen-1-ol	a,b,f	0.28
41	(Z)-8-Tetradecen-1-ol	a,b,c,f	1.32
5	(Z)-3-Nonen-1-yl formate	a,b,c	[b]
12	(Z)-4-Decen-1-yl formate	a,b,c,d	0.11
19	(Z)-5-Undecen-1-yl formate	a,b,c,d,f	0.34
28	(Z)-6-Dodecen-1-yl formate	a,b,c,d,f	2.07
36	(Z)-5-Tridecen-1-yl formate	a,b,c,d	0.17
38	(Z)-7-Tridecen-1-yl formate	a,b,c,d,f	1.13
47	(Z)-8-Tetradecen-1-yl formate	a,b,c,d,f	7.91
55	(Z)-9-Pentadecen-1-yl formate	a,b,c,d,f	0.31
60	13-Methyl-(Z)-8-pentadecen-1-yl formate	a,b,c,d,e	[c]

TABLE 9. Continued

No. in Figure 1	Compounds	Analytical methods	Quantity (ng/animal)
15	(Z)-4-Decen-1-yl acetate	a,b,c,d	0.38
24	(Z)-5-Undecen-1-yl acetate	a,b,c,d,f	0.49
33	(Z)-6-Dodecen-1-yl acetate	a,b,c,d,f	1.00
44	(Z)-7-Tridecen-1-yl acetate	a,b,c,d,f	1.43
52	(Z)-8-Tetradecen-1-yl acetate	a,b,c,d,f	10.20
59	[d]-Pentadecen-1-yl acetate	a,b,c	[c]
61	(Z)-9-Pentadecen-1-yl acetate	a,b,c,d,f	2.89
67	(Z)-8-Hexadecen-1-yl acetate	a,b,c,d,f	0.97
68	(Z)-10-Hexadecen-1-yl acetate	a,b,c,d,f	1.05
71	(Z)-9-Heptadecen-1-yl acetate	a,b,c,d	0.53
10	(Z)-5-Undecenal	a,b,d,f	0.02
16	(Z)-6-Dodecenal	a,b,c,d	0.55
23	(Z)-7-Tridecenal	a,b,d,f	0.27
34	(Z)-8-Tetradecenal	a,b,d,f	1.10
30	(4Z,7Z)-4,7-Tridecadien-1-ol	a,b,c,f	1.60
40	(5Z,8Z)-5,8-Tetradecadien-1-ol	a,b,c	5.80
49	(6Z,9Z)-6,9-Pentadecadien-1-ol	a,b,c,f	7.63
57	(7Z,10Z)-7,10-Hexadecadien-1-ol	a,b,c	1.18
25	(3Z,6Z)-3,6-Dodecadienyl-1-formate	a,b	0.05
35	(4Z,7Z)-4,7-Tridecadienyl-1-formate	a,b,c,f	1.25
45	(5Z,8Z)-5,8-Tetradecadienyl-1-formate	a,b,c,d	6.87
53	(6Z,9Z)-6,9-Pentadecadienyl-1-formate	a,b,c,f	40.37
62	(7Z,10Z)-7,10-Hexadecadienyl-1-formate	a,b,c,d	1.86
69	(8Z,11Z)-8,11-Heptadecadienyl-1-formate	a,b,c,d	7.57
74	(9Z,12Z)-9,12-Octadecadienyl-1-formate	a,b,c,f	0.30
42	(4Z,7Z)-4,7-Tridecadien-1-yl acetate	a,b,c,f	4.88
50	(5Z,8Z)-5,8-Tetradecadien-1-yl acetate	a,b,c,d	14.24
58	(6Z,9Z)-6,9-Pentadecadien-1-yl acetate	a,b,c,d,f	16.83
66	(7Z,10Z)-7,10-Hexadecadien-1-yl acetate	a,b,c,d	35.72
73	(8Z,11Z)-8,11-Heptadecadien-1-yl acetate	a,b,c,d	7.06
77	(9Z,12Z)-9,12-Octadecadien-1-yl acetate	a,b,c,f	4.70
22	(4Z,7Z)-4,7-Tridecadienal	a,b,c,f	0.94
32	(5Z,8Z)-5,8-Tetradecadienal	a,b,c,d	13.30
43	(6Z,9Z)-6,9-Pentadecadienal	a,b,c,f	18.67
51	(7Z,10Z)-7,10-Hexadecadienal	a,b,c	0.55

[a] a, low-resolution EI mass spectral data; b, CI(CH_4) mass spectral data; c, CI(NO) mass spectral data; d, EI mass spectral data of dimethyl disulfide derivative; e, published data; f, retention time comparison.

[b] Unresolved peak; total quantity 0.04 ng.

[c] Unresolved peak; total quantity 2.26 ng.

[d] Position and configuration of the double bound uncertain.

The formates were prepared by esterification of the corresponding alcohols with formic acid at room temperature, and the acetates were obtained by acetylation of the alcohols with acetyl chloride and pyridine in ether. The unsaturated aldehydes were prepared by oxidation of the corresponding alcohols with pyridinium chromate on silica gel in dichloromethane in the presence of a small quantity of acetic acid.

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VOLATILES EMITTED BY DIFFERENT COTTON VARIETIES DAMAGED BY FEEDING BEET ARMYWORM LARVAE

JOHN H. LOUGHRIN,[1] ARA MANUKIAN, ROBERT R. HEATH, and JAMES H. TUMLINSON*

Insect Attractants, Behavior and Basic Biology Research Laboratory
Agricultural Research Service, United States Department of Agriculture
Gainesville, Florida 32604

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Abstract—Volatile compounds elicited by insect herbivore feeding damage in five cotton cultivars and one naturalized cotton variety were examined by allowing beet armyworm larvae to feed overnight on leaves and collecting volatiles from the plants in situ. Of 23 compounds identified from larval damaged leaves, terpenes and lipoxygenase–hydroperoxide lyase-derived volatiles predominated. No pronounced differences in the levels of volatile emission were noted from leaves of undamaged plants of the different varieties. However, average volatile emission from damaged leaves of the naturalized variety was almost sevenfold higher than from damaged leaves of the commercial cultivars. This was despite the fact that larvae preferred feeding on the leaves of commercial cultivars over those of the naturalized variety in choice tests.

Key Words—Beet armyworm, *Spodoptera exigua*, cotton, *Gossypium hirsutum*, volatile compound, terpene, plant–insect interaction.

INTRODUCTION

The chemical composition of the cotton plant, *Gossypium hirsutum* L. (Malvaceae) has been extensively studied because of its economic importance and its susceptibility to a wide array of pests and diseases. Numerous investigations have been concerned with the volatile terpenoids found in the leaves and flower buds (Thompson et al., 1971; Chang et al., 1988) that may serve as attractants and/or feeding deterrents (Harborne, 1988) for pests. In the past, isolation of

*To whom correspondence should be addressed.
[1]Present address: Department of Entomology, University of Kentucky, S225 Agricultural Science Center North, Lexington, Kentucky 40546-0091.

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these compounds usually relied on solvent extraction and/or steam distillation, which may have given unrepresentative aroma profiles due to artifact formation and cellular compartment disruption. Newer technology relies on the use of dynamic headspace techniques in which volatile compounds are entrained in a stream of air and adsorbed on porous polymers like Tenax or Porapak Q. These techniques allow volatiles to be collected from living plants and thus provide more accurate assessments of the composition of volatiles emitted by plants in response to various stimuli (Heath and Manukian, 1992; Surburg et al., 1993).

Recently, we analyzed the volatile compounds emitted by cotton (var. McNair 235) seedlings (with four to six leaves) damaged by feeding corn earworm, *Helicoverpa zea* (Boddie) larvae (McCall et al., 1994). Volatiles emitted by damaged seedlings that had been cut and detached from the base of the stem were found to serve as cues for the location of hosts by the parasitoid *Microplitis croceipes* (Cresson), which attacks corn earworm larvae (McCall et al., 1993).

Given the increasing awareness of the role that plant volatiles may play in herbivore/predator relationships and a need to study these phenomena in situ, we undertook the present study to determine whether significant differences might occur in volatile emissions from more mature specimens of different commercial cotton cultivars subjected to feeding damage by lepidopterous larvae. For this study we chose four varieties commonly planted in the coastal plain and piedmont areas of Georgia. For comparison we also chose a nectariless variety and a naturalized variety from south Florida. Beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) larvae were used as test insects. They are pests of considerable economic importance in the southern United States on a variety of agronomic and horticultural crops such as beets, corn, and soybeans in addition to cotton.

METHODS AND MATERIALS

Cotton Varieties and Insects. Cotton varieties examined in the present study were Delta Experiment Station 119 (DES119), Delta Pineland 90 (DPL90), Stoneville 453 (SV453), Stoneville 213 nectariless (SV213), KC311, and a south Florida naturalized cotton, Texas accession no. 2259 (TX2259). Plants were grown in 30-cm pots in a 1 : 1 : 1 mixture of topsoil, vermiculite, and peat moss. Plants analyzed in these experiments were mature plants that had set squares (flower buds). Beet armyworm larvae were reared by the method of King and Leppla (1984).

Volatile Collections from Damaged Leaves of Cotton Varieties. Larvae were allowed to feed on cotton leaves overnight by enclosing a single leaf in a lightweight muslin bag in the late afternoon along with two late second-instar beet armyworms. A fully expanded leaf occupying a position near the apical

end of a branch was used in all studies. The following morning, about 2 hr after sunrise, the bag and larvae were removed from the plant. The branch holding the damaged leaf, still attached to the plant, was inserted into a 15-cm-diameter × 40-cm-tall glass sleeve sampling chamber of the volatile collection apparatus (Heath and Manukian, 1992; Heath et al., 1992). Air was forced through multiple layers of charcoal-infused fabric at the top of the sleeve, and the purified air passed down over the branch at a rate of 40 liters/min. This high flow rate was required to prevent intrusion of unfiltered air into the bottom of the sampling apparatus, which was not constricted. Volatiles were collected on 50 mg of Super Q adsorbent (Alltech Associates, Deerfield, Illinois) contained in a glass volatile collection trap (4 mm ID × 40 mm long) (Heath and Manukian, 1992) placed downstream from and adjacent to the damaged leaf. Air was pulled through the trap at a rate of 2 liters/min by a vacuum pump. Volatiles were collected for 2 hr beginning approximately 2 hr after sunrise. The experiments were replicated six times for each variety to obtain mean values. Leaves from an undamaged plant were sampled in a similar fashion, replicated four times for each variety, for comparison.

Feeding Choice Tests with Cotton Varieties. Larval preference for feeding on the different cotton varieties was tested by placing two second-instar beet armyworms in an 18-cm covered dish with a mature leaf of TX2259 and one of the commercial cultivars. Between 3:00 and 3:30 PM the leaves were cut from undamaged plants at the midpoint of the petiole, weighed, and the ends of the petioles wrapped in moistened cotton to prevent desiccation. Immediately, the larvae were placed in the dishes with the leaves and allowed to feed overnight. At about 7:00 AM the next day the larvae were removed and the leaves were weighed again to determine percent mass of the leaf eaten. The experiments were repeated six times for each cotton cultivar.

Analytical Methods. At the end of the sampling period, volatiles were eluted from the Super Q in each volatile collection trap with 150 μl of methylene chloride, 600 ng each of *n*-octane and *n*-nonyl acetate were added as internal standards, and each sample was analyzed by capillary gas chromatography on a methyl silicone column (Quadrex, New Haven, Connecticut, 50 m × 0.25 mm ID, with a 0.25-μm-thick bonded stationary phase) in a Varian model 3400 gas chromatograph equipped with a split/splitless injection system and a flame ionization detector. Injections were made in the splitless mode, and the injector was purged after 30 sec. The column was connected to the injector with a 5-m × 0.25-mm deactivated fused silica retention gap and operated under the following conditions: 50°C for 3 min, then programmed at 5°C/min to 180°C, injector 220°C, detector 240°C, helium linear flow velocity 21 cm/sec.

Volatiles from each cotton variety were analyzed by gas chromatography–mass spectroscopy using a 50-m methyl silicone column, operated as before, and interfaced to a Finnigan-MAT ITS 40 (ion trap) mass spectrometer operated

in the electron impact mode. Compounds were identified by comparison of spectra with those in the EPA-NIH data base and by comparison of GC retention times with those of authentic standards on the methyl silicone column and on a 50-m × 0.25-mm CPS-1 column (Quadrex, New Haven, Connecticut) operated under the following conditions: 30 sec splitless delay after injection, oven temperature 60°C for 1 min then programmed at 5°C/min to 180°C, injector 220°C, detector 260°C, helium linear flow velocity 19 cm/sec.

RESULTS AND DISCUSSION

The predominant components in the volatiles collected from leaves of undamaged plants of the various cotton cultivars were terpenoids, including the monoterpenes α-pinene, myrcene, and (*E*)-β-ocimene, the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene, and the sesquiterpene caryophyllene (Table 1). There were no pronounced differences in the composition of the volatiles collected from undamaged leaves of the commercial cultivars and the naturalized cotton TX2259. These results are in good agreement with those of McCall et al. (1994) for detached cotton plants, in that monoterpenes comprised the majority of compounds collected. Similarly, Chang et al. (1988) reported that the monoterpenes were the most abundant compounds in the volatiles collected from detached cotton flower buds. Thus, it appears that detached flower buds and leaves may not differ from intact living plants in volatile emission. However, it is possible that the release of some or all of these compounds in our experiments was caused by slight damage incurred when the branch was placed in the sampling apparatus.

Compounds emitted by leaves that had been damaged by beet armyworm larvae feeding overnight are presented in Table 2 and are summarized by biosynthetic class in Figure 1. A total of 23 compounds, representing approximately 90% of the trapped volatiles, were identified. A number of other compounds, notably sesquiterpene hydrocarbons, were not identified because of insufficient spectral data and/or lack of reference standards. The majority of the volatile components consisted of monoterpene and sesquiterpene hydrocarbons, with the most abundant components being the monoterpenes α-pinene and (*E*)-β-ocimene and the sesquiterpene caryophyllene. More unusual terpenes identified were (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. These compounds, which are usually referred to as homomonoterpenes and homosesquiterpenes, result from oxidative cleavage of the sesquiterpenoid nerolidol and diterpenoid geranyllinalool, respectively (Gäbler et al., 1991).

Most cotton cultivars accumulate large amounts of terpenoids in specialized glands present in the leaf and flower buds (Elzen et al., 1985). Therefore, it is

TABLE 1. VOLATILE COMPOUNDS COLLECTED FROM UNDAMAGED LEAVES OF COTTON CULTIVARS

Compound	Cotton variety, mean yield of compound (ng/hr)[a]					
	DES119	DPL90	KC311	SV213	SV453	TX2259
(*Z*)-3-Hexenal	5.3 ± 4.9	10.1 ± 11.9	3.2 ± 1.3	4.8 ± 2.7	[b]	5.0 ± 1.9
(*Z*)-3-Hexenyl acetate	2.2 ± 1.5	19.3 ± 16.5	20.1 ± 18.0	4.9 ± 7.0	7.8 ± 4.5	16.7 ± 11.7
Hexyl acetate	[b]	[b]	2.8 ± 2.9	[b]	[b]	[b]
Indole	21.6 ± 30.1	[b]	[b]	[b]	[b]	5.1 ± 4.1
α-Pinene	6.8 ± 3.6	12.9 ± 4.6	12.8 ± 7.3	4.7 ± 3.7	6.1 ± 5.5	7.9 ± 7.0
β-Pinene	[b]	2.7 ± 1.2	2.8 ± 1.3	[b]	[b]	2.5 ± 1.3
Myrcene	9.2 ± 5.9	6.3 ± 2.9	6.9 ± 5.0	8.2 ± 5.4	3.7 ± 1.7	9.2 ± 8.0
Limonene	2.2 ± 2.6	2.2 ± 1.1	[b]	9.2 ± 6.4	[b]	3.8 ± 2.1
(*E*)-β-Ocimene	2.5 ± 1.7	6.0 ± 3.4	5.2 ± 5.0	[b]	[b]	13.5 ± 12.9
Terpinene	[b]	[b]	[b]	[b]	[b]	3.2 ± 3.3
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	13.5 ± 15.6	4.6 ± 3.7	4.5 ± 6.6	6.8 ± 3.7	[b]	20.1 ± 22.2
(*E*,*E*)-4,8,12 Trimethyl-1,3,7,11-tridecatetraene	[b]	[b]	[b]	[b]	[b]	2.7 ± 3.2
Caryophyllene	2.2 ± 1.4	11.7 ± 6.1	11.7 ± 6.1	7.0 ± 9.9	3.6 ± 1.3	[b]
α-Humulene	[b]	3.7 ± 2.0	3.7 ± 2.0	[b]	[b]	[b]

[a] Mean of four determinations ± standard deviation.
[b] Compound not detected or production less than 2 ng/hr.

TABLE 2. VOLATILE COMPOUNDS COLLECTED FROM LEAVES OF COTTON CULTIVARS SUBJECTED TO BEET ARMYWORM FEEDING DAMAGE OVERNIGHT

Compound	Cotton variety, mean yield of compound (ng/hr)[a]					
	DES119	DPL90	KC311	SV213	SV453	TX2259
(*Z*)-3-Hexenal	222 ± 154	59.6 ± 82.6	76.5 ± 58.9	87.7 ± 56.4	117 ± 188	307 ± 363
(*E*)-2-Hexenal	110 ± 59.6	39.2 ± 53.1	68.7 ± 35.6	81.3 ± 46.1	46.4 ± 41.0	122 ± 125
(*Z*)-3-Hexenol	149 ± 57.0	41.4 ± 54.1	91.9 ± 137	132 ± 117	71.4 ± 70.5	321 ± 347
(*E*)-2-Hexenol	43.1 ± 38.1	5.8 ± 6.5	20.3 ± 11.1	23.2 ± 13.5	11.4 ± 10.8	46.1 ± 53.6
Hexanol	21.5 ± 13.0	7.4 ± 7.4	15.4 ± 13.7	16.2 ± 8.7	9.5 ± 8.7	23.1 ± 26.4
(*Z*)-3-Hexenyl acetate	662 ± 483	206 ± 111	452 ± 369	333 ± 180	492 ± 226	1710 ± 1448
Hexyl acetate	27.1 ± 10.7	13.6 ± 9.3	33.3 ± 23.3	18.2 ± 11.3	17.2 ± 9.1	65.7 ± 65.7
(*Z*)-3-Hexenyl butyrate	25.7 ± 17.1	8.4 ± 5.0	15.6 ± 17.7	10.3 ± 11.4	10.8 ± 4.0	36.7 ± 30.1
(*E*)-2-Hexenyl butyrate	8.5 ± 3.0	5.0 ± 4.5	7.8 ± 5.0	5.7 ± 4.4	4.0 ± 3.8	18.4 ± 13.5
(*Z*)-3-Hexenyl-2-methylbutyrate	3.0 ± 2.2	[b]	2.6 ± 2.2	2.9 ± 4.2	3.9 ± 4.4	6.1 ± 5.0
(*E*)-2-Hexenyl-2-methylbutyrate	4.8 ± 3.0	[b]	3.2 ± 4.7	4.2 ± 5.1	2.9 ± 3.6	3.6 ± 3.6
(*Z*)-Jasmone	3.3 ± 4.1	8.7 ± 4.4	2.1 ± 4.0	12.6 ± 15.6	3.5 ± 7.3	19.3 ± 13.7
Indole	70.9 ± 91.8	23.1 ± 16.7	59.3 ± 118	30.6 ± 40.2	44.7 ± 53.6	69.2 ± 111
α-Pinene	87.8 ± 54.1	24.5 ± 25.8	115 ± 73.6	123 ± 79.7	59.8 ± 34.5	913 ± 625
β-Pinene	15.8 ± 9.9	5.2 ± 5.3	23.0 ± 12.1	22.3 ± 13.6	9.8 ± 5.9	211 ± 146
Myrcene	59.5 ± 31.6	14.6 ± 13.1	73.3 ± 57.7	50.8 ± 45.1	42.3 ± 22.9	1170 ± 922
Limonene	8.2 ± 4.4	2.7 ± 2.7	11.4 ± 4.2	9.2 ± 6.4	5.2 ± 2.7	215 ± 152
(*E*)-β-Ocimene	143 ± 143	36.5 ± 18.6	95.7 ± 65.6	47.4 ± 30.7	82.6 ± 55.7	954 ± 548
Terpinene	[b]	[b]	[b]	[b]	[b]	620 ± 478
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	194 ± 209	51.3 ± 24.5	71.4 ± 78.2	54.6 ± 49.3	96.4 ± 46.4	215 ± 105
(*E*,*E*)-4,8,12 Trimethyl-1,3,7,11-tridecatetraene	3.3 ± 4.1	5.5 ± 11.3	[b]	[b]	5.0 ± 3.5	9.4 ± 8.5
Caryophyllene	99.8 ± 82.9	15.2 ± 8.5	122 ± 153	82.9 ± 55.2	45.9 ± 24.6	1640 ± 1010
α-Humulene	25.4 ± 21.9	3.2 ± 1.9	32.7 ± 43.5	22.4 ± 18.4	17.3 ± 7.9	412 ± 253

[a] Mean of six determinations ± standard deviation.
[b] Compound not detected or production less than 2 ng/hr.

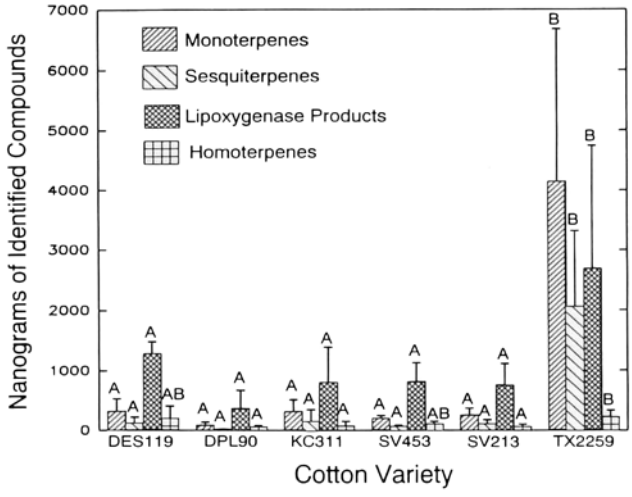

FIG. 1. Total identified volatiles, divided by biosynthetic class, from cotton varieties damaged by beet armyworm larvae. Data represent the means of six experiments ± standard deviation. Within a biosynthetic class, bars with the same letter are not significantly different by Duncan-Waller's multiple range comparison at $P = 0.05$.

possible that the terpenes released by the damaged plants in this study came from glands that were physically disrupted by the larvae. However, many of these terpenes, including (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene have previously been shown to be emitted in response to arthropod feeding damage in corn and lima bean (Turlings et al., 1990; Dicke et al., 1990). In a subsequent study (Loughrin et al., 1994) we found that larval damaged plants released significantly greater quantities of acyclic terpenes such as ocimene and the homoterpene and homosesquiterpene during the mid- to latter part of the photophase than during the scotophase or early photophase. However, cyclic terpenoids such as α-pinene and caryophyllene were not released in significantly greater amounts during the photophase than during the scotophase. Therefore, the time of the photoperiod during which volatiles are collected from a plant may have a significant effect on the composition of the volatile blend. In this study, volatiles were collected from all the cultivars at the same time of the photoperiod to allow valid comparison of the composition and quantities of the volatiles.

For most of the cotton cultivars studied, the greatest quantity of volatiles emitted by damaged plants consisted of lipoxygenase–hydroperoxide lyase products (Vick and Zimmerman, 1987). This class of compounds, formed by the

hydroperoxidation of fatty acids containing a *cis,cis*-1,4-pentadiene moiety, are known to be induced by damage (Hatanake et al., 1987) and are largely responsible for the odor of cut grass. While the majority of the lipoxygenase-derived volatiles identified in the present study are commonly associated with damaged leaves, a number of less commonly encountered esters of (*Z*)-3-hexen-1-ol were also identified in the volatiles from the leaves damaged by larvae. These were previously identified in volatiles collected from detached cotton seedlings damaged by *Helicoverpa zea* Boddie larvae (McCall et al., 1994). In the latter work it was also demonstrated that the emission of esterified forms of the lipoxygenase products was more favored from leaves with old damage than from freshly damaged plants. While the butanoic acid esters of (*Z*)-3-hexenol are less common than the acetate esters, (*Z*)-3-hexenyl butanoate and related esters are common floral headspace components (Knudsen et al., 1993).

Another compound identified in the volatiles from damaged cotton leaves, *cis*-jasmone, likely resulted from the action of a hydroperoxide dehydrase (Vick and Zimmerman, 1987) rather than hydroperoxide lyase on the hydroperoxide formed by lipoxygenase. This compound probably resulted from the β-oxidation and subsequent decarboxylation of jasmonic acid, a plant messenger known to be synthesized in response to insect damage and responsible for mediating responses such as the accumulation of proteinase inhibitors (Farmer and Ryan, 1990). Thus, this compound may have been formed in direct response to the damage incurred by the insect feeding.

When the quantities of compounds emitted by damaged leaves of the different cotton cultivars were summed by biosynthetic class and subjected to statistical analysis as shown in Figure 1, no significant differences were seen among the commercial cotton cultivars. However, TX2259 emitted substantially more volatiles of all biosynthetic classes, except the homoterpenes, than the other varieties.

The standard deviations noted in these experiments were quite large. These may have been caused by variations in the amount of damage done by the larvae, differences in environmental parameters such as light and relative humidity for each individual experiment, and/or variations in the stage of development of each individual leaf. Although visual inspection indicated that a similar amount of damage was inflicted by the larvae on all the varieties, we were not able to determine accurately the amount of damage to each plant. Therefore, to determine whether the higher levels of volatile emission from the variety TX2259 could have been due to greater feeding damage by the larvae to this variety, feeding choice tests were conducted. Larvae were given the opportunity to feed on a leaf of either a commercial cultivar or a TX2259 leaf. The results of these experiments (Figure 2) indicate that beet armyworm larvae consistently preferred to feed on leaves of the commercial cultivars when given a choice. Whether these results are due to greater amounts of terpenoids and other secondary metab-

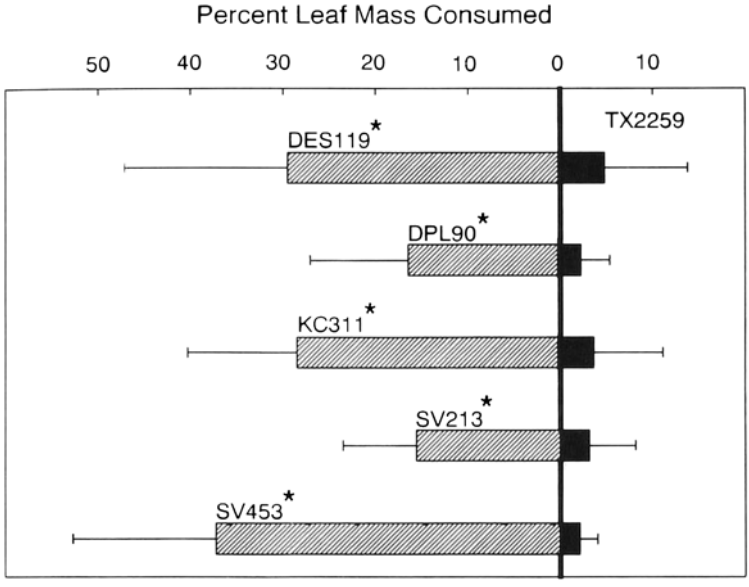

FIG. 2. Beet armyworm preference for feeding on leaves of commercial cotton cultivars versus those of TX2259 leaves. Two second-instar beet armyworms were provided with a mature leaf of TX2259 and a mature leaf of one of the commercial cultivars in an 18-cm covered dish. Solid bars to the right indicate mean percent of leaf mass of TX2259 consumed and hatched bars to the left indicate mean percent of leaf mass of the commercial cultivar leaf consumed in each choice test. $N = 6$ for each feeding choice test. Error bars represent standard deviation. An asterisk after a variety name indicates significant preference for feeding on the leaves of that variety (Student's t test).

olites or the presence of a specific feeding deterrent in TX2259 is unknown. However, the results suggest that beet armyworm larvae are not likely to cause more damage by feeding on TX2259 than on the other varieties, and thus the greater volatile emission by damaged TX2259 leaves was probably not due to greater damage incurred by this variety.

The volatiles released by cotton leaves damaged by feeding larvae may serve as indicators of the presence of hosts to potential biological control agents such as parasitic wasps (Elzen et al., 1984; Tumlinson et al., 1992). Of course, previous research has also implicated cotton volatiles in the attraction of pests such as the boll weevil (Gueldner et al., 1970; Dickens, 1990). Even given the low levels of terpenes released by undamaged leaves, the air over a cotton field should contain detectable amounts of these compounds. More likely, insect herbivore-induced compounds (Loughrin et al., 1994), which are released in

much greater amounts from herbivore-damaged plants than from mechanically damaged or undamaged plants, act as a beacon to draw parasitoids to plants with potential hosts.

In summary, the leaves of the cotton varieties we studied emitted only relatively low quantities of volatiles unless damaged by feeding larvae. This was true even for a naturalized cotton variety, but the latter emitted markedly greater amounts of terpenes from damaged leaves than the commercial cultivars studied. The implication for these findings may be that biological control using predators and parasitoids would be enhanced by developing strains of cotton that release greater amounts of volatile compounds when attacked by insect herbivores.

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Book Review

Beetle–Pathogen Interactions in Conifer Forests. T.D. Schowalter and G.M. Filip, (eds.). London: Academic Press, 1993. UK £50.00 hardcover. 252 pp. ISBN 0-12-628970-0. Applied Botany and Crop Sciences Series. Series editors R.W. Snaydon and J.M. Barnes.

This book contains an integrated synthesis of the current research and knowledge base developed by a multidisciplinary group of scientists organized under the former United States Department of Agriculture Regional Research Project W-110. The original 23-year project consisted of over 30 technical experts and explored the role of bark beetles and pathogens (predominantly fungal) as agents of stress on conifers in North American forest ecosystems. The text summarizes the contributions of 24 of these experts and their colleagues to that project. The project was renewed for an additional five-year period in late 1994 as Regional Research Project W-187: "Interactions Among Bark Beetles, Pathogens, and Conifers in North American Forests."

The book's twelve chapters are organized into six sections. Excluding the first and last sections, which introduce and summarize the book, the section topics advance sequentially from organismal to ecological and managerial levels (e.g., systematics, factors influencing interactions, effects of interactions, and management of interactions). The authors and editors have generously supplemented the work with black-and-white photos, figures, and tables. The photos appropriately illustrate the text, and many were photographed, and apparently chosen by the editors themselves. Schowalter and Filip also wisely chose to present the scientific and common names of all of the organisms discussed in the text in four appendices. The appendices pertaining to trees, insects, and pathogens (fungi, bacteria, nematodes, and parasitic higher plants) are all based on accepted checklists or reference works of nomenclature. An index based on key words and common and scientific names has been included and is very useful. An index of authors of the literature cited throughout the text would have been a useful addition to the book.

The theme of the text is that species interactions are of paramount importance to the productivity and stability of integrated forest ecosystems. The 252-page volume focuses on the systematics, physiology, biology, and ecology of scolytid bark beetles, ascomycete and deuteromycete fungi, and, to some extent, their pinaceous host plants. Nonetheless the effects of predacious and parasitic insects, mites, nematodes, and bacteria on the colonization of host conifers by

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bark beetles and pathogenic fungi are also considered in this treatment of forest ecology. Furthermore, the effects of biotic and abiotic agents that predispose host trees to bark beetle and fungal colonization are also discussed. All of these species and factors are considered to be integral to the function of forested terrain from the ecosystem perspective.

With the current focus on environmental health and biodiversity issues in ecology, the value of the material in this book to ecologists is self-evident. This well-studied and integrated system provides an ecological case study of considerable breadth and depth. The book serves as an entry point into an ecological literature that is often avoided by students because of the sheer number of research papers on bark beetle–fungal interactions. Some of the ecological relationships described in the book are simply stunning. Two species of tarsonemid mites, which are flightless associates of the southern pine beetle, *Dendroctonus frontalis*, possess specialized morphological structures to carry ascospores of species of plant pathogenic *Ophiostoma* fungi, and this fungal–acarial interaction ostensibly assists *D. frontalis* in its colonization of its primary host, loblolly pine, *Pinus taeda* (Stephen et al., p. 142).

To chemists or chemical ecologists, the value of this book is more subtle. Chapters entitled "Environmental Stress and Whole-Tree Physiology," "Strategies and Mechanisms of Host Colonization by Bark Beetles," and "Host Response to Bark Beetle and Pathogen Colonization" all contain significant portions devoted to chemical mediation of the beetle–pathogen–host triangle. In the latter chapter, Nebeker et al. (pp. 164–166) not only discuss a variety of phytochemicals involved in the induced defensive response of conifers to fungal colonization, but also review the literature related to a group of hydroxy isocoumarins that are thought to be toxic fungal metabolites involved in overcoming tree resistance and eventually killing the tree. They follow this (p. 168) with an interesting discussion of iron chelation chemistry that might be involved in the blue sapwood discoloration that is the signature of blue-stain fungi ubiquitous in trees colonized by phloeophagous and xylophagous insects.

Moreover, the book is replete with biological and ecological vignettes that could provide the basis for many studies of chemical ecology. For example, Stephen et al. (pp. 138–141) discuss the phenomenon of phoresy by wingless mites that ride on bark beetles or other flying associates to disperse to new subcortical tissue of conifers. Since the phoretic relationship between mite and bark beetle host can sometimes be quite species-specific, these interactions might provide interesting models for the study of host recognition through contact chemoreception between the arthropodous classes Arachnida and Insecta. Although Stephen et al. do not raise the subject in their discussion of bark beetle–fungal mutualism (pp. 142–144), one could envision a role for ingested fungal cellulose-degrading enzymes in digestion of phloem and xylem tissue by

scolytids. This would be analogous to the elegant work on xylophagous cerambycid beetles and siricid woodwasps in the laboratory of M.M. Martin. In another example, conifers that are damaged by lightning strike or fire exhibit elevated levels of bark beetle and pathogen colonization (Paine and Baker, pp. 64–67). When compared to controls, lightning-struck *P. taeda* have different relative amounts of specific monoterpenes as well as other biochemical differences (Nebeker et al., p. 160). One wonders which biochemical changes, and in particular which monoterpenes, are primarily responsible for the enhanced colonization rate by bark beetles following a lightning strike and whether the same chemical phenomena apply to fire-damaged trees. Lastly, both Raffa et al. (p. 111) and Nebeker et al. (pp. 157–158) review the behavioral basis for feeding stimulation in bark beetles and preliminary attempts at chemical characterization of the relevant semiochemicals. This is an area that is woefully understudied and deserves the attention of insect behaviorists and chemists skilled in modern preparative and analytical techniques. Clearly, the curious chemical ecologist would find fertile subject matter for research among the organismal interactions introduced and explored in this book.

In general the chapters are interwoven quite skillfully by the many authors. They frequently and smoothly allude to each other's chapters throughout the book. However, problems inherent with chapters written by teams of experts do surface from time to time. For example, Stephen et al. (p. 137) write "All parasitoids of bark beetles are wasps (Hymenoptera)." However, two pages later in a discussion of mites associated with bark beetles, the same authors write "Although **mite parasitoids** tend to be the most specific, practically all ride more than one host (bark beetle) species." Nonetheless, due perhaps to excellent technical reviews of the entire book by R.L. Edmonds and D.L. Wood, these occurrences are rare in a text that amalgamates the input of a large number of experts. The positive effect of the collective expertise far outweighs any minor oversights of coordination.

As a chemical ecologist interested in the colonization behavior of bark beetles and the role of aggregation pheromones in that behavior, I dwelled on Chapter 6: "Strategies and Mechanisms of Host Colonization by Bark Beetles." The chapter, written by K.F. Raffa and two colleagues, is a masterful and complete update of a 1982 review of the same topic by D.L. Wood. Undoubtedly this chapter will be the new classic of citation for the next generation of bark beetle chemical ecologists. A section on pheromone biosynthesis by scolytids is particularly well written. While the authors emphasize conversion of host monoterpenes to monoterpene alcohol and ketone pheromone components by bark beetles, they also discuss the possibility that cyclic ketal and some monoterpene alcohol pheromone components might arise from *de novo* synthesis. The open-minded approach of this review is critical to its enduring nature,

as recent results in our laboratory demonstrate that the model species *Ips paraconfusus* and *I. pini* can both produce hydroxylated monoterpenes *de novo* from radiolabeled acetate.

As I read this book I noted that it has a marked regional emphasis. Nineteen of the 24 authors are from the western and southern regions of the United States, and the book is centered on model organisms and systems that occur in these regions. There is less coverage of the Lake States (central) region and even less of the eastern region. For example, Filip and Schowalter (p. 231) summarize the significance of bark beetles and root pathogens to human utilization of timber resources by stating that they cause a loss of volume of 0.9–1.1 billion m^3/year in **western** North America. This regional influence likely resulted because the membership of Project W-110 was drawn primarily from the western and southern regions of the United States, which in turn reflects the economic impact of coniferous forests in these regions and the research priorities of the relevant state agricultural experiment stations.

It is likely that some portions of *Beetle–Pathogen Interactions in Conifer Forests* will not capture the attention of all chemical ecologists. Chapters on topics such as abiotic and biotic predisposition of conifers to attack by bark beetles and pathogenic fungi, computer modeling of beetle–pathogen–tree interactions, and management of root disease and bark beetle impacts on forest resources, while interesting, are typically not in the mainstream reading of nonforest ecologists. However, enough of this book deals with chemically related ecological issues and interactions to justify careful scrutiny by the readers of the *Journal of Chemical Ecology*. The book, and especially selected chapters (1, 5, 6, 7, 8, 9, and 12), would be very suitable as discussion matter for a graduate level seminar or supplementary course reading in general ecology or chemical ecology. This book provides a valuable window into a voluminous and often intimidating primary literature that will aid research scientists, instructors, and students in chemical ecology, general ecology, forestry, and resource management.

Steven J. Seybold
Department of Biochemistry
University of Nevada, Reno
Reno, Nevada 89557-0014

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Announcement

BIOLOGY OF COMMUNICATION AWARD

The Jean-Marie Delwart Foundation will award in 1996 a prize for research or a series of researches, individual or collective, in the field of chemical communication. The award will recognize the discovery of a new signaling function of one or several substances particularly in the relations between animal species (including humans) as well as between animal and plants.

The research or researches should be fundamental and original.

The prize of $10,000 will be awarded for works written in or translated into French or English.

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Belgium

by December 31, 1995.

The jury is composed of members of the scientific committee of the Jean-Marie Delwart Foundation and of members of the Académie Royale des Sciences de Belgique.

The prize will be awarded in December 1996 at the Public Session of the Académie Royale des Sciences de Belgique.

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PRESERVING LEAVES FOR TANNIN AND PHENOLIC GLYCOSIDE ANALYSES: A COMPARISON OF METHODS USING THREE WILLOW TAXA

COLIN M. ORIANS[1]

Department of Biology, Williams College
Williamstown, Massachusetts 01267

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Abstract—Members of the Salicaceae often produce phenolic glycosides and condensed tannins. There is much debate on the best method for the preservation of leaf material prior to chemical analysis. Published results indicate freeze-drying, a method commonly used for tannin analysis, may be inappropriate for phenolic glycosides, unless done in a manner to prevent thawing during the drying process. Another commonly employed method, air-drying, is appropriate for phenolic glycosides but inappropriate for condensed tannins. I present evidence using willow leaves that demonstrates that: (1) leaves freeze-dried in external flasks without temperature control contain lower concentrations of phenolic glycosides (salicortin and 2′-cinnamoylsalicortin), (2) air-dried leaves have reduced concentrations of condensed tannins, while (3) vacuum-dried fresh leaves have high concentrations of both phenolic glycosides and condensed tannins. Freeze-drying caused salicortin and 2′-cinnamoyl salicortin concentrations to drop by 20 mg/g and 4 mg/g, respectively. Salicin, a product of salicortin and 2′-cinnamoyl salicortin degradation, is absent in vacuum-dried leaves, present in air-dried leaves and very high in freeze-dried leaves. Thus, the presence of salicin in this system is an artifact of the preservation technique. Condensed tannin concentrations dropped nearly 20 mg/g when leaves were air-dried. Thus, vacuum-drying fresh leaves allows researchers to quantify phenolic glycosides and condensed tannins from the same leaf material.

Key Words—Salicaceae, willows, phenolic glycosides, salicortin, 2′-cinnamoyl salicortin, salicin, condensed tannins, leaf preservation.

INTRODUCTION

Willows and other Salicaceous plant species produce two main secondary chemicals: phenolic glycosides and/or condensed tannins (Julkunen-Tiitto, 1986,

[1]Present address: Dept. of Biology, Tufts University, Medford, MA 02155.

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1989; Lindroth et al., 1987). The concentrations of the phenolic glycosides may vary among individuals (Nichols-Orians et al., 1993) and in some cases influence the susceptibility of plants to both insect and mammalian herbivores (Rowell-Rahier, 1984; Tahvanainen et al., 1985; Basey et al., 1988; Lindroth and Peterson, 1988; Lindroth et al., 1988; Clausen et al., 1989; Lindroth, 1992). Condensed tannins are bioactive as well, especially against microbes (Schultz, 1989; Nichols-Orians, 1991a).

Salix sericea Marshall and *Salix eriocephala* Michx. produce different secondary chemicals. *S. sericea* produces two main phenolic glycosides, salicortin and 2′-cinnamoylsalicortin (Nichols-Orians et al., 1992) and very low concentrations of condensed tannins. *S. eriocephala* produces high concentrations of condensed tannins and no phenolic glycosides. Interestingly, these species hybridize and *S. sericea* × *S. eriocephala* hybrids produce both tannins and phenolic glycosides at intermediate concentrations (Orians and Fritz, 1995). When studying the bioactivity of these secondary chemicals, it is critical that leaves be preserved in a manner that prevents degradation.

Salicortin is quite labile and when improperly preserved it degrades to salicin (Lindroth and Pajutee, 1987; Julkunen-Tiitto and Meier, 1992). Even relatively nonlabile chemicals like tannins are lost when improperly preserved (Hagerman, 1988). Given the bioactivity of both these chemical types, it is of interest to determine the method of preservation that will prevent their degradation (Lindroth and Pajutee, 1987; Hagerman, 1988; Julkunen-Tiitto and Gebhardt, 1992; Julkunen-Tiitto and Meier, 1992).

Julkunen-Tiitto and Meier (1992) have suggested that air-drying at low temperatures is sufficient to prevent the degradation of phenolic glycosides. However, air-drying leaves is known to result in the degradation of tannins (Hagerman, 1988). Van Sumere et al. (1983) suggest that lyophilization (freeze-drying) is inappropriate for low- and intermediate-molecular weight phenolics. When drying leaves for tannin analysis, Hagerman (1988) recommends fresh extraction or freeze-drying prior to extraction. The exact method depends upon the species and time of year. Thus, the method appropriate for one set of chemicals is often inappropriate for another. The lack of a standard technique has made it difficult to compare studies and has hindered researchers in their efforts to determine the bioactivity of foliar chemicals. This study was initiated after preliminary results indicated that vacuum-drying was appropriate for both phenolic glycosides and tannins.

Here I compare three methods of preservation (air-drying, freeze-drying, and vacuum-drying). Based on studies by Hagerman (1988), Van Sumere et al. (1983), and Julkunen-Tiitto and Meier (1992), I expected concentrations of phenolic glycosides to be lowest in freeze-dried leaves and concentrations of condensed tannins to be lowest in air-dried leaves. The results indicate that air-drying results in a loss of tannins, freeze-drying causes extensive conversion of

salicortin and 2′-cinnamoylsalicortin to salicin, while vacuum-drying preserved both the phenolic glycosides and the tannins. Thus, of the three methods, vacuum-drying is superior for both chemicals.

METHODS AND MATERIALS

Plants. Salix sericea Marshall and *Salix eriocephala* Michx. are abundant in the northeastern United States and eastern Canada (Argus, 1986). They produce leaves continuously from May until September at our field site near Milford, New York, and adult plants reach a height of 3–4 m. *S. sericea* × *S. eriocephala* hybrids are abundant at the field site.

Cuttings from field plants at the study site were brought back to Williams College. Cuttings were rooted, potted, and then placed in a common garden on Williams College campus. I used a total of 36 one-year old cuttings for this study (12 of each plant species). Four plants of each species were randomly assigned to each treatment. Thus all the leaves removed from one plant were processed the same way.

Leaf Material. Two adjacent leaves (the first two fully mature leaves) from eight different shoots were collected from each plant. The methods of collection were as follows. For freeze-drying, leaves were immediately frozen in liquid nitrogen, transported to the lab, placed in flasks and attached to a Labconco (model 77500) freeze-drier. Care was taken to limit thawing once frozen. This freeze-drier has external flasks (that cannot be temperature regulated) attached to a central vacuum. Therefore it was impossible to prevent thawing completely prior to sublimation.

For air-drying, leaves were put into a cooler containing ice, transported back to the lab, and air-dried at 60°C for 24 hr.

For vacuum-drying, leaves were put into the ice cooler, transported back to the lab, and immediately vacuum dried (a freeze-dryer was used but the leaves were never frozen). Once dry, leaves were ground in a Wiley mill (size 30 mesh), weighed, and stored in the freezer until chemical analyses were performed.

Chemical Analysis. The phenolic glycosides were assayed using standard techniques (Nichols-Orians et al., 1992, 1993). Briefly, leaf powder (30 ± 3 mg) was extracted in cold MeOH (10 mg leaf powder/1 ml MeOH) with sonication for 10 min. Cold water was constantly flushed through the sonicator to prevent the heating of samples. Extracts were centrifuged and filtered (0.45 μm) then placed in crimp-top vials. Extracts were kept in the freezer until analysis (48 hr or less). The concentration of glycosides was quantified with an HPLC (Hewlett-Packard) and a UV detector set at 274 nm. A reverse-phase Nova-Pak C_{18} (4 μm) column (Waters) and a gradient system of distilled water and MeOH

was used. The samples were injected through an LS-3200 automated sampler (SGE). 1,3-Dimethoxybenzene was used as the internal standard. Purified standards of salicin, salicortin, and 2′-cinnamoylsalicortin were used to determine concentrations.

The condensed tannins also were extracted using standard techniques (see Nichols-Orians, 1991a,b). Briefly, approximately 300 mg leaf powder was washed with ether and extracted with 70% acetone for 3 hr in a 40°C water bath. Acetone was removed under reduced pressure and all extracts were diluted to 8 ml with distilled water. Two techniques were used to quantify condensed tannin concentrations: the butanol/HCl method and the vanillin method (Bate-Smith, 1977; Butler et al., 1982; Hagerman and Butler, 1989). The concentration of the condensed tannin (milligrams per gram dry leaf) in each species was determined using purified tannins collected from each of the species.

RESULTS AND DISCUSSION

The method of preservation had a statistically significant effect on the concentrations of the phenolic glycosides (see treatment effect in Table 1). As expected, freeze-dried *S. sericea* or hybrid leaves had the lowest concentrations of salicortin and 2′-cinnamoylsalicortin (Figure 1A and B) (*S. eriocephala* does not produce phenolic glycosides). The difference in phenolic glycoside concentration between air-dried and vacuum-dried leaves was not significant. However, the presence of a breakdown product, salicin, is the best indicator of salicortin or 2′-cinnamoylsalicortin degradation. No salicin was present in the vacuum-dried leaves but was present in both the air-dried and freeze-dried *S. sericea* leaves (Figure 1C).

TABLE 1. SIGNIFICANCE OF EFFECTS OF LEAF PRESERVATION TECHNIQUE ON CONCENTRATIONS OF PHENOLIC GLYCOSIDES IN *S. sericea* AND HYBRID LEAVES USING TWO-WAY ANALYSIS OF VARIANCE WITH PLANT SPECIES AND PRESERVATION TECHNIQUE AS MAIN EFFECTS[a]

Source of variation	Phenolic glycoside					
	Salicortin		2-Csalicortin[b]		Salicin	
	F	*P*	*F*	*P*	*F*	*P*
Taxon	34.95	<0.001	5.95	0.025	165.98	<0.001
Treatment	13.33	<0.001	4.02	0.036	432.62	<0.001
Taxon × Trtmt	1.34	NS	0.01	NS	71.35	<0.001

[a]Concentrations were LN transformed prior to analyses.
[b]2-Csalicortin = 2′-cinnamoylsalicortin.

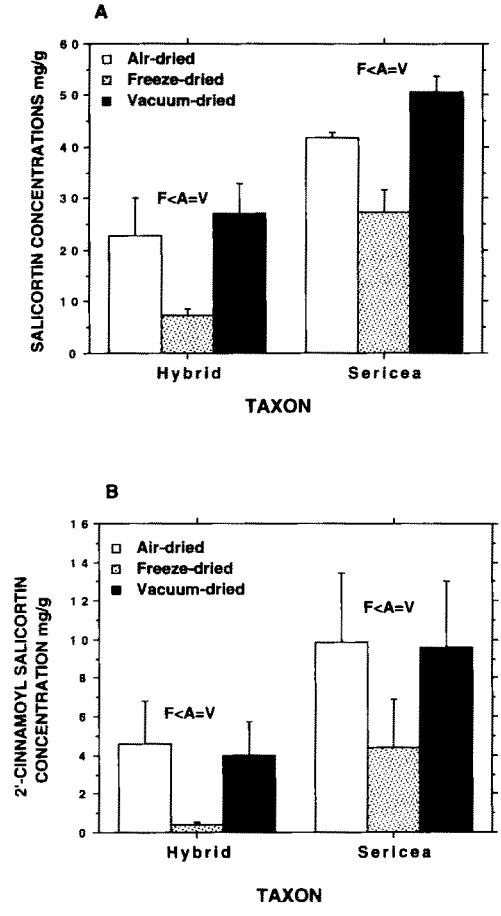

FIG. 1. The concentration (milligrams per gram dry leaf) of (A) salicortin, (B) 2'-cinnamoylsalicortin, and (C) salicin as a function of plant taxon and preservation treatment. Data were ln transformed prior to analyses and the Bonferroni method was used to determine which treatments were significantly different. A = air-dried, F = freeze-dried, V = vacuum-dried.

When vacuum-dried, the mean concentration of salicortin was 50 mg/g in *S. sericea* leaves and 27 mg/g in hybrid leaves. When freeze-dried, the mean concentration dropped to approximately 27.5 mg/g in *S. sericea* and 7.4 mg/g in hybrid leaves. Salicin concentrations increased from zero in vacuum-dried leaves to approximately 21 mg/g and 10 mg/g in *S. sericea* and hybrid freeze-dried leaves, respectively.

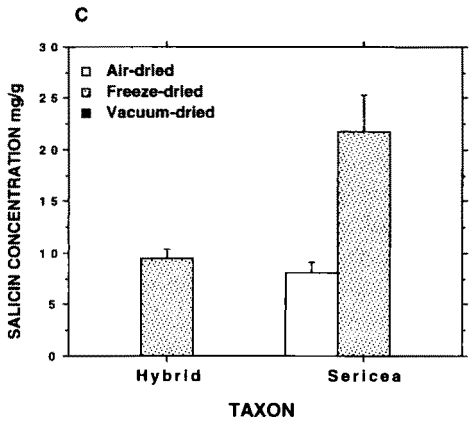

FIG. 1. Continued.

If samples are placed in a freeze-dryer with a temperature-regulated drying chamber (set below freezing), degradation can be prevented (Lindroth, personal communication). It is critical, therefore, not to use freeze-driers with external flasks when analyzing phenolic glycosides. Many researchers have reported the presence of salicin in their study species, yet my results show that its presence may be merely an artifact of the preservation technique.

Condensed tannin concentrations also varied with preservation technique (Table 2). As expected, both the vanillin and the butanol/HCl method show that concentrations are lowest in air-dried leaves and highest in the freeze- and

TABLE 2. SIGNIFICANCE OF EFFECTS OF LEAF PRESERVATION TECHNQIUE ON CONCENTRATIONS OF CONDENSED TANNINS IN LEAVES USING TWO-WAY ANALYSIS OF VARIANCE WITH PLANT TAXON AND PRESERVATION TECHNIQUE AS MAIN EFFECTS[a]

Source of variation	Condensed tannin method			
	Vanillin method		Butanol/HCl method	
	F	*P*	*F*	*P*
Taxon	260.49	<0.001	149.64	<0.001
Treatment	4.35	0.023	8.55	0.001
Taxon × Trtmt	0.86	NS	1.42	NS

[a]Concentrations were LN transformed prior to analyses.

vacuum-dried leaves (Figure 2A and B). The loss of tannin with air-drying is consistent with the results of Hagerman (1988). It is standard methodology to freeze-dry leaves prior to tannin analysis. I found no difference in concentration between freeze-dried and vacuum-dried leaves. Clearly vacuum-drying is an excellent method when quantifying the concentrations of condensed tannin.

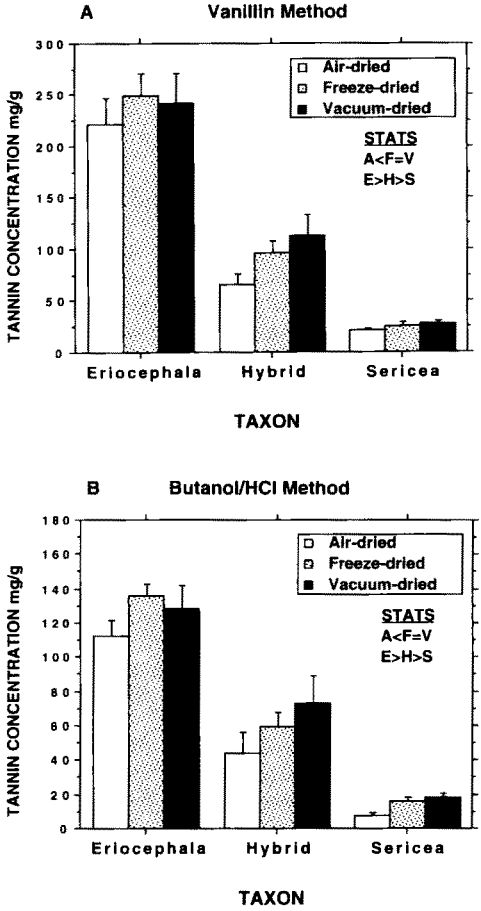

FIG. 2. The effects of plant taxon and preservation treatment on the concentration of condensed tannin (milligrams per gram dry leaf) as determined with the (A) vanillin method and (B) butanol/HCl method. Data were transformed prior to analyses and the Bonferroni method was used to determine which taxa and treatments were significantly different. A = air-dried, F = freeze-dried, V = vacuum-dried, E = *S. eriocephala*, H = hybrid and S = *S. sericea*.

Overall, this technique allows researchers to quantify both phenolic glycosides and condensed tannins from the same leaf material. For plants that contain both chemical types, such as *S. sericea*, *S.s.* × *S.e.* hybrids, and most *Populus* sp., this technique would be ideal. Further study will be required to determine if vacuum-drying will be the method of choice for other chemicals, such as hydrolyzable tannins and alkaloids. Van Sumere et al. (1983) suggest that some phenolics might be lost during freeze-drying under high-vacuum conditions because many of the volatile phenolics are concentrated on the ice trap. Whether these same chemicals would be lost in the absence of freezing is unknown. If vacuum-drying proves to be generally appropriate, it would alleviate the need for liquid nitrogen and facilitate research in remote sites. I conclude that researchers studying salicaceous species should adopt this preservation technique as it prevents the degradation of both phenolic glycosides and condensed tannins and avoids the need for liquid nitrogen—a cooler of ice and a vacuum pump will do.

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SECONDARY CHEMISTRY OF HYBRID AND PARENTAL WILLOWS: PHENOLIC GLYCOSIDES AND CONDENSED TANNINS IN *Salix sericea*, *S. eriocephala*, AND THEIR HYBRIDS

COLIN M. ORIANS[1,*] and ROBERT S. FRITZ[2]

[1]*Department of Biology, Williams College*
Williamstown, Massachusetts 01267

[2]*Department of Biology, Vassar College*
Poughkeepsie, New York 12601

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Abstract—*Salix sericea* and *S. eriocephala* differ markedly in secondary chemistry. *S. sericea* produces phenolic glycosides, salicortin and 2′-cinnamoylsalicortin, and low concentrations of condensed tannin. In contrast, *S. eriocephala* produces no phenolic glycosides, but high concentrations of condensed tannins. Hybrid chemistry is intermediate for both types of chemicals, suggesting predominantly additive inheritance of these two defensive chemical systems from the parental species. However, there is extensive variation among hybrids. This variation may be due to genetic variation among parental genotypes, which genes were passed on, or to subsequent back-crossing. The differences in chemistry are likely to exert a strong effect on the relative susceptibility of hybrid and parental willows to herbivores.

Key Words—Salicaceae, willows, hybrids, hybridization, phenolic glycosides, salicortin, 2′-cinnamoylsalicortin, condensed tannins.

INTRODUCTION

Hybridization among plants is very common (Barton and Hewitt, 1985; Rieseberg and Brunsfeld, 1992; Reiseberg and Ellstrand, 1993). The ubiquity of hybrids has important implications to plant speciation and could affect the evolution of plant phytophages. In fact, the study of herbivores in plant hybrid

*To whom correspondence should be addressed. Present Address: Department of Biology Tufts University Medford, MA 02155.

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zones has recently gained attention (Whitham, 1989; Boecklen and Spellenberg, 1990; Whitham et al., 1991, 1994; Aguilar and Boecklen, 1992; Floate and Whitman, 1993; Paige and Capman, 1993; Fritz et al., 1994). While some have argued that hybrids typically have higher densities of herbivores and thus may serve as ecological and evolutionary sinks (sensu Whitham 1989), others have argued that the relative abundance of herbivores and hybrids and parental taxa is more variable; densities may be greater, less, or equal to the parental species (Boecklen and Spellenberg, 1980; Aguilar and Boecklen, 1992; Fritz et al., 1994).

The different patterns of herbivore abundance could be due to the specifics of the plant system; Boecklen and colleagues study oaks, Whitham and colleagues study cottonwoods, and we have been studying willows. The morphology and chemistry of hybrids will differ among systems (Meier et al., 1989; O'Donoughue et al., 1990; Levy and Milo, 1991; Reiseberg and Ellstrand, 1993). However, differences in abundance could merely be due to variation among herbivores in their response to plant traits (Rowell-Rahier, 1984; Tahvanainen et al., 1985a; Marquis, 1990).

The effects of hybridization on plant morphology are well researched (Whitham, 1989; Boecklen and Spellenberg, 1990; O'Donoughue et al., 1990; Levy and Milo, 1991; Reiseberg and Ellstrand, 1993), but the effects of hybridization on foliar chemistry are less understood (Rabotyagov and Akimov, 1987; Huesing et al., 1989; Meier et al., 1989; Reiseberg and Ellstrand, 1993). Willows and other salicaceous plant species produce two main secondary chemicals: phenolic glycosides and condensed tannins (Julkunen-Tiitto, 1986, 1989; Lindroth et al., 1987). In this system, *S. sericea* produces high concentrations of phenolic glycosides, especially salicortin (Nichols-Orians et al., 1992, 1993) and phenolic glycosides are well known to influence the susceptibility of plants to both insect and mammalian herbivores (Rowell-Rahier, 1984; Tahvanainen et al., 1985a,b; Basey et al., 1988; Lindroth and Peterson, 1988; Lindroth et al., 1988; Clausen et al., 1989; Denno et al., 1990; Kolehmainen et al., 1994). Preliminary results indicated that *S. sericea* produces low concentrations of condensed tannins, while *S. eriocephala* produces high concentrations of condensed tannins, but no phenolic glycosides. Since condensed tannins can be bioactive as well, especially against microbes (Zucker, 1983; Schultz, 1989), the patterns of inheritance of these two chemicals could have strong effects on the relative resistance of hybrids to phytophages.

Here we report on the concentrations of condensed tannin and phenolic glycosides in *Salix sericea*, *Salix eriocephala*, and hybrids of the two species. To avoid confounding environmental effects (Bryant et al., 1987; Price et al., 1989; Julkunen-Tiitto et al., 1993), we grew the hybrids and the two parental taxa in a common garden.

METHODS AND MATERIALS

Plants. Both *Salix sericea* Marshall and *Salix eriocephala* Michx. are abundant in the northeastern United States and eastern Canada (Argus, 1986). They produce leaves continuously from May until September at our field site near Milford, New York, and adult plants reach a height of 3–4 m. Hybrid and parental plants are abundant within our field site. In March 1993 we took leafless budsticks (cuttings) from seven *S. eriocephala*, eight hybrid, and eight *S. sericea* field plants. These plants were far apart from one another and therefore likely represent different genotypes.

Cuttings were dipped in Rootone and planted in a soiless medium until roots formed. In May the plants were transplanted into pots containing soil, peat moss, and vermiculite (3 : 1 : 1). For this experiment one cutting from each of the field plants was used. Plants were replaced in a common garden on the Williams College campus, and fertilized weekly with 120 ml of 3.2 g/liter 20–20–20 Agway complete fertilizer. On July 20, leaves were collected for chemical analyses.

Leaf Material. Leaf developmental age can have pronounced effects on leaf chemistry (unpublished data). Therefore it is important to compare leaves of similar age. The results reported here are based on the chemistry of the first fully mature leaf. In order to get enough leaf material for both tannin and phenolic glycoside analyses, we collected 10 different leaves (the first fully mature leaf on 10 different shoots). Leaves were cut at the petiole, placed on ice, transported to the laboratory and vacuum-dried. Vacuum-drying of leaves has been shown to prevent the loss of both condensed tannins and phenolic glycosides (Orians, 1995).

Chemical Analysis. The phenolic glycosides were assayed using standard techniques (Nichols-Orians et al., 1992, Orians, 1995). Briefly, leaf powder (30 $\pm$ 3 mg) was extracted in cold MeOH (10 mg leaf powder/ml MeOH) with sonication for 10 min. Cold water was constantly flushed through the sonicator to prevent the heating of samples. We centrifuged and filtered (0.45 μm filter) each sample before placing extracts in crimp-top vials. Extracts were kept in the freezer until analysis (48 hr or less). We quantified the concentration of glycosides with an HPLC (Hewlett-Packard) and a UV detector set at 274 nm. A reverse-phase Nova-Pak C_{18} (4 μm) column (Waters) and a gradient system of distilled water and MeOH was used. We injected samples through an LS-3200 automated sampler (SGE). 1,3-Dimethoxybenzene was used as the internal standard. Standard curves were determined for salicortin and 2′-cinnamoylsalicortin.

The condensed tannins also were extracted using standard techniques (Orians, 1995). Briefly, approximately 300 mg leaf powder was washed with ether

and extracted with 70% acetone for 3 hr in a 40°C water bath. Acetone was removed under reduced pressure, and all extracts were diluted to 5 ml with distilled water. The butanol/HCl method was used to quantify tannin concentrations (Hagerman and Butler, 1989). The concentration of the condensed tannin (milligrams per gram dry leaf) was determined using purified tannins collected from the two willow species and the hybrids. For reference we have included results using quebracho tannin as a standard.

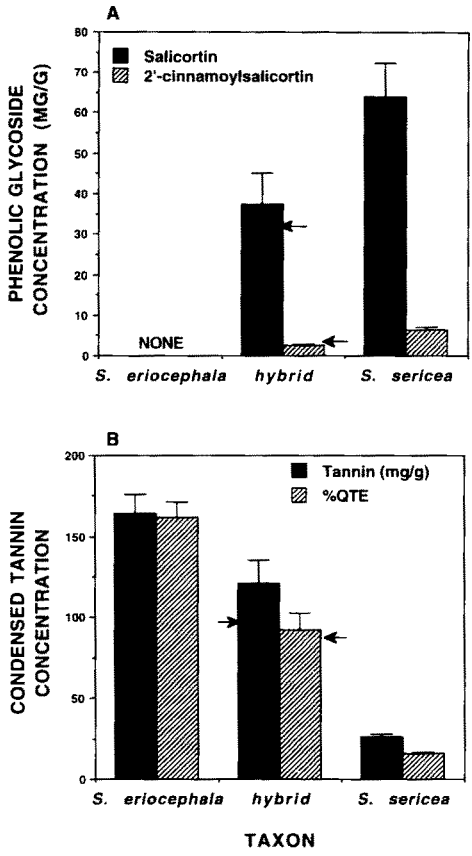

FIG. 1. The concentration (milligrams per gram dry leaf weight) of (A) phenolic glycosides and (B) condensed tannins in parental and hybrid willows. Arrows represent midpoint concentrations.

RESULTS AND DISCUSSION

The concentrations of condensed tannins and phenolic glycosides differ among the three taxa (ANOVA, $P \leq 0.03$) (Figure 1). *S. sericea* contains high concentrations of salicortin and 2′-cinnamoylsalicortin and low concentrations of condensed tannins. Conversely, *S. eriocephala* produces high concentrations of condensed tannins but no phenolic glycosides. The concentrations of these two types of chemicals are intermediate in the hybrids. We performed contrasts to determine whether the concentrations in hybrids are statistically different from the parental midpoint. We found no significant difference for condensed tannins ($P \geq 0.06$), salicortin ($P = 0.51$) or 2′-cinnamoylsalicortin ($P = 0.39$). The intermediate concentrations of these chemicals suggests additive inheritance [a result consistent with other studies (Meier et al., 1989; Reiseberg and Ellstrand, 1993] or balanced ambidirectional dominance. Deviation from the midpoint would have indicated some directional dominance.

The concentrations of both condensed tannins and phenolic glycosides among hybrids are quite variable. In general, the coefficients of variation resemble *S. eriocephala* for tannin concentration and *S. sericea* for phenolic glycoside concentration (Table 1). Thus, overall, hybrids are more variable and the lack of significant differences, on average, from the mid-parent reflects the plants we chose to study. Condensed tannin concentrations in hybrids range from 78 to 171 mg/g dry leaf weight, and salicortin and 2′-cinnamoylsalicortin concentrations range from 10 to 70 and from 0.75 to 5.6 mg/g dry leaf weight, respectively.

The variation among hybrids may reflect parental variation in chemistry.

TABLE 1. COEFFICIENT OF VARIATIONS FOR CONCENTRATIONS OF PHENOLIC GLYCOSIDES AND CONDENSED TANNINS IN *S. sericea*, HYBRID AND *S. eriocephala* LEAVES

	Taxon		
Chemical	*S. sericea*	Hybrid	*S. eriocephala*
Phenolic glycosides			
Salicortin	46.1	42.5	NA
2′-Cinnamoylsalicortin	59.5	39.9	NA
Condensed tannin			
Absolute concentration	3.5	38.6	28.9
%QTE*	2.0	32.1	30.4

* %QTE = % quebracho tannin equivalents.

A hybrid may produce a low concentration of 2′-cinnamoylsalicortin if its *S. sericea* mother does. We know that phenolic glycoside concentrations vary extensively within *S. sericea* (Nichols-Orians et al., 1993), and have found that *S. sericea* female #61 and its *S. sericea* progeny contain low concentrations of 2′-cinnamoylsalicortin (unpublished data). We are currently testing the correlation between parental and offspring chemistry for both intraspecific and interspecific crosses. There could also be between-progeny variation because a parent passes on half its genes to each offspring. Finally, variation among hybrids could be due to whether the hybrid is F_1, F_2, or back-crossed to one of the parental species. We do not know the status of these hybrids, but are currently collaborating with Stephen Brunsfeld to evaluate the effects of back-crossing on the chemistry.

Interestingly, we found an inverse correlation between tannin and phenolic glycoside concentration among the hybrids (Figure 2A and B). (No negative correlation was found within *S. sericea*.) This negative phenotypic correlation indicates that it may be impossible for hybrids to produce high concentrations of both phenolic glycosides and condensed tannins (sensu Berenbaum et al., 1986). Future studies will evaluate the basis of this trade-off and its effect on hybrid–herbivore interactions.

Differences in chemistry can strongly affect the resistance of salicaceous plants to herbivores (Rowell-Rahier, 1984; Tahvanainen et al., 1985a; Basey et al., 1988; Lindroth and Peterson, 1988; Lindroth et al., 1988; Clausen et al., 1989; Denno et al., 1990; Kolehmainen et al., 1994). For example, Tahvanainen et al. (1985a) found that herbivory by several willow-feeding beetles varied as a function of the phenolic glycoside content of different willow species. They found that the response of each beetle species depended upon its adaptations to phenolic glycosides. Previously we reported (Fritz et al., 1994) that many herbivore species differ in their response to *S. eriocephala*, *S. sericea*, and *S.e.* × *S.s.* hybrids. Depending on the herbivore, hybrids were less resistant, intermediate in resistance, or as resistant as one of the parental taxa. Since we know phenolic glycosides and tannins differentially affect herbivores (Rowell-Rahier, 1984; Tahvanainen et al., 1985a; Schultz, 1989; Denno et al., 1990), plant chemistry may be one factor that explains the varied herbivore responses. There is, however, no a priori reason to expect hybrids to be more or less resistant than either of the parental taxa.

The apparent additive inheritance of these secondary chemicals in hybrids has further implications for the resistance of hybrids to herbivores. Whitham (1989) suggests that back-crossing might lead to the further breakdown of resistance traits of backcross plants compared to parents or F_1s. However, if a resistance trait were additively inherited, back-crossing to the parent with the higher concentration should lead to the recovery of resistance traits of the parent. This

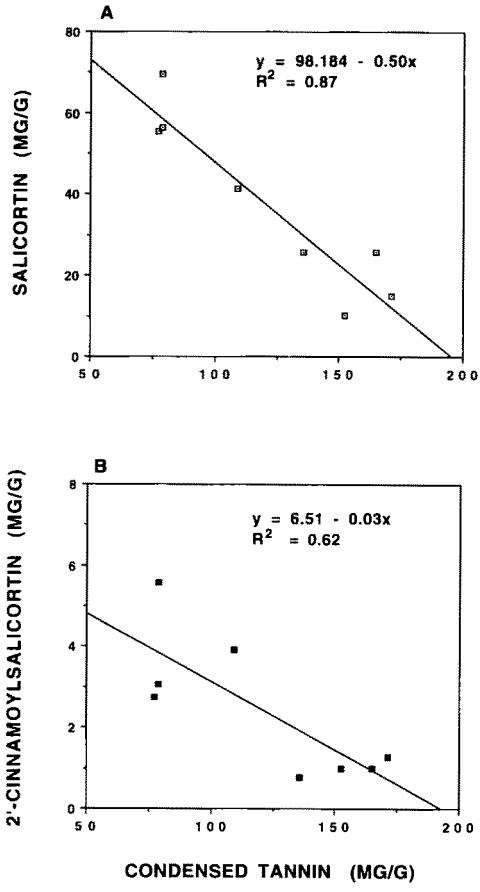

FIG. 2. The relationship between (A) condensed tannin and salicortin concentration, and (B) condensed tannin and 2'-cinnamoylsalicortin concentration in hybrid leaves.

in turn should result in a parallel herbivore response rather than further breakdown. We are currently studying both the inheritance of chemical variation and resistance in backcross plants to test this hypothesis.

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ANTIFEEDANT AND TOXIC EFFECTS OF SESQUITERPENES FROM *Senecio palmensis* TO COLORADO POTATO BEETLE

AZUCENA GONZALEZ-COLOMA,[1,*] MATIAS REINA,[1] RAIMUNDO CABRERA,[2] PEDRO CASTAÑERA,[3] and CARMEN GUTIERREZ[4]

[1]*Instituto de Productos Naturales y Agrobiología, CSIC*
Adva. Astrofísico F. Sánchez 2
38206 La Laguna, Tenerife, Spain

[2]*UDI Fitopatología, Dpto. Biología Vegetal*
Universidad de La Laguna, Tenerife, Spain

[3]*Centro de Investigaciones Biológicas, CSIC*
Velázquez 144, 28006 Madrid, Spain

[4]*C. Ciencias Medioambientales, CSIC*
Serrano 115-dpdo., 28006 Madrid, Spain

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Abstract—A bioassay-guided fractionation of the aerial parts of *Senecio palmensis* resulted in the isolation of two sesquiterpenes, 2,10-bisaboladien-1-one and 11β-acetoxy-5-angeloyloxy-silphinen-3-one. The bisabolene and the silphinene represented 0.012% and 0.024% of the plant dry weight, respectively. Both compounds showed antifeedant activity against *Leptinotarsa decemlineata* larvae and adults in short-term choice and no-choice bioassays. Both compounds were also tested against different species of phytopathogenic fungi. The beetles were more sensitive to these compounds in choice than in no-choice assays, with a gradient of increasing sensitivity from second instars to adults. Bisabolene was 45 times less active as an antifeedant than juglone, which was tested as a positive control. The silphinen was more active than the bisabolene, with a range of activity similar to juglone. Furthermore, exposure of fourth instars to these compounds over a 24-hr period resulted in reduced feeding and growth rates. To distinguish between antifeedant and toxic effects, growth efficiencies were calculated as the slope of the regression of relative growth rate on relative consumption rate. The comparison of these

*To whom correspondence should be addressed at: C. Ciencias Medioambientales, CSIC, Serrano 115-dpdo., 28006 Madrid, Spain.

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results with those of antifeedant simulation and contact toxicity bioassays indicates that feeding inhibition is the primary mode of action of the bisabolene, while the silphinene shows both antifeedant and toxic effects.

Key Words—*Senecio palmensis*, Asteraceae, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, antifeedant, toxic, sesquiterpenes.

INTRODUCTION

The chemical control of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say.), with synthetic insecticides has induced a rapid development of resistance of bettle populations to most of these chemicals, including some natural insecticides such as avermectins and the δ-endotoxin of *Bacillus thuringiensis* (Brattsten, 1991; Whalon et al., 1993).

Insect antifeedants of plant origin may provide an attractive alternative to control this insect since their use may reduce the development of cross-resistance. Furthermore, substances that exert both behavioral (antifeedants) and physiological (toxins) effects would be more useful in reducing the development of resistance (Jermy, 1990).

Plant sesquiterpenes and other terpenoids are major determinants of insect-plant interactions (references in Mullin et al., 1991). Inhibitory cyclic sesquiterpenes and diterpenes for insect herbivores have been identified from at least 28 genera of the terpenoid-rich Asteraceae (Mullin et al., 1991). Additionally, species belonging to the Asteraceae family are an important source of CPB antifeedants (Jermy et al., 1981; Hough-Goldstein, 1990). This family also offers numerous examples of insecticidal and/or antifeedant plant metabolites with additional fungicidal activity (Picman, 1986; Miles et al., 1990). As a result of our ongoing search for natural agrochemicals among Canarian endemic plant species in the Asteraceae, we found that an ethanolic extract of *Senecio palmensis* (Chr. Sm.) had a strong antifeedant activity against *Leptinotarsa decemlineata* larvae.

Here we report the isolation of two sesquiterpenes from a biologically active fraction of *S. palmensis*, along with their antifeedant and antifungal activity against *L. decemlineata* larvae and adults and six species of phytopathogenic fungi. Juglone, a natural compound with insecticidal, antifeedant (Hassanali and Lwande, 1989; Bernays and Cornelius, 1992), and antifungal activity (Marston et al., 1993) was included in the experiments as a positive control. We also report on the effects of those compounds on feeding and growth of fourth-instar *L. decemlineata* larvae and the study of their mode(s) of action through antifeedant simulation.

METHODS AND MATERIALS

Plant Material

Flowering plants were collected in Arico, Tenerife (July 1992). A voucher specimen has been deposited in the Centro de Investigación y Tecnología Agraria (CITA), Tenerife.

General

IR spectra were recorded in CH_2Cl_2 solutions using a Perkin-Elmer spectrometer. ^1H and ^{13}C NMR spectra in $CDCl_3$ were measured in a Bruker AMX 400 MHz spectrometer (chemical shifts reported are relative to residual $CDCl_3$, 7.26 ppm for ^1H and 77.0 ppm for ^{13}C). Mass spectra were obtained at 70 eV on a VG micromass ZAB-2F instrument. Optical rotations were recorded with a Perkin Elmer 241 polarimeter. HPLC chromatography was performed on a Beckman System Gold apparatus. The GC-MS analysis was done on a Perkin-Elmer Sigma 3B with a Supelco capillary silica column SPB-1 (30 m × 0.25 mm and 0.25 μm mesh) and the conditions were as follows: temperature gradient 110–250°C and 150–280°C with 5°C/min increments for compounds **1** and **2**, respectively.

Isolation of Compounds

Dried aerial parts of *S. palmensis* (4.7 kg) were exhaustively extracted with EtOH in a Soxhlet apparatus. The extract (808.75 g) was chromatographed on a silica gel vacuum liquid chromatography column (VLC column 9 × 5 cm, packed with silica gel 5–40 μm mesh) with an *n*-hexane–ethyl acetate–methyl alcohol gradient to give one biologically active fraction A (6.35 g, *n*-hexane–EtOAc, 9:1). This fraction was further chromatographed on silica gel (VLC column 7 × 5 cm, packed with silica gel 5–40 μm mesh) and eluted with an *n*-hexane–EtOAc gradient to provide two biologically active fractions, A1 (1.41 g, *n*-hexane–EtOAc, 6:4) and A2 (2.10 g, *n*-hexane–EtOAc 2:8). Both fractions were subsequently purified by preparative normal-phase HPLC using a 250 × 20 mm silica column (Gasukuro Kogyo Inertsil ODS-2, 5 μm particle size) and an isocratic system of Cl_2CH_2–CH_3CN, 93:7, at a flow rate of 12 ml/min. Peaks were detected at 254 nm. Two pure bioactive compounds (**1**, 475 mg; and **2**, 850 mg) were obtained from fractions A1 and A2, respectively, as a result of the HPLC purification.

(6S)-2,10-bisaboladien-1-one **(1).** Oil, $[\alpha]_D = -34°$ (EtOH; c 0.34); EI-MS (70 eV, *m/z*, rel. int.): 220 $[M]^+$, 205 (7), 163 (13), 137 (100), 135 (55), 110 (44), 109 (35), 95 (27), 82 (31), 67 (15), 55 (13), 53 (15). ^1H NMR

($CDCl_3$, 400 MHz): δ, 5.86 (1H, q, J = 1.2 Hz, H-2), 5.06 (1H, brt, J = 5.8 Hz, H-10), 2.37 (1H, m, H-7), 2.31 (2H, m, H-4), 2.14 (1H, ddd, J = 12.0, 4.2 Hz, H-6), 2.01 (1H, dt, J = 7.2 Hz, H-9), 1.91 (1H, m, H-5α), s-1.89 (3H, s, H-14), 1.76 (1H, m, H-5β), 1.63 (3H, d, J = 1.0 Hz, brs, H-13), 1.55 (3H, brs, H-12), 1,24 (2H, dt, J = 7.0 Hz, H-8), 0.79 (3H, d, J = 6.8 Hz, H-15). ^{13}C NMR ($CDCl_3$, 100 mHz): 200.89 (C-1), 126.96 (C-2), 161.05 (C-3), 30.77 (C-4), 22.32 (C-5), 49.73 (C-6), 30.18 (C-7), 34.57 (C-8), 26.12 (C-9), 124.4 (C-10), 131.2 (C-11), 17.55 (C-12), 25.9 (C-13), 24.01 (C-14), 15.56 (C-15).

11β-Acetoxy-5-angeloyloxy-silphinen-3-one **(2).** Oil, $[\alpha]_D$ = −66.6° (EtOH; c 0.15); IR ($CDCl_3$) 1735 (ester), 1706, 1603, 1458, 1377, 1238, 1131, 1086 cm^{-1}. EI-MS (70 ev, *m/z*, rel. int.): 374 (M^+) (10), 291 (24), 275 (51), 231 (18), 215 (90), 203 (10), 189 (14), 187 (16), 173 (13), 161 (20), 145 (10), 83 (100), 55 (57).

Insect Bioassays

The *L. decemlineata* colony was reared on potato foliage (cv. Desirée) and maintained at 24 ± 1°C, >70% relative humidity with a photoperiod of 16:8 hr (L:D) in a growth chamber.

Short-Term Choice and No-Choice Feeding Assays: These experiments were conducted with a newly emerged second (L2) and fourth instar (L4) *L. decemlineata* larvae and adults. Potato leaf disks (1 cm^2) were treated on the upper surface with 10 μl of the test substance. For choice assays, four treated and four control disks were arranged alternatively on five 2.5% agar-coated Petri dishes (9 cm diameter); for no-choice assays, eight treated or control disks were used per dish. Ten second instars, three fourth instars, or three adults were placed in each dish following three hours of starvation and allowed to feed in a growth chamber (environmental conditions as described above). Feeding was terminated after consumption of 50–75% of the control disks, and the uneaten leaf disk surfaces were measured according to Escoubas et al. (1993) with a computer-interfaced scanner (Escoubas, personal communication).

Calculations of amounts of treated and control disks eaten were made by subtracting the surface area of the remaining portion from the initial surface for the appropriate test. Percent feeding reduction (%FR) was determined for each arena by the equation %FR = [1 − (treatment consumption/control consumption)] × 100 (Bentley et al., 1984). For the crude extract and its fractions, an arbitrary level of %FR > 75 was considered indicative of a strong feeding deterrency.

The two isolated compounds and commercial juglone (Sigma) were tested in a dose–response experiment to calculate their relative potencies (EC_{50} values, the effective dose for 50% feeding reduction), which were determined from log

probit analysis (Finney, 1971). Two dose series were used: 10, 25, 50, 75, and 100 $\mu g/cm^2$ for compound **1** and 0.1, 0.25, 0.50, 0.75, 1.0, 10 and 25 $\mu g/cm^2$ for compound **2** and juglone.

Long-Term Feeding Assays. These experiments were performed under the same environmental conditions as above. One L4 larva (less than 24 hr old) and eight potato leaf disks (1 cm^2) were placed on 2.5% agar-coated Petri dishes (9 cm diam.). The upper surfaces of the disks were treated with either 10 μl of the test solution or the solvent carrier alone (acetone control), and 15 plates per treatment were used in all feeding experiments.

To distinguish between the antifeedant and toxic effects of these compounds, antifeedant simulation assays with different levels of starvation and dose–response assays were run simultaneously (Blau et al., 1978). At the end of the feeding experiments larval feeding indexes and growth efficiencies were calculated.

Feeding Indexes and Growth Efficiency. The feeding indexes were calculated on a dry weight basis. Initial larval dry weights were estimated from the regression of fresh on dry weight of additional newly molted L4 larvae. Initial dry weight of food was calculated similarly using additional leaf disks. At the end of the feeding experiments, the remaining food and larvae were lyophilized and weighed.

The relative consumption rate (RCR) and the relative growth rate (RGR) were calculated according to Farrar et al., (1989). Growth efficiency (GE) was calculated as the slope of the regression of RGR on RCR, assuming a common intercept determined by the RGR of the starved control larvae (Blau et al., 1978). The relative chemical consumption (RC) was estimated as RCR $\times$ dose (in milligrams) (Liu et al., 1990).

Antifeedant Simulation. Fifteen individually weighed *L. decemlineata* L4 larvae were fed for 24 hr on zero, two, four, six, and eight preweighed potato leaf disks (1 cm^2). At the end of the experiment, the weights of larvae and remaining leaf disks were recorded. With these data, a calibration curve was constructed by calculating the regression of RGR on RCR ranging from total starvation to abundance.

Dose–Response Experiments. Fifteen individually weighed L4 CPB larvae were fed during 24 hr on eight preweighed leaf disks treated with 0, 10, 25, 50, 75, and 100 $\mu g/cm^2$ of each terpene. At the end of the experiment, uneaten food and live larval weights were recorded. To study the posttreatment growth, the same larvae were allowed to feed ad libitum on untreated potato foliage for another 24 hr and then reweighed.

Contact Toxicity. The contact toxicity of the compounds was investigated by topical application using 15 preweighed L4 larvae. A drop (2 μl) of each compound (20 μg) dissolved in acetone was applied to the back of each larva. Treated larvae were fed on eight preweighed potato leaf disks (1 cm^2) for 24

hr, and then reweighed, along with the remaining food, to calculate their relative consumption and growth rates as above.

Antifungal Activity Assays

The antifungal activity of the terpenes isolated was tested against six species of plant pathogens (*Fusarium moniliforme*, *F. oxysporum*, *F. solani*, *Botrytis cinerea*, *Phytophthora syringae*, and *Ascochyta lentis*) and estimated as mycelial growth inhibition (Murabayashi et al., 1991). The test substances were dissolved in acetone and incorporated in a PDA culture medium (5% final concentration of solvent). For each experiment, 2-cm-diameter disks were cut from the corresponding culture medium and inoculated with the appropriate fungal species. Eight replicates of each culture were incubated at 27°C in darkness and the colony diameters measured after 48 hr. Control experiments consisted of inoculated untreated culture-medium incubated under the same conditions. EC_{50} values were determined from log probit analysis (Finney, 1971). Two dose series were used: 0.01, 0.05, 0.1, 0.25, and 0.5 mg/ml for compounds **1** and **2**; and 0.005, 0.01, 0.025 mg/ml plus the same as above for juglone.

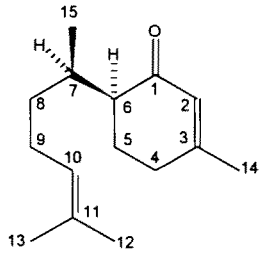
Statistical Analysis

All dry weight measures were log-transformed prior to ANOVA analysis to test for treatment effects on the feeding indexes, chemical consumption, and contact toxicity. Differences between treatment means were checked with Duncan's multiple range test. The toxicity of these compounds was determined by regressing RGR on RCR and comparing each treatment slope with the control slope (antifeedant simulation experiment) (Blau et al., 1978; Berenbaum and Feeny, 1981; Miller and Feeny, 1983). The slopes of the regression lines were compared with a *t* test for parallelism (Tallarida and Murray, 1981). The basic assumption of this analysis is that the RGR of starved larvae does not differ among treatments within an experiment. Treatments in which the slope of the regression is significantly lower than that of the calibration curve can be categorized as toxins. The slope of the regression of RGR on RCR is one estimate of growth efficiency (GE) on any particular treatment.

RESULTS

Bioassay-Guided Isolation of Compounds. The crude alcoholic extract of the aerial parts of *S. palmensis* was a strong antifeedant against *L. decemlineata* fourth instars in choice tests (97 ± 3% feeding reduction at 100 $\mu g/cm^2$). A bioassay-guided fractionation of the extract resulted in the isolation of two active sesquiterpenes (**1** and **2**, Figure 1) by VLC column, normal-phase column, and

Compound 1



Compound 2

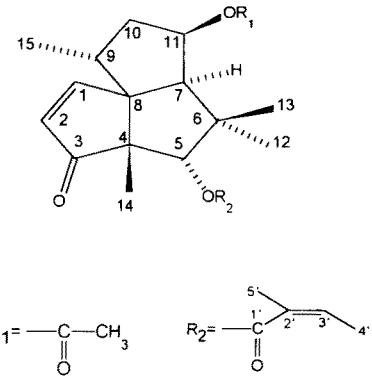


FIG. 1. Structures of compounds isolated from *S. palmensis*.

normal-phase HPLC chromatography. Their structures were established by IR, mass (EI-MS) GC-MS, and ^{1}H and ^{13}C NMR spectroscopic experiments, including two-dimensional methods (HMCQ and HMBC) (Table 1), and by comparison with previously reported data.

Compound **1** was identified as (6*S*)-2,10-bisaboladien-1-one, previously isolated from *Stevia purpurea* and *Cymbopogon citratus* (Asteraceae) (Bohlmann et al., 1976; Abegaz and Yohannes, 1983). Compound **2,** two times more abundant in the plant than **1,** was identified as 11β-acetoxy-5-angeloyloxy-silphinen-3-one (see Table 1), previously isolated from *Cineraria geifolia* var. glabra (Asteraceae) (Jakupovic and Abraham, 1985).

TABLE 1. ^{1}H, ^{13}C, HMQC, AND HMBC NMR DATA OF **2** (400 MHz, $CDCl_3$)[a]

Proton		Correlated carbon	
		HMQC	HMBC
1	7.56 d (7.6)	167.8 d	C-8, C-4, C-2
2	6.12 d (7.6)	131.9 d	C-8, C-4, C-3
		211.1 s (C-3)	
		63.9 s (C-4)	
5	5.43 s	86.3 d	C-13,C-1′(167.5),
		42.5 s (C-6)	C-3, C-8
7	2.23 d (4.0)	61.6 d	C-5, C-13
		57.1 s (C-8)	
9	2.61 ddq (12.5, 6, 7)	35.1 d	C-15, C-8, C-4
10α	1.60 dt (13.8, 2.6)	41.7 t	
10β	2.12 dd (12.8, 6)		C-7
11α	5.25 t (3.4)	71.6 d	
12	0.90 s	25.5 q	C-6, C-7
13	0.95 s	23.7 q	C-11
14	1.24 s	19.9 q	C-8, C-4, C-3
15	0.93 d (7.2)	15.9 q	C-10, C-7
3′	6.11 q (7.2)	138.7 d	C-1′ (167.5)
4′	2.04 dd (7.2)	15.8 q	C-2′, C-3′
5′	1.94 t (1.4)	20.7 q	C-2′, C-3′, C-1′
C=O		170.1 s	
CH$_3$	2.16 s	21.7 q	

[a]Carbon multiplicities were established by DEPT pulse sequence. The coupling constants in parenthesis are in Hz.

Antifeedant Effects of Sesquiterpenes. Both chemicals strongly deterred feeding of CPB larvae (98% feeding reduction at 100 $\mu g/cm^2$ in choice assays). Their relative potencies (EC_{50}) are presented in Table 2 along with that of juglone. The degree of antifeedant activity of the chemicals tested varied with the insect's developmental stage, in agreement with previous reports (Bentley et al., 1988; Liu et al., 1989; Hough-Goldstein, 1990). In general, a gradient of increasing sensitivity from second instars to adults was shown. Larvae and adults generally appeared more selective in choice than in no-choice experiments, in contrast with the effect of limonin (Alford et al., 1987), and similar to the effect of polygodial and warbuganal (Caprioli et al., 1987).

Compound **1** showed the lowest antifeedant activity (Table 2) with a relative potency range similar to limonin, a known CPB antifeedant with EC_{50}

TABLE 2. RELATIVE ANTIFEEDANT POTENCIES OF COMPOUNDS **1** AND **2** AND JUGLONE (JU) AGAINST *L. decemlineta*

Test[a]	L2	L4	Adults
1 A	20.34 (17.03, 24.29)[b]	4.00 (2.73, 5.86)[b]	14.64 (3.55, 60.40)[b]
B	35.21 (21.0, 59.05)[b]	29.86 (26.55, 33.6)[b]	11.66 (1.65, 82.04)[b]
2 A	12.55 (4.87, 32.34)[b]	1.69 (1.36, 2.11)[b]	0.27 (0.16, 0.46)[b]
B	30.90 (16.62, 57.45)[b]	14.43 (3.01, 69.14)[b]	2.67 (1.38, 5.18)[b]
JU A	—	0.83 (0.25, 2.80)[b]	0.32 (0.06, 1.81)[b]
B	—	20.41 (16.93, 24.59)[b]	3.14 (0.04, 9.42)[b]

[a]Test A, choice; test B, no-choice.
[b]Lower and upper 95% confidence intervals of EC_{50}.

values of 34.92 and 9.34 $\mu g/cm^2$ against L4 larvae in choice and no-choice tests, respectively (Alford et al., 1987).

Compound **1** decreased *L. decemlineata* consumption and growth rate. The responses were not dose-dependent, but significant reductions of RGR at 100 $\mu g/cm^2$ and RCR at 75 and 100 $\mu g/cm^2$ were observed (Table 3). The effects of compound **1** on posttreatment growth rate and consumption were similar (Table 3).

The growth efficiencies of CPB larvae treated with different concentrations of **1** were similar to those of the starved control (Table 3). Each treatment dose gave significant linear relationships between RCR and RGR (0 $\mu g/cm^2$: $r = 0.88$; $P < 0.00001$; 10 $\mu g/cm^2$: $r = 0.87$; $P < 0.00001$; 25 $\mu g/cm^2$; $r = 0.71$, $P < 0.00001$; 50 $\mu g/cm^2$: $r = 0.78$; $P < 0.00001$; 75 $\mu g/cm^2$: $r = 0.57$, $P = 0.00075$; 100 $\mu g/cm^2$: $r = 0.88$, $P < 0.00001$) and parallel to the calibration curve (Figure 2, Table 3).

Since the growth efficiencies of larvae treated with **1** were similar to those of the starved control, we conclude that feeding inhibition at the behavioral level is the primary mode of action of **1**. Additionally, this compound did not show contact toxicity (Table 4).

The antifeedant effect of the 24-hr assay with compound **1** (50% feeding inhibition at 100 $\mu g/cm^2$) was lower than that of short-term (<6 hr) no-choice

TABLE 3. FEEDING INDEXES, GROWTH EFFICIENCY, POSTTREATMENT GROWTH AND CONSUMPTION MEAN VALUES AND STANDARD ERRORS (SE) OF FOURTH-INSTAR CPB LARVAE (N = 15) FEEDING ON COMPOUND **1**.

Dose (μg/cm^2)	RCR[a]	RGR[b]	post-RGR[c]	RC1[d]	GE[e]
0	0.88 (0.13)	0.36 (0.05)	0.49 (0.03)	0.00 (0.01)	0.482
10	1.04 (0.11)	0.22 (0.08)	0.42 (0.06)	0.080a (0.009)	0.443
25	1.00 (0.07)	0.26 (0.04)	0.43 (0.02)	0.185b (0.01)	0.344
50	0.77 (0.07)	0.22 (0.02)	0.42 (0.02)	0.309c (0.03)	0.399
75	0.62 (0.09)	0.15[f] (0.08)	0.40 (0.03)	0.374d (0.06)	0.297
100	0.44[f] (0.08)	0.02[f] (0.04)	0.35[f] (0.03)	0.356d (0.06)	0.438
Starved control					0.371
F[g]	5.387	4.910	1.376	19.287	
P	0.0002	0.0005	0.24	<0.0000 1	

[a]RCR = $I/(BI) \times T$, I = food consumed (mg), T = feeding period (days), BI = initial insect weight (mg).
[b]RGR = $\Delta B/(BI) \times T$, ΔB = change in insect body weight (mg).
[c]Growth efficiency (GE), calculated as the slope of the regression of RGR on RCR.
[d]RGR of insects fed for 24 hr on untreated food after the treatment.
[e]Relative consumption (RC) of compound **1**: RCR × dosage (mg). Values followed by a different letter are significantly different (Duncan's multiple range test, $P = 0.05$).
[f]Denotes a significant difference from the control, Duncan's multiple range test, $P = 0.05$.
[g]ANOVA parameters (F statistic and probability level, P).

tests (95% feeding reduction at 100 μg/cm^2). An explanation of the decreased effect of antifeedant **1** with longer exposure time (over 6 hr) could be a behavioral desensitization. A similar effect has been described for behavioral desensitization of *Spodoptera frugiperda* to aristolochic acid in no-choice situations (Raffa and Frazier, 1988).

Compound **2** showed higher activity levels than compound **1** (Table 2). Compound **2** strongly deterred adult beetles from feeding and had relative potencies within the range of juglone. When compared with limonin, compound **2** was 23 times more active against L4 larvae in choice tests, but less active in no-choice tests, with an effective dose of **2** that was 1.5 times higher than

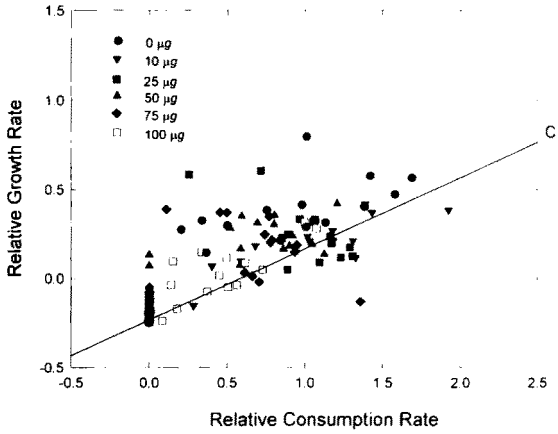


FIG. 2. Plot of the relative growth rate (RGR) on the relative consumption rate (RCR) for *L. decemlineata* L4 larvae fed for 24 hr on leaf disks treated with compound **1**. The line represents the calibration curve ($y = 0.371x - 0.220$, $r = 0.84$, $P < 0.0001$).

limonin (Alford et al., 1987). The activity of juglone was also stronger than limonin (42 and 2.2 times stronger against L4 larvae in choice and no-choice tests, respectively) (Alford et al., 1987). Therefore, commercial juglone can be considered an effective CPB antifeedant suitable for use as a positive control.

Compound **2** showed a strong and significant reduction in feeding and growth of *L. decemlineata* larvae with increasing concentrations, with 100%

TABLE 4. FEEDING INDEXES MEAN VALUES AND STANDARD ERRORS OF FOURTH-INSTAR CPB LARVAE ($N = 15$) TOPICALLY TREATED WITH 20 μg OF COMPOUNDS **1** AND **2**

Treatment	RCR[a]	RGR[b]
1	0.75 (0.03)	0.12 (0.01)
2	0.65 (0.07)	0.09 (0.02)
Control	0.72 (0.04)	0.13 (0.02)
F[c]	1.028	1.397
P	0.366	0.266

[a] As in Table 3.
[b] As in Table 3.
[c] See footnote *g* in Table 3.

feeding inhibition at the maximum dose tested (Table 5). The posttreatment larval relative growth rate decreased without following a dose response pattern and was significantly lower than the control starting from a dose threshold of 25 $\mu g/cm^2$ (Table 5). The chemical consumption of **2** peaked at 50 $\mu g/cm^2$, decreasing to zero at 100 $\mu g/cm^2$ (Table 5).

The growth efficiencies of the larvae treated with **2** were significantly lower than the starved control, also starting from a dose threshold of 25 $\mu g/cm^2$. We could not calculate the growth efficiency for the highest dose of **2** (100 $\mu g/cm^2$) since the corresponding RCR was zero (Table 5). For compound **2**, the linear relationships between RCR and RGR were not always significant (0 $\mu g/cm^2$: $r = 0.51$, $P = 0.003$; 10 $\mu g/cm^2$: $r = 0.91$, $P < 0.00001$; 25 $\mu g/cm^2$: $r = 0.61$, $P < 0.001$; 50 $\mu g/cm^2$: $r = 0.40$, $P = 0.001$; 75 $\mu g/cm^2$: $r = 0.22$, $P = 0.14$), and their slopes were lower than the slope of the calibration curve at a dose of 25 $\mu g/cm^2$ (Figure 3, Table 5), indicating that compound **2** has a toxic action.

TABLE 5. FEEDING INDEXES, GROWTH EFFICIENCY, POSTTREATMENT GROWTH AND CONSUMPTION MEAN VALUES AND STANDARD ERRORS OF FOURTH-INSTAR CPB LARVAE ($N = 15$) TREATED WITH COMPOUND **2**[a]

Dose ($\mu g/cm^2$)	RCR[a]	RGR[a]	post-RGR[a]	RC2[a]	GE[a]
0	0.62 (0.13)	0.32 (0.03)	0.52 (0.03)	0.00	0.320
10	0.64 (0.10)	0.20[a] (0.02)	0.46 (0.07)	0.05a (0.008)	0.350
25	0.33[a] (0.05)	−0.05[a] (0.01)	0.34[a] (0.02)	0.04a (0.007)	0.174[b]
50	0.18[a] (0.03)	−0.09[a] (0.01)	0.34[a] (0.03)	0.08b (0.014)	0.125[b]
75	0.05[a] (0.03)	−0.06[a] (0.01)	0.34[a] (0.02)	0.04a (0.02)	0.165[b]
100	0.00[a]	−0.01[a] (0.01)	0.34[a] (0.03)	0.00	
Starved control					0.358
F[a]	14.519	76.633	2.832	7.357	
P	<0.00001	<0.00001	0.020	<0.00001	

[a] See footnotes *a* to *g* in Table 3.
[b] Significantly different from the starved control (t-test, $p < 0.05$).
[c] Slope could not be calculated.

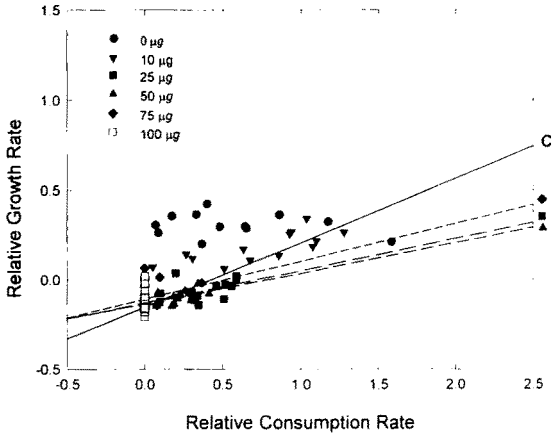


FIG. 3. Plot of the relative growth rate (RGR) on the relative consumption rate (RCR) for *L. decemlineata* L4 larvae fed for 24 hr on leaf disks treated with compound **2.** Represented are the lines of the calibration curve C ($y = 0.376x - 0.156$, $r = 0.82$, $P < 0.00001$) and the treatment lines with slopes significantly different from the control C.

Antifungal Activity: Both terpenes were tested against soil-borne (*Fusarium* sp.) and foliar (*Botrytis*, *Phytophthora*, and *Ascochyta* sp.) phytopathogenic fungi. They showed only mild activity against *B. cinerea* (EC_{50} values of 0.15 and 0.03 for **1** and **2**, respectively) and *A. lentis* (EC_{50} values of 0.19 and 0.33 for **1** and **2**, respectively). Juglone showed the highest overall antifungal activity (EC_{50} values of 0.007 and 0.04 for *B. cinerea* and *A. lentis*, respectively).

DISCUSSION

Two effective CPB sesquiterpene antifeedants have been isolated from *S. palmensis*. Compound **1** belongs to the chemical class of bisabolenes and has an antifeedant action lower than juglone but similar to limonin. Compound **2** is a silphinene sesquiterpene with an activity level similar to juglone and higher than limonin. Both compounds showed mild antifungal activity, suggesting a target-specific interaction.

Bisabolenes are a class of sesquiterpenoids mainly isolated from Asteraceae plants (Kreiser, 1991). In contrast, silphinenes have only been described in the genera *Cineraria* and *Callilepis* (Bohlmann and Zdero, 1982; Jakupovic and Abraham, 1985) and in *Silphium* sp. along with some bisabolenes (Bohlmann and Jakupovic, 1980).

This is the first report of an antifeedant silphinene (compound **2**). In con-

trast, some natural bisabolenes are effective antifeedants. For example bisabolangelone, isolated from *Angelica silvestris*, has strong antifeedant properties against several insect species including *L. decemlineata* (Muckenstrum et al., 1981; Nawrot et al., 1984).

Another bisabolene structurally related to compound **1**, zingiberene, appears to be responsible for the toxicity of the wild tomato plant *Lycopersicon hirsutum* f. *hirsutum* to CPB and represents a potential new source of insect resistance in the cultivated tomato *L. esculentum* (Carter et al., 1989). The search for structurally related natural bisabolenes could be a promising source of new leads for the development of CPB host-plant resistance.

These two compounds were more effective in choice that in no-choice tests, indicating behavioral avoidance, in addition to their deterrent effect. The greater effect that both compounds had against adult CPB indicates that these chemicals may alter the host selection process through behavioral avoidance by the adults, which are more mobile and are the primary finders of hosts (Hough-Goldstein, 1990).

Furthermore, compound **1** and the starvation simulation test both had a similar effect of reduction in the growth and feeding of the larvae, which suggests an antifeedant effect at the behavioral level. The association between larval feeding and growth reduction caused by compound **2** did not parallel that of the starved control, indicating both antifeedant and toxic effects. Additionally, larval contact with compound **2** was associated with detrimental effects. We also observed a decrease in larval posttreatment growth rate that did not correlate with chemical consumption. These observations suggest that the toxic action of **2** is not postingestive, in contrast to limonin, an antifeedant and toxic limonoid with a negative postingestive effect on *L. decemlineata* larvae associated with relative chemical consumption (Mendel et al., 1991). Compound **2**, with antifeedant and toxic activity, could be more promising than compond **1** as a control agent against CPB because both activities together require behavioral and physiological adaptations in order to develop resistance (Jermy, 1990). Further research is needed in order to understand the toxic mode of action of **2**.

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EFFECTS OF BENZOIC AND CINNAMIC ACIDS ON MEMBRANE PERMEABILITY OF SOYBEAN ROOTS

R. BAZIRAMAKENGA,[1] G.D. LEROUX,[1] and R.R. SIMARD[2,*]

[1]*Laval University, Department of Phytology*
Ste-Foy, Québec, Canada, G1K 7P4

[2]*Agriculture and Agri-Food Canada*
Soils and Crops Research Center
2560 Hochelaga Boulevard,
Ste-Foy, Québec, Canada, G1V 2J3

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Abstract—Benzoic (BEN) and cinnamic (CIN) acids are commonly found in soils and are considered as strong allelochemicals. Published information suggest that BEN and CIN and other phenolic acids decrease plant growth in part by suppressing nutrient absorption. However, studies on the mechanism of action were not conclusive. We examined the effects of BEN and CIN on the cell plasma membrane in intact soybean (*Glycine max* L. cv. Maple Bell) seedlings. Treating intact root systems with BEN or CIN rapidly increased electrolyte leakage and ultraviolet absorption of materials into the surrounding solution. After 12 hr of treatment, BEN and CIN lowered the extracellular sulfhydryl group content in roots. The two allelochemicals induced lipid peroxidation, which resulted from free radical formation in plasma membranes, inhibition of catalase and peroxidase activities, and sulfhydryl group depletion. Oxidation or cross-linking of plasma membrane sulfhydryl groups is the first mode of action of both compounds. The BEN- and CIN-induced decrease in soybean nutrient absorption may be a consequence of damage to cell membrane integrity caused by a decrease in sulfhydryl groups followed by lipid peroxidation.

Key Words—Allelochemicals, lipid peroxidation, sulfhydryl groups, leakage, plasma membrane, superoxide dismutase, catalase, peroxidase.

INTRODUCTION

Numerous phenolic derivatives of benzoic acid (BEN) and cinnamic acid (CIN) have been identified in soil, and several of these compounds markedly inhibit

*To whom correspondence should be addressed; Contribution #505 of the Soils and Crops Research Centre.

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growth and development of plants (Rice, 1984; Vaughan and Ord, 1991). Several investigations have found a lower mineral content in plants treated with phenolic acids (Glass, 1974; Lee, 1977; Baziramakenga et al., 1994). Because a membrane is the first living tissue that comes in contact with any allelochemical, it is likely to be the first damaged. Allelochemicals can damage cell membranes through direct interaction with a constituent of the membrane or as a result of an impairment of some metabolic function necessary to the maintenance of membrane function (Rice, 1984). An impairment of lipid or protein metabolism by phytotoxins could lead to membrane disturbance (Keck and Hodges, 1973), but studies of this type have not been conclusive.

BEN induced K^+ and NO_3^- leakage from roots of *Hordeum vulgare* L. (Lee, 1977). Salicylic acid was reported to induce K^+ efflux from yeast (Scharff and Perry, 1976; Hoeberichts et al., 1980). Glass (1974) presented evidence suggesting that the inhibition of mineral uptake by phenolic derivatives of BEN and CIN is linked to their lipid-solubilizing capacity. Studies conducted with artificial lipid membranes showed that phenolic compounds caused disruption of the integrity of the phosphatidylethanolamine monolayers (Kaye and Proudfoot, 1971) and increased fluidity of lipid alkyl chains in phospholipids (Singer and Wan, 1977).

On the other hand, it was established that phenolic compounds applied exogenously stimulated hydrogen peroxide production in the cell wall of *Armoracia lappathifolia* Gilibb (Gross et al., 1977). Oxidation of phenolic compounds can generate superoxide and hydroxyl radicals with high reactivity for biological substances such as unsaturated lipids and proteins, causing enzyme inactivation, membrane lipid peroxidation, and strand break in DNA (Appel, 1993).

Ion transport across membranes is thought to be mediated by intrinsic membrane proteins and the altered ion uptake in phenolic-treated plants may be due to a disruption of the functions of these proteins (Vaughan and Ord, 1991). Sulfhydryl groups play an essential role in the function of transport proteins (Nelson et al., 1975; Lucas and Alexander, 1980; Ramaiah and Mookerjee, 1981). Numerous environmental toxins such as heavy metals and toxic chemicals can reduce the sulfhydryl group content (Spring and Hager, 1982; Ayers et al., 1986). Depletion of the sulfhydryl groups could therefore lead to the inactivation of enzymes, which depend on such reduced sulfhydryl groups.

The objective of our work was to determine the effects of BEN and CIN on electrolyte leakage, lipid peroxidation, and depletion in sulfhydryl groups in roots of *Glycine max* L. cv. Maple Bell. BEN and CIN were selected because they have been frequently isolated in soil (Chou and Patrick, 1976; Baziramakenga et al., 1995) and exudates of plants (Yu and Matsui, 1994). Our previous investigation indicated that they alter ion uptake by roots of soybean (Baziramakenga et al., 1994), but their mechanisms of action have not been elucidated.

Information of this kind will provide a better understanding of how these compounds reduced mineral uptake by plants.

METHODS AND MATERIALS

Plant Materials. Soybean (*Glycine max* L. cv. Maple Bell) seeds were surface-sterilized in 5% hypochlorite for 10 min, soaked for 6 hr in distilled water, and allowed to germinate on sterilized vermiculite saturated with 1 mM $CaSO_4$. Plants were grown in a controlled environment chamber with a 28:20°C day–night temperature, a light–dark regime of 16:8 hr, irradiance of 280 μmol/m^2/sec, and 70% relative humidity. After three days, seedlings of uniform size were transferred to 2-liter buckets filled with nutrient solution (μM): 750 KNO_3, 325 $Mg(NO_3)_2$, 10 KH_2PO_4, 1000 $CaSO_4$, 8 H_3BO_3, 0.2 $MnSO_4$, 0.2 $ZnSO_4$, 0.2 $CuSO_4$, and 0.2 $(NH_4)_6Mo_7O_{24}$ at pH 5.8 (Cakmak and Horst, 1991). Seedlings were allowed to acclimate to hydroponic conditions for three days and were subsequently transferred to similar medium supplemented with various concentrations of BEN or CIN. The roots were exposed to allelochemicals for 12, 24, 48, and 96 hr. Controls received no allelochemical. The solutions were changed daily.

Leakage. Changes in membrane permeability due to benzoic or cinnamic acids were assayed by measuring leakage of solutes and ultraviolet absorbing materials. After washing for 10 min in total with four changes of deionized water (50 ml each), the roots were resuspended in 20 ml of deionized water for 4 hr to test leakage at room temperature. Conductivity of the solution bathing intact roots was measured with a Dorian conductivity bridge. The leaked ions were analyzed for K^+ and Ca^{2+} by atomic absorption spectrometry and for Cl^-, NO_3^-, PO_4^{3-}, and SO_4^{2-} by ion chromatography. The UV-absorbing materials (e.g., amino acids, nucleotides, polypeptides, etc.) were determined by measuring the absorbance at 260 nm on a Varian DMS 100 spectrophotometer.

Lipid Peroxidation. The level of lipid peroxidation was followed by measuring malondialdehyde (MDA) accumulation, a product of unsaturated fatty acid peroxidation, by the method of Dhindsa et al. (1981). Samples consisting of root tips (250 mg) were homogenized in 5 ml of 0.1% of trichloroacetic acid (TCA) in sodium phosphate buffer (pH 7.2) with a Polytron (Kinematica CH-600 Kriens W) and centrifuged at 15,000*g* for 15 min. An aliquot of 1 ml of the supernatant was added to 4 ml of 0.5% of thiobarbituric acid (TBA) in 20% TCA. The mixture was placed in a water bath at 90°C for 10 min and then quickly cooled on an ice-bath for 15 min. Samples were centrifuged at 15,000*g* for 5 min. The A_{532} of the supernatant was measured on a Hitachi U-1000 Spectrophotometer, and this value was subtracted from the nonspecific A_{600}

reading value. The MDA contents were calculated with the extinction coefficient value of 155 mM^{-1} cm^{-1}. MDA values were expressed as millimoles per gram fresh weight of root tissue.

Enzyme Activities. Root tips (500 mg) were quickly homogenized in 10 ml of 50 mM HEPES-KOH buffer (pH 7.8) containing 0.1 mM EDTA with a Polytron (Cakmak and Horst, 1991). All operations were performed at 0–4°C. The homogenate was centrifuged at 15,000*g* for 15 min and the supernatants were stored at −20°C until enzyme activity determination.

Superoxide dismutase (SOD) activity was assayed by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) by the procedure of Beauchamp and Fridovich (1971). The assay medium (3 ml) contained 50 mM HEPES-KOH buffer (pH 7.8), 0.1 mM EDTA, 50 mM Na_2CO_3, 12 mM L-methionine, 2 μM riboflavin, 75 μM NBT, and 300 μl of crude root extract. Riboflavin was added last. The tubes were shaken and placed under two 25-W fluorescent lamps at room temperature. The reaction was initiated and terminated by turning the light on and off. The A_{560} was measured on a Hitachi U-1000 Spectrophotometer. Tubes containing the assay mixture without the root extract were illuminated to determine maximum A_{560}. Each root extract A_{560} value was subtracted from the control A_{560} value; the mathemical difference was then divided by the maximum and multiplied by 100 to obtain the percentage of inhibition of NBT reduction.

Peroxidase activity was measured at 460 nm using guaiacol as hydrogen donor (Yamanoto and Tani, 1986). The reaction mixture contained 2.5 ml of potassium phosphate buffer (pH 6.80), 0.125 ml of 120 mM guaiacol, and 0.125 ml of crude extract. The reaction was initiated by the addition of 0.250 ml of 0.1% H_2O_2 in potassium phosphate buffer. The absorbance was measured after 5 min, and the activity expressed as percentage of the control.

Catalase activity was assayed by following the disappearance of H_2O_2, its substrate from the assay mixture (Chance and Machly, 1955). The reaction mixture contained 25 mM potassium phosphate buffer (pH 6.8) and 10 mM H_2O_2. Root extract (0.1 ml) was added to 2.9 ml of the reaction mixture to initiate the reaction. The tubes were shaken for 10 min at room temperature and the decrease in H_2O_2 was measured as the decline in A_{240} on a Varian DMS 100 spectrophotometer. Catalase activity was expressed as percentage of the control.

Sulfhydryl Groups. The concentration of sulfhydryl groups was determined by Ellman's reagent, 5,5′-dithio-bis-2 nitrobenzoic acid (DTNB) according to the method of Welch and Norvell (1993). Seedlings treated with the allelochemicals were soaked in 100 ml of the reaction mixture containing 0.2 M Tris HCl and 0.2 M Na EDTA (pH 8.2). One milliliter of 10 mM DTNB in methanol was added to the reaction mixture. The control reaction mixture received 1 ml of methanol. After 15 min, the roots were removed from reaction buffer, and

2.0 ml of the reaction buffer was assayed for reactive sulfhydryl groups at 412 nm on a Hitachi U-1000 Spectrophotometer. The coefficient of extinction used for measuring the sulfhydryl group was 1.36×10^4 M^{-1} cm^{-1} (Prudêncio-Ferreira and Arêas, 1993).

Statistical Analysis. All experiments were conducted as completely randomized designs. Treatments were replicated three times except in the lipid peroxidation (four) and sulfhydryl group (five) studies. Each experiment was conducted three times. Data were subjected to analysis of variance and treatment means ± standard error were determined.

RESULTS AND DISCUSSION

BEN and CIN induced electrolyte leakage as determined by the conductivity of bathing medium (Figure 1). After 96 hr of treatment, the conductance of

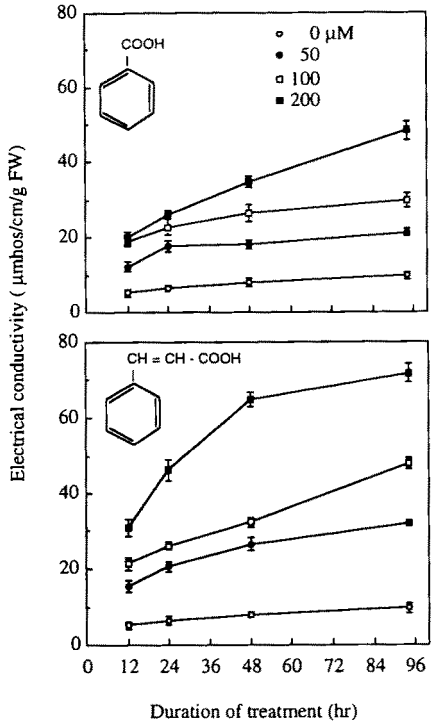

FIG. 1. Effect of different concentrations of BEN and CIN on electrolyte leakage as determined by conductivity of bathing medium.

the bathing solution of treated roots was two to eight times more than that of the control plants. Leakage of electrolytes from treated seedlings increased with time, indicating that cell permeability is affected by time of exposure of the tissues to the allelochemicals. Roots treated with CIN showed more electrolyte leakage than those treated with BEN. After 12 hr of treatment, the loss of electrolytes in CIN-treated tissue was 1.5 times greater than that of the roots treated with BEN.

Roots of BEN- or CIN-treated soybean seedlings leaked larger amounts of K^+ (Figure 2) and Cl^- (Figure 3) than did roots of control plants. Exposure to BEN or CIN for 12 hr, even at high concentrations, did not markedly affect efflux of K^+ and Cl^-. The increase of efflux became more distinct after 24 hr of treatment. Leakage of Ca^{2+}, PO_4^{3-}, and SO_4^{2-} from treated roots occurred at approximately 1/10th of the rate of K^+ leakage (data not shown). No NO_3^- efflux was detected under BEN or CIN treatments. K^+ and NO_3^- leakage have

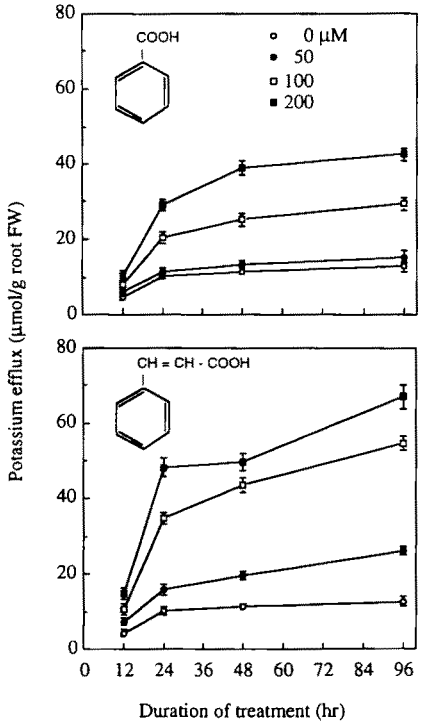

FIG. 2. Time course of K^+ release from soybean roots treated with different concentrations of BEN and CIN.

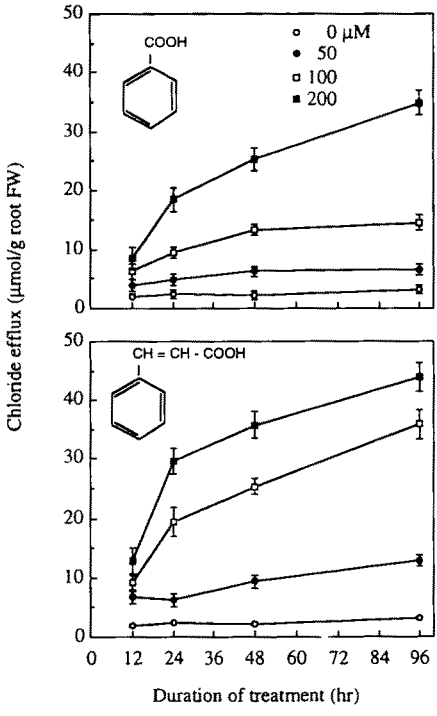

FIG. 3. Time course of Cl^- release from soybean roots treated with different concentrations of BEN and CIN.

been noted in intact roots of barley treated with BEN (Lee, 1977). The leakage of NO_3^- in barley and not in soybean is difficult to explain. This may be a situation in which species differences are involved. Leakage of Cl^-, assumed to be accumulated within cell vacuoles (Drew and McLaren, 1970), is clear evidence that change in cell permeability due to BEN or CIN is not limited to epidermal cells but extends deeply into the tissue. Our data support the hypothesis of membrane disruption by phenolic compounds (Rice, 1984). Chipley (1982) proposed that the undissociated form of BEN may diffuse freely through the cell membrane and then ionize in the cell to yield protons that delocalize the negative charge of ions within the interior of cell. If the pH-controlling capacity of the cytoplasm is exceeded, its pH may decrease sufficiently to inhibit active uptake of ions and their normal retention by the cell (Lee, 1977).

Leakage of ultraviolet-absorbing materials from seedlings treated with BEN or CIN followed a pattern similar to that of the electrolytes (Figure 4). At 200

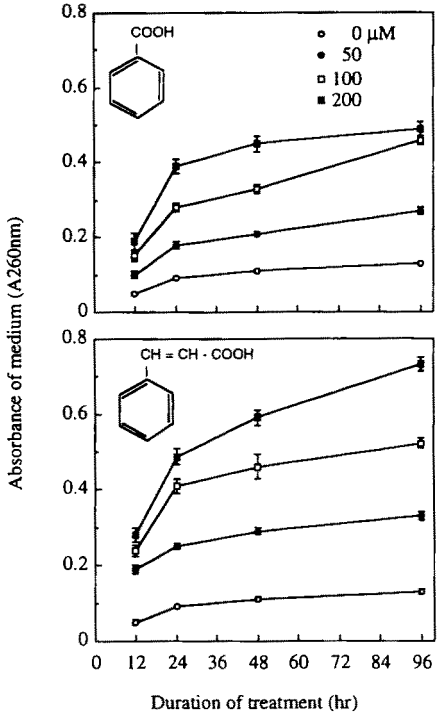

FIG. 4. Effect of varying concentrations of BEN and CIN on the loss of UV-absorbing materials from soybean roots.

μM, the leakage of organic components as measured by the absorbance of the medium at 260 nm increased five and seven times over the control when tissues were treated with BEN and CIN, respectively. The efflux of nonelectrolytes evidences a loss of membrane integrity in soybean roots.

We may postulate two categories of alterations in membrane function and structure following BEN or CIN treatment: (1) changes in membrane lipids and (2) changes in membrane proteins. In fact, solute transport across biological membranes is based on diffusion through lipids via aqueous pores or in association with carriers and on transport systems such as pumps, carriers, or channels (Novacky, 1983; Iswari and Palta, 1989; Briskin, 1994).

Changes in the membrane lipids involve solubilization of undissociated acids within the lipid of membrane or lipid degradation (Jackson and St. John, 1980). We investigated the effects of the two allelochemicals on the lipid degradation by means of lipid peroxidation. The level of TBA-reactive materials,

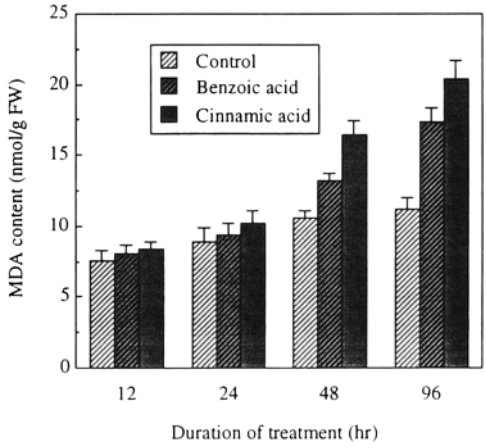

FIG. 5. Lipid peroxidation assayed as malondialdehyde (MDA) content in soybean root treated with 100 μM of BEN and CIN.

mainly MDA, in roots of seedlings treated with BEN and CIN is expected to be higher than in the control. The effect of BEN or CIN on lipid peroxidation became more apparent after incubation for 48 hr (Figure 5) and was concentration dependent (Figure 6). The striking increase in MDA seen in roots of treated

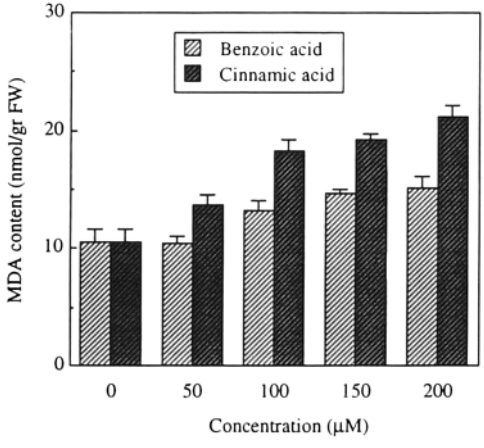

FIG. 6. Lipid peroxidation in root tips as dependent on the concentration of BEN and CIN after 48 hr of treatment.

plants suggests changes in peroxide-scavenging activity. In view of this, radical- and peroxide-scavenging enzymes were assayed. SOD, peroxidase, and catalase activities were affected by BEN or CIN (Figure 7). SOD activity in treated seedlings was slightly higher than that in controls. Peroxidase activity was stimulated at 50 μM of CIN, but inhibited at 200 μM for both allelochemicals. Catalase activity was reduced at all concentrations, and this effect was more pronounced for CIN than for BEN.

Increased SOD activity suggests an increased production of peroxides in allelochemical-treated root tips. The inactivation of peroxidase (at high concentrations) and catalase suggests that H_2O_2 might accumulate in the presence of the allelochemicals. This could induce damage in the cell either directly or

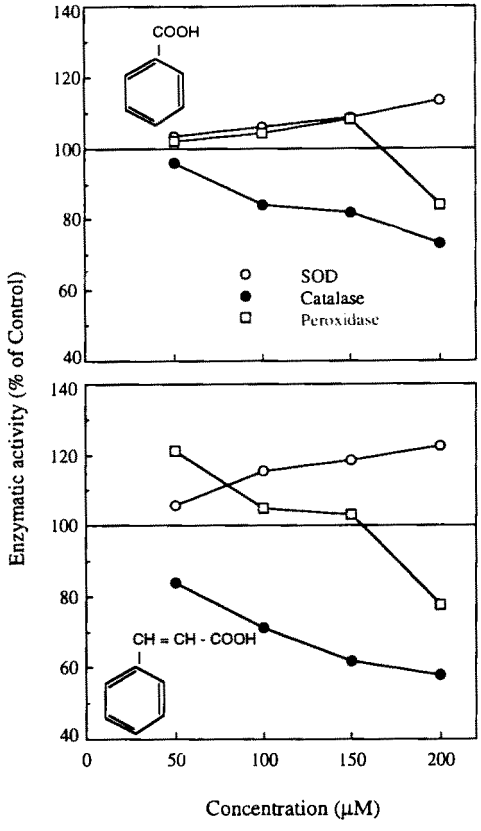

FIG. 7. Effect of BEN and CIN on activities of superoxide dismutase (SOD), catalase, and peroxidase in root tips of soybean plants after 48 hr of treatment.

through the formation of hydroxyl radicals or other oxygen radicals, leading to peroxidation of the unsaturated fatty acids in the cellular membrane (Mehdy, 1994). It is well established that phenolic acids catalyze peroxide formation (Elstner, 1987). In *Escherichia coli* cell cultures, SOD activity was increased by the addition of quinones at concentrations of more than 100 μM (Hassan and Fridovich, 1979). Our results do not support the hypothesis that BEN can serve as a scavenger of free radicals (Chipley, 1982; Epperlein et al., 1986). However, derivatives of BEN, and specifically salicylic acid, inhibit catalase activity and induce an increase of H_2O_2 in tobacco leaves (Chen et al., 1993). It is likely that BEN is converted to biologically active phenolic compounds (Pridham, 1965). Peroxidase activity was inhibited by *p*-coumaric and *p*-hydroxybenzoic in wheat roots (Vaughan and Malcolm, 1979) and by *p*-hydroxybenzoic acid in *Nicotiana tabacum* tissue cultures (Pickering et al., 1973). Gamborg et al. (1961) reported no effect of CIN on horseradish and pea epicotyl peroxidase activities. The reduced enzyme activity may be related to a reduction of solubility of the enzyme by forming insoluble protein–phenolic complexes or direct inhibition of the enzymes by forming a soluble but inactive enzyme inhibitor complex (Firenzuoli et al., 1969).

It has been suggested that phenolic compounds can interfere directly with carrier proteins (Booker et al., 1992). We examined the influence of the two allelochemicals on sulfhydryl groups in root. Roots treated with BEN or CIN for 12 hr contained lower concentrations of sulfhydryl groups than roots of control seedlings (Figure 8). Since the plasma membrane is relatively imperme-

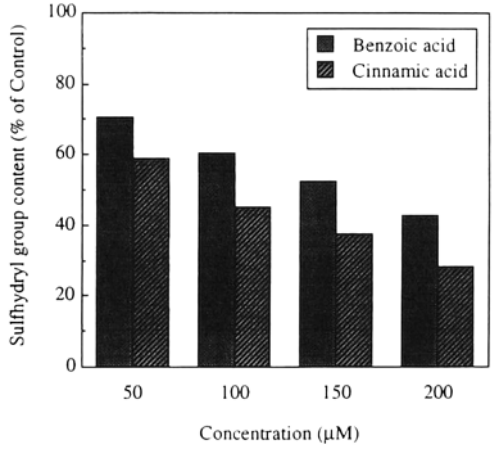

FIG. 8. Effect of BEN and CIN on sulfhydryl group contents in root of soybean plants after 12 hr of treatment.

able to the DTNB (Welch and Norvell, 1993), there is evidence that BEN and CIN affect essential sulfhydryl groups located at the plasma membrane.

A change in ion transport following BEN or CIN treatment could be explained in terms of reaction with sulfhydryl groups of membrane carrier proteins, ion pumps, or channels that may be required for the maintenance or modification of the plant cell membrane potential or the proton electrochemical gradient involved in the transport of ions or solutes. Studies with Ca^{2+}-ATPase and Na^+, K^+-ATPase of the sarcoplasmic reticulum indicated the participation of sulfhydryl groups in ATP recognition (Schoner et al., 1982). Sulfhydryl groups are implicated in phosphate transport (Jeanjean et al., 1975; Poder and Penot, 1986). Phenolic acids and their oxidation products can effectively tie up proteins, modify enzymatic active sites and decrease their activity, and even create artifactual enzymes by blocking ionic amino acid residues (Chalker-Scott and Fuchigami, 1989). Allelochemicals such as sesquiterpene lactones were reported to bind irreversibly to SH groups involved in auxin–auxin receptor (Spring and Hager, 1982) or to the sulfhydryl groups of enzymes (Fisher and Quijano, 1985).

Comparative physiological changes occurring in roots of soybean treated with BEN or CIN indicate that the earliest detectable effects of the two allelochemicals are the decline of sulfhydryl groups. In this regard, it seems likely that CIN and BEN cause intramolecular changes in protein sulfhydryl that could result in enhanced permeability of cellular membranes and subsequent electrolyte leakage. It has been suggested that depletion of cellular sulfhydryl groups implies a lowered internal defense against free radicals (Ayers et al., 1986), which in turn may lead to enhanced peroxidation of membrane lipids and loss of cell integrity. In contrast to other measured parameters that were markedly affected after 12 hr, 48 hr were necessary to observe an effect of BEN and CIN on lipid peroxidation. Thus, the leakage and lipid peroxidation observed in our experiments could be interpreted as secondary responses in the phytotoxic actions of BEN or CIN.

However, given the high reactivity of most allelochemicals with soil matrix, it should not be difficult to appreciate the significance of our data in terms of field conditions in the absence of data on the fate of BEN and CIN in soil environment. A sufficient pool of allelochemicals could be maintained in the rhizosphere through exudation from living plant tissues, decomposition of plant residues, or microbial metabolism. In this view, Haider and Martin (1975) concluded that after one week 39–63% of phenolic acids added to a sandy loam soils was decomposed. Although the recovery of phenolic acids from soils varied with soil type, horizon, and the particular kind of phenolic acid added, 30–75% was recovered after 32 days (Dalton et al., 1989).

The work presented here provides evidence that BEN and CIN alter ion uptake by affecting membrane properties. We propose that BEN- or CIN-induced

damage to the permeability of roots can be ascribed to direct action on membrane sulfhydryl groups. The intramolecular changes by oxidation or cross-linking in plasma membrane protein sulfhydryls is the first damaging effect of phenolic acids. Increased electrolyte leakage and lipid peroxidation, coupled with reduced of peroxidase and catalase activities, is a secondary response.

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BIOLOGICAL ACTIVITY OF CHEMICALS IDENTIFIED FROM EXTRACTS AND VOLATILES OF MALE *Rhagoletis cerasi*[1]

D. RAPTOPOULOS,* G. HANIOTAKIS, A. KOUTSAFTIKIS,[2] D. KELLY,[3] and V. MAVRAGANIS

Biology Department, N.R.C. "Demokritos" Aghia Paraskevi Attiki, Greece

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Abstract—Fractions obtained by chromatographic separation of extracts and volatiles from male *Rhagoletis cerasi* flies were tested for biological activity on females under laboratory conditions. During the bioassays, the number of incoming flies as well as the time spent by individual flies on the area of stimulus were taken under consideration. Two distinct types of female behavior were observed, i.e., attraction and arrestant behavior. GC-MS analysis of biologically active fractions resulted in tentative identification of 75 compounds. EAG screening for 27 of these compounds was performed, and subsequent laboratory bioassays resulted in the confirmation of arrestant activity for various mixtures of eight fatty acids (octadecanoic, nonadecanoic, eicosatetraenoic, eicosapenaenoic, eicosaenoic, heneicosanoic, docosahexenoic, and docosanoic). Following EAG tests and laboratory bioassays, a possible mode of chemical communication of this species with sex pheromones is proposed.

Key Words—*Rhagoletis cerasi*, cherry fruit fly, Diptera, Tephritidae, EAG, volatiles, extracts, sex pheromone.

INTRODUCTION

Cherries constitute a crop of economic importance for a number of regions in Greece. They are harvested in spring, a period during which no other fruit is in season, and consequently they are sold at high prices. A limiting factor in cherry

*To whom correspondence should be addressed.

[1](Diptera: Tephritidae).

[2]Present address: University of Patras, Departement of Biology, Patras, Greece.

[3]Present address: Department of Chemistry, University of Wales, College of Cardiff, P.O. Box 912, CF1 3TB, Cardiff Wales, UK.

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production is the cherry fruit fly, *Rhagoletis cerasi*, Linne (Diptera: Tephritidae).

The conventional control method for this pest is cover sprays with organophosphorus insecticides. The short period between the time of effective spray applications and fruit harvesting frequently results in detectable levels of pesticide on the fruit.

A number of alternative possibilities for the control of this pest have been explored with encouraging results, but for various reasons none has been adopted by cherry growers. The use of behavior modifying agents for the development of alternative control methods is at the center of attention. Mass trapping with visual traps, which are commercially available today, may effectively protect the crop (Russ et al., 1973). Mass trapping with insecticide-treated wood boards baited with food attractants and bait sprays are also effective (Haniotakis et al., 1989, 1991). Oviposition-deterring pheromones identified in this species have been tested for control with promising results (Katsoyannos, 1975; Boller et al., 1987; Boller and Aluja, 1992.). The existence of sex pheromones has also been reported (Katsoyannos, 1982). Sex pheromones and host kairomones seem to play an important role in the ecological behavior of the species, including host selection, mating site selection, and oviposition site selection. Host odors, for example, act as long-range attractants for sexually mature males and females to the host plant, where sex pheromones are responsible for mating. This combination of kairomones and male-produced sex pheromones is considered as a highly specialized recognition system, which is an important element for both evolution and speciation (Metcalf, 1990).

We report here a detailed analysis of the extracts and volatiles emitted by the male cherry flies and results of laboratory behavioral bioassays and EAG studies to determine biological activity of some of the compounds identified.

METHODS AND MATERIALS

Insects. The insects used in this study were collected as larvae from infested cherries from the regions of Pelion and Crete (Greece).

Headspace Sampling. Biologically active compounds were collected using the total condensation cold trap (Browne et al., 1974; Haniotakis et al., 1977). Approximately 100 live 4-day-old males were placed in cylindrical air-tight glass chambers (20 cm × 12 cm ID). Sugar and water were provided as food. Incoming air was purified through two filters: one of activated charcoal (Fluka AG, Buchs SG), and the other of Porapak Q. The outlet of the insect chamber was connected via glass tubing to a cylindrical flask (30 cm × 9 cm ID) immersed in liquid nitrogen. The system was tested without insects to ensure its proper functioning. Volatiles were collected from insects from the 6th to the 10th hour

of the photophase each day, during their mating period (Katsoyannos, 1982). A total of 150 ml liquefied air was collected each day. Subsequently the air was allowed to evaporate slowly at 25°C, and, prior to its total evaporation, 50 ml methylene chloride was slowly added. The collection procedure lasted for six consecutive days. In this way, it was calculated that a total of 2400 male-hour equivalents were collected. The end product (0.5 liters of methylene chloride solution) was dried by adding anhydrous magnesium sulfate, and evaporated under a Vigreux column (13 cm length) to a total volume of 1 ml, which was kept at 4°C.

Extraction Procedure. Two hundred sexually mature males were extracted in methylene chloride–methanol (3:1), for 4 hr at room temperature at the seventh hour of photophase on the sixth day of their adult life. The extract (20 ml) was evaporated under a Vigreux column to a volume of 1 ml and kept at 4°C.

Laboratory Bioassays. Bioassays were performed in a Plexiglass cage (30 × 50 × 80 cm), with its two small sides covered with wire screen (1-mm mesh). During the bioassays, 100 virgin, sexually mature females one to two weeks old were present in the cage. The bioassays were conducted between the 7th and the 10th hour of the photophase in an insect-free well-ventilated room, under uniform light (3000 lux), 25 ± 2°C, and 60 ± 5% relative humidity. Samples of the crude extract or male volatiles, and the fractions obtained therefrom during the purification procedure (estimated to ten male-hour equivalents), as well as samples (10 μg) of the compounds identified and various combinations of them, were pipetted onto a piece (2 × 2 cm) of Whatman No. 1 filter paper that was suspended upstream about 3 cm below the top of the cage. A weak airstream was directed through the cage by means of a small fan (Mazomenos and Haniotakis 1985). The bioassays lasted for 5 min. Ten minutes was allowed between two consecutive bioassays for full recovery of the insects. Filter paper impregnated with solvent only was used as control. Two types of behavior, i.e., attraction and arrestment, as well as the overall behavior of the females in the cage were recorded, e.g., excessive insect mobility. Bioassays were video-taped and the time spent by individuals on the filter paper was measured by replaying tapes of the experiments. Incoming and outgoing individuals were timed, and during replay the elapsed time was calculated.

EAG Studies. Preliminary EAGs were obtained from antennae excised from male and female *R. cerasi* heads. Since no difference was observed in the degree of response between males and females, replications for statistical analysis were limited to females only. For this purpose, the indifferent glass capillary microelectrode was positioned in the hemocoel of the cranial cavity, while the recording electrode made contact with the distal end of the funiculus. The microelectrodes used (GC150F-10, Clark Electromedical Instruments) contained Ag electrodes, and 0.1 N aqueous solution of KCl as electrolyte (van der Pers et

al., 1984). The electrodes were connected to a high-impedance amplifier (Un-03 Syntech General Research Instruments, Borneolaan 4, NL-1217 HA Hilversum, Netherlands). Aliquots (10 μl) containing 10 μg of the compounds tested were pipetted on filter paper (1 × 2 cm), which, after solvent evaporation, was placed inside a Pasteur pipet. Air purified by means of an activated charcoal trap was "puffed" through the pipet by an air pump (CS-27, Syntech), and onto the preparation. Stimulus duration was adjusted to 6 liters/min for 0.7 sec, and the constant air current was 5 liters/min. A valve diverted the air current into the pipet at the onset of the signal. The antenna signals were recorded, digitized, stored, and analyzed on a PC, using the EAG v.3 program (Syntech). The various stimuli were randomly presented to the insect. An interval of 60 sec between two consecutive stimuli was found to be necessary for full recovery of the antenna. Each compound was tested on at least five individuals.

Extracts of male insects and male insect volatiles were tested. In addition, a series of compounds (aldehydes, ketones, terpenes) that were either identified in the volatiles of the male insects or are known to be part of the "general green leaf volatile complex" (Guerrin et al., 1982; Jang et al., 1989; Levinson et al., 1990; Light et al., 1988, 1992), or compounds identified from cherry volatiles (Mattheis et al., 1992), and pheromone compounds of other tephritids (Mazomenos and Haniotakis, 1981; Heath et al., 1991) were also examined.

The insects' response to the compounds tested was evaluated by the maximum negative deflection (−mV) produced by every compound after subtracting the control. The final values obtained in this way (mV) were transformed to percentages (%) relative to 6-methyl-5-hepten-2-one (100%). This compound was selected because it was identified both in cherry (Mattheis et al., 1992) and insect volatiles, and its EAG response was similar in magnitude to that of the male volatiles.

Isolation and Identification of Components of Biologically Active Samples. For the determination of the polarity of the active compounds present in the male extracts and their initial separation, TLC was used. A system of diethyl ether–hexane (40:60) was used to elute Silica Gel 60 TLC plates (Merck). Four fractions were collected: TLC-1 at R_f0.0, TLC-2 between R_f0.0 and 0.5, TLC-3 between R_f0.5 and 0.75, and fraction TLC-4 between R_f0.75 and 1.

Large-scale separation of the male-produced volatiles was obtained using HPLC (Binary LC Pump 250 Perkin Elmer) with a Techsil silica 10 column (250 mm × 4.6 mm ID), using methylene chloride–methanol (99:1) as solvent system. The HPLC was operated isocratically for 40 min with a flow rate of 1 ml/min. Subsequently, the ratio of methanol in the system was increased up to 40%, with the same flow rate. Column effluent was monitored by a UV/Vis LC-235 diode array detector (Perkin Elmer) at λ = 210 nm and 235 nm. Four fractions were collected, HPLC-1 to HPLC-4, each containing 20 ml. All fractions were concentrated in a Vigreux column to a total volume of 1 ml and kept at 4°C.

The active fractions obtained by either TLC or HPLC were analyzed on a Varian model 3300 capillary gas chromatograph equipped with flame ionization detector and an on-column injector. The fractions were injected onto DB-35, 30 m × 0.32 mm ID and DB-17, 30 m × 0.32 mm ID fused silica columns (J&W Scientific, Folsom, California). The column temperature program for DB-5 was 50–180°C at 15°C/min, held for 10 min, and then programmed to 250°C at 25°C/min. For DB-17 the temperature program was 50–190°C at 8°C/min. The injector temperature was set at 200°C and the detector temperature at 260°C. Helium was the carrier gas, the column flow rate was adjusted to 2 ml/min. and the make-up flow rate to 30 ml/min. Sample aliquots (2 μl) of each active fraction were injected, and the retention times of the components on the two columns were compared to those of the available synthetic compounds.

Tentative mass spectral identification of the components of active fractions was performed on a GC-MS VG-Trio-1000 (VG-MassLab, Manchester, UK) spectrometer system in the electron impact (EI) mode, equipped with a DB-5, 30 m × 0.32 mm ID chromatographic capillary column (J&W Scientific), held at 50°C for 1 min., then programmed at 25°C/min to 250°C.

RESULTS AND DISCUSSION

EAG Results. Figure 1 shows the EAG responses of female antennae to various amounts of male extracts and male volatile emissions ranging from 10^{-3} to 5 male equivalents, and 10^{-3} to 5 male-hour equivalents, respectively. Unfortunately, experiments with higher concentrations of male equivalents were not

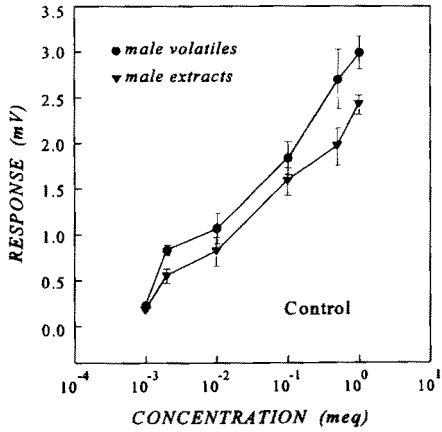

FIG. 1. EAG response of the female antenna to various concentrations of male produced volatiles, and male extracts.

possible due to the unavailability of biological material. These figures show that female flies have the ability to detect chemicals present in male volatiles at very low concentrations.

Laboratory Bioassays. The fractions collected from the TLC (male solvent extracts) and HPLC (male trapped volatiles) were tested for biological activity under laboratory conditions. Table 1 summarizes the results obtained from the bioassays and shows that there are two distinct groups of compounds: those acting as "attractants," attracting large numbers of females (such is the case of HPLC-2), and the second acting as "arrestants," stimulating incoming individuals to stay for a long time on the area of the stimulus (such is the case of HPLC-4, and TLC-1). Solvent extraction of the male seems to remove compounds belonging to the arrestant group, while in the case of volatile collection, compounds belonging mainly to the attractant group are obtained.

Analysis of Male Extracts and Headspace Volatiles. The GC-MS analysis of the male extracts and the male produced volatiles is summarized in Table 2. The various compounds identified were arbitrarily arranged into three abundance classes (major, intermediate, and trace), based upon visual examination of the reconstructed gas chromatograms from individual GC-MS runs.

Laboratory Bioassays of Synthetic Compounds. Based mainly on ranking of EAGs, 44 chemicals were tested in bioassays. A list of the compounds tested for their behavioral activity under laboratory conditions is given in Tables 2 and 3.

TABLE 1. *Rhagoletis cerasi* FEMALES ATTRACTED IN LABORATORY BIOASSAYS TO VARIOUS NAUTRAL PRODUCTS AND TIME SPENT BY INDIVIDUALS[a]

Test[b]	Attracted (N), $X_n \pm$ SE	Time (sec), $X_t \pm$ SE	BR(X_n/X_t)
Control	9.0 ± 1.0 a	8.7 ± 1.8 a	1.03
Male extracts	20.3 ± 2.8 b	17.9 ± 3.4 b	1.13
Male volatiles	32.4 ± 3.4 b	9.3 ± 1.4 a	3.48
HPLC 1	11.7 ± 2.1 a	8.2 ± 1.7 a	1.43
HPLC 2	41.6 ± 4.6 c	10.2 ± 2.3 ab	4.08
HPLC 3	9.3 ± 1.9 a	5.8 ± 2.2 a	1.60
HPLC 4	20.5 ± 2.8 b	23.1 ± 2.8 bc	0.89
TLC 1	17.0 ± 2.9 b	35.3 ± 4.6 c	0.48
TLC 2	10.6 ± 1.8 a		
TLC 3	14.2 ± 2.3 ab		
TLC 4	12.0 ± 1.6 a		

[a]Means of six replicates. Behavioral ratio: (BR) > 1.0 = attractant; (BR) < 1.0 = Arrestant. HPLC 1–4, refer to fractions of male produced volatiles, obtained by HPLC separation. TLC 1–4, refer to fraction of male extracts, obtained by TLC separation. Means followed by the same letter do not vary significantly. (Tukey's test, $P = 0.05$).

TABLE 2. CHEMICAL COMPONENTS IDENTIFIED FROM EXTRACTS AND VOLATILES EMITTED BY MALE *Rhagoletis cerasi* AND THEIR BIOLOGICAL ACTIVITY BASED ON EAG RESPONSES

Compounds[a]	FIT[b]	*RT*[c]	Purity (%)[d]	EAG[e] ± SEM
Major				
2-Ethoxy-2-methylbutane	970	4.36		
2-Pentanone	900	4.76		
4-mMethyl-3-penten-2-one	939	5.01[f]	90	50.4 ± 6.2
3-Hexen-2-one	944	5.03		
2-Hexanone	969	5.16[f]	99	100.5 ± 11.1
2-Hexanol	946	5.45		
3-Heptanone	962	7.43[f]	99	145.3 ± 9.0
3-Heptanol	937	7.95		
Tetrahydro-5,6 dimethyl-2H-pyran-2-one	925	9.69		
2-Ethylhexanol	969	12.43		
Limonene[g]	939	12.63[f]	97	44.0 ± 6.5
β-Phellandrene[g]	982	13.07[f]		79.7 ± 12.5
Nonanal[g]	979	13.45[f]	95	69.8 ± 5.1
Decanal	968	13.48		
Methyl heptanoate[h]	826	22.31		
Geranyl acetate[g]	986	25.28[f]	98	64.9 ± 4.4
Intermediate				
2-Methylpentanal	934	4.63		
Hexanal	920	5.06[f]	98	29.4 ± 6.0
3-Hexanone[g]	934	5.28[f]	98	101.2 ± 9.1
2-Methylcyclopentanol	944	5.61		
2-Methyl-2-pentanol	934	5.66		
3-Hexanol	957	7.21		
2-Ethylhexanal	899	9.53		
α-Pinene[g]	934	9.70[f]	97	24.6 ± 5.5
Benzaldehyde[g,i]	849	10.00[f]	99	100.4 ± 9.8
Octanal[g]	980	10.80[f]	99	62.6 ± 3.7
β-Pinene[g]	925	11.05[f]	95	27.9 ± 2.6
6-Methyl-5-hepten-2-one[g]	952	11.16[f]	99	100.0 ± 5.7
(Z)-2-Nonenal	828	12.63[f]	97	81.7 ± 2.7
Phenylacetaldehyde[i]	970	13.16		
Octanol	942	13.43		
2-Nonenol	829	14.50		
2-Ethylhexanoic acid[i]	939	16.51		
Camphor[g]	969	16.86[f]	98	65.4 ± 3.4
Dodecanal[g]	897	19.83[f]	95	63.0 ± 5.2
3-Hydroxy-4-methylbenzaldehyde	910	20.86		
Tridecanal	902	22.34[f]	90	57.4 ± 5.5
γ-Nonalactone[g]	945	24.78[f]	96	34.5 ± 5.6
Tetradecanal	955	26.46		
Methyl-8-methyldecanoate[h]	955	28.46		
Tetradecanoic acid (Myristic acid)[h]	869	29.86		
Hexadecanoic acid (Palmitic acid)[h]	865	34.45		

TABLE 2. Continued

Compounds[a]	FIT[b]	*RT*[c]	Purity (%)[d]	EAG[e] ± SEM
Trace				
3-Methyl-3-buten-2-one	876	3.63		
2-Methoxy-2-methylbutane	937	5.06		
Dihydro-5,5-dimethyl-2(3H)-furanone	752	5.48		
3-Methoxybutyl acetate	936	6.01		
4-Hydroxy-4-methyl-2-pentanone	955	6.11		
3-Methylbutanoic acid[f]	823	7.53		
Heptanal[g]	972	8.01[f]	95	48.2 ± 5.1
2-Nonanone[g]	849	9.35[f]	99	100.4 ± 11.0
4-Decanone[g]	889	10.20[f]	98	92.5 ± 8.0
p-Cymene	902	11.08[f]	95	23.5 ± 6.5
Ocimene	991	11.38		
3-Carene	954	12.48[f]	95	37.5 ± 3.0
4-Hydroxybenzaldehyde	959	13.05		
4-Methyl,1-methylethyl-bicyclo[3.1.0]hex-2-ene	990	13.06		
β-Terpinene	944	13.16		
Linalool[g]	771	14.92[f]	90	37.4 ± 3.8
2-Undecenal	944	16.03[f]	90	73.1 ± 4.6
Borneol[g]	922	17.41[f]	96	37.8 ± 6.1
Octanoic acid (Caprylic acid)[f]	759	18.32[f]	98	
5-Dodecenol	953	19.83		
Nonanoic acid[f]	968	20.48		
Dodecanol	948	21.35		
Decanoic acid (capric acid)[h]	826	22.38		
Linalyl acetate[f]	899	24.68		
Dodecanoic acid (lauric acid)[h]	884	26.32		
α-Farnesene	917	28.97		
9-hexadecenoic acid[h]	756	33.89		
$C_{18}H_{28}O_2$ (MW 252)[h]		37.00		
9-Octadecenoic acid[h]	800	37.50		
Octadecanoic acid (Stearic acid)[h]	901	37.98		
Ethyl nonadecanoate[h]	809	40.08		
$C_{20}H_{32}O_2$ (MW 304)[h]		40.40		
$C_{20}H_{40}O_2$ (MW 312)[h]		42.10		

[a]Compounds identified were arbitrarily arranged into three abundance classes (major, intermediate, and trace) based upon visual examination of the reconstructed gas chromatograms from individual GC-MS runs.

[b]Degree of fitness out of possible 1000 of the various sample components to the respective component of the system's library.

[c]Retention time.

[d]Purity (%) of the compounds used for identification and EAGs.

[e]EAG response of the female antenna based on the response to 6-methyl-5-hepten-2-one (100%). Compounds with no response indication were not tested.

[f]Compounds identified by GC using standards.

[g]Compounds tested in laboratory bioassays.

[h]Compounds identified in the male extracts.

[i]Compounds identified in both extracts and volatiles. Unmarked compounds were identified in volatiles.

TABLE 3. COMPOUNDS[a] AND COMBINATIONS TESTED IN LABORATORY BIOASSAYS AND FEMALE *Rhagoletis cerasi* RESPONSE

Compound	Response[b]
1. Octanoic acid (caprylic)	−
2. Decanoic acid (capric)	−
3. Dodecanoic acid (lauric)	−
4. Tetradecanoic acid (myristic)	−
5. 9-Hexadecenoic acid (palmitoleic)	−
6. Hexadecanoic acid (palmitic)	−
7. 9-Octadecenoic acid (oleic)	−
8. 9,12-Octadecadienoic acid (linoleic)	−
9. 9,11,13-Octadecatrienoic acid (linolenic)	−
10. Octadecanoic aid (stearic)	−
11. Nonadecanoic acid	−
12. 5,8,11,14,17-Eicosapentaenoic acid	−
13. 5,8,11,14-Eicosatetraenoic acid	−
14. 11-Eicosenoic acid	−
15. Eicosanoic acid (arachidic)	−
16. Heneicosanoic acid	−
17. 4,7,10,13,16,19-Docosahexenoic acid	−
18. Docosanoic acid	−
Combinations	
5 + 7 + 14	−
6 + 7 + 8 + 9 + 10 + 14	−
1 + 2 + 3 + 4 + 6	−
10 + 11 + 15 + 16 + 18	+
12 + 13 + 17	+
5 + 6 + 7 + 10 + 15	−
12 + 13 + 15 + 16 + 17 + 18	+

[a]Aldrich was the source of tested compounds.
[b]Positive sign (+) refers to arrestant behavior. Negative sign (−) refers to no response.

None of the compounds marked in Table 2 elicited any behavioral response to the female flies similar to that of the male volatiles or extracts when tested individually. Indication for biological activity appeared when 3-heptanone and 4-decanone were tested. Females seemed excited and flew vigorously, but did not land on the filter paper.

Arrestant behavior was observed when combinations of fatty acids were tested in bioassays (Table 3). Of these acids, octadecanoic was identified in fractions TLC-1 and HPLC-4. Gas chromatograms with standards of the acids listed in Table 3 indicated the possible presence of these acids in the polar fractions TLC-1 and HPLC-4, but since no reliable mass spectra were obtained, they could not be positively identified. However, bioassays with these acids and

some of their combinations were carried out. Ohinata et al. (1977) and Levinson et al. (1990) reported the existence of a similar group of acids in the male emitted volatiles of *Ceratitis capitata*, and their combination elicits a behavioral response by the females.

Some of the compounds identified have been also reported as components of the pheromone blend of other tephritids. Geranyl acetate and α-*E*-*E*-farnesene, for example, are constituents of the pheromone blend of *Ceratitis capitata* (Heath et al., 1991), and α-pinene, of *Bactrocera oleae* (Mazomenos and Haniotakis, 1981).

In summary, we believe that two different types of precopulatory behavior in *R. cerasi* are governed by chemical stimuli. One group of compounds attracts females to the stimulus area, and the other has arrestant properties. The fatty acids tested belong to the second group of compounds and may play a significant role in the communication system of this insect.

More work needs to be done to identify the compound(s) responsible for the attraction observed.

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SPATIAL DISTRIBUTION OF PHEROMONE IN VINEYARDS TREATED FOR MATING DISRUPTION OF THE GRAPE VINE MOTH *Lobesia botrana* MEASURED WITH ELECTROANTENNOGRAMS

GERHARD KARG[1,*] and ARNE E. SAUER

University of Kaiserslautern
67653 Kaiserslautern, Germany

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Abstract—The spatial distribution of the pheromone of the grape vine moth, *Lobesia botrana* (Lepidoptera: Tortricidae), was measured in vineyards treated for mating disruption by using an electroantennogram technique (EAG). Five hundred dispensers per hectare, each containing 0.1 g of the main component of the sex pheromone (*E,Z*)-7,9-dodecadienyl acetate (*E7,Z9*-12:Ac) were evenly distributed in the experimental vineyards. The EAG amplitudes measured in the experimental plots were transformed into relative pheromone concentrations by means of a calibration curve. Mean relative pheromone concentrations in the center of a treated plot reached 2.31×10^{-4} relative units. No significant differences in the mean relative pheromone concentrations were found between replicate plots ($P > 0.01$). The mean relative pheromone concentrations measured within one plot along a transect at 5-m intervals also showed no significant differences between the sites. These results indicate that inside the borders of treated areas the pheromone was evenly distributed. No sites with significantly lower pheromone concentrations, frequently assumed to be the cause for higher trap catches in some areas, were found. However, the mean relative pheromone concentration rapidly declined more than 100-fold outside the border of the treated plot. At 10 m from the treated area, the EAGs showed no significant difference compared to the EAGs recorded in an untreated area. A rapid drop in the mean relative pheromone concentration was also found on a vertical transect through the canopy of the vineyard. Measurements in an untreated control block gave a mean antennal response approximately 1000-fold lower than in a nearby pheromone treated plot. The significance of the variation in the pheromone distribution for the success of the mating-disruption method is discussed.

*To whom correspondence should be addressed
[1]Present address: HortResearch, P.O. Box 51 Lincoln, Canterbury, New Zealand.

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INTRODUCTION

Pest control by mating disruption has been applied successfully for control of a number of pest insects worldwide (Ridgeway et al. 1990; Jutsum and Gordon, 1989; Suckling, 1993). The aim of this method is to reduce the mating success of the pest insects by applying large quantities of its sex pheromone, or parts of the sex pheromone blend, in the area to be protected. While the effectiveness of the method has been proven for a number of pest insects (e.g., Arn et al., 1979; Charmillot and Vickers, 1991; Suckling and Shaw, 1992), mating disruption has failed to function as an appropriate means for pest control for other species or in other situations. The reasons for these differences in the success are not always fully understood and are subject of investigation.

Methods widely used to monitor the success of mating disruption are male catch in traps baited with synthetic pheromone baits or virgin females and crop damage assessment. In the first case, the number of males caught indicates the effectiveness of the method, assuming that male insects would not be capable of reaching the pheromone baited traps if the mating disruption was successful. However, the number of male insects caught is not always correlated to fruit damage. In addition, the number of males caught within a treated area can differ significantly between traps, so the question arises whether the higher number of trap catches in some traps is caused or supported by a reduced pheromone concentration in the vicinity of that trap. Monitoring mating disruption through crop damage assessment gives a good indication of the success of the pheromone application but has the disadvantage that any potential problems in the control of the pest insect that arise during the pheromone application will not become obvious until harvest and therefore cannot be addressed. The use of a field EAG apparatus (Baker and Haynes 1989; Sauer et al.; 1992; Karg and Sauer, 1992; Bengtsson et al., 1994; Karg et al., 1994; Suckling et al., 1994) is useful as an additional monitoring method. Its application could contribute to improving the success of the mating disruption technique in two ways. Firstly, field EAG could function as a monitoring system for (a) the pheromone concentration achieved in mating disruption plots during the season, (b) the spatial and temporal distribution of the pheromone, and (c) the longevity of the dispensers. Secondly, information concerning the spatial distribution of the pheromone in the area to be protected contributes to knowledge regarding the mechanisms underlying mating disruption by describing the olfactory environment in which a male insect has to find his partner. This information can be used to adjust the pheromone application method to the specific circumstances during the flight period of the pests.

The primary aim of this work was to measure relative pheromone concentrations and gain insight into the three-dimensional spatial distribution of the pheromone applied in vineyards under ambient conditions. We aim to describe the olfactory environment in which a male moth has to find his female counterpart. Consequences for the mechanisms of mating disruption are discussed.

METHODS AND MATERIALS

Insects. Grape vine moths, *Lobesia botrana* (Lepidoptera: Tortricidae), were reared in the laboratories of the Landwirtschaftliche Versuchsanstalt of BASF, Limburgerhof, Germany, and supplied as pupae. The purpae were sexed and, after eclosion, kept in a refrigerator at 9°C until used. All experiments were performed with antennae from 2 to 4-day-old males.

Pheromones. For calibration purposes, the main component of the pheromone of *Lobesia botrana* (*E,Z*)-7,9-dodecadienyl acetate ((*E,Z*)-7,9–12 : Ac) (Buser et al., 1974) was used. Different pheromone concentrations were obtained by dissolving different amounts of pheromone in paraffinoil (Merck, No. 7161, Uvasol). The vials containing the different pheromone–paraffinoil mixtures (from 10^{-2} to 10^{-7}) were kept inside 20-ml glass syringes. The equilibrium concentrations of the volatile pheromone inside these syringes above the pheromone–paraffinoil mixture depended on the concentrations of pheromone in the solutions (Raoult's law). While not in use, the pheromone solutions were kept in a freezer at −20°C. The pheromone dispensers in the field were standard pheromone dispensers as used by BASF, containing 0.1 g (*E,Z*)-7,9–12 : Ac.

Experimental Setup. The dispersion and the concentration of pheromones were measured by using electroantennograms (EAG). The measuring system and evaluation of the data are described in more detail by Sauer et al. (1992), Bengtsson et al. (1994), and Karg et al. (1994). A suction pump generated a continuous airstream across an antenna, which was mounted on an antenna holder, inside a glass chamber isolated from ambient air in the vineyard. A removable charcoal filter with a low flow-resistance was attached to the inlet of the measuring chamber to remove pheromones and other odors from the incoming ambient air. This filtering system was necessary for measuring the resting potential of the antenna in clean air and during calibration. For calibration purposes, 0.75 ml of air from a syringe loaded with a known pheromone concentration (10^{-4}) was injected into a continuous airstream (42 ml/sec) by a step-motor-driven device in a 0.2-sec puff. The pheromone concentration reaching the antenna was called the relative pheromone concentration, as this reflects the pheromone concentration above the pheromone–paraffinoil mixture as transported to the antenna by the main airstream. Different relative pheromone concentrations, necessary to measure the dose–response curve of the antenna to pheromone, were generated with different pheromone–paraffinoil mixtures in the

syringes. In order to measure the relative pheromone concentration in the ambient air, the charcoal filter was removed. Thus the ambient air containing pheromone was sucked across the antenna. Removal of the charcoal filter alone did not affect the flow rate over the antennae, as monitored with a flowmeter.

EAG signals were amplified, frequency modulated, and recorded on a tape recorder (see Sauer et al., 1992). To prevent contamination of the apparatus, glass parts were washed in acetone and heated with a Bunsen burner between subsequent measurements.

Estimation of Pheromone Concentrations. The measurement of three to five calibration pulses was followed by at least three measurements of the airborne pheromone concentration. The mean antennal response to the calibration pulses and to the ambient air measurements was determined and normalized by dividing the mean response to the ambient air measurements by the mean response to the calibration pulses (this equals one measurement), giving the normalized EAG amplitude. Normalization was necessary because of differences between antennae in the absolute antennal sensitivity to pheromone, as well as to compensate for the loss of responsiveness of individual antennae due to signal degradation and antennal fatigue. This procedure was repeated 3–15 times at every site. A single antenna was used for all related measurements, if possible. Antennae were replaced when only approximately 30% of the initial amplitude to a calibration pulse could be measured.

The relative pheromone concentration in the field was estimated by comparing the normalized EAG amplitudes generated by the ambient air with the normalized EAG amplitude elicited by the calibration pulses. The normalized EAG amplitude measured in ambient air could be transformed to the normalized calibration curve and converted to the corresponding relative pheromone concentration value (see Figure 1 below).

Field Experiments. All experiments were carried out in summer (1989 and 1990) during the flight season of the second generation of *Lobesia botrana*, in vineyards near Billigheim and Wachenheim, Rhineland-Palatinate, Germany. The plot sizes were 0.3–5 ha. Five hundred dispensers per hectare were applied, each containing 0.1 g of (*E*,*Z*)-7,9-12 : Ac. During the measurements the field EAG was always placed in the center of the four nearest pheromone dispensers. All related measurements began in an untreated control vineyard of the same grape variety (Scheurebe) approximately 1 km (Wachenheim) or 350 m (Billigheim) upwind from the pheromone-treated fields. The control vineyards had been treated conventionally with insecticides, ensuring that the possible interference of pheromones related by calling females was minimized. The EAG system was then transported to the pheromone-treated plots for subsequent measurements.

For comparison of the mean relative pheromone concentrations of different plots, the field EAG system was placed close to the center of the treated areas. At least seven antennae were used in the center of every plot. The measurement

of the transect along an axis of a treated plot began at the upwind edge (see Fig. 5 below, 2 m on x axis, 17 m on y-axis). Measurements were taken initially following one row. The field EAG system was transported towards the center of the plot, with measurements taken at 5-m intervals. On two occasions (at 17 and 32 m inside the treated vineyard) the EAG system was transported in an orthogonal direction (right angle to the previous axis), again with measurements taken in 5-m intervals, with the distance varying slightly depending on the interrow distances. For all related measurements, only one antenna was used. Measurements were repeated with five antennae. The measurements of the vertical distribution of the pheromone began above the canopy and continued in steps through the canopy. Measurements were taken at approximately 3.1, 2.8, 2.3, 1.8, 1.3, and at 0.8 m above ground level.

RESULTS

Calibration of Antennal Response. Figure 1 shows the mean normalized calibration curve of antennae of male *Lobesia botrana* to pheromone stimuli

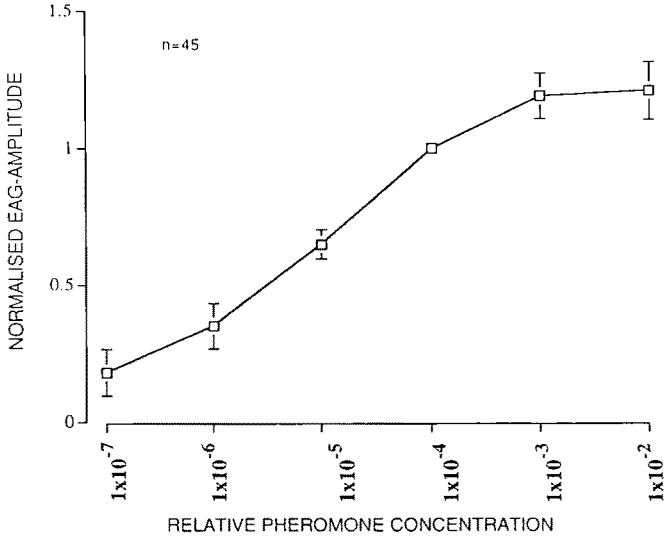

FIG 1. Dose—response curve of male antenna of *Lobesia botrana* to sex pheromone (*E,Z*)-7,9-dodecadienyl acetate. The dose-response curves of 45 antennae of male insects were normalized ot the antennal response to a stimulus of 1×10^{-4} relative pheromone concentration (pheromone–paraffin oil). The bars indicate the standard deviation. The normalized EAG amplitude elicited by the ambient air (y axis) can be converted to the corresponding relative pheromone concentration value (x axis) with the help of the normalized calibration curve.

from syringes containing vials with different pheromone–paraffinoil mixtures from 1×10^{-7} to 1×10^{-2}. The dose–response curves of 45 antennae were normalized to the response of a stimulus of 1×10^{-4} (relative pheromone concentration) and then averaged. With the help of the calibration curve, the normalized EAG amplitude measured in a pheromone-treated plot could be transferred to its corresponding relative pheromone concentration (Figure 1).

Comparison of Pheromone-Treated and Untreated Fields. The comparison of the mean relative pheromone concentrations measured in the center of a treated plot (Billigheim) and an untreated control plot, approximately 350 m upwind, is shown in Figure 2. The measurements were carried out in August 1989, with temperatures of 22°C and at a mean wind speed of <2 m/sec. The relative pheromone concentration measured in the treated area ranged from 4.5×10^{-5} (relative units) to 3.0×10^{-4} with a mean relative pheromone concentration of 2.31×10^{-4} ($N = 24$). The antennal response to the ambient air in this field thus had the same amplitude as it would have had to a calibration pulse from a syringe with a 2.31 : 1000 pheromone–paraffinoil solution. The EAG amplitude elicited in an untreated area was significantly lower (approximately 1000-fold) ($P < 0.001$) compared to the treated area. The mean relative pheromone concentration calculated from the EAG amplitude here reached a mean value of 1.98×10^{-7} ($N = 22$).

The EAG measured in untreated plots cannot be elicited by pheromone molecules but is the antennal response to other volatiles present in the ambient

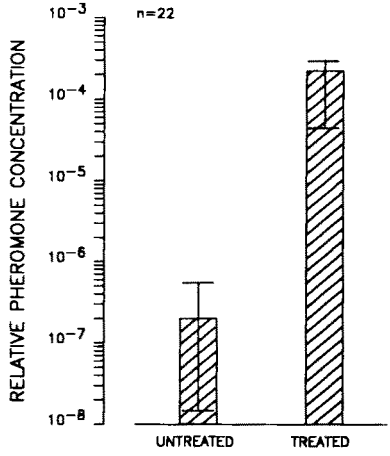

FIG. 2. Comparison of the relative pheromone concentrations in a treated area (Billigheim) and an untreated control plot (350 m upwind). The mean relative pheromone concentrations were 2.31×10^{-4} ($N = 24$) in the treated area and 1.98×10^{-7} ($N = 22$) (relative units) in the untreated area. The bars indicate the range of the measured values.

air, such as plant volatiles or other environmental odors. This is corroborated by results of Suckling et al. (1994) and Rumbo et al. (1995). The EAG elicited by these odors appears at first to be rather high, but if one takes the logarithmic relationship between the pheromone concentration and the elicited EAG into account, the contribution of environmental odors can be neglected (see Sauer et al., 1992).

Comparison of Pheromone Concentrations Measured in Replicate Pheromone-Treated Plots. Figure 3 shows the mean relative pheromone concentrations measured in replicate treated plots in Rhineland-Palatinate in 1989. Plots 1 and 2 were both located in the center of a 5-ha treated vineyard in Wachenheim. Plot 3, located in Billigheim, had a size of approximately 1.2 ha and was situated on a slope (6%). Mean relative pheromone concentrations in the center of three plots were measured on several days under similar weather conditions, with temperatures of 20–23°C and mean wind speeds of <2.5 m/sec.

The mean relative pheromone concentration estimated according to the dose–response curve (Figure 1) was 3.31×10^{-5} ($N = 24$) in plot 1 in Wachenheim, with measurements ranging between 2.3×10^{-5} and 6.9×10^{-5} (relative units). In plot 2 in Wachenheim the mean relative pheromone concentration was 5.25×10^{-5} ($N = 9$) (range 2.25×10^{-5}–12.8×10^{-5}) and in

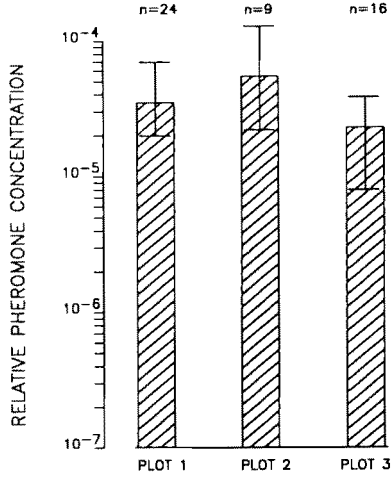

FIG. 3. Comparison of the relative pheromone concentrations in different treated plots in Billigheim and Wachenheim. The mean relative pheromone concentrations were 3.31×10^{-5} ($N = 24$) in plot 1 in Wachenheim, 5.25×10^{-5} ($N = 9$) in plot 2 in Wachenheim and 2.18×10^{-5} in the center of plot 3 in Billigheim ($N = 16$). There was no significant difference in the mean relative pheromone between the plots. The bars indicate the range of the values.

the center of plot 3 in Billigheim a mean relative pheromone concentration of 2.18×10^{-5} (range 0.94×10^{-5}–3.81×10^{-5}) was measured ($N = 16$). There was no significant difference in the mean relative pheromone concentration between the three replicate plots ($P > 0.01$).

Measurement of Pheromone Concentrations at Different Sites within Treated Plots. The spatial distribution of pheromone was repeatedly measured at different sites within the boundaries of treated plots along an axis. The results of the measurements carried out in Billigheim on August 3, 1989, are shown in Figure 4. The mean relative pheromone concentrations measured at the different sites ranged from 1.74×10^{-5} at the edge of the treated plot to 4.51×10^{-5} at 30 m inside the plot. There was no significant difference in the mean relative pheromone concentration within the treated plot ($P > 0.01$). In Figure 5 the mean relative pheromone concentrations measured inside a pheromone-treated plot in Billigheim on July 11, 1990, are shown. The mean relative pheromone concentrations again were not significantly different between the measurements ($P > 0.01$).

These results suggest that the pheromone is rather homogeneously distributed within the treated areas. No reductions in the mean pheromone concentration, frequently assumed to be the cause for higher trap catches in some areas, were found on any occasion.

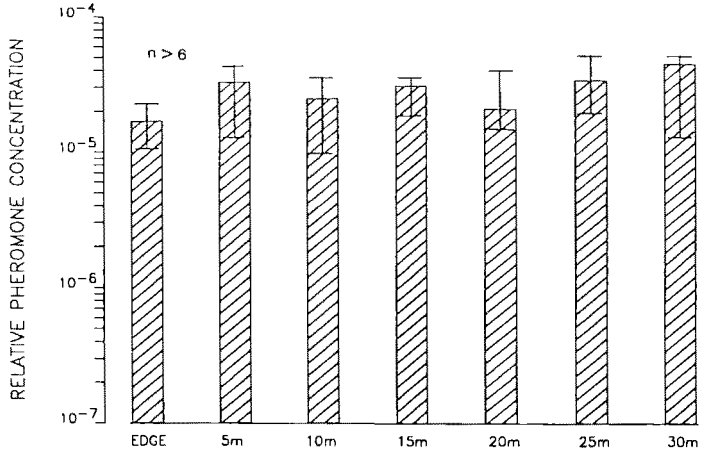

FIG. 4. Relative pheromone concentration within a treated area in Billigheim on August 3, 1989. The mean relative pheromone concentrations ranged from 1.74×10^{-5} to 4.51×10^{-5}. There was no significant difference in the mean relative pheromone concentrations measured within the boundaries of this treated area. The bars indicate the range of the values.

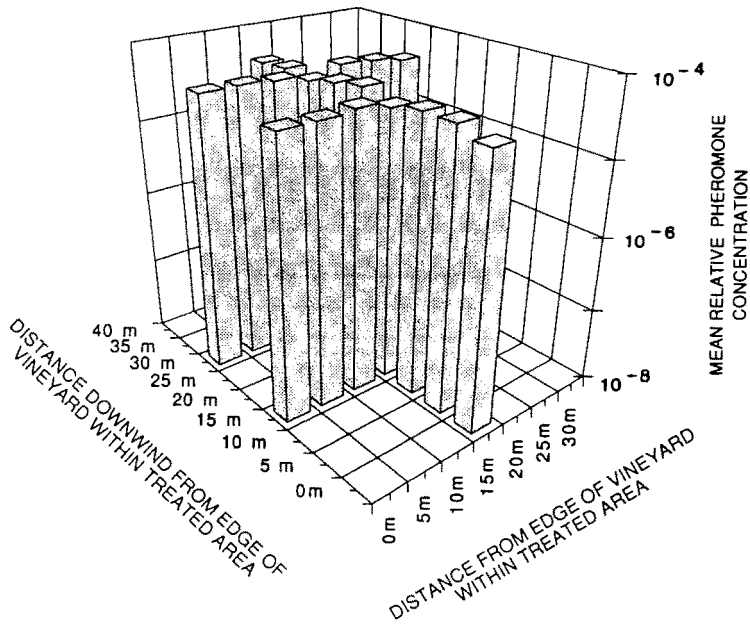

FIG. 5. Relative pheromone concentration within the treated area in Billigheim on July 11, 1990. The values ranged from 1.95×10^{-5} to 2.83×10^{-5}. There was no significant difference between the mean relative pheromone concentrations measured on several axes in the pheromone-treated vineyard. Distances are in meters.

Pheromone Concentrations outside the Borders of a Treated Vineyard. The mean relative pheromone concentration rapidly declines with increasing distance from the edge of the treated area (Figure 6). The mean relative pheromone concentrations measured inside a treated area on July 20, 1989, in Wachenheim was 8.43×10^{-5} (range $1.36 \times 10^{-5} - 8.6 \times 10^{-5}$) and dropped significantly to 3.41×10^{-7} (range $2.1 \times 10^{-7} - 1.1 \times 10^{-6}$) just 10 m outside the plot ($P < 0.001$) (Figure 6A). The antennal response at 10 m outside the treated plot was comparable to an EAG elicited in an untreated area. Measurements were repeated several times with similar results. Figure 6B and C show the results of measurements from August 8, 1989, and from July 11, 1990 in Billigheim. The decline of the mean relative pheromone concentration took place in all directions from the treated area, independent of wind direction, although the pheromone concentration seemed to be slightly biased towards the downwind direction.

Pheromone Concentrations along a Vertical Transect through a Treated Plot. The decline of the mean relative pheromone concentration with height is shown in Figure 7. The highest mean relative pheromone concentration of

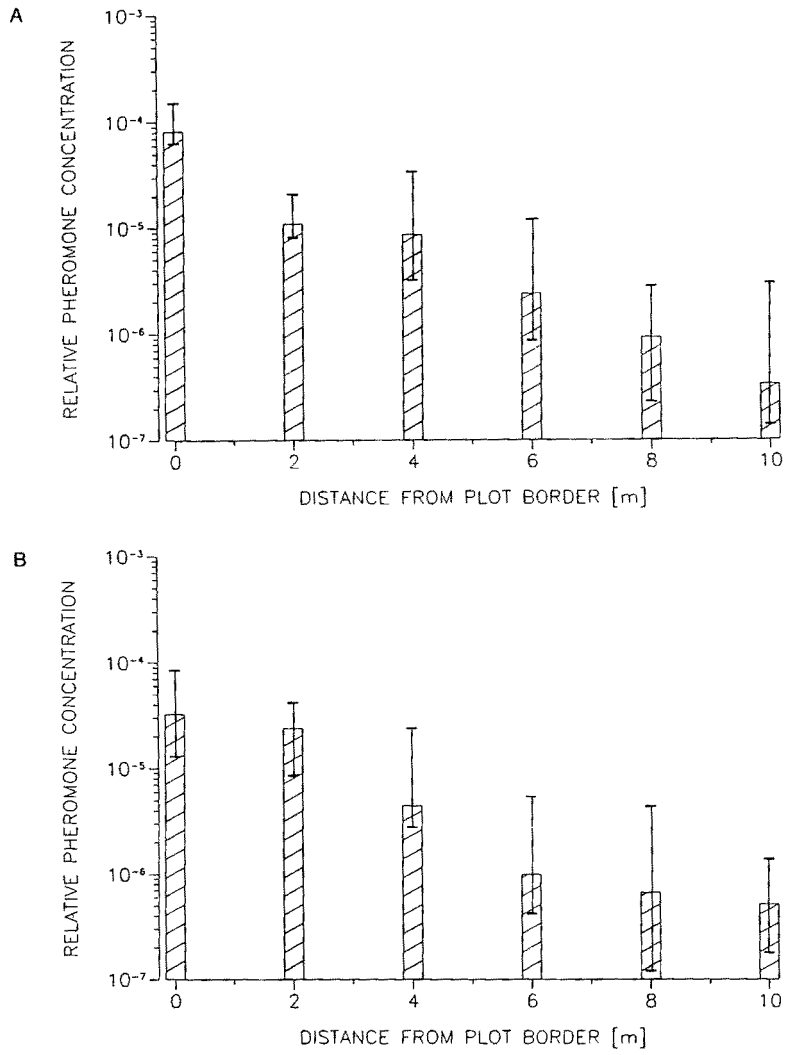

FIG. 6. Decline of the mean relative pheromone concentration with increasing distance from the edge of the treated plot. The mean relative pheromone concentration measured at the edge of a treated plot on July 20, 1989, in Wachenheim was 8.43×10^{-5} and dropped significantly at 3.41×10^{-7} only 10 m, outside the plot ($P < 0.001$) (A). This antennal response was comparable to an EAG elicited in untreated areas. The response measured at increasing distances did not change significantly. (B and C) The results of the measurements from August 21, 1989 in Billigheim and from July 11, 1990, in Billigheim.

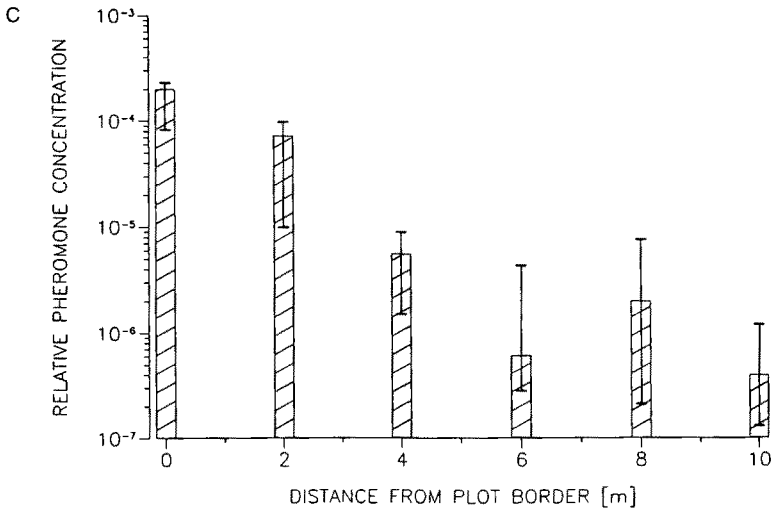

FIG. 6. Continued.

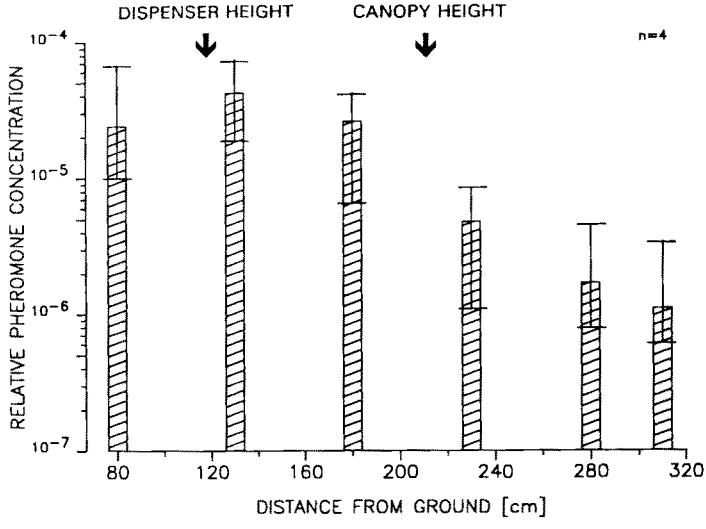

FIG. 7. Decline of the mean relative pheromone concentration along a vertical transect through a treated vineyard. The mean relative pheromone concentration declines rapidly approximately 30-fold from 2.42×10^{-5} to 7.42×10^{-7} at 3.1 m above the canopy of the treated area.

4.22×10^{-5} (range 2.3×10^{-5}–6.7×10^{-7}) was measured at 1.3 m, the same height the pheromone dispeners had been applied. The mean relative pheromone concentration was lower both below and above 1.3 m and declined approximately 30-fold with increasing height to 7.42×10^{-7} at 3.1 m.

DISCUSSION

Information concerning the distribution of airborne pheromone in mating disruption trials will be helpful in understanding the mechanisms on which mating disruption is based. Three different techniques are commonly used to measure pheromones in the field: (1) sampling of ambient air with collection of airborne volatiles on filters (e.g., Tenax) and subsequent chemical analysis (e.g., Caro et al., 1980, 1981) (2) field EAG measurements (e.g., Baker and Haynes, 1989; Sauer et al., 1992; Karg and Sauer 1992; Bengtsson et al., 1994; Karg et al., 1994; Suckling et al., 1994), and (3) single sensillum recordings (van der Pers and Minks, 1993).

Absolute, but time averaged, pheromone concentration can probably most accurately be measured by using air-sampling methods, followed by chemical analysis (Caro et al., 1980, 1981). This approach has not been attempted for *Lobesia botrana*, but preliminary results from another species (*Cydia nigricana*) show that the estimated aerial pheromone concentration from areas treated for disruption, as measured with EAG, compare closely with the value achieved by chemical analysis of field samples collected on a Tenax filter (Witzgall, unpublished data.).

The advantage of the field EAG system is the instantaneous nature of the measurements (on-line measurements) and the high temporal resolution (Karg et al., 1990a; Karg and Sauer, 1992; Bengtsson et al., 1994), which gives insight into the possible spatial and temporal fluctuations of the pheromone concentration. In addition, the portability of the EAG system allows repetitive measurements and measurements at different sites within only a short time period, and the high sensitivity of the antenna to pheromones enables us to gain information concerning the three-dimensional distribution of pheromone in mating disruption trials. However, the method has some disadvantages that hinder the interpretation of electroantennogram measurements (Sauer et al., 1992; Bengtsson et al., 1994; van der Pers and Minks, 1993). The major drawbacks are the interaction of pheromone and host-plant detection (Karg et al., unpublished data, Rumbo et al., 1995), the variance between individual antennae (Suckling et al., 1994), and the nonlinearity of the detector. The importance of these problems varies from species to species. In the case of *Lobesia botrana*, the response to environmental volatiles or host-plant volatiles are of minor importance (Sauer et al., 1992). Separation of the antennal preparation from the ambient air through

the surrounding glassware in the EAG system of Sauer et al. (1992) has the advantage that repetitive calibration of the antennal response in clean air is possible. Continuous sampling of ambient air through the glassware with a constant flow rate by a suction pump possibly distorts the EAG signal. However, fluctuations in an artificial pheromone plume, generated with the help of a fan and pheromone plumes released from a dispenser in an open field can readily be measured (Karg and Sauer, unpublished data).

In single sensillum recording techniques (van der Pers and Minks, 1993) neural responses of individual pheromone-specific sensilla are recorded. The advantage of this technique compared to the EAG method is that the presence of environmental volatiles does not usually interfere with the recordings from pheromone receptors. The major disadvantage of this technique is the comparably more difficult and fragile preparation that hampers its use in field measurements. In addition, the information acquired from recordings made from single olfactory hairs might be insufficient for the interpretation of sensory input (van der Pers and Minks, 1993), especially when the insects use a pheromone blend.

Our results show that the spatial distribution of the pheromone in the treated plots can best be described as a dense pheromone cloud, which is characterized by a high relative pheromone concentration. The pheromone cloud has well-defined edges outside which the pheromone concentrations decrease rapidly, and there are comparatively small internal variations in the mean pheromone concentration. Distinct trails generated by pheromone dispensers, often assumed to be present in treated vineyards, were not found.

The high relative pheromone concentration with little internal structure has two causes. Firstly, the higher temperatures in summer lead to higher release rates by the pheromone dispensers. Secondly, the plant canopy has a strong effect on the pheromone concentration obtained (Karg et al., 1990b). The foliage reduces mean windspeeds within the canopy (Uchijiama, 1988) and thereby decreases pheromone transport. In addition adsorption and release of pheromone by foliage has a significant impact on the atmospheric pheromone concentration in mating disruption trials (Karg et al., 1994) and probably also contributes to the even distribution of the pheromone within the treated area. Areas with significantly lower pheromone concentrations, often assumed to be responsible for increased trap catches or infestation (Krieg and Netter, personal communication), were not detected. The rapid decline of relative pheromone concentration outside the boundaries of the treated area is one possible cause for failure of mating disruption. Immigration of females mated in the surrounding areas with low pheromone concentration can lead to high infestation and fruit damage, even though disruption is successful within the treated area. Therefore, it is important to treat a sufficient distance outside the area to be protected with pheromone. The decline above the vineyard is less critical, as flight and ori-

entation takes place only within the canopy of the vineyards (Schneickhart, 1989; Netter, 1993).

Male *Lobesia botrana* attempting to find a female partner in a "pheromone cloud" as described above are most likely to undergo adaptation and/or habituation processes, which require sustained and/or repetitive simulation of the olfactory system (Rothschild, 1981; Bartell, 1982; Cardé, 1990). In our experiments discrimination between adaptation and habituation was not possible. Both sensory adaptation and habituation elicit changes in male behavior that can be registered in field observations. Only limited flight activity of *Lobesia botrana* males was found in pheromone-treated areas, whereas rather high flight activity was recorded in untreated fields (Netter, 1992; Schneickart, 1989; Karg, unpublished data). This reduction of male activity probably leads to a decrease in mate contact and can be responsible for the success of mating disruption of male *Lobesia botrana*. If distinct odor plumes or trails were present in the treated area, then sensory adaptation alone would be very unlikely to lead to disruption (Cardé, 1990) since the receptor neurons are known to regain their initial sensitivity to the pheromone within less than a second if returned to clean air (Rumbo, 1981). However, in a pheromone cloud characterized by high concentrations and a lack of fluctuations, sensory adaptation alone could explain the success of mating disruption.

Our results do not support the false trail following hypothesis as an explanation for the success of mating disruption of the second generation of *Lobesia botrana*. According to the false trail following hypothesis, pheromone plumes generated by the pheromone dispensers in the mating disruption trails compete with the plumes generated by calling females (Sanders, 1981; Bartell, 1982). The reduction of mating success is assumed to be due to the increased number of plumes (both artificial and natural), thus reducing the rate of success in finding a partner. In our experiments, no such odor plumes were ever found inside the borders of the treated fields.

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CHEMICALLY MEDIATED OVIPOSITIONAL BEHAVIORS OF THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (Lepidoptera: Pyralidae)[1]

B.F. BINDER,[2,*] J.C. ROBBINS,[2] and R.L. WILSON[3]

[2]*USDA-ARS, Corn Insects Research Unit, and Department of Entomology Genetics Lab., c/o Insectary Bldg. Iowa State University, Ames, Iowa 50011*

[3]*USDA, Agricultural Research Service North Central Regional Plant Introduction Station Iowa State University, Ames, Iowa 50011*

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Abstract—The volatile plant sesquiterpenoids farnesene, nerolidol, and farnesol were tested to determine their effect on European corn borer, *Ostrinia nubilalis*, oviposition during the first six nights of the adult stage. Adult European corn borers were released into cages designed to encourage oviposition on eight glass plates randomly arranged on top of the cage: four coated with either 80 $\mu g/cm^2$ farnesene, nerolidol, or farnesol, and four coated with the solvent methylene chloride. The setup was used as a binary choice test. Farnesene was preferred by females because they deposited significantly more egg masses on plates coated with the compound. Nerolidol had no effect. Farnesol, by contrast, deterred oviposition: the number of egg masses was significantly lower on plates covered with that compound. These data show that structural modification at a single terminal functional group in these compounds affects the ovipositional behavior of European corn borer females.

Key Words—Farnesene, farnesol, nerolidol, European corn borer, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, oviposition, behavior, egg masses, digital analysis technique.

INTRODUCTION

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is a highly mobile, polyphagous, primary pest of corn, and females deposit an average of two to

*To whom correspondence should be addressed.

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three egg masses each night throughout their ovipositional period, which ranges from 9 to 14 days, depending on environmental conditions (Caffrey and Worthley, 1927). Unidentified volatiles of injured corn plants repelled ECB oviposition and odors of undamaged plants attracted females (Schurr and Holdaway, 1970), but little is known about how individual volatile compounds might influence ECB oviposition. Volatile emission of the corn natural products (*E*)-β-farnesene and (*E*)-nerolidol increased due to larval feeding of the beet armyworm, *Spodoptera exigua* (Hübner) (Turlings and Tumlinson, 1992). A combination of these and other herbivore-induced plant odors attracted the host-seeking parasitic wasp *Cotesia marginiventris* (Cresson), and the authors suggested that release of host plant volatiles after damage also might deter host-seeking phytophagous insects from ovipositing on plants (Turlings et al., 1990). While farnesene acts as an ovipositional stimulant and larval attractant for the codling moth *Laspeyresia pomonella* (L.) (Sutherland and Hutchins, 1973; Wearing and Hutchins, 1973), its impact on ECB females is unclear. We evaluated the effects of the structurally related sesquiterpenoids farnesene, nerolidol, and farnesol on the ovipositional behaviors of ECB in the laboratory and found that these compounds could mediate ECB ovipositional behaviors.

METHODS AND MATERIALS

Insects. ECB were reared according to the methods of Reed et al. (1972), with modifications for the adult stage as described. Adults eclosed in specially designed cages (58.7 × 58.7 × 63.7 cm) made of angle and strap aluminum frame (1.9 cm and 2.3 cm, respectively) covered on the sides and bottom with 16 × 18 mesh brass cloth. Each cage was constructed so the wire cloth, which discourages oviposition, covered the inside surface of the cage, except for a section in the front, which was replaced by a cloth sleeve (Figure 1). The top of the cage was covered with 5-mesh galvanized cloth, which permitted oviposition on the underside of surfaces placed on top of the cage. Two feeding stations were included in each cage. One feeder was a cotton pad suspended from a brass wire 19.5 cm from the top of the cage and moistened daily with water. The other feeder, a molded plastic unit (10.3 cm^2) with 16 wells (3 ml), was located on the bottom of the cage and its wells were filled with 1.4% (w/v) agar gel containing 39.4% sucrose (w/v). Adult feeding was not restricted during the tests.

Oviposition Tests. The ovipositional tests were designed to examine ECB reproductive behaviors during the peak ovipositional period in a laboratory environment that simulated natural conditions in Iowa (16L : 8D, 24.5 : 18.5°C, 96% humidity). Females and males eclosed in the emergence cages; mating probably occurred soon after adult emergence. Within 15 hr of eclosion, 100 each of males and females were collected separately by using a small vaccum apparatus

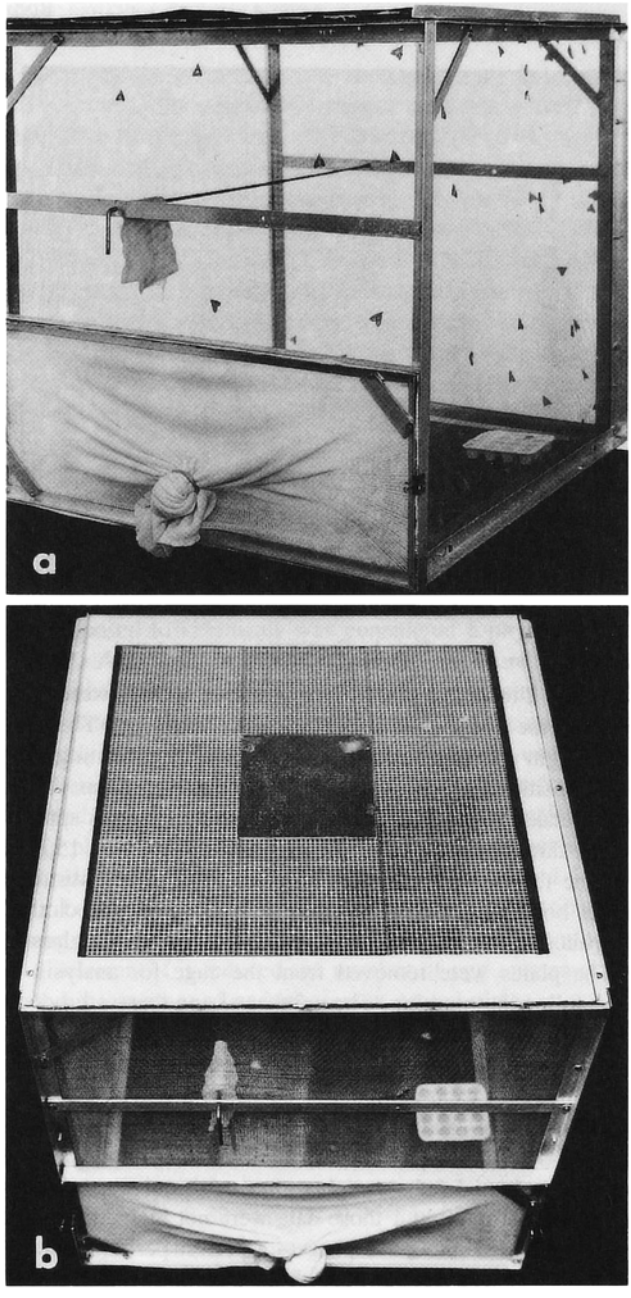

FIG. 1. Oviposition cage for *O. nubilalis* adults: (a) front view, (b) top view showing the position of the glass oviposition plates, (c) one oviposition plate covered with egg masses.

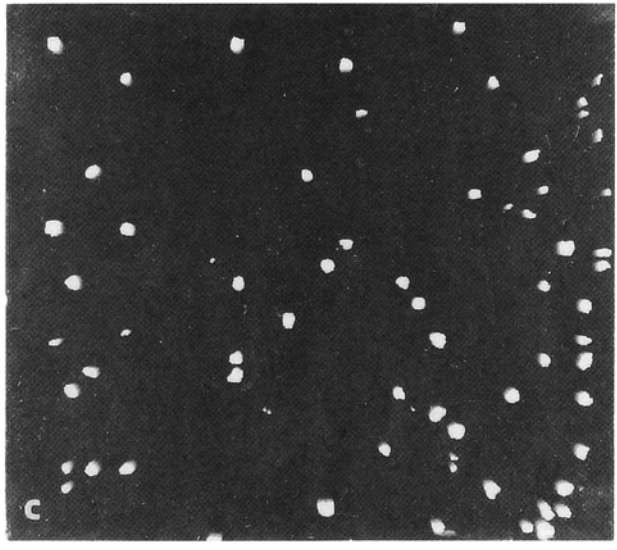

FIG. 1. Continued.

and released inside a test cage placed in a chamber. Adults were free to walk, fly, or mate within the ample confines of the cage (Figure 1a). These large cages allowed limited flight and ample mobility during posturing (mating movements), mating, and oviposition, which maximized reproductive efforts. There was no noticeable preference by the adults for any side, and the adults apparently were dispersed evenly throughout the cage. Eight glass plates (17 × 15.3 × 0.3 cm) positioned on the top of the cage were used as oviposition substrates (Figure 1b). A Plexiglas border and the central plate were covered with cloth to prevent oviposition on those surfaces. Females deposited eggs on the glass during the scotophase. The plates were removed from the cage for analysis during the photophase the following morning. The number of egg masses produced nightly and their size were monitored with a digital image analysis system described below. The plates were randomly arranged for each replicate (cage), and three replicates were made for each compound.

Farnesene, nerolidol, and farnesol were tested separately to determine if they stimulated or deterred oviposition or had no effect. Ovipositional behaviors for females were evaluated for six nights after eclosion, but because few egg masses were deposited on night 1 those data were not included in the analysis. The analysis was divided into three phases: a pretest period without chemical on night 2, a binary choice test period with chemicals on nights 3 and 4, and a posttest period without chemical on nights 5 and 6. The pretest period on night 2 following eclosion monitored uncoated plates to determine two factors: (1)

the total number of egg masses produced, which was expected to fall in a predicted range of 250–400 on that night if females were healthy, and (2) whether the number of egg masses distributed between plates 1–4 and 5–8 was equal, indicating that there was no bias in the random positioning of the plates. If the number and distribution of egg masses were as expected, the analysis advanced to the binary choice test period with chemical. Each chemical, dissolved in methylene chloride, was coated (80 $\mu g/cm^2$) on plates 1–4, and methylene chloride was coated on plates 5–8. The plates were monitored on nights 3 and 4 to determine if the plant compound influenced oviposition, as indicated by the number of egg masses deposited on the coated plates. The posttest period monitored oviposition on uncoated plates again on nights 5 and 6 to determine if there was a residual effect from the compound that might keep total oviposition reduced or elevated and to distinguish behavioral responses if oviposition was as expected.

The effect of the compound on oviposition was interpreted as toxicological if the total number of egg masses was significantly below the number expected from the same number of females not exposed to chemicals. A number of egg masses equal to or greater than the number expected from the same number of females not exposed to chemicals was considered a physiological response to the compound. A change in oviposition from exposure to a compound was interpreted as a behavioral response if two criteria were met: (1) the total number of egg masses was as expected on nights 3 and 4, but the number of egg masses was significantly higher or lower than expected on plates coated with farnesene, farnesol, or nerolidol; and (2) the total number of egg masses was as expected on nights 5 and 6. This type of response showed that females could not distinguish among plates before exposure to the compound, but were behaviorally sensitive to plates coated with chemical during nights 3 and 4. Additional evidence for a behavioral response occurred if females distributed egg masses evenly among the uncoated plates on nights 5 and 6. A compound was described further as a deterrent if it reduced the number of egg masses or as a stimulant if it increased the number of egg masses on coated plates.

Some volatile compounds could have indirectly affected oviposition by causing the premature death of adults. Therefore, mortality was monitored throughout the experiment.

Chemicals. The purity of each sample was analyzed by gas chromatography–mass spectrometry (GC-MS) by using a Hewlett Packard 5892 Chem Station, including a Wiley data base of spectra for reference. Each sample was injected on a 30-m × 0.32-mm-ID, 0.25-μm film thickness, DB-225 50% cyanopropylphenyldimethylpolysiloxane column (J & W Associates, Folsom, California); gas flow rate for He: 1.0 ml/min; temperature program: 35°C for 1 min then to 200°C at 20°C/min; EI at 70 eV. Analysis of the samples showed that farnesene was composed of two isomers and several unidentified impurities,

while there were four isomers of farnesol and two isomers of nerolidol. Evaporation and decomposition of farnesene, farnesol, and nerolidol was evaluated by gas chromatography–flame ionization detection (GC-FID). Glass plates were coated with each sample, stored in the conditions as described for oviposition tests, and extracted with methylene chloride after 0, 24, and 48 hr. An aliquot of each sample was injected on a 30-m × 0.25-mm-ID, 0.25-μm film thickness, DB-5 5% phenyl methyl silicone column (J & W Associates); gas flow rate for He: 1.3 ml/min; temperature program: 100°C for 1 min then to 200°C at 20°C/min. The integrated areas of each sample at 0, 24, and 48 hr were compared to standard curves to determine the amount of evaporation or decomposition.

Analysis. Each egg mass deposited on the glass plates was measured with a digital analyzer system (Decagon Industries Monochrome Ag Vision Imaging System, Pullman, Washington). A glass plate containing the egg masses was positioned under the camera of the digital analyzer and lighted from below. The resulting image appeared on the video monitor as a bright background with the egg masses showing as dark spots. This system digitized the video camera image (CCD 512 × 488 pixels) for each egg mass, and these data were stored as a separate record in a common-separated value (CSV) file for each glass plate. CSV files were then converted into an XLS file of the MS-DOS-driven spreadsheet program, Microsoft Excel. Once the data were transferred to the spreadsheet program, further statistical analyses were completed. Egg masses varied in size and therefore number of eggs. Egg masses with fewer than five eggs were not considered in this analysis because often the digital analyzer would mistake imperfections in the glass (nicks, scores, etc.) for egg masses, and small egg masses were sometimes missed entirely by the analyzer.

Statistical Analyses. Effects of each chemical on oviposition were analyzed with a two-tailed t test (Ott, 1984). Values were expressed as the mean and standard deviation (SD) ($N = 3$).

RESULTS

Female ECB were attracted to smooth surfaces for oviposition and deposited most of their egg masses on the underside of the glass plates resting on the top of the cage. Glass plates were convenient for the application of the test compounds and the easy quantification of egg masses by digital analysis. A few egg masses were deposited on the smooth surface of the plastic feeder. These spurious masses represented less than 1% of the total number of egg masses produced and were disregarded. No egg masses were deposited on the central plate or on the perimeter border.

The egg masses typically appeared evenly distributed on the plates (Figure 1c). Females deposited few eggs on the night following eclosion, but thereafter

females produced two to three egg masses/night/female for approximately one week. After one week, the insects' fecundity markedly declined.

Females distinguished between plates coated with the sesquiterpenoid plant volatiles and plates coated with the solvent methylene chloride for two of three compounds tested. Use of sesquiterpenoids modified at the terminal position provided three structurally related analogs: farnesene with a congugated terminal unsaturation, nerolidol with an unconjugated terminal unsaturation and an adjacent tertiary hydroxyl, and farnesol with a terminal hydroxyl moiety. There was substantial evaporation and decomposition of the compounds during the exposure period of the test, but even after 48 hr some of each compound was still detectable by gas chromatography (Table 1). While females responded to each of the compounds differently, there was little mortality from exposure to the compounds on nights 3–4 (Figure 2).

Plates coated with farnesene were moderately preferred by ECB females. Females deposited significantly more egg masses on plates coated with 80 $\mu g/cm^2$ of farnesene (plates 1–4) during nights 3 and 4 following eclosion (Table 2). There was no bias due to the position of the coated plates because on night 2, when none of the plates were coated, there were equal numbers of egg masses deposited on plates 1–4 and on plates 5–8; these plates were maintained in the same position throughout the experiment whether or not they had been coated with volatile plant compounds. When the coated plates were removed from the system and uncoated plates tested again on nights 5 and 6, females could not distinguish among the plates and deposited as many egg masses on plates 1–4 as on plates 5–8 (Table 2).

Females showed no preference for plates coated with nerolidol. There was no significant increase or decrease in the number of egg masses deposited on plates 1–4 coated with 80 $\mu g/cm^2$ nerolidol (Table 3). No bias was detected due

TABLE 1. TOTAL RECOVERY OF FARNESENE, NEROLIDOL, AND FARNESOL FROM GLASS PLATES EXPOSED TO AIR FOR VARIED TIMES[a]

Exposure time (hr)	Amount recovered (μg)		
	Farnesene	Nerolidol	Farnesol
0	2.4	1452.3	2530.0
24	3.4	1.6	167.0
48	1.8	1.5	71.6

[a]Each plate was coated with 20.8 mg of a compound. Amount recovered was measured by GC-FID, limit 50.0 ng/μl farnesene, 3.0 ng/μl nerolidol, and 22.0 ng/μl farnesol. Incubation conditions were as described for oviposition tests in Methods and Materials.

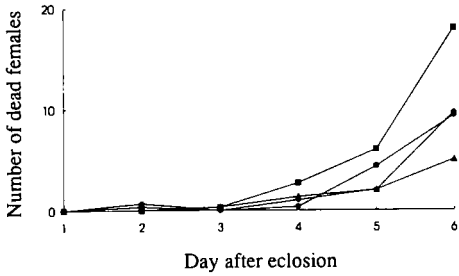

FIG. 2. Mortality of newly eclosed *O. nubilalis* females before, during, and after exposure to the plant volatiles farnesene (square), nerolidol (triangle), and farnesol (diamond) on nights 3 and 4. An untreated group (circle) is also shown. Because 100 pairs were used for each test, the values also represent percent mortality. Each value is the average of three replicates.

to the random positioning of the plates on nights 2, 5, and 6; there was no significant increase or decrease in the number of egg masses deposited on plates 1–4 (Table 3).

Farnesol deterred the females. Significantly fewer egg masses were deposited on plates 1–4 on nights 3 and 4 (Table 4). No difference in egg mass placement was detected from the random positioning of the plates on night 2,

TABLE 2. EFFECT OF FARNESENE ON OVIPOSITION OF EUROPEAN CORN BORER, *Ostrinia nubilalis*[a]

Night after eclosion	Glass treatment	Egg masses observed (mean ± SD)[b]		
		Plates 1–4	Plates 5–8	Total
2	No	137 ± 20 Farnesene	131 ± 36 Control	267 ± 54
3	Yes	211 ± 16	145 ± 23	356 ± 20
4	Yes	178 ± 24	115 ± 8	293 ± 28
5	No	108 ± 16	97 ± 14	205 ± 28
6	No	88 ± 30	72 ± 28	160 ± 57

[a] Oviposition was observed on nights 2–6. Plates were treated on nights 3 and 4 with farnesene (20.8 mg/plate) or solvent only (control).

[b] Few egg masses were observed on night 1 because of mating and premating behaviors, so they were not included. Oviposition was significantly higher on farnesene-treated plates (two-tailed t test, $\alpha = 0.05$, $N = 3$).

TABLE 3. EFFECT OF NEROLIDOL ON OVIPOSITION OF EUROPEAN CORN BORER, *Ostrinia nubilalis*[a]

Night	Glass treatment	Egg masses observed (mean ± SD)[b]		
		Plates 1–4	Plates 5–8	Total
2	No	180 ± 44 Nerolidol	187 ± 17 Control	367 ± 27
3	Yes	200 ± 26	235 ± 46	435 ± 48
4	Yes	169 ± 9	153 ± 33	322 ± 28
5	No	148 ± 20	147 ± 36	295 ± 18
6	No	122 ± 7	134 ± 32	256 ± 36

[a]Oviposition was observed on nights 2–6. Plates were treated on nights 3 and 4 with nerolidol (20.8 mg/plate) or solvent only (control).

[b]Few egg masses were observed on night 1 because of mating and premating behaviors, so they were not included. Oviposition was not significantly higher or lower on nerolidol-treated plates (two-tailed t test, $\alpha = 0.05$, $N = 3$).

but on nights 5 and 6 there was a significant reduction in the number of egg masses deposited on plates 1–4. This indicated that there was some residual effect due to the farnesol after the plates coated with the chemical were removed (Table 4).

TABLE 4. EFFECT OF FARNESOL ON OVIPOSITION OF EUROPEAN CORN BORER, *Ostrinia nubilalis*[a]

Night	Glass treatment	Egg masses observed (mean ± SD)[b]		
		Plates 1–4	Plates 5–8	Total
2	No	196 ± 32 Farnesol	200 ± 26 Control	396 ± 57
3	Yes	212 ± 5	306 ± 24	518 ± 25
4	Yes	176 ± 8	234 ± 16	409 ± 24
5	No	162 ± 30	202 ± 15	364 ± 43
6	No	124 ± 13	171 ± 5	295 ± 17

[a]Oviposition was observed on nights 2–6. Plates were treated on nights 3 and 4 with farnesol (20.8 mg/plate) or solvent only (control).

[b]Few egg masses were observed on night 1 because of mating and premating behaviors, so they were not included. Oviposition was significantly lower on farnesol-treated plates and on subsequent nights for plates in positions where farnesol had been present during nights 3 and 4 (two-tailed t test, $\alpha = 0.05$, $N = 3$).

DISCUSSION

Volatile emissions detected from corn leaves are composed of complex mixtures of aliphatic aldehydes, ketones, alcohols, and esters as well as terpenoids and other aromatic compounds (Buttery and Ling, 1984). These volatiles mark the aroma of the plant and are used as cues by the parasitic wasp *Cotesia marginiventris* to recognize appropriate host sites (Turlings et al., 1990). In this study, we found that farnesene, one of the chemicals in the blend that is attractive to *C. marginiventris*, enhanced ECB oviposition. Use of analogs of farnesene modified at the terminal functional group to make either farnesol or nerolidol affect ECB adults by reducing oviposition or having no influence, respectively. Thus, we could mediate oviposition positively or negatively simply by exposing the adults to closely related compounds with different terminal functional groups.

Farnesene is a common sesquiterpenoid plant natural product (Teranishi et al., 1993) and its release is induced at rates as high as 120 ng/hr following injury from the armyworm, *Spodoptera exigua* (Turlings and Tumlinson, 1992). Even though the high rate of release of farnesene is not sustained for more than 24 hr (Turlings and Tumlinson, 1992), we used it as a guide to determine the quantity of mixed isomers of farnesene to apply on the plates in our oviposition assay. Herbivore-damaged corn plants might release as much as 2.9 μg in a 24-hr period. Our tests, conducted with 80 $\mu g/cm^2$, represent a total exposure of 83 mg/cage/night or an approximately 29,000-fold higher amount over that expected from exposure to the corn plant. Since the isomers readily evaporate or decompose from the plate at a rate as high as 0.86 mg/hr and leave as little as 1.6 mg on each plate after 24 hr, we believe the females are exposed to similar amounts of the compounds on plates in the laboratory as they might experience from an injured plant. Therefore, their movements and activities during oviposition probably reflect the same responses to chemical stimuli as those expected on exposure to the injured plant itself.

Farnesene readily influenced ECB oviposition but females do not distinguish among plates once the chemical is removed. Farnesol, by contrast, has a residual effect because ECB females distinguished among the plates, even two days after exposure to the chemical. A simple explanation to account for this observation is that some of the compound dispersed on to the metal screen of the cage, was present during subsequent days during the test, and females were still able to perceive the deterring compound by olfaction or gustation or both. Residual amounts of the compound could have remained on the cage for several days because we found that farnesol evaporated or decomposed less readily than the other compounds during a 48-hr period.

Physiological effects from exposure to either farnesene or farnesol also can never be completely eliminated as a cause of the changes in ECB oviposition, but neither farnesene nor farnesol increased mortality and, even though females

were exposed to chemicals, they produced the expected total number of egg masses during the experiment. Because there were not overt symptoms of a physiological problem, we interpreted the results of our studies as behavioral modulation of ECB oviposition.

Farnesene, farnesol, and nerolidol are important molecules in insect communication and development. Stereoisomers of farnesene are alarm pheromones for numerous species of aphids (Bowers et al., 1972; Montgomery and Nault, 1977; Pickett and Griffiths, 1980), trail pheromones for ants (Attygalle and Morgan, 1984), and feeding stimulants for larvae and attractants for adults of the codling moth *Cydia pomonella* (Sutherland and Hutchins, 1973; Wearing and Hutchins, 1973). Farnesene and farnesol are feeding stimulants for the sand fly *Lutzomyia longipalpis* (Tesh et al., 1992). Farnesol and nerolidol possess high juvenilizing activity against the mealworm, *Tenebrio molitor*, while reduced farnesol, the analog without unsaturation, and farnesane lose all activity (Bowers and Thompson, 1963). Farnesol, a powerful juvenoid, acts as a gonadotropic hormone in some adult insects (Slama, 1985), but its role in the physiology of ECB adults during this study seems to be minimal.

Commercial samples of farnesene, farnesol, and nerolidol evaluated by GC-MS showed that they are composed of several isomers, and for farnesene, several impurities. Isomers and structural analogs of these compounds are known to stimulate different activities in insects. For instance, geometrical stereoisomers of 3,7,11-trimethyldodeca-2,6,10-triene, structurally related analogs of the natural aphid alarm pheromone, (*E*)-β-farnesene, show differential activity: (*E*)-2,6,10-trimethylundeca-1,5,9-triene is approximately 1000-fold more active than its stereoisomer (*Z*)-2,6,10-trimethylundeca-1,5,9-triene against the aphid *Acyrthosiphon pisum* (Harris), but both are less effective at triggering an alarm response than the natural pheromone (Nishino and Bowers, 1976; Nishino et al., 1976). Farnesene- and farnesol-related structural isomers of sesquiterpene ovipositional stimulants, (+)-(*E*)-*endo*-β-bergamoten-12-oic acid and (−)-(*E*)-*endo*-α-bergamoten-12-oic acid, which differ only in the position of a double bond, elicit contrasting responses from gravid females of *Helicoverpa zea* (Boddie): (+)-(*E*)-*endo*-β-bergamoten-12-oic acid is more attractive to females than (−)-(*E*)-*endo*-α-bergamoten-12-oic acid (Coates et al., 1988). These studies illustrate that differences in biological activities are evoked from stereochemical changes in molecular structure and we speculate that structural isomers and stereoisomers of farnesene, farnesol, nerolidol, and related compounds may similarily modulate ovipositional behaviors of ECB females.

Prominent differences in ovipositional behaviors of ECB females from exposure to farnesene and farnesol may arise because they vary the use of olfactory and gustatory sensory mechanisms to distinguish between plates coated with the sesquiterpenoid plant volatiles and plates coated only with solvent. A combination of olfaction and gustation are used during resource selection and

host location in many insects (Ahmad, 1983; Bell, 1991; Metcalf and Metcalf, 1992; Renwick and Chew, 1994), but further studies on ECB behaviors such as searching, orientation, and landing behavior are needed. Females also respond to volatile aromatics (Cantelo and Jacobson, 1979), and nonvolatile carbohydrates on the leaf surface of corn (Derridj et al., 1993). Studies of interactions of the ECB adults at the corn leaf surface will show if herbivore-induced release of these compounds will affect their orientation and ovipositional behaviors on a natural host and if they have potential to be employed to disrupt oviposition.

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INTERFAMILY VARIATION IN COMB WAX HYDROCARBONS PRODUCED BY HONEY BEES

MICHAEL D. BREED,[1,*] ROBERT E. PAGE JR.,[2] BRUCE E. HIBBARD,[3,4] and LOUIS B. BJOSTAD[3]

[1]*Department of Environmental, Population, and Organismic Biology The University of Colorado, Boulder, Colorado 80309-0334*

[2]*Department of Entomology, The University of California, Davis Davis, California 95616*

[3]*Department of Entomology, Colorado State University Fort Collins, Colorado 80523*

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Abstract—The hydrocarbons of honeybee comb wax vary significantly between colonies. This variation is explained in part by genetic (familial) differences among colonies. Even though significant differences in wax hydrocarbons exist among families, there is a high level of consistency within and among families in a correlation analysis, indicating structural constancy in comb wax. The significance of these results in interpreting the potential role of comb wax in the nestmate recognition system of the honeybee is discussed.

Key Words—Honeybee, comb wax, hydrocarbons.

INTRODUCTION

The comb wax of the honeybee serves as a source of recognition cues that are used in discriminating nestmates from nonnestmates (Breed et al., 1988ab, 1995; Breed and Stiller, 1992). Exposure to wax for periods as brief as 5 min radically alters the recognition phenotype of a bee (Breed et al., 1988b), superimposing the wax-derived cues on cues found on the cuticle of bees (Breed, 1983). The purpose of this paper is to present an analysis of the hydrocarbon component of comb wax and to test the hypothesis that comb wax could convey genetic information that might be used in nestmate recognition.

*To whom correspondence should be addressed.

[4]Current address: USDA-ARS, Rm. 103 Curtis Hall, University of Missouri, Columbia, Missouri 65211.

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Comb wax is chemically complex (Hepburn, 1986; Tulloch, 1980), containing hydrocarbons, esters, free acids, and alcohols. It may also serve as a repository for compounds brought into the colony with foodstuffs. For example, Breed et al. (1988a) showed that anise extract, when placed in a waxy medium, could be used by honeybees as a recognition cue. Bowden and Breed (unpublished) have recently shown that a number of other compounds found in flowers, such as anethole, citral, and linalool, can function as recognition cues. Breed and Stiller (1992) showed that the alkanes hexadecane and octadecane can serve as recognition cues. Our focus in this paper on the hydrocarbons is justified by the biological activity shown by these alkanes; subsequent studies (Breed et al., 1995) will deal with other comb wax components.

In addition to the investigations of Tulloch (1980) and Hepburn (1986) on the chemistry of comb wax, Carlson (1988) compared the comb waxes of European and African honeybees, with the objective of developing a method of identifying Africanized bees. The finding of interracial variation in comb wax composition suggests genetic variation. Blomquist et al. (1980) investigated the synthetic pathways leading to bee's wax.

There is, however, considerable information available on honeybee cuticular hydrocarbons. Cuticular hydrocarbons have been implicated in nestmate recognition in a number of social insect species (reviews: Howard, 1993; Smith and Breed, 1995). Most relevant to our study is the work of Page et al. (1991), who showed that there is a genetic component of the variation among bees in cuticular hydrocarbon profiles. Other studies include those of Carlson and Bolton (1984) and Smith (1990), who compared African and European bees, and Francis et al. (1985, 1989), who characterized the cuticular hydrocarbons of European bees. Singer and Espelie (1992) implicated cuticular hydrocarbons in nestmate recognition in social wasps, and Clement et al. (1987) made a similar argument for an ant. Cuticular hydrocarbons also have been hypothesized as signals in species or sex identification in nonsocial insects (Howard, 1993; Markow and Toolson, 1990).

We obtained comb wax from 40 colonies representing nine families. We analyzed the results by chromatographic analyses of the wax to determine the extent of variation within and among families and to assess whether adequate variation exists for wax hydrocarbons to function as recognition cues. We then placed our findings in the context of other studies of recognition cue chemistry.

METHODS AND MATERIALS

Comb Production. Combs produced by sets of sister colonies were collected from the University of California, Davis, apiaries. Colonies for comb production were derived from 18 colonies headed by naturally mated parental queens. Nine

queens were designated queen mothers (Q1–Q9), and nine other queens were designated drone mothers (D10–D18). Virgin queens and drones were raised, and virgin queens from each queen mother were mated to a single, different drone from each colony, using the following scheme: Q1 × D10, Q2 × D11, Q3 × D12, etc. This resulted in families designated 1–9.

Parental queens were naturally mated. We assumed that sister queens were half-sisters, rather than supersisters, because queens mate naturally with an average of about 17 drones (Adams et al., 1977). We also assumed that all queen and drone mothers were unrelated. With these assumptions, workers within colonies had pedigree coefficients of relatedness (Pamilo and Crozier, 1982) of $G = 0.75$. Workers between colonies with sister queens (same family) were related to $G = 0.31$, and workers of different families were unrelated. The mean number of colonies sampled per family was 4.4 (SE = 0.58) and the range was 2–7.

To collect comb samples, instrumentally inseminated queens were installed in single-story Langstroth hives in a single apiary containing nine standard frames and about 8000 workers. Colonies were allowed to develop for at least 60 days, during which time the original workers died and were replaced by worker progeny of the queens. After this time a single comb constructed on an empty frame without foundation was removed from the boxes and colonies were stimulated to construct a new comb. Empty, newly constructed combs were collected and frozen for analysis. Comb from a single colony is considered, in this paper, to be a sample. Each colony is represented by a single sample in our dataset.

Extraction Procedures for Comb Wax. Comb wax (50.0 mg) was added to a clean 4-ml glass vial with a Teflon liner on the lid, 2.0 ml of dichloromethane was added, and the sample was mixed thoroughly and allowed to sit over night. The sample was then mixed thoroughly again and 80 μl added to 0.5 ml dichloromethane. To remove undissolved materials and other high-molecular-weight material, the contents of the second vial were passed through a silica gel column. The column was made from a 14.6-cm disposable Pasteur pipet containing a glass wool plug to which a slurry of dichloromethane and silica gel was added to within 5 cm of the top. Dichloromethane was added to the column until a total of 4 ml of eluate was collected. An internal standard (20 μg *n*-octadecane) was added to the 4-ml solution. After this procedure, the solution contained 1 μg/μl of total wax (based on the amount of wax at start) and an internal standard (*n*-octadecane) at a concentration of 5 ng/μl. In preliminary results, chromatograms from wax solutions that had gone through silica gel were identical to those that had not, indicating that volatile materials were not left on the silica gel.

Gas Chromatography. Chemical separations were made with an RSL-150 methyl silicone capillary column (25 m × 0.25 mm ID, 0.25 μm film) in a

Hewlett-Packard 5890 gas chromatograph with splitless injector and flame ionization detector. The injector temperature was set at 250°C and the detector temperature at 290°C. The inlet purge of the splitless injector was turned on at 0.75 min. The initial oven temperature was set at 150°C for 1 min, then programmed at 10°C/min to a final temperature of 280°C. These conditions resulted in good separation of volatile wax components within a time frame of 25 min. Larger compounds (alkanes > C_{29}) may not have been detected in the same proportion as lower-molecular-weight compounds; this factor does not affect the analysis as each peak is used as an independent variable.

Statistical Analyses. All analyses were performed using the Statview II statistical package. After using the octadecane standard to standardize the data, separate analyses of variance (ANOVA) were performed between families for each compound. To determine levels of association between colonies, parametric correlation analyses were performed using the 25 peaks as independent measures for each colony; this procedure yielded a matrix of between-colony correlation coefficients. For example, each colony in family 1 was represented by a correlation coefficient with each other colony in family 1, as well as by correlation coefficients with each colony in the other families. An ANOVA was then used to test whether the between-colony correlation coefficients were higher within families than among families. Correlation coefficients were also calculated between peaks; in this analysis the correlations between peak 1 and peak 2, peak 1 and peak 3, and so on, were calculated.

RESULTS

Peak Separation. Twenty-five peaks were resolved, in addition to the internal standard (Table 1). Mean concentrations, in nanograms per microgram of wax, varied from 0.3 to 27.9. The standard errors are not large with respect to the mean. The small standard errors indicate a relatively high level of homogeneity among the samples. The major *n*-alkenes were identified and are indicated in Table 1.

ANOVAs of Wax Concentration Variation among Families. Each of the 25 compounds resolved was subjected to an analysis of variance to determine whether significant differences in concentration existed among families (Table 1). Of the 25 ANOVAs, seven, representing compounds 6, 8, 9, 12, 20, 23, and 26, were significant. Compounds 1, 5, 6, 14, 18, and 24 were present either in very low concentrations or were not detected in many samples. Six other ANOVAs yielded probabilities between 0.05 and 0.10 (compounds 3, 4, 10, 11, 13, and 25), suggesting that further significant variation may have been resolved with larger sample sizes. There is no obvious relationship between the magnitude of the mean peak for a given compound (Table 1) and the significance

TABLE 1. SELECTED PEAK IDENTIFICATIONS, MEAN CONCENTRATION, AND ANOVA AMONG FAMILIES FOR 25 PEAKS ANALYZED[a]

Peak	Peak Identification	Concentration (ng/μg wax) Mean	SE	F	P
1	heptadecane	0.13	0.024	1.16	0.35
2	octadecane (internal standard)	5.00	0.000		
3	nonadecane	0.26	0.020	1.85	0.10
4	eicosane	0.62	0.193	1.93	0.09
5		0.03	0.008	1.58	0.17
6		0.09	0.013	2.65	0.02*
7	tricosane	2.31	0.110	1.50	0.20
8		0.18	0.010	2.52	0.03*
9		0.33	0.030	2.74	0.02*
10	pentacosane	6.45	0.375	1.88	0.10
11		0.82	0.033	1.86	0.10
12		0.31	0.024	2.58	0.03*
13	heptacosane	27.86	1.225	2.10	0.07
14		0.09	0.011	0.74	0.65
15		0.63	0.027	0.53	0.83
16		1.03	0.062	1.56	0.18
17	nonacosane	15.80	0.639	0.71	0.68
18		0.04	0.014	0.29	0.96
19		0.53	0.050	1.27	0.29
20		0.02	0.013	3.56	>0.01*
21		3.24	0.204	1.20	0.33
22		3.26	0.192	0.80	0.60
23	eitriacontane	12.18	0.536	2.47	0.03*
24		0.34	0.032	1.01	0.45
25		11.02	0.470	2.11	0.06
26	tritriacontane	1.73	0.114	4.85	>0.01*

[a] N = 40 samples for each peak. F values are calculated based on 8 degrees of freedom in the denominator and 31 in the numerator, and a total sample size of 40 colonies. Probabilities <0.05 are indicated with an asterisk.

of the ANOVA. The number of significant ANOVAs indicates substantial differentiation among samples and supports the hypothesis of genetic differentiation in wax among families.

Correlation Analyses. Two types of correlation analyses were performed. The first is between compounds, i.e., does the variation in concentration of one compound have value in predicting the variation in other compounds or are the concentrations of the different compounds unrelated? Second, correlations were

calculated between colonies to determine if the familial similarity shown in the ANOVAs was reflected in higher correlations among colonies within families than among colonies between families. These correlations tested the level of association among colonies and are analyzed to determine if intrafamilial association is higher than interfamilial association.

Correlations among Compound Concentrations. Is the variation in any one compound associated with the variation in any other compound? The result of this analysis is a 25 × 25 half matrix. Ninety-seven of the 600 (ignoring autocorrelations) correlation coefficients were significant. The first four peaks had only five significant correlations, while the remaining peaks were more highly intercorrelated.

Important to the interpretation of correlations is a consideration of the percentage of variation explained by the correlations (r^2); the critical value for significance of the correlation coefficients in this analysis was 0.40, which explained only 15.7% of the variation. Relatively highly correlated (more than 50% of the variation explained) compounds are listed in Table 2. Compounds

TABLE 2. RELATIVELY HIGHLY CORRELATED ($r^2 > 50\%$) COMPOUND PAIRS[a]

Compound	Compound	% variation explained ($r^2 \times 100$)
9	6	57.5
9	7	70.1
10	7	60.5
11	7	52.6
11	10	61.5
13	7	56.0
13	10	79.4
13	11	68.1
16	15	56.4
17	15	85.4
17	16	64.0
20	13	64.6
21	16	67.7
21	17	52.3
22	15	57.3
22	16	70.6
22	17	69.2
22	21	80.6
23	17	54.9
25	17	56.7
25	22	51.4
26	23	61.3

[a]Pairs are presented with the compound having the higher retention time first.

with close retention times are highly associated in the data set (Spearman rank correlation between the retention times in Table 2, $r = 0.93$, $P < 0.0001$).

Intercolonial Correlations. Are related colonies similar in their wax hydrocarbon profiles? The second correlation analysis, between colonies, yielded much higher correlation coefficients. All but five of the correlations in this 40 × 40 half matrix were 0.90 or higher; coefficients this high explained 81% or more of the variation in the relationship. The five lower coefficients (between 0.80 and 0.89) involved the same colony and may indicate a pathology in that colony or a problem in the handling of that particular sample. An ANOVA between the within and between correlations showed no significant difference between the within-family correlations (mean = 0.974 ± 0.003, $N = 80$) and among-family correlations (mean = 0.973 ± 0.0009) ($F = 0.297$, $P = 0.58$, $N = 800$). This level of homogeneity precluded further analysis.

DISCUSSION

Intercolony variation in wax hydrocarbons appears to be adequate for these compounds to function as nestmate recognition cues. However, the statistical significance among family groups for any given compound is a little difficult to interpret in terms of functional significance in the recognition system. How large a concentration difference is necessary for perceptual differences to exist? Which of these compounds are actually used by bees in the recognitive context? And how do the bees make efficient use of the many possible recognition cues that are present in wax? Much additional work will be needed to elucidate the role of these compounds in nestmate recognition (Getz and Page, 1991).

Page et al. (1991) performed a similar analysis of surface hydrocarbons on bees that had not been exposed to comb as adults. They analyzed the 20 largest gas chromatographic peaks obtained from hexane extracts run through a methylsilicone column. These 20 peaks included 11 alkanes (C_{23} to C_{33}) and nine unidentified compounds—"probably . . . unsaturated and methylated derivative compounds" (Page et al., 1991). Smith (1990) identified cuticular hydrocarbons from European and African honeybees; he found that the smaller peaks associated with the alkanes were primarily alkenes, with the exception of two prominent C_{33} alkadienes. Based on his analysis and our mass spectrometry, the peaks immediately preceding each alkane are probably the corresponding alkenes, although we did not determine the location of the double bonds in these compounds. These identifications have some importance, as three of the seven significant differences among families involve these compounds. We have also analyzed polar compounds in the wax (Hibbard et al., unpublished); long-chain free fatty acids are a significant wax component (Blomquist et al., 1980).

Page et al. (1991) found that a significant proportion of the variation among bees in surface hydrocarbons was attributable to genetic factors. This led to the

conclusion that adequate information was available from surface hydrocarbons for kin recognition to take place. Page et al. (1991) analyzed correlation coefficients between colonies for cuticular hydrocarbons, as we have done here for wax hydrocarbons. Similar to our results, they found high correlation coefficients both within and between genetic groups (0.90 and higher). This high level of uniformity among samples for cuticular hydrocarbons and wax hydrocarbons may be due either to genetic canalization of the biosynthetic pathways or to the function that these compounds have in the biology of the honeybee. On the surface of an insect, large deviations from the norm might result in failure of cuticular waterproofing (Hadley, 1989); the physical properties of the comb may also be sensitive to major changes in composition.

In addition to similarity among individuals driven by waterproofing or structural functions of the hydrocarbons, similarity may also be affected by their use as signals. Under certain circumstances selection can favor loss of diversity in kin recognition cues and a consequent loss of signal information (Ratnieks, 1991). Conversely, random genetic variation could yield usable signals in the absence of selection for a signal function. Bees are capable of using a wide variety of compounds as recognition cues—alkanes, acids, esters, terpenoids—and they seem to exploit whatever cues are available.

Unlike the present findings, Page et al. (1991) found correlation coefficients for cuticular hydrocarbons were significantly higher within bee families than between families. Our correlation analysis indicates that sets of chemically similar compounds (e.g., an alkane and the alkenes of the same chain length) often have highly correlated concentrations in wax. This suggests that if these compounds are used as recognition cues, the information conveyed is redundant within these groupings. This is particularly true of the group of compounds from C_{29} to C_{33}.

The inability of the correlation analysis to yield predictive factors concerning familial relationship is interesting. However, it should be noted that nestmate recognition depends on intercolonial differences and optimally would function equally well, whether colonies are related or not.

Behavioral experiments (Breed et al., 1988a, 1995; Breed and Stiller, 1992) have clearly shown that whatever cues are present in the cuticular hydrocarbons are overridden by cues acquired from the comb in the nestmate recognition context. There is some evidence that the cuticular hydrocarbon recognition cues persist and are used in other behavioral contexts (e.g., Oldroyd et al., 1991). Given the close correspondence between comb wax and cuticular hydrocarbons, the issue is raised as to the salient differences (see Page et al., 1991, for a listing of the cuticular compounds). Breed et al. (1995) have shown that certain compounds, such as the C_{27} alkane, the C_{29} alkane, and tetradecanoic acid, are relatively easily transferred from comb to the bees, while others, such as the

C_{31} alkane, do not transfer in statistically significant amounts. This suggests compounds that might be tested for activity in recognition bioassays.

The chemistry of honeybee nestmate recognition is quite complicated, with a plethora of candidate compounds from diverse sources present in the bee's environment. The results of this paper support the suggestion that comb wax is a possible source of compounds for use as recognition cues and points to compounds that differ enough in concentration among colonies to make them worthwhile targets of further testing.

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PLATYPHYLLOSIDE: METABOLISM AND DIGESTIBILITY REDUCTION *IN VITRO*

KERSTIN SUNNERHEIM-SJÖBERG[1,*] and PER-GÖRAN KNUTSSON[2]

[1]*Department of Chemistry, Uppsala University*
Box 531, S-751 21 Uppsala, Sweden

[2]*Department of Animal Physiology*
Swedish University of Agricultural Sciences
Box 7045, S-750 07 Uppsala, Sweden

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Abstract—The metabolism of platyphylloside [(5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-O-β-D-glucopyranoside]—known to reduce digestibility—was studied *in vitro* in sheep rumen liquor. Platyphylloside is hydrolyzed to 5-hydroxy-3-platyphyllone [(5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone], which is reduced to centrolobol [1,7-bis-(4-hydroxyphenyl)-3-heptanol], via 3-platyphyllone [7-bis-(4-hydroxyphenyl)-3-heptanone]. The digestibility-reducing effect was shown to be correlated with the concentration of centrolobol.

Key Words—Phenols, platyphylloside, 5-hydroxy-3-platyphyllone, 3-platyphyllone, centrolobol, metabolites, digestibility, rumen liquor, birch.

INTRODUCTION

Birch—like other plants—produces a large number of secondary metabolites, some of which can be regarded as part of a chemical defense against foraging animals. Defense compounds can have pheromonal properties, and they can be toxic, unpalatable, or digestibility reducing (Harborne, 1988, and references cited therein). Hares fed solely on birch twigs lose weight and have a negative sodium balance (Pehrson, 1983). Caged rabbits fed on fine birch twigs or a commercial diet sprayed with a phenolic birch extract showed increased sodium losses in the urine, and the *in vitro* digestibility of hay in rumen liquor from birch-fed goats was severely reduced as compared to hay-fed controls (Palo,

*To whom correspondence should be addressed.

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1987). In a previous study (Sunnerheim-Sjöberg *et al.*, 1988), it was shown that the phenol platyphylloside [(5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-O-β-D-glucopyranoside] isolated from birch twigs (*Betula pendula* Roth.) could explain about 80% of the observed depression of *in vitro* organic matter digestibility (IVOMD). Platyphylloside also reduces palatability and digestibility *in vivo* (Palo, personal communication).

Preliminary studies have shown that platyphylloside (I) is hydrolyzed to 5-hydroxy-3-platyphyllone (II) in rumen liquor and that platyphyllane [1,7-bis-(4-hydroxyphenyl)-heptane] is excreted with feces from moose and goat fed on birch (Palo, 1987; Palo *et al.*, 1989; Sunnerheim-Sjöberg, 1991). Platyphyllane was also detected after enzymatic hydrolysis of urine from rabbits and hares fed on birch (Palo, 1987). It was not shown, however, how II is further metabolized or if platyphyllane actually is a metabolite from I, and if so, whether it is formed in the rumen or later in the digestive system.

Platyphylloside (I) and 5-hydroxy-3-platyphyllone (II) were first isolated from *Betula platyphylla* by Terasawa et al. (1973). They named II platyphyllonol. Sasaya and Izumiyama (1974) and Nomura et al. (1981) isolated II from *Alnus hirsuta* Turcz. and *Alnus japonica*, respectively, and called it hannokinin.

The aims of the present study were to: (1) study the metabolism and identify the metabolites of platyphylloside *in vitro* in rumen liquor, and (2) identify the compound/compounds—platyphylloside or any of its metabolites—correlated to IVOMD inhibition in rumen liquor.

METHODS AND MATERIALS

Digestibility Experiment

IVOMD was determined according to the method originally described by den Braaver and Eriksson (1967) and modified by Palo (1985). Rumen liquor was sampled from a canulated sheep fed on hay (800 g/day plus mineral supplement).

Sample Preparation. Three types of samples were prepared: (1) with hay and with I: to each of 18 tubes containing 500 mg milled hay, 2.0 ml of a solution of I in 96% aqueous ethanol (12.00 g/liter) was added; (2) with hay but without I (control samples): to each of 18 tubes containing 500 mg milled hay, 2.0 ml of 96% aqueous ethanol was added; (3) without hay but with I: to each of 5 tubes, 2.0 ml of a solution of I in 96% aqueous ethanol (12.00 g/liter) was added.

The solvent was allowed to evaporate at 40°C for 12 hr. To each of the 41 tubes, 50 ml buffer (pH 6.8 ± 0.1) (den Braaver and Eriksson, 1967) and 1.0 ml rumen liquor were added. Tubes were incubated at 37°C.

After incubation (6, 12, 24, 48, 72, 96 hr), hay residues were removed

from the liquid by suction filtration. The hay residue was washed twice with 25 ml acetone. The combined solutions were evaporated to dryness, and resuspended in 10.0 ml 48% aqueous ethanol and stored at −20°C for one to four days, filtered through cotton, and analyzed with high-performance liquid chromatography (HPLC).

Chromatography

Thin-layer chromatography (TLC) was performed on Merck HF-254 silica gel plates. Column chromatography was performed on silica gel (Riedel-de-Haën, 0.032–0.063 mm). The following eluents were used for TLC: (I) chloroform–methanol–water, 240 : 15 : 1; (II) toluene–acetonitrile, 2 : 1; (III) ligroin–ethyl acetate–acetic acid, 4 : 2 : 1. The TLC-plates were inspected under UV light at 254 nm. Spray reagents used were: (i) 4-diazobenzene-sulfonic acid (Fluka AG, CH 9470) dissolved in 10% aq. sodium carbonate, followed by 50% sulfuric acid (v/v) (for phenols) and (ii) dinitrophenylhydrazine reagent (for carbonyl groups).

HPLC was performed on a Merck-Hitachi chromatograph equipped with a UV detector. Detection was made at 280 nm. The column was a LichroCart 125-4 Lichrospher 100 RP-18 (5 μm) (Merck). The mobile phase consisted of solvent A: phosphate buffer (0.01 M, pH 2.8) and solvent B: acetonitrile. The gradient program was 1–3 min: 5% B, 3–50 min: linear gradient from 5 to 50% B. The flow rate was 1.5 ml/min; 10 μl/sample was injected. Peak retention times and areas were monitored and integrated automatically.

Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 300 MHz on a Varian XL-300 and/or at 400 MHz on a Varian VXR-400 spectrometer. Chemical shifts (δ) are given relative to solvent peak as internal reference. Mass spectra were recorded at 70 eV with a Finnigan 4000 spectrometer using a direct insertion probe. Optical rotation was recorded at 22°C with a Perkin-Elmer 241 polarimeter.

Statistical Methods

For multivariate analysis, partial least square regression (PLSR), Unscrambler 4.0, CAMO AA/S, Norway, was used.

Isolated Substances

Platyphylloside (I), [(5S)-5-hydroxy-1,7-bis-(4-hydroxyphenol)-3-heptanone-5-O-β-D-glucopyranoside]. This was isolated from the inner bark of *Betula pendula* according to Smite et al. (1993) and was identical with previously isolated platyphylloside (Sunnerheim-Sjöberg et al., 1988). ^{1}H NMR:

(CD_3OD)δ: 1.75(m, 1H, 6-H), 1.83 (m, 1H, 6-H), 2.59 (m, 3H) and 2.75–2.84 (m, 5H), (1, 2, 4, and 7-H), 4.17 (m, 1H, 5-H), 6.67 (two d, 4H, J = 8.5 Hz, Ar-H), 6.99 (d, 2H, J = 8.5 Hz, Ar-H), 7.01 (d, 2H, J = 8.6 Hz, Ar-H), glucose moiety: 3.14 (1H, 2-H), 3.25 (1H), 3.70 (dd, 1H, J = 12 and 5 Hz), 3.86 (dd, 1H, J = 12 and 2 Hz), 4.29 (d, 1H, J = 8 Hz, H-1'), one H hidden under the solvent peak. ^{13}C NMR: (CD_3OD) δ: 29.8, 31.4, 38.5, 46.4, 62.7, 71.6, 75.2, 76.2, 77.8, 78.0, 103.5, 116.0, 116.2, 130.3, 130.4, 133.2, 134.3, 156.3, 156.6, 211.9.

5-Hydroxy-3-platyphyllone (II), [(5S)-5-Hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone]. This was obtained from enzymatic hydrolysis of platyphylloside and was identical with that previously isolated (Sunnerheim-Sjöberg et al., 1988). TLC; eluent I: R_f = 0.30, eluent II: 0.38; eluent III color with reagent (i): a red center with blue–green contour. Color with reagent (ii): yellow. ^1H NMR (CD_3OD) δ: 1.65 (m, 2H, 6-H), 2.48–2.66 (m, 4H, 4-H and 7-H), 2.73 (m, 4H, 1-H and 2-H), 4.00 (m, 1H, 5-H), 6.67 (d, 2H, J = 8.5 Hz, 3'-, 5'-H), 6.68 (d, 2H, J = 8.4 Hz, 3"-, 5"-H), 6.98 (d, 4H, J = 8.4 Hz, 2'-, 6'-, 2"-, 6"-, 2"-H, and 6"-H). ^{13}C NMR (CD_3OD) δ: 29.8 (I-C), 31.9 (7-C), 40.5 (6-C), 46.4 (2-C), 51.3 (4-C), 68.3 (5-C), 116.1 and 116.2, (3'-, 5'-, 3"-, and 5"-C), 130.2, 130.3 and 130.4 (2'-, 6'-, 2"-, and 6"-C), 133.3 (1'-C), 134.1 (1"-C), 156.4 (4'-C), 156.6 (4"-C), 211.9 (3-C).

3-Platyphyllone [1,7-Bis-(4-hydroxyphenyl)-3-heptanone] (III) and Centrolobol [1,7-bis-(4-hydroxy-phenyl)-3-heptanol] (IV). These were isolated from rumen liquor samples that had been incubated for 48 hr. The rumen liquor was concentrated by evaporation below 40°C, and the two compounds were obtained by flash chromatography on silica gel; two columns: length = 18 cm, ID = 5 cm, eluent I, followed by one column; length = 29 cm, ID = 1.5 cm, eluent II. The progress of the separation was followed by TLC with eluents I and II.

Compound III was further purified by flash chromatography on silica gel; length = 29 cm, ID = 1.5 cm, eluent II. Ten milligrams with small amounts of impurities was obtained. TLC: Eluent I R_f = 0.57, eluent II R_f = 0.68. Color with reagent (i): a red center with blue–green contour. Color with reagent (ii): yellow. ^1H NMR ($CDCl_3$) δ: 1.55 (m, 4H, 5- and 6-H), 2.38 (t, 2H, J = 6.8 Hz), 2.51 (t, 2H, J = 6.9–7.3 Hz), 2.67 (t, 2H, J = 7.5 Hz) and 2.81 (t, 2H, J = 7.3–5 Hz) (1-, 2-, 4-, and 7-H), 6.73 and 6.74 (each: d, 2H, J = 8.4 Hz, Ar-m-H), 6.99 and 7.03 (each: d, 2H, J = 8.4 Hz, Ar-o-H). ^1H NMR (CD_3OD) δ: 1.49 (m, 4H, 5- and 6-H), 2.39 (t, 2H), 2.46 (t, 2H), 2.66 (m, 2H) and 2.73 (m, 2H) (1-, 2-, 4-, and 7-H), 6.67 (two d, 4H, Ar-m-H), 6.94 and 6.97 (each: d, 4H, Ar-o-H). MS data were in accordance with those published by Nagai et al. (1990).

Compound IV was further purified by flash chromatography on silica gel; column 1: length = 28 cm, ID = 1.5 cm, eluent II, column 2: length = 26 cm, ID = 1.5 cm, eluent I. TLC: eluent I R_f = 0.26, eluent II R_f = 0.56. By

this means, 12 mg of IV containing small amounts of impurities was obtained. Color with reagent (i): blue–green contour with an orange center. No reaction with reagent (ii). ^{1}H NMR (CD_3OD) δ: 1.33 (m, 1H, 5-H), 1.45 (m, 3H, m, 4-, 5-H), 1.55 (m, 2H, 6-H), 1.64 (m, 2H, 2-H), 2.49 (m, 3H, 1-, 7-H), 2.64 (m, 1H, 1-H), 3.50 (m, 1H, 3-H), 6.67 (d, 2H, J = 8.80 Hz, Ar-m-H), 6.68 (d, 2H, J = 8.40 Hz, Ar-m-H), 6.96 (d, 2H, J = 8.40 Hz, Ar-o-H), 6.98 (d, 2H, J = 8.80 Hz, Ar-o-H). MS data were in accordance with those published by Craveiro et al. (1970). The isolated centrolobol had no optical activity when dissolved in methanol.

RESULTS AND DISCUSSION

Metabolism of Platyphylloside

Samples with Hay. When exposed to rumen liquor and hay *in vitro*, platyphylloside could not be detected after the initial 6 hr. The concentration of the hydrolysis product, 5-hydroxy-3-platyphyllone, was at its maximum after 12 hr of incubation. 3-Platyphyllone started to form between 6 and 12 hr and reached a maximum after 24 hr. Centrolobol emerged between 12 and 24 hr and increased throughout the experiment. Centrolobol is the final degradation product *in vitro* (Figure 1).

The analysis of the time dependency of the concentrations of the four compounds involved suggests the following main metabolic pathway of platyphylloside *in vitro* in rumen liquor: Platyphylloside → 5-hydroxy-3-platyphyllone → 3-platyphyllone → centrolobol (Figure 2.)

3-Platyphyllone (III) and centrolobol (IV) have previously been isolated from plant material as glycosides. Nagai et al. (1986, 1990) isolated two glycosides of III and one glycoside of IV from *Acer* and obtained the aglycone III by acid hydrolysis. They named III "acerogenin G." Craveiro et al. (1970) found the two enantiomers of the aglycone IV from two *Centrolobium* species. Smite et al. (1993) isolated a trisaccharide of IV from *Betula pendula*. The indicated configurations are solely based upon comparison of optical rotation

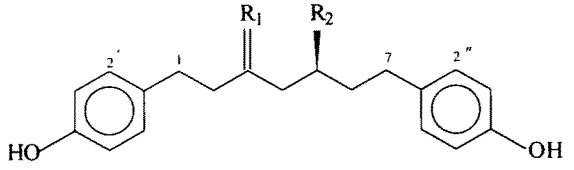

FIG. 1. The structure of compounds I, II, III, and IV. I: R_1 = O, R_2 = O-β-D-glucopyranoside; II: R_1 = O, R_2 = OH; III: R_1 = O, R_2 = H; IV: R_1 = H, OH, R_2 = H

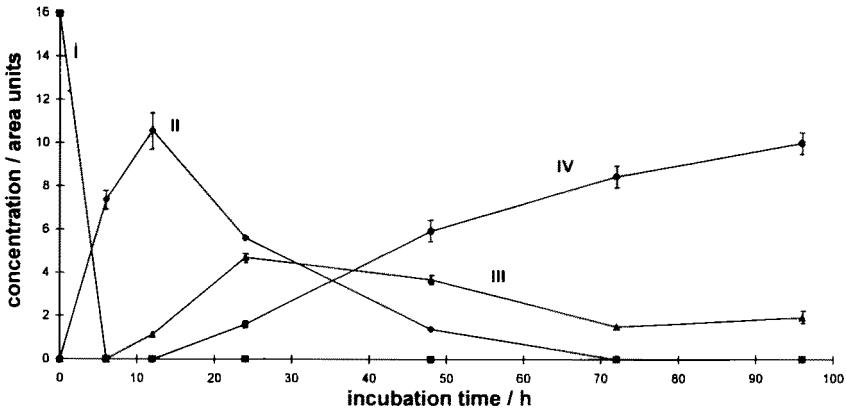

FIG. 2. Changes in relative concentrations with incubation time. I incubated with rumen liquor plus hay. I = platyphylloside, II = 5-hydroxy-3-platyphyllone, III = 3-platyphyllone, IV = centrolobol. Mean (±SD) of three replicates. (Some of the standard deviations are so small that they are not observable on this scale). *Note:* Response factors are not identical for the different compounds.

and ^{13}C NMR data with results of Ohta et al. (1985). Assignment of the absolute configuration of I was based on the application of the glycosidation shift rule of Seo et al. (1978). There has been some debate about the absolute configuration of IV (Nagai et al., 1986).

Samples without Hay. The hydrolysis of I in the samples without hay was as rapid as in the samples with substrate, but the subsequent reactions were slower. After 72 hr, considerable amounts of II remained, three times higher than the concentration of III. No IV was formed without substrate. Probably the microflora became exhausted with time due to lack of the hay substrate, and the reactions were thus slowed down.

Unidentified Metabolite. There was another unidentified metabolite, both in the samples with and without hay. The compound had a relatively high UV absorbance, but ^{1}H NMR showed that the concentration was very low. It reached a maximum after 6 hr and was not detectable after 24 hr. During that time, digestibility was not affected (see below). For this reason one can assume that this unidentified compound is not the active one.

Platyphyllane. Platyphyllane, 1,7-bis-(4-hydroxyphenyl)-heptane, found in feces from moose and goat fed on birch (Palo 1987; Palo et al., 1989) could not be detected in any of the samples in this *in vitro* experiment, which might indicate that a reduction of centrolobol to platyphyllane takes place after passage through the rumen.

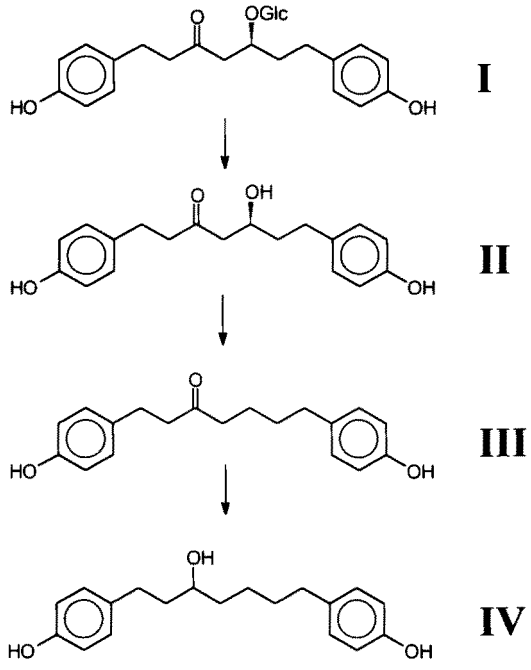

FIG. 3. Metabolism of platyphylloside *in vitro* in rumen liquor.

Digestibility Inhibition

IVOMD. During the initial 12 hr, no digestion difference between samples and controls was observed; in both, 67% of the organic matter was left. From 12 to 72 hr, however, there was a notable difference; in the control tubes organic matter decreased to 30%, while in the samples with platyphylloside 58% of the organic matter was left (Figure 3). The average digestion rate was 0.13%/hr in the platyphylloside-containing samples compared to 0.62%/hr in the control tubes. At the end of the experiment, the enzymatic activity might have decreased, leading to some uncertainty in the IVOMD values at 96 hr.

During the first 12 hr, when no digestibility difference between samples and controls was observed, no centrolobol could be detected. As centrolobol emerged, the rate of digestion decreased. As centrolobol concentration increased, the effect on digestion was more pronounced (Figures 1 and 3). The concentration of centrolobol was well correlated with digestibility reduction (Figure 4), measured as the IVOMD difference between samples and controls. It seems reasonable to conclude, but has yet to be proved, that centrolobol is the com-

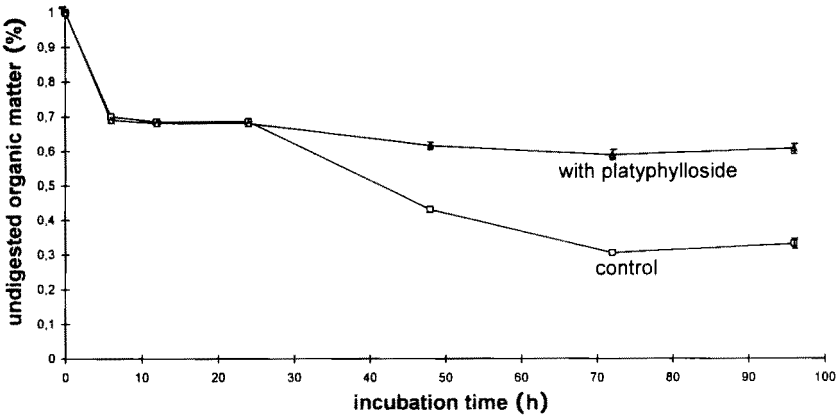

FIG. 4. Change in in vitro organic matter digestibility with incubation time. Mean (±SD) of three replicates. (Some of the standard deviations are so small that they are not observable on this scale).

pound responsible for the decreased digestibility. The slight deviation from a linear relation might be explained by the decreased activity of the microflora with time.

Multivariate Analysis. A partial least squares regression (PLSR) model confirms the correlation between centrolobol and digestibility reduction. Con-

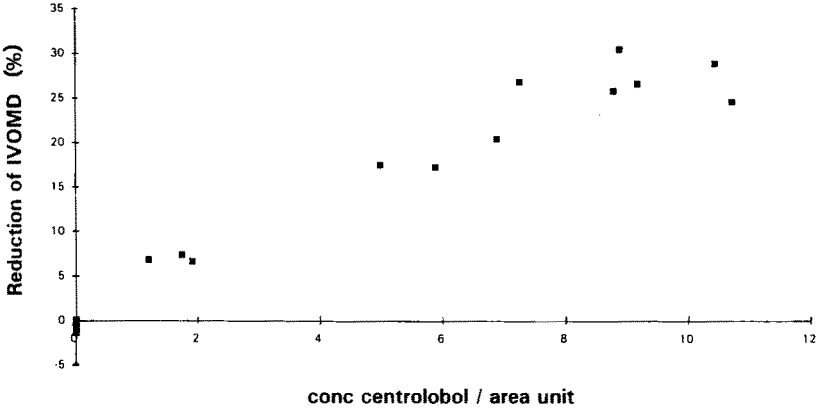

FIG. 5. Relation between concentration of centrolobol and reduction of IVOMD (differences between digestibility with and without platyphylloside).

centration of centrolobol had the highest positive loading, i.e., showed a strong correlation with IVOMD reduction, while 3-platyphyllone had a weak correlation and the other two compounds had small negative loadings, i.e., were weakly negatively correlated to IVOMD reduction. PLSR shows relations between the dependent variable (here IVOMD reduction) and all independent variables (here concentrations of all metabolites) simultaneously. [For a short presentation of multivariate analysis, see Sunnerheim-Sjöberg and Hämäläinen (1992) and references cited therein.]

Stereochemistry. Centrolobol isolated from the rumen liquor samples showed no measurable optical rotation, which was unexpected since most digestion reactions are enzymatic reactions. However, the sample was not absolutely pure, and the impurities might accidentally have the opposite optical rotation of the same magnitude. Further studies are in progress.

CONCLUSIONS

Platyphylloside is rapidly hydrolyzed to 5-hydroxy-3-platyphyllone *in vitro* in sheep rumen liquor, followed by reduction to 3-platyphyllone and finally to centrolobol. The reduction of digestion *in vitro* is correlated with the concentration of centrolobol in sheep rumen liquor.

Acknowledgments—This investigation was supported by a grant from The Swedish Research Council of Forestry and Agriculture, which is gratefully acknowledged. We thank Mrs. E. Fuchs for skillful technical assistance and Dr. E. Smite and Dr. L. Lundgren for providing a sample of platyphylloside-containing extract.

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RESPONSES OF BEAVER (*Castor canadensis* KUHL) TO PREDATOR CHEMICALS

AXEL ENGELHART[1] and D. MÜLLER-SCHWARZE*

SUNY College of Environmental Science and Forestry
Syracuse, New York 13210

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Abstract—Free-ranging beaver (*Castor canadensis*) in two different beaver populations in New York State were exposed to predator chemicals to test feeding inhibition. Solvent extracts of feces were applied to stem sections of aspen, the preferred food tree of beavers, permitting smelling and tasting the samples. Predator odors were from wolf (*Canis lupus*), coyote (*Canis latrans*), dog (*Canis familiaris*), black bear (*Ursus americanus*), river otter (*Lutra canadensis*), lynx (*Lynx canadensis*), and African lion (*Panthera leo*). The experiment was repeated. The predator odors reduced feeding compared to untreated or solvent-treated controls. One population consumed 17.0% of the samples with predator odor and 27.0% of the controls in summer, and 48.4% and 60.0%, respectively, in autumn. The other population accepted 3.15% of the predator odor samples and 11.05% of the controls in summer. Coyote, lynx, and river otter odors had the strongest effects. Diesel oil and bitter-tasting neem extract had weaker effects. Predator odors are promising as feeding repellents for beaver.

Key Words—Adirondacks, Allegany State Park, allopatric, aspen, beaver, *Castor canadensis*, feeding inhibition, *Populus tremuloides*, predator odor, repellent, sympatric.

INTRODUCTION

Populations of the North American beaver (*Castor canadensis* Kuhl, 1820) are currently increasing because low pelt prices discourage fur trapping. The growing beaver populations cause considerable wildlife damage by flooding roads, forests, and fields and by cutting down valuable trees. This intensifies the need for effective beaver management. Among other means, environmentally com-

*To whom correspondence should be addressed.
[1]Current address: Schulstr. 14, 71296 Heimsheim, Germany.

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patible biological repellents are needed to keep beavers from sensitive areas or single trees. An obvious candidate for a natural repellent would be a potent predator odor (Müller-Schwarze, 1990).

Predator "sign," i.e., droppings, urine, scent marks, scrapes on the ground, scratches on objects, or rubbing sites, inform potential prey species about the presence, recent activity, and possibly diet of predators in an area. While avoiding predators, the prey species still has to operate in the same area. The use of predator odors as repellents is predicated on the assumptions that: (1) the prey species is merely alerted by the odor and avoids the particular spot or area in expectation of encountering a predator, and/or (2) the predator odor itself is aversive. In an application, the first case requires occasional reinforcement by an actual encounter with a predator. The second situation suggests a more lasting repellent effect, which also could be more general, across predator species. Sulfur-containing compounds, metabolites derived from protein, have been postulated to be the aversive agent common to many predator odors (Mason, et al., 1994; Nolte et al., 1994). Trimethyl thiazoline from feces of red fox (*Vulpes vulpes*) is aversive to rats (Vernét-Maury, 1992). Even plants rich in sulfur compounds, such as onion or leek, are avoided by herbivores such as black-tailed deer, *Odocoileus hemionus columbianus*, or goats (personal observations).

Previous predator odor studies on vertebrates have ranged from fish (Mathis and Smith, 1993) to mammalian herbivores such as black-tailed deer (Müller-Schwarze, 1972; Sullivan *et al.*, 1985b), white-tailed deer (Swihart *et al.*, 1991), red deer (Abbott *et al.*, 1990), and sheep (Arnould, 1992, Arnould and Signoret, 1993). Weldon (1990) has summarized the known responses of vertebrates to predator odors. Particularly relevant here are studies in lagomorphs (Sullivan and Crump, 1984; 1986; Sullivan *et al.*, 1985a; Boag and Mlotkiewicz, 1991, 1994), and rodents (Bäumler *et al.*, 1990; Sullivan *et al.*, 1988a,b, 1990a,b; Vernét-Maury, 1992).

The primary mammalian predators of North American beavers are the wolf (*Canis lupus*) and coyote (*C. latrans*), particularly during spring and summer (Theberge et al., 1978; Potvin *et al.*, 1988; Fuller, 1989) and when preferred prey such as white-tailed deer (*Odocoileus virginianus*) are less abundant (Pimlott *et al.*, 1969).

It is thought that the decline of the beaver population on Isle Royale in Lake Superior around 1980 was caused by increased wolf predation (Shelton and Peterson, 1983). In northern Manitoba, wolves have been observed to stalk beaver (Nash, 1951). In several studies various amounts of beaver remains were found in wolf scats. On Isle Royale in Lake Superior, Mech (1966) reported that about 11% of wolf scats contained beaver hair. This compares with 7% in the Rocky Mountain national parks of Canada (Cowan, 1947) and 10.5% in Ontario (Peterson, 1955). In Algonquin Provincial Park, Ontario, up to 62.8% of wolf scats contained beaver hair in summer, but only 11.5% in winter (The-

berge *et al.*, 1978). In Minnesota, however, 20–47% of wolf scats contained beaver remains in April and May, but only 2% in June and July (Fuller, 1989). In southern Québec, beaver constituted 29–44% of biomass in the diet of wolves from May through November but only 1–3% during the remainder of the year (Potvin et al., 1988). Indirect evidence for wolf predation on beaver comes from experimental wolf reduction in Québec, which resulted in an increase in beaver numbers and a decrease after the wolf population was allowed to grow again (Potvin *et al.*, 1992).

Coyotes have been observed to hunt and kill beaver in Rocky Mountain National Park, Colorado (Packard, 1940). In the Adirondacks, New York, coyotes also prey on beaver (Brundage and Brocke, personal communication).

At Allegany State Park, New York, our group observed black bears (*Ursus americanus*) taking beaver out of live-traps on at least two occasions (Houlihan, unpublished; Schulte, 1993). Observations of black bears trying to dig into beaver colonies were "fairly common" in northern Saskatchewan (Gunson, 1970). Substantial predation by black bears has only been reported from an island in Lake Superior (Smith et al., 1994). For grizzly bears (*Ursus arctos*) in Denali National Park, Alaska, Murie (1981) had "no observations suggesting bear predation on beavers." They may serve only as "occasional taste of carrion for the bears."

Fox (*Vulpes vulpes*), bobcat (*Lynx rufus*), and lynx (*Lynx canadensis*) prey little on beaver (Saunders, 1988). In a study in Maine, less than 5% of bobcat carcasses contained remains of beaver (Litvaitis *et al.*, 1986). Despite claims in the past, the river otter (*Lutra canadensis*) is not a predator of beaver. Beaver are rare in the diet of otter. In a northeast Alberta area with high beaver and otter densities, Reid (1984) found only five otter scats among 1140 (0.4%) that contained beaver remains. Otter may have fed on beaver carcasses. No beaver remains were found in seven other studies of otter droppings, cited by Reid (1984).

The exclusively herbivorous beaver most prefers bark and leaves of aspen (*Populus tremuloides* and *P. deltoides*) among tree species. In our study areas they also feed on beech (*Fagus grandifolia*), speckled alder (*Alnus rugosa*), red maple (*Acer rubrum*), yellow birch (*Betula alleghaniensis*), American hornbeam (*Carpinus caroliniana*), and other deciduous tree species. Conifers are rarely taken. Beaver also feed on grass, apples, ferns, and aquatic vegetation, depending on the season.

Beavers are monogamous animals that live in families (for a review see Chapman and Feldhamer, 1983). A typical family consists of an adult male and female and their offspring of the current and the past year. A family at a particular site created by damming up a stream is termed a "colony." For this study, a colony of beaver was the unit of experimental subjects.

In this study we seek answers to the following questions: Do predator odors

on or near food inhibit feeding by beaver? Are odors from certain predators more potent inhibitors than others? If so, are odors of predators sympatric with beaver more active than those from allopatric species?

METHODS AND MATERIALS

The study areas were Allegany State Park (ASP) in southwestern New York State and the Huntington Wildlife Forest (HWF) in the Adirondack Park in northern New York. The natural vegetation in both study areas is northeastern hardwood, with differences in the composition of the forest.

All odor samples except lion odor were prepared from excrement. The lion odor sample was prepared from a commercial lion urine extract (Imperial Chemical Industries PLC, Brachnell, England). All feces, except dog (a pet female black Labrador), were collected at the Burnet Park Zoo, Syracuse. For all samples, 1 part feces (by weight) was suspended in 2 parts pure methanol for 2 hr and then filtered with a vacuum filter. The filtrate was used for the experiment, the solid phase discarded. The odor samples were stored in glass jars with alcohol-proof plastic lids in a refrigerator.

The aspen sticks used as carriers for the odors were cut from aspen saplings in the study area. Stems with a diameter of 1–2 cm and with as few lateral twigs as possible were selected. The sticks were cut fresh on the first day of each trial, pruned, and cut into sections 30 cm long. To facilitate uptake of the methanol solutions, the bark of all sticks, except the "intact" control, was perforated by rolling them on a nailboard. The sticks were dried at room temperature for 2 hr and then scented by dipping them to two thirds of their length into the extracts for 2 sec (except those left blank). In addition to intact and perforated blanks, some sticks were soaked in bitter-tasting extract of seeds (main component azadirachtin) of the neem tree, *Azadirachta indica*, which inhibits feeding by small rodents (Bäumler *et al.*, 1990). Some sticks were soaked in diesel oil, also a repellent for small rodents (Bäumler *et al.*, 1990), and others were soaked in pure methanol as a solvent control. The sticks were dried for 2 hr before further handling.

For each trial we used 10 randomly chosen colonies. Twelve sticks, arranged in a row, were placed at the bank of each pond parallel to the water's edge near feeding places that were frequently visited by beavers. One stick each carried one of the seven predator odors, one neem extract, and diesel oil. The remaining three sticks served as controls: one was perforated and methanol-soaked, one perforated and untreated (blank), and one unperforated and untreated (intact). The distance between the sticks was 30 cm for the trials in June and July in ASP and in HWF. For the trials in October and November in ASP, the distance was increased to 1 m to determine whether distance affects the ability of the

beavers to distinguish the odors. The sticks were placed in a random order, determined by lot and the same pattern used for all 10 colonies. Each trial was five nights long, i.e., five beaver activity periods, without replacing any missing or altered sticks. This time period was long enough to assure the beaver had encountered the samples and short enough to minimize random influences such as heavy rains. First, a baseline trial with 12 intact, i.e., unperforated, untreated aspen sticks was run for three nights at 10 ASP colonies. At HWF, two such baseline trials were run (total number of sticks for baseline trials in both areas: 360). For treated samples, a total number of 2108 sticks were used. Their distribution over summer and fall, and ASP and HWF, the predator odors used, and the numbers of replications and colonies are listed in Table 1.

The results of the nocturnal beaver activity were recorded each morning. Sticks were classified into completely peeled, partially peeled, left intact and moved, and left intact and in place. Missing sticks (swept away or sunken) were regarded as consumed by beaver (aspen wood does not float on water for a long time). Beaver sample sticks and often discard partially peeled sticks. Sticks that were peeled less than 80% were regarded as rejected, as were sticks that were not taken after five activity periods.

TABLE 1. EXPERIMENTAL DESIGN: NUMBERS OF SAMPLES (NUMBERS OF TRIALS × NUMBERS OF COLONIES) USED AT TWO BEAVER POPULATIONS AND DURING DIFFERENT SEASONS

	Population and Season			
Treatment	ASP summer (5 × 10)	HWF summer (5 × 10 + 1 × 4)	ASP fall (8 × 10)	Total
River otter		54	80	134
Lynx	50	54	80	184
Coyote	50	54	80	184
Wolf	50	54	80	184
Dog	50	54	80	184
Black bear	50	54	80	184
Lion	50	54	80	184
Diesel oil	50	54	80	184
Neem	50	54	80	184
Methanol control	50	54	80	184
Intact		54	80	134
Blank	50	54	80	184
Number of treatments	10	12	12	
Total number of sticks	500	648	960	2108

As statistical tests of significance in the differences of numbers eaten vs. not eaten, we used the non-parametric Cochran and McNemar tests. They retain the pairwise comparison of simultaneous samples. χ^2 tests were also used.

RESULTS

Allegany State Park (ASP). In a three-nights-long baseline test at the beginning of the trials, 74 of 120 (i.e., 12 sticks at each of 10 ponds) or 61.7% of intact aspen sticks were taken. For the trials run in June and July (sample size: 50 for each of 10 treatments) the percentages of experimental sticks accepted by the beaver after one to five activity periods are given in Figure 1. Acceptance ranged from 10.0% for coyote odor to 32.0% for the perforated aspen control ($\chi^2 = 7.30$; $P < 0.01$). (Intact, i.e., untreated, unperforated aspen logs, and

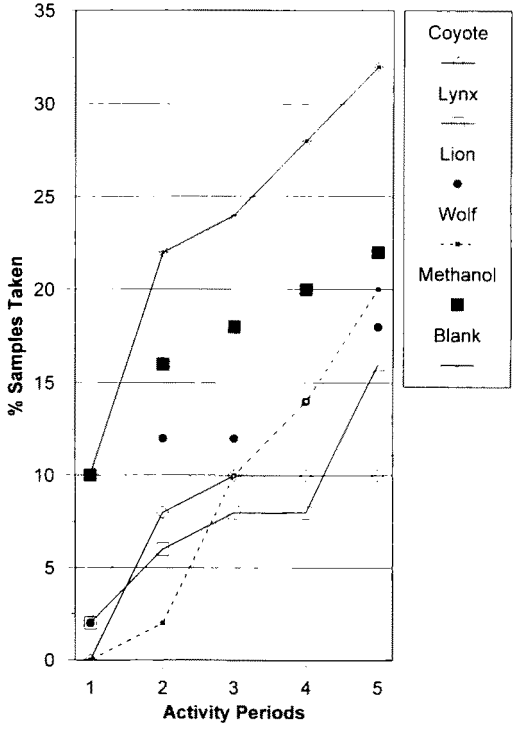

FIG. 1. Responses by beaver (% samples taken) at Allegany State Park in June/July 1993.

river otter odor had not been used at that time.) After coyote odor, the most active predator odors were lynx and black bear (16% of each accepted; χ^2 = 0.236 vs. control; NS; black bear data not shown in Figure 1). Thus, coyote odor inhibited feeding significantly, compared with untreated controls.

In the October/November trials, significantly more sticks were consumed (Figure 2). This was a time of provisioning for the winter and reinforcing lodge and dams. A total of 690 sticks were used. When only intact aspen sticks, 1 m apart, were offered, 48.7% were taken after three days. The most avoided odor was river otter (28% acceptance after three days; χ^2 = 10.98; $P < 0.001$; 42.5% after five days), and the most frequently taken samples were the perforated and methanol-treated aspen controls (63.7% each; difference between otter and each control: χ^2 = 7.26; $P < 0.01$). The wider spacing of 1 m between the sticks had no effect on the preference for the controls. The ratio between the numbers of predator odor samples and controls that were consumed was

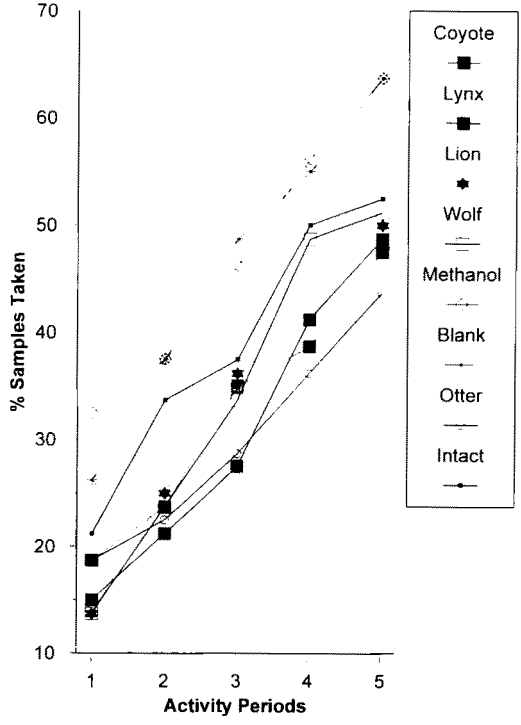

FIG. 2. Responses by beaver at Allegany State Park in October/November 1993.

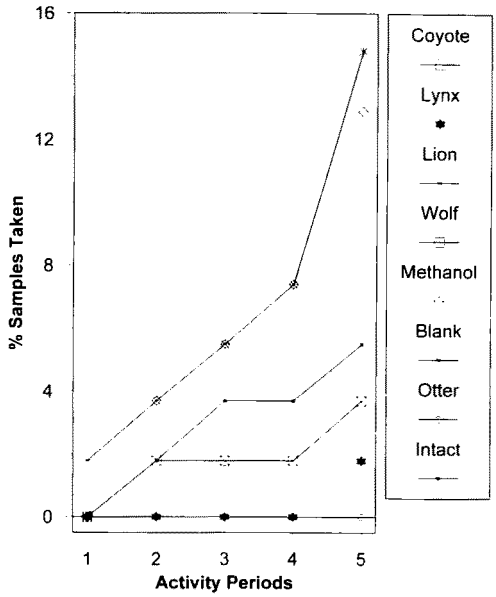

FIG. 3. Responses by beaver at Huntington Wildlife Forest in August/September 1993.

0.81 in October/November. For an interstick distance of 30 cm, this relation was 0.49 for ASP in June/July and 0.30 for HWF in August/September (difference not significant).

Huntington Wildlife Forest (HWF). At HWF (experiments during August/September) the beaver consumed fewer sticks than at ASP. The acceptance rate ranged from 0 for river otter odor to 14.8% for the perforated aspen control (Figure 3; $\chi^2 = 8.64$; $P < 0.025$). This compares with 32.5% and 34.2% in two trials with only untreated perforated aspen sticks as baseline test (difference to otter: $P < 0.001$).

The ratio between the percentages of consumed predator-treated and control sticks at HWF was 0.30. Because the data were collected during the summer, the experiment was influenced by the fact that the beaver at HWF could choose from a great variety of available food, notably large beds of floating watershield (*Brassenia* sp.) leaves.

Both Study Areas. Figure 4 summarizes the responses in all trials in both areas. The average acceptance rate of five predator odors (those shown in the graphs) changed from 8.3% on the first day to 26.6% on the fifth, while the average for the three controls was 14.7% on day 1 and 38.5% on day 5. The average acceptance of five predator odors as percent of the average of the three

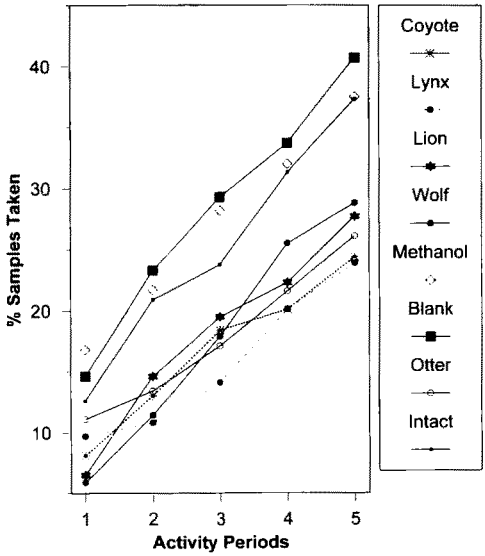

FIG. 4. Summary of all responses in both study areas. (No data for river otter and intact controls for June/July).

controls increased slightly from 56.5% on day 1 to 68.1% on day 5. The most avoided predator odor (different odors in different blocks of trials) as percent of the average for the three controls increased gradually from 40.1% on the first day to 67.7% on the fifth. Table 1 summarizes the experimental design for all replicates in both areas, and Table 2 summarizes the results for all replicates in both areas.

The significance levels of the differences in the responses of the beavers to the different samples after five activity periods for all trials are shown in Table 3. The greatest statistical differences ($P < 0.001$) between predator odors and controls were as follows: all seven predator odors vs. perforated, untreated sticks; coyote, dog, otter, lynx, and lion vs. neem; black bear, coyote, dog, and otter vs. methyl alcohol; and coyote, otter, and lynx vs. diesel oil. Also significant ($P < 0.01$) were: wolf, lynx, otter, and lion vs. methyl alcohol; bear and wolf vs. neem; and lion vs. diesel oil. Among the controls, only blanks (unperforated, untreated) were taken significantly less often than untreated, but perforated sticks ($P < 0.01$).

Generally, there were no significant differences between different predator odors. The exception is the odor of otter, which was slightly, but consistently, more potent ($P < 0.1$) than all other predator odors except lynx. Although river

TABLE 2. SUMMARY OF ALL RESPONSES IN BOTH AREAS (Cumulative % Eaten)

	Night				
Odors	1	2	3	4	5
Lynx	9.7	10.8	14.1	20.1	23.9
Coyote	8.1	13.0	18.4	20.1	24.4
Lion	8.1	14.1	17.9	19.0	25.5
River otter	11.1	13.4	17.1	21.6	26.1
Black bear	10.3	14.6	18.4	23.3	26.6
Dog	7.6	12.5	16.8	23.6	27.7
Wolf	5.9	11.4	17.9	25.5	28.8
Diesel oil	14.4	20.6	26.0	28.8	33.6
Neem	11.4	18.4	25.5	29.3	35.3
Methanol control	16.8	26.0	30.9	31.7	35.8
Intact	12.6	20.9	23.8	31.3	37.3
Blank	14.6	21.1	29.3	33.1	40.2

otter odor was the most avoided odor, there were treatments that were accepted less on average because river otter odor was not used for the June/July trials in ASP, when relatively few samples were taken. The only other slight differences among other predator odors occurred between coyote and lion odors ($P < 0.1$) and between lynx and lion odors ($P < 0.1$). The acceptance rates for neem and diesel oil were intermediate between predator odors and aspen controls (Table 2).

TABLE 3. SIGNIFICANCE LEVELS OF DIFFERENCES IN RESPONSES TO SAMPLES AFTER FIVE ACTIVITY PERIODS FOR ALL TRIALS[a]

	Wolf	Coyote	Dog	Otter	Lynx	Lion	D	N	I	M	B
Bear	--	–	–	*	–	–	*	**	–	***	***
Wolf		–	–	*	–	–	*	**	–	**	***
Coyote			–	*	–	*	***	***	–	***	***
Dog				*	–	–	*	***	–	***	***
Otter					–	*	***	***	*	***	***
Lynx						*	***	***	–	**	***
Lion							**	***	–	**	***
Diesel oil								–	–	–	*
Neem									–	–	*
Intact										*	**
Methanol											–

[a]Abbreviations for odors: D: Diesel oil; N: neem; I: intact; M: methanol; B: blank (perforated). ***$P < 0.001$; **$P < 0.01$; *$P < 0.1$; –: $P > 0.1$

DISCUSSION

The responses toward the samples show that free-ranging beaver are able to distinguish between different odor treatments of food, which in this experiment included the opportunity to taste the samples. They did so in the presence of abundant alternate food. This type of field experiment contrasts with captivity or laboratory studies in which the experimenter provides the only food available and manipulates its amount. Examples are experiments with cattle (Pfister *et al.*, 1990), sheep (Arnould and Signoret, 1993; Pfister *et al.*, 1990), goats (Weldon *et al.*, 1993), or mountain beaver (Epple *et al.*, 1993). The three questions posed at the outset can be answered as follows. At least some predator fecal extracts inhibit feeding (the actual concentrations of active compounds are unknown at this point). The effects of different extracts did not differ significantly from one another. From this it follows that there was no significant difference between chemicals of predators that are sym- or allopatric with beaver.

The different responses to the samples by the beavers in the two regions may result from genetic differences between the two populations or environmental factors such as experience with predators or the composition of the food supply. Originally there were two different subspecies in the two respective study areas (Hall, 1981). The original subspecies in the Adirondacks was *Castor canadensis acadicus* Bailey and Doutt (Hall, 1981). The subspecies for western New York State, including ASP, is the nominate race. The beaver had been almost extirpated in the entire State of New York in the 19th century. The Adirondack population was restocked in 1905 with seven beaver from Canada (nominate race), and again in 1907, when 14 specimens from Yellowstone National Park (subspecies *C. c. missouriensis* Bailey) were released at Old Forge. After their extirpation, the first beavers at ASP were recorded in 1937 (Shadle and Austin, 1939). These beaver had come north from Pennsylvania where animals from Wisconsin had been introduced in 1917 and subsequent years (Hilfiker, 1991). Still, the genetic distance between the two current study populations is not known.

Since the two study areas also differ in the presence of potential predators, the effect of learning could be important. In general, ASP has fewer different predators. Felidae are totally missing (personal communication with local wildlife managers) and so are wolves. Black bear, coyote, and river otter occur in the park (personal observation of animals and tracks by both authors). By contrast, all North American predators whose odors were used, except wolf, occur in HWF. The lynx was extirpated in the Adirondacks in the late 19th century and recently reintroduced (Brocke *et al.*, 1991), but is still very rare. The closely related bobcat (*Lynx rufus*) also occurs in the Adirondacks (Saunders, 1988). Black bear tracks and droppings around beaver ponds have been seen in HWF by the author, as have three specimens of river otter and several coyotes. The

barking and howling of coyote packs was often heard. The abundance of predators in HWF could explain why the predator odors were avoided so strongly, as were the nearby control samples. Nevertheless, beavers did not avoid the odors of sympatric species significantly more than the odor of the allopatric lion. However, the slightly more avoided odors were those of species that are sympatric with each particular beaver population used in this study, such as river otter and lynx in HWF.

Other studies of predator odor effects on feeding by herbivores show consistent effects (e.g., Müller-Schwarze, 1972; Sullivan and Crump, 1984). Dog odor has no significant effect on the feeding behavior of snowshoe hare (Sullivan *et al.*, 1985a), but coyote odor decreases browsing by black-tailed deer significantly (Sullivan *et al.*, 1985b). Black-tailed deer avoid the odors of sympatric predator species more than those of allopatric species. This behavior may have a genetic basis but might be refined by learning (Müller-Schwarze, 1972). In this study beavers avoided the odors of sympatric predators such as river otter and coyote that actually occur within the range of the tested beaver slightly more than those of originally sympatric, but now absent, species such as the wolf. Lynx are in the same category as wolf (except for recent local reintroductions) (Brocke *et al.*, 1991), but their odor was still avoided. Furthermore, beaver do not discriminate between the odors of the allopatric lion and the sympatric wolf, nor did they distinguish between wolf and dog odor. Similarly, Mason et al. (1994) found that urine odors from dog, mink, bobcat, and coyote inhibited feeding by mountain beaver, *Aplodontia rufa*, equally well. These authors postulate that to herbivores, sulfur compounds constitute a general predator cue across carnivore species.

We have evidence that beavers actually encounter predators in our study areas. The authors observed river otters in beaver ponds in both regions. Black bear footprints and droppings close to beaver ponds were found in ASP and HWF. In our team's studies, a black bear took a beaver out of a Hancock live-trap in ASP on two occasions (Houlihan, unpublished; Schulte, 1993). Beavers might learn to avoid predator odors through direct encounters or witnessing attacks on other beaver. A beaver might also be conditioned by warning signals from conspecifics, even though the signal is associated only with a predator odor, not an encounter itself.

The seasonal differences in sample acceptance are most likely correlated with the changing abundance of palatable food. In summer beavers feed very little on tree bark. Beavers prefer to eat leaves and chew the bark of a twig only after all leaves are consumed. They also eat grass and forbs. This might explain the low interest in the aspen sticks in general at both ASP and HWF in summer. In September the situation changes dramatically. The beavers cut many trees to build food caches for the winter. Furthermore, beavers working on bulky food items such as branches might entirely ignore the relatively small aspen samples.

During this period they spend more time than usual on the shore and are more exposed to predators. They might be more alert and are consequently avoiding the predator odor more for both of these reasons. The responses at HWF dropped towards zero acceptance from the middle of September onwards. In late autumn the growing season is over, and beavers depend almost exclusively on bark in their diet. The aspen samples might then become more attractive again and the beaver less selective.

When the distance between the sticks was increased from 30 cm to 1 m, the beaver did not discriminate controls more from predator odors. Thus "olfactory overshadowing" may not have been important with our arrangement of the samples.

During some days in late October the ponds were frozen, and the beaver tended to either ignore or accept all samples. The ice cover affected the beavers' activities, and the low temperature of air and sticks could have impaired their ability to distinguish between different odors. The different amounts of rainfall in the course of the year could also play an important role. Since the predator odors were applied as alcoholic solutions, they are easily washed away by rain. Sticks with predator odor that had lain in the water for some time were eventually chewed. The same was true for initially rejected sticks with diesel oil. In other studies, such as that of black-tailed deer (Sullivan et al., 1985b), predator odors also became less effective after heavy rains. The strong feeding responses after rainfall indicate that the odor was washed away. This might be different for each odor, depending on original odor strength and other compounds present. For instance, wolf odor was more avoided than any other odor on the first day (Figure 1) but had no strong repellent effect later on. The reverse was true for river otter odor. That taste, in addition to olfaction, may be involved is shown by the fact that beaver often consumed more than half of the bark on a stick and then abandoned the rest.

Any application of repellents has to take into account which predator species are present in the area. Beaver exposed to a more diverse predator guild may avoid more strongly repellents based on predator odors. For lasting effects, the learning ability of beaver requires that other stimuli be used to reinforce the avoidance of predator odors. Furthermore, odors applied directly to food may trigger responses different from odors placed in the vicinity of food, and the senses involved determine the stimuli used. For an effective beaver repellent to last in the elements, a formulation with a hydrophobic carrier will be necessary. The next step is the chemical isolation and identification of the active compounds.

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ALLELOPATHIC POTENTIAL OF *Anthoxanthum odoratum* FOR INVADING *Zoysia*-GRASSLAND IN JAPAN

YOSHITO YAMAMOTO

Department of Ecology
National Grassland Research Institute
Nishinasuno Tochigi 329-27, Japan

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Abstract—The growth of *Zoysia japonica* surrounding *Anthoxanthum odoratum* on *Zoysia*-grassland in Japan was investigated in June 1990. The stem density of *Z. japonica* tended to decrease with short distances between two *A. odoratum* plants. This showed that the growth of *Z. japonica* surrounding *A. odoratum* was reduced. Basic, neutral, and acidic fractions extracted from *A. odoratum* plants inhibited the seedling growth of lettuce. In particular the neutral fraction showed the strongest activity among the three fractions. The main inhibitory compound obtained at R_f 0.6–0.7, on the thin-layer chromatogram of the neutral fraction, was isolated and identified as coumarin by means of GC-MS. Coumarin solution inhibited seedling growth of *Z. japonica* in low concentrations but, conversely, promoted seedling growth of *A. odoratum*. Coumarin was contained in all parts of *A. odoratum* and its concentration varied with the season and from one individual plant to another. In particular, coumarin was highly concentrated in the leaves, accounting for more than 2.5% of dry leaf weight in June. The inhibitory effect of these aqueous extracts was correlated to the amount of coumarin in *A. odoratum* leaves and coumarin was considered to be the main inhibitory compound.

Key Words—*Anthoxanthum odoratum*, allelopathy, coumarin, *Zoysia japonica*, allelochemicals, grassland.

INTRODUCTION

Zoysia japonica is one of the important native grasses mainly dominant in the seminatural grassland of Japan, but recently, the author observed that *Anthoxanthum odoratum* has invaded *Zoysia*-type vegetation and formed a pure stand. Judging from the distribution pattern of *A. odoratum* in *Zoysia*-type vegetation, the author hypothesized that allelopathy contributed to its aggressiveness. *A.*

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odoratum was one of the pasture plants introduced from Europe as horse feed about 100 years ago, but it has escaped from pasture and spread all over Japan except for the Okinawa region. Several studies have been made on allelopathy of *A. odoratum*; for example, it was reported that the root exudate from *A. odoratum* affected the growth and phosphorus uptake (Newman and Rovia, 1975; Newman and Miller, 1977). However, the effects of allelochemicals and the allelopathic role of *A. odoratum* in grassland communities have not yet been studied. Therefore, the author started this research on the allelopathy of *A. odoratum*.

In this study, the growth of *Z. japonica* surrounding *A. odoratum* was investigated, and the main allelopathic substances of *A. odoratum* were isolated and identified. In addition, the allelochemical content of *A. odoratum* was measured, and the effects of this allelochemical on the growth of *Z. japonica* and *A. odoratum* were assessed.

METHODS AND MATERIALS

Field Survey of Growth of Zoysia japonica. The growth of *Z. japonica* surrounding *Anthoxanthum odoratum* in *Zoysia*-type vegetation was investigated in June 1990. First, two *A. odoratum* plants were selected in *Zoysia*-type vegetation, on the condition that there was no *A. odoratum* within a circle the diameter of which was the distance between the two plants. The stem numbers of the two *A. odoratum* plants were counted (Figure 1). A quadrat (10 × 10 cm) was set between the two *A. odoratum* plants, and the stem numbers of *Z. japonica* within the quadrat were counted. This investigation was carried out on a total of 22 circles.

Extraction of Active Substances from A. odoratum and TLC Fractionation. The plant top of *A. odoratum* was collected in March and freeze-dried. The extraction procedure used is shown in Figure 2. Thin-layer chromatography was carried out on a 20-cm × 20-cm silica gel plate (Whatman, Silica gel 60A PK6F) 1 mm thick using benzene–MeOH–acetic acid (45 : 8 : 4 v/v/v) as a developing solvent. After the TLC plate was dried, it was divided into 10 parts from the origin to front. Each fraction was eluted with MeOH for 12 hr at 25°C, and the eluates were filtered. These fractions were bioassayed using lettuce seedlings. Thirty seeds were placed on filter paper moistened with 1.6 ml of the test solution in a 20-mm-high × 55-mm-ID glass Petri dish. Lettuce seeds were incubated for four days in a growth chamber at 25°C in darkness. The average hypocotyl length of 10 germinated plants was compared with that of controls grown in distilled water.

Isolation and Identification of Allelochemical. An active compound separated by TLC was isolated and identified using a Shimadzu GCMS-QP1000S

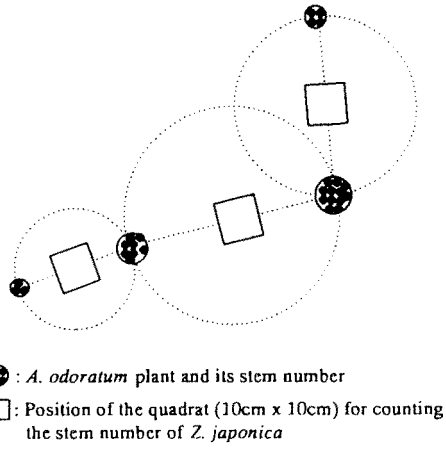

FIG. 1. Diagram showing procedure used to quantify stem density of *Z. japonica* and *A. odoratum* in *Zoysia*-type vegetation. The two *A. odoratum* plants were selected so that there was no *A. odoratum* within a circle the diameter of which was the distance between two plants. The distance between the two plants was measured, and then the stem number of each plant was counted. Next, the stem density of *Z. japonica* located between the two plants was counted.

gas chromatograph–mass spectrometer. The analytical conditions were as follows: column—Shimadzu fused silica capillary column CBP1-M25-0.25 (25 m length × 0.25 mm ID); carrier gas—He, 40 ml/min; temperature—column, 50–250°C, 5°C/min; injection, 250°C; detector, 250°C.

Seedling Growth Bioassay. Thirty seeds of *Z. japonica* or *A. odoratum* were placed on filter paper moistened with 2 ml of the known concentrations of the test water solution in a 20-mm-high × 55-mm-ID glass Petri dish. *Z. japonica* seeds were incubated in a growth chamber for 10 days under a cycle of 12 hr light (7.5×10^3 ergs/cm^2/sec) at 35°C and 12 hr dark at 25°C. *A. odoratum* seeds were also incubated for eight days in light at 30°C and in dark at 20°C. The percentage of germination of the 30 seeds and average length of shoot and root of 10 germinated plants were compared with those of controls grown in distilled water.

Measurement of Content of Allelochemical in A. odoratum. Four plants of *A. odoratum* were collected every month from April to November 1993 in a field plot. In this plot, cutting and nitrogen fertilization (1.42 g N/m^2/yr) were performed in March, July, and September. Each of the collected plants was separated into head, leaf, stem, stubble, root, and standing dead litter and then each part was freeze-dried and powdered. Each 1 g of powder was extracted

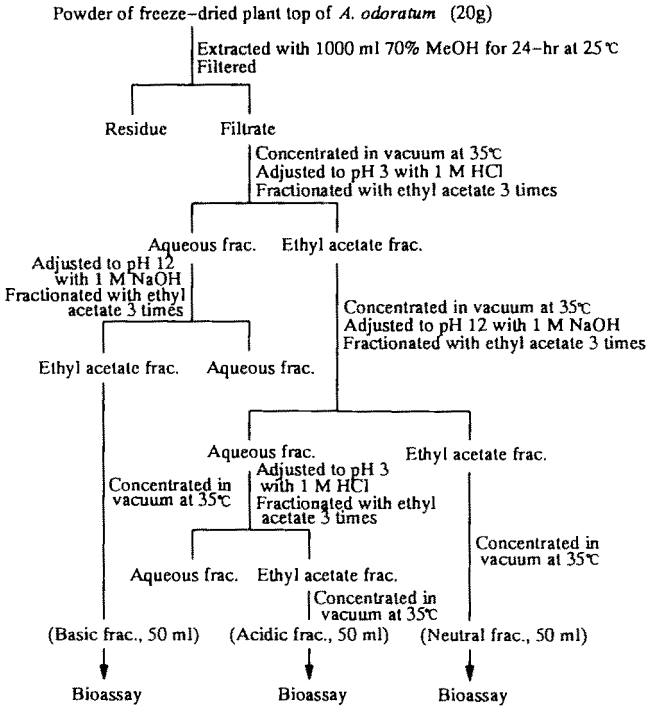

FIG. 2. Extraction procedure of active substances from *A. odoratum*.

with 50 ml MeOH for 12 hr at 25°C, and the extracts were filtered. The concentration of coumarin in these extracts was measured using a Shimadzu GC-15A gas chromatograph. The analytical conditions were as described in the section "*Isolation and Identification of Allelochemical.*" In addition, aqueous extracts of the leaf freeze-dried samples were bioassayed using *Z. japonica* seeds, as described above.

RESULTS

Field Survey of Growth of Zoysia japonica. The relationship of the distance between two *Anthoxanthum odoratum* plants and the stem density of *Zoysia japonica* between the two plants was unclear, but the effect of *A. odoratum* on the growth of *Z. japonica* seemed to depend on plant size of *A. odoratum*. When total stem numbers of the two *A. odoratum* plants were between 15 and 19, stem density of *Z. japonica* located between the two plants tended to decrease the shorter the distance between the two *A. odoratum* plants (Figure 3). This

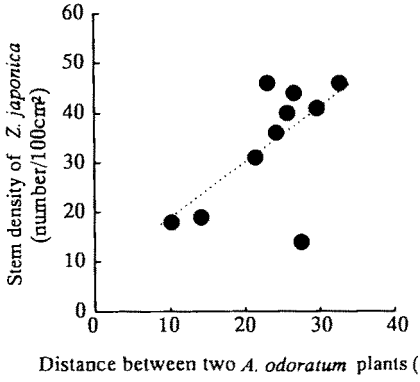

FIG. 3. Relationship between the distance of two *A. odoratum* plants and stem density of *Z. japonica* located between the two plants.

result showed that the stem density of *Z. japonica* surrounding *A. odoratum* was reduced.

Isolation and Identification of Allelochemical. Basic, neutral, and acidic fractions extracted from the plant top of *A. odoratum* inhibited the seedling growth of lettuce (Figure 4). Neutral and acidic fractions showed strong inhibitory activity at high concentrations, especially concentration 1, which completely inhibited germination of lettuce seeds. The neutral fraction showed the

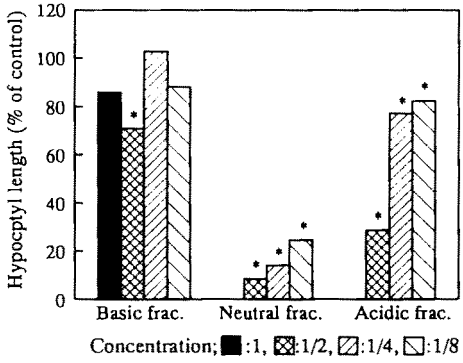

FIG. 4. Effect of the plant top extracts of *A. odoratum* on growth of lettuce. Concentration 1 was the 50-ml fraction obtained from 20 g of plant top of *A. odoratum* as shown in Figure 2. *Significant at $P < 0.05$ as compared with the distilled water control by using Student's *t* test. Lettuce did not germinate in concentration 1 of neutral and acidic fractions.

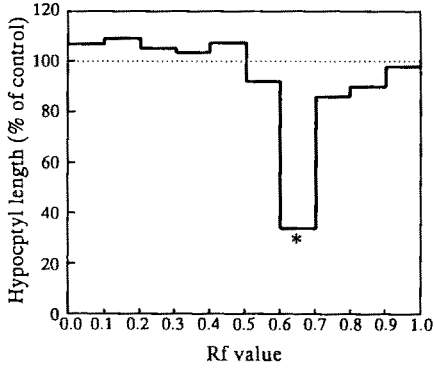

FIG. 5. Effect of thin-layer chromatogram fractions of neutral fraction on the growth of lettuce. Adsorbent: silica gel; developing solvent: benzene–MeOH–acetic acid (45 : 8 : 4, v/v/v). *Significant at $P < 0.05$ as compared with the silica gel control by using Student's t test.

strongest inhibitory activity of the three fractions. From the results of the bioassay using lettuce, significant growth inhibitory activity was exhibited by the neutral fraction at R_f 0.6–0.7 from the thin layer chromatogram (Figure 5). The main inhibitory compound at R_f 0.6–0.7 was isolated and identified as coumarin by GC-MS.

Seedling Growth Bioassay. Coumarin, which was identified as the main allelopathic compound from *A. odoratum*, inhibited the growth of *Z. japonica* and *A. odoratum*. The compound inhibited the seedling growth of *Z. japonica* even in low concentrations (Figure 6). In particular, root length was inhibited

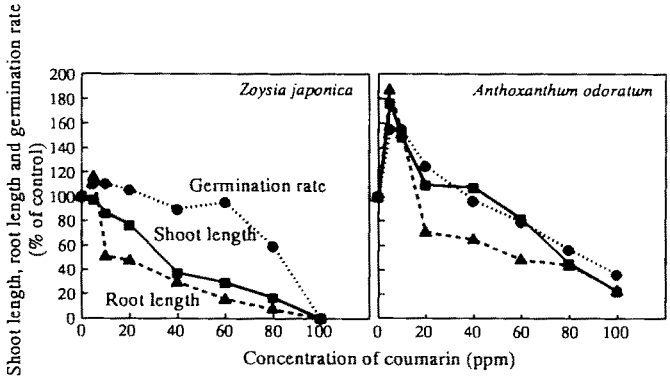

FIG. 6. Inhibitory effect of coumarin on the growth of *Z. japonica* and *A. odoratum*.

TABLE 1. SEASONAL CHANGES IN CONTENT OF COUMARIN IN *Anthoxanthum odoratum*

	Coumarin content (μg/g dry sample ± SD)					
Month	Root	Stubble	Leaf	Stem	Standing dead litter	Head
April	55 ± 13	860 ± 462	3998 ± 1078	2587 ± 727	93 ± 56	1306 ± 391
May	99 ± 41	151 ± 64	8988 ± 3302	2456 ± 1437	20 ± 9	252 ± 256
June	461 ± 62	1179 ± 401	26577 ± 4531	6558 ± 2112	281 ± 136	
July	291 ± 145	382 ± 337	7188 ± 2832	5599 ± 786	92 ± 46	
August	369 ± 218	522 ± 67	8199 ± 1158	4691 ± 728	440 ± 115	
September	163 ± 32	435 ± 116	6458 ± 1567	5397 ± 1913	348 ± 81	
October	51 ± 24	454 ± 196	7013 ± 1680	5597 ± 1104	354 ± 81	
November	47 ± 13	235 ± 183	2773 ± 956	2327 ± 98	75 ± 44	

by as little as 10 ppm coumarin solution. The seedling growth of *A. odoratum* was also inhibited by high concentrations of coumarin solution, but at low concentrations it was, conversely, promoted.

Measurement of Content of coumarin in A. odoratum. The concentration of coumarin in the head, leaf, stem, stubble, root, and standing dead litter of *A. odoratum* was quantified (Table 1). The concentration of coumarin contained

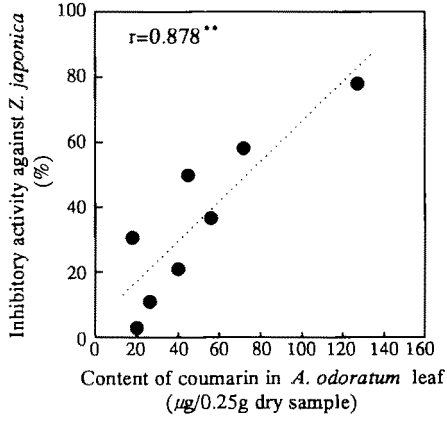

FIG. 7. Relationship between the content of coumarin of *A. odoratum* leaf collected from April to November and inhibitory activity of aqueous extracts against the root growth of *Z. japonica*. Inhibitory activity was calculated by subtracting treatment root length (as percentage of the control root length) from 100%. Correlation coefficient, 0.878, was significant at $P < 0.01$.

in all plant parts of *A. odoratum* varied with the month. Coumarin content in all parts tended to be highest during summer. The leaf and stem showed the highest concentrations and the leaf, in particular contained more than 2.5% dry weight of coumarin in June. Aqueous extracts of these leaves also inhibited the root length of *Z. japonica*.

The aqueous extracts of leaves with high coumarin content tended to show a high inhibitory effect (Figure 7). The correlation coefficient between inhibitory effect and content of coumarin was 0.878 ($P < 0.01$). Therefore, the coumarin contained in *A. odoratum* was considered to be the main inhibitory compound against *Z. japonica*.

DISCUSSION

Generally, *Zoysia japonica*, which dominated the experimental site, is the dominant species in seminatural grazing land in Japan. *Z. japonica* is a stoloniferous plant tolerant of grazing or cutting and deteriorates without these forms of management (Numata, 1975; Itow, 1975). However, it was found from a field survey that *Anthoxanthum odoratum* tended to invade *Zoysia* vegetation, and the stem density of *Z. japonica* surrounding *A. odoratum* was confirmed to be reduced (Figure 3). Regarding the mechanism of aggressiveness of *A. odoratum*, both allelopathy and competition for light, soil moisture, or plant nutrients were considered. As both *A. odoratum* and *Z. japonica* were grazed by cattle, there was less competition between these species for light. *Z. japonica* is drought resistant and is able to grow in sterile land without fertilizing. Therefore, it was possible that allelopathy contributes to the aggressiveness of *A. odoratum*. Allelopathy is well known to play a significant role in the dominance of grassland communities (Rice, 1984; Heisey and Delwiche, 1985; Chou, 1987; Chou and Lee, 1991).

Coumarin was isolated and identified as an inhibitory compound from the most active fraction of the extracts of *A. odoratum* (Figures 4 and 5). Coumarin is a known constituent of many kinds of plant species (Putnam, 1988). It is possible that coumarin in the germinated seeds of *Myroxylon peruiferum* could play an important role as an allelopathic factor (Oniki and Valio, 1992). *A. odoratum* contained coumarin in high concentrations, particularly in leaves (Table 1). Coumarin inhibited root growth in *Phleum pratense* (Avers and Goodwin, 1956) and *Hordeum vulgare* (Hu, 1985) and inhibited germination of *Medicago sativa*, *Lolium multiforum*, and *Abutilon theophrasti* (Dornbos and Spencer, 1990). In exudates from *Avena fatua* roots the compound also inhibited growth of *Triticum aestivum* (Perez and Ormeno-Nunez, 1991). In this study, coumarin inhibited the growth of *Z. japonica*, but promoted the growth of *A. odoratum*, at low concentrations (Figure 6). It was interesting that the sensitivity of the

two species to coumarin was different. The data suggested that the aggressiveness of *A. odoratum* might be due to the allelopathic effect of coumarin. Newman and Rovia (1975) reported that the growth of *A. odoratum* was promoted by its own root leachates compared with controls or with other species. If coumarin were leached from the root of *A. odoratum*, the promotion of the growth of *A. odoratum* can be understood. The amount of coumarin in the leaf of *A. odoratum* and the inhibitory effect of these aqueous extracts on the growth of *Z. japonica* were correlated. Therefore, coumarin was considered to be the main inhibitory compound in *A. odoratum*.

The aggressiveness of *A. odoratum* in *Zoysia* grassland may be the result of many factors, but judging from the results above, it is clear that coumarin in *A. odoratum* could play an important role in terms of its allelopathic qualities and its aggressiveness in *Zoysia* grassland.

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EFFECTS OF *neo*-CLERODANE DITERPENES FROM *Teucrium* ON FEEDING BEHAVIOR OF COLORADO POTATO BEETLE LARVAE

FÉLIX ORTEGO,[1] BENJAMÍN RODRÍGUEZ,[2] and PEDRO CASTAÑERA[1,*]

[1]*Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain*

[2]*Instituto de Química Orgánica, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*

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Abstract—Treatment of potato leaf disks with four *neo*-clerodane diterpenes from *Teucrium* (eriocephalin, teucrin-A, teucvin, and teuscorolide) significantly reduced feeding by larvae of *Leptinotarsa decemlineata* (Colorado potato beetle). Choice and no-choice tests suggest that teuscorolide acts as a feeding deterrent, whereas the antifeedant activity of teucrin-A, teucvin, and eriocephalin is likely associated with a toxic mode of action. Nutritional tests, antifeedant simulation assays, and posttreatment studies confirmed that teucrin-A can be categorized by its mode of action as a toxin, rather than as a feeding deterrent.

Key Words—*Leptinotarsa decemlineata*, Colorado potato beetle, *Teucrium*, clerodane diterpenes, feeding behavior, antifeedants, toxins, feeding deterrents.

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is an economically important pest currently resistant to most classes of synthetic insecticides (Forgash, 1981, 1985). Despite widespread development of resistance, chemical insecticides remain the major control method (Casagrande, 1987; Hare, 1990). We are now in a situation where alternative pesticides are not always available, which may lead to a decline in potato production. Recently, interest has grown

*To whom correspondence should be addressed

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in the search of antifeedants and toxins of plant origin as a promising source of new control agents (Alford et al., 1987; Hough-Goldstein, 1990).

Clerodane diterpenes have attracted interest in recent years on account of their biological activities against some economically important pests (review in Merritt and Ley, 1992). The most abundant sources of this kind of compounds are the plants belonging to the genus *Teucrium* (Labiatae), from which about 150 *neo*-clerodanes have been isolated (reviews in Piozzi et al., 1987; Merritt and Ley, 1992). Natural *neo*-clerodanes from *Teucrium* and some of their structural derivatives reduced feeding in larvae of some economically important lepidopterous pests (Miyase et al., 1981; Blaney et al., 1988; Simmonds et al., 1989; Simmonds and Blaney, 1992; Rodríguez et al., 1994) and the African migratory locust, *Locusta migratoria* (Hanson et al., 1982).

We report here the effects of four natural *neo*-clerodanes from *Teucrium* (eriocephalin, teucrin-A, teucvin, and teuscorolide) on the feeding behavior of larvae of the Colorado potato beetle. Choice and no-choice tests were used to assess the potency of each compound as an antifeedant. Teucrin-A was further investigated with the help of nutritional tests, antifeedant simulation assays, and posttreatment studies to distinguish between a feeding deterrent or toxic mode of action.

METHODS AND MATERIALS

Chemicals

The *neo*-clerodane diterpenes eriocephalin, teucrin-A, teuscorolide, and teucvin (Figure 1) were available from previous studies.

Insects

A colony of Colorado potato beetle was established by collecting over 50 adults from a potato field located in Zaragoza, Spain. The laboratory colony

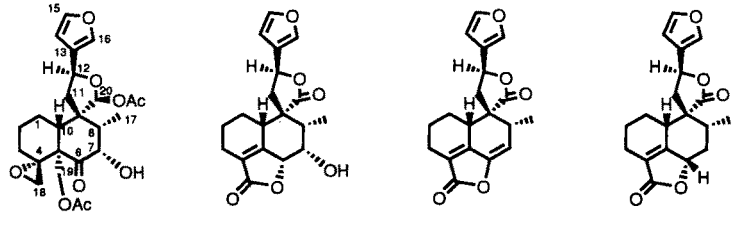

FIG. 1. Molecular structures of *neo*-clerodanes tested against Colorado potato beetle larvae.

was reared on potato plants, *Solanum tuberosum* cv. Kennebec, at 22 ± 2°C, 90 ± 10% relative humidity, and 16 L:8 D photoperiod in an environmental chamber. Fourth-instar larvae from the first generation were used in all feeding experiments.

Feeding Experiments

General. The arena for the assays consisted of plastic Petri dishes (15 × 90 mm), coated on their bottom half with about 20 ml of a 2.5% agar solution (Escoubas et al., 1993). Potato leaf disks (1.77 cm^2) were cut with a cork borer (No. 15) and each disk was fitted into a hole punched in the agar layer. The disks were treated on the upper surface with 10 μl of an acetone solution containing the test compound or the solvent carrier alone. After complete evaporation of the solvent, newly emerged fourth-instar larvae (less than 24 hr old and starved for 6 hr) were placed in each dish in a growth chamber at 26 ± 0.5°C and 85 ± 10% relative humidity, where they were allowed to feed.

Feeding and nutritional indexes were calculated on a dry weight basis. Initial and final larval dry weights were estimated from the ratio of fresh to dry weight calculated with 50 newly molted fourth-instar larvae. Initial dry weight of the leaf disks was estimated similarly using 100 leaf disks to calculate the ratio. At the end of the experiment, the uneaten leaf disks were oven dried at 60°C for two days and weighed.

Choice and No-Choice Feeding Assays. Potato leaf disks were treated with 0, 100, 300, or 1000 ppm concentrations of the test compounds. For the no-choice assays, six treated or control disks were placed in each arena. In the choice assays, three treated and three control disks were alternately arranged per arena. Arenas that contained six untreated leaf disks were simultaneously set and compared with the arenas of the choice assay (choice/no-choice test). Two newly emerged fourth-instar larvae were placed in each dish. In the choice assay, feeding was terminated after the consumption of 50% of the disks (control plus treated) or after 24 hr, whichever occurred first, and after the consumption of about 75% of the control disks in the no-choice assay (4 hr). Assays were conducted with 10 replications per treatment.

The antifeedant index (AI) was calculated in the no-choice assay by the equation $[(C - T)/C] * 100\%$ (Bentley et al., 1984) and in the choice assay by the equation $[(C - T)/(C + T)] * 100\%$ (Simmonds et al., 1989), where C and T represent the consumption of control and treated disks, respectively. The suppression index (SI) was determined in the choice/no-choice assay by the equation $[(C - T)/C] * 100\%$, where T represents consumption of both treated and untreated leaf disks in test arenas and C the consumption in control arenas that contain untreated leaf disks only (Raffa and Frazier, 1988).

Nutritional Indexes. Twenty newly emerged fourth-instar larvae were individually fed for 24 hr on eight potato leaf disks treated with 0, 10, 30, 100,

300, and 1000 ppm concentrations of teucrin-A. At the end of this period, 10 larvae per treatment were allowed to continue feeding on eight untreated potato leaf disks for another 24 hr. Nutritional indexes were calculated for the 24 hr that the larvae were feeding on treated leaf disks (treatment period), and for the following 24 hr that the larvae were feeding on untreated leaf disks (posttreatment period).

The relative consumption rate (RCR) and the relative growth rate (RGR) were calculated according to Farrar et al. (1989). Growth efficiency (GE) was calculated as the slope of the regression of RGR on RCR, assuming a common intercept determined by the RGR of starved larvae (Blau et al., 1978). The relative chemical consumption (RCC) was estimated as RCR $\times$ dosage, according to Liu et al. (1990).

An antifeedant simulation assay (Blau et al., 1978) was run simultaneously to distinguish between the deterrent or toxic effects of teucrin-A. A calibration curve for the effect of RCR on RGR at different levels of starvation was built by feeding larvae with different amounts of food, ranging from total starvation to surplus of food. Twenty larvae were individually presented for 24 hr with one of five different arrangements: zero, two, four, six and eight potato leaf disks.

Statistical Analysis

Significant differences between the consumption in control and treatment arenas in the no-choice assays and between the consumption in control (only untreated leaf disks) and test (with treated and untreated leaf disks) arenas in the choice/no-choice assays were tested using the Dunnett two-tailed test. Significant differences between the amount of control and treated potato disks eaten in choice tests were determined by Wilcoxon's matched pair test. Significant differences among nutritional index means (RCR, RGR, and RCC) were tested using the Student-Newman-Keuls test.

The potential antibiosis or antixenosis of teucrin-A was determined by regressing RGR on RCR and comparing each treatment slope with the slope of the calibration curve, assuming a common intercept determined by the RGR of starved larvae (Blau et al., 1978). Curves for deterrent substances would fall on the calibration line, although concentrated in the range of low consumption rate. Treatments in which the slope of the regression is significantly lower than that of the calibration curve (test of homogeneity of slopes) can be categorized as toxins.

We found that RCR, RGR, and GE were inversely proportional to the larval weight. Since the weight of the larvae after 24 hr feeding on treated leaf disks was different for each concentration, it was necessary to exclude the differential effect this would cause from the nutritional indexes in the posttreatment

period. We did this by calculating the regression of nutritional indexes (RCR, RGR, and GE) obtained over the posttreatment period on the larval weight at the end of the treatment period. From the regression the expected post-RCR, post-RGR, and post-GE were then calculated for each specimen according to its weight at the beginning of the posttreatment period. The difference between the observed and the expected postnutritional indexes are the residual postnutritional indexes, which represent the portion of the observed index that can not be explained by the initial weight and therefore should be due to the posttreatment effect of teucrin-A. Significant differences between residual means were tested using the Student-Newman-Keuls test.

RESULTS AND DISCUSSION

The four *neo*-clerodanes tested affected the feeding behavior of Colorado potato beetle larvae, showing some degree of antifeedant activity. The term "antifeedant" is used here as the capacity to reduce feeding, independently of a deterrent (antixenosis) or toxic (antibiosis) mode of action.

Teuscorolide significantly reduced feeding at 1000 and 300 ppm in the choice assay, but only at 1000 ppm in the no-choice assay, and it lacked suppression activity in the choice/no-choice assay (Table 1). The highest antifeedant activity in choice, as opposed to no-choice, tests suggests that this compound acts as a feeding deterrent, since it is able to deter larvae in a choice situation, but the larvae will feed on it when other resources are not available. Similar feeding behavior responses have been reported for other feeding deterrents tested with larvae and adults of Colorado potato beetle (Caprioli et al., 1987; Hough-Goldstein, 1990).

Teucrin-A and teucvin caused a significant dosage-dependent feeding reduction in the no-choice assay at all concentrations tested, indicating a potent antifeedant activity (Table 1). However, consumption of treated disks was only significantly reduced at the highest dose (1000 ppm) in the choice test, indicating low discrimination between treated and control disks (Table 1). A significant suppression of feeding by these two compounds at 300 and 1000 ppm in the choice/no-choice test suggest a noxious effects of these compounds (Table 1). The results observed in the choice situations might then be explained by the antibiosis of these compounds. When the larvae ingest a quantity of treated leaves large enough to evoke toxic responses, feeding activity decreases or ceases and the reduction in the amount of treated disks eaten would be rather small, since no control disk consumption occurred either. Only at 1000 ppm was there a significant reduction, because at this concentration the larvae only fed when control disks were found, but stopped feeding for several hours after they eat a small portion of a treated disk. The tetranortriterpene limonin, which

TABLE 1. EFFECT OF FOUR *neo*-CLERODANE DITERPENES ON FEEDING BEHAVIOR OF FOURTH-INSTAR LARVAE OF COLORADO POTATO BEETLE IN CHOICE, NO-CHOICE, AND CHOICE/NO-CHOICE TESTS

Concentration (ppm)	No-choice, AI[a]	Choice, AI[b]	Choice/no-choice, SI[c]
Eriocephalin			
1000	60.1 ± 3.2*	23.8 ± 5.0*	18.7 ± 6.0
300	33.4 ± 6.3*	6.2 ± 6.9	6.9 ± 3.5
100	−7.7 ± 11.1	−7.8 ± 5.4	8.9 ± 5.9
Teucrin-A			
1000	75.0 ± 5.9*	34.0 ± 7.6*	76.3 ± 1.3*
300	51.0 ± 7.8*	8.4 ± 3.6	62.6 ± 2.3*
100	35.6 ± 9.3*	11.6 ± 4.6	18.3 ± 11.9
Teuscorolide			
1000	39.0 ± 5.2*	32.9 ± 6.2*	20.9 ± 6.6
300	7.3 ± 5.3	25.2 ± 4.8*	4.3 ± 9.1
100	−10.1 ± 6.7	14.0 ± 8.4	12.3 ± 6.4
Teucvin			
1000	77.8 ± 5.3*	25.1 ± 5.8*	80.9 ± 2.8*
300	59.4 ± 2.6*	8.1 ± 4.6	52.9 ± 7.8*
100	45.6 ± 7.7*	5.5 ± 7.2	15.0 ± 8.8

[a] Average antifeedant index $[(C - T)/C] \times 100\% \pm$ standard error ($N = 10$). *Significant differences between the consumption in control (C) and treatment (T) arenas (Dunnett two-tailed test $P \leq 0.05$).

[b] Average antifeedant index $[(C - T)/(C + T)] \times 100\% \pm$ standard error. ($N = 10$). *Significant differences between the consumption of control (C) and treatment (T) disks within each arena (Wilcoxon's matched pair test $P \leq 0.05$).

[c] Average suppression index $[(C - T)/C] \times 100\% \pm$ standard error ($N = 10$). *Significant differences between the consumption of both treated and untreated disks in test arenas (T) and control arenas (C) that contain untreated leaf disks only (Dunnett two-tailed test $P \leq 0.05$).

has been reported to act as a toxin against larvae of Colorado potato beetle, elicited similar responses in these types of tests (Alford et al., 1987; Mendel et al., 1991).

Eriocephalin significantly reduced feeding on treated leaf disks at 300 and 1000 ppm in no-choice assays, but only at 1000 ppm in choice assays, and lacked suppression activity in choice/no-choice assays (Table 1). The results from the no-choice test suggest an antifeedant activity for this compound, but the choice test indicates low discrimination between treated and control disks, similar in both cases to the results obtained with teucrin-A and teucvin. However, with eriocephalin, there was no suppression of overall consumption in choice/no-choice tests. These results suggest some degree of antibiosis associated with eriocephalin. This is exhibited only in no-choice tests after the larvae

have eaten several treated leaf disks. In choice/no-choice tests the assays are finished before the larvae have fed on sufficient treated disks to display any adverse effects.

Structure–activity studies have suggested that the C-12 β-substituted furan ring and the 4α,18-epoxide function of most natural *neo*-clerodanes from *Teucrium* are essential to keep the feeding reduction activity against larvae of *Spodoptera littoralis*. (Blaney et al., 1988; Simmonds et al., 1989; Rodríguez et al., 1994). We have found that eriocephalin, a *neo*-clerodane with both molecular moieties, also reduced feeding in larvae of the Colorado potato beetle. However, it was less active than teucrin-A and teucvin, two 19-*nor*-*neo*-clerodanes with a 18,6α-γ-lactone. Simmonds and Blaney (1992) found that at 100 ppm, eriocephalin and teucvin reduced feeding in larvae of *S. littoralis* and *S. frugiperda*, whereas teuscorolide was effective only against *S. littoralis* and teucrin-A lacked activity. Likewise, Simmonds et al. (1989) found that, at the same concentration, eriocephalin reduced feeding against *S. littoralis* but not against *H. armigera*, whereas teucrin-A lacked activity against both species. The differences of specificity observed among insect species (even within the same family) reveal the importance of targeting a broad range of insect pests in structure–activity studies.

The effect of teucrin-A on feeding behavior was further studied with the help of nutritional indexes to distinguish between a feeding deterrent or toxic mode of action. Teucrin-A was selected over the other *neo*-clerodanes because of its potent antifeedant activity, similar to that of teucvin, and the availability of this compound.

Feeding during 24 hr on potato leaf disks treated with teucrin-A significantly decreased consumption and larval growth at concentrations over 30 and 100 ppm, respectively (Table 2). The responses were dose-dependent and correlated with the chemical consumption (RCR vs RCC: $r = 0.57$, $P \leq 0.0001$; RGR vs RCC: $r = 0.68$, $P < 0.0001$). The growth efficiencies of larvae feeding on leaves treated with 300 and 1000 ppm of teucrin-A were significantly lower than that predicted by an antifeedant simulation–calibration curve with different levels of starvation (Figure 2 and Table 2). Since growth efficiencies for deterrent substances would fall on the calibration line and treatments with lower growth efficiencies can be categorized as toxins (Blau et al., 1978), our data suggest that toxic effects are the primary mode of action of this compound.

The larvae fed for 24 hr on potato leaf disks treated with teucrin-A were maintained on untreated leaf disks for another 24 hr to study if they were able to overcome the adverse effects observed during the treatment period. The resulting nutritional indexes were inversely proportional to the larval weight (Figure 3), and the weight of the larvae after 24 hr of feeding on treated leaf disks was different for each concentration (Table 3). Thus we excluded the effects of the different larval weights at the beginning of the posttreatment period by calcu-

TABLE 2. NUTRITIONAL INDEXES OF FOURTH-INSTAR LARVAE OF COLORADO POTATO BEETLE (N = 20) FEEDING DURING 24 HOURS ON POTATO LEAF DISKS TREATED WITH TEUCRIN-A

Concentration (ppm)	LW[a]	RCR[b]	RGR[c]	RCC[d]	GE[e]
1000	43.5 ± 1.2a[f]	0.45 ± 0.05a	−0.01 ± 0.02a	4.11 ± 0.45a	0.16*
300	43.6 ± 1.4a	0.96 ± 0.12b	0.17 ± 0.02b	2.61 ± 0.33b	0.22*
100	44.4 ± 1.7a	1.38 ± 0.09c	0.49 ± 0.03c	1.25 ± 0.08c	0.39
30	45.0 ± 2.0a	2.53 ± 0.19d	0.89 ± 0.08d	0.69 ± 0.05cd	0.38
10	44.6 ± 1.8a	3.15 ± 0.09e	0.91 ± 0.06d	0.29 ± 0.01d	0.33
Control	44.5 ± 1.5a	3.28 ± 0.19e	1.01 ± 0.06d	0d	0.33

[a] Average initial larval weight ± standard error (mg fresh weight).
[b] Average relative consumption rate ($I/W \times T$) ± standard error, where I = leaf disks consumed (mg dry weight), W = initial larval weight (mg dry weight), T = feeding period (days).
[c] Average relative growth rate ($\Delta W/W \times T$) ± standard error, where ΔW = increment in larval weight during the feeding period (mg dry weight).
[d] Average relative chemical consumption [RCR × D] ± standard error, where D = dosage of tested compound (μg compound/mg dry weight of leaf tissue).
[e] Growth efficiency calculated as the slope of the regression of RGR on RCR, assuming a common intercept determined by the RGR of starved larvae.* Significantly different from the slope of the calibration curve (0.32) (homogeneity of slopes test, $P \leq 0.05$).
[f] Column means followed by the same letter are not significantly different from each other (Student-Newman-Keuls test $P \leq 0.05$).

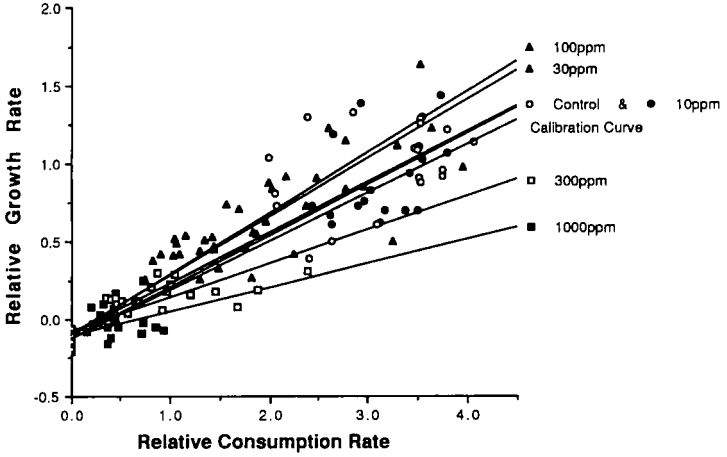

FIG. 2. Plot of relative growth rate (RGR) against relative consumption rate (RCR) for fourth-instar larvae of Colorado potato beetle feeding for 24 hr on potato leaf disks treated with different concentrations of teucrin-A (1000, 300, 100, 30, 10 ppm, and control) and different amounts of food (calibration curve). A common intercept was determined by the RGR of starved larvae.

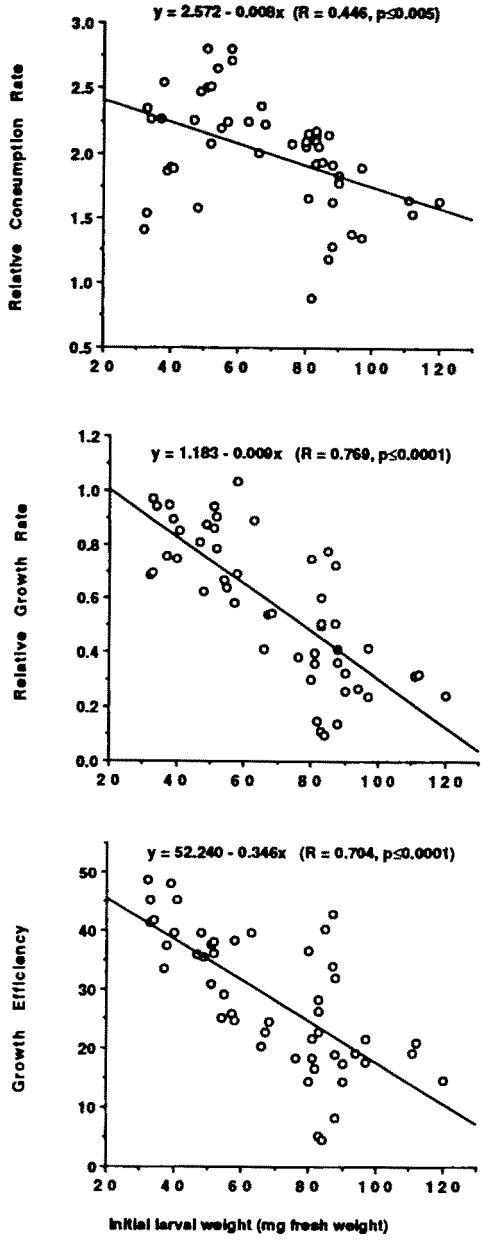

FIG. 3. Plots of relative consumption rate, relative growth rate, and growth efficiency against initial larval weight for fourth-instar larvae of Colorado potato beetle feeding for 24 hr on potato leaf disks.

TABLE 3. NUTRITIONAL INDEXES DURING 24-HOUR POST TREATMENT PERIOD OF FOURTH-INSTAR LARVAE OF COLORADO POTATO BEETLE (N = 10), FED DURING PREVIOUS 24 HOURS WITH POTATO LEAF DISKS TREATED WITH TEUCRIN-A[a]

Concentration, ppm	LW[b]	Post-RCR[c]	Post-RGR[d]	Post-GE[e]
1000	44.6 ± 3.4ab[f]	−1.07 ± 0.12a	−0.58 ± 0.02a	−20.08 ± 1.49a
300	52.6 ± 2.4b	−1.06 ± 0.09a	−0.46 ± 0.03b	−10.49 ± 3.02b
100	68.7 ± 3.1c	−0.99 ± 0.13a	−0.31 ± 0.02c	−1.28 ± 3.42c
30	83.1 ± 4.5d	−0.63 ± 0.08b	−0.16 ± 0.04d	−1.79 ± 1.68c
10	82.8 ± 3.8d	−0.08 ± 0.10c	0.04 ± 0.04e	2.06 ± 1.99c
Control	84.9 ± 4.2d	0.01 ± 0.10c	0.04 ± 0.04e	1.58 ± 2.25c
Starved	38.8 ± 2.7a	−0.05 ± 0.17c	−0.06 ± 0.05de	−2.77 ± 2.86c

[a] Residual postnutritional indexes were calculated as the difference between the observed and the expected ones. The observed post-RCR and post-RGR were calculated as in Table 2, and the observed post-GE was equal to (RGR/RCR) × 100. Expected post-RCR, post RGR and post-GE were calculated according to their initial larval weight by the regressions in Figure 3.

[b] Average larval weight at the beginning of the post-treatment period ± standard error (mg fresh weight).

[c] Average residual post-RCR ± standard error.

[d] Average residual post-RGR ± standard error.

[e] Average residual post-GE ± standard error.

[f] Column means followed by the same letter are not significantly different from each other (Student-Newman-Keuls test $P \leq 0.05$).

lating the residual post-RCR, post-RGR, and post-GE as the difference between the observed and the expected ones, according to the larval weight at the beginning of the posttreatment period (Table 3). We found that the residual post-RCR and post-RGR of treated larvae become more negative with increasing concentrations of teucrin-A and significantly different from those of control and starved larvae at concentrations over 30 ppm. A similar effect was found on residual post-GE, but was significantly different from the control and starved larvae only at 300 and 1000 ppm. These results showed that starved larvae reached the usual nutritional indexes once they started eating, whereas larvae feeding on leaf disks treated with 300 or 1000 ppm of teucrin-A for 24 hr were not able to recover during the following 24 hr. No mortality occurred when we continued rearing the larvae on untreated leaf disks until pupation, but the length of the instar of larvae treated with 1000 ppm of teucrin-A (5.6 ± 0.2 days) was significantly longer than that of starved larvae (5.1 ± 0.1 days) ($P \leq 0.05$). These data indicate that although ingestion of potato leaves treated with teucrin-A for 24 hr does not result in death, teucrin-A produces a postingestive toxic effect that affects nutritional indexes and delays larval growth.

In summary, we have found that four *neo*-clerodane diterpenes from *Teucrium* exhibit antifeedant activity against larvae of the Colorado potato beetle.

Antifeedant simulation assays and posttreatment studies indicate that teucrin-A can be primarily categorized as a toxin by its mode of action. Further research is in progress to elucidate which physiological functions are altered by the toxic action of teucrin-A.

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POTENTIAL AVERSIVE COMPOUNDS IN LEAFY SPURGE FOR RUMINANTS AND RATS[1,2]

S.L. KRONBERG,[3,*] W.C. LYNCH,[4] C.D. CHENEY,[5] and J.W. WALKER[5,6]

[3]*Department of Animal and Range Sciences*
South Dakota State University, Brookings, South Dakota 57007

[4]*Department of Psychology*
Montana State University, Bozeman, Montana 59717

[5]*Department of Psychology*
Utah State University, Logan, Utah 84322

[6]*USDA-ARS, U.S. Sheep Experiment Station*
HC 62 Box 2010, Dubois, Idaho 83423

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Abstract—Several wild and domestic ruminant species and horses apparently will not consume leafy spurge (*Euphorbia esula*) while grazing range and pasture lands. It has been demonstrated that leafy spurge can elicit conditioned food aversions in cattle and sheep, and the aversion-eliciting capacity of leafy spurge may account for why cattle seldom graze this nutritious plant and why sheep may not readily consume it at some locations. The identity of the aversive compound(s) in leafy spurge is unknown, but several different diterpenoid ingenol esters have been isolated from its tissues, and we suspect that one or more ingenol esters may be aversion-eliciting compounds in leafy spurge. The objectives of this study were to determine whether or not leafy spurge is aversive to laboratory rats and if a crude acetone extract of leafy spurge, presumably containing ingenol esters and other phytochemicals, could generate an aversive response in sheep and laboratory rats. An additional objective was to determine whether or not a particular ingenol monobenzoate, which may be similar to ingenol esters in leafy spurge, might also elicit an aversive response from rats. Rats exhibited food aversions associated with leafy spurge ($P < 0.05$). An acetone extract of leafy spurge induced condi-

*To whom correspondence should be addressed.

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tioned food aversions in both sheep and rats ($P < 0.01$). The ingenol 3-monobenzoate also induced conditioned food aversions in rats ($P < 0.01$). Our interpretation of these data is that rats can be used as a model for cattle and sheep with respect to their aversion to leafy spurge ingestion. Additionally, we suggest that one or more ingenol esters may be aversion-inducing agents in leafy spurge. However, others may exist in leafy spurge that are also aversive or are the only or prime aversive chemicals.

Key Words—Leafy spurge, *Euphorbia esula*, ingenol esters, diterpenes, weeds, conditioned food aversion, diet selection, cattle, sheep.

INTRODUCTION

Leafy spurge (*Euphorbia esula*), which is indigenous to Eurasia, has become a common member of many plant communities in the northern Great Plains and Intermountain regions of North America and is replacing native vegetation on over one million acres of range and pasture land in these regions. This forb has high levels of crude protein and low levels of indigestible fiber; therefore, animals that will consume it can realize valuable nutritional benefits (Landgraf et al., 1984; Fox et al., 1991; Hanson et al., 1994). However, cattle (*Bos tarus*), bison (*Bison bison*), elk (*Cervus elaphus*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and horses (*Equus caballus*) consume little if any leafy spurge (Marlow et al., 1984; Lacey et al., 1985; Lym and Kirby, 1987; Sullivan et al., 1988; Hein and Miller, 1992). In contrast, domestic goats (*Capra hircus*) readily consume leafy spurge in various locations (Hanson and Kirby, 1992, Hanson et al., 1994; Walker and Kronberg, 1992). Domestic sheep (*Ovis aries*) graze it more readily in some areas than in others (Landgraf et al., 1984; Lacey et al., 1984; Kronberg et al., 1992). It has been demonstrated that leafy spurge can elicit conditioned food aversions in cattle, and leafy spurge from an area where sheep generally graze it reluctantly has induced conditioned food aversions in sheep (Kronberg et al., 1993a; Kronberg and Walker, 1993; Kronberg et al., unpublished data). This may explain why cattle seldom consume this plant and why sheep will not readily consume it at some locations.

The identity of the aversive compound(s) in leafy spurge is unknown; however, several different diterpenoid ingenol esters have been isolated from leafy spurge tissues (Kupchan et al., 1976; Upadhyay et al., 1978; Seip and Hecker, 1982; Sorg and Hecker, 1982). One or more ingenol esters may be aversion-eliciting compounds in leafy spurge, possibly due to the capacity of ingenol or ingenol esters to activate the phosphorylating enzyme protein kinase C (PKC) by substituting for, with greater potency, the natural activator diacylglycerol (Aitken, 1987). Protein kinase C is ubiquitous in mammalian tissues (Smith and Meldrum, 1992), with relatively large amounts in the brain, and mediates cellular signal transduction for a large class of hormones and neuro-

transmitters whose effects are mediated through hydrolysis of phosphatidylinositol phosphates (Aitken, 1986, 1987). Adrenergic, histaminergic, muscarinic–cholinergic, and vasopressinergic receptors are involved in phosphatidylinositol hydrolysis (Aitken, 1986).

Activation of the hypothalamic–pituitary–adrenal (HPA) axis appears to be involved in the induction of food aversions by one or more chemicals in leafy spurge (Kronberg et al., 1993b) as well as by other compounds such as lithium (Ader, 1976; Hennessey et al., 1976; Smotherman, 1985), and there are several reports that link the activation of PKC with HPA activation. For example, the release of adrenocorticotropin (ACTH) from the ovine pituitary through stimulation by arginine vasopressin involves activation of PKC, and ACTH secretion appears to be partly regulated by PKC (Liu et al., 1992). Furthermore, the PKC pathway appears to be involved in regulation of gene expression for corticotropin releasing hormone (Van, 1993), which stimulates the release of ACTH. Additionally, dexamethasone, a synthetic glucocorticoid that can inhibit activity of the HPA axis, inhibits both the tumor-promoting and inflammatory activities of phorbol esters (Aitken, 1986) and food aversions caused by lithium and radiation (Hennessey et al., 1976, Revusky and Martin, 1988, Cairnie and Leach, 1982). Phorbol esters are PKC activators. Ingenol esters are closely related to the phorbol esters.

Laboratory rats have been used effectively to study the interactions of phytotoxins in larkspurs (*Delphinium* spp.) and snakeweeds (*Gutierrezia* spp.) with grazing ruminants (Olsen, 1977; Edrington et al., 1993). Therefore, laboratory rats may be a suitable animal model for studies of interactions of leafy spurge with ruminants.

The objectives of this study were to: (1) determine whether or not leafy spurge can elicit food aversions in laboratory rats; (2) determine if a crude acetone extract of leafy spurge, presumably containing diterpenoids along with other chemicals, could cause an aversive feeding response in sheep and rats; and (3) determine whether or not an ingenol monobenzoate with known capacity to activate PKC might also elicit a food aversion in rats.

METHODS AND MATERIALS

Trial 1. Twenty-four laboratory rats (Sprague-Dawley) were randomly divided into four groups (6/group). The subjects were 90 days old and weighed an average of 365 g. Each was individually housed and had continuous access to standard laboratory rat food (Purina Rat Chow)[7] throughout the study.

Subjects were allowed *ad libitum* access to water and food for three days after arrival. Water was then provided at the same time each day for 15 min

[7] Mention of trade names or companies does not constitute an implied warranty by the authors or by the USDA, Utah State University, or Montana State University.

per day for nine days. The final three days were used to compute a baseline on water intake. On the 10th day, saccharine (1.6 g) was stirred into the usual daily fresh water ration prior to the regular access time. Following 15 min of access to this saccharine-water, the treatment subjects were individually anesthetized (see below) and treated with 2 (treatment group 1; T1) or 4 ml (treatment group 2; T2) of a spurge-water slurry. Ground leafy spurge was administered by gavage via a polyethylene tube (2 mm outer diameter) attached to a 20-ml syringe. Thirty milliliters of spurge was suspended in warm tap water (1 : 1 v/v) to form a slurry of leafy spurge for administration.

The volume of liquid and care required for intubation was such that treatment could best be managed with the subjects lightly anesthetized. Each rat was placed in a 20 × 20 × 60-cm clear plastic box and CO_2 was delivered as the animal was closely observed. When the subject became immobile, it was quickly removed, treated, and returned to its home cage and observed during recovery. This process required approximately 3 min per animal and none was lost. Control subjects (treatment group 3; T3) were given saccharine-water on treatment day and were then gavaged with 2 ml of plain water. Subjects in the fourth treatment group (T4) were never offered saccharine-water but were treated with 2 ml of leafy spurge after drinking plain water on the treatment day.

Two days of baseline conditions were allowed for recovery following treatment before tests were conducted. The test session consisted of offering all subjects in all groups except T4 a single bottle of saccharine-water (plain water for T4) of the same composition for the same time as on the treatment day. Again, bottles were weighed and amount consumed was the dependent measure of any effects of the treatment.

Baseline and test intakes were compared within and between treatments by a split-plot design where individuals were nested within their treatment groups, and day (baseline intake on day 1, test intake on day 2) was the subplot treatment. When appropriate, individual means were compared with the protected LSD procedure (SAS, 1988).

Acetone Extraction. Rat chow and leafy spurge were extracted with acetone, and the residues from these extractions were used in trials 2, 3, and 4 (extracted rat chow for trial 2 and extracted leafy spurge for trials 3 and 4). The following procedure was used to prepare the extracts: the rat chow or leafy spurge was ground to pass a 1-mm screen and then stirred with acetone (1 : 5 w/v, substrate to acetone). The mixtures were stirred in a covered container for 24 hr at room temperature, then any fibrous material was removed by filtration and the soluble filtrate was placed under a fume hood until all acetone evaporated, leaving a greasy residue.

Trial 2. Five laboratory rats (Sprague-Dawley; average weight 340 g) were used to determine if an acetone extract of a known nonaversive material (i.e., rat chow) were aversive. Rats were housed individually and had continuous

access to standard rat chow throughout the trial. During the training period, fresh water was provided at the same time each day for 15 min per day for seven days. On the first day of the test period, which immediately followed the training period, saccharine-flavored water (0.10% saccharine, w/v) was offered to the rats for 15 min at the same time as for the training period. Intake of saccharine-water during this period was measured. Rats were then gavaged with the residue from the acetone-extracted rat chow, which was mixed with corn oil at a ratio of 1:10 (w/w) extracted residue to corn oil. This mixture was gavaged at a dosage of approximately 0.002 ml/g of body weight for each rat. On the second day of the test period, saccharine-water was offered to the rats again for 15 min at the normal time, and their intakes of the fluid were measured. Intakes of saccharine-water on both days of the test period were compared, using a *t* test (SAS, 1988), to determine if the rats had an aversive response to the novel-flavored fluid on the second day.

Trial 3. Fifteen laboratory rats (Sprague-Dawley; average weight 300 g) were used in a seven-day trial to determine if an acetone extract of leafy spurge would induce an aversive feeding response in rats. The rats were randomly divided into two groups with eight rats in the treatment group. The rats were housed individually and had continuous access to standard rat chow throughout the trial. During the training period, water was provided for 30 min per day at the same time each day for five consecutive days. On the first day of the test period, which immediately followed the training period, saccharine-water (0.10% saccharine, w/v) was offered to the rats for 30 min at the same time as for the training period. Rats were then gavaged with either the leafy spurge extract in corn oil or the corn oil vehicle (ca. 0.002 ml/g of body weight). The extract was mixed with corn oil (1:10 w/w, extracted residue to corn oil). After completion of the gavage procedure, all rats were offered water for 30 min to equilibrate their thirst since rats drank different amounts of the saccharine-water earlier in the day. On the second day of the test period, the saccharine-water was offered to the rats again for 30 min at the normal time, and their intake of the saccharine-water was measured to determine if the acetone extract of leafy spurge induced an aversion to it.

Intake of saccharine-water was compared using a split-plot design where individuals were nested within their treatment groups, and day was the subplot treatment. When appropriate, individual means were compared with the protected LSD procedure (SAS, 1988).

Trial 4. Fourteen ewes (average weight 60 kg) were used in a nine-day trial to determine if an acetone extract of leafy spurge would induce an aversive feeding response in sheep. The sheep were randomly divided into two equal-sized groups. They were housed individually and had continuous access to water throughout the trial. During the training period (first six days), alfalfa pellets were provided *ad libitum* from 0800 to 1800 hr each day. On the first day of

the test period, rolled oats were offered to all sheep from 0800 to 0900 hr. A gelatin capsule containing the acetone extract of leafy spurge was then placed down the throat of treatment sheep. Control sheep received an empty capsule. The dosage of extract given to each animal was based on the equation: dosage = body weight × 0.15% (aversive dosage for air-dried spurge; Kronberg et al., unpublished data) × 4.5% (acetone-extract residue recovered from air-dried leafy spurge, w/w). After completion of this procedure by 0930 hr, the sheep were offered alfalfa pellets *ad libitum* until 1700 hr. On the second day of the test period, the sheep were offered rolled oats from 0800 to 0900 hr, and their intake of the oats was measured. Intakes of oats on both days of the test period were compared to determine if pairing ingestion of rolled oats with introduction of the leafy spurge extract caused the sheep to develop an aversion to the oats. Data were analyzed as described for trial 3 except oat intake was the dependent variable for this trial with sheep.

Trial 5. Nine laboratory rats (BD-1V Hooded rats; average weight 170 g) were used to determine if an ingenol 3-monobenzoate would induce an aversive feeding response in rats. The rats were randomly divided into two groups with five in the treatment group. The rats were housed individually and had continuous access to standard rat chow throughout the trial. During the training period, water was provided for only 30 min per day at the same time each day for four consecutive days. On the first day of the test period, which immediately followed the training period, saccharine-water (0.10% saccharine, w/v) was offered to the rats for 30 min at the same time as used in the training period. Rats were then gavaged with either the ingenol ester or the ethanol vehicle. Four milligrams of the ingenol ester was dissolved in 8 ml of ethanol and this solution, as well as the vehicle alone, were gavaged at a dosage of 0.001 ml/g of body weight. After completion of the gavage procedure (about half an hour after completion of the drinking period), all rats were offered water for 30 min to equilibrate their thirst. On the second day of the test period, the flavored water was offered to the rats again at the normal time for 30 min to determine if the ingenol ester had induced an aversion to the novel fluid. Data were analyzed as described for trial 3.

RESULTS

Trial 1. Leafy-spurge treatment reduced ($P < 0.01$) saccharine-water intake relative to the control group of rats (Figure 1). The 4-cc dose of spurge (treatment group T2) was more effective ($P = 0.03$) than the 2-cc dose (treatment group T1) at reducing saccharine-water ingestion. Rats in the control group (T3) did not show ($P = 0.40$) suppressed ingestion of saccharine-water during test

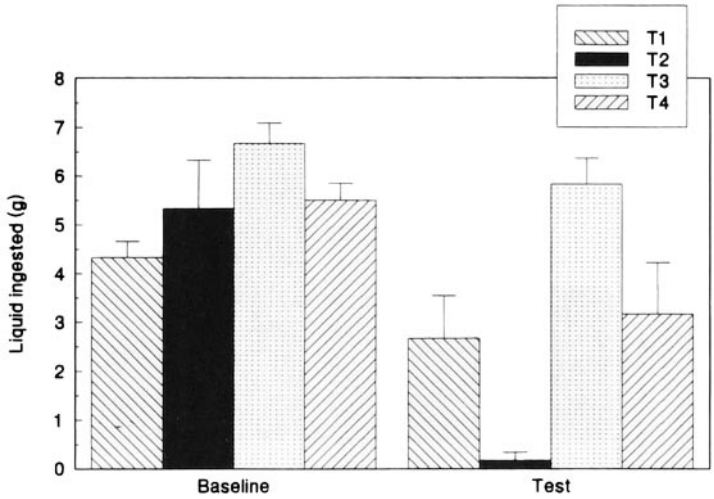

FIG. 1. Ingestion by rats of saccharine-water before and after receiving either 2 ml of leafy spurge (treatment 1, T1), 4 ml of leafy spurge (treatment 2, T2), or 2 ml of plain water (treatment 3, T3) and ingestion by rats of plain water before and after receiving 2 ml of leafy spurge (treatment 4, T4).

sessions. Rats that received spurge after drinking plain water (treatment group T4) reduced ($P = 0.02$) their subsequent intake of plain water.

Trial 2. The residue from acetone-extracted rat chow did not induce a food aversion in the rats. Rats consumed 13.0 (SE = 0.85) and 15.8 (SE = 1.3) ml of saccharine-flavored water on the first and second days of the test period, respectively, and these means were not different ($P = 0.11$).

Trial 3. Rats in treatment and control groups ingested similar ($P = 0.80$) amounts of the saccharine-water on the first day of the test period (Figure 2). However, rats that received a gastric infusion of the acetone extract of leafy spurge (treatment group SPUEXT) after ingesting the saccharine-water ingested less ($P < 0.01$) of this liquid than did control rats (treatment group CNTL) when it was offered to them a second time. The control rats increased ($P < 0.01$) their intake of the saccharine-water from the first to second days, but the treatment rats did not ($P = 0.28$).

Trial 4. Sheep in treatment and control groups ingested similar ($P = 0.99$) amounts of the rolled oats on the first day of the test period (Figure 3). Sheep that received a capsule containing the acetone extract of leafy spurge (treatment group SPUEXT) after ingesting the rolled oats ate less ($P < 0.01$) of them than

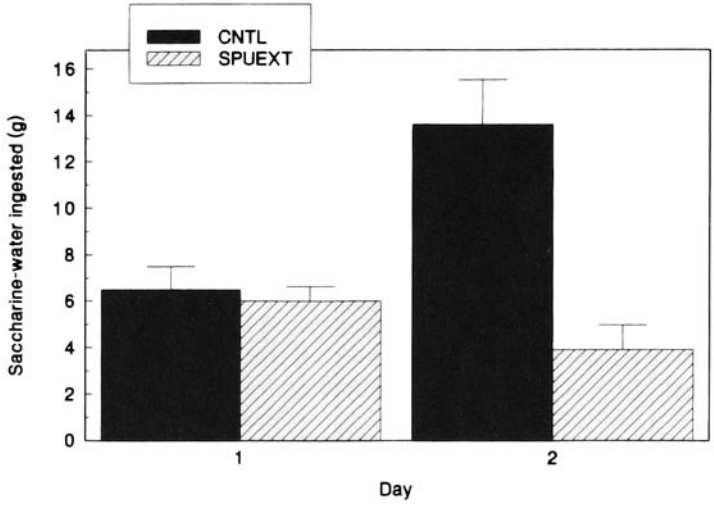

FIG. 2. Ingestion by rats of saccharine–water before and after they were gavaged with either an acetone extract of leafy spurge (SPUEXT) or only the vehicle (CNTL).

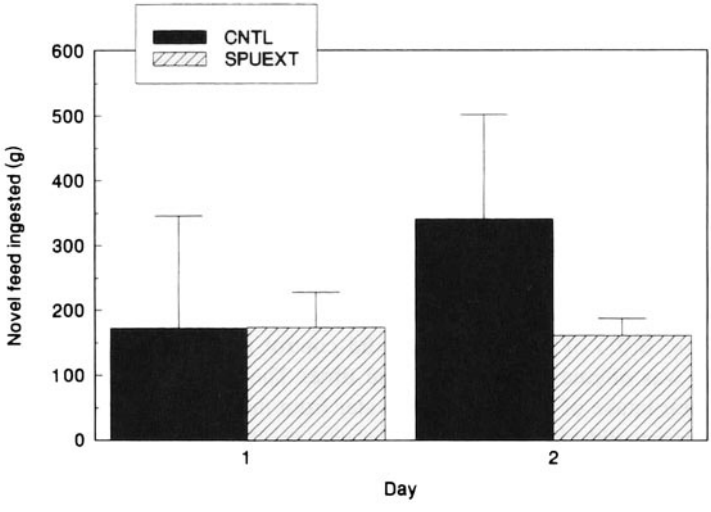

FIG. 3. Ingestion by sheep of rolled oats before and after they were bolused with either a gelatin capsule containing an acetone extract of leafy spurge (SPUEXT) or an empty gelatin capsule (CNTL).

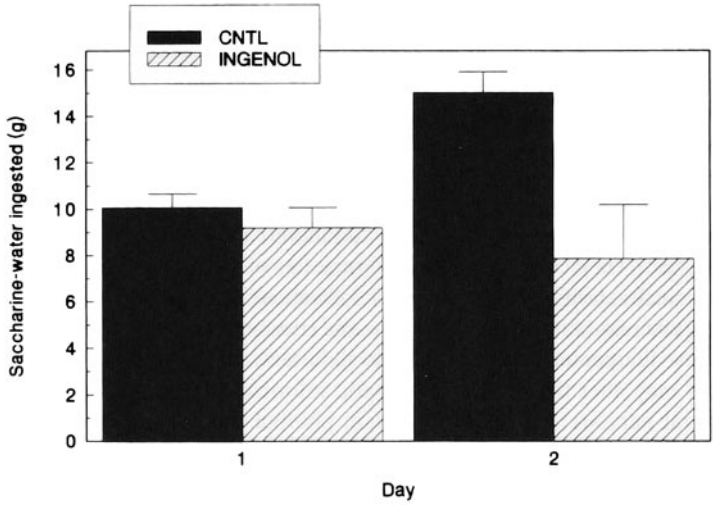

FIG. 4. Ingestion by rats of saccharine water before and after they were gavaged with either ingenol 3-monobenzoate (INGENOL) or only the vehicle (CNTL).

the control sheep (treatment group CNTL) when they were offered this food a second time.

Trial 5. Rats in treatment and control groups ingested similar ($P = 0.67$) amounts of the saccharine-water on the first day of the test period (Figure 4). However, rats that received a gastric infusion of ingenol 3-monobenzoate after ingesting the saccharine-water (treatment group INGENOL) ingested less ($P < 0.01$) of this solution than did control rats (treatment group CNTL) when it was offered to them a second time. The control rats increased ($P < 0.05$) their intakes of the liquid from the first to the second days, but the treatment rats did not ($P < 0.50$).

DISCUSSION

We interpret the results from the trials to suggest that leafy spurge can elicit an aversive feeding response in rats as it does in cattle and sheep (Kronberg et al., 1993a; Kronberg and Walker, 1993). Additionally, an acetone extract of leafy spurge can elicit an aversive feeding response in both rats and sheep; therefore, the aversive compounds in leafy spurge are apparently soluble in acetone. Given the results from the rat trials, we suggest that rats may be effective models for cattle and sheep with respect to studies on phytochemicals in leafy spurge that influence the feeding behavior of these livestock. Results

from the fifth trial indicate that an ingenol ester may be an aversion-inducing component of leafy spurge.

Ingenol and its esters vary considerably in their biological activity. Ingenol, the parent alcohol, has no to weak activity (Hirata 1975; Hirota et al., 1980; Hasler et al., 1992), certain ingenol esters exhibit inflammatory effects or tumor promotion (Upadhyay et al., 1978; Seip and Hecker, 1982; Sorg and Hecker, 1982), and another ingenol ester has antitumor activity (Kupchan et al., 1976). Hirota et al. (1980) found considerable variation in the toxicity of various ingenol esters to fish. They concluded that acyl moieties of ingenol ester were necessary for piscicidal activity. Certain ingenol 3,20-diesters had the greatest piscicidal activity; whereas certain ingenol mono- and triesters had weak toxicity. Ingenol, the parent diterpenoid, was not toxic to their fish. In addition to these findings, ingenols may alter the transfer of genetic information or have other effects on the central nervous system as do certain phorbol esters (Speroni et al., 1991; Cahill and Perlman, 1992; Emanuel et al., 1992; Van, 1993).

The type of ingenol esters contained in leafy spurge appears to vary as a function of environmental factors (Seip and Hecker, 1982) such that leafy spurge growing at two locations and/or times may exhibit different effects on the animals that ingest it. This may explain why the palatability of leafy spurge to sheep appears to vary as a function of location of the leafy spurge (Kronberg et al., 1992).

The capacity of ingenol and different ingenol esters to activate PKC is quite variable. Hasler et al. (1992) found that ingenol binds to and activates PKC weakly with a K_i of 30 μM. In contrast, ingenol 3-monobenzoate had an apparent K_i of 0.14 $\pm$ 0.04 nM for PKC, while ingenol 3,20-dibenzoate had a K_i of only 240 $\pm$ 9 nM for PKC (Winkler et al., 1993). This may explain why ingenol 3,20-dibenzoate did not elicit an aversion in sheep when it was injected into their blood stream after ingestion of a novel feed (Kronberg and Walker, 1993).

Lithium has induced food aversions in a large variety of animals (Riley and Tuck, 1985). Lithium chloride and phorbol 12-myristate 13-acetate (PMA, a compound that activates PKC and has a structure similar to that of the ingenol esters) appear to share a similar effect in certain mammalian cells. Both lithium and PMA inhibit gene expression for phosphoenolpyruvate carboxykinase and thereby inhibit gluconeogenesis (Chu and Granner, 1986; Bosch et al., 1992). Perhaps lithium and ingenol esters elicit aversion through related mechanisms. This may help to explain why both leafy spurge and lithium elevate blood cortisol levels and condition food aversions in certain mammals (Hennessey et al., 1976; Smotherman et al., 1976; Smotherman, 1985; Kronberg et al., 1993b).

Kronberg et al. (1993b) and others (Ader, 1976; Braveman, 1977; Smotherman, 1985) have argued that stress and activity of the hypothalamic-pituitary-adrenal (HPA) axis is involved in the development of conditioned aversions. Therefore, if ingenol esters exist in leafy spurge that can activate PKC, then

activity of corticotropin releasing hormone is likely stimulated (Van, 1993) and, consequently, the HPA axis is likely activated (Kronberg et al., 1993) when leafy spurge is ingested and ingenol esters are absorbed into the blood stream from the digestive tract. This process may lead to avoidance of leafy spurge by the offended animal.

In summary, we interpret our data to suggest that leafy spurge can elicit food aversions in rats as it does in cattle and sheep; consequently, rats can be used to model certain interactions of leafy spurge with these ruminant species. We also suggest that one or more ingenol esters may exist in leafy spurge that can elicit aversions in certain mammals and cause these mammals to avoid future ingestion of leafy spurge. However, other compounds may exist in leafy spurge that can also elicit conditioned food aversions or may be the only or primary aversive chemicals.

Acknowledgments—We thank J.D. Winkler for his generous gift of the ingenol monobenzoate used in the fifth trial and E. Carter for his assistance in conducting some of the trials. We also thank C. Dwivedi, D.C. McFarland, and anonymous reviewers for their comments on the manuscript.

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VOLATILE KETONES FROM INTERDIGITAL GLANDS OF BLACK-TAILED DEER, *Odocoileus hemionus columbianus*

WILLIAM F. WOOD,* TONY B. SHAFFER, and AYA KUBO

Department of Chemistry,
Humboldt State University
Arcata, California 95521

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Abstract—Three volatile ketones, 2-tridecanone, (*E*)-3-tridecen-2-one and (*E*)-4-tridecen-2-one were identified in interdigital gland extracts from black-tailed deer, *Odocoileus hemionus columbianus*. The major compound, (*E*)-3-tridecen-2-one, inhibited the growth of several species of fungi and gram-positive bacteria. The bacterium, *Propionibacterium acnes*, and the fungus, *Trichophyton mentagrophytes* required a minimum concentration (MIC) of 12.5 μg/ml and 25 μg/ml, respectively, for inhibition. (*E*)-3-Tridecen-2-one represents a new class of lipid antibiotics found on animal skin. The second major compound is this secretion, 2-tridecanone, was not active against any of the organisms tested.

Key Words—Antibiotic lipids, antimicrobial activity, Cervidae, deer, gram-positive bacteria, fungi, interdigital gland, *Odocoileus hemionus columbianus*, 2-tridecanone, (*E*)-3-tridecen-2-one, (*E*)-4-tridecen-2-one.

INTRODUCTION

Interdigital glands are found in most species of deer (Cervidae), antelope (Bovidae), and pronghorns (Antilocapridae). There have been few investigations on the chemical composition of the secretion from this gland. A single Old World cervid, the reindeer, *Rangifer tarandus tarandus* (Andersson et al., 1979; Brundin et al., 1978; Brundi and Andersson, 1979; Sokolov et al., 1974) and one African antelope, the bontebuck, *Damaliscus dorcas dorcas* (Burger et al., 1976, 1977) have been investigated.

*To whom correspondence should be addressed

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It has long been speculated that interdigital glands are involved in semiochemical communication. Lydekker (1898) suggested their function was "to leave a strong scent upon the ground over which their owner has passed." Research on reindeer interdigital gland secretion indicates that one function may be track-laying and track-following. Müller-Schwarze et al. (1978) showed that reindeer actively investigate interdigital gland secretion of conspecifics, and Brundin et al. (1978) indicated response to two short-chain acids of this secretion, isovaleric and isobutyric acids. The compounds identified from the interdigital (pedal) gland of the bontebuck have not been the subject of a comprehensive behavioral study. In preliminary tests, intense interest in these compounds was reported to have been elicited in two captive animals (Burger et al., 1976), but a later report indicated limited response of conspecifics to interdigital gland secretion or to its components (Bigalke and Novellie, 1979).

Black-tailed deer (*Odocoileus hemionus columbianus*) are a subspecies of mule deer that inhabit the Pacific coastal mountain ranges of North America from central California to central British Columbia and have well-developed interdigital glands in fore- and hind-feet. We investigated the volatile compounds of these glands and identified three ketones (Figure 1). Due to the difficulty of doing behavioral experiments on free-ranging animals and the fact that Müller-Schwarze (1971) had not observed any specific behavior associated with deer smelling the hoof prints of conspecifics, we investigated other possible natural functions of these compounds. Hoof rot has been reported to have a serious effect on the mortality of black-tailed deer in areas where they are forced to concentrate at muddy springs (Tabor and Dasmann, 1958). Since, to our knowledge, antimicrobial properties of mammalian exocrine gland secretions have been little investigated, we tested the two major compounds for activity. One compound, (*E*)-3-tridecen-2-one, showed antibiotic activity against fungi and gram-positive bacteria.

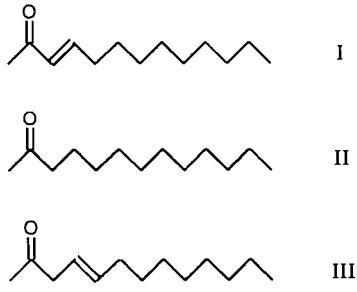

FIG. 1. The ketones identified from black-tailed deer interdigital glands: (*E*)-3-tridecen-2-one (I), 2-tridecanone (II), and (*E*)-4-tridecen-2-one (III).

METHODS AND MATERIALS

The interdigital gland secretion from five male and three female deer were obtained from animals taken by sport hunters or under depredation permits for crop damage. All animals were from Humboldt County, California. One was a yearling and the others were at least 2 years old.

Soon after harvesting, the secretion was scraped from the glands and extracted with dichloromethane. The resulting solution was filtered through glass wool and then analyzed by gas chromatography–mass spectroscopy (GC-MS). Fore- and hind-foot glands were examined separately to see if there was a difference in the secretions. As a control, samples of hair from fore- and hind-legs, twice the weight of the material from the gland, were removed 5 in. above the gland from two males and two females. This hair was extracted and analyzed as described for material from the gland.

GC-MS was performed using a Hewlett-Packard gas chromatograph (model 5890) fitted with a mass selective detector (model 5970) using a 12-m cross-linked methyl silicone capillary column (HP-1). The gas chromatography was programmed so the oven temperature was kept at 40°C for 4 min, then increased to a final temperature of 250°C at a rate of 30°C/min and kept at this temperature for 4 min. Mass spectral fragments below $m/z = 35$ were not recorded. The relative amount of each component is reported as the percent of the total ion current.

(*E*)-3-Tridecen-2-one (I) was synthesized by the method of Kologrivova and Belnov (1958). (*E*)-4-Tridecen-2-one (III) was a minor by-product in the synthesis of 3-tridecen-2-one. A pure sample was obtained by preparative gas chromatography using a Varion Aerograph model 90P TC gas chromatograph fitted with a 2-m × 8 mm 5% SE-30 column.

FT-IR was performed on a Perkin-Elmer 1600 and NMR on a Bruker QE Plus. Microozonolysis was accomplished as described by Beroza and Bierl (1966) and products were identified by GC-MS. The isomerization of (*E*)-4-tridecen-2-one to (*E*)-3-tridecen-2-one was effected by adding one drop of 12 M HCl to 2 mg of (*E*)-4-tridecen-2-one in 20 ml of diethyl ether. GC-MS analysis was executed at 1, 10 and 24 hr.

(*E*)-3-Tridecen-2-one and 2-tridecanone (Pfaltz & Bauer) were tested against the following strains of microorganisms purchased from the American Type Culture Collection (Rockville, Maryland): *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598 (a methicillin-susceptible strain), *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 9637, *Proteus vulgaris* ATCC 133315, *Saccharomyces cerevisiae* ATCC 7754, *Candida utilis*

ATCC 9226, *Pityrosporum ovale* ATCC 14521, *Penicillium chrysogenum* ATCC 10106, and *Trichophyton mentagrophytes* ATCC 18748.

Except for *S. mutans*, bacterial cultures were grown in 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco), and 0.1% glucose (NYG broth). *S. mutans* was cultured in 3.7% brain–heart infusion broth (Difco). All fungi, except *P. ovale* and *T. mentagrophytes*, were cultured in 2.5% malt extract broth (BBL). *P. ovale* was cultured in 1% bactopeptone (Difco), 0.5% yeast extract, 1% glucose, and 0.1% corn oil. For *T. mentagrophytes*, the culture medium consisted of 1% bactopeptone and 4% glucose.

Freeze-dried samples of microorganisms were prepared for testing as follows: *B. subtilis*, *S. cerevisiae*, *C. utilis*, and *P. ovale*, were cultured in shake flasks for two days at 30°C. *P. chrysogenum* and *T. mentagrophytes* were cultured in shake flasks for five days at 30°C. *B. ammoniagenes* and *E. aerogenes* were grown stationary at 30°C. *S. aureus*, *S. mutans*, *P. acnes*, *P. aeruginosa*, *E. coli*, and *P. vulgaris* were grown stationary at 37°C.

The minimum inhibitory concentration (MIC) of the 3-tridecen-2-one and 2-tridecanone was determined using a twofold serial broth dilution. Each test compound was dissolved in DMF and 20 μl of this sample was dissolved in 3 ml of the appropriate medium. A 30 μl sample of the previously described culture of each microorganism was added to the various media solutions. After two days the cultures of *B. subtilis*, *S. cerevisiae*, *C. utilis*, *B. ammoniagenes*, *E. aerogenes*, *S. aureus*, *S. mutans*, *P. acnes*, *P. aeruginosa*, *E. coli*, and *P. vulgaris* were examined for turbidity (OD at 660 nm). The fungi, *P. ovale*, *P. chrysogenum*, and *T. mentagrophytes*, were examined visually for growth at three days (*P. ovale*) and five days (*P. chrysogenum* and *T. mentagrophytes*). The MIC was determined to be the lowest concentration of each compound for which no growth was observed. The highest concentration used in these tests was 800 μg/ml.

RESULTS

The secretion is an unctuous material that covers the inner surface of the gland and adheres to the hair growing in the gland. It exudes from the gland and stains the hair on the dorsal surface of the foot and the hair between the hooves. GC-MS analysis of dichloromethane extracts of this secretion from both male and female showed the same major compound with a retention time of 9.45 min. The relative amount of compound varied considerably, but not consistently, between fore- and hind-foot extracts and between male and female. These amounts ranged from 8% to 57% of the volatiles, with an average of 31%.

This compound exhibited a molecular ion at $m/z = 196$, which suggested

a compound with a molecular formula of $C_{13}H_{24}O$, containing two sites of unsaturation. The base peak at $m/z = 43$ indicated that one site of unsaturation is due to a methyl ketone (CH_3-CO-). A fragment at $m/z = 69$ implied that a double bond is adjacent to the ketone ($CH_3-CO-CH=CH-$). A possible candidate compound, 3-tridecen-2-one, was synthesized (Kologrivova and Belnov, 1958) and found to have identical retention time and mass spectrum as the major compound in the gland. The natural sample of 3-tridecen-2-one was shown to be the *E*-isomer by the ^1H NMR coupling constant of 15.9 Hz for the olefinic protons and an IR absorption at 980 cm^{-1}. The spectral properties of this liquid are 300 MHz ^1H NMR ($CDCl_3$) $\delta = 6.81$ (dt, 1H, $J = 15.9$ Hz, 6.9 Hz), 6.06 (dt, 1 H, $J = 15.9$ Hz, $J = 1.48$ Hz), 2.24 (s, 3H), 2.22 (quart, 2H), 1.46 (m, 2H), 1.26 (m, 12H) and 0.88 (t, 3H); 75 MHz ^{13}C NMR ($CDCl_3$) $\delta =$ 198.80, 148.69, 131.24, 32.47, 31.85, 29.46, 29.37, 29.27, 29.17, 28.07, 26.79, 22.65, and 14.09; FT-IR (neat) 2925, 2854, 1700, 1677, 1628, 1467, 1360, 1253, 1189 and 980 cm^{-1}; and EI-MS $m/z = 196$ (M^+, 2), 181 (8), 97 (31), 96 (14), 83 (22), 81 (20), 71 (34), 69 (31), 55 (65), 43 (100), 41 (44). This is the first identification of 3-tridecen-2-one from a natural source.

A second compound with a retention time of 9.24 min was found in all samples and was the second most abundant volatile. The relative amount of the second compound varied considerably, but not consistently, between fore- and hind-foot extracts and between male and female. The amount ranged from a trace to 30% of the volatiles, with an average of 9.6%. This compound had a molecular ion at $m/z = 198$ that indicated a molecular formula of $C_{13}H_{26}O$ and one site of unsaturation. The base peak at $m/z = 43$ suggested a methyl ketone (CH_3-CO-). A major fragment at $m/z = 58$ due to a McLafferty rearrangement ($CH_3-CO-CH_3$) corroborates a methyl ketone. A possible candidate compound, 2-tridecanone, had the same mass spectrum and retention time as the compound from the deer's gland. The EI-MS of this compound was $m/z =$ 198 (M^+, 1.3), 96 (3.7), 85 (6.2), 71 (23), 69 (4.2), 59 (32), 58 (92), 55 (13), 43 (100), 41 (32) and 39 (8.1).

The number of other compounds seen in the gas chromatogram varied with each different extract. At the low end, one extract had three volatile components and at the high end there were 45 detectable compounds. While the number of volatile compounds varied considerably, there was no consistency between fore- and hind-foot or male and female. The identities of these minor compounds were not investigated except for a compound with a retention time of 9.21 min. This compound was observed in all samples but one and ranged in amount from a trace to 5% of the volatiles, averaging 1.2%.

This minor compound exhibited a molecular ion at $m/z = 196$ and a base peak at $m/z = 43$, suggesting it was an isomer of (*E*)-3-tridecen-2-one. The mass spectrum and retention time of this compound were identical to a minor component observed in the preparation of (*E*)-3-tridecen-2-one. A synthetic

sample of the compound was isolated by preparative gas chromatography. The FT-IR spectrum showed an unconjugated carbonyl absorption at 1718 cm^{-1} and an absorption at 968 cm^{-1}, indicating a *trans*-double bond. The double bond was shown to be at the 4 position by ozonolysis. Nonanal, one product expected on ozonolysis of a double bond at this position, was identified by comparison with an authentic sample using GC-MS. Also as expected, (*E*)-4-tridecen-2-one isomerized to the more stable (*E*)-3-tridecen-2-one on treatment with hydrochloric acid. The spectral properties of this liquid are FT-IR (neat) 2924, 2854, 1718, 1653, 1457, 1356, and 969 cm^{-1}, and EI-MS m/z = 196 (M^+, 0.4), 96 (4), 69 (5), 55 (10), 54 (6), 43 (100) and 41 (12). 4-Tridecen-2-one has not been reported previously in the chemical literature.

GC-MS analysis of extracts from the control samples of hair removed from the leg above the interdigital gland showed none of the ketones identified in the interdigital gland secretion. Clearly, the source of these compounds is the interdigital gland secretion.

In order to determine whether the two major interdigital gland ketones were active against microorganisms, these compounds were tested with various bacteria, yeast, and fungi. (*E*)-3-Tridecen-2-one showed activity [minimum inhibitory concentration (MIC)] against the gram-positive bacteria *Bacillus subtilis* (100 μg/ml), *Brevibacterium ammoniagenes* (100 μg/ml), *Staphylococcus aureus* (50 μg/ml), *Streptococcus mutans* (25 μg/ml), and *Propionibacterium acnes* (12.5 μg/ml). This compound was not active (MIC > 800 μg/ml) against the gram-negative bacteria *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Escherichia coli*, and *Proteus vulgaris*. Activity against yeast was mixed; no activity (MIC > 800 μg/ml) was observed against *Saccharomyces cerevisiae* and *Candida utilis*, although *Pityrosporum ovale* was moderately inhibited (MIC, 100 μg/ml). Weak activity was observed with the fungus *Penicillium chrysogenum* (MIC, 800 μg/ml), while the fungus *Trichophyton mentagrophytes* was strongly inhibited at 25 μg/ml. 2-Tridecanone showed no activity against these microorganisms.

DISCUSSION

The ketones, (*E*)-3-tridecen-2-one, (*E*)-4-tridecen-2-one, and 2-tridecanone, from the interdigital gland of the black-tailed deer are similar to many compounds identified from other species. 1-Hydroxy-7-methyl-3-octanone, 7-methyl-1-octen-3-one, 7-methyl-3-octanone, and 6-methyl-2-heptanone are reported as constituents in the interdigital gland secretion of the reindeer (Andersson et al., 1979). The seasonal variation of three of these ketones was later studied by Brundin and Andersson (1979). Eight short-chain carboxylic acids were also found in reindeer interdigital gland secretions (Brundin et al., 1978).

The only other ungulate interdigital gland secretion that has been investigated is that from the bontebok, a rare antelope from South Africa (Burger et al., 1976, 1977). The major volatile constituent found in this gland was (*Z*)-5-undecen-2-one. Minor components from this gland included four methyl ketones: 2-heptanone, 2-nonanone, 2-undecanone, and 2,5-undecanedione.

It is widely believed that a major function of the interdigital glands is semiochemical communication with conspecifics. Two studies have shown that reindeer actively investigate interdigital gland secretion and that it may act as a trail pheromone for single animals separated from the herd (Müller-Schwarze et al., 1978; Brundin et al., 1978). Preliminary biological tests of synthetic compounds of bontebok interdigital gland secretion elicited intense interest by two captive animals (Burger et al., 1976.) A later report indicated limited response to interdigital gland secretion or to its components but suggested it might be used by conspecifics to indicate use or presence of other animals (Bigalke and Novellie, 1979).

In a report on the pheromones of black-tailed deer, Müller-Scharze (1971) observed deer investigating deep foot prints of conspecifics but saw no specific reaction to this behavior. There may be a connection between the interdigital gland secretion and forehead rubbing, another behavior reported by Müller-Schwarze. Black-tailed deer of both sexes mark dry tips of branches with their forehead. These scent-marked twigs are actively investigated by other deer and may help to mark territories, since introduced deer retreat upon smelling the twigs marked by inhabitants of the area. Müller-Schwarze observed deer rubbing their hind leg over their forehead. Since the secretion of the hind-foot interdigital gland stains the dorsal surface of the foot, it may be transferred to the head and become part of the forehead pheromone. We did not undertake behavioral studies of these compounds, and further studies are needed to determine the semiochemical role of these ketones.

The major compound from the interdigital gland shows a broad range of activity against microorganisms, suggesting that a major function of interdigital gland secretions might be to produce antibiotics. The speculation that the function of ungulate interdigital gland secretion is used primarily for semiochemical marking may need to be reinvestigated. Interdigital gland compounds may serve a dual function as semiochemical markers and antibiotics. It is also possible that antimicrobials may be included in animal exocrine gland secretions to keep semiochemicals from being metabolized by microorganisms.

Antibiotic lipids have previously been identified from the skin of animals. These compounds are fatty acids and their monoglycerol esters (Kabara et al., 1977; Bibel et al., 1989) and glycerosphingolipids and phopholipids (Bibel et al., 1989). To the best of our knowledge, (*E*)-3-tridecen-2-one represents a new class of lipid antibiotics found on animal skin. This ketone is water insoluble and would be adsorbed on skin, hair, and hoof. It is of note that *T. mentagro-*

phytes, the organism responsible for athlete's foot in humans and that also infects other animals, is strongly inhibited by this compound.

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IDENTIFICATION OF AN ATTRACTANT FOR THE CANEBORER *Sesamia grisescens* WALKER (LEPIDOPTERA: NOCTUIDAE)

C.P. WHITTLE,[1,*] R.A. VICKERS,[1] L.S. KUNIATA,[2] T.E. BELLAS,[1] and E.R. RUMBO[1]

[1]*CSIRO Division of Entomology, GPO 1700, Canberra, A.C.T., Australia.*

[2]*Ramu Sugar Ltd., Gusap, PO Box 2183 Lae, Papua New Guinea*

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Abstract—The composition of the sex pheromone of *Sesamia grisescens* was investigated using gas chromatography, electroantennograms, and field trapping. (*Z*)-11-Hexadecenyl acetate and (*Z*)-11-hexadecenol were identified in field tests as the major attractants. Trapping trials identified a 3:2 blend of these compounds as the most effective bait. Gas chromatography indicated the presence of hexadecyl acetate, (*Z*)-9-hexadecenyl acetate, (*Z*)-9-hexadecenol, and (*E*)-11-hexadecenyl acetate in the pheromone gland, but these compounds had no significant effect on trap catches when added to the major components. Traps baited with the major components in a 1:1 ratio caught more male moths than traps baited with virgin females.

Key Words—Caneborer, sugarcane, trapping, electroantennogram, sex pheromone, attractant, Insecta.

INTRODUCTION

The noctuid borer, *Sesamia grisescens*, has been recorded from sea level to 1600 m above sea level on the Papua New Guinea (PNG) mainland and offshore islands (Szent-Ivany and Ardley, 1962; Bourke, 1968; Anon., 1969; Bourke et al., 1973). Known host plants include sugarcane, *Saccharum officinarum* L.; edible "pitpit," *Saccharum edule* Hassk; wild "pitpit," *Saccharum robustum* Brandes and Jeswiet ex Grassl (Bourke, 1968; Anon., 1969; Bourke

*To whom correspondence should be addressed.

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et al., 1973; Young, 1982; Li, 1985); *Saccharum spontaneum*, *Pennisetum purpureum*, and *Panicum maximum* (Young and Kuniata, 1992).

S. grisescens is the most important insect pest of sugarcane in the Ramu Valley of Papua-New Guinea. Although the extent of damage varies from year to year, losses are always significant, and in 1987 were estimated at $A10 million (P. Sweet, Ramu Sugar Ltd., personal communication, 1991). Adult females deposit clusters of ca. 120 eggs beneath tightly folded leaf sheaths, from whence newly hatched larvae bore into the cane stems in which they complete their larval development and pupate before emerging as adults. Thus, no immature stage is amenable to control with contact insecticides. The systemic insecticide Furadan has proven ineffective and, so far, attempts at biological control with the larval parasite *Cotesia (=Apantales) flavipes* (Hymenoptera: Braconidae) have been of limited value. Lack of a suitable means of control, a risk of entry into Australia, and a desire by the Australian Quarantine Inspection Service for an effective means of detecting the presence of *S. grisescens* led to an attempt to identify the insect's pheromone, which could then be employed in traps for quarantine purposes and be evaluated as a mating disruptant for control.

Attractants or pheromones are known for four *Sesamia* species. *S. cretica* is attracted to a mixture of (Z)-9-tetradecenyl acetate (Z9-14:OAc) and (Z)-9-tetradecenol (Z9-14:OH) (Arsura et al., 1977). These two compounds, together with (Z)-11-hexadecenyl acetate (Z11-16:OAc) and (Z)-11-hexadecenol (Z11-16:OH), have been found in the pheromone gland of *S. calamistis* (Zagatti et al., 1988). Furthermore, Z11-16:OAc and Z11-16:OH have been found to be components of the pheromone of both *S. nonagrioides* (Sreng et al., 1985; Rotundo et al., 1985; Mazomenos, 1989) and *S. inferens* (Nesbitt et al., 1976; Wu and Cui, 1986; Zhu *et al.* 1987). It was considered likely that a combination of some or all of these compounds would also be attractive to *S. grisescens*.

MATERIALS AND METHODS

Insect Material. Pupae were collected from sugar cane at Ramu Sugar Ltd. plantations at Gusap in Papua New Guinea during the first week of August 1991. The male and female pupae (100 of each) were separated and packed in sealed containers, which were hand delivered to quarantine in Canberra, Australia (Whittle et al., 1993). The timing of the collection was chosen as an additional precaution against accidental release and establishment of the pest in Australia since it is unlikely that it could survive under the climatic conditions in Canberra at this time of year. Moreover, there are no known host plants in the vicinity of Canberra.

Male and female pupae were placed in separate cages in an environment cabinet with a relative humidity of 70–80% and temperatures of 25°C and 20°C, respectively, at 12L:12D. At the beginning of each photophase, emerged adults

were collected and held individually in waxed paper cups fitted with clear plastic lids and a feeding tube containing 5% honey solution. At no time were the insects permitted to mate. At the end of the investigation, apart from voucher specimens lodged with the Australian National Insect Collection, all insect material was destroyed.

Identification of Pheromone Gland Components. Observation of mating behavior in *S. grisescens* has shown that the moths become sexually active 1 hr before dawn (L.S. Kuniata, unpublished data). Ovipositors were excised from 3-day-old female moths 3–4 hr before the onset of photophase. The ovipositor was placed in a small test tube drawn from 4-mm-ID glass tubing and allowed to stand for 15 min in 15 μl of hexane (Waters Associates, HPLC grade). The hexane extract (and washings, 5 μl) was removed with a syringe and injected in 3-μl aliquots into a small glass tube packed with glass beads (60–80 mesh, 250 mg). Hexane was evaporated from each aliquot in a gentle stream of nitrogen (60 sec, 20 ml/min). Pheromone components were then evaporated from the glass beads under helium (5 ml/min) at 135°C for 10 min, and the effluent was trapped in a cooled (Dry Ice) glass capillary connected by glass-lined stainless steel tubing and PTFE sleeve. The trapped volatiles were washed from the capillary by injecting and then recovering a bead of hexane (2 × 10 μl). A reference mixture of normal hydrocarbons (C_{18}, C_{19}, C_{20}, C_{22}, C_{24}; or C_{17}, C_{19}, C_{21}, C_{23}; 10 ng/component) was added. The mixture was then examined by gas chromatography using Varian 3300 and Carlo Erba HRGC 5300 instruments fitted with splitless injectors and capillary columns (SGE BP20, polyethylene glycol, bonded phase 1.0 μm, 25 m × 0.32 mm, 170°C and SGE BP5, 5% phenyl siloxane, 95% dimethyl siloxane, bonded phase 1.0 μm, 12 m × 0.32 mm, 210°C, respectively; carrier gas was He). Injections were made at 40°C and isothermal conditions maintained for 4 min (inlet purged after 2 min). The column oven was then rapidly heated (50°C/min) to the operating temperature (170°C or 210°C) and the remainder of the run completed isothermally at that temperature. Under these conditions the log/linear relationship between retention time and carbon number still applies and computed Kovats indices are reproducible to ±0.3 units (Bellas, 1975; Guardino et al., 1976). Data were collected with a Hewlett Packard HP3392A integrator and a DAPA data system (DAPA Scientific Pty. Ltd., Western Australia). Peaks were assigned by coincidence with those obtained from known compounds.

Electrophysiology. Electroantennogram measurements were carried out as previously described (Rumbo, 1988), with a few small differences. Instead of using a glass electrode to connect to the tip of the antenna, the stump remaining after cutting off a few terminal segments from the tip of the antenna was covered with a conductive gel (Lectrogel, an electrosurgical preparation from Valleylab) and a Ag–AgCl electrode inserted into the gel. Lectrogel was similarly used to cover a slit made in the abdomen of the insect for the ground electrode.

Sets of sources containing 10 μg of *Z* and *E* isomers of C_{14} and C_{16} alkenyl

acetates were tested against the male antennae. A blank source was added to each set and sources within a set were presented at random as prompted by a microcomputer. Each set of sources was presented to the same insect three or four times depending on the life of the antenna. A different insect was used for each set. To compensate for aging effects and varying sensitivity between antennae, the measurements were normalized to an average response of unity.

Preparation and Analysis of Baits. All synthetic acetates and alcohols were of >99% purity by gas chromatography and obtained from the Research Institute for Plant Protection, Wageningen, The Netherlands, and Shin-Etsu Chemical Co. Ltd., Japan. Synthetic pheromone baits were prepared by dispensing 10 μl doses from stock solutions of candidate components onto 20 mm lengths of surgical rubber tubing (4.5 mm ID, 6.5 mm OD). The stock solutions contained the candidate mixture as a 10% v/v solution in toluene. Rubber tubing dosed with 10 μl of toluene alone provided blanks. The particular compounds or mixtures, together with ratios, are indicated in Tables 1 and 3 below.

In order to measure the volatiles released from a synthetic pheromone bait, five baits were threaded on a wire and placed in a glass tube through which was passed a stream of nitrogen at 60 ml/min. Volatiles in the nitrogen stream were trapped at the outlet with a small tube packed with glass beads (described above). After a collection time of at least 24 min, the trapped volatiles were desorbed and analyzed by gas chromatography as described above using an SGE BP10 column (14% cyanopropylphenyl siloxane, 86% dimethyl siloxane, bonded phase 0.5 μm, 12 m × 0.32 mm, 160°C).

Field Trials. Field trials were carried out using traps similar in design to the Pherocon 1CP trap (Zoecon Company, Palo Alto, California), hereinafter called wing traps. Synthetic pheromone baits were placed centrally on the bases of traps. Virgin female traps were baited with 2- to 3-day-old females obtained from field-collected pupae. They were placed individually in plastic tubes (75 × 26 mm) whose bases and lids had been replaced with fine copper mesh. One such tube was placed centrally on the base of each wing trap. Over the eight days of the trial, there were sufficient females available for a total of 14 trap nights, compared with 40 trap nights for each of the synthetic pheromone blends tested.

Field trials were carried out at Ramu Sugar Ltd. plantations at Gusap, Papua New Guinea. In all trails there were five replicates of each treatment, and the treatments were assigned randomly to traps set 10.5 m apart within and between rows of cane at a height of 1.3–1.5 m. Catches were recorded and cleared daily, and all traps were moved forward one position daily. The first trap in each row was at least 10.5 m from the edge of the crop. Representative moths caught at each treatment were retained for species identification.

Trial 1, using empirical blends (Table 1 below) was carried out in a 16.1-ha plot of variety Cadmus cane between July 25 and August 2, 1991. For trial

2 (Table 3 below), blends were evaluated in a field of variety Q127 cane between September 20 and October 10, 1991. Because of a diminishing population in that field, the trial was reset in another field of the same variety between October 15 and November 4, 1991. In trial 3 (Table 3 below), two blends were compared in a field of H56752 cane between January 1 and March 18, 1992 (trial 3A) and repeated between May 30 and June 8, 1992 (trial 3B). Trial 4 was carried out in fields of Q135 (April 5–June 10, 1993) and Q127 (June 11–August 10, 1993) cane. No blank or virgin female traps were incorporated in trials 2, 3A, 3B, or 4.

In a fifth trial wing traps were compared with more robust plastic delta and Hara traps (Hara Products, Swift Current, Saskatchewan, Canada). All were baited with 1 μl of a 1:1 ratio of Z11–16:OAc and Z11–16:OH and were evaluated in a field of Q127 cane from September 20 to October 10, 1991. Because of diminishing population levels, the trial was moved to another field of the same variety for the period October 15–November 4, 1991.

Trap data were subjected to an analysis of variance, and the significance of differences between means determined using Duncan's MRT. Missing values, which occasionally occurred when rats removed baits from some traps, were replaced with the mean catch of the remaining replicates for the night that the bait was missing. Means shown in Table 1 have been back-transformed following a reciprocal transformation of the original data.

RESULTS

Initial field trials were carried out with a range of compounds and mixtures based on known pheromones or attractants for *Sesamia* species. The results are shown in Table 1. Most moths were caught at a 1:1 combination of Z11–16:OAc and Z11–16:OH, although there was no significant difference between mean catch per trap with this bait and at the 3:1 ratio of the same components ($P < 0.05$). Combinations of Z9–14:OAc and Z9–14:OH were generally unattractive, and none of the single-component baits caught any *S. grisescens*. Over the period of these trials, virgin female traps were put out for a total of 14 trap nights (i.e., the sum of the number of traps on each night that trapping took place), during which three male *S. grisescens* were caught, for a mean of 0.2 males/trap/night. On the same nights, two synthetic blends, 3:1 and 1:1 ratios of Z11–16:OAc and Z11–16:OH, caught 0.5 and 0.4 males/trap/night, respectively. There were insufficient data to analyze for differences between these means.

Electrophysiological and chemical studies were made to confirm the active components of the pheromone and to determine the ratio of these materials in the sex pheromone gland. Electroantennogram responses from male antennae to

TABLE 1. MEAN CATCH PER TRAP 25 JULY–2 AUGUST 1991 OF ADULT *Sesamia grisescens* AT VARIOUS MIXTURES OF ALKENYL ACETATES AND ALCOHOLS (TRIAL 1)

Compound(s)	Ratio	Mean catch/trap ($N = 5$)[a]
Z9–14:OAc		0
Z9–14:OH		0
Z9–14:OAc/Z9–14:OH	19:1	0
	3:1	2.5b
	1:1	1.0a
	1:3	0
	1:19	0
Z11–16:OAc		0
Z11–16:OH		0
Z11–16:OAc/Z11–16:OH	19:1	1.2a
	3:1	7.8cd
	1:1	16.0d
	1:3	3.6bc
	1:19	2.3b
Blank		0

[a]Means not followed by the same letter are significantly different (Duncan's MRT, $P < 0.05$).

compounds of different carbon chain length, double bond position, and isomeric configuration produced significant profiles for tetradecenyl and hexadecenyl acetates (Figure 1). The lower response variability, as indicated by the standard deviations, for the hexadecenyl acetates and the greater response to Z11–16:OAc and E11–16:OAc suggests that one or both of these compounds may be present in the pheromone. The response from Z9–14:OAc is likely to be due to binding to the same active site on the antenna.

The gas chromatographic analyses of ovipositor extracts from individual virgin female moths showed peaks coincident with those for the compounds listed in Table 2. The average amount of the major component (Z11–16:OAc) per gland was 44 ng; however, amounts ranged from 4 to 176 ng. The corresponding alcohol, Z11–16:OH, occurred in significant quantities in all of the extracts examined, although the relative amounts ranged widely (142–2728) about the average value shown in Table 2. The relative amounts of hexadecanol (*n*16:OH) were also found to be extremely variable. This compound was not detectable in 18 of 21 analyses, but in one analysis *n*16:OH was found to be twice as abundant as Z11–16:OAc. A small peak consistent with E11–16:OAc was observed in four of 21 analyses. Similarly E11–16:OH was not usually detectable, but a small peak consistent with this compound was seen in two analyses. Analysis on the BP5 column showed peaks consistent with Z11–16:OAc, Z11–16:OH, and hexadecyl acetate (*n*16:OAc) as the major com-

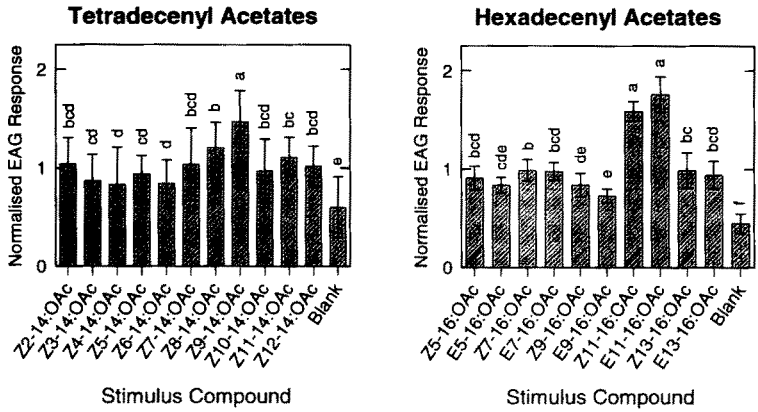

FIG. 1. Electroantennogram (EAG) responses of male antennae from *S. grisescens* to tetradecenyl (14-carbon) and hexadecenyl (16-carbon) acetates. Within each set, results are normalized to an average value of 1.0 and are shown for each double-bond position and isomer tested. Error bars are ± 1.0 SD. Responses not marked by the same letter are significantly different (Duncan's MRT $P < 0.05$).

ponents, although on this column *E* and *Z* isomers are not resolved. Half of one extract was treated with lithium aluminum hydride, which resulted in the disappearance of the acetate peaks in the gas chromatogram when compared to the untreated half of the extract.

The results from the field trial of empirical blends (trial 1, Table 1) sug-

TABLE 2. COMPOUNDS DETECTED IN PHEROMONE GLAND OF *S. grisescens* BY GLC ON POLYETHYLENE GLYCOL COLUMN

Component	Average relative amount ($N = 21$)[a]	Range[a,b]	Kovats index ±0.3
*n*16:OAc	206	nd-572	2318.2
Z9-16:OAc	6	nd-39	2348.4
*E*11-16:OAc	9	nd-112	2354.3
Z11-16:OAc	1000	4-176ng	2362.6
*n*16:OH	122	nd-2277	2404.7
Z9-16:OH	19	nd-57	2437.2
*E*11-16:OH	1	nd-11	2441.8
Z11-16:OH	953	142-2728	2449.9

[a] Values normalized to 1000 for Z11-16:OAc
[b] nd = not detected, values for Z11-16:OAc given in nanograms.

gested that the optimum ratio of Z11-16:OAc to Z11-16:OH lies somewhere between 3:1 and 1:1, while gland content analysis indicated that the addition of Z9-16:OAc, Z9-16:OH, and *n*16:OAc might enhance the attractiveness of the two component mixture. Further trials with the blends shown in Table 3 were undertaken to identify the optimum blend for trapping purposes.

A 3:2 ratio of Z11-16:OAc/Z11-16:OH caught significantly more moths than the 1:1 ratio or the 1:1 blend with the addition of minor components ($P < 0.05$) (trial 2, Table 3). While the addition of the minor components to 3:2 and 1:1 blends of the major components resulted in fewer moths being caught than were taken at the two-component blends alone (trials 2, 3A, and 3B, Table 3), the differences were not significant. Because of the significant EAG response to *E*11-16:OAc and the detection of a peak corresponding to this compound in the gas chromatographic analyses of gland components, 3:2 blends of Z11-16:OAc/Z11-16:OH with and without a small amount of *E*11-16:OAc were compared (trial 4). There was no significant difference between mean catches at the two blends. The ratio of components emitted from baits loaded with a 3:2 mixture of Z11-16:OAc and Z11-16:OH was measured daily for 12 days. The emitted ratio was found to average 10:9.1 ± 0.8 SD.

TABLE 3. TRIALS 2, 3A, 3B, AND 4[a]

Compound/s	Ratio	Mean catch ($N = 5$)	
Trial 2			
Z11-16:OAc/Z11-16:OH	1:1	8.6(b)	
Z11-16:OAc/Z11-16:OH	3:2	13.8(a)	
Z11-16:OAc/Z11-16:OH/ *n*16:OAc/Z9-16:OAc/ Z9-16:OH	9:9:2:0.6:0.2	5.6(b)	
Trial 3		A	B
Z11-16:OAc/Z11-16:OH	3:2	6.2(a)	5.6(a)
Z11-16:OAc/Z11-16:OH/ *n*16:OAc/Z9-16:OAc/ Z9-16:OH	6:4:1:0.3:0.1	3.4(a)	3.3(a)
Trial 4			
Z11-16:OAc/Z11-16:OH	3:2	2.2(a)	
Z11-16:OAc/Z11-16:OH/ *E*11-16:OAc	3:2:0.3	3.0(a)	

[a] Mean catch of *Sesamia grisescens* at various mixtures of alkenyl acetates and alcohols. Within trials, means not followed by the same letter are significantly different (trial 2: $P < 0.05$, Duncan's MRT; trials 3 and 4: $P < 0.10$, t test).

In a fifth trial the wing traps were tested against plastic delta traps and Hara traps (using 1:1 Z11-16:OAc/Z11-16:OH as bait) and mean catches (±SD) of 6.8 (2.1), 4.4 (2.0), and 0.8 (1.3), respectively, were obtained. Mean catch at the wing and delta traps were not significantly different from one another, but both were significantly greater than the mean catch at the Hara traps.

In addition to *S. grisescens*, other moths were occasionally caught in the traps. For all trials involving mixtures of Z11-16:OAc and Z11-16:OH, the species caught were a pyralid, *Chilo terenellus* Pagenstecher (16), and three noctuids, *Sesamia inferens* (Walker) (3), a *Mythimna* sp. (2), and a *Hydrillodes* sp. (9).

DISCUSSION

The results from the field trials and chemical/electrophysiological investigations are consistent with the presence of Z11-16:OAc and Z11-16:OH as the principal components of the sex pheromone of *S. grisescens*. In this respect *S. grisescens* is similar to three of the four *Sesamia* species for which information is available. *S. cretica*, which is attracted to a mixture of Z9-14:OAc and Z9-14:OH is the exception. Although Z11-16:OAc is the predominant pheromone component, *S. grisescens* differs in having a far higher proportion of Z11-16:OH compared to the other *Sesamia* species. The gas chromatographic (GLC) analyses of individual pheromone gland contents suggested that the two major components are produced, on average, in a ratio close to 1:1. However there were large differences between individual moths, which indicated that male moths might respond to a range of mixtures, and in the field trials it was found that although baits loaded with a 3:2 ratio were the most attractive to male moths, significant catches were also obtained with 1:1 and 3:1 blends. The GLC analyses of the effluvia from the 3:2 baits showed an OAc-OH ratio of 10:9, close to the average ratio found in the gland and well within the range of values found in the pheromone glands for individual moths (Table 2). Nevertheless, the best definition of the optimum blend will require a study of the effluvia emitted by calling females, a study that was beyond the scope of the present investigation.

The saturated acetate *n*16:OAc was also identified as a pheromone gland component, but there was no evidence for any biological activity for this compound. Similarly, several compounds present in trace amounts were tentatively identified but, when added to the two principal components, failed to enhance the attractancy of the synthetic blend. This was unexpected, particularly with respect to *E*11-16:OAc, which elicited a very strong electrophysiological response from antennae of male *S. grisescens*. It may be that *S. grisescens* needs to discriminate between the *Z* and *E* isomers to ensure its reproductive isolation

from a sympatric species. However, if this is so, it would also be expected that the *E* isomer would have some inhibitory effect. For example, the tortricid *Epiphyas postvittana* shows strong EAG responses to both *E*11-14:OAc and *Z*11-14:OAc. While the *E* isomer is the major component of the pheromone blend, the *Z* isomer is not present in the pheromone gland and is inhibitory when mixed with the *E* isomer at 10% or greater (Rumbo et al., 1993). *E*11-16:OAc was not observed to be inhibitory for *S. grisescens*, and it is not yet known whether the male moth has separate sensory cells for the *Z* and *E* isomers.

The observation that the addition of secondary components to a 3:2 blend of *Z*11-16:OAc and *Z*11-16:OH had little effect on trap catches, and in fact marginally reduced them in one trial, can be compared with the results of Mazomenos (1989). He reported that while the addition of individual secondary components to the major pheromone component of *S. nonagrioides* (*Z*11-16:OAc) reduced male capture, a combination of the major and all secondary components was highly attractive. Although it is possible that further adjustment of ratios or addition of minor components in suitable proportions might provide an improved attractant for *S. grisescens*, the attractancy of the 3:2 blend is sufficient to provide a bait for monitoring purposes. Certainly the synthetic blend was found to be more attractive than a caged virgin female moth. The availability of the synthetic attractant will permit monitoring in Australia for accidental introduction of the pest and allow the natural distribution of the species to be mapped in PNG. Little is currently known about the distribution of *S. grisescens* and, hence, the climatic extremes across its range are unknown. Using weather data for Ramu Sugar Ltd. plantations at Gusap in Papua New Guinea and for the Australian mainland, a climate matching computer program (CLIMEX; Maywald and Sutherst, 1991) indicates that cane-growing areas in the north of Australia might provide a suitable habitat for *S. grisescens*. This model will be refined when further data on the range of the pest becomes available.

The use of the synthetic attractant in monitor traps for either quarantine or survey purposes will require further research. During trapping trials in PNG, other species that were occasionally found in traps included *S. inferens*, a *Mythimna* sp., and more often *C. terenellus* and a *Hydrillodes* sp. *S. inferens* has a distribution that includes New Guinea and has been found to be a pest on sugarcane, but this insect has not been a problem at the Ramu Sugar Ltd. plantations where its population density is very low. A total of three *S. inferens* were trapped during all trials at Gusap, PNG. In a preliminary trial in Australia the synthetic blend attracted single specimens of a noctuid, *Athenis reclusa* (Walker), and a geometrid, *Petelia medardaria* H-Sc. No other moths were trapped, but further trials will be necessary to determine the specificity of the monitor traps in Australia.

The use of synthetic attractants in traps to monitor populations of *S.*

grisescens in PNG may have only limited value in pest control strategies as the pest occurs in sugarcane in discrete generation cycles that are readily determined by sampling egg and larval numbers. However, there is potential for use of a synthetic blend as a mating disruptant, and trials are currently being planned to assess the suitability of a two component blend for this purpose. Control of *S. grisescens* may also assist in the containment of the weevil borer *Rhabdoscelus obscurus* Boisd, the larvae of which enter cane stalks through holes made previously by *S. grisescens*.

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VARIATION AMONG AND WITHIN MOUNTAIN BIRCH TREES IN FOLIAGE PHENOLS, CARBOHYDRATES, AND AMINO ACIDS, AND IN GROWTH OF *Epirrita autumnata* LARVAE

JANNE SUOMELA,[1,*] VLADIMIR OSSIPOV,[1,2] and ERKKI HAUKIOJA[1]

[1]*Laboratory of Ecological Zoology, Department of Biology, and Kevo Subarctic Research Station University of Turku, FIN-20500 Turku, Finland*

[2]*Institute of Forest Krasnoyarsk, 660036, Akademgorodok, Russia*

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Abstract—Leaf quality of the mountain birch (*Betula pubescens* ssp. *tortuosa*) for herbivores was studied at several hierarchical levels: among trees, among ramets within trees, among branches within ramets, and among short shoots within branches. The experimental units at each level were chosen randomly. The indices of leaf quality were the growth rate of the larvae of a geometrid, *Epirrita autumnata*, and certain biochemical traits of the leaves (total phenolics and individual phenolic compounds, total carbohydrates and individual sugars, free and protein-bound amino acids). We also discuss relationships between larval growth rate and biochemical foliage traits. Larval growth rates during two successive years correlated positively at the level of tree, the ramet, and the branch, indicating that the relationships in leaf quality remained constant between seasons both among and within trees. The distribution of variation at different hierarchical levels depended on the trait in question. In the case of larval growth rate, ramets and short shoots accounted for most of the explained variation. In the case of biochemical compounds, trees accounted for most of the variance in the content of total phenolics and individual low-molecular-weight phenolics. In the content of carbohydrates (total carbohydrates, starch, fructose, glucose, and sucrose) and amino acids, variation among branches was generally larger than variation among trees. Variation among ramets was low for most compounds. No single leaf trait played a paramount role in larval growth. Secondary compounds, represented by phenolic compounds, or primary metabolites, particularly sugars, may both be

*To whom correspondence should be addressed.

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important in determining the suitability of birch leaves for larvae. If phenols are causally more important, genet-specific analyses of foliage chemistry are needed. If sugars are of primary importance, within-genet sampling and analysis of foliage chemistry are necessary.

Key Words—Amino acids, *Betula pubescens* ssp. *tortuosa*, carbohydrates, *Epirrita autumnata*, larval growth, leaf biochemical traits, phenolics, sugars, within-tree variation.

INTRODUCTION

The plant modular structure (Harper, 1977, 1981; White, 1979; Vuorisalo and Tuomi, 1986) and partial physiological autonomy (Watson and Casper, 1984; Watson, 1986; Sprugel et al., 1991) of plant parts has been assumed to have ecological and evolutionary implications for plant growth patterns, resource allocation, and reproduction (Watson and Casper, 1984; Jerling, 1985; Tuomi et al., 1988a,b; Tuomi and Vuorisalo, 1989; Marquis, 1992). Modularity and partial autonomy may also modify interactions between plants and their herbivores and pathogens by increasing within-individual variation in plant quality (Whitham and Slobodschikoff, 1981; Denno and McClure, 1983; Gill and Halverson, 1984; Marquis, 1988; Edwards et al., 1990; Haukioja, 1991a; Suomela and Ayres, 1994; Suomela and Nilson, 1994).

Since food quality is important for herbivore growth, survival, and reproductive capacity (for reviews, see Mattson, 1980; Scriber and Slansky, 1981; Denno and McClure, 1983; Slansky and Rodriguez, 1987), among- and within-tree variation in leaf quality may affect performance of herbivores. Variation may also affect adult oviposition behavior (Thompson, 1988; Renwick and Chew, 1994) and larval search and feeding behaviors (Schultz, 1983). For researchers, variation has implications with regard to experimental design and sampling procedures (Suomela and Ayres, 1994).

In earlier studies we have demonstrated the occurrence of variation in the performance of larvae of the autumnal moth (*Epirrita autumnata*) and of sawfly species on leaves of the mountain birch (*Betula pubescens* ssp. *tortuosa*). There is considerable variation both among years (Hanhimäki et al., 1995) and according to the previous defoliation history of the trees (Hanhimäki, 1989; Ruohomäki et al., 1992). The performance of *E. autumnata* larvae on the mountain birch also varies both among trees growing at the same site and within individual trees. There are systematic within-tree differences in larval growth rate, e.g., among leaves of different sizes and on different positions on the crown (Suomela et al., 1995). In addition, even same-aged short shoot leaves, randomly sampled from a restricted part of the birch canopy, show pronounced within-tree variation in suitability as a food for *E. autumnata* larvae (Suomela and Nilson, 1994).

This may be related to the within-tree variation found in mountain birch leaf water content, specific weight, and toughness (Suomela and Ayres, 1994). Although these traits are only crude measures of plant quality, all of them have previously been claimed to be important for herbivore performance and have often been used to characterize foliage quality (Mattson, 1980; Scriber and Slansky, 1981; Coley, 1983; Ayres and MacLean, 1987; Ayres et al., 1987; Alliende, 1989; Basset, 1992). Detailed analyses of within-tree variation in the biochemical characteristics of mountain birch foliage have not been published.

In general, studies of within-tree variation among randomly or haphazardly sampled same-aged leaves are few (but see Schultz, 1982; Baldwin et al., 1987). However, within-individual variation in characters of the leaves and performance of the herbivores has been measured between leaves of different age or size (e.g., Whitham, 1981; Raupp and Denno, 1983; Meyer and Montgomery, 1987; Howard 1990; Stamp and Bowers, 1990; Bogacheva, 1994; Hikosaka et al., 1994) and between different parts of tree canopies (Jensen, 1988; Watt, 1992; Quiring, 1993).

In this paper we: (1) study the consistency of among- and within-tree variation in the growth rate of larvae of *Epirrita autumnata* on the same trees, ramets, and branches during two successive years; (2) compare patterns of among- and within-tree variation in different biochemical characters of the foliage in the same trees on which the larvae were reared, and (3) discuss the relationship between larval growth and the biochemical characters measured.

METHODS AND MATERIALS

Study Site and Organisms. Mountain birch [*Betula pubescens* Ehrh. ssp. *tortuosa* (Ledeb.) Nyman] forms the treeline in northwestern Europe. The trees (genets) are usually polycormic with several trunks (ramets), most of which originate from dormant basal buds (Kauppi et al., 1987). The growth type of mountain birch is indeterminate with two types of shoots: short shoots and long shoots (Clausen and Kozlowski, 1965). The short shoot leaves (usually three leaves per shoot) burst simultaneously in the spring and are thus of equal age. This is in contrast with the long shoot leaves, which are produced at the branch tips up to the late summer.

The experimental trees were located in a mountain birch forest on the slope of the Skallovaara fell, near the Kevo Subarctic Research Station (69°45′N, 27°E), in northern Finland.

The autumnal moth, *Epirrita autumnata* (Bkh.), is a univoltine polyphagous geometrid. The mountain birch is its most abundant host species in our study area, although the larvae also grow well on several other deciduous trees (Neuvonen et al., 1987; Haukioja et al., 1988a). The adults fly in the autumn

and the females oviposit on trees, where the eggs overwinter and hatch next spring at the time of birch budbreak. The larvae feed for about one month, after which they pupate in ground litter. *Epirrita autumnata* populations fluctuate cyclically at about 10-year intervals and can reach outbreak densities (Tenow, 1972; Haukioja et al., 1988a).

Larval Growth Experiments. We bioassayed leaf quality in the summers of 1990 and 1991 by measuring the growth rates of *E. autumnata* larvae at different hierarchical levels of the birch canopies. At the end of June 1990, we marked 20 mountain birches in a homogeneous semiopen mountain birch forest. We selected only trees that were 2–4 m tall and that had at least three ramets and two branches per ramet. Of these 20 birches, we randomly selected five trees for the experiment, using a random-numbers table. From each tree we randomly selected two ramets, from each ramet two branches, and from each branch a certain number of short shoots. To reduce effects of systematic within-canopy variation (Suomela et al., 1995), we used only branches that were at a height of 1–2 m from the ground and had at least 30 short shoots.

The larvae originated from a stock reared at the Kevo Station. They had always been reared on birch foliage; field-caught adult males were regularly used in matings to prevent inbreeding. During both study years the larvae were taken from several broods and randomly allocated to sample units. During the preexperimental period, larvae were reared individually in plastic vials, with birch leaves collected from trees growing outside the experimental plot.

At the beginning of July 1990 and 1991, we conducted growth trials with fifth-instar *E. autumnata* larvae, rearing them both years on leaves of the same trees, ramets, and branches. In both years we randomly sampled four short shoots per branch. We used different shoots in 1990 and 1991, since damage to leaves in an individual short shoot reduces the size of leaves in that shoot (but not in adjacent shoots) the next year (Ruohomäki et al., unpublished manuscript).

The leaves were excised at the middle of the petiole just before the beginning of the growth trial. The leaf lamina was not damaged, to avoid damage-induced responses in the foliage (Haukioja, 1991b). The leaves were taken to the laboratory in sealed plastic vials (with the leaves of each branch in a separate vial), protected from sunlight. We fed the *E. autumnata* larvae on the two largest leaves per short shoot (one larva per leaf) in the lab for about 24 hr. The number of larvae and leaves was thus 160 (five trees, two ramets per tree, two branches per ramet, four short shoots per branch, and two leaves per shoot) for each experiment. This means that, for very practical reasons, we had a large number of replicates at the lowest levels of the hierarchy but only five replicate trees.

Solitary larvae of *E. autumnata* are more sensitive to variation in food plant quality than larvae reared in groups (Haukioja et al., 1988b). For this reason we reared the larvae individually in 48-ml plastic vials. Leaf turgor was main-

tained by a 1-cm substrate of moistened plaster of Paris. We used larvae that had just molted to the fifth (ultimate) instar; they had, however, started to feed and had filled their guts.

At the start of the trial, each larva was weighed. Both the order of weighings and the order of leaves and vials on the rearing trays was randomized in each experiment. After 24 hr, the larvae were reweighed in the same order. The times of all weighings were recorded to an accuracy of 1 min.

Illumination was continuous, corresponding to natural summer light conditions at the latitude of Kevo. The temperature was +18°C in 1990 and during the first half of the experiment in 1991, but was reduced to +10°C for the second half of the 1991 trial to prevent the larvae from exhausting the leaves too quickly. Otherwise the methods used were similar in the two years.

As an index of leaf quality, we used the relative growth rate of the larvae. It was calculated as follows:

$$\text{Relative growth rate} = [\ln(m_e) - \ln(m_b)]/\text{time}$$

where m_b and m_e are the dry mass of a larva at the beginning and the end of the growth trial, respectively. Larval dry mass (DM) was calculated from the fresh mass (FM) as $DM = 0.125 \times FM^{1.113}$ (Neuvonen and Haukioja, 1984).

Biochemical Measurements. In 1991, we analyzed the contents of phenolics, carbohydrates, and amino acids in the birch leaves. From each branch used to feed the larvae, we clipped 25–30 short shoot leaves at the same time that we collected the leaves for the larval growth experiment. The leaves were collected on ice, transported immediately to the laboratory and freeze-dried.

The analyses were branch-specific. The shoot level had to be omitted, since the leaf mass on individual short shoots was too small to permit the collection of enough leaves for analyses at the shoot level.

The plant material was lyophilized, homogenized to a powder, and stored at +4°C. One gram of the resulting powder was suspended in 10 ml of 70% aqueous ethanol, allowed to stand for 3 hr at room temperature with continuous stirring, and centrifuged at 1500*g* for 15 min. The pellet was reextracted three times. The combined extracts were evaporated under low pressure at 40°C. The residue was redissolved in 10 ml of water and centrifuged (referred to henceforth as water extract).

The total content of phenolic compounds was measured using the Folin-Denis method as described in Marigo (1973) with gallic acid as a standard.

The contents of individual low molecular weight phenolics were determined by high-performance liquid chromatography (HPLC), using a Milichrom-4 chromatograph with a Separon C-18 column. The chromatographic data were used as the "fingerprints" of individual phenolics, since their contents were expressed in relative units: peak height (in millimeters) on the chromatogram per 1 g of dry matter.

The total content of carbohydrates was analyzed using the phenol–sulfuric acid method (Dubois et al., 1956). The contents of glucose, fructose, and sucrose were determined by gas–liquid chromatography. Two millimiters of the water extract containing 10–15 mg of soluble carbohydrates was evaporated at 40°C at low pressure. One milliliter of pyridine was stirred in, and the mixture was desiccated by evaporating three times under the same conditions. The residue was dissolved in 2 ml of pyridine for 12 hr and 0.6 ml of hexamethyldisilasane and 0.3 ml of trimethylchlorsilane (Sigma) were added and stirred for 10 min. After evaporation of pyridine, the residue was dissolved in 2 ml of *n*-hexane.

The contents of the sugars were measured with the Biochrom gas chromatograph, equipped with a flame ionization detector and a capillary column (20 m × 0.32 mm ID) with liquid phase SE-30. The column temperature was increased from 145° to 250°C at a rate of 8°C/min. The detector and injector temperatures were 280° and 190°C, respectively. Helium was used as the carrier gas. The internal standard was sorbitol.

Starch content was measured by the method of Humphreys and Kelly (1961).

To measure free amino acids, 6 ml of the water extract was applied to a column of Dowex HCR-S (70 × 15 mm ID, H^+-form; Sigma). The amino acids retained on the column were eluted with 3 N NH_4OH. The eluate was evaporated and dissolved in water (2.5 ml). The contents of free amino acids were determined by a standard procedure using a Microtechna amino acid analyzer T-339 M. Protein-bound amino acids were analyzed by the method of Lunderstädt (1976).

All biochemical measurements for each powder sample (representing the branch level) were repeated three times, and the mean was used in the statistical analyses. Since we took only one sample per branch, the reliability of the branch level values was checked beforehand by analyzing five separate samples taken from a homogeneous powder. The coefficients of variation among the samples were 2% for total phenolics, 4–23% for individual low-molecular-weight phenolics, 3% for total carbohydrates, 7–9% for sugars, 3–13% for free amino acids, and 0–6% for protein-bound amino acids.

Statistical Analysis. Due to the hierarchical design, the data were analyzed with nested ANOVA (Proc Nested, SAS Institute, 1990). In the larval growth trials the lowest hierarchical level (error variance) included variation within shoots, among larvae, and the measurement error. In the biochemical analyses, the lowest level included variation among branches within ramets plus variation within branches and measurement error.

The distribution of variation among different hierarchical levels was evaluated by comparing the proportions of total variance and the coefficients of variation. The proportions of total variance express the relative contributions of different hierarchical levels to the total variance. The coefficient of variation reveals variation as a proportion of the mean and is used to compare actual

variation between different characters. It was calculated in terms of percentages of estimated standard deviation from the mean (derived from the nested ANOVA model, Proc Nested, SAS Institute, 1990).

The relationships between larval growth and biochemical characters were tested with Pearson's correlation analysis and multiple regression analysis (Proc Corr and Proc Reg, SAS Institute, 1990).

We verified the assumption of normality of the data by evaluating the residuals of the second lowest level (short shoots and ramets in larval growth rate and contents of biochemical compounds, respectively) in nested ANOVA by Shapiro-Wilk's test (Proc Univariate, SAS Institute, 1990). Higher levels were generally not tested, since means follow a normal distribution better than individual values (Sokal and Rohlf, 1981). The distributions of a few biochemical compounds deviated from normal and could not be corrected by any transformations. The homogeneity of variances was tested with Cochran's test (Winer 1971); in a few biochemical compounds the variances differed significantly. There were, however, no deviations from normality or homoscedasticity in those compounds or at those hierarchical levels in which significant differences were found.

RESULTS

Larval Growth Experiments

Ramets (within trees) and short shoots (within branches) accounted for most of the explained variation in the growth rate of *E. autumnata* larvae (Table 1). Variation among trees and branches was smaller, whereas error variance accounted for over half of the total variance. Differences were significant at the shoot level, but insignificant at other levels (Table 1).

TABLE 1. RESULTS OF ANOVA, PROPORTION OF TOTAL VARIANCE (%), AND COEFFICIENTS OF VARIATION (CV) FOR DIFFERENCES AMONG TREES, AMONG RAMETS WITHIN TREES, AMONG BRANCHES WITHIN RAMETS, AND AMONG SHORT SHOOTS WITHIN BRANCHES IN GROWTH RATE OF *E. autumnata* LARVAE IN 1990 AND 1991

Source of variation	Error term	*df*	1990				1991			
			F	*P*	%	CV	F	P	%	CV
Trees	Ramets	4	2.1	ns	12	3.8	0.8	ns	0	0.0
Ramets	Branches	5	3.2	0.06	15	4.2	2.3	ns	15	6.3
Branches	Shoots	10	1.3	ns	3	2.0	1.9	0.06	10	5.3
Shoots	Error	60	1.5	0.04	15	4.1	1.6	0.03	17	6.8
Error		80			55	8.0			57	12.5

The coefficients of variation at other levels than among trees were higher in 1991 than in 1990. The proportion of the variance for branches was also higher in 1991, whereas the reverse was true for trees. The proportion of the variance for ramets and shoots was similar between years (Table 1).

The correlation coefficients in larval growth rates (Figure 1) between the years were positive (branch level, $r = 0.48$, $P < 0.05$, $df = 18$; ramet-level, $r = 0.60$, $P < 0.07$, $df = 8$; tree-level, $r = 0.87$, $P = 0.056$, $df = 3$). Because different shoots were used each year (see Methods and Materials), a correlation at the shoot level was not possible.

Biochemical Traits

Phenolic Compounds. In leaf total phenolic content, trees accounted for most of the variance (Table 2). The coefficients of variation at different levels ranged from 7 to 14% (Table 2). The differences were significant both among trees ($F_{4,5} = 5.4$, $P < 0.05$) and among ramets within trees ($F_{5,10} = 3.9$, $P < 0.05$). Detailed data are presented in Table 3.

Altogether 27 low-molecular-weight phenolic compounds were detected in the leaves (a representative HPLC trace is shown in Figure 2). Ten of these compounds could be identified using preparative HPLC and ^1H and ^{13}C NMR (Ossipov et al., 1995). The compounds identified were 1-*O*-galloyl-(2-*O*-acetyl)-glucopyranoside (no. 1), (+)-catechin (no. 6), chlorogenic acid (no. 8), 3-*O*-*p*-coumaroyl-D-quinic acid (no. 12), myricetin-3-*O*-(acetyl)-rhamnopyranoside (no. 21), quercetin-3-*O*-arabinofuranoside (no. 22), kaempferol-3-*O*-glucopyranoside (no. 23), quercetin-3-*O*-(4′-*O*-acetyl)-rhamnopyranoside (no. 24),

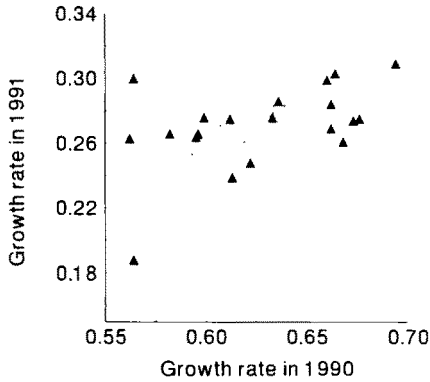

FIG. 1. Mean relative growth rate of *E. autumnata* larvae on individual trees (plus signs), on ramets within trees (squares), and on branches within ramets (triangles) during two consecutive years.

TABLE 2. PROPORTIONS OF TOTAL VARIANCE AND COEFFICIENTS OF VARIATION IN CONTENTS OF TOTAL PHENOLICS AND INDIVIDUAL LOW-MOLECULAR-WEIGHT PHENOLICS AT DIFFERENT HIERARCHICAL LEVELS, AND GRAND MEAN (MEAN OF ALL VALUES) FOR EACH COMPOUND

Source of variation	Total phenolics	Low-molecular-weight phenolics																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Proportion of total variance (%)																												
Trees	64	99	94	99	98	95	86	78	97	67	94	94	96	86	90	91	74	10	90	82	89	85	79	89	89	86	99	78
Ramets	21	0	0	0	0	0	3	0	0	0	0	3	0	8	1	0	19	28	0	5	0	0	0	0	0	0	1	0
Branches and error	15	1	6	1	2	5	11	22	3	31	6	3	3	6	9	9	7	63	10	14	11	15	21	11	11	14	0	22
Coefficient of variation (%)																												
Trees	14	120	52	91	99	50	29	49	50	13	56	36	47	47	74	36	17	4	53	37	70	21	27	31	32	27	164	20
Ramets	8	0	0	0	7	0	5	0	3	0	2	6	2	14	9	0	8	7	0	9	0	0	0	0	0	0	14	0
Branches and error	7	14	13	11	14	11	11	26	9	9	14	6	9	12	23	11	5	10	18	15	24	9	14	11	11	11	7	10
Grand mean[a]	63	370	39	14	42	21	43	74	656	42	37	31	184	9	33	28	41	30	17	21	26	209	82	66	75	41	42	66

[a] Units: mg/g dry mass for total phenolics; height of peaks on chromatogram in mm/g dry mass for low-molecular-weight compounds.

TABLE 3. CONTENTS OF TOTAL PHENOLICS AND 27 LOW-MOLECULAR-WEIGHT PHENOLICS IN LEAVES OF MOUNTAIN BIRCH[a]

Compound	Tree no. 1	2	3	4	5
Total phenolics (mg/g dry mass)	53 (42–61)	69 (61–75)	58 (53–63)	59 (52–63)	77 (72–80)
Peak no. (height of chromatogram peak mm/g dry mass)					
1	101 (90–123)	1146 (1056–1240)	319 (252–404)	179 (166–190)	104 (95–124)
2	17 (14–20)	61 (52–70)	30 (24–36)	61 (60–63)	27 (22–33)
3	7 (6–8)	36 (33–38)	9 (7–11)	11 (9–11)	6 (5–7)
4	5 (5–6)	107 (100–123)	30 (22–41)	55 (52–60)	11 (10–11)
5	11 (10–12)	38 (34–40)	14 (12–14)	22 (21–23)	22 (21–23)
6	29 (28–30)	35 (30–38)	38 (32–45)	52 (47–56)	60 (54–69)
7	50 (40–57)	138 (121–160)	63 (10–93)	75 (72–79)	46 (43–49)
8	1211 (1086–1320)	484 (432–564)	519 (488–592)	381 (356–400)	687 (644–712)
9	39 (34–41)	40 (36–45)	36 (31–39)	49 (47–52)	45 (43–47)
10	21 (19–22)	70 (60–79)	43 (36–48)	27 (22–34)	23 (21–27)
11	50 (46–55)	30 (28–35)	24 (23–25)	21 (19–23)	31 (29–32)
12	212 (176–228)	194 (184–216)	169 (155–188)	54 (48–60)	293 (280–320)
13	16 (13–19)	11 (9–13)	5 (4–6)	7 (7–7)	7 (6–7)
14	40 (31–50)	73 (56–90)	28 (21–35)	9 (7–11)	17 (14–23)
15	39 (34–44)	24 (21–27)	28 (25–31)	14 (13–14)	36 (33–40)
16	35 (29–42)	49 (46–53)	37 (34–39)	35 (34–36)	50 (49–50)
17	29 (28–30)	32 (28–39)	33 (29–38)	31 (27–34)	27 (24–32)
18	24 (19–28)	10 (8–13)	18 (15–22)	28 (24–31)	6 (6–7)
19	24 (20–27)	33 (26–40)	21 (18–27)	17 (16–19)	11 (10–12)

TABLE 3. CONTINUED

Compound	Tree no.				
	1	2	3	4	5
20	13 (11-15)	57 (47-67)	22 (17-28)	14 (13-15)	23 (22-24)
21	157 (136-172)	168 (150-184)	227 (200-264)	265 (260-272)	225 (216-236)
22	62 (51-70)	93 (81-107)	109 (92-133)	90 (87-91)	57 (55-58)
23	58 (50-64)	102 (89-114)	61 (53-72)	48 (47-49)	62 (58-66)
24	91 (81-106)	45 (40-51)	81 (74-91)	100 (92-109)	55 (50-58)
25	36 (31-42)	23 (20-26)	49 (45-56)	46 (43-50)	49 (45-54)
26	19 (17-22)	3 (2-4)	17 (13-20)	5 (4-7)	164 (150-174)
27	80 (74-90)	79 (70-87)	62 (56-72)	49 (45-53)	62 (55-67)

"Tree means and values of branches with the lowest and highest value for each tree (in parentheses) are shown.

and kaempferol-glycosides (nos. 26 and 27). The contents of different compounds are given in relative units (peak height in millimeters per 1 g of dry matter). According to chromatographic observations at different wavelengths (250, 280, and 340 nm; data not shown), the main phenolic compounds were numbers 1, 8, 12, 21, 22 and 23 (Figure 2).

The 27 phenolic compounds were found in all leaf samples, i.e., there were no qualitative differences among or within trees. Trees accounted in general for most of the total variance in different phenolics (Table 2). In 24 of the 27 compounds, trees accounted for over 75% (in most cases over 90%) of the total variance.

Coefficients of variation among trees ranged from 4% (no. 17) to 164% (no. 26), being less than 50% for two thirds of the compounds. Coefficients of variation among ramets were low, ranging from 0% to 14%. Coefficients of variation among branches (including error variance) ranged from 6% to 24% (Table 2).

Differences among trees were significant in all compounds (in most cases at the $P < 0.001$ level, $F_{4,5}$ values ranging from 8 to 869), except for no. 17, in which most of the total variance was accounted for by branches (including error variance). Differences among ramets were low and mostly insignificant. Only in compounds 13, 16, and 26 were there significant differences among

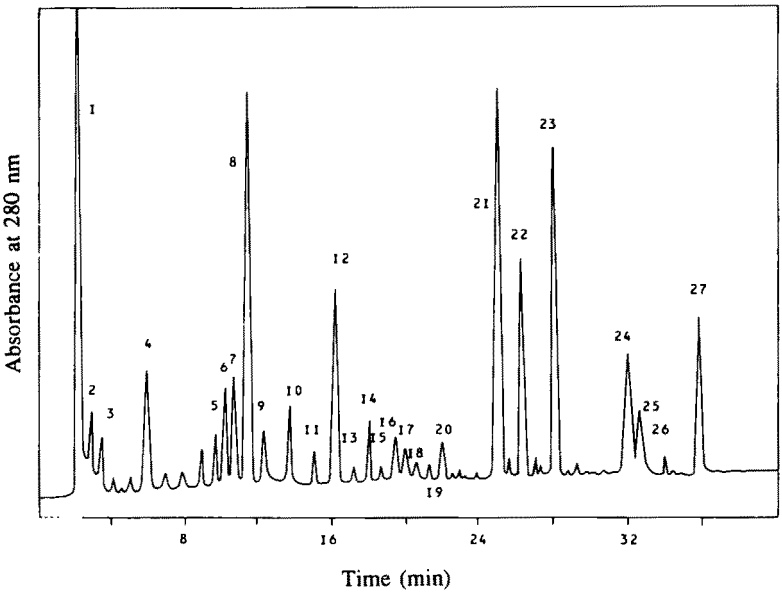

FIG. 2. A typical trace of HPLC analysis of individual low-molecular-weight phenolics (nos. 1–27) from birch leaves: 1, 1-*O*-galloyl-(2-*O*-acetyl)-glucopyranoside; 6, (+)-catechin; 8, chlorogenic acid; 12, 3-*O*-*p*-coumaroyl-D-quinic acid; 21, myricetin-3-*O*-(acetyl)-rhamnopyranoside; 22, quercetin-3-*O*-arabinofuranoside; 23, kaempferol-3-*O*-glucopyranoside; 24, quercetin-3-*O*-(4′-*O*-acetyl)-rhamnopyranoside; 26 and 27, kaempferol-glycosides. Separon C-18 column (64 × 2 mm I.D., 5 μm) at a flow rate of 50 μl/min. Two solvents were used: A, 5% aq. acetic acid; B, 96% aq. ethanol. The elution profile was 0–42 min, 0–95% B in A (linear gradient). Detection wavelength of 280 nm.

ramets within trees (at the $P < 0.05$ level, $F_{5,10}$ values ranging from 3.8 to 9.1).

Carbohydrates. In the total carbohydrate content of leaves, trees accounted for 40% and branches (including error variance) for about half of the total variance (Table 4). The coefficients of variation were low at every level (Table 4), and there were no significant differences at the $P < 0.1$ level. In leaf starch content, neither trees nor ramets differed significantly, while branches (including error variance) accounted for most of the total variation.

In monosaccharides and sucrose, and in their pooled contents (Table 4), branches (including error variance) accounted for most of the variation. Variation among trees was also considerable, but variation among ramets was low or nonexistent. Actual variation, at both the tree and the branch level, was highest in sucrose content and lowest in glucose content. The only significant differ-

TABLE 4. PROPORTIONS OF TOTAL VARIANCE AND COEFFICIENTS OF VARIATION OF TOTAL CARBOHYDRATE, STARCH, AND SUGAR CONTENTS AT DIFFERENT HIERARCHICAL LEVELS, AND GRAND MEAN FOR EACH COMPOUND

Source of variation	Total	Starch	Fructose	Glucose	Sucrose	Glucose + fructose	Glucose + fructose + sucrose
Proportion of total variance (%)							
Trees	40	0	35	46	21	40	14
Ramets	10	22	5	0	0	0	0
Branches and error	50	78	58	54	79	60	86
Coefficient of variation (%)							
Trees	5	0	19	15	23	15	5
Ramets	2	13	7	0	0	0	0
Branches and error	6	25	25	16	44	18	13
Grand mean (mg/g dry mass)	210	19	13	27	23	40	63

ence in the sugars was in leaf glucose content among trees ($F_{4,5} = 8.17$, $P < 0.05$). Detailed data on carbohydrates in each of the five trees are presented in Table 5.

Free Amino Acids. Glutamic acid was the most abundant free amino acid in all the trees. Branches (including error variance) accounted for most of the total variance in most amino acids. Exceptions were arginine, cisteic acid, citrulline, ethanolamine, γ-amino-*n*-butyric acid, and glutamic acid, in which most of the variance was accounted for by trees, and lysine and phenylalanine, in which about half of the variance was accounted for by ramets and half by branches (Table 6).

Coefficients of variation ranged from 0% to 69% among trees, from 0% to 28% among ramets, and from 15% to 64% among branches (Table 6). Differences among trees were significant in arginine, cisteic acid, citrulline, ethanolamine, and γ-amino-*n*-butyric acid (mostly at $P < 0.01$ level, $F_{4,5}$ values ranging from 10.2 (ethanolamine) to 27.2 (cisteic acid). The only significant difference among ramets was in glutamic acid ($F_{5,10} = 3.8$, $P = 0.03$). Details of amino acid contents in the five trees are presented in Table 7.

Protein-Bound Amino Acids. Glutamic acid, leucine, and lysine were the three most abundant protein-bound amino acids (Table 8). Branches (including

TABLE 5. CONTENTS OF CARBOHYDRATES IN LEAVES OF MOUNTAIN BIRCH[a]

Compound (mg/g dry mass)	Tree				
	1	2	3	4	5
Total carbohydrate	196 (190–203)	208 (194–215)	213 (94–228)	230 (214–245)	203 (194–223)
Starch	21.7 (15.0–29.8)	16.9 (12.0–22.8)	19.5 (16.7–21.7)	21.0 (14.4–26.5)	16.0 (7.8–20.9)
Fructose	15.7 (11.4–21.6)	9.7 (7.0–14.7)	17.4 (11.3–21.9)	13.3 (10.5–16.1)	11.4 (10.3–12.4)
Glucose	27.5 (20.6–36.0)	26.3 (23.4–30.7)	32.8 (27.5–35.5)	26.7 (24.4–29.7)	20.1 (18.9–21.4)
Sucrose	19.8 (13.2–26.2)	32.8 (5.8–47.4)	13.9 (7.2–27.4)	25.6 (22.0–30.4)	24.2 (19.6–26.7)

[a] Tree means and values of branches with the lowest and highest value for each tree (in parentheses) are shown.

error variance) accounted for most of the total variance in all the protein-bound amino acids except for cysteine and methionine. Variation among trees ranged from 0 to 56% of the total variance, being in most cases lower than 25%. Variation among ramets was low or nonexistent in compounds other than cysteine. Coefficients of variation were, in general, highest among branches and lowest among ramets. The only significant difference was in methionine content among trees ($F_{4,5} = 10.4$, $P = 0.012$). The protein-bound amino acids in leaves from the five trees are shown in Table 9.

Relationships between Biochemical Characters and Larval Growth

Although our experiment was not designed to test for relationships between leaf biochemicals and larval growth, we did a tentative multiple regression analysis. Since the number of independent replicates (=trees) was five (see Hurlbert, 1984), we could include only a few biochemical compounds as independent variables into the model. Consequently, we included those two compounds that separately had the highest correlation coefficients with larval growth: (+)-catechin ($r = -0.87$ $P = 0.053$) and monosaccharides (=fructose + glucose) ($r = 0.87$, $P = 0.057$).

(+)-Catechin had a negative effect and monosaccharides a positive effect on larval growth. Neither of the compounds was quite significant, however (Table 10), possibly due to low sample size.

TABLE 6. PROPORTIONS OF TOTAL VARIANCE AND COEFFICIENTS OF VARIATION OF FREE AMINO ACIDS AT DIFFERENT HIERARCHICAL LEVELS AND GRAND MEAN FOR EACH COMPOUND

	Amino acids[a]																							
Source of variation	aab	ala	arg	asp	cis	cit	cys	eta	gab	gln	glu	gly	his	ile	leu	lys	met	mhl	orn	phe	pro	ser	thr	tyr	val
Proportion of total variance (%)																									
Trees	9	14	83	25	57	82	7	67	70	3	49	0	11	39	30	0	0	32	0	0	26	0	0	47	23
Ramets	3	38	3	21	0	9	15	0	0	3	30	0	12	2	24	48	8	8	0	53	27	0	25	0	12
Branches and error	88	48	14	54	43	9	78	33	30	94	21	100	77	59	46	52	92	60	100	47	48	100	75	53	65
Coefficient of variation (%)																									
Trees	21	10	69	24	50	66	14	29	49	7	27	1	11	16	12	0	0	19	0	0	27	0	0	15	12
Ramets	11	17	13	22	0	22	20	0	0	7	22	0	11	4	11	18	13	9	0	17	28	0	12	0	9
Branches and error	64	19	28	35	43	22	45	21	32	39	18	34	27	20	15	19	43	26	42	16	37	22	21	16	20
Grand mean (μg/g dry mass)	3	69	102	15	24	16	42	54	43	26	338	7	8	5	6	7	2	5	8	28	20	58	14	11	10

[a]Explanations for abbreviations: aab = α-amino-*n*-butyric acid, ala = alanine, arg = arginine, asp = aspartic acid, cis = cisteic acid, cit = citrulline, cys = cysteine, eta = ethanolamine, gab = γ-amino-*n*-butyric acid, gln = glutamine, glu = glutamic acid, gly = glycine, his = histidine, ile = isoleucine, leu = leucine, lys = lysine, met = methionine, mhl = 1-methylhistidine, orn = ornithine, phe = phenylalanine, pro = proline, ser = serine, thr = threonine, tyr = tyrosine, val = valine.

TABLE 7. CONTENTS OF FREE AMINO ACIDS IN LEAVES OF MOUNTAIN BIRCH[a]

Compound (μg/g dry mass)	Tree				
	1	2	3	4	5
aab	2.9 (1.8–3.7)	4.5 (3.9–5.5)	3.0 (1.1–8.5)	2.5 (1.6–4.2)	1.4 (0.8–2.9)
ala	53.6 (44.2–66.6)	61.5 (40.8–95.6)	85.0 (80.8–91.4)	66.1 (45.1–81.5)	77.4 (64.3–102.2)
arg	32.1 (24.8–42.1)	63.2 (38.8–93.8)	197.9 (160.8–237.0)	58.1 (47.9–68.9)	159.0 (134.0–240.8)
asp	16.6 (9.5–26.6)	20.0 (13.7–25.5)	16.9 (10.5–29.9)	11.0 (8.2–12.8)	8.0 (4.6–9.7)
cis	15.0 (11.3–19.4)	14.2 (9.8–20.0)	25.2 (21.2–30.8)	22.3 (15.2–30.4)	45.1 (27.3–65.2)
cit	6.7 (5.2–8.7)	7.5 (3.6–11.8)	34.1 (23.8–46.4)	14.2 (13.1–15.0)	18.1 (16.7–18.8)
cys	27.1 (18.7–34.3)	38.4 (28.6–55.6)	46.0 (31.6–56.0)	60.7 (27.2–94.1)	37.0 (17.5–81.7)
eta	36.1 (32.7–41.7)	40.2 (31.1–55.5)	55.7 (50.0–62.9)	57.8 (48.7–72.1)	77.6 (62.3–95.0)
gab	20.0 (17.2–24.0)	29.0 (18.4–42.5)	58.8 (48.3–66.9)	36.9 (29.9–53.1)	72.6 (51.7–99.1)
glu	201.8 (165.3–241.5)	260.8 (193.9–372.1)	438.0 (261.0–559.0)	331.0 (317.6–339.1)	457.5 (386.3–591.8)
gln	25.7 (13.2–51.1)	20.4 (11.1–27.5)	34.6 (28.9–45.4)	22.6 (17.4–31.4)	25.6 (16.3–31.0)
gly	7.7 (5.9–11.8)	8.8 (5.3–12.1)	6.6 (4.8–9.2)	7.2 (6.6–8.0)	6.3 (2.4–7.8)
his	6.9 (5.1–8.9)	8.8 (4.7–11.6)	9.3 (6.7–11.4)	6.0 (4.8–7.6)	9.1 (5.0–11.1)
ile	3.6 (3.0–4.3)	5.2 (3.3–7.5)	5.6 (5.4–5.7)	4.9 (4.4–6.1)	6.3 (5.6–7.9)
leu	4.7 (4.0–5.3)	5.3 (3.9–7.4)	6.9 (6.8–7.1)	6.0 (5.2–7.6)	6.8 (5.9–8.8)
lys	5.7 (4.6–7.8)	7.6 (4.0–9.9)	6.8 (6.0–7.7)	6.7 (5.6–8.8)	7.1 (5.8–8.1)
met	1.4 (1.1–1.8)	1.7 (1.2–2.6)	1.9 (1.2–2.5)	2.2 (1.5–3.2)	1.9 (0.6–3.6)
mhl	3.7 (2.3–6.0)	5.6 (4.3–8.6)	6.1 (5.5–6.8)	3.6 (3.1–4.6)	4.8 (3.7–5.5)
orn	7.3 (4.3–14.6)	9.8 (4.1–13.9)	7.7 (5.1–10.1)	8.6 (8.3–8.8)	8.9 (6.0–11.7)
phe	22.7 (18.7–27.0)	28.8 (22.7–38.0)	32.5 (25.3–37.3)	27.9 (21.0–36.0)	27.4 (22.0–35.1)
pro	13.2 (10.6–14.6)	17.8 (10.0–25.0)	31.4 (10.9–50.3)	13.8 (10.9–18.9)	25.1 (14.3–35.5)
ser	60.7 (51.3–67.2)	61.0 (45.5–88.4)	52.2 (39.8–60.4)	61.5 (49.0–68.3)	56.4 (35.3–71.2)

TABLE 7. CONTINUED

Compound (μg/g dry mass)	Tree 1	2	3	4	5
thr	16.1 (11.4–21.0)	15.6 (11.7–22.1)	12.7 (10.2–15.5)	12.0 (9.5–14.8)	13.7 (10.2–16.8)
tyr	9.7 (8.6–10.7)	10.6 (8.0–14.1)	12.1 (11.3–13.4)	9.4 (7.4–11.8)	13.7 (11.9–15.6)
val	7.1 (6.2–8.5)	9.2 (7.0–12.9)	11.1 (9.7–12.3)	10.3 (8.7–13.6)	10.8 (8.8–14.2)

"Tree means and values of branches with the lowest and highest value for each tree (in parentheses) are shown.

DISCUSSION

Variation in Larval Growth Rate. Ramets within trees and short shoots within branches accounted for most of the explained variation in larval growth rate. The variation was distributed similarly among the different levels during both years. Earlier findings show that during successive years individual mountain birches retain their relative rankings in chemical and morphological characters of the leaves, and as a diet for *E. autumnata* larvae (Haukioja et al., 1985a; Senn et al., 1992; Hanhimäki et al. 1995). The present results indicate that the same is also true at the level of the ramet and the branch. It also confirms that the methods used in this study, as well as earlier (Suomela and Nilson, 1994), are sufficiently reliable and reproducible for this kind of work.

Compared to earlier experiments (Suomela and Nilson, 1994), in which short shoots accounted for about 7% of total variance, the relative proportion of the short shoots in the present study was higher. As a proportion of the mean (coefficient of variation), however, variation among short shoots was lower or about the same as in the earlier experiments.

The constancy of leaf quality between successive years may result from intrinsic or extrinsic factors. Consistent differences may be due to somatic mutations (Whitham and Slobodchikoff, 1981; Antolin and Strobeck, 1985; but see Slatkin, 1985) or other intrinsic sources, such as the proximity of strong physiological sinks (Marshall, 1990; Wardlaw, 1990), which may remain relatively constant between years in different parts of the tree. They may also be due to persistent external factors, such as variation in solar irradiation or in shading by other branches or trees (see Suomela and Nilson, 1994).

Variation in Biochemical Characters. To our knowledge, these are the first findings partitioning variance in biochemical characteristics among and within trees, using same-aged, randomly chosen leaves. Our main finding was that the

TABLE 8. PROPORTIONS OF TOTAL VARIANCE AND COEFFICIENTS OF VARIATION OF PROTEIN-BOUND AMINO ACIDS AT DIFFERENT HIERARCHICAL LEVELS, AND GRAND MEAN FOR EACH COMPOUND

	Amino acids[a]																
Source of variation	ala	arg	asp	cys	glu	gly	his	ile	leu	lys	met	phe	pro	ser	thr	tyr	val
Proportion of total variance (%)																	
Trees	8	7	11	38	26	20	2	6	5	12	56	21	26	2	0	22	8
Ramets	0	0	0	28	0	0	0	0	0	0	0	0	0	0	0	0	0
Branches and error	92	93	89	33	74	80	98	94	95	88	44	79	74	98	100	78	92
Coefficient of variation (%)																	
Trees	3	3	2	11	6	6	2	3	3	4	22	5	9	2	0	5	4
Ramets	1	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0
Branches and error	11	11	6	10	9	12	11	11	14	12	20	10	15	12	13	10	12
Grand mean (mg/g dry mass)	8	7	5	1	15	7	3	6	11	9	1	6	6	6	5	4	8

[a] Explanations for abbreviations, see Table 6.

TABLE 9. CONTENTS OF PROTEIN AMINO ACIDS IN LEAVES OF MOUNTAIN BIRCH[a]

Compound (mg/g dry mass)	Tree				
	1	2	3	4	5
ala	7.9 (7.3-8.3)	8.6 (8.3-8.7)	7.9 (7.3-8.1)	8.6 (7.8-9.2)	7.4 (6.3-9.9)
arg	7.0 (6.5-7.4)	7.6 (7.3-7.8)	6.8 (6.2-7.4)	7.1 (6.5-7.7)	6.9 (5.8-8.7)
asp	5.3 (5.1-5.5)	5.4 (5.3-5.5)	5.1 (4.9-5.3)	5.5 (5.2-5.9)	5.1 (4.7-5.9)
cys	0.7 (0.6-0.8)	0.5 (0.5-0.5)	0.7 (0.6-0.7)	0.7 (0.6-0.7)	0.6 (0.5-0.7)
glu	14.9 (13.9-15.5)	15.8 (15.3-16.1)	14.8 (13.7-15.3)	16.2 (14.8-17.2)	13.6 (12.0-17.2)
gly	6.6 (6.1-6.9)	7.1 (6.8-7.2)	6.6 (6.2-6.9)	7.3 (6.8-7.8)	6.1 (5.0-8.2)
his	2.7 (2.4-2.8)	2.9 (2.8-3.0)	2.6 (2.4-2.8)	2.7 (2.4-2.9)	2.6 (2.2-3.3)
ile	6.0 (5.5-6.4)	6.5 (6.2-6.6)	5.9 (5.5-6.2)	6.2 (5.5-6.6)	5.8 (4.9-7.5)
leu	10.6 (9.8-11.3)	11.5 (11.0-11.7)	10.7 (9.9-11.1)	11.0 (7.5-12.6)	9.7 (8.4-12.4)
lys	8.7 (8.0-9.2)	9.1 (8.8-9.4)	8.7 (8.0-9.1)	9.2 (8.2-10.0)	7.9 (6.6-10.5)
met	0.5 (0.4-0.6)	0.6 (0.5-0.7)	0.4 (0.4-0.5)	0.5 (0.4-0.6)	0.3 (0.2-0.4)
phe	6.3 (5.9-6.8)	6.9 (6.6-7.1)	6.3 (5.8-6.6)	6.6 (5.9-7.2)	5.8 (5.1-7.3)
pro	6.2 (5.8-6.5)	6.8 (6.5-7.0)	6.4 (6.0-6.7)	5.5 (4.9-6.2)	5.3 (4.4-7.8)
ser	5.3 (5.0-5.5)	5.7 (5.5-5.8)	5.3 (4.9-5.5)	5.7 (5.2-6.2)	5.2 (4.5-7.0)
thr	5.0 (4.7-5.2)	5.3 (5.1-5.4)	4.8 (4.5-5.0)	5.3 (4.8-5.7)	5.1 (4.3-7.1)
tyr	4.0 (3.7-4.3)	4.4 (4.2-4.6)	3.7 (3.4-4.1)	4.0 (3.7-4.3)	3.9 (3.4-4.8)
val	7.5 (6.9-7.9)	8.1 (7.7-8.3)	7.4 (6.7-7.7)	8.0 (7.1-8.7)	7.1 (6.3-9.4)

[a]Tree means and values of branches with the lowest and highest value for each tree (in parentheses) are shown.

proportion of variation attributable to different hierarchical levels and the coefficients of variation varied depending on the character. In total phenolics and individual low-molecular-weight phenolics, most of the variance was accounted for by trees. In carbohydrates and amino acids, it was the branch level (including

TABLE 10. MULTIPLE REGRESSION ANALYSIS ON EFFECTS OF (+)-CATECHIN AND MONOSACCHARIDES ON GROWTH RATE OF *E. autumnata* LARVAE ($R^2 = 0.96$)

Variable	*df*	Parameter estimate	Standard error	*T* for H0: Parameter = 0	$P > \|T\|$
(+)-Catechin	1	−0.000635	0.000191	−3.33	0.08
Monosaccharides	1	0.001171	0.000359	3.26	0.08
Intercept	1	0.251482	0.020247	12.42	0.006

error variance) that generally accounted for most of the variance. In sugars and certain amino acids, a considerable part of the variation was also accounted for by trees (Tables 4, 6, and 8).

As indicated previously, variation among branches cannot be distinguished in the biochemical data from error variance, since we could only analyze one sample per branch. To estimate the reliability of branch-level measurements based on a single sample, we had checked beforehand the extent of variation in measurements among different samples derived from the same homogeneous powder (see Methods and Materials). In carbohydrates and amino acids, the coefficients of variation among different samples were clearly lower than coefficients of variation at the branch level (which include the measurement error) in the actual experiment (Tables 4, 6, and 8). This suggests that in carbohydrate and amino acid contents, in which variation was high at the lowest hierarchical level, a considerable proportion of variation was actually accounted for by the branches, not only by error variance.

Of the different biochemical compounds, the among-tree coefficients of variation were highest for the contents of phenolic compounds and lowest for protein-bound amino acids (Tables 2, 4, 6, and 8). The most extreme example of among-tree variation was the kaempferol-glycoside (phenolic no. 26), with a 50-fold difference among extreme trees (coefficient of variation 164%). At the ramet level, the coefficients of variation were generally low, being highest for free amino acids.

At the branch level, the coefficients of variation were highest for free amino acids and lowest for phenolics and protein-bound amino acids. Although the branch level relatively accounted for most of the total variance in protein-bound amino acids but for only a minor proportion in low-molecular-weight phenolic compounds, the coefficients of variation at this level were of about the same magnitude in both compound groups (on average 11.7% and 12.5% for amino acids and phenolics, respectively). This shows that it is essential to evaluate both the relative contributions of different hierarchical levels to the total variation

and the actual magnitude of variation (i.e., standard deviations or coefficients of variation).

The coefficients of variation for most biochemical compounds were clearly higher than those for larval growth rate, particularly at the levels of the tree and the branch. This suggests that, at least for short periods, the generalist *E. autumnata* larvae are able to handle relatively large biochemical variation in their food plants. This may be due, e.g., to changes in feeding rate or to flexibility of the digestive and detoxification systems of the larvae (Slansky, 1993 and references therein). It is possible, however, that younger larvae are more sensitive to biochemical variation than the late-instar larvae used in the present study (Gould, 1984; Slansky, 1993).

There are also some other studies in which variation among conspecific plant individuals has been estimated. The coefficients of variation of different phenolic compounds among trees in our study were of about the same magnitude as in the bark of winter-dormant *Betula pendula* seedlings representing several origins (Tahvanainen et al., 1991). Variation among individuals of several *Artemisia* species was also of the same magnitude (Wilt et al., 1992). In sugar contents the coefficients of variation among trees were lower than or about the same magnitude as in the bark of birch seedlings in Tahvanainen et al. (1991).

Generalizing, our findings indicate that there are differences in the among- and within-tree variation for the biochemical characteristics. Some compounds have a larger range than others, and the distribution of variance depends on the character. The large among-tree variation in the low-molecular-weight phenolics suggests that contents of these compounds in mountain birch leaves depend mainly on genetic factors and/or on relatively invariant microenvironmental or phenotypic factors. The contents of carbohydrates and amino acids seem to vary more than phenolics for phenotypic reasons, since in these compounds there was considerable variation within trees. This is consistent with the status of carbohydrates and amino acids as active metabolites, whose concentrations will vary depending, for instance, on the source-sink status of a particular plant part (Wardlaw, 1990). Furthermore, within-canopy differences in light conditions (DeJong and Doyle, 1985) or other environmental factors may have increased within-tree variation.

Since all 27 individual phenolic compounds were found in all 20 branches, the results do not lend support to the hypothesis of somatic mutations as an important source of qualitative variation in secondary chemistry within individual birches (Whitham and Slobodschikoff, 1981; Antolin and Strobeck, 1985).

Biochemical Characters and Larval Growth. Both phenolics [particularly (+)-catechin] and sugars seemed to affect larval growth, although the results were not quite significant. (+)-Catechin has earlier been found to have an adverse effect on bollworm (Noctuidae) larvae (Guerra et al., 1990). This com-

pound is a precursor of condensed tannins, and its negative effect on larval growth may take place via its interaction with proteins.

Although sugars have been largely overlooked in plant-herbivore studies, the contents of glucose and fructose (Zou and Cates, 1994), sucrose (Clancy, 1992), or total sugar content (Jensen, 1988) have been found to correlate positively with insect performance. Most of the experiments have been conducted with coniferous trees (Shaw et al., 1978; Jensen, 1988; Clancy, 1992) or with artifical diets (Rahbé et al., 1988; Clancy, 1992; Zou and Cates, 1994). Our results suggest that sugars may be important in deciduous trees also.

Total phenolic content of the mountain birch leaves has earlier been found to correlate negatively with growth of *E. autumnata* larvae on the mountain birch (Haukioja et al., 1985b). Also in the present study, there was a negative correlation between leaf total phenolic content and larval growth ($r = -0.74$, $P = 0.15$). However, the correlation between monosaccharides and total phenolics was negative ($r = -0.84$, $P = 0.07$). Thus, it is impossible to evaluate if total phenolics affected larval growth also in the present study but did not enter into the regression model due to their intercorrelation with sugars.

In summary, the relationships between biochemical characters and larval growth in the present study should be interpreted as suggestive only, since the number of trees in our study was low and we could include only few compounds into the model. However, the results indicate that both secondary metabolites, represented here by one phenolic compound, and primary metabolites, represented here by sugars, may be important for the growth of *E. autumnata* larvae.

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HERBIVORE AVOIDANCE OF DIGITALIS EXTRACTS IS NOT MEDIATED BY CARDIAC GLYCOSIDES

DALE L. NOLTE,[1,*] KEVIN L. KELLY,[2] BRUCE A. KIMBALL,[2] and JOHN J. JOHNSTON[2]

[1]*United States Department of Argiculture*
Animal and Plant Health Inspection Service
Denver Wildlife Research Center
9701 Blombreg Street, Olympia, Washington 98512

[2]*United States Department of Argiculture*
Animal and Plant Health Inspection Service
Denver Federal Center
Denver, Colorado 80225

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Abstract—This study was conducted to determine whether avoidance of digitalis (*Digitalis purpurea*) by mountain beaver (*Aplodontia rufa*) is induced by toxic cardiac glycosides. High-performance liquid chromatography and behavioral assays were used to relate animal responses with the presence of common cardiac glycosides in several digitalis extracts. Statistical analyses of multiple-choice tests showed no correlation between cardiac glycoside content and mountain beaver avoidance of apple cubes treated with digitalis extracts. Therefore, we concluded that known toxic cardiac glycosides were not responsible for chemosensory cues that inhibited intake of food treated with digitalis extracts. These results suggest that digitalis is a source of an effective nontoxic herbivore repellent.

Key Words—*Aplodontia rufa*, avoidance, cardiac glycoside, digitalis, *Digitalis purpurea*, herbivore, mountain beaver, repellent

INTRODUCTION

Human and wildlife interactions are becoming more frequent, often with negative effects on both humans and wildlife. Current preventive measures such as trapping and lethal control are often not feasible or inappropriate in many management situations. Nonlethal repellents may provide viable alternatives.

*To whom correspondence should be addressed.

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Plants that are naturally avoided may be a source of aversive agents to inhibit browsing (Cardellina, 1988). For example, digitalis (*Digitalis purpurea*) plants are rarely harvested by mountain beaver (*Aplodontia rufa*), and prior studies indicate that even a preferred food is avoided when treated with water extracts of digitalis (Nolte et al., 1995). Unfortunately, toxins may render digitalis undesirable as a source of nonlethal aversive agents. The aversive cues, however, are not necessarily the toxic cardiac glycosides. Gustatory or olfactory cues of a food, and the specific compounds that cause aversive feedback, need not be synonymous (Provenza and Balph, 1990). We conducted a series of chemical and behavioral assays to determine whether the toxic glycosides common in digitalis are the aversive cues avoided by mountain beaver.

METHODS AND MATERIALS

Chemicals. High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and hexane were obtained from Fisher Scientific (Fair Lawn, New Jersey), and HPLC grade chloroform was obtained from Baxter (McGaw Park, Illinois). Water was either HPLC grade (Baxter) or purified in-house using the Milli-Q+ purification system (Millipore, Molsheim, France). Digitoxin and gitoxin were obtained from Sigma (St. Louis, Missouri).

Stimuli. Digitalis was collected in the vicinity where test animals were subsequently trapped. Samples were frozen and lyophilized for 48 hr. The lyophilized material was ground through a 20-mesh Wiley mill, mixed uniformly, and stored at 4°C.

Subjects. Experimentally naive (not previously tested) adult mountain beavers were trapped in the Capital State Forest, Grays Harbor County, approximately 30 km from Olympia, Washington. Animals were penned individually in outdoor pens (3 × 3 m) and given free access to pelleted feed (X-Cell Feed Company, Tacoma, Washington) and water throughout the trials. All animals were given a minimum of four days to adjust to captivity and the test regime prior to the onset of trials.

Extract Preparation. To derive a series of liquid–liquid extracts, two hot water digitalis extracts were prepared identically by placing 60 g of ground digitalis in 1.5 liters of water maintained at 85°C. The extraction was performed for 4 hr with periodic stirring followed by filtration through Whatman 114 coarse filter paper (Whatman International Ltd., Maidstone, Kent, U.K.). The liquid–liquid extracts were subsequently prepared by sequentially "washing" one of the filtered hot water digitalis extracts with 1.2 liters of hexane and 1.2 liters chloroform in a separatory funnel by manual shaking for 20 min. The hexane (L-L:H1) and chloroform (L-L:C2) phases, as well as the resultant filtrate (RF:A) were collected. This process was repeated with the other hot water

digitalis extract but the order of the hexane and chloroform washes was switched. Again, the chloroform (L-L:C1) and hexane (L-L:H2) phases were collected as was the resultant filtrate (RF:B).

Room temperature liquid–solid extracts were prepared by combining 60 g of ground digitalis with methanol (L-S:M), hexane (L-S:H), chloroform (L-S:C), or water (L-S:W) and shaking on a horizontal mechanical shaker for 30 min prior to filtration. Approximately 1.25 liters of filtrate was collected from each of the extraction processes.

Digitalis extracts were applied to apple cubes (1 cm^3) by submerging the cubes in the extracts for 1 hr immediately prior to tests. Apple cubes were similarly treated with the respective solvents for trials to determine whether mountain beaver were responding to cues emitted from digitalis or merely avoiding the solvents. Peeled apples were selected as the test food because they are readily ingested by mountain beavers.

Behavioral Assays. A series of multiple-choice tests were used to determine mountain beaver responses to extracts derived from digitalis. First, mountain beaver were given a choice of apple cubes treated with the liquid–liquid extracts (L-L:H1, L-L:H2, L-L:C1, L-L:C2) and the resultant filtrates (RF:A, RF:B) along with a water control. Response of mountain beavers to the liquid–solid extracts (L-S:H, L-S:C, L-S:M, L-S:W), one of the resultant filtrates (RF:B), and a water control was assessed in the second test. A third behavioral assay was conducted with the solvents (methanol, hexane, chloroform, and a water control) used to prepare the extracts.

During each of the tests, mountain beaver ($N = 10$) were presented with 20 untreated apple cubes placed in weigh-boats spaced at even intervals along the perimeter of their pens for four days of pretreatment. The number of apple cubes in each weigh-boat and within the immediate area (< 30 cm) was assessed after 24 hr. At that time, all remaining cubes were removed and another 20 fresh cubes were placed in the weigh-boats. A four-day treatment period immediately followed pretreatment. Treatment procedures were identical to those described for pretreatment except apples cubes were treated with the extracts as described above. Three tests were conducted sequentially as described above, and all treatments (extracts) within a test were offered simultaneously. Treatment locations were randomly selected each day.

Chemical Assays. All extracts were analyzed for digitoxin and gitoxin with a Hewlett Packard 1090M HPLC equipped with a diode array detector (Hewlett Packard Co., Palo Alto, California). Ultraviolet (UV) detection of the analytes was achieved at 220 nm. The analytical column was a 250 × 4.6 mm Keystone Octyl-H with a 10 × 4-mm guard of the same stationary phase (Keystone Scientific, Inc., Bellefonte, Pennsylvania). The mobile phase consisted of 68% water and 32% acetonitrile for 10 min, followed by a 15-min linear gradient of increasing acetonitrile concentration until a composition of 60% water and 40%

acetonitrile was achieved. For chloroform extracts only, the mobile phase consisted of a linear gradient from 100% water to 50% methanol/50% acetonitrile over 65 min. The flow rate was 1.0 ml/min and the injection volume was 10 μl.

Standards of digitoxin and gitoxin, prepared in methanol, were used for identification and quantitation.

Statistical Analyses. The data for each behavioral assay were assessed separately in single factor analyses of variance (ANOVA). A randomized block design was used where mountain beaver were blocks and food with the respective extracts were treatments. Tukey tests (Winer, 1971) were used to isolate significant differences among means subsequent to the omnibus procedures ($P < 0.05$).

RESULTS

Mountain beaver ($N = 9$) responses varied among treatments in the first behavioral assay ($P < 0.0001$) (Figure 1). One animal was not included in the

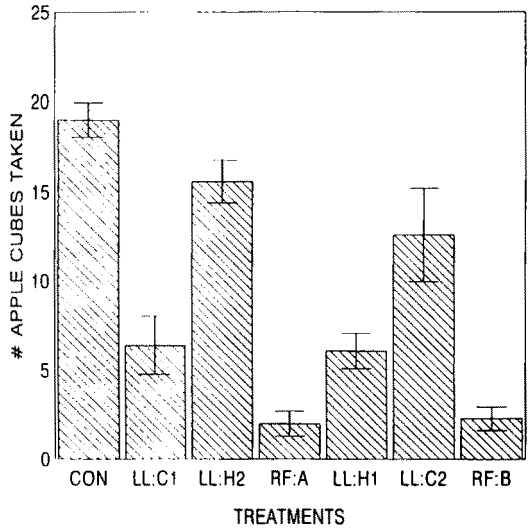

FIG. 1. Number of apple cubes take by moutain beaver during a multiple-choice test when the test food (20 apple cubes) was treated with one of six digitalis extracts prepared by a two-step liquid–liquid extraction or untreated (CON). The resultant filtrate (RF:A) was first collected after hexane (LL:H1) was followed by chloroform (LL:C2). Next the resultant filtrate (RF:B) was collected after chloroform (LL:C1) was followed by hexane (LL:H2).

analysis because it failed to ingest any treatment or control cubes throughout the assay. Mountain beaver took fewer ($P < 0.05$) apple cubes treated with either of the resultant filtrates (RF:A, RF:B) or the initial phase L-L extracts (L-L:H1, L-L:C1) than they did control cubes or cubes treated with the second phase liquid–liquid extracts (L-L:H2, L-L:C2). Mountain beaver responses were similar ($P > 0.05$) to L-L:H2 and L-L:C2 and to L-L:H2 extract and control cubes, but they took fewer ($P < 0.05$) cubes treated with the L-L:C2 extract than control cubes. Subsequently, we rated the mountain beavers' relative avoidance of each extract as: high, greater than control; low, similar to control; or moderate, greater than control but less than extracts rated as high (Table 1).

Responses of mountain beavers also varied with treatments in the second behavioral assay ($P < 0.0001$) (Figure 2). Animals ingested similar numbers of cubes treated with the hexane (L-S:H) or chloroform (L-L:C) extracts as they did control cubes ($P > 0.05$). Response to the water extracts (L-S:W or RF:B) and the methanol (L-S:M) extract were similar, and all of these extracts were substantially more aversive than the other treatments ($P < 0.05$). Mountain beavers' relative avoidance of these extracts was also rated as described above (Table 1).

None of the solvents used to prepare extracts were aversive. Mountain

TABLE 1. SUMMARY OF MOUNTAIN BEAVER RESPONSES TO TEST EXTRACTS AND ABSENCE OR PRESENCE OF CARDIAC GLYCOSIDES

Extract	Cardiac glycosides	Relative avoidance
Liquid–solid		
L-S:W	present	high
L-S:M	absent	high
L-S:C	absent	low
L-S:H	absent	low
Liquid–liquid		
L-L:C1	present	high
L-L:H2	absent	low
R-F:A	absent	high
L-L:H1	present	high
L-L:C2	present	moderate
R-F:B	absent	high
Solvents		
Water	absent	low
Methanol	absent	low
Chloroform	absent	low
Hexane	absent	low

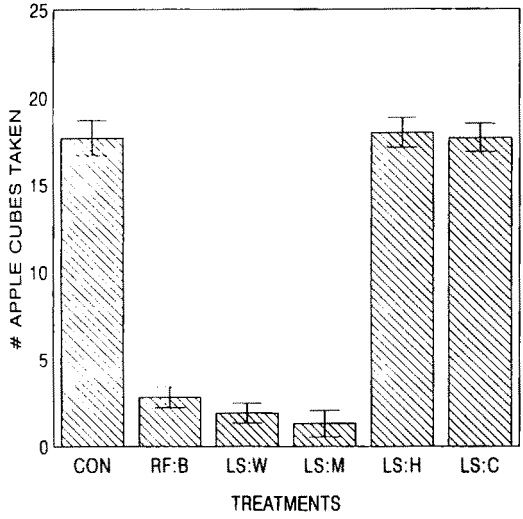

FIG. 2. Number of apple cubes taken by mountain beaver during a multiple-choice test when the test food (20 apple cubes) was treated with one of four liquid–solid digitalis extracts prepared with methane (LS:M), chloroform (LS:C), hexane (LS:H), water LS:W), or treated with the resultant filtrate (RF:B) from a chloroform/hexane liquid–liquid extract, or untreated (CON).

beaver ingested similar numbers of apple cubes in the third assay regardless of treatment ($P > 0.3$). The mean number of the 20 apple cubes treated with methanol, chloroform, hexane, or water taken by mountain beaver was 19.2, 19.7, 17.8, and 18.0, respectively.

Chemical Assays. All extracts were analyzed for digitoxin and gitoxin to determine if the cardiac glycosides were present (Table 1). Digitoxin and gitoxin were detected in the hot water liquid–solid extract prior to being subjected to the liquid–liquid extraction. HPLC analysis also indicated that digitoxin and gitoxin were present in both chloroform liquid–liquid extracts (L-L:C1 and L-L:C2), as well as the initial hexane liquid–liquid extract (L-L:H1) (Figure 3). Cardiac glycosides were not present in the second hexane L-L extract (L-L:H2) or either of the resultant filtrates (RF:A and RF:B) (Figure 4). Although the water liquid–solid extract contained digitoxin and gitoxin, neither of these cardiac glycosides were detected in the other liquid–solid extracts (L-S:M, L-S:H, L-S:C).

The detection limits, defined as the analyte concentration required to produce a chromatographic response equal to three times the chromatographic noise,

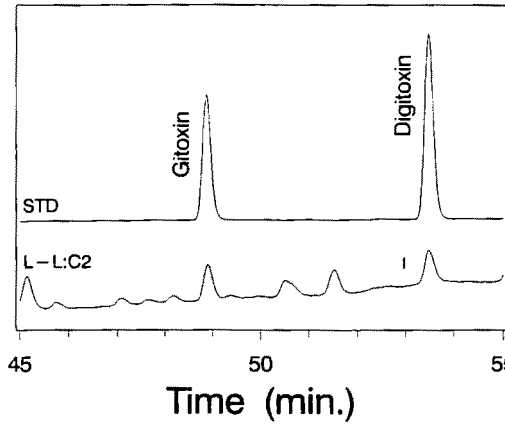

FIG. 3. Chromatograms of an extract (L-L:C2) in which digitoxin and gitoxin were detected and a standard mixture of gitoxin and digitoxin (STD). The y scale of L-L:C2 was amplified by fourfold for clarity. Chromatographic conditions are provided in the text.

were found to be 0.67 μg/ml and 0.48 μg/ml for digitoxin and gitoxin, respectively.

DISCUSSION

Aversive cues emitted by foxglove do not appear to originate from digitoxin or gitoxin. The methanol liquid–solid extract (L-S:M) and both resultant filtrates

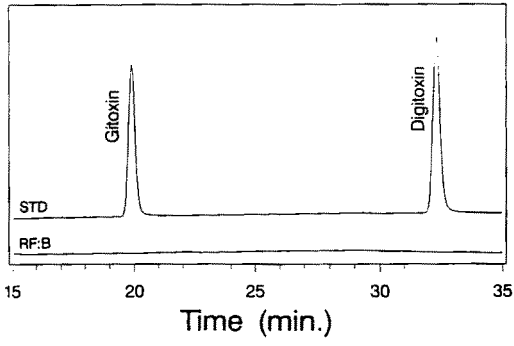

FIG. 4. Chromatograms of a resultant filtrate (RF:B) in which digitoxin and gitoxin were not detected and a standard mixture of digitoxin and gitoxin (STD). Chromatographic conditions are provided in the text.

were avoided by mountain beaver, although digitoxin and gitoxin were absent. In addition, avoidance of one of the liquid–liquid extracts that contained both digitoxin and gitoxin was only moderate.

The absence of cardiac glycosides in extracts was based on chromatographic analysis of the extracts for digitoxin and gitoxin, although other cardiac glycosides are known to be present in digitalis (Fujii et al., 1989). Digitoxin and gitoxin, however, should be good indicators of the cardiac glycosides present in digitalis. Digitoxin and gitoxin are two of the three main secondary glycosides formed from the primary glycosides that are present in living, undamaged digitalis plants. The polarities and solubilities of all the primary and secondary glycosides are similar. Therefore, we used the presence of digitoxin and gitoxin as "markers" for the presence of all possible cardiac glycosides. If neither digitoxin nor gitoxin were detected in an extract, it was assumed that no cardiac glycosides were present in the extract at our detection limit.

The combined assays indicate that the chemical cues avoided by mountain beaver were polar. Extracts prepared with polar solvents were aversive to mountain beaver, while extracts prepared with nonpolar solvents were generally not avoided. For example, in the second behavioral assay, apple cubes treated with the nonpolar hexane (L-S:H) and chloroform (L-S:C) extracts were taken, while cubes treated with the polar water (L-S:W) and methanol (L-S:M) extracts were avoided. Furthermore, the aversive resultant filtrates (RF:A, RF:B) were also derived from polar extracts of digitalis.

Generally, the polar extracts were avoided by mountain beaver on the first day of trials. Repellents that elicit initial avoidance are generally either irritants (e.g., capsaicin) or those that evoked "fear" response (e.g., predator scents) (Mason and Clark, 1992). The initial avoidance in these trials, however, may have reflected a conditioned food aversion. Conditioned food aversions occur when ingestion of a novel food is paired with gastrointestinal distress (Garcia and Koelling, 1966; Garcia, 1989). The mountain beavers in our trials likely had encountered digitalis prior to capture. Since the glycosides in digitalis induce nausea, it is possible that these animals learned to avoid digitalis through pairing the nontoxic cues with the toxins. Gustatory or olfactory cues of a food, and the specific compounds that cause aversive feedback, need not be and probably rarely are synonymous (Provenza and Balph, 1990).

Management Implications. Digitalis extracts may provide an effective source of aversive agents to inhibit herbivore damage in the Pacific Northwest. Nontoxic cues derived from the plant are avoided by mountain beaver. However, it is unclear whether the avoidance reflects a conditioned aversion. Regardless, the abundance of digitalis throughout the Pacific Northwest virtually ensures that if learning is required, animals will recognize its aversive properties through prior "natural" experiences. Further, herbivore familiarity with digitalis may

reduce habituation to its aversive cues. Animals that ignore cues normally emitted by the plant would risk exposure to its toxic properties.

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VOLATILE COMPOUNDS INDUCED BY HERBIVORY ACT AS AGGREGATION KAIROMONES FOR THE JAPANESE BEETLE (*Popillia japonica* NEWMAN)

JOHN H. LOUGHRIN,[1] DANIEL A. POTTER,[1,*] and THOMAS R. HAMILTON-KEMP[2]

[1]*Department of Entomology, S-225 Agricultural Science Center North University of Kentucky, Lexington Kentucky 40546*

[2]*Department of Horticulture and Landscape Architecture, N-323 Agricultural Science Center North University of Kentucky, Lexington Kentucky 40546*

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Abstract—The Japanese beetle is a polyphagous insect that typically aggregates on preferred host plants in the field. We studied the response of Japanese beetles to artificial damage, fresh feeding damage, and overnight feeding damage to test the hypothesis that beetles are attracted to feeding-induced volatiles. Crabapple leaves that had been damaged overnight by Japanese beetles or fall webworms attracted significantly more Japanese beetles than did undamaged leaves. Artificially damaged leaves or leaves freshly damaged by Japanese beetles, however, were not significantly more attractive than undamaged leaves. Leaves that had been damaged overnight by Japanese beetles or fall webworms produced a complex mixture of aliphatic compounds, phenylpropanoid-derived compounds, and terpenoids. In comparison, artificially damaged leaves or leaves with fresh Japanese beetle feeding damage generated a less complex blend of volatiles, mainly consisting of green-leaf odors. Feeding-induced odors may facilitate host location and/or mate finding by the Japanese beetle.

Key Words—*Popillia japonica*, Coleoptera, Scarabeidae, apple, *Malus* × *domestica*, attractant, induction, plant–insect interaction, semiochemical, terpene.

*To whom correspondence should be addressed.

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INTRODUCTION

Odors induced by arthropod herbivory on higher plants have recently been implicated as attractants of predators and parasitoids (Turlings et al., 1990). In the cases so far examined, volatiles were induced by arthropod feeding in a delayed manner, and the induced odors were typically a complex blend of aliphatics, terpenoids, and/or phenylpropanoid-derived compounds. The induced blends are often floral in character, which may increase the likelihood of predation on lepidopteran larvae in that many adult parasitic Hymenoptera are dependent on nectar for food (Leius, 1960).

Little is known, however, about the effects of these induced volatiles on the herbivores themselves. Still, evidence has been obtained for the additive or synergistic effects of host volatiles along with sex pheromones on insect aggregation. For example, female mountain pine beetles, *Dendroctonus ponderosae* Hopkins, convert the host-produced monoterpene α-pinene into *trans*-verbenol, which is an aggregation pheromone for this species. Verbenone, which is produced by microbial symbionts of *D. ponderosae*, serves as an anti-aggregation pheromone and terminates mass attacks on individual host trees (Borden, 1984; Hunt and Borden, 1990). In another example, fruit volatiles released by feeding of the green June beetle, *Cotinus nitida* L., were shown to be highly attractive to other green June beetles (Domek and Johnson, 1988). To our knowledge, however, no studies have examined feeding-induced volatile compounds from the leaves of woody angiosperms and their effect on aggregation of herbivorous insects.

The adult Japanese beetle (*Popillia japonica* Newman: Scarabaeidae) is a highly polyphagous insect that feeds upon nearly 300 plant species in 130 families (Fleming, 1972). Since its accidental introduction into New Jersey about 80 years ago, the beetle has become one of the most destructive pests of shade trees, fruit and vegetable crops, and ornamental plantings in the eastern United States. One of the more curious aspects of this insect's behavior is its tendency to congregate on individual hosts, often nearly defoliating them, while other suitable hosts in the vicinity often suffer only minimal damage (Van Leeuwen et al., 1928; Fleming, 1972). Males of this species are strongly attracted to the female sex pheromone, (Z)-5-(1-decenyl)-dihydro-2-(3H)-furanone (Tumlinson et al., 1977), but this alone cannot explain the aggregative behavior because females are normally mated on the ground at emergence, and mated females do not attract males at long range (Ladd, 1970). This has led to speculation of an aggregation pheromone (Adler and Jacobson, 1971; Iwabuchi and Takahasi, 1983).

We speculated that volatile compounds induced by feeding on leaves could act as aggregation kairomones for the Japanese beetle. The present study using

crabapple leaves (*Malus* × *domestica*), a highly preferred host of the Japanese beetle (Fleming, 1972), was undertaken to test this hypothesis.

METHODS AND MATERIALS

Insects and Plants. Japanese beetles were captured in the field using traps baited with commercial food-type lures (1 : 1.1 : 2.2 ratio of geraniol –phenethyl propionate–eugenol (Trécé Inc., Garwood, New Jersey). Fall webworms (*Hyphantria cunea* Drury: Arctiidae) were reared on wheat germ medium (Yearian et al., 1966) and then transferred to a diet of crabapple leaves at least one day before use in experiments. Third instars were used for the bioassays. Crabapple trees (cv. Dolgo) were maintained in nursery plots at the University of Kentucky's South Farm in Lexington, Kentucky.

The afternoon before an experiment, six to eight crabapple branches with an average of 12–15 leaves each were collected, immediately placed in clear plastic trays with lids, and held overnight. For investigation of the volatiles induced by overnight feeding damage, each tray received about 150 Japanese beetles or fall webworms. No insects were added to undamaged controls or leaves reserved for artificial or fresh beetle damage. In the case of artifical damage, leaves were damaged immediately before experiments by dragging a razor blade parallel to the midvein along the length of the leaf five times. For fresh beetle damage, about 150 beetles were allowed to feed in the trays for 2 hr prior to pitfall assays or volatile collections.

Pitfall Bioassays. Attraction of Japanese beetles to damaged or intact leaves was measured in a pitfall bioassay system because the beetles' extreme phototropism rendered use of a conventional wind tunnel impractical. Assays were conducted in choice-test arenas (Figure 1) consisting of six 125-ml Erlenmeyer flasks (mouth diam. 3.0 cm) in plastic trays (32 × 26 cm, 10 cm deep). The trays were filled with dry sand until the sand was level with the mouths of the flasks. Leaf treatments, consisting of a single damaged or undamaged leaf per flask, were placed in the flasks and the flasks were situated such that the beetles could enter by walking on the surface of the sand. Five comparisons were performed daily: a single crabapple leaf versus no leaf and the four types of damaged leaves (i.e., artificially damaged, freshly damaged by Japanese beetles, leaves with overnight feeding damage by Japanese beetles, and leaves with overnight feeding damage by fall webworms) versus undamaged crabapple leaves.

Preliminary trials indicated that male and female Japanese beetles did not differ significantly in their responses to leaves in the bioassay system. We therefore used both sexes in the pitfall bioassays in the same ratio (about 1 : 1) that

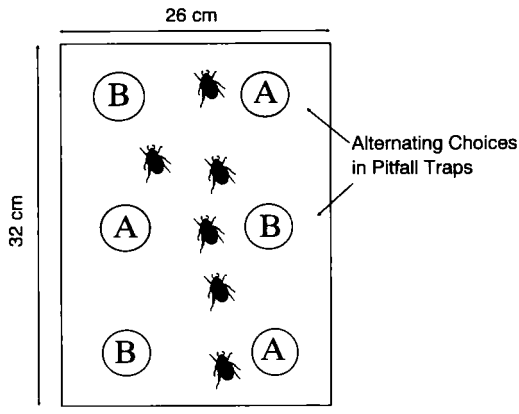

FIG. 1. Arena used for testing Japanese beetle attraction to damaged or undamaged leaves.

was captured in the traps baited with commercial food-type lures. This was intended to simulate attraction of a mixed-sex population as occurs to hosts in the field. Beetles were captured daily using traps baited with food lures and then held overnight without food. For each bioassay, 30 beetles were placed in the middle of the trays and allowed to choose between the two treatments. The bioassays were conducted at room temperature using overhead fluorescent lighting. Bioassays were allowed to proceed for 3 hr or until 25 beetles within a tray had been captured. Beetle counts were pooled for the three flasks of a particular treatment within each tray. Each comparison was replicated five times and numbers of beetles responding to each treatment were analyzed within treatment combinations by Wilcoxon signed-rank tests at $\alpha = 0.05$ (Hollander and Wolfe, 1973).

Volatile Comparisons. In the morning, about 5 g of leaves (detached at the base of the petiole) were collected from each tray of damaged or undamaged foliage. All insect–damage comparisons included 10 beetles or caterpillars that were placed in the volatile collection chambers along with the leaf samples.

The leaves were placed in a push–pull collection apparatus modified from the design of Cross (1980), with eight chambers for samples. Each chamber was a glass sleeve (40 cm long, 8 cm diam.) closed at each end with a no. 14 neoprene stopper adapted for copper tubing (0.635 cm OD), which served as air inlet or vacuum lines. The face of each stopper was covered with a Plexiglas plate attached with machine screws to reduce emission of volatiles from the neoprene. The air inlet side of the apparatus was furnished by the house air supply, which was passed through separate charcoal (500-ml) and Tenax (150-ml) filters for purification and then through an air scrubber filled with deionized

water to humidify the airstream and reduce desiccation of the leaves. From the air scrubber, the air passed through a manifold system to supply each chamber of the collection apparatus. The vacuum side of the apparatus was supplied by house vacuum via a manifold. Each port of the manifold was controlled by a separate flow controller. From the flow controllers, Tygon tubing led to glass tubes that served as collection traps and that contained 50 mg of Super Q absorbent (Alltech Associates, Deerfield, Illinois). The glass tubes were fitted to the collection chambers with Swagelok fittings and were rinsed prior to volatile collections with 2 ml of high purity CH_2Cl_2. During volatile collections, air was pulled through each chamber at 250 ml/min, and the entire system was kept slightly above atmospheric pressure as measured by a flow tube attached by means of a vent at the vacuum end of one of the volatile collection chambers.

After 4 hr, the collection traps were removed from the apparatus and compounds were eluted from the traps with 180 μl of an 80:20 v/v mixture of high purity hexane–CH_2Cl_2. Cumene (1000 ng) was added as an internal standard and compounds were quantitated by 2.0-μl injections onto a gas chromatograph operated in splitless mode and equipped with a 60-m × 0.32-mm Supelcowax 10 (polyethylene glycol) column. GC operating conditions were as follows: oven temperature 60°C for 1 min and then programmed at 2°C/min to 220°C; injector 220°C; FID 240°C; He carrier linear flow rate 21 cm/sec. Levels of individual compounds were pooled for individual treatments and contrasted between different treatments by Fisher's protected least significant difference test at $P = 0.05$ (Sokal and Rohlf, 1981).

Gas chromatography–mass spectroscopy was performed on a Hewlett-Packard mass ion detector operated in electron impact mode and interfaced to a GC equipped with a 30-m × 0.25-mm DB-5 column operated as follows: oven temperature 60°C for 1 min and then programmed at 2°C/min to 220°C; injector 220°C; He carrier linear flow velocity 21 cm/sec. Compound identification was performed by computer library searches and retention time matches of the natural plant products with authentic standards on the Supelcowax 10 column and a 30-m × 0.32-mm DB-5 column operated as described for GC-MS analyses. Authentic samples of volatile compounds were obtained from commercial sources except for (*E*)-4,8-dimethyl 1,3,7-nonatriene, which was donated by J.H. Tumlinson (USDA, Gainesville, Florida), (*E*,*E*)-α-farnesene isolated from the hydrocarbon fraction of ylang-ylang oil (Gaydou et al., 1986), and linalool oxide synthesized by the method of Cooper et al. (1990).

RESULTS AND DISCUSSION

For the pitfall bioassays to have value as a tool for investigating Japanese beetle discrimination between differing plant materials, one should be able to

demonstrate the beetle's ability to locate leaves of a preferred host within the bioassay system. Significantly more beetles were captured in flasks that contained a single undamaged leaf than in those that contained no leaf ($\alpha = 0.021$, Wilcoxon signed rank test, Figure 2). Moreover, significantly more beetles were attracted to leaves that had been damaged overnight by either Japanese beetles or fall webworms than to undamaged leaves ($\alpha = 0.022$ and 0.034, respectively). Leaves that had been artificially damaged or leaves with fresh Japanese beetle feeding damage were not significantly more attractive than undamaged leaves.

Representative chromatograms show that crabapple leaves that had been damaged overnight by Japanese beetles produced a more complex blend of volatiles than did undamaged or artificially damaged leaves (Figure 3). Nineteen compounds were identified from the insect-damaged leaves (Table 1).

The fact that a wider variety of volatile compounds was isolated from leaves that had received overnight feeding damage than from freshly damaged leaves parallels the findings of previous researchers (Turlings et al., 1995). Odor induction from angiosperm leaves due to insect feeding seems to occur in a delayed manner or perhaps requires that damage occurs at a certain time of the day (Turlings et al., 1990; Loughrin et al., 1994). In addition, the role of the insect in the production of the blend of induced odors should be stressed since artificial

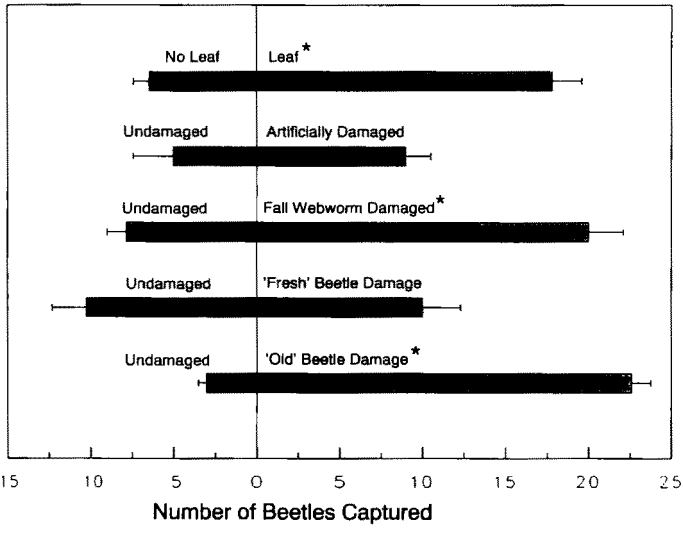

FIG. 2. Capture of Japanese beetles in choice tests. Data represent the mean of five replications ± standard error. Asterisk indicates a significant preference for indicated treatment by Wilcoxon signed rank test at $\alpha = 0.05$.

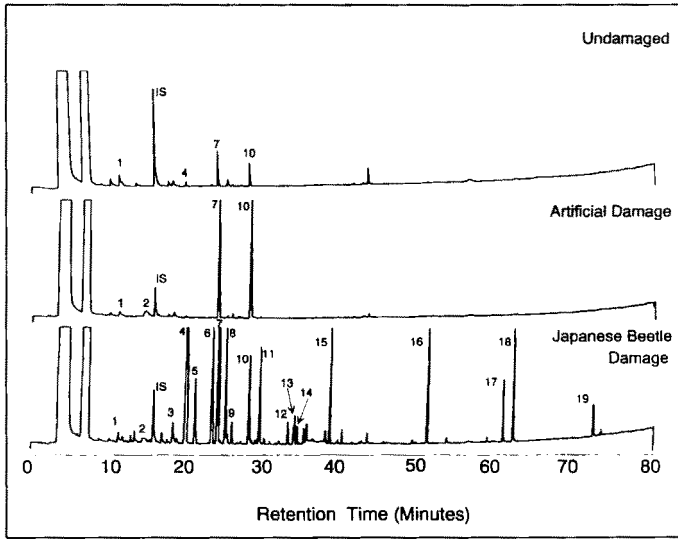

FIG. 3. Chromatograms of undamaged crabapple leaves, crabapple leaves subjected to artificial damage, and crabapple leaves subjected to overnight Japanese beetle feeding damage. Peak identities: 1, hexanal; 2, (*Z*)-3-hexenal; 3, (*E*)-2-hexenal; 4, (*E*)-β-ocimene; 5, hexyl acetate; 6, (*E*)-4,8-dimethyl 1,3,7-nonatriene; 7, (*Z*)-3-hexenyl acetate; 8, (*E*)-2-hexenyl acetate; 9, hexanol; 10, (*Z*)-3-hexenol; 11, (*E*)-3-hexenol; 12, (*Z*)-3-hexenyl butyrate; 13, a furan linalool oxide; 14, (*E*)-2-hexenyl butyrate; 15, linalool; 16, (*E*,*E*)-α-farnesene; 17, phenethanol; 18, phenylacetonitrile; 19, (*Z*)-3-hexenyl benzoate; IS = cumene internal standard.

damage alone does not seem to produce the same blend of volatiles as insect damage regardless of the age of the damage (Turlings et al., 1990).

Differences in induced volatiles may explain the beetle's attraction to leaves damaged by either Japanese beetles or fall webworms and their lack of preference for freshly damaged or artificially damaged leaves. Many of the compounds isolated from leaves that had received overnight feeding damage were either floral in character (e.g., phenethanol, linalool) or fruit-like [e.g., (*Z*)-3-hexenyl butyrate, (*Z*)-3-hexenyl benzoate). In contrast, artificially damaged leaves or leaves that had been freshly damaged by the beetles produced volatiles that were much more green-leaf in character (Hatanaka, 1993). Of the many essential oils and individual compounds that have been evaluated as Japanese beetle lures, those best described as floral or fruity are most effective (Schwartz and Hamilton, 1969).

The odors released by feeding damage on leaves of *Duranta repens* have been implicated as aggregation kairomones for adults of another scarabaeid

TABLE 1. VOLATILES COLLECTED FROM UNDAMAGED CRABAPPLE LEAVES AND CRABAPPLE LEAVES SUBJECTED TO VARIOUS FORMS OF DAMAGE

Compound	Compound collected (ng/g fresh wt)[a]				
	Undamaged	Artificially damaged	Fall webworm damage	Fresh Japanese beetle damage	Overnight Japanese beetle damage
Aliphatics					
Hexanal	[b]a	61.2 ± 16.0 b	87.6 ± 11.5 b	85.1 ± 22.9 b	84.4 ± 2.0 b
(*E*)-2-Hexenal	14.9 ± 4.2 a	65.7 ± 27.2 bc	149 ± 43.3 bc	56.9 ± 26.5 b	144 ± 28.4 c
(*Z*)-3-Hexenal	a	726 ± 385 b	a	584 ± 171 b	a
Hexyl acetate	6.4 ± 4.6 a	17.9 ± 11.6 a	66.0 ± 28.2 a	22.8 ± 22.8 a	188 ± 44.2 b
(*Z*)-3-Hexenyl acetate	374 ± 127 a	6800 ± 1620 c	538 ± 65 a	979 ± 192 a	2070 ± 246 b
(*E*)-2-Hexenyl acetate	a	4.2 ± 2.2 a	72.8 ± 25.5 b	a	429 ± 85.0 b
(*Z*)-3-Hexenyl butyrate	5.6 ± 2.5 a	62.0 ± 22.4 b	12.0 ± 6.1 a	16.2 ± 8.9 a	99.0 ± 23.6 b
(*E*)-2-Hexenyl butyrate	a	17.8 ± 11.1 a	41.9 ± 11.2 a	a	215 ± 44.1 b
Hexanol	7.6 ± 5.3 a	82.4 ± 31.0 b	71.9 ± 37.0 b	a	105 ± 22.5 b
(*Z*)-3-Hexenol	123 ± 52 a	6820 ± 1760 c	245 ± 61 b	387 ± 93 b	452 ± 30 b
(*E*)-2-Hexenol	a	57.2 ± 24.3 b	118 ± 47.9 b	a	462 ± 91.9 c
Terpenoids					
(*E*)-β-Ocimene	36.0 ± 16.0 a	77.0 ± 22.0 a	474 ± 99.0 b	20.0 ± 5.8 a	1930 ± 345 b
(*E*)-4,8-Dimethyl 1,3,7-nonatriene	49.0 ± 13.0 a	157 ± 25.0 b	361 ± 109 b	330 ± 78.0 b	1020 ± 85.0 c
Linalool oxide isomer	a	a	a	a	90.1 ± 31.5 b
Linalool	a	a	82.9 ± 37.3 b	a	381 ± 92.3 c
(*E*, *E*)-α-Farnesene	149 ± 38.0 ab	251 ± 54.0 b	1770 ± 273 c	101 ± 31.0 a	5370 ± 877 d
Aromatics					
Phenethanol	a	a	39.2 ± 6.2 b	26.1 ± 7.7b	217 ± 13.4 c
Phenylacetonitrile	a	a	58.5 ± 11.0 b	a	405 ± 39.7 c
(*Z*)-3-Hexenyl benzoate	a	a	76.7 ± 11.2 b	a	363 ± 14.4 c

[a] Data represent the mean of five determinations ± standard error. Means within rows followed by the same letter are not significantly different by Fisher's protected least significant difference test at $P = 0.05$.

[b] Blanks: compound present at less than 5 ng/g.

beetle, *Maladera matrida* Argaman (Harari et al., 1994). In this case, however, the chemistry of induction was not studied, and the authors assumed the insects were responding to general green-leaf odor components, such as leaf alcohols, aldehydes, and their derivatives. If so, this would be in contrast to our findings for the Japanese beetle, where attractiveness of leaves increased only after overnight feeding damage.

The terpene hydrocarbons (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E*,*E*)-α-farnesene were the most abundant of the compounds induced by insect feeding on crabapple. However, since compounds such as the various induced esters and phenylpropanoids have previously been shown to be attractive to Japanese beetles (Schwartz and Hamilton, 1969), the terpene hydrocarbons may not be important components of the beetle's response to damaged leaves.

Attraction of Japanese beetles to fall webworm damaged foliage was tested in order to compensate for possible residual sex or aggregation pheromone that might be present on the beetle-damaged leaves. A higher overall proportion of beetles was indeed attracted to the beetle-damaged leaves than to the fall webworm-damaged leaves (Figure 2, $\chi^2 = 11.03$ with 1 *df*, $P < 0.001$). This is likely explained, however, because leaves that had received beetle feeding damage were usually more heavily damaged than were webworm-damaged leaves, with overall volatile production about three times higher.

The Japanese beetle's tendency to congregate on individual host plants had led researchers to propose aggregation pheromone production by this insect (Adler and Jacobson, 1971; Iwabuchi and Takahashi, 1983). For instance, Iwabuchi and Takahashi found that either sex of Japanese beetles tethered singly to a grape leaf for 30 min attracted more males than did leaves alone. Ether extracts of females also attracted males to leaves. Extracts of males or visual cues (male or female dummies on a leaf) were not attractive. They suggested, from these observations, that an airborne substance is responsible for aggregation of males. Aggregations also included some females, but the basis of their response was not clarified.

We suggest that the argument for an aggregation pheromone in Japanese beetles is weakened by the fact that in Iwabuchi and Takahashi's study (1983), neither sex attracted additional beetles when tethered on yellow cardboard or on clear plastic plates attached to leaves. Similarly, traps baited with groups of mated females or males, without leaves, were no more attractive to other beetles than unbaited traps (Ladd, 1970). Iwabuchi and Takahashi (1983) did not indicate whether their tethered beetles produced feeding damage on the substrate leaves during the test period. Beetles used in their study were collected from a grape vineyard each morning and used immediately in tethering assays or were held overnight on grape leaves before being tethered or extracted. It is therefore also possible that the males contained traces of residual female sex pheromone or that both sexes carried traces of induced volatiles from grape leaves.

Several reasons may exist for the Japanese beetle's exploitation of damage-induced odors. Among these may be increased efficiency for mate finding. Although females cease production of sex pheromone shortly after their first mating (Ladd, 1970) and apparently do not require subsequent matings to produce a full complement of eggs (Ladd, 1987), they will frequently remate while feeding on host plants between oviposition bouts (Fleming, 1972). Ladd (1966) demonstrated that sperm displacement occurs in the Japanese beetle so that sperm contributed by a female's most recent partner is used to fertilize her eggs when she leaves a host plant to oviposit in the soil. Beetles often remain on damaged foliage overnight. So, by responding to induced plant odors, males may increase their chances of locating nonvirgin females and females may benefit by selectively remating with high-quality males.

Induced odors may also serve as reliable cues to beetles of a high-quality food source. Japanese beetles are attracted to a wide array of plant species regardless of their acceptability as hosts (authors' unpublished data). Induced odors may serve as more reliable indicators of host suitability than volatiles released constituitively from plants.

In summary, we have demonstrated odor induction due to insect herbivory on crabapple leaves and the delayed nature of this induction. The induced odors are attractive to Japanese beetles and may explain the aggregative behavior of this insect on its preferred host plans.

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EARLY WARNING IN THE PREDATION SEQUENCE: A DISTURBANCE PHEROMONE IN IOWA DARTERS (*Etheostoma exile*)

BRIAN D. WISENDEN,* DOUGLAS P. CHIVERS, and R. JAN F. SMITH

Department of Biology, University of Saskatchewan 112 Science Place, Saskatoon, SK, S7N 5E2, Canada

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Abstract—The probability of prey avoiding a predator's attack should increase if the predator's presence is detected at an early stage in the predation sequence. In this study, we tested whether threatened Iowa darters (*Etheostoma exile*) release disturbance pheromones that warn conspecifics of the presence of predation threat. Pairs of aquaria were visually isolated from one another, but connected chemically by water circulating between them. Darters in one aquarium were observed before and after darters in the other aquarium were chased with a model predator. In control tests, the model was moved in the same manner but there were no darters in the upstream tank. Darters receiving water from threatened fish increased vigilance behavior and decreased movement. Vigilant fish raised their head above the substratum, propping themselves up on their pectoral fins and/or arching their neck dorsally, pointing the snout upward. Exposure to water from disturbed darters suppressed exploratory behavior and resulted in movement by short rapid hops that ended abruptly in a rigid, alert posture. This study suggests that Iowa darters release a disturbance pheromone that can provide conspecifics with an early warning of predation risk.

Key Words—Disturbance pheromone, alarm signal, predator-prey, predator avoidance, vigilance, Iowa darter, *Etheostoma exile*.

INTRODUCTION

In many situations in aquatic habitats, chemical information is more salient than visual information. For example, visual information may be of little use in highly

*To whom correspondence should be addressed. Current address: Center for Ecology, Evolution and Behavior, Morgan School of Biological Sciences, University of Kentucky, Lexington, KY 40506-0225.

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structured habitats, turbid water conditions, or at night. Furthermore, water serves as an excellent medium in which to dissolve and disperse chemical substances (Hara, 1992).

Chemical alarm signals occur in a wide range of aquatic taxa (gastropod mollusks, Atema and Stenzler, 1977; opisthobranchs, Sleeper et al., 1980; anemones, Howe and Sheik, 1975; decapods, Appleton and Palmer, 1988; fishes, Smith, 1992). Many fish have a well developed system of chemical alarm signaling (Smith, 1992). To date, study of alarm pheromones in fishes has focused on substances contained within the epidermis and released only by injury, including the *Schreckstoff* of ostariophysan species (von Frisch, 1938; reviewed in Pfeiffer, 1977; Smith, 1992). Conspecific skin extract invokes antipredator behavior in most ostariophysan fishes tested thus far and in nonostariophysan species such as brook stickleback, *Culaea inconstans* (Mathis and Smith, 1993b), darters (Smith, 1979, 1982), sculpins (Hugie et al., 1991), cyprinodontids (Reed, 1969) and gobies (Smith, 1989; Smith and Lawrence, 1991; Smith et al., 1991). Chemicals released by an injured conspecific serve as a reliable indicator of predation risk. Fathead minnows that have been "warned" by chemical alarm signals are significantly better at surviving in the presence of a predator (Mathis and Smith, 1993a).

Prewarned prey may be able to avoid an encounter with a predator. Prey that are subsequently detected by the predator are better able to avoid an attack by being in a state of readiness. The greater the degree of advance warning of a predator's presence, the greater the advantage to the prey. Therefore, there should be a selective advantage to individuals that can gain information about predation risk from a predator "disturbance" of a conspecific before the encounter escalates to a predator attack.

In birds and mammals, there are examples of visual, auditory, and chemical preinjury alarm signals that occur before prey capture (Caro, 1986b; Caro, 1986a,b; Klump and Shalter, 1984; Müller-Schwarze et al., 1984). In fishes, there are examples of preinjury visual alarm cues (e.g., goby bobbing, Smith, 1989; Smith and Smith, 1989; Sweatman, 1984) and auditory cues (e.g., squirrel fish, *Holocentrus rufus*, Winn et al., 1964; cod, *Gadus callarias*, Brawn 1961). However, among fishes, there are no documented cases of chemical alarm signals sent by uninjured individuals. Rainbow trout (*Oncorhynchus mykiss*) subjected to strong electric shock and other "stressors" release "chemical alarm signals" that increase stress in receivers, but no behavioral responses were measured (Lebedeva et al., 1994). Malyukina et al. (1983) reported that cod (*Gadus morhua*) detect and avoid chemical stimuli from stressed conspecifics. Tester (1963) found that grey sharks (*Caracharhinus menisorrah*) and black tipped sharks (*C. melanopterus*) were attracted to water from "agitated" and "distressed" groupers (*Epinephelus merra*), and black tipped sharks were attracted to distressed mullet (*Mugil cephalus*). These latter studies suggest that

distressed fish may release distinctive chemical stimuli, but they do not show a link between predation threat and the release of such chemicals or their detection and associated changes in vigilance by conspecifics.

Crayfish (*Orconectes virilis*) and hermit crabs (*Calcinus laevimanus*) respond to "disturbance pheromones" from conspecifics with antipredator behavior (Hazlett, 1985, 1990b). In addition, individuals of *O. virilis* respond to disturbance pheromones produced by other species of crayfish (*O. rusticus*, *Cambarus diogenes*), leeches (*Macrobdella decora*), newts (*Notopthalamus viridescens*), rock bass (*Ambloplites rupestris*), and the Iowa darter (*Etheostoma exile*) (Hazlett, 1985, 1989; 1990a).

If Iowa darters produce chemical stimuli when stressed (but not injured) to which crayfish respond, it follows that Iowa darters, themselves, may be able to detect these chemicals and respond with antipredator behavior. We conducted a laboratory experiment to investigate whether Iowa darters respond to chemical stimuli produced by disturbed conspecifics. We predicted that test individuals would increase vigilance behavior and suppress conspicuous behavior (movement) in response to chemical cues produced by sender conspecifics that were chased by a model predator in a separate, visually isolated aquarium.

METHODS AND MATERIALS

Two variations of this experiment were conducted at separate times by different investigators using fish from two different populations.

Experiment 1. We captured Iowa darters by seine in Eagle Creek, a tributary of the North Saskatchewan River, at a site approximately 50 km west of Saskatoon, Saskatchewan. Mean $\pm$ SE total lengths (TL) of a subsample of 15 females and 10 males were 43.1 $\pm$ 0.09 mm TL and 44.0 $\pm$ 0.11 mm TL, respectively. They were held in the laboratory at 15°C and fed a mixture of frozen brine shrimp and live food, including cladocerans, copepods, and mosquito larvae.

We placed pairs of 37-liter aquaria (50 $\times$ 30 $\times$ 25 cm high) end to end, separated by a small space and an opaque barrier. We used eight pairs in rotation. We connected the tanks by siphons at the abutting ends. Water was pumped from the distal end of the receiver tank into the distal end of the sender tank so that water flowed continuously through the siphons from senders to receivers. Flow rates averaged 1.2 liter/min. Each tank contained a substratum of several centimeters of silica sand.

Data were collected in May 1992 during daylight hours. Three darters (receivers) were placed in the receiver tank at least three days before the observation day. In experimental trials, 12 darters were placed in the sender tank at least three days before observation to ensure that they were well adjusted to the

laboratory setting. Both senders and receivers need to be in an undisturbed state for observers to detect a behavioral response to chemical cues (Hazlett, 1989; Smith, 1979). We buried a predator model in the sand of the sender's tank at least three days prior to the observations. The model was a fish lure 15 cm long and 2.5 cm high with hooks removed. A metal disc was glued to the underside of each model with silicone cement. The disc provided a heavy, stable platform to prevent the model from rolling or lifting off the substrate during the trial. The model was attached to monofilament line that led from the tank to the observation location. There was always live food present in both tanks on the observation day, and we fed the fish to satiation on the day before the observations. Darters do not respond to conspecific skin extract when they are hungry (Smith, 1981). Fish were used only once as receivers but were occasionally used more than once as senders. We drained and washed tanks between observations.

We quietly observed fish from about 1.5 m from the receiver tank. For 15 min before the fish model stimulus, we recorded the number of moves and head-up posture. In addition, we noted the time during which at least one of the three receivers was in the head-up posture. We then pulled the model out of the sand in the sender tank and moved it a short distance at 30-sec intervals for 5 min. No observations were made during this period. The above behaviors were similarly recorded for 15 min following exposure of the senders to the model predator. We conducted 10 experimental trials in which senders were present in the upstream tank. In 10 control trials, the upstream tank contained no sender fish, but we manipulated the model in the same manner as in experimental trials. This controlled for any stimuli produced by the movement of the model.

Iowa darters normally rest on the bottom. A move was scored each time a fish changed its location. A head-up posture was scored if a darter was stationary on the substratum with either the anterior part of its body propped up by its pectoral fins or if it arched its neck dorsally, pointing its snout upwards, or both. Head-up behavior is a component of the fright reaction of Iowa darters (Smith, 1982). Iowa darters are small, elongate benthic fish. Their body shape and habit leave these fish with a limited vantage point from which to view the surrounding area for predators and to sample water-borne chemical cues above the boundary layer (Moore and Atema, 1991; Moore et al., 1994). Head-up behavior would thus appear to be a cryptic, low-risk form of vigilance behavior.

Experiment 2. We collected adult and subadult darters (TL of subsample $= 48 \pm 1.5$ mm, $N = 4$) from a 1-ha pond on the University of Saskatchewan campus using minnow traps and a seine net. This population originated from the South Saskatchwan River in 1959. Darters in the laboratory holding tanks were provided with live cladocerans, mosquito larvae, and copepods ad libitum, supplemented occasionally with frozen brine shrimp.

We set up six pairs of 37-liter aquaria in the same manner as described for

experiment 1. The mean ± SE rate of flow was 2.9 ± 0.06 liters/min (N = 6 paired tanks). Food coloring added to the far end of upstream tanks was visible in the downstream tank to a human observer within 35 ± 7.7 sec (N = 6). Live food was always present in both tanks during the trials and for at least 1 hr before observations began.

Data were collected in June and July 1994 during daylight hours. We placed three receiver darters into the downstream tank. For experimental trials (N = 9), we placed 12 sender darters into the upstream tank. For control trials (N = 10), no darters were added to the upstream tank. All receiver fish were naive to the experiment and used only once as receivers. Some sender and receiver fish were used as senders in subsequent trials. Tanks were drained and washed between trials.

We videotaped all trials. Each trial lasted 35 min. At 10-sec intervals for 15 min, we scored the number of receiver fish with their head elevated above the substratum. After 15 min, a previously concealed model predator emerged from under a piece of black plastic in the upstream tank. In control trials the upstream tank contained no fish, but in experimental trials it contained sender darters. The model predator was similar to the one used in experiment 1. The model was attached to monofilament line through a system of pulleys, which enabled us to manipulate its position remotely (6 m from the receiver fish). The predator model moved from one end of the tank and back again every 15 sec, for 5 min. We then recorded the number of receiver fish in the head-up posture every 10 sec for 15 min.

Statistical Analyses. For statistical comparisons, we tested three behaviors (head-up posture, duration of time when at least one of three receivers was in head-up posture, and move frequency). We used the total frequency of each behavior exhibited by all three receivers in each trial to produce a single, independent datum before and after stimulus presentation per trial. We used Wilcoxon signed-rank tests (Siegel and Castellan, 1988) to compare darter behavior before and after model stimulus in the number of receiver fish performing head-ups for experimental and control trials separately.

RESULTS

Experiment 1. Darters significantly increased (Wilcoxon T^+ = 47, P = 0.048, two-tailed, N = 10) the frequency of their head-up postures from 15.7 ± 3.6 (mean ± SE) per 15 min before presentation of the predator model to 24.7 ± 3.7 per 15 min after model presentation, during which they were exposed to water from threatened conspecifics (Figure 1). In control trials, darters performed 17.7 ± 3.8 head-ups in the 15 min before the model emerged from the sand compared to 19.1 ± 3.2 during the final 15 min of each trial, which were

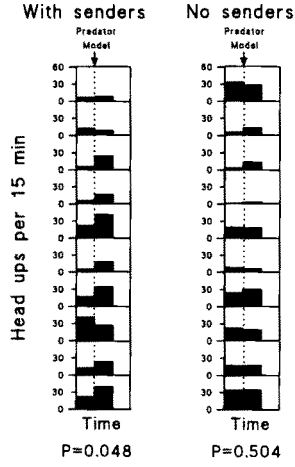

FIG. 1. Frequency of head-up posture by three receiver darters for 15 min before and after presentation of a model predator in an adjacent but visually isolated aquarium. Each cell represents a single trial. In experimental trials (left-hand column) sender darters were present in the adjacent aquarium with the model. In control trials (right-hand column) no sender fish were in the adjacent tank with the model. Below each column, significance of change between pre- and postpredator head-up frequency (Wilcoxon signed-rank test).

not significantly different (Wilcoxon $T^+ = 23.5$, $P = 0.504$, two-tailed, $N = 8$ excluding ties).

The duration of time in which at least one of the three receiver darters performed a head-up behavior significantly increased (Wilcoxon $T^+ = 33.5$, $P = 0.032$, two-tailed, $N = 8$ excluding ties) in experimental trials from 3.2 ± 0.8 min before the model was presented to 5.2 ± 0.8 min after sender darters were exposed to the model predator, but remained virtually unchanged (5.2 ± 0.5 vs. 4.7 ± 0.5 min) in control trials (Wilcoxon $T^+ = 13$, $P = 0.546$, two-tailed, $N = 8$ excluding ties, Figure 2).

In control trials, the number of moves per 15 min by the three receiver fish significantly increased (Wilcoxon $T^+ = 47.5$, $P = 0.044$, two-tailed, $N = 10$) from 97.7 ± 23.5 to 116.8 ± 20.9 after the model presentation. However, in experimental trials, move frequency did not change between the experimental treatments (115.8 ± 13.6 vs. 101.7 ± 11.1, Wilcoxon $T^+ = 35.5$, $P = 0.146$, two-tailed, $N = 9$ excluding ties, Figure 3).

Experiment 2. The number of receiver darters observed in the head-up posture significantly increased when exposed to water containing chemical cues of disturbed conspecifics (Figure 4). Overall, the number of individuals in the

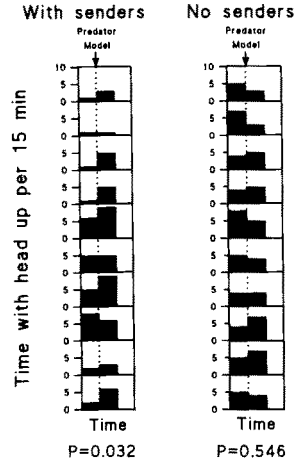

FIG. 2. Time, in minutes, in which at least one of the three receiver darters maintained a head-up posture before and after presentation of a model predator in the adjacent tank in the presence or absence of sender fish. Figure format as described for Figure 1.

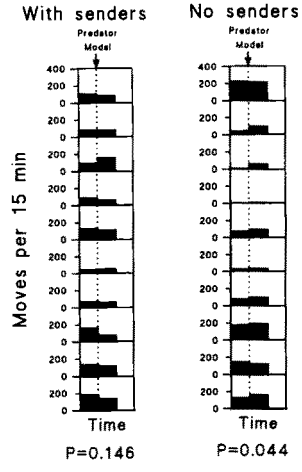

FIG. 3. Move frequency by three receiver darters before and after presentation of a model predator in an adjacent tank with or without sender fish. Figure format as described for Figure 1.

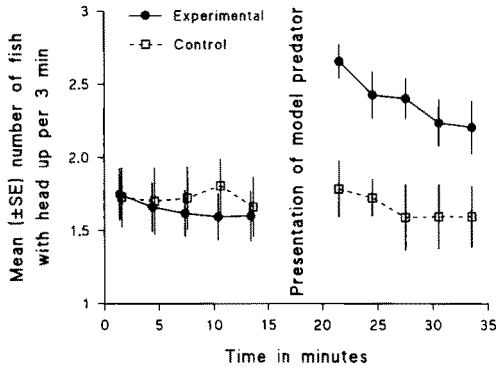

FIG. 4. Mean (±SE) number of fish exhibiting the head-up posture during each 3-min interval of the 15-min prepredator period and the 15-min postpredator period, separately. Data were not recorded during the 5-min interval while the model predator was active in the upstream tank.

head-up posture in the experimental trials increased from 1.64 ± 0.15 to 2.38 ± 0.15 (Wilcoxon $T^+ = 44$, $P = 0.008$, two-tailed, $N = 9$ excluding ties). In control trials, the average number of receivers in the head-up posture was 1.72 ± 0.21 before the model predator, and 1.66 ± 0.16 for the 15 min afterwards (Wilcoxon $T^+ = 22$, $P = 0.625$, two-tailed, $N = 10$).

Although we did not quantify the frequency of moves in the second experiment, we noted qualitative changes in fish movement during the postpredator period. Receiver fish in experimental trials changed position on the tank bottom with short rapid hops ending abruptly in a rigid, alert posture. These hops never took the fish very far above the substratum and the hops were separated by long intervals of motionlessness. In one extreme case, a fish buried itself in the sand during the 5-min interval when the model was being manipulated in the upstream tank and did not emerge until 6 min into the postpredator period. During the postpredator period, the behavior of control fish was similar to control and experimental fish in the prepredator period. Control fish moved about slowly, often swimming up into the water column and then gliding back to the tank bottom.

DISCUSSION

In both replications of this experiment, Iowa darters responded to water that had first passed over threatened conspecifics by increasing the frequency and duration of their head-up (vigilance) behavior. In a head-up position, darters

would likely be better able to view the surrounding area and sample chemical information.

We are reasonably confident that the disturbance signal was transmitted by a chemical cue. The two test tanks containing sender and receiver individuals were visually isolated and separated by a small space. Mechanical vibrations caused by movement of the model were present in both experimental and control trials. However, it is conceivable (we think remotely so) that darters may emit an alarm sound that could travel through the water in the siphon hose connecting the two test tanks. Further experiments are needed before this alternative modality can be excluded completely.

Motionlessness, or freezing, is a component of darter antipredator behavior (Radabaugh, 1989; Rahel and Stein, 1988; Smith, 1979). Darters exposed to water from threatened conspecifics did not decrease their frequency of movement. However, they did not increase movement as the control fish did (Figure 3). Movement by control fish may have been stimulated by the model's activity in the adjacent tank, perhaps stirring up particles and odors in the sand substratum. Thus, increased movement by control fish may represent exploratory behavior. The suppression of a similar increase in experimental fish may reflect a trade-off between information-gathering and cost of conspicuous behavior when under risk of predation.

Disturbance pheromones do not require injury and thus indicate a lower level of predation threat to receivers than injury-released pheromones. In crayfish, this is reflected by a behavioral response of lower intensity (Hazlett, 1985). In this study, response to disturbance pheromone did not include freezing behavior, characteristic of the response of darters to skin extract (Smith, 1979) or predators (Radabaugh, 1989) or of crayfish to conspecific hemolymph (Hazlett, 1994).

In nature, the release and detection of disturbance pheromones could occur before the predator detects the prey, hence the emphasis on crypsis. Head-up behavior is a relatively cryptic method of gathering information about predation risk. Decreased movement is a form of crypsis. Disturbance pheromones themselves may be a cryptic warning signal if the origin of a chemical signal is more difficult to localize than a visual or auditory signal, thus minimizing the signaler's conspicuousness to predators.

Hazlett (1989, 1990a) speculated that the disturbance pheromone in crayfish is ammonia, excreted under duress in urine or through the gills during periods of increased (metabolic) activity. The same may be true for Iowa darters. However, prestressed Iowa darters lose their ability to produce disturbance pheromones (Smith, 1979), suggesting that the pheromone supply can be exhausted. This evidence suggests that disturbance pheromone may be urinary in origin, rather than a metabolite excreted continuously from the gills.

The disturbance signal may not necessarily be an intentional warning to other fish but rather a normal physiological response to stress to which other individuals have become attuned. Urine released by nonthreatened individuals would constitute a false alarm. Some level of experience may be required for darters to associate predation threat with a sudden pulse of nitrogenous metabolites. In this study we compared the behavior of the recipient fish before and after the stimulus and thus controlled for any response of recipients to background level of urine released by nonstressed sender fish.

The evolution of a disturbance signal would involve selection pressures similar to the evolution of injury-released alarm signals (Smith 1992). There are greater possibilities for the signal sender to benefit from postsignal effects of a disturbance signal, because the sender of such a signal has a better chance of survival than a sender of injury-released pheromones. A possible postsignal benefit to the sender may be to induce group crypsis, which would reduce the chance of detection by the predator, thus benefiting both the signaler and receivers (Dawkins, 1976). Signal-senders may benefit by warning and increasing the survival of group members because retaining group members may reduce future predation risk to the sender (Smith, 1986). Reducing predator success in the signaler's home range may discourage future hunting in the region (Trivers, 1971). A disturbance alarm signal may also serve as an attack deterrent by informing the predator that prey are alerted to its presence and that further hunting is unlikely to succeed (Caro, 1986a,b).

From what is known about injury-released alarm pheromones, we predict that fish may be able to use disturbance pheromones to acquire predator recognition (Chivers and Smith, 1994a,c; Magurran, 1989) and avoid risky areas (Chivers and Smith, 1995) and may respond to disturbance pheromones produced by heterospecifics with which they share habitat and predators (Chivers and Smith, 1994b; Mathis and Smith, 1993b). Crayfish respond to disturbance pheromones from a range of heterospecifics (Hazlett, 1990a). Documenting the existence of disturbance pheromones increases the scope of chemosensory information in predator–prey relationships in fishes. Disturbance pheromones provide conspecifics, and possibly heterospecifics within the same prey guild, with an early warning of predation threat in advance of prey capture and hence earlier than the release of injury-released alarm pheromones. This should increase the probability that prey can avoid an encounter with a predator by either fleeing the area or employing crypsis. Increased vigilance will result in improved chances of escape should a predator initiate an attack.

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RATES OF URINE EXCRETION BY HOUSE MOUSE (*Mus domesticus*): DIFFERENCES BY AGE, SEX, SOCIAL STATUS, AND REPRODUCTIVE CONDITION

LEE C. DRICKAMER

Department of Zoology
Southern Illinois University
Carbondale, Illinois 62901

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Abstract—A series of five experiments was used to test hypotheses about factors affecting excreted urine output per day in male and female house mice (*Mus domesticus*). Urine was collected in metabolism cages over a 24-hr period. Male house mice excrete urine at a rate 1.5–2.0 times that of females. Daily average urine output increases with age for both sexes and for mice of the same age; urine output per day is correlated with body mass. Females in estrus produce more urine than females in diestrus. Urine output per day increases during the latter two thirds of pregnancy and remains high throughout lactation. Density does not influence urine output per day for either sex over the range of densities tested. Castration reduces urine output per day in male mice, but ovariectomy in females does not alter rates of urine production. Dominant males produce more urine than subordinate males, but there are no similar effects for female mice. The findings have potential implications for our understanding of the functional and ecological uses of urine by house mice.

Key Words—House mouse, *Mus domesticus*, urine, social status, urine marking.

INTRODUCTION

Many rodents use urinary odor cues as part of a complex system of signals that influences reproductive and social biology (Eisenberg and Kleiman, 1972; Brown, 1985a,b; Halpin, 1986; Hurst, 1987). For example, various rodents can use olfactory cues for recognition of gender, familiarity, kinship, stress, and individual identification (Bowers and Alexander, 1967; Carr et al., 1970; Davies and Bellamy, 1974; Hayashi and Kimura, 1974; Cox, 1984; Kareem and Bar-

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nard, 1986; Winn and Vestal, 1986). Among such rodents, house mice have received, perhaps, the greatest attention by researchers (Brown, 1985a,b). Urine odor cues of house mice affect a variety of functions, including reproductive physiology, marking behavior, spatial relations, and possibly locating mates (Rose and Drickamer, 1975; Bronson, 1976; Harrington, 1976; Brown, 1979). Male house mice utilize urine to convey information about dominance status and aggression (Jones and Nowell, 1973a,b, 1977).

House mice engage in urine marking and countermarking under a variety of circumstances (Wolff and Powell, 1984) and produce accretions of urine termed urine posts by Welch (1953). Desjardins et al. (1973) reported that the patterns of urine marking were affected by dominance status. Male house mice urine mark more often (100–200 marks/hr) than females (25–95 marks/hr) (Maruniak et al., 1974; Powell and Wolff, 1982). Females mark more often in the presence of intact males than they do in the presence of other females or castrated males, but Maruniak et al. (1974) reported that there are no apparent variations in the amount of urine marking during different stages of the estrous cycle. Both males and females are attracted to urine odors from the opposite gender (Rowe, 1970; Davies and Bellamy, 1974; Drickamer, 1995). Female mice in estrus can discriminate the urine of dominant males from urine of subordinate males (Drickamer, 1992). Male mice discriminate male from female urine odor and also discriminate and prefer urine from females in estrus over females in diestrus (Rose and Drickamer, 1975; Sokolov and Kotenkova, 1976; Drickamer, 1995). Urine and related odors from pregnant and lactating females are avoided by most other mice, except for adult males (Drickamer, 1995). This avoidance is more pronounced for juvenile females and males and for estrous females.

Recently, Hurst conducted extensive studies on urine marking and related phenomena in house mice living in a poultry house and in laboratory enclosures (Hurst, 1989, 1990a–c). Adult males typically did not pay much attention to or countermark over urine from other adult males, although subadult males thoroughly investigated adult male urine. Adult males, adult females, and subadult females all countermarked female urine stimuli. Female urine was given great attention by all males and females, except by pregnant and lactating females. Females were attracted to urine from the resident dominant male, but avoided marks from males of neighboring territories or those of unfamiliar males.

One of the questions raised by these data on the use of urine cues by house mice concerns excreted urine output per day. Although mice must excrete urine to rid themselves of wastes, there may be differences in urine output per day attributable to a variety of other factors. How much urine do house mice of different genders and ages generate? What factors influence urine output per day by house mice?

Urine quantities have been recorded for rodents and other mammals, but

almost always in the context of metabolic studies. Two reports provide data on daily urine production under various conditions for albino laboratory strains of *Mus*; Bing and Mendel (1931) found excretion rates of 2.7–4.4 ml/day, and Chew and Hinegardner (1957) reported urine production of 10.5–12.0 ml/day, with a decrease with age and no gender differences. Data regarding urine production in deermice, *Peromyscus* (Chew, 1951, 1965), indicate that they urinate at a rate of 5.4–6.8 ml/day, with some of the variation attributable to differences in diet or ambient conditions.

These previous findings led me to an interest in possible variation in urinary output per day for house mice, and in particular, to consideration of a series of hypotheses regarding the factors that might affect such output. The first hypothesis concerned the possible differences by gender and age with regard to urine output per day. A second hypothesis concerned whether there were differences in urine output per day according to reproductive condition among females. Third, I tested whether there were any effects of density on urine output per day. Fourth, I asked whether gonadectomy affected urine output per day in either males or females. Finally, I tested the effects of social dominance status on urine output per day.

METHODS AND MATERIALS

General Methods. The mice used in these experiments were second through fourth generation from a stock of wild house mice (*Mus domesticus*) bred from animals trapped on farms in the area of Williamstown, Berkshire County, Massachusetts. Mice were housed in opaque polypropylene cages (28 × 15 × 15 cm deep) with fitted wire lids. A bedding of wood shavings was changed once each week. Food (Wayne Lab Blox) and water were provided ad libitum. Mice for the urine collection experiments were produced by individual female–male pairings. Mice were weaned at 23 days of age and housed individually until used in the experiments.

Urine collection was carried out in a series of four polycarbonate metabolism cages (overall dimensions = 28 cm diameter × 48 cm high; living area for mouse = 20 cm diameter × 14 cm; Maryland Plastics) placed on a table with wooden visual barriers between them. Food and water were available in small side chambers such that the mouse stuck its head, but not its hindquarters, into an opening to eat or drink. Each collection involved placing an individual mouse in the metabolism cage between 0600 and 0900 hr and leaving it there for 24 hr. After 24 hr, the urine in the receptacle was drawn into 1-ml and 5-ml hypodermic syringes and measured to the nearest 0.1 ml. Each metabolism apparatus was then cleaned thoroughly and dried before being used again.

Two methods issues must be addressed. First, how much could evaporation

from the surface of the urine that collected in the receptacle affect the results? Since the surface area from which urine could evaporate was the same at all times, this factor could result in disproportionately diminished estimates of urine quantity for mice with smaller amounts than those with larger amounts. Second, could seasonal differences in relative humidity in the room used for urine collection differentially affect the amount of evaporation from the surface of the urine collection receptacle? In the laboratory there is a tendency to have lower relative humidity in the winter and higher humidity during summer months. I attempted to alleviate both problems by placing 0.2 ml of vegetable oil in the receptacle at the outset of each collection period. This oil then floated on the surface of the urine as it collected and eliminated any significant evaporation. When the urine quantities were measured, I subtracted 0.2 ml from each value to account for the oil. For the second problem I also proceeded by testing mice for collection of urine in each treatment of the various experiments across all four seasons of the year; no more than 50% of the subjects were tested in any treatment during either the months of December through February (low humidity) or June through August (high humidity).

All colony cages and the urine collection metabolism apparatus were housed in a single room maintained at 21–25°C and 30–60% relative humidity on a 12-hr dark–12-hr light daily photoperiod regimen with overhead fluorescent lights on from 0600 to 1800 hr.

Experiment I: Gender and Age Effects. First, descriptive data concerning the quantities of urine according to age and gender were obtained. A total of 192 mice were used, 96 of each gender. Mice were tested at 25, 35, 45, 55, 75, 100, 125, and 150 days of age (N = 12 mice/treatment); different mice were used for each age period. Mice were tested at ± 2 days from the designated age on an as-available basis. Body mass was obtained for each mouse just prior to testing. No two mice of the same gender were tested from any single litter at the same age.

Initially, I used a two-way analysis of variance on urine quantity with two genders and six ages as main effects. Post-hoc analyses of treatment mean differences were tested using a Tukey-Kramer procedure with $\alpha = 0.02$ (Sokal and Rohlf, 1981); I elected to set the alpha level lower than the traditional 0.05 because of the large number of post-hoc tests conducted in the course of the various experiments and analyses. In addition, for this experiment, I tested whether there was any relationship between body mass and urine output per day. These data were used, in conjunction with the information on urine quantity, for each mouse in separate correlation analyses for each age and sex group.

Experiment II: Female Reproductive Condition. To test the effects of female reproductive condition on urine output per day, I used 112 adult female mice 110–170 days of age. There were eight treatments as follows (N = 14 mice/treatment): (1) females in estrus, (2) females in diestrus, (3) females in the first

six days of pregnancy, (4) females in the second six days of pregnancy, (5) females in the third six days of pregnancy, (6) females in the first week of lactation, (7) females in the second week of lactation, and (8) females in the third week of lactation. Different mice were used for each treatment. To obtain mice for treatments 1 and 2 individually caged females were monitored daily for reproductive cycle condition via wet-mount vaginal smears (Rugh, 1968; Drickamer, 1982). Only mice clearly in estrus or diestrus were placed in the metabolism cage for urine collection. To obtain females for treatments 3–5, females were placed with adult males and checked daily for vaginal plugs. Pregnancies were timed from the day of discovery of the vaginal plug. In some cases ($N = 9$ across the first two pregnancy treatments), I only determined after the fact that the female was not actually pregnant. Data from those mice were discarded and additional females were tested to obtain the required sample size. For treatments 6–8, pregnant females were monitored until parturition. The intervals for urine collection during lactation were timed from parturition until the completion of the three-week lactation period. Data were subjected to a one-way analysis of variance and post-hoc testing using a Tukey-Kramer procedure with $\alpha = 0.02$.

Experiment III: Crowding Effects. To test the possible effects of density on urine output per day, I used 576 mice, 288 of each gender. Adult mice, 130–170 days of age, were used in one of six treatments ($N = 12$ cages of each type for each gender): (1) housed individually, (2) housed 2 mice/cage, (3) housed 3 mice/cage, (4) housed 4 mice/cage, (5) housed 6 mice/cage, and (6) housed 8 mice/cage. Mice were placed into these housing conditions for 3 to 4 weeks prior to their use in urine collection. Cages with grouped mice were comprised of nonsiblings. For urine collection, only one mouse, selected at random, was tested from each cage. Data were subjected to a two-way analysis of variance with the two genders and six density treatments as the main factors.

Experiment IV: Gonadectomy Effects. To explore the effects of gonadectomy on urine output per day, I used 28 male and 28 female mice, all aged 125–165 days of age. There were four treatment groups ($N = 14$ cages of each type): (1) singly-caged intact males, (2) singly-caged castrated males, (3) singly-caged intact females, and (4) singly-caged ovariectomized females. Gonadectomies were performed under ether anesthesia when the mice were 110–140 days of age, and all operated mice were housed individually and given three weeks to recover prior to initiation of the treatment conditions. Data from males and females were subjected to separate t tests.

Experiment V: Social Status Effects. Possible effects of social status on urine output per day were tested using 12 groups of four mice each for males and for females. All mice were 110–140 days of age when dominance testing began. Groups were comprised of nonsiblings. In four instances, all with cages of males, aggression became so severe that I eliminated the group from the

experiment, substituted a new group of males, and separated the males in the original cage. Each mouse in a group received a small colored mark (red, green, blue, black) on its back at the base of the tail from a series of permanent ink marking pens. Marks were refreshed on the third and sixth days of observation. These identification marks were used during twice-daily 1-hr observations of the mice in their home cage to determine dominance status relationships. These observations were conducted for a minimum of five and a maximum of eight days to ascertain and confirm the dominance positions of the mice in each group. In all cases it was possible to assign the top and bottom rankings, but there was some question about assignment of the second and third ranking mice in some cages of each gender. Therefore, I elected to limit the treatment conditions to three: (1) most dominant individuals, (2) middle ranking mice, including both the second and third positions in the hierarchy, and (3) most subordinate individuals. Urine collection from all four mice in a cage was carried out on the same day, and the group was not reconstituted afterwards. Data from males and females were subjected to separate one-way analyses of variance and post-hoc testing for males was carried out using a Tukey-Kramer procedure with $\alpha = 0.02$.

RESULTS

Experiment I: Gender and Age Effects. Both the main treatment factors for gender ($F = 240.47$; $df = 1,176$; $P < 0.001$) and age ($F = 31.49$; $df = 7,176$; $P < 0.001$), as well as the interaction term ($F = 8.12$; $df = 7,176$; $P < 0.001$) were significant. Mean urine output per day within males was significantly lower at 25–45 days of age than at all subsequent ages (Table 1). Within females, there was an overlapping pattern of significant differences in mean urine output per day; in general, older females had higher output (Table 1). Males had higher mean urine output per day than females at all ages. The significant interaction was due to the fact that the ratio of urine output per day by males to that by females was approximately 1.5 : 1 at younger ages and shifted at about 2 : 1 at later ages. Correlations between body mass and urine output per day were significant for males at all ages (35 days of age: $r = 0.766$; $P = 0.0037$; 55 days of age: $r = 0.811$; $P = 0.0014$; 100 days of age: $r = 0.719$; $P < 0.0084$; 150 days of age: $r = 0.835$; $P < 0.0007$) and for females at all ages (35 days of age: $r = 0.923$; $P < 0.001$; 55 days of age: $r = 0.763$; $P = 0.0039$; 100 days of age: $r = 0.907$; $P < 0.001$; 150 days of age: $r = 0.910$; $P < 0.001$).

Experiment II: Female Reproductive Condition. There were significant differences in urine output per day according to reproductive condition ($F = 6.20$; $df = 7,104$; $P < 0.001$). Post-hoc comparisons revealed that females in diestrus had significantly lower urine output per day than all other treatments, including females in estrus (Table 2). There was a significant pattern within pregnant

TABLE 1. MEAN VALUES (± 1 SEM) OF URINE OUTPUT PER DAY BY MICE OF BOTH SEXES AND EIGHT DIFFERENT AGES[a]

Age (days)	Urine output (ml/day)	
	Males	Females
25	1.7 (0.2)A	1.1 (0.1)A
35	2.1 (0.2)A	1.4 (0.1)AB
45	2.4 (0.2)A	1.8 (0.2)ABC
55	3.6 (0.2)B	2.1 (0.2)BC
75	4.2 (0.2)B	2.1 (0.2)BC
100	4.4 (0.2)B	2.1 (0.2)BC
125	4.2 (0.2)B	2.0 (0.1)BC
150	4.4 (0.3)B	2.4 (0.2)C

[a]Those means in each column not marked with the same letter are significantly different at the $\alpha = 0.02$ level. $N = 12$ mice/treatment.

females; urine output per day was significantly less during early pregnancy compared to the final days of pregnancy.

Experiment III: Density Effects. The main treatment effect for gender was significant ($F = 174.31$; $df = 1,132$; $P < 0.001$), but neither the main treatment effect for density ($F = 1.98$; $df = 5,132$; P = NS) nor the interaction term ($F = 1.96$; $df = 5,132$; P = NS) were significant. As in experiment I, males had significantly higher urine output per day than females (Table 3).

TABLE 2. MEAN VALUES (±1 SEM) OF URINE OUTPUT PER DAY BY FEMALE MICE IN TWO DIFFERENT STAGES OF ESTRUS, THREE DIFFERENT INTERVALS DURING PREGNANCY, AND THREE DIFFERENT INTERVALS DURING LACTATION[a]

Treatment	Urine output (ml/day)
Female in estrus	2.6 (0.1)BC
Female in diestrus	1.8 (0.1)A
Pregnant female (days 1–6)	2.4 (0.2)B
Pregnant female (days 7–12)	2.9 (0.2)BCD
Pregnant female (days 13–18)	3.2 (0.2)CD
Lactating female (days 1–7)	3.1 (0.3)CD
Lactating females (days 8–14)	3.0 (0.2)CD
Lactating females (days 15–21)	3.0 (0.2)CD

[a]Those means not marked with the same letter are significantly different at the $\alpha = 0.02$ level. $N = 14$ mice/treatment.

TABLE 3. MEAN VALUES PER MOUSE (±1 SEM) OF URINE OUTPUT PER DAY BY MICE OF BOTH SEXES AND SIX DIFFERENT DENSITY TREATMENTS (N = 12 MICE/TREATMENTS)

	Urine output (ml/day)	
Density	Males	Females
1/cage	3.7 (0.2)	2.0 (0.2)
2/cage	4.0 (0.3)	2.4 (0.1)
3/cage	4.1 (0.2)	2.2 (0.2)
4/cage	3.8 (0.4)	2.4 (0.2)
6/cage	3.9 (0.5)	2.4 (0.2)
8/cage	4.4 (0.5)	2.6 (0.2)

Experiment IV: Gonadectomy Effects. For males, there was a significant effect of gonadectomy (t = 4.375; df = 26; $P < 0.001$) on urine output per day. Castration significantly reduced urine output (mean = 4.3 ± 0.3 ml/day for intact males and mean = 2.9 ± 0.2 ml/day for castrated males). For females, there was no effect of gonadectomy on urine output per day (t = 0.303; df = 26; P = NS) (mean = 2.4 ± 0.3 ml/day for intact females and mean = 2.5 ± 0.2 ml/day for ovariectomized females).

Experiment V: Social Status Effects. For males, there was a significant effect of dominance status on urine output per day (F = 40.22; df = 2,45; $P < 0.001$). The post-hoc analysis revealed that top ranked males had significantly higher urine output per day than middle ranking males, which, in turn, had significantly higher urine output per day than the lowest ranking males (Table 4). For females, there was no effect of dominance status on urine output per day (F = 2.00; df = 2,45; P = NS).

TABLE 4. MEAN VALUES (±1 SEM) OF URINE OUTPUT PER DAY BY MICE OF BOTH SEXES AND THREE DIFFERENT LEVELS OF DOMINANCE STATUS[a]

	Urine output (ml/day)	
Treatment (N)	Males	Females
Top ranked (12)	5.4 (0.2)C	2.7 (0.2)
Second and third ranked (24)	3.7 (0.2)B	2.3 (0.1)
Fourth ranked (12)	2.7 (0.2)A	2.2 (0.2)

[a]Those means in the first column not marked with the same letter are significantly different at the α = 0.02 level.

DISCUSSION

The foregoing experiments provide support for the following conclusions: (1) Male house mice excrete urine at a rate 1.5–2.0 times that of females. (2) Urine output per day increases with age for both genders. (3) For mice of the same age, urine output per day is correlated with body mass. (4) Females in estrus produce more urine per day than females in diestrus. (5) Urine output per day increases during pregnancy and remains high throughout lactation. (6) Density does not influence urine output per day for either gender over the range of densities tested. (7) Castration reduces urine output per day in male mice, but ovariectomy in females does not alter the rate of daily urine production. (8) Dominant males produce more urine per day than subordinate males, but there are no similar effects for female mice. These results can be interpreted in terms of the social biology and ecology of the mice, and, in some cases, in terms of their physiology.

Finding that urine output per day increases with age and with body mass is not surprising. The general rate of urine excretion is similar to the values from Bing and Mendel (1931), and the age-related changes reported here are in agreement with Chew and Hinegardner (1957). I do not have any explanation for my values being considerably less than those recorded by Chew and Hinegardner (1957), except possibly that the average body mass of their mice was higher than of the mice I tested. The significant difference between males and females in urine output per day differs from the findings of Chew and Hinegardner (1957), exceeds what would be expected based on any possible gender differences in body mass, and is consistently present at all ages tested. A key problem then is to explain the possible proximate and ultimate reasons for this gender difference.

That males excrete more urine per day than females is in accord with earlier reports that males urine mark at roughly twice the rate of females (Maruniak et al., 1975; Powell and Wolff, 1982). The extra marking by males could be related to the fact that they are more likely to be using urine to mark territory boundaries than are females. The higher levels of urine excretion by males appear to be testosterone dependent, as the volume drops to levels comparable to those recorded for females after males have been castrated. Further evidence of the role of testosterone comes from the data on dominance status and urine output per day. Since aggressiveness and dominance are related to testosterone in house mice (Barkley and Goldman, 1977a–c), we can predict that social dominance is associated with the tendency to establish and maintain a larger territory, corresponding with the need for more urine to mark the territory. Subordinate males, or those that are castrated and thus have lower testosterone, would have lower urine output per day and would mark less. Thus, higher testosterone and higher dominance status are linked (cause and effect cannot be readily separated) and, in some combination, they lead to greater quantities of urine production.

It is noteworthy that females in estrus excrete more urine than those in diestrus. If urine of females signals conspecifics about reproductive condition, then we might expect females in estrus to excrete additional urine to attract potential mates. This is supported by earlier reports that males prefer the odors of estrous to diestrous females (Rose and Drickamer, 1975; Sokolov and Kotenkova, 1976; Drickamer, 1995) and by Hurst's (1990b) finding that female mice advertise their status and condition through urine marks. Although dominant females may mark more (Hurst, 1990b), my findings indicate that, contrary to my previous logic concerning males, dominant females do not produce a significantly higher volume of urine per day than lower ranking females. I have no thorough explanation for the positive effects of dominance in males on their urine production versus that there is no such effect in females, although there are at least two possibilities. The first is that higher urine volume per day is related to higher levels of testosterone in male mice. Second, in the polygynous mating system of the house mouse and with a key function of urinary chemosignal cues in female mice being to signal their reproductive status, the subordinate females, when they come into estrus, may be competing for mates and thus they could have urine output per day similar to those of higher ranking females.

The somewhat higher rates of urine output per day for females that are pregnant may be due to purely physiological reasons. This notion is supported by the fact that females in the latter two thirds of gestation excrete more urine per day than those early in gestation. Females later in gestation are carrying larger fetuses and are providing the excretory function for those fetuses. Higher urine excretion rates for lactating females could, in addition to physiological reasons relating to the process of lactation and facilitation of excretion by the young, contribute to their use of that urine as a signal to other conspecifics to maintain their distance. Beginning near parturition and continuing through lactation, female mice are quite aggressive toward conspecifics, presumably as a measure of protection for their litter (Svare, 1981; Svare and Mann, 1983). Deposition of urine as a cue indicating their lactational status and as a warning to conspecifics could be an efficient means of avoiding more overt conflicts. These findings fit with evidence for avoidance of urine cues from pregnant and lactating females (Brown, 1985a; Drickamer, 1995). To my knowledge, no one has yet studied the rates of urine marking by pregnant or lactating female mice.

That density does not influence the rate of urine excretion per mouse is curious. Based on earlier work linking higher density with stress, and stress, in turn, with less urine excretion via the effects of vasopressin (Zikmund and Lichardus, 1961; Scharrer and Scharrer, 1963; Christian and Davis, 1964; Levine and Treiman, 1964; Levin et al., 1974; Drickamer and McIntosh, 1980), I might have predicted urine output per day per mouse to decrease with increasing density for one or both genders. I do not have an explanation for the lack of a

density effect, other than to suggest that it is likely that a number of interacting factors, including density-related stress and social status relationships among the mice could be affecting urine output per day. This suggestion concerning social status effects is supported, to some degree, by the fact that the standard errors for the mean daily urine output of male, but not female, mice in higher density cages are larger than for mice at lower cage densities. For males, the larger amount of variation could be attributable to the fact that there are dominance relationships among the males, with some, higher ranking males, excreting more urine and other, lower ranking males, excreting smaller amounts. It could also be the case that some critical or threshold density must be reached before density effects on urine output per day occur. This explanation seems less likely given the very high densities that already exist under the caging conditions used relative to any densities obtained in free ranging mice (Anderson, 1961).

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SUBSTRATE SPECIFICITY OF ACETYLTRANSFERASE AND REDUCTASE ENZYME SYSTEMS USED IN PHEROMONE BIOSYNTHESIS BY ASIAN CORN BORER, *Ostrinia furnacalis*[1]

CHENG-HUA ZHAO,[2,3] FANG LU,[2] MARIE BENGTSSON,[4] and CHRISTER LÖFSTEDT[3,*]

[2]*Institute of Zoology, Chinese Academy of Sciences*
100 080 Beijing, China

[3]*Department of Ecology, Lund University*
S-223 62 Lund, Sweden

[4]*Department of Plant and Forest Protection*
Swedish University of Agricultural Sciences
Ecology Building, S-223 62 Lund, Sweden

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Abstract—The substrate specificity of the acetyltransferase and the reductase enzyme systems used by *Ostrinia furnacalis* (Lepidoptera: Pyralidae) in pheromone biosynthesis was studied in vivo by topical application of precursors to pheromone glands. Each of the tetradecenols, varying in double bond position (from 7 to 13) and geometry of the double bond, was converted to the corresponding acetate by the acetyltransferase. The similarity in the conversion rates of all tested fatty alcohols indicated that the acetyltransferase has a low substrate specificity. Most of the corresponding tetradecenoic acids could also be converted to the respective acetates. However, very different conversion rates among the tested fatty acids demonstrated that the reductase system has a higher substrate specificity than the acetyltransferase. The conversion rates of most *E* isomers were higher than those of the corresponding *Z* isomers, except for the (Δ)-11-tetradecenoic acids, in which much more *Z* isomer was converted to the product. Saturated tetradecanoic acid was converted to the corresponding acetate at a high rate; the shorter homolog, tridecanoic acid, was converted at a lower rate (56%), and conversion to the respective acetates of the longer homolog, pentadecanoic and hexadecanoic acids, was insignificant (<5%). The results from the present study showed that specificity of pheromone production is to a large extent controlled by the pheromone gland reductase system.

*To whom correspondence should be addressed

[1]Guenée, Lepidoptera: Pyralidae

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INTRODUCTION

Many moths use *E/Z* isomers of monounsaturated acetates in specific ratios in their sex pheromones. The sex pheromone of *Ostrinia furnacalis* Guenée (Lepidoptera: Pyralidae), consists of (*E*)- and (*Z*)-12-tetradecenyl acetate (*E*12- and *Z*12-14:OAc) in a 47:53 ratio (Cheng et al., 1981). Two pheromone strains of the European corn borer, *Ostrinia nubilalis* Hübner, both have (*E*)- and (*Z*)-11-tetradecenyl acetate (*E*11- and *Z*11-14:OAc) as pheromone components but in different ratios: an *E/Z* ratio of 99:1 was found in *E* strain, whereas the same compounds were produced in a 3:97 ratio in the *Z* strain (Kochansky et al., 1975; Klun and Cooperators, 1975). Studies of the pheromone biosynthesis in both species showed a difference between isomeric ratios of acetates and the corresponding fatty acid precursors in the pheromone glands. In *O. furnacalis*, the ratio of (*E*)- and (*Z*)-12-tetradecenoate (*E*- and *Z*12-14:OAcyl) is about 12:88 (Zhao et al., 1990). Both the *E* and *Z* strains of *O. nubilalis* have a 70:30 *E/Z* ratio of Δ11-tetradecenoate (Δ11-14:Acyl) (Wolf and Roelofs, 1987; Jurenka and Roelofs, 1989). The specific *E/Z* ratio of acetates in *O. nubilalis* and *O. furnacalis* may thus be produced from the immediate fatty acid precursors by the selectivity of the postulated last two steps in the pheromone biosynthetic pathway: reduction and acetylation (Wolf and Roelofs, 1987; Zhao et al., 1990).

Recently, in vivo experiments demonstrated that the acetylation step has a very low substrate specificity in three noctuid species (Teal and Tumlinson, 1987; Bestmann et al., 1987; Dunkelblum et al., 1989). In contrast, an in vitro enzyme assay showed that the acetyltransferase exhibited some selectivity for the *Z* isomer of Δ11-tetradecenol (Δ11-14:OH) in *Argyrotaenia velutinana* Walker and three other species of tortricid moths, but not in *E*, *Z* and hybrid strains of *O. nubilalis*. Thus, in the later case, the *E/Z* acetate ratios produced may be due to the selectivity of the reduction step, whereas in *A. velutinana* the specific *E/Z* ratio may be controlled by the coupling of the reductase and the acetyltransferase (Jurenka and Roelofs, 1989). In general, the reductase system should be very important for the production of a specific ratio of acetates in many moth species, including tortricid moths (Morse and Meighen, 1987; Jurenka and Roelofs, 1989). However, so far the substrate specificity of the reductase in moth pheromone biosynthesis systems has not been experimentally investigated.

In the present study, we investigated the selectivity of the acetyltransferase and the reductase enzymes in *O. furnacalis* based on in vivo experiments using

a series of tetradecenols (Δ14 : OH) and tetradecenoic acids (Δ14 : Acid) as substrates.

METHODS AND MATERIALS

Chemicals. Tetradecenols, varying in double bond position from 7 to 13 as well as in geometry of the double bond, were purchased from the Research Institute for Plant Protection (Wageningen, The Netherlands). Gas chromatographic analyses using a BP-20 capillary column showed that the purity of the alcohols ranged from 96.3 to 99.4% and that the corresponding geometric isomer in each compound was less than 1%. Tetradecanol (14 : OH) and tridecanoic acid (13 : Acid) were available in our laboratory. [14,14,14-$^2H_3$]Tetradecanoic acid ([14-D_3]14 : Acid), [15,15,15-$^2H_3$]pentadecanoic acid ([15-D_3]15 : Acid), and [16,16,16-$^2H_3$]hexadecanoic acid ([16-D_3]16 : Acid) were purchased from Larodan Fine Chemicals, Malmö, Sweden.

Each of the tetradecenoic acids was prepared from the corresponding alcohol by a method modified from Corey and Smidt (1979): 45 μl of an alcohol, 600 mg pyridinium dichromate (PDC), and 2 ml dry dimethylformamide (DMF) were added to an 8-ml vial with a Teflon-lined screw cap. After 12 hr at 25°C without stirring, the reaction was stopped and the rest of the steps were performed as described by Corey and Smidt (1979).

The products were purified by centrifugal chromatography. Petroleum ether (60–90°)–ether–acetic acid (90 : 10 : 1) was used as the developing solvent at 3 ml/min flow rate. The fractions containing tetradecenoic acids were combined, and the solvent was removed with a stream of nitrogen. Capillary GC analyses of the fatty acids after conversion to methyl esters indicated that all were at least 96% pure and no corresponding alcohol or aldehyde was detected (the limit of detection was 0.1%). The yield for the different acids varied between 50 and 70%.

Deuterium-labeled pheromone precursors were synthesized by the procedures described below. High-resolution mass spectra of final products were recorded on a Jeol SX-102 mass spectrometer. ^1H NMR spectra were recorded on a Varian XL-300 spectrometer in $CDCl_3$ solutions with Me_4Si as the internal reference. Products were purified by flash chromatography (Taber, 1982) on TLC-Silica gel 60 H supplied by Merck and argentation liquid chromatography (Houx et al., 1974). DMPU was purchased from Fluka AB. Immediately before use, it was distilled over CaH_2 at reduced pressure and kept over 4 Å molecular sieves under an argon atmosphere.

(*Z*)-[14,14,14-$^2H_3$]12-Tetradecenoic acid (**1**) ([14-D_3]*Z*12–14 : Acid) (Figure 1) was prepared from 1-(2-tetrahydropyranyloxy)-dodecyne (6.0 g, 21 mmol) (**3**) (Smith and Beumel, 1974; Rossi et al., 1980) *n*-butyl lithium (11 ml, 2.09

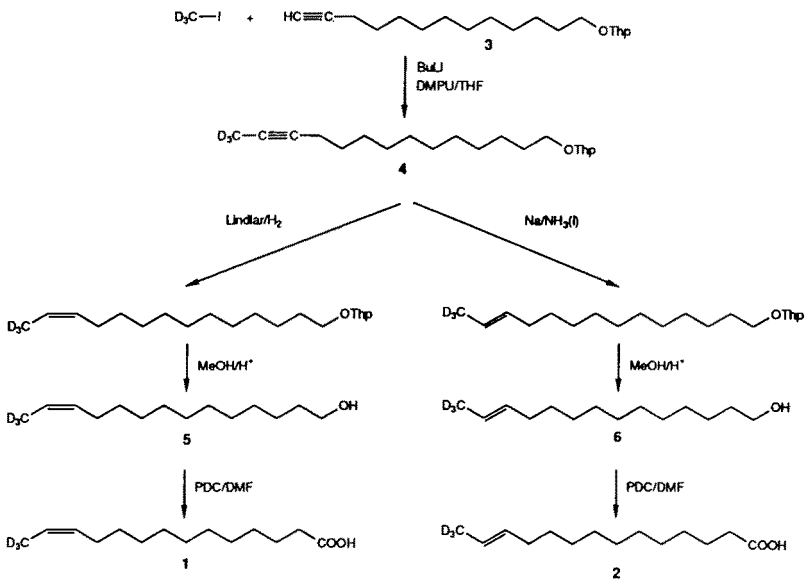

FIG. 1. Scheme for the synthesis of (*Z*)- and (*E*)-[14,14,14-$^2H_3$]12-tetradecenoic acid.

M in hexane) in dry THF (21 ml) and [$^2H_3$]methyl iodide (4.1 g, 34 mmol) in DMPU (36 ml) according to a method previously described for similar systems (Bengtsson and Liljefors, 1988) affording 5.6 g (88%) of the product (**4**) (Figure 1) after flash chromatography. Reduction with Lindlar catalyst (Leznoff et al., 1977; Wong et al., 1984) gave the *Z* monoene, which was converted to the corresponding alcohol (**5**) by treatment with *p*-toluenesulfonic acid in methanol. The fatty alcohol (**5**) was oxidized by the same method used in the synthesis of Δ14:Acid and purified with liquid chromatography.

The final product (**1**) was obtained in 60% overall yield. *m/z* 229 (M^+, 11%), 211(31), 169(17), 137(13), 124(20), 110(31), 97(49), 83(60), 69(100), 58(77), 55(97), 41(51). High-resolution mass spectra: $[M]^+_{calc}$ = 229.2121, $[M]^+_{obs}$ = 229.2114. ^1H NMR (300 MHz); δ 1.27–1.35 (m, 14H, CH_2CH_2), 1.58–1.65 (m, 2H, CH_2—C—COOH), 1.99–2.05 (m, 2H, CH_2—C=C), 2.35 (t, 2H, CH_2—COOH), 5.36–5.41 (m, 2H, CH=CH). Capillary GC analyses showed that the purity of (*Z*) [14,14,14-$^2H_3$]12-tetradecenol ([14-D_3]*Z*12-14:OH) was 97.4%, and no *E* isomer was detected (the limit of detection was 0.1%). The purity of [14-D_3]*Z*12-14:Acid was 97.4%, with no corresponding alcohol detected.

(*E*)-[14,14,14-$^2H_3$]12-Tetradecenoic acid (**2**) ([14-D_3]*E*12-14:Acid) was prepared as described above for the corresponding *Z* isomer (**1**), except that the

reduction of the triple bond was instead performed with sodium in liquid ammonia (Warthen and Jacobson, 1973) to give the desired *E* monoene. *m/z* 229 (M^+, 11%), 211(27), 193(4), 169(15), 138(12), 123(19), 110(31), 97(46), 83(57), 69(88), 58(90), 55(100), 43(42), 41(59). High-resolution mass spectra: $[M]^+_{calc}$ = 229.2121, $[M]^+_{obs}$ = 229.2115. ^1H NMR (300 MHz); δ 1.23–1.28 (m, 14H, CH_2CH_2), 1.60–1.65 (m, 2H, CH_2—C—COOH), 1.92–1.98 (m, 2H, CH_2—C=), 2.32 (t, 2H, CH_2—COOH), 5.39–5.42 (m, 2H, CH=CH). Capillary GC analyses showed that the purity of (*E*)-[14,14,14-$^2H_3$]12-tetradecenol ([14-D_3]*E*12-14:OH) was 94.0%, and the corresponding *Z* isomer was 0.9%. The purity of [14-D_3]*E*12-14:Acid was 95.7%, and no corresponding alcohol was detected.

Insect Source, Application of Fatty Alcohol or Fatty Acid to Pheromone Glands. Insects were reared as previously described (Zhao et al., 1990). Pupae were sexed and then kept under a 16:8 hr light–dark cycle. The application procedure was as follows, unless otherwise specified: 5–8 hr after dark, 24 to 48 hr old female moths were used for topical application (Bjostad and Roelofs, 1981; Zhao et al., 1990) of a tested compound. (*E*)-11-Tetradecenol (*E*11-14:OH) and (*E*)-11-tetradecenoic acid (*E*11-14:Acid) were chosen as metabolic standards for studying substrate specificity of the acetyltransferase and the reductase, respectively. The glands were exposed by gently squeezing the female abdomen. To each pheromone gland we applied 0.05 μl dimethyl sulfoxide (DMSO) containing 500 ng of a mixture of a standard and the compound to be tested in an approximate 1:1 ratio. About 15 min after application, the pheromone glands (5 glands/batch for the acetyltransferase study and 10 glands/batch for the reductase study) were extracted with hexane containing 6 ng of pentadecyl acetate as an internal standard for about 1 hr.

Chain length specificity of the reductase was investigated in two separate experiments. In the first experiment, a mixture of 13:Acid, [14-D_3]14:Acid, and [15-D_3]15:Acid was applied to glands of 3–9 females. Conversion rates in each batch (replicate) were calculated relative to that of [14-D_3]14:Acid. In the second experiment, a mixture of 13:Acid and [16-D_3]16:Acid was applied to glands of 5–10 females.

Gas Chromatography and Combined Gas Chromatography–Mass Spectrometry. Gas chromatographic (GC) analyses were performed on a Pye Unicam 204 GC equipped with a splitless capillary injector, a flame ionization detector, and an HP 3394 integrator. The linear velocity of the carrier gas (hydrogen) was 50 cm/sec and the purge valve was opened 0.25 min after injection. The following columns were used for the separation of various positional and geometric isomers of tetradecenyl acetates in the extracts: (A) 25 m × 0.22 mm ID BP-20 (Pye Unicam), temperature program: 1 min at 80°C and then 3°/min to 200°C; (B) 25 m × 0.22 mm ID BP-5 (SGE), temperature program: 1 min at 80°C and then 4°/min to 200°C; (C) 50 m × 0.22 mm ID BP-20 (SGE), temperature program: 1 min at 80°C and then 2°/min to 200°C; and (D) 51 m

× 0.22 mm ID CPSIL 88 (Chrompack), temperature program: 1 min at 80°C and then 2°/min to 200°C.

Gas chromatographic–mass spectrometric analyses (GC-MS) were performed using an Hewlett-Packard 5970B GC-MS system (electron impact, 70 eV) equipped with a 59970B computer system and interfaced with an Hewlett-Packard model 5890 GC. The diagnostic ions *m/z* 194.20 and *m/z* 197.20 were selected in an acquisition program to monitor unlabeled and D_3-labeled tetradecenyl acetates, respectively. Other acetates were also monitored at ions corresponding to [M-60].

Relative Conversion Factors. To define substrate specificity, a relative conversion factor was calculated for each compound tested. The relative conversion factor (RCF) for a specific compound was calculated as:

$$RCF = (\text{Test:OAc}/E11\text{–}14\text{:OAc}) \times (\text{Met stand/test compound})$$

Test:OAc/*E*11–14:OAc is the ratio between the acetate generated from the test compound and *E*11–14:OAc generated from the metabolic standard in gland extracts after incubation as determined by GC or GC-MS analyses. Met stand/test compound is the GC-determined ratio between the amounts of the compound to be tested and the metabolic standard in the solution that was applied to the gland (this ratio was always close to 1 but varied slightly between mixtures, which made this correction necessary).

Synthetic samples of the naturally occurring pheromone precursors, (*E*)- and (*Z*)-12-tetradecenol (*E*12- and *Z*12–14:OH), were initially used to determine the relative conversion factors for these isomers. The amount of *E*12- or *Z*12–14:OAc from the corresponding applied precursor was calculated by subtraction of the average amount of natural pheromone in a control group (no acids applied). The amount of tetradecanyl acetate (14:OAc) produced from applied 14:OH was calculated in a similar way (a normal ratio between native 14:OAc and one of the pheromone components was determined in control groups); thus, the relative conversion factor of 14:OH was calculated. The deuterated precursors [14-D_3]*E*12- and [14-D_3]*Z*12–14:OH were synthesized in the course of the study, and the relative conversion factors of *E* and *Z*12–14:OH were confirmed by the application of these compounds.

The relative conversion factors of (*E*)- and (*Z*)-12-tetradecenoic acid (*E*12- and *Z*12–14:Acid) were also determined with both unlabeled and labeled acids ([14-D_3]*E*- and [14-D_3]*Z*12–14:Acid, compounds **2** and **1** and Figure 1), using the same approach as in the acetyltransferase study.

RESULTS

Dosage for Topical Application. In an initial experiment, 100 ng of (*Z*)-11-tetradecenoic acid (*Z*11–14:Acid) was applied to the pheromone glands. One

batch of 10 female glands was incubated for 15 min, and another one was incubated for 30 min. Gas chromatographic analyses showed that the average recovered amount of Z11-14:OAc was significantly larger after 15 min of incubation than after an incubation time of 30 min (3.03 and 1.16 ng, respectively; t test, $P \leq 0.05$, $N = 5$). Thus, 15 min of incubation was used in all of the following experiments. Glands incubated with larger amounts of Z11-14:Acid produced slightly more Z11-14:OAc. It was evident from this experiment that the amount of the product may vary considerably between replicates (Figure 2). We subsequently used a dosage of 250 ng of a tested fatty acid or a tested fatty alcohol throughout the study.

Acetyltransferase Specificity. In the study of the acetyltransferase specificity, each of the tetradecenols tested, as well as the saturated 14:OH, was converted to the corresponding acetate with similar relative conversion factors (Figure 3) with one exception. The exception was (Z)-7-tetradecenol (Z7-14:OH), for which the smallest relative conversion factor (0.56) was determined. The relative conversion factors for the other fatty alcohols were in the range of 0.72 to 1.01. For each pair of geometric isomers the E isomer was normally converted at a higher rate than the Z isomer. A typical mass chromatogram showed that [14-D_3]E12-14:OH and E11-14:OH were converted to the corresponding acetates in approximately equal amounts (Figure 4A). Very similar relative conversion factors of E- and Z12-14:OH were obtained from both unlabeled and labeled precursors (Figure 3).

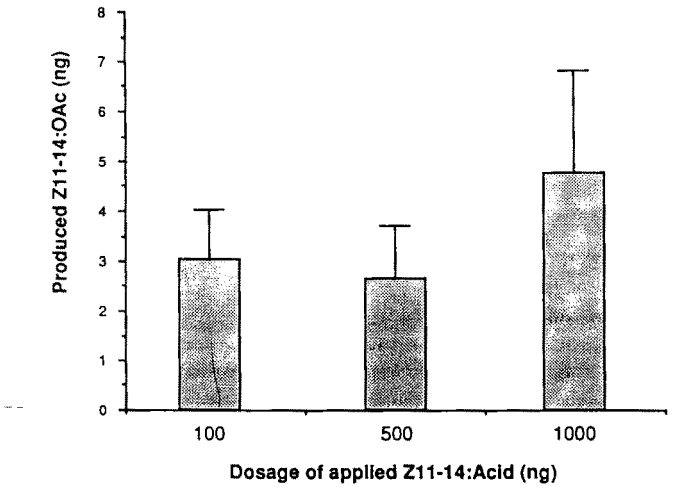

FIG. 2. Titer of Z11-14:OAc in pheromone glands of *O. furnacalis* as determined by GC analysis after topical application of different amounts of Z11-14:Acid and 15 min of incubation ($N = 5$, each batch consisted of 10 female glands).

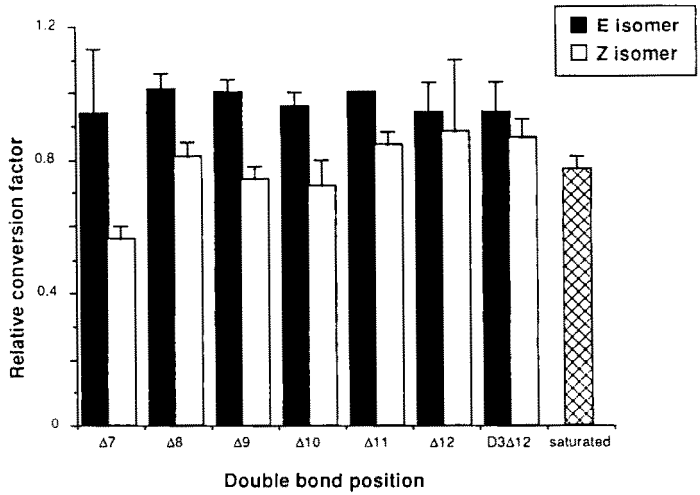

FIG. 3. Substrate preference of the acetyltransferase in *O. furnacalis* for 14-carbon alcohols. All conversion factors are normalized to that of *E*11–14:OH, which thus becomes 1.0 ± 0.0 by definition. The relative conversion factors of Δ7, Δ11, Δ12, and saturated 14:OH were determined by GC analyses of gland extracts from five females ($N = 6$). The relative conversion factors of Δ8, Δ9, Δ10, and [14-D_3]Δ12–14:OH (D3Δ12) were determined by GC-MS analyses of individual females ($N = 4$). Vertical bars indicate standard error of the mean.

Reductase Specificity. In the study of the reductase specifity, most of the tetradecenoic acids varying in double bond position (from 7 to 13) and geometry of the double bond, as well as saturated [14-D_3]14:Acid, were converted to the corresponding acetates, but with very different conversion factors (Figure 5).

The relative conversion factors of the *E* isomers of the tested Δ14:Acids were larger than those of the corresponding *Z* isomers, except for Δ11–14:Acid, in which the relative conversion factor of the *Z* isomer is much larger than that of the *E* isomer (Figure 5). The smallest conversion factor was found for the (*Z*)-7- and (*Z*)-8-tetradecenoic acids, from which the corresponding acetates could not be detected by GC upon incubation with pheromone glands. A typical mass chromatogram showed that [14-D_3]*E*12–14:Acid and *E*11–14:Acid were converted to the corresponding acetates at different rates (Figure 4B). The relative conversion factor for [14-D_3]*E*12–14:Acid was 2.6 ± 0.2, while the factor for [14-D_3]*Z*12–14:Acid was 1.4 ± 0.1. Thus, the relative conversion factor for the *E* isomer pheromone precursor was almost two times larger than that of the *Z* isomer. This relation was also found for unlabeled pheromone precursors (Figure 5).

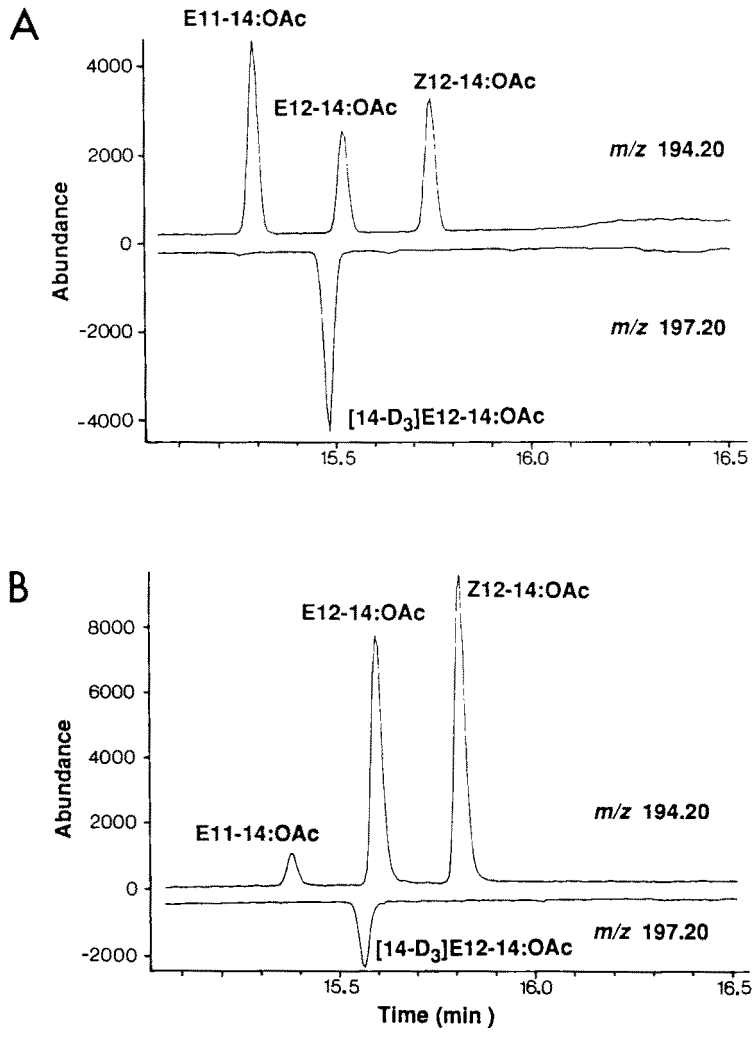

FIG. 4. Mass chromatograms from analyses of the pheromone gland extracts of *O. furnacalis* showing labeled and native pheromone components after application of labeled pheromone precursors. (A) The acetates produced from the extract of a gland after application of [14-D_3]E12-14:OH and *E*11-14:OH. (B) The acetates produced from the extract of two glands after application of [14-D_3]*E*12-14:Acid and *E*11-14:Acid.

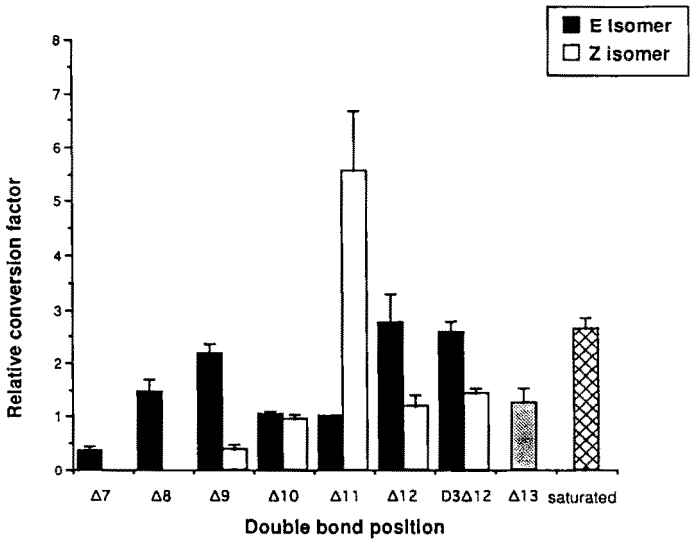

FIG. 5. Substrate preference of the fatty acid reductase in *O. furnacalis* for 14-carbon acids. All conversion factors are normalized to that of *E*11-14:Acid, which thus becomes 1.0 ± 0.0 by definition. The relative conversion factors of [14-D_3]Δ12-14:Acid (D3Δ12) were determined by GC-MS analyses of gland extracts from two females (N = 6). The relative conversion factors of other fatty acids including [14-D_3]14:Acid (saturated) were determined by GC analyses of pooled gland extracts from 10 females (N = 6). The relative conversion factors of *Z*7- and *Z*8-14:Acid were below the limit of detection. Vertical bars indicate standard error of the mean.

Conversion of 13:Acid to the corresponding acetate was less than 60% of that obtained with [14-D_3]14:Acid (Table 1). For the longer homolog, [15-D_3]15:Acid, conversion to acetate was less than 5% of that observed with the 14-carbon substrate, and with [16-D_3]16:Acid the production of acetate was below the limit of detection.

Production of Specific Ratios. *E/Z* ratios of the pheromone precursors and components obtained by GC-MS analyses of methanolized and acetylated gland extracts were compiled from two earlier investigations (Zhao and Wang, 1990; Zhao et al., 1990). It is apparent that an approximate 50:50 ratio of *E*12- and *Z*12-14:OAc is produced by the reductase and acetyltransferase from a very different ratio of *E*12- and *Z*12-14:Acyl found in the pheromone gland (15:85 and 24:76, in the two investigations, respectively). Furthermore, the ratio of *E*12- and *Z*12-14:Acyl was produced from another ratio of (*E*)- and (*Z*)-14-

TABLE 1. CONVERSION OF TOPICALLY APPLIED SATURATED FATTY ACIDS TO ACETATES IN PHERMONE GLANDS OF *O. furnacalis*

Substrate	Relative conversion	*N*
13 : Acid	56[a]	6
[14-D_3]14 : Acid	100[b]	6
[15-D_3]15 : Acid	< 5[c]	6
[16-D_3]16 : Acid	0[d]	4

[a] Range of observed values was 47.0–60.7.
[b] Conversion rates were calculated relative to that of [14-D_3]14 : Acid, which thus always became 100 by definition.
[c] Observed values were below the limit of quantification, but were always less than 5% of [14-D_3]14 : OAc.
[d] [16-D_3]16 : OAc was below the limit of detection in all experiments.

hexadecenoate (*E*14- and *Z*14–16 : Acyl, ca. 75 : 25 in the two studies) by a β-oxidase for chain shortening (Figure 6). When a 13 : 87 ratio of [14-D_3]*E*- and [14-D_3]*Z*12–14 : Acid (the ratio is similar to that found in the gland) was applied to the glands, an average ratio 28 : 72 ± 8.5 of [14-D_3]*E*- and [14-D_3]*Z*12 : OAc was obtained from the gland extracts ($N = 6$). The results implied that an approximate 50 : 50 ratio of *E*12- and *Z*12–14 : OAc cannot be produced by the reductase from the ratio of *E*12- and *Z*12–14 : Acyl found in the gland (Figures 5 and 6).

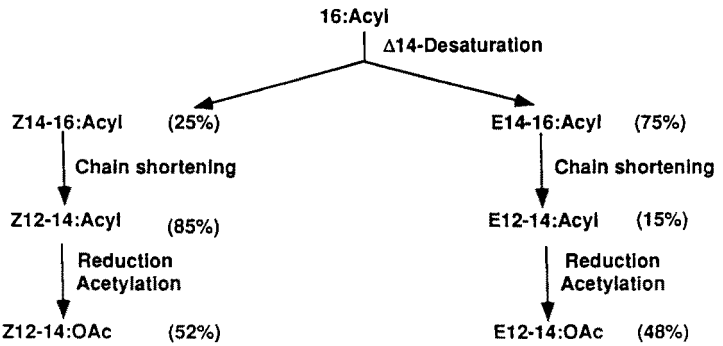

FIG. 6. Biosynthetic pathways for the production of pheromone components in *O. furnacalis* (after Zhao et al., 1990) with data added on the isomeric composition at each level.

DISCUSSION

The production of specific ratios between pheromone components in moths may be controlled by the selectivity of several postulated biosynthetic reactions. In the current study we have demonstrated a high chain-length specificity for the conversion of exogenously applied fatty acids to corresponding acetates in *O. furnacalis*. A highly selective conversion of positional and geometric isomers of tetradecenoic acid to the respective acetates was also shown. In contrast, the postulated final step in the pheromone biosynthesis, the acetylation of alcohols, is essentially nonselective with respect to the geometry and position of the double bond in the tetradecenol substrates. Our interpretation of these results is that the specificity of one or several reaction steps involved in the reduction of fatty acid precursors to alcohols is responsible for the production of a specific ratio between the pheromone components *E*12- and *Z*12-14:OAc.

Ideally it should be possible to study pheromone biosynthesis in individual insects to minimize the need for insect material and labeled precursors and to allow investigations of the biosynthetic basis for individual variation in pheromone composition. In the present study, we made some efforts to optimize the protocol for topical application and in vivo studies of the metabolism of pheromone precursors. We found that in *O. furnacalis*, variation of the dosage of the applied fatty acid between 100 and 1000 ng had no significant influence on the amount of product formed. This result is similar to what was found in *A. velutinana* for the application of tetradecanoic acid. When Bjostad and Roelofs (1986) increased the amount of saturated precursor from 100 to 4000 ng, the production of labeled (*E*)- and (*Z*)-11-tetradecenoate as well as labeled pheromone components increased only slightly. We subsequently used a standard dosage of 250 ng of both acid and alcohol precursors throughout our studies. Obviously, only a small percentage of the topically applied precursor is converted into pheromone components, and this fraction was even lower after 30 than after 15 min of incubation. The 15-min incubation time used in our studies of the acetyltransferase and the reductase in *O. furnacalis* was much shorter than that reported for the study of the actylation of alcohols in *Chrysodeixis chalcites* (Esper) (Dunkelblum et al., 1989) and *Hydraecia micacea* (Esper) (Teal and Tumlinson, 1987), but similar to what was used in the study of acetylation in *Mamestra brassicae* (L.) (Bestmann et al., 1987). In *M. brassicae*, the acetate could be found 5 min after the corresponding alcohol was applied to the pheromone gland. We believe that metabolism of precursors is generally very fast and 15 min of incorporation should normally be sufficient for in vivo studies of moth pheromone production. The decrease in titer of the products after longer periods of incubation could be due to release of pheromone by calling females or to recycling of labeled products, possible explanations that were not further investigated.

The calculation of a relative conversion factor in the present study is similar to the approach previously used by Dunkelblum et al. (1989). *E*11-14:OH and *E*11-14:Acid were chosen as metabolic standards for the acetyltransferase and the reductase study, respectively. These compounds are not naturally present in the gland but they are readily metabolized. The high variation between replicates in the formation of product, whether due to the performance of the insects or to the procedures used, makes the use of a metabolic standard important for every quantitative study of pheromone biosynthesis.

The results from the acetyltransferase study demonstrated low selectivity for tetradecenols with different position and geometry of the double bond. Low substrate specificity of the acetyltransferase was also observed in *H. micacea* (Teal and Tumlinson, 1987), *M. brassicae* (Bestmann et al., 1987), and *C. chalcites* (Dunkelblum et al., 1989). In all these noctuid species, in vivo experiments demonstrated that the acetylation was not specific with respect to carbon chain length, degree of unsaturation, or geometry of double bonds. In vitro experiments showed that 1.5–2.0 times more *Z* isomer of Δ11-14:OH was converted to the corresponding acetate by the acetyltransferase in *A. velutinana*, and similar results were reported for three other species of Tortricidae (Jurenka and Roelofs, 1989). However, in the same study no significant substrate selectivity with respect to *E*11- and *Z*11-14:OH was found in the *E*, *Z*, and hybrid strains of *O. nubilalis*, similar to what we report for *O. furnacalis*. We therefore conclude that the observed differences in substrate specificity among species are not due to the use of different techniques, but rather reflect real differences in insect biochemistry.

In contrast to the low selectivity of the acetyltransferase, high selectivity of the reductase system with respect to chain length and to double bond position and geometry was demonstrated by the experiments with different acid precursors. Because the acetyltransferase has low specificity for the series of corresponding tetradecenols as demonstrated above, the differences in relative conversion factors of the tested fatty acids should be due to high specificity of the reductase or other enzymes involved in the reduction of acyl precursors. To date little is known about the actual pathways for reduction of acids to aldehydes and alcohols in insects (Morse and Meighen, 1987). The fatty acid reductase elucidated in luminescent bacteria is part of a multienzyme complex (500 kDa), the so-called fatty acid reductase complex (for a review, see Meighen, 1993). In moth pheromone biosynthesis, the fatty acid reductase should generally be coupled to an aldehyde reductase, as fatty alcohols and not aldehydes are the normal product of the reduction (Morse and Meighen, 1987). Thus any one or several of the reaction steps taking place in this reductase complex could be responsible for the observed selectivity, In experiments with the European corn borer, *O. nubilalis*, we actually found that reduction of labeled aldehydes, postulated transient intermediates in sex pheromone biosynthesis, showed almost

the same selectivity as the overall conversion of free fatty acids to pheromone components, suggesting that the postulated aldehyde reductase alone may account for the selectivity (Zhu et al., 1995).

Moths do not normally make pheromones from exogenously applied precursors and the fatty acid precursors occur as acyl derivatives rather than free acids in the pheromone glands. Thus the observed selective conversion of precursors to acetates could theoretically be due to differences in uptake between different compounds, selective transport between cell compartments, or selective hydrolysis. However, the current study clearly demonstrates that hexadecanoic acid is not converted to the corresponding acetate 16:OAc, although earlier experiments with *O. furnacalis* (Zhao, et al. 1990) showed that topically applied hexadecanoic acid is readily desaturated, chain-shortened, and finally incorporated into the pheromone components. Thus we find it very unlikely that differences in uptake account for the pattern observed in the current study and favor an explanation involving the selectivity of the reductase complex or enzymes operating in close connection with this. The final proof of this hypothesis awaits the isolation and characterization of the different enzymes involved.

As mentioned above, hexadecanoic acid was not at all converted to the corresponding acetate. Neither was Z7–14:Acid, which is especially interesting as native (*Z*)-7-tetradecenoate is present in gland extracts at a titer comparable to that of (*E*)-12-tetradecenoate, whereas neither (*Z*)-7-tetradecenol nor Z7–14:OAc is (Zhao and Wang, 1990).

The reductase system had a preference for the *E* isomer of most of the Δ14:Acids tested in the present study. The exception from this pattern was Z11–14:Acid, which had the largest relative conversion factor among all the fatty acids tested. The efficient reduction of a fatty acid that does not naturally occur in *O. furnacalis* was surprising to us, but it may indicate a close relationship between this species and the *Z* strain of *O. nubilalis* or another corn borer species in which Z11–14:OAc is a major pheromone component. The productive interaction with Z11–14:Acid may be an evolutionary "memory-effect," an ability that is still there just because it has not been selected against.

The difference in *E/Z* ratios between the pheromone components and precursors can not be entirely accounted for by the twofold higher relative conversion factor for *E*12–14:Acid compared to the *Z* isomer. In *A. velutinana*, it was suggested that the fatty acyl precursors for reduction and acetylation are selected from a fresh supply of phospholipid acyl groups, whereas the unused portion is incorporated into triacylglycerols (Bjostad et al., 1987). More recently, it was proposed that phospholipids are not directly involved in pheromone biosynthesis, but that the biosynthetic enzymes interact with CoA derivatives. Once the intermediates are incorporated into a lipid class, they are no longer available for conversion to a pheromone component (Jurenka and Roelofs, 1993; Russell Jurenka, personal communication). Similarily, one may hypothesize that in

O. furnacalis, the pheromone is biosynthesized from a pool of fatty acids in which the *E/Z* ratio of Δ12–14 : Acyl is different from that found in total lipid extracts of the gland. Unfortunately, it seems as if pheromone biosynthesis in the gland proceeds so fast that the *E/Z* isomeric ratio of Δ12–14 : Acyl from the "fresh pool" can not be determined by in vivo experiments. The titer of deuterium-labeled pheromone was high 10 min after the application of deuterium-labeled palmitic acid to the glands. In *O. furnacalis*, the *E/Z* ratio of Δ12–14 : Acyl available for pheromone production is not only influenced by the geometric specificity of the desaturase, but also by the selectivity of the β-oxidase responsible for chain shortening from a Δ14–16 : Acyl (Figure 6). Therefore, in *O. furnacalis*, the specific *E/Z* ratio of the pheromone is influenced in concert by at least three different enzyme systems in the pheromone biosynthesis.

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FOLIAR OXIDATIVE STRESS AND INSECT HERBIVORY: PRIMARY COMPOUNDS, SECONDARY METABOLITES, AND REACTIVE OXYGEN SPECIES AS COMPONENTS OF INDUCED RESISTANCE

J.L. BI and G.W. FELTON*

Department of Entomology
University of Arkansas
Fayetteville, Arkansas 72701

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Abstract—Oxidative responses of plants to pathogens and other environmental stresses have received considerable recent attention. We propose that an oxidative response also occurs following attack by herbivores. Our data strongly indicate a shift in the oxidative status of soybean following herbivory by the insect *Helicoverpa zea*. Herbivory caused significant increases in lipid peroxidation and ·OH radical formation. The activity of several oxidative enzymes including lipoxygenases, peroxidase, diamine oxidase, ascorbate oxidase, and NADH oxidase I increased after herbivory on soybean. The enhanced production of phenolic compounds is indicated by an increase in the activity of phenylalanine ammonia lyase in wounded tissues. On the other hand, the level of soybean foliar antioxidants such as ascorbic acid, total carotenoids, nonprotein thiols, and catalase decreased significantly following herbivory. These results implicate primary compounds (e.g., ascorbic acid, proteins), secondary metabolites (e.g., phenolics), and reactive oxygen species (e.g., hydroxyl radical, hydrogen peroxide) as multiple components of induced resistance. The oxidative changes in the host plant correspond with increased oxidative damage in the midgut of insects feeding on previously wounded plants. Decreases in nonprotein thiols and reduced ascorbic acid occurred in midgut epithelial tissue from insects feeding on wounded plants compared to the insects on control plants. In contrast, midgut hydroperoxides and dehydroascorbic acid concentrations were greater in insects on wounded plants compared to their counterparts on control plants. We conclude that oxidative responses in soybean may have both positive and negative effects upon the host plant: a decrease in herbivory and an increase in oxidative damage to the plant. The salient benefit to the plant, in terms of insect resistance, is the relative balance between these opposing effects.

*To whom correspondence should be addressed.

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Key Words—Oxidative stress; induced resistance, reactive oxygen species, soybean, midgut, ascorbic acid, *Helicoverpa zea*, lipoxygenase, peroxides.

INTRODUCTION

The role of oxidative stress in plant–pathogen interactions has received considerable attention during the last decade (Sutherland, 1991; Mehdy, 1994). It is now recognized that one of the most rapid responses in the plant cell following invasion by pathogens is an oxidative burst of reactive oxygen species (Doke, 1983; Apostol et al., 1989; Paxton and Groth, 1994; Mehdy, 1994). Reactive oxygen species include hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and superoxide radical (O_2^-). Bursts of reactive oxygen species in plant tissues occur in response to viruses (Doke and Ohashi, 1988), bacteria (Adam et al., 1989; Devlin and Gustine, 1992), fungi (Doke, 1983; Montalbini, 1991; Davis et al., 1993), and nematodes (Zacheo and Bleve-Zacheo, 1988). These oxygen intermediates, particularly $\cdot OH$, are highly reactive and detrimental to living cells due to their ability to induce lipid peroxidation and DNA and protein oxidation (Fridovich, 1978; Halliwell and Gutteridge, 1985; Imlay et al., 1988). Furthermore, H_2O_2 may be an important signal for eliciting several plant defensive responses associated with systemic acquired resistance (Apostol et al., 1989; Chen et al., 1993).

The oxidative plant responses to attack by arthropod herbivores are comparatively unknown (Hildebrand et al., 1986a,b; Felton et al., 1994a,b). Spider mite damage on soybean foliage increased lipid peroxidation, lipoxygenase (LOX), and peroxidase (POX), but did not affect the levels of the antioxidant enzymes catalase (CAT) and superoxide dismutase (Hildebrand et al., 1986b). Foliar levels of antioxidant carotenoids declined with increasing mite populations (Hildebrand et al., 1986a). Phloem-feeding by aphids increased the levels of the antioxidant enzyme glutathione reductase in wheat and barley (Argandona, 1994), induced lipid peroxidation in alfalfa foliar tissues (Dillwith et al., 1991), and increased O_2^- formation in excised lucerne foliage (Jiang and Miles, 1993). Feeding damage to soybean by the phloem-feeding, three-cornered alfalfa hopper increased the activities of LOX, POX, polyphenol oxidase (PPO), and ascorbate oxidase (AOX) (Felton et al., 1994a).

Our laboratory has been examining the oxidative plant response to foliar feeding herbivores (Felton et al., 1994a,b). Herbivory by the corn earworm *Helicoverpa zea* (Lepidoptera: Noctuidae) on soybean increased LOX activity and lipid peroxidation (Bi et al., 1994; Felton et al., 1994b). Oxidative damage to foliar protein from wounded plants was indicated by a loss of free thiols (Bi et al., 1994). Felton et al. (1994a) reported that herbivory by the bean leaf beetle *Ceratoma trifurcata* also caused increases in LOX levels but did not affect POX or PPO in soybean.

The effects of herbivory on other reactive oxygen species, antioxidants, and many oxidative enzymes (e.g., NADH oxidase, diamine oxidases, etc.) have not been demonstrated. We initiated this study to gain a more complete understanding of the oxidative status of the host plant following foliar feeding. The system chosen for this study includes soybean and *H. zea*, a major pest in the mid-Atlantic region of the southeastern United States (Smith and Brim, 1979; Fitt, 1989). Our specific objectives were to: (1) assess the impact of herbivory on the oxidative status of soybean, and (2) assess the impact of induced responses in soybean on insect growth and on the oxidative status of the insect midgut.

METHODS AND MATERIALS

Plants and Insects. Eggs of *H. zea* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on a wheat germ-based artificial diet until used in the experiment (Chippendale, 1970).

Soybean seeds (cv. Forrest or Hutcheson) were soaked in water for 6 hr and then incubated at 28°C for 24 hr. Preliminary experiments indicated that both cultivars showed similar oxidative responses to herbivory. Germinated seeds were sown in 430-ml polystyrene foam cups filled with soil mixture (Redi Earth Peat-Lite Mix) in the greenhouse. Plants were used in experiments at the V3 stage unless otherwise noted (Fehr et al., 1971). The cups were arranged in a completely randomized experimental design. Plants were watered every two days. Fertilizer (N/P/K = 20:20:20) was used weekly. Conditions were as follows: (1) 14-hr photophase, using high-pressure sodium light (1000 W) and (2) day temperature, 33 ± 2°C and night temperature, 20 ± 2°C.

Induction of Oxidative Responses. To determine if feeding by *H. zea* larva causes oxidative responses, soybean plants were grown in the greenhouse, treated with insects, and assayed for antioxidants and oxidants. A single fourth-instar *H. zea* was placed on each of five plants, and plants were placed individually in screen cages to prevent larval escape. Five control plants were placed in cages without larvae. After three days, fully expanded leaflets were excised from the uppermost node and assayed for enzymes and antioxidants as described below. Leaflets from the treated plants contained both damaged and undamaged leaflets. Preliminary results indicated that no significant differences existed between these leaflets in the variables assayed. A period of three days was initially chosen to investigate the oxidative changes because maximal systemically induced resistance to insects occurred within this period (Bi et al., 1994; Felton et al., 1994a). Percent defoliation was from ca. 20% by visual estimation using calibrated standards (Kogan and Kuhlman, 1982). The soybean cultivar Forrest was used for LOX, PPO, and nonprotein thiol determinations, whereas Hutcheson was

used for POX, AOX, ascorbate peroxidase (APX), ascorbic acid (ASC), dehydroascorbic acid (DHA), CAT, diamine oxidase (DAO), NADH oxidase I, carotenoids, lipid peroxidation, and ·OH assays. Assays were replicated three times, and the data were analyzed by one-way completely randomized ANOVA and mean differences were determined by LSD at $P = 0.05$.

Time Course for Induction of Oxidative Enzymes. To determine the time course of oxidative changes following feeding by *H. zea*, V4-stage soybean plants (cv. Hutcheson) were grown in the greenhouse, treated with insects, and assayed as described above. The levels of LOX (pH 5.5), POX, and AOX were determined after 24, 48, 72, and 96 hr of feeding by larvae. Six plants per treatment were used for each time period.

Plant Enzyme Assays. To assay for foliar enzymes, 1 g of foliage was homogenized in 10 ml 0.1 M ice-cold K Pi buffer, pH 7.0, containing 1% PVP and 0.5 mM EDTA. The homogenate was centrifuged at −2°C for 20 min at 10,000*g*, and the supernatant was used immediately as the enzyme source for the assays described below. A SLM-AMINCO 3000 diode array spectrophotometer with rate analysis software was used for all assays.

To assay for LOX, 0.25 mM linoleic acid was used as a substrate, and the rate of change in A_{234} was measured (Grayburn et al., 1991). The reaction mixture contained 50 μl of enzyme homogenate and 1 ml of substrate. LOX activity was assayed at pH 5.5, 7.0 (0.1 M K Pi), and 8.5 (0.1 M Na borate) to account for the activities of several isozymes. An extinction coefficient of 27 mM^{-1} cm^{-1} was used.

To assay for POX activity, guaiacol was used as the hydrogen donor according to the procedure of Ridge and Osborne (1970). Fifty microliters of enzyme solution was mixed with 1.0 ml of substrate containing 1 mM H_2O_2 and 2 mM guaiacol in 0.1 M K Pi, pH 7.0. POX activity was estimated from the increase in A_{470}.

To determine PPO activity, the procedure of Kahn and Miller (1987) was followed. Twenty-five microliters of enzyme solution was mixed with 200 μl of 20 mM Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) in 0.1 M K Pi buffer, pH 7.0, and PPO activity was estimated from A_{420}.

NADH oxidase I activity was measured as a decrease in A_{340} due to the loss of NADH (Brightman et al., 1991). The reaction mixture contained 100 μl enzyme extract in 2.9 ml 20 mM Tris Mes, pH 7.0, containing 1 mM KCN and 300 μM NADH. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used.

DAO activity was measured with 10 mM putrescine (diaminobutane) in 0.1 M K Pi, pH 7.0 (Federico et al., 1985). The reaction was initiated with 1 ml enzyme extract in 3 ml of substrate. After incubation at 37°C for 10 min, the reaction was stopped by adding 0.5 ml of 10% (w/v) TCA followed by 0.05 ml (10 mg/ml) *o*-aminobenzaldehyde in 95% ethanol. The absorbance of the Δ^1-pyrroline complex was measured at A_{430} after removal of protein by centrif-

ugation at 3000*g* for 20 min. An extinction coefficient of 1.86 mM^{-1} cm^{-1} was used (Federico et al., 1985).

AOX activity was measured according to the procedures of Felton and Summers (1993). The reaction mixture contained 100 μl enzyme homogenate, 1 ml of 0.1 M K Pi buffer (pH 7.0) with 0.15 mM ASC, 0.5 mM EDTA, and 0.002% metaphosphoric acid. The decrease in A_{265} of ASC was monitored, and an extinction coefficient of 14.7 mM^{-1} cm^{-1} was used.

APX activity was measured following Mittler and Zilinskas (1991) with the addition of 1 mM ASC to the extraction buffer. The reaction mixture contained 0.50 mM ASC, 50 mM K Pi buffer, pH 7.0, 0.1 mM H_2O_2, and 50 μl enzyme homogenate in a 1.5-ml total volume. Corrections were made for ASC disappearance due to nonenzymatic oxidation and H_2O_2-independent oxidation. An extinction coefficient of 2.8 mM^{-1} cm^{-1} for A_{290} was used.

CAT activity was monitored by following the loss of H_2O_2 at 240 nm (Aebi, 1984). The substrate was 0.059 M H_2O_2 in 0.05 M K Pi buffer, pH 7.0, containing 1 mM EDTA. The reaction was initiated by adding 100 μl enzyme homogenate to 2 ml of substrate, and enzyme activity was determined from the linear portion of the rate curve (A_{450} to A_{400}).

To assay for phenylalanine ammonia lyase (PAL) activity, the enzyme extract was prepared following Degousee et al. (1994). Briefly, 1 g fresh weight of soybean foliage was homogenized in 10 ml Na borate buffer, pH 8.8, containing 2.5% PVP and 15 mM 2-mercaptoethanol and then centrifuged at 20,000*g* for 15 min. The reaction mixture contained 100 μl supernatant from the enzyme extract and 1 ml 20 mM L-Phe. Reaction mixture was incubated for 1 hr at 40°C, and the change in A_{290} was measured against a blank without substrate (Degousee et al., 1994). An extinction coefficient of 10 mM^{-1} cm^{-1} was used.

Chemical Antioxidants. Carotenoid analysis followed the procedure of Hildebrand et al. (1986a). One gram of fresh foliage from each plant was lyophilized and then pulverized in a mortar and pestle. The pulverized sample was extracted with 8 ml of petroleum ether containing 0.45 mM butylated hydroxytoluene. The resulting mixture was incubated for 15 min at 30°C in a water bath, vortexed, and centrifuged at 2000*g* for 30 min. The A_{450} of the supernatant was measured, and an $E^{1\%}$ (Extinction coefficient of 1% concentration) value of 25,000 was used for carotenoid content.

The concentrations of foliar ASC and DHA were determined by a spectrophotometric assay (Law et al., 1983). Total ASC (ASC + DHA) was measured after reduction of DHA by DTT. Reduced ASC was estimated in the absence of DTT. DHA was estimated from the difference of total ASC and reduced ASC. Five hundred milligrams of foliage was homogenized in 5 ml of 5% metaphosphoric acid and centrifuged at 10,000*g* for 20 min. The reaction mixture for total ASC contained 0.02 ml of tissue aliquot, 0.05 ml of 150 mM Na Pi (pH 7.4) containing 5 mM EDTA, and 0.01 ml of 10 mM DTT. After 10

min at room temperature, 0.01 ml of 5 mM *N*-ethylmaleimide was added to remove DTT. Color was developed with 0.04 ml of 10% TCA, 0.04 ml of 44% orthophosphoric acid, 0.04 ml of 4% α,α'-dipyridyl in 70% ethanol, and 0.02 ml of 3% $FeCl_3$. The color development was processed at 25°C for 3 hr, and the A_{525} was read. The assay for reduced ASC omitted the DTT and *N*-ethylmaleimide, using 0.02 ml water as a replacement. L-ASC was used as a standard.

To assay for nonprotein thiols, 500 mg foliage was homogenized in 5 ml 5% metaphosphoric acid and centrifuged at 10,000*g* for 20 min. The color was developed by adding 0.1 ml of supernatant to 2.5 ml of 2.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.2 M K Pi buffer, pH 8.0 (Anderson and Wetlaufer, 1975). The A_{412} was recorded and concentrations determined from a standard curve for glutathione.

Plant Hydroxyl Radical Formation, Lipid Peroxidation, and Total Foliar Peroxides. To assay for ·OH formation in soybean, DMSO was used as a specific detector for ·OH formation in vivo (Babbs et al., 1989; Popham and Novacky, 1991). DMSO may also react with the potent oxidant peroxynitrite (Crow et al., 1994). The wounded DMSO treatment contained five greenhouse-grown plants treated with one larva per plant and watered with 0.5% DMSO. The control DMSO treatment contained five plants that were treated identically except that no larvae were placed on the plants. The remaining 10 control plants contained five plants with larvae and five without. Twelve hours prior to infesting plants with larvae, the DMSO-treated plants were watered to saturation with 0.5% DMSO and control plants were watered with tap water. Larvae were placed on treatment plants for 24 hr. The watering procedure was repeated three times during the experiment. Foliage was then excised from the two uppermost nodes (2 g/plant), quickly frozen with dry ice, and then ground with a mortar and pestle. The resulting slurry was extracted with distilled water and centrifuged to remove solid materials (Babbs et al., 1989). Removal of interfering chemicals and the color reaction with fast blue BB dye was as described (Babbs et al., 1989). The A_{420} of the methane sulfinic acid product was read, and concentrations were calculated based upon a standard curve. The A_{420} of the appropriate water-treated controls was subtracted from the DMSO treatments. Nonspecific absorbance in the water treatments was 28% of the DMSO treatments.

Lipid peroxidation was assayed using the thiobarbituric acid assay (Dhindsa and Matowe, 1981). One gram of fresh foliage was homogenized in 5 ml H_2O, and 5 ml of 5 mM thiobarbituric acid in 20% TCA was added. The mixture was heated to 95°C for 30 min and then quickly cooled in an ice-bath. The cold mixture was centrifuged at 10,000*g* for 20 min, and the A_{532} and A_{600} were recorded. The nonspecific A_{600} was subtracted from the A_{532}. Thiobarbituric-reactive substances were expressed as malondialdehyde equivalents using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Total hydroperoxides (ROOH) were measured following Jiang et al. (1992). The method is based on the hydroperoxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic condition. The Fe^{3+} forms a Fe^{3+}–xylenol orange complex measured at 560 nm. The extinction coefficients for lipid hydroperoxides and H_2O_2 are very similar in this assay (Jiang et al., 1991). Foliar samples were homogenized in the same buffer used for preparation of enzyme extract, but with the addition of 2.5% TCA and 1 mM BHT. After centrifugation of the homogenate, 100 μl of the sample was incubated with a 900-μl reaction mixture containing 100 μM xylenol orange, 250 μM Fe^{2+}, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol. After a 30-min incubation, the A_{560} was recorded and concentrations of hydroperoxides were calculated based on a standard curve with H_2O_2.

Effect of Induced Responses on Oxidative Status of Insect Midgut. To determine the effect of induced responses in soybean plants on larval growth and the oxidative status of the midgut epithelium, V6-stage soybean plants (cv. Hutcheson) were grown in cages in the field (Felton et al., 1994a). Three third-instar *H. zea* were placed on each treatment plant for three days. After this time period, the third instars were removed from plants and discarded. Five plants from each treatment were assayed for POX, LOX (pH 5.5), and LOX (pH 7.0). Newly molted fifth instars were then placed on each remaining control and treatment plant. Initial weights of these larvae were not significantly different ($P = 0.514$). Mean weight of larvae placed on the treatment plants was 125.7 $\pm$ 6.5 mg, and mean weight of larvae on control plants was 121.5 $\pm$ 5.0 mg. A total of 30 larvae per treatment was tested.

After three days, larvae were removed from each plant and weighted. The midgut epithelium was removed, weighed, and homogenized (Summers and Felton, 1994). Midgut tissue was pooled with three larvae per replicate providing a total of 10 replicates per treatment. ASC and ROOH were assayed using the assays described above and modified following Summers and Felton (1994). Nonprotein thiols were determined with DTNB in absolute methanol (Hu, 1994). Because of the movement of larvae between plants in their respective cages, the relative growth rate (milligrams per day/per milligram of larva) was calculated using the mean initial weights rather than individual larval weights (Felton et al., 1994a).

RESULTS

Foliar Oxidative Response to Herbivory. Larval *H. zea* feeding on soybean increased the activities of several foliar oxidative enzymes. LOX activities increased by 2.5-fold when measured at pH 5.5 ($P < 0.001$), 2.81-fold at pH 7.0 ($P < 0.001$), and 3.34-fold at pH 8.5 ($P < 0.001$) compared with the control foliage (Table 1). The feeding also significantly increased the activities of several other foliar oxidases (Table 1) such as POX by 1.6-fold ($P = 0.006$),

TABLE 1. EFFECT OF HERBIVORY ON FOLIAR ENZYME ACTIVITIES IN SOYBEAN

Enzyme	Control	Wounded	LSD (0.05)
AOX			
(nmol/min/g fresh weight)	219.0 (±33.3)[a]	468.1 (±89.3)	95.2
APX			
(μmol/min/g fresh weight)	2.95 (±0.49)	4.442 (±0.51)	0.76
CAT			
(μmol/min/g fresh weight)	143 (±30)	92 (±25)	45.1
DAO			
(μmol/min/g fresh weight)	447 (±77)	704 (±104)	92.0
LOX (pH 5.5)			
(nmol/min/g fresh weight)	420 (±63)	1050 (±148)	160
LOX (pH 7.0)			
(nmol/min/g fresh weight)	309 (±89)	867 (±165)	177
LOX (pH 8.5)			
(nmol/min/g fresh weight)	210 (±68)	701 (±171)	204
NADH oxidase I			
(nmol/min/g fresh weight)	72.6 (±33.4)	112.9 (±17.7)	34.3
PAL			
(nmol/min/g fresh weight)	9.9 (±5.1)	23.3 (±11.5)	9.03
POX			
(nmol/min/g fresh weight)	23.0 (±5.5)	36.8 (±6.1)	8.5
PPO			
(Δ OD/min/g fresh weight)	6.5 (±0.7)	8.5 (±1.2)	NS

[a]Numbers in parentheses are standard deviations.

DAO by 1.57-fold ($P < 0.001$), AOX by 2.14-fold ($P = 0.006$), and NADH oxidase I by 1.56-fold ($P = 0.026$) (Table 1). PAL activity was increased 2.35-fold ($P = 0.001$) by herbivory, but PPO was not significantly affected ($P = 0.06$) (Table 1). The level of the antioxidant enzyme CAT decreased 36% ($P = 0.03$) in damaged foliage compared to control foliage; however, another antioxidant enzyme, APX, increased 1.5-fold ($P = 0.002$) following herbivory (Table 1).

Feeding by *H. zea* in soybean also resulted in significant changes in foliar antioxidants as indicated by a 26% loss of ASC ($P = 0.003$) and 104% higher DHA ($P = 0.02$) level compared to controls (Table 2). The ratio of ASC to DHA was reduced from 11.68 to 3.62 reflecting the significant change in redox status. Herbivory resulted in a greater than 33% decrease in carotenoid concentration ($P < 0.001$) and 8% loss of nonprotein thiols ($P < 0.001$) in comparison with those in control foliage (Table 2).

TABLE 2. EFFECT OF HERBIVORY ON FOLIAR ANTIOXIDANTS IN SOYBEAN

Antioxidant	Control	Wounded	LSD (0.05)
Total ASC			
(μg/g fresh weight)	621.5 ($\pm$50.1)[a]	462.8 ($\pm$40.5)	70.6
Reduced ASC			
(μg/g fresh weight)	572.5 ($\pm$50.2)	362.6 ($\pm$41.0)	71.6
DHA			
(μ/g fresh weight)	49.0 ($\pm$11.6)	100.2 ($\pm$39.5)	42.4
ASC:DHA	11.68	3.62	
Total carotenoids			
(μ/g fresh weight)	126 ($\pm$20)	84 ($\pm$12)	21.0
Nonprotein thiols			
(ng/g fresh weight)	279 ($\pm$5.8)	257 ($\pm$4.8)	7.7

[a] Numbers in parentheses are standard deviations.

Folivory by *H. zea* caused an increase in ·OH flux by 3.87-fold (P = 0.038) in wounded soybean foliage compared to the undamaged control treatment (Table 3). Lipid peroxidation increased in wounded tissue as indicated by a 17% increase in malondialdehyde equivalents (P = 0.009) (Table 3). Total hydroperoxide levels were increased by 2.26-fold (P = 0.002) in the wounded tissue compared to the control treatment (Table 3).

Time Course for Induction of Oxidative Enzymes. Herbivory caused significant increases in LOX after 24 hr of feeding, which continued throughout the sampling period ($P < 0.05$; Figure 1). Maximal increase occurred at 72 hr for LOX and for POX, but the POX increase was significant ($P < 0.05$) only

TABLE 3. EFFECT OF HERBIVORY ON FOLIAR LIPID PEROXIDATION, TOTAL HYDROPEROXIDES, AND HYDROXYL RADICAL (·OH) LEVELS IN SOYBEAN

Oxidant	Control	Wounded	LSD (0.05)
Malondialdehyde equivalents			
(μmol/g fresh weight)	1.35 ($\pm$0.07)	1.58 ($\pm$0.19)	0.17
Total hydroperoxides			
(nmol/g fresh weight)	71.9 ($\pm$47.1)	162.4 ($\pm$26.3)	50.6
Hydroxyl radical			
(nmol/g fresh weight)	1.75 ($\pm$0.90)	6.78 ($\pm$1.36)	2.45

[a] Numbers in parentheses are standard deviations.

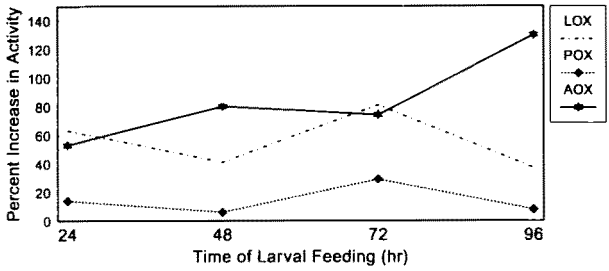

FIG. 1. Effect of length of herbivory on oxidative enzymes in soybean foliage. AOX = ascorbate oxidase; LOX = lipoxygenase measured at pH 5.5; and POX = peroxidase.

at 72 hr of herbivory. AOX was significantly increased throughout all time periods ($P < 0.05$) and was maximal after 96 hr of larval feeding.

Effect of Induced Responses on Oxidative Status of Insect Midgut. The oxidative shift in soybean plants caused by insect feeding was indicated by a 2.09-fold increase in LOX (pH 5.5), a 1.44-fold increase in LOX (pH 7.0), and a 1.42-fold increase in POX (Table 4). The ingestion of previously wounded plant tissue by larvae affected relative growth rate and the oxidative status of the midgut. The relative growth rate of larvae feeding on the wounded plants was reduced by more than 62% compared to larvae on control plants ($P < 0.001$; Table 4). Evidence that oxidative damage occurred in larvae ingesting wounded tissue was indicated by the fact that total ASC was decreased by ca. 15% ($P = 0.002$), reduced ASC was decreased by more than 20% ($P < 0.001$), and the oxidized form of ASC, DHA, was increased by 2.25-fold ($P = 0.005$). Accordingly, the ratio of midgut ASC to DHA decreased from 26.50 in larvae on control plants to 9.33 in larvae feeding on wounded plants. The levels of nonprotein thiols were significantly decreased in the wounded treatment by 48% ($P < 0.001$). Total hydroperoxides, an indicator of oxidative stress, were more than 66% higher in larvae feeding on the wounded plants compared to larvae on control plants ($P = 0.035$).

DISCUSSION

Plant chemical defense against insects is largely ascribed to plant secondary metabolites (Rosenthal and Berenbaum, 1991) and to a lesser extent primary metabolites such as amino acids, water, vitamins, etc. (Slansky, 1990). Our data indicate that the induced responses to herbivory in soybean entail a three-fold, integrated response (Figure 2) that includes: (1) enhanced secondary metabolite production (e.g., phenylalanine ammonia lyase; Liu et al., 1992;

TABLE 4. EFFECT OF HERBIVORY IN SOYBEAN ON FOLIAR OXIDATIVE ENZYMES, LARVAL GROWTH, AND OXIDATIVE STATUS OF INSECT MIDGUT EPITHELIUM

Chemical	Control	Wounded	LSD (0.05)
Plant enzymes			
LOX (pH 5.5)			
(nmol/min/g fresh weight)	189 (±31)[a]	395 (±79)	88
LOX (pH 7.0)			
(nmol/min/g fresh weight)	138 (±16)	199 (±35)	46
POX			
(Δ OD/min/g fresh weight)	29.6 (±3.9)	41.9 (±4.3)	6.6
Insect factors			
Relative growth rate			
(mg/da/mg larva)	0.204 (±0.07)	0.077 (±0.05)	0.02
Midgut total ASC			
(mg/g fresh weight)	1.10 (±0.08)	0.93 (±0.11)	0.09
Midgut reduced ASC			
(mg/g fresh weight)	1.06 (±0.09)	0.84 (±0.08)	0.08
Midgut DHA			
(mg/g fresh weight)	0.04 (±0.01)	0.09 (±0.03)	0.02
Midgut ASC:DHA	26.50	9.33	
Midgut nonprotein thiols			
(μmol/g fresh weight)	5.03 (±1.37)	2.61 (±1.04)	1.34
Midgut hydroperoxides			
(μmol/g fresh weight)	0.36 (±0.07)	0.60 (±0.17)	0.20

[a] Numbers in parentheses are standard deviations.

Table 1); (2) a loss in the quantity and/or quality of essential nutrients and antioxidants (e.g., protein, Bi et al., 1994; ascorbic acid, Table 2); and (3) the production of reactive oxygen species (e.g., hydroxyl radical, Table 3; hydrogen peroxide, superoxide radical). However, at present it is not known if these oxidative responses are the direct result of herbivory or the result of microbes entering open wounds caused by insect feeding. Nonetheless, we suggest that the net impact upon the herbivore involves both nutritional and oxidative stress (Figure 2).

The role of reactive oxygen species has been largely overlooked as a component of plant defense (Norris and Liu, 1992; Ahmad, 1992; Appel, 1993; Norris, 1994) for several reasons. First, there are no clearly defined pathologies or diseases associated with oxidative stress in insects as have been defined for mammals and humans (e.g., amytrophic lateral sclerosis, atherosclerosis, etc.; Felton, 1995). Second, because many consider oxidative stress to be a chronic phenomenon in animals, some scientists have assumed that insects are too short-

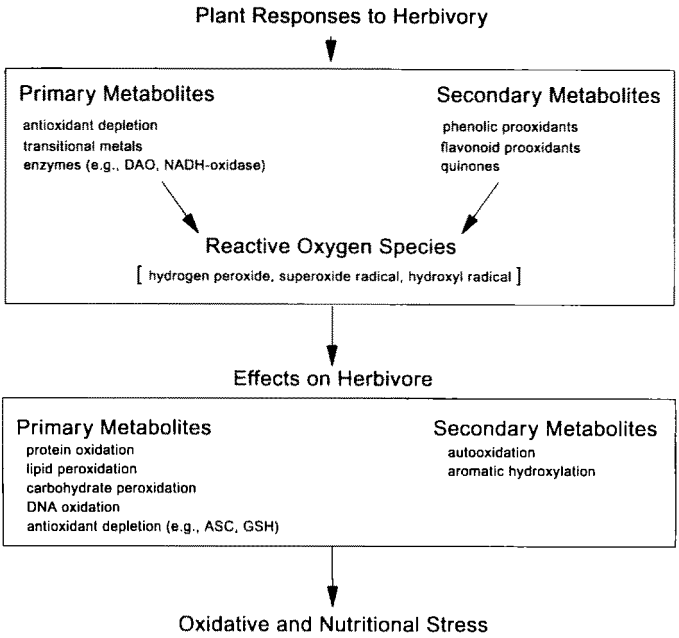

FIG. 2. Threefold response of soybean foliage to insect herbivory. Numerous interactions may take place among the metabolites produced in response to herbivory. For example, phenolic prooxidants may produce reactive oxygen species or may bind to proteins. Alternatively, phenolics may be reduced by primary metabolites (e.g., ascorbate, cysteine) or be oxidized by enzymes requiring reactive oxygen species such as hydrogen peroxide.

lived to be affected by oxidative stress. However, it has been recently shown that even short-lived insects such as *Drosophila melanogaster* can greatly benefit by additional antioxidant protection (Orr and Sohal, 1994). Third, the methodologies for measuring reactive oxygen species (e.g., ESR spectroscopy and spin traps) have been relatively difficult and expensive. Furthermore, these methodologies have been difficult or impossible to deploy for many *in situ* or *in planta* determinations. Fourth, because of the highly transitory behavior of reactive oxygen species, it is essentially impossible to use artificial diets to bioassay their effect directly. Thus, chemical fractionation of plant tissues to isolate and identify potential sources of chemical resistance may fail to account for not only the activities of plant enzymes (e.g., polyphenol oxidase; Felton et al., 1989; peroxidase, Liu et al., 1993), but also for potential contribution of reactive oxygen species. The problems in causally linking insect herbivore performance with oxidative stress are difficult but not insurmountable. Decades of intensive

research in the biomedical field have encountered similar difficulties, but the use of selective inhibitors of antioxidant systems and the ability to genetically modify the expression of antioxidant systems has contributed to rapid progress in recent years.

Several lines of evidence suggest that oxidative stress may be an important factor in insect biology and a potential mechanism of plant resistance to insects. First, insect life-span and fitness can be increased by elevated levels of chemical antioxidants, as well as the overexpression of antioxidant enzymes in transgenic insects (Orr and Sohal, 1994; Felton and Summers, 1995).

Second, selective modification of the antioxidant status of insects has demonstrated the detrimental effects of dietary prooxidants. For instance, specific inhibition of antioxidant enzymes (e.g., superoxide dismutase) decreases insect performance on certain host plants or artificial diets rich in prooxidants (Pritsos et al., 1991; Ahmad, 1992). Elevated levels of dietary chemical antioxidants such as vitamin E, carotenoids, and ascorbate decrease the toxicity of prooxidant compounds (Aucoin et al., 1990; Summers and Felton, 1994).

Third, insects feeding on previously wounded foliage show evidence of oxidative damage in the midgut (Table 4). The toxicological effect of the oxidative stress in the midgut is unknown, but disruptions in absorption, transport, and membrane-bound enzymes may be likely (Yu, 1994). The oxidative change in foliage may alter the nutritional quality of foliage to herbivores as well. The loss of nutrients such as ASC, free fatty acids (e.g., linoleic acid), and carotenoids that occur in damaged foliage may disrupt normal growth and development of insects (Dadd, 1973). Bi et al. (1994) also showed that prior herbivory reduced the nutritional quality of foliar protein to *H. zea*.

Fourth, herbivory causes increases in reactive oxygen species, namely, ·OH (Table 3) in soybean foliage. The ·OH radical is the most reactive known and can attack and damage virtually any molecule found in living cells (Halliwell, 1991). ·OH reacting with lipids, proteins, or DNA can initiate chain reactions that involve lipid peroxidation, protein oxidation and fragmentation, and substantial alteration of DNA (Halliwell, 1991). Several enzymatic systems may contribute to elevated ·OH levels. The ·OH may be formed from H_2O_2 during metal-catalyzed reactions (Halliwell, 1991). Increases in H_2O_2 may arise from several biochemical pathways in different cellular compartments. Our results (Tables 1 and 4) indicate that H_2O_2 may arise from several enzymes that produce reactive oxygen species as primary or secondary products including NADH oxidase, DAO, POX, and LOX (Kanofsky and Axelrod, 1986; Chamulitrat et al., 1991; Sutherland, 1991; Vianello and Macri, 1991; Liu et al., 1993). Alternatively, H_2O_2 accumulation could result from the decreased levels of several scavenging agents such as CAT, ASC, or thiols (Tables 1, 2, and 4). However, the H_2O_2-scavenging enzyme APX increased following herbivory (Table 1). We have not differentiated the chloroplastic vs. cytosolic APX isozymes, and thus

do not know the relative importance of differences in H_2O_2 synthesis and degradation between different cellular compartments. Herbivory caused a decline in CAT levels, which have also been implicated in plant–pathogen interactions (Chen et al., 1993). The inhibition of CAT was due to induction of salicylic acid, a potent CAT inhibitor (Chen et al., 1993). Presumably levels of H_2O_2 increase following CAT inhibition and then act as an elicitor of plant defenses (Chen et al., 1993). It is unknown if the decrease in CAT following herbivory is due to enhanced salicylic acid biosynthesis.

Fifth, increases in oxidative enzymes and other oxidative changes in soybean may serve in several manners to enhance insect resistance (Liu et al., 1993; Felton et al., 1994a,b). Increased production of H_2O_2 by DAO (Federico and Angelini, 1986, 1988; Angelini and Federico, 1989; Angelini et al., 1990) may provide substrate for POXs. NADH oxidase occurs in the plasma membrane of plant cells and oxidizes NADH or NADPH to form O_2^- that then dismutates to H_2O_2 (Doke et al., 1991; Brightman et al., 1991). POXs are important in cell wall strengthening, including lignification (Goldberg et al., 1985; Lagrimini et al., 1987), suberization (Espelie and Kolattukudy, 1985; Espelie et al., 1986), and cross-linking of hydroxyproline-rich cell wall protein (Fry, 1986). These cross-linking processes may ultimately decrease the digestibility of plant tissues for herbivores. PAL provides phenolic precursors for these reactions (Goldberg et al., 1985). Moreover, oxidative products of enzymes (e.g., phenolic prooxidants, quinones, lipid hydroperoxides, aldehydes) can impose considerable toxicological assault upon herbivores (Duffey and Felton, 1991; Appel, 1993; Summers and Felton, 1994; Barbehenn and Martin, 1994). Increases in PAL activity (Table 1) may lead to increased levels of phenolic prooxidants such as caffeic acids that are toxic to larvae (Summers and Felton, 1994). The volatile aldehyde products of LOX are repellent to certain insect herbivores including bean bugs *Riptortus clavatus*, strawberry leafy beetles *Galerucella vittaticollis*, and false melon beetles *Atrachya menetriesi* (Mohri et al., 1990). Moreover, the induction of LOXs may be important in defense signal transduction because LOXs are involved in the initial phases of jasmonic acid biosynthesis (Ueda and Kato, 1980; Vick and Zimmerman, 1987; Enyedi et al., 1992). The jasmonates induce the synthesis of several plant defensive proteins or chemicals, including proteinase inhibitors, chalcone synthase, proline-rich cell wall proteins, PAL, and alkaloids (Creelman et al., 1992; Enyedi et al., 1992; Hamberg and Gardner, 1992).

Oxidative responses in plants may have both positive and negative effects upon the host plant: a decrease in herbivory but an increase in oxidative damage to the plant. Oxidative stress is an important factor in plant responses to several environmental stresses including chilling temperatures (Prasad et al., 1994; Gupta et al., 1993), phytopathogens (Devlin and Gustine, 1992; Aver'yanov et al., 1993), NaCl injury (Gossett et al., 1994), and environmental pollutants (Badiani

et al., 1993; Guzy and Heath, 1993). Our data provide more substantive evidence that plant responses to herbivory also include changes in the oxidative status of foliage. Consequently, herbivory may predispose the plant to damage by other environmental stresses that impose oxidative stress to the plant. Conversely, the oxidative changes may be a component of plant defense against herbivores and other pests. The salient benefit to the plant, in terms of insect resistance, may represent the relative balance between these opposing effects.

In summary, we propose that at least five potential functions for oxidative stress in plant–herbivore interactions exist including: (1) direct oxidative injury to the herbivore (Appel, 1993; Felton et al., 1994b; Summers and Felton, 1994); (2) indirect injury to the herbivore through oxidative damage to dietary nutrients and antioxidants (Felton et al., 1989, 1992; Bi et al., 1994; Barbehenn and Martin, 1994); (3) obstruction of access to plant protein and nutrients via rapidly induced oxidative cross-linkage of cell wall proteins (Bradley et al., 1992); (4) formation of volatile compounds that are repellent to herbivores (Mohri, 1990); and (5) signal transduction for elicitation of plant defenses (Apostol et al., 1989; Norris and Liu, 1992; Creelman et al., 1992; Farmer et al., 1992; Chen et al., 1993; Norris, 1994). Thus, induced resistance in certain soybean cultivars may be composed of a threefold, integrated response involving primary metabolites, secondary metabolites, and reactive oxygen species (Figure 2). Further research is needed to determine if these results are generalizable to other plant cultivars and species.

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A FLAVONOID GLUCOSIDE, PHELLAMURIN, REGULATES DIFFERENTIAL OVIPOSITION ON A RUTACEOUS PLANT, *Phellodendron amurense*, BY TWO SYMPATRIC SWALLOWTAIL BUTTERFLIES, *Papilio protenor* AND *P. xuthus*: THE FRONT LINE OF A COEVOLUTIONARY ARMS RACE?

KEIICHI HONDA* and NANAO HAYASHI

Study of Environmental Sciences
Faculty of Integrated Arts and Sciences
Hiroshima University, Higashihiroshima 739, Japan

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Abstract—We studied the chemical basis for the differential acceptance of a rutaceous plant, *Phellodendron amurense*, by ovipositing females of two sympatric *Papilio* butterflies, *P. protenor* and *P. xuthus*, that have a very similar host range. *P. amurense*, on which larvae of both species perform well, was rejected by *P. protenor* females but was marginally accepted by *P. xuthus*, even though water-soluble fraction of this plant elicited significant ovipositional responses from the two species. A prenylated dihydroflavonol glucoside, 3,5,7,4′-tetrahydroxy-8-(3-methylbut-2-enyl)flavanone-7-*O*-β-glucoside (phellamurin), was identified as a dominant active substance that deters oviposition. *P. protenor* responded highly sensitively to phellamurin, whereas *P. xuthus* was less susceptible and was affected conspicuously only at higher concentrations than that found in living plants, thereby accounting for their differential responses to *P. amurense*. In *P. protenor*, the deterrent effect of phellamurin was considered to outweigh the activity of coexisting stimulant(s) at the concentrations actually present in the foliage, resulting in avoidance of this plant. The phellamurin content in the foliage amounted to as much as 1.8% of the fresh weight, which is far higher than the average levels of flavonoids present in other major host plants of *Papilio*. The dose-response experiments demonstrated that unacceptability of *P. amurense* for *P. protenor* was attributable largely to such a high concentration of phellamurin. These results appear to provide information on possible coevolutionary interactions between herbivores and plants and also on the potential for shifts in host affiliation.

*To whom correspondence should be addressed.

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Key Words—Oviposition deterrent, *Phellodendron amurense*, Rutaceae, phellamurin, dihydroflavonol glucoside, papilionid butterfly, *Papilio protenor*, *Papilio xuthus*.

INTRODUCTION

Two Rutaceae-feeding oligophagous papilionid butterflies, *Papilio protenor* and *P. xuthus*, occur sympatrically in most localities in Japan and are pests of various cultivated *Citrus* plants, their major host plants in nature. Occasionally, and in certain cases frequently, they utilize some rutaceous plants in other genera, e.g., *Zanthoxylum*, *Poncirus*, *Fortunella*, in the field. Several rutaceous mountainous plants, *Orixa japonica*, *Fagara ailanthoides*, *Skimmia japonica*, and *Phellodendron amurense*, are also potential hosts, but although the larvae sometimes feed on *F. ailanthoides* and *S. japonica*, *O. japonica* is not accepted. Thus the host range of *P. protenor* and *P. xuthus* is almost overlapping. The acceptability of *P. amurense* for ovipositing females differs, however, between the two species. *P. protenor* females never lay eggs on *P. amurense* in nature nor under experimental conditions, whereas the plant is barely acceptable for *P. xuthus* females. However, the plant can be found infested by *P. xuthus* in the field. Previous work (Honda and Hayashi, 1995) has demonstrated that despite the presence of oviposition stimulant(s) in *P. amurense*, coexisting oviposition deterrent(s) severely inhibit *P. protenor* from ovipositing.

It is widely accepted that host utilization of herbivorous insects is mediated to a large extent by plant secondary metabolites serving as stimulants or deterrents (e.g., Dethier, 1982; Miller and Strickler, 1984). Studies on the chemical cues implicated for host recognition by papilionid butterflies have exclusively concentrated on oviposition stimulants, which are perceived by foretarsal chemoreceptors upon contact with leaf surface (Ohsugi et al., 1985, 1991; Honda, 1986, 1990; Nishida et al., 1987; Nishida and Fukami, 1989; Feeny et al., 1988; Papaj et al., 1992; Sachdev-Gupta et al., 1993a; Nishida, 1994) or by antennal chemoreceptors (Feeny et al., 1989; Baur et al., 1993). However, information on phytochemicals responsible for deterring oviposition by papilionids is quite insufficient. To our knowledge only one publication (Nishida et al., 1990) has dealt with the identification of an oviposition deterrent. A flavonol glycoside (a xylosyl derivative of rutin) was isolated from *O. japonica* and claimed to be one of the deterrents of *P. xuthus*. Thus, it is essential to study further the behaviorally or physiologically active substances involved in plant–herbivore associations in order to clarify the evolutionary processes of host specificity.

This paper addresses the isolation and identification of a flavonoid oviposition deterrent involved with the differential acceptance of *P. amurense* by the two *Papilio* butterflies.

METHODS AND MATERIALS

Insects. Adults of *P. protenor* and *P. xuthus* used for the behavioral bioassays were 3- to 10-day-old gravid females derived from laboratory stock cultures. These originated from a natural population principally infesting cultivated *Citrus* plants in Kanagawa Prefecture. The larvae were reared at 25°C under a 16-hr light–8-hr dark regime on fresh leaves of *Citrus unshiu*. Females were fed daily with 15% aqueous sucrose solution.

Extraction and Fractionation of Plant Materials. Young foliage of *P. amurense* (820 g) collected in early summer in Kanagawa Prefecture was extracted with methanol (3 liters) at room temperature for one month. The methanolic extract was 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 fraction). The chloroform and isobutanol fractions were evaporated to dryness, and each residue was dissolved in acetone and in methanol, respectively. On the other hand, the water-soluble fraction, once lyophilized, was stored in 50% aqueous methanol.

Bioassay for Ovipositional Response. To evaluate stimulatory or deterrent activity of test samples, behavioral bioassays were carried out by a method given in a previous paper (Honda, 1986) employing artificial green plastic leaves as ovipositional substrates. Before testing, females were screened daily to assess their responsiveness, and only those that showed positive responses to *Citrus* foliage and negative responses to water alone (control) were chosen. The rate of response was defined as the mean response rate of the individual females, represented by the equation 1:

$$\text{Rate of response} = \frac{\Sigma R_i}{N} \tag{1}$$

$$R_i = \frac{\Sigma S_j}{n} \tag{2}$$

where N is the total number of females tested, and R_i denotes the response rate of an individual, which in turn was calculated by expression (2). S_j and n are the scores for a single trial of a given female and the number of trials, respectively. Trials were replicated five to seven times. S_j was scored as 1.0 for actual egg-laying or an equivalent behavior, 0.5 for half-curling the abdomen with continuous drumming, and 0.0 for drumming only with no positive response. For all trials, merely alighting on ovipositional substrates without drumming was not included.

Instrumentation for Structural Elucidation. ^{13}C NMR (CMR) spectra were measured at 100 MHz in acetone-d_6 on a Jeol JNM-A400 FT-NMR spectrometer

with tetramethylsilane as the internal standard. Chemical shifts were represented by δ unit. Multiplicities of signals determined by DEPT are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q).

FAB-mass spectra (FAB-MS) were recorded with a Jeol JMS-DX 303 mass spectrometer using a mixture of NaI and glycerine as a matrix.

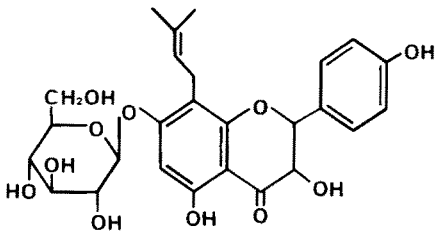
RESULTS

Previous work (Honda and Hayashi, 1995) and preliminary experiments revealed that the aqueous fraction derived from a methanolic extract of *P. amurense* strongly evoked positive responses from ovipositing females of both *P. protenor* and *P. xuthus*, suggesting the presence of oviposition stimulant(s), and hence, the potential acceptability of this plant for both species. By contrast, the isobutanol fraction, in which the key substance(s) regulating differential oviposition was found to be localized, exerted conspicuous deterrent activity that is potent for *P. protenor* and moderate for *P. xuthus* (Honda and Hayashi, 1995). Thus, the isobutanol fraction was chemically examined in this study.

The isobutanol solution, when evaporated to dryness, gave pale brown crystals, which, after recrystallization from acetone–water, afforded pale yellow needles (compound **1**). Since compound **1** was found to singly suppress the stimulatory activity of the aqueous fraction, structural elucidation of compound **1** was undertaken. Compound **1** (mp: 199–201°C) was presumed to be a flavonoid because its aqueous methanolic solution gave a reddish purple coloration when treated with Mg-HCl. Spectral evidence of FAB-MS: $[M+Na]^+$ at *m/z* 541, combined with elemental analysis: found, C, 60.26; H, 5.80, definitely determined the molecular formula of the compound to be $C_{26}H_{30}O_{11}$ (calcd., C, 60.23; H, 5.83). In addition, the CMR spectrum, δ(ppm): 17.9(q), 22.3(t), 25.9(q), 62.5(t), 71.2(d), 73.4(d), 74.6(d), 77.9(d), 78.0(d), 84.3(d), 96.5(d), 101.5(d), 102.8(s), 110.5(s), 115.9(d, 2C), 123.6(d), 129.2(s), 130.2(d, 2C), 131.4(s), 158.8(s), 160.1(s), 162.7(s), 164.7(s) and 199.4(s), shows the presence of a glucosyl and a prenyl moiety and also the lack of methoxyl groups in the molecule. From all these data, compound **1** was concluded to be a C-prenyl derivative of 5,7,4′-trihydroxydihydroflavonol. Another CMR measurement without proton decoupling was attempted to determine the position (C-6 or C-8) of the prenyl substituent by observing long-range ^{13}C–^{1}H coupling between C-6 (C-8) and a hydroxyl proton attached to C-5 (C-5-O**H**). The resonance at 110.5 ppm, assigned to a prenylated carbon, showed a simple singlet, while the signal of unsubstituted carbon at 96.5 ppm was a double doublet (J = 164.2, 7.7 Hz) coupled both with a directly attached proton and with C-5-O**H**, implying that C-6 is not substituted and that the prenyl group, therefore, is attached to C-8 (Shirataki et al., 1985). This was further corroborated by the fact that the

multiplicity of C-6 resonance apparently changed to doublet by the addition of D_2O, although the line width was somewhat broadened. This phenomenon can reasonably be explained by the small value of J_{CD} that is theoretically known to be 1/6.51 of that of J_{CH}. Consequently, compound **1** was concluded to be 3,5,7,4′-tetrahydroxy-8-(3-methylbut-2-enyl)flavanone-7-*O*-glucoside, which is identical with phellamurin (Figure 1), reported by Sakai and Hasegawa (1974). The stereochemistry of the anomeric carbon of the glucosyl group, appearing at 101.6 ppm, was concluded to be in the β-configuration, following chemical shift values of β-glucosylated flavonoids (Harborne, 1982).

The deterrent effect of phellamurin on oviposition by the two *Papilio* species was investigated in combination with the aqueous fraction that induces positive ovipositional response. The results are shown in Table 1. Phellamurin exerted remarkable deterrence in oviposition by *P. protenor* at concentrations higher than 0.1%, and at doses over 0.25% oviposition was completely inhibited. In contrast, *P. xuthus* was less influenced by this compound, and considerable reduction of positive response occurred only at 0.5% (and at higher doses, probably). Such differences in responsiveness appear to reflect clearly the acceptability of *P. amurense* for each species. In fact, the response rates of *P. protenor* and *P. xuthus* to 0.3% phellamurin sample are congruent with those to the foliage of *P. amurense* (0 and 0.73, respectively) (Honda and Hayashi, 1995). Upon composition analysis, water-soluble substances contained in the foliage of the plant amounted to ca. 3.7% of the fresh weight, and the relative quantities of isobutanol-soluble substances and phellamurin were approximately 2.0% and 1.8%, respectively. Since the quantity of phellamurin in the foliage was estimated at about one half that of the hydrosoluble substances, a mixed solution composed of 0.25% phellamurin and 0.5% aqueous fraction is regarded as equivalent to an extract from the foliage. Accordingly, avoidance of *P. amurense*



PHELLAMURIN

FIG. 1. Oviposition deterrent to *Papilio protenor* and *P. xuthus* identified from *Phellodendron amurense*.

TABLE 1. EFFECT OF PHELLAMURIN ON OVIPOSITIONAL RESPONSES OF *Papilio protenor* AND *P. xuthus* TO AQUEOUS FRACTION[a] OF *Phellodendron amurense*

Species	Responses to phellamurin added (%)					
	0	0.1	0.2	0.25[b]	0.3	0.5
P. protenor[c]	0.96[d]	0.50	0.10	0	0	0
P. xuthus[e]	1.00[d]	1.00	1.00	0.89	0.78	0.28

[a]Concentration: 0.5%.
[b]The proportion of phellamurin to water-soluble substances in this sample approximates to that in living plants.
[c]$N = 20$.
[d]Response to the aqueous fraction only.
[e]$N = 18$.

by *P. protenor* seems to be well explained solely by the quantitative balance of phellamurin and stimulant(s) in the plant. In *P. xuthus* as well, phellamurin is likely to play a dominant role in assessing the suitability of the plant by females, although there still remains the possibility of involvement of other deterrents. In addition, females of *P. protenor* from a Hiroshima (ca. 650 km distant from Kanagawa Prefecture as the crow flies) population also rejected *P. amurense*, while *P. xuthus* females inhabiting the same locality accepted it. Therefore, the geographical differences in their responsiveness to the plant seem to be negligible, at least in Japan.

DISCUSSION

Quantitative relationships between the oviposition deterrent (phellamurin) and stimulant(s) (water-soluble substance) present in *P. amurense* make it possible to account for rejection of this plant by *P. protenor*, in which positive activity of co-occurring stimulant(s) is entirely masked by potent deterring action of phellamurin. On the other hand, *P. xuthus*, which is less susceptible to this compound, is permitted to accept this plant marginally. In other words, *P. xuthus* is narrowly adapted for *P. amurense*, whereas *P. protenor* is not at all at present. Our previous work (Honda and Hayashi, 1995) has revealed that the total content of flavonoids in *P. amurense* (ca. 2%) is approximately two to four times as high as those present in *C. unshiu*, *F. ailanthoides*, and *O. japonica*, which are the major host plants of *Papilio* butterflies in Japan, and that phellamurin comprises 90% of the total flavonoids. Such an extraordinary level of a single compound suggests the possibility that phellamurin might function as a defensive

chemical of the plant, which has possibly been elaborated through a coevolutionary arms race with herbivores. The present results permit the conclusion that if phellamurin content in *P. amurense* were less than ca. 0.7% (an intermediate value among flavonoid levels of other major host plants), even *P. protenor* might be able to colonize this plant. Furthermore, *P. xuthus* as well as *P. protenor* might be expelled from the plant if the phellamurin content were to exceed ca. 3.6%, unless they overcome the chemical constraint. Larval preadaptation for *P. amurense* (Honda and Hayashi, 1995) and the occurrence of oviposition-stimulatory substance(s) and profuse accumulation of phellamurin in this plant, when taken altogether, suggest the feasibility of ancient or future colonization on this plant by *P. protenor*. These facts, therefore, also seem to provide some information on possible coevolutionary interaction, whether pairwise or diffuse, between plants and herbivores and also on host shifts.

In Pieridae, certain cardiac glycosides from *Erysimum cheiranthoides*, a plant accepted by *Pieris napi* but not by *P. rapae*, have been shown to exert deterrent effects in varying degrees on egg-laying by both species (Sachdev-Gupta et al., 1990; Huang et al., 1993). Not only females but also larvae of *P. rapae* refuse the plant. Cardenolides have been reported to be responsible for deterring oviposition and larval feeding (Sachdev-Gupta et al., 1990, 1993b). However, in *P. protenor* and *P. xuthus*, females' adaptability for *P. amurense* is not correlated with larval fitness for the plant. The dissection of larval performance and oviposition preference has also been documented for other *Papilio* species (e.g., Wiklund, 1975; Thompson et al., 1990; Thompson and Pellmyr, 1991). Such differential adaptation of "mother and child" to a given plant would be a powerful driving force to facilitate shift to a novel host.

For all three *Papilio* butterflies (*P. xuthus*, *P. protenor*, and *P. polyxenes*) for which oviposition stimulants have so far been elucidated, flavonoids or their derivatives are known as common (distinct compounds being involved) active substances implicated for stimulating oviposition (Ohsugi et al., 1985; Honda, 1986; Feeny et al., 1988). In contrast, the present work and a previous report (Nishida et al., 1990) have substantiated that flavonoids do not always serve as stimulants, but act as deterrents in some cases. How can we provide a reasonable and convincing explanation for the dual bioactivities of flavonoids to *Papilio* species? Flavonoids, which are ubiquitous in the plant kingdom and are generally postulated to be defensive substances produced by plants, are highly likely to have originally displayed a deterrent effect on egg-laying by *Papilio* butterflies. Behavioral adaptation to and speciation onto a particular plant would have given rise to the change of sensorial responsiveness to specific phytochemicals (e.g., flavonoids), hence, the eventual conversion of the unacceptable into the acceptable. *P. xuthus* appears to be overcoming the chemical barrier of phellamurin, which is considerably different from *Citrus* flavonoids in chemical structure.

It is intriguing that *P. amurense* is a primary host of *Papilio maackii* and is also utilized frequently by *P. bianor* as a secondary host (Endo and Nihira, 1990). In this context, our findings are helpful to understanding the chemical basis for host selection and host shifts in *Papilio* species. Further investigation is being undertaken on the action of phellamurin on ovipositing females of other *Papilio* species that are successfully adapted for *P. amurense*.

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A NEOLIGNOID FEEDING DETERRENT AGAINST *Luehdorfia puziloi* LARVAE (LEPIDOPTERA: PAPILIONIDAE) FROM *Heterotropa aspera*, A HOST PLANT OF SIBLING SPECIES, *L. japonica*

KEIICHI HONDA,* TAKASHI SAITOH, SHIGEKI HARA and NANAO HAYASHI

Study of Environmental Sciences
Faculty of Integrated Arts and Sciences
Hiroshima University, Higashihiroshima 739, Japan

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Abstract—A feeding deterrent against larvae of a papilionid butterfly, *Luehdorfia puziloi* (Parnassiinae), a specialist on *Asiasarum* plants (Aristolochiaceae), was isolated from another aristolochiaceous plant, *Heterotropa aspera*. Antifeedant activity was exhibited by the *n*-hexane-soluble fraction that proved to contain at least two active components. One of the deterrents was identified as a neolignan compound, asatone. The concentration of asatone in *H. aspera* was estimated at approximately 225 ppm, and larval feeding of *L. puziloi* was significantly deterred at concentrations over 90 ppm. By contrast, asatone was not detectable (< 1 ppm, if any) in its host plant, *Asiasarum sieboldii*.

Key Words—Feeding deterrent, *Luehdorfia puziloi*, *L. japonica*, Papilionidae, Parnassiinae, Zerynthiini, neolignoid, asatone, *Heterotropa aspera*, *Asiasarum sieboldii*, Aristolochiaceae.

INTRODUCTION

Two papilionid butterflies, *Luehdorfia puziloi* and *L. japonica* (subfamily Parnassiinae, tribe Zerynthiini), share a unique univoltine life history (Hidaka et al., 1971; Tabuchi, 1974) and are regarded as sibling species. In Japan *L. puziloi* inhabits the central to northern districts, while *L. japonica* occurs in the central to southwestern districts of the main island (*Honshû*). Their geographical distribution is distinctly partitioned off along the boundary known as the "Luehdorfia Line," although several patches of common habitat spread around this

*To whom correspondence should be addressed.

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line (Tabuchi, 1974). Another distinctive characteristic between the two species is manifested by the difference in host plants. *L. japonica* feeds on a large variety of *Heterotropa* plants (Aristolochiaceae, tribe Sarumeae) in the field and is also able to perform well on other aristolochiaceous plants such as *Asiasarum* or *Asarum* (Sarumeae). In contrast, *L. puziloi* is restricted to *Asiasarum* plants. It never deposits eggs on and usually fails to complete larval development on most plants of other genera even under experimental conditions.

Numerous investigations of the mechanism of host selection by herbivorous insects indicate that phytochemical information perceived by contact with plants ultimately serves as a primary determinant of host selection and that a qualitative and quantitative balance of stimulants and deterrents finally governs acceptance or rejection of plants by herbivores (e.g., Dethier, 1982; Miller and Strickler, 1984; Renwick and Chew, 1994).

In Papilionidae, an array of oviposition stimulants (Ohsugi et al., 1985, 1991; Honda, 1986, 1990; Nishida et al., 1987; Nishida and Fukami, 1989; Feeny et al., 1988; Papaj et al., 1992; Sachdev-Gupta et al., 1993a; Baur et al., 1993; Nishida, 1994) and oviposition deterrents (Nishida et al., 1990; Honda and Hayashi, 1995b) has come to light during the last decade. Chemical bases for differential acceptance of some rutaceous plants by ovipositing *Papilio* females have also been examined (Honda and Hayashi, 1995a). However, the chemistry of larval discrimination between host and nonhost plants is little known.

We report here on the isolation and identification of an antifeedant to *L. puziloi* larvae from *H. aspera*, which is one of the host plants accepted by western populations of *L. japonica*.

METHODS AND MATERIALS

Insects. Eggs of *L. puziloi inexpecta* (a subspecies inhabiting *Honshû*), usually deposited in a cluster on the underside of the foliage of *A. sieboldii*, were collected in Nagano Prefecture or obtained from females of stock cultures maintained in our laboratory. Larvae were reared on fresh leaves of *A. sieboldii* at 17°C under a 16-hr light–8-hr dark regime.

Extraction and Fractionation of Bioactive Substances. Young foliage of *H. aspera* (650 g) collected in the spring in Osaka and Hiroshima Prefectures was extracted with *n*-hexane (2 liters) at room temperature for a month. The *n*-hexane extract was concentrated in vacuo below 50°C. An aliquot (100 mg) of the residue was subjected to flash column chromatography (FCC) with silica gel (Whatman, LPS-1, 13-24 μm), and eluted stepwise with *n*-hexane (50 ml, fraction 1), benzene (80 ml, fraction 2), chloroform (200 ml, fraction 3), methanol (90 ml, fraction 4), and methanol (250 ml, fraction 5). Subsequent fractionation of an active fraction (114 mg) was carried out by reversed-phase FCC on ODS-

silica gel (Whatman, LRP-2, 37–53 μm), using 95% aq. methanol (120 ml, fraction 41), methanol (220 ml, fraction 42), and acetone (210 ml, fraction 43), successively. Isolation and purification of active substances were achieved by preparative TLC [2-mm-thick silica gel plate (Merck Art. 5745); chloroform–acetone (25 : 1) or chloroform] and finally by HPLC. Fresh leaves of *A. sieboldii* collected in Nagano Prefecture were also extracted with *n*-hexane, and a sample for chemical analysis was prepared by an analogous chromatographic procedure. Concentrations of test samples were expressed as weight/volume percent.

Bioassay for Feeding Deterrence. Deterrent activities of test samples were evaluated by binary-choice assays with young leaves of *A. sieboldii.* As the foliage of this plant is very thin and can be severely damaged by physical treatment, intact foliage was used without cutting. A pair of leaves of similar size (ca. 20 ± 10cm^2) were laid at the bottom of a cylindrical assay chamber (19 cm diameter × 7 cm high) that was lined with wet paper towels. One leaf was soaked in a given concentration of a test sample in dichloromethane for a split second and air-dried. The other leaf (control) was similarly treated with dichloromethane alone. Since the two leaves were often not of the same size, the larger one was designated as the control. Twenty fourth- or fifth-instar larvae, which had been starved beforehand for 6 hr, were placed near or on the sample-treated leaf and were allowed to feed freely. After the control leaf had been thoroughly consumed (usually 30 min later), the remaining area of the treated leaf was measured. Feeding deterrence was indexed (DI) by the following formula: $DI = A_R/A_0 \times 100$ (%), where A_R represents the remaining area of treated leaf, and A_0, its original area. Bioassays were replicated four times for each sample.

Instrumentation for Chemical Analyses and Structural Elucidation. HPLC analyses were carried out using a Jasco 880-PU liquid chromatograph equipped with a ODS column (Merck, LiChrospher 100 RP-18, 5 μm). Samples were eluted with acetonitrile and water (70 : 30) at a flow rate of 1 ml/min. The column eluate was monitored at 275 nm.

^{13}C NMR (CMR) spectra were measured at 100 MHz in $CDCl_3$ on a Jeol JNM-A400 FT-NMR spectrometer with tetramethylsilane as the internal standard. Chemical shifts were given as δ units. Multiplicities of signals determined by DEPT are abbreviated as follows: singlet (s), doublet (d), triplet (t), and quartet (q).

EI-mass spectra (70 eV) were recorded with a Jeol JMS-DX 303 mass spectrometer.

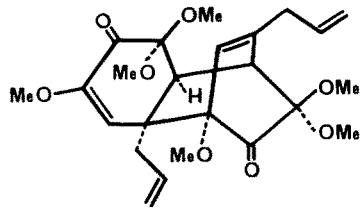
RESULTS

Larvae of *L. puziloi*, when given a choice of the foliage of *A. sieboldii* and *H. aspera*, fed on the former only and were reluctant to ingest the latter even when

starved. This behavior was strongly suggestive of the presence of some feeding deterrent(s) in *H. aspera*. In preliminary experiments, a methanolic extract of *H. aspera*, that was found to exhibit potent deterrent activity when applied to the foliage of *A. sieboldii*, was evaporated and partitioned with chloroform–water. Deterrence was obtained only with the chloroform fraction, which, after removal of the solvent, was extracted with *n*-hexane. Since apolar substances soluble in *n*-hexane exerted conspicuous deterrent activity, *n*-hexane was used thereafter instead of methanol to extract active substances from living plants.

n-Hexane extract (100 mg) of *H. aspera* was subjected to normal-phase FCC with silica gel, yielding fractions 1–5. Bioassays with these fractions at a concentration of 0.1% revealed that only fraction 4 (23 mg) was active with a DI of 69. Thus, fraction 4 (114 mg) was further fractionated on ODS-silica gel into three fractions (fractions 41–43). Among these, fraction 41 (80 mg) exhibited deterrent activity as high as DI 86 at 0.1%. This fraction, when analyzed by TLC with silica gel and developing with chloroform–acetone (25 : 1), gave five spots: A (R_f = 0.80), B (R_f = 0.69), C (R_f = 0.34), D (R_f = 0.24), E (R_f = 0.18). Since fraction B appeared to be still contaminated with minor components, it was further separated by TLC with chloroform to afford a major (B-II, R_f = 0.18) and two minor (B-I, R_f = 0.38; and B-III, R_f = 0.11) components. Individual fractions, obtained by preparative TLC in a similar manner, were assayed (0.1%) for feeding deterrence except for B-I, which was presumed to be an artifact. Comparison of DIs (0.1%) of A (21), C (11), D (34), E (61), B-II (70), and B-III (6) clearly indicated that the principal compounds responsible for feeding deterrence reside in fractions E and B-II. However, fraction E, was not pursued further in this study, because it was found to be accompanied by a large quantity of ubiquitous aliphatic acids. Final purification of fraction B-II was achieved by reversed-phase HPLC. A peak appearing at R_t = 8.2 min was collected to yield compound **1**.

Compound **1** (mp 100–102°C), upon MS measurement, gave M^+ at *m/z* 448 (rel. int., 1%) and diagnostic fragment ions at *m/z* 416(13), 384(16), 343(17), 224(67), 193(27), 181(37), 165(37), 105(100). The CMR spectrum of **1**, δ(ppm): 39.0(t), 41.3(t), 43.9(d), 44.5(d), 49.1(q), 49.8(s), 50.1(q), 50.2(q), 50.4(q), 54.8(q), 55.6(q), 92.1(s), 93.3(s), 98.7(s), 116.7(d), 116.9(t), 118.2(t), 121.8(d), 133.5(d), 135.3(d), 145.0(s), 150.6(s), 188.7(s) and 201.4(s), suggested the presence of at least 24 carbon atoms. It was evident that this compound has two carbonyl and six methoxyl moieties in its molecule. Moreover, resonances observed from 116.7 ppm to 150.6 ppm were suggestive of eight olefinic carbons, two of which are assignable to terminal methylenes. This spectral information suggested a possible molecular formula of **1** as $C_{24}H_{32}O_8$ and fits with the chemical structure of a neolignoid named asatone (Yamamura and Terada, 1975). Comparison of the spectral data with those of an authentic sample isolated from *Asarum asperum* (=*H. aspera*) (Hayashi et al., 1976) corroborated the identity of compound **1** as asatone (Figure 1).



ASATONE

FIG. 1. Chemical structure of a feeding deterrent against *Luehdorfia puziloi* larvae identified from *Heterotropa aspera*.

A dose–response relationship of antifeedant activity of asatone against *L. puziloi* larvae was examined by the same method of bioassay. Fresh leaves of *A. sieboldii* were impregnated with known concentrations of asatone in dichloromethane and air-dried. The amount of asatone adhering to the foliage, expressed as parts per million to the fresh weight of the foliage, was estimated on the basis of the volume of the solution applied, and the average of 10 replicates was obtained. The results are shown in Figure 2. DIs at 36, 90, 180, and 360 ppm were 4.3, 56, 73, and 91, respectively. Thus, larval feeding was significantly deterred at a concentration of 90 ppm or higher.

Quantitative determination of asatone in *H. aspera* and *A. sieboldii* by HPLC analyses revealed that the average content of asatone in *H. aspera* amounted to 225 ppm of the fresh weight, whereas asatone was not detected from *A. sieboldii* (detection limit, 1 ppm).

DISCUSSION

L. puziloi is a specialist, relying only on *Asiasarum* plants for larval growth. The fact that the two sibling species, *L. puziloi* and *L. japonica*, utilize distinct plant genera even in common habitats has long been the center of interest for entomologists and amateurs from the viewpoint of phylogeny or cladogenesis. On the one hand, many publications have dealt with essential oil constituents of *Heterotropa* and related genera in terms of chemotaxonomy (e.g., Endo and Nagasawa, 1974; Hayashi et al., 1984, 1990). In this context, earlier work has reported on feeding attractants for *L. japonica* larvae, in which three phenylpropanoid components, safrole, methyleugenol, and elemicin, were listed as active compounds (Takaishi et al., 1969). Very recently, a flavonoid oviposition stimulant for *L. japonica* was identified from *H. aspera* (Nishida, 1994).

In *L. puziloi*, however, nothing is known to date about the phytochemicals mediating differential acceptance of actual and potential host plants. A neolignoid, asatone, which proved to be a potent antifeedant to *L. puziloi* larvae,

seems to be specific to some, but not all, species of *Heterotropa* (Yamamura et al., 1976; Hayashi et al., 1976). This compound is, therefore, highly likely to be one of the key substances that regulate host selection by *L. puziloi*. Several neolignans have been shown to have growth-inhibitory or toxic action on some insects (e.g., Nitao et al., 1991; Gonzalez-Coloma et al., 1994).

Differential responses of papilionid larvae to plant metabolites have also been demonstrated in studies on *glaucus* members of *Papilio* butterflies. Sesquiterpene lactones in *Liriodendron tulipifera* are suspected to be responsible for larval avoidance or toxicity of *P. canadensis* (formerly *P. glaucus canadensis*) (Lindroth et al., 1986), and phenolic glycosides, such as tremulacin in *Populus tremuloides*, are toxic to larvae of *P. glaucus* (formerly *P. glaucus glaucus*) (Scriber et al., 1989). A few neolignan compounds (magnolol and/or a certain allyl-substituted biphenyl ether) contained in *Magnolia virginiana* were found to be toxic to Lauraceae-feeding *P. palamedes* and *P. troilus* larvae and significantly reduced the survival of their neonates (Nitao et al., 1992). In addition, recent investigations on a pierid butterfly, *Pieris rapae*, have documented that larval feeding on *Erysimum cheiranthoides* or *Iberis amara* was notably deterred by cardenolides or cucurbitacins (Sachdev-Gupta et al., 1993b,c).

The content of asatone in *H. aspera*, combined with the results given in Figure 2, leads to the conclusion that about 75% of the total deterrent activity of *H. aspera* against *L. puziloi* larvae may be attributed to asatone. Unidentified constituents present in fraction E would account for the remainder of the activity. The implication of these or other substances for both larval feeding and female oviposition is currently under investigation.

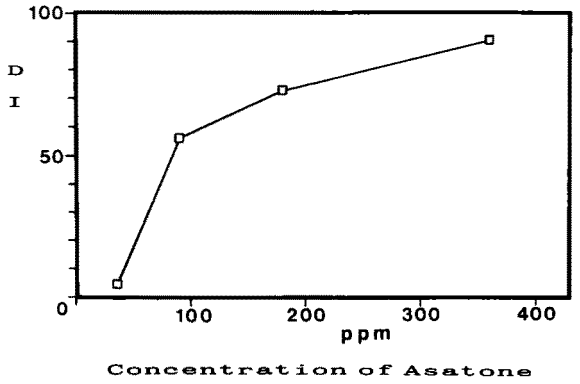


FIG. 2. Deterrent effect of asatone on feeding by *Luehdorfia puziloi* larvae. DI denotes deterrent index (see text).

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AGGREGATION PHEROMONE OF COCONUT RHINOCEROS BEETLE, *Oryctes rhinoceros* (L.) (COLEOPTERA: SCARABAEIDAE)

REBECCA H. HALLETT,[1,*] ALICE L. PEREZ,[2] GERHARD GRIES,[1] REGINE GRIES,[1] HAROLD D. PIERCE JR.,[2] JUNMING YUE,[2] A. CAMERON OEHLSCHLAGER,[2] LILLIANA M. GONZALEZ,[3] and JOHN H. BORDEN[1]

[1]*Centre for Pest Management*
Department of Biological Sciences
[2]*Department of Chemistry*
Simon Fraser University
Burnaby, British Columbia, Canada V5A 1S6
[3]*Chem Tica International, Apdo 159-2150*
San Jose, Costa Rica

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Abstract—Male coconut rhinoceros beetles, *Oryctes rhinoceros* (L.), produce three sex-specific compounds, ethyl 4-methyloctanoate, ethyl 4-methylheptanoate, and 4-methyloctanoic acid, the first of which is an aggregation pheromone. Synthesis of these compounds involving conjugate addition of organocuprates to ethyl acrylate is reported. In field trapping experiments, (4*S*)-ethyl 4-methyloctanoate and the racemic mixture were equally attractive and 10 times more effective in attracting beetles than ethyl chrysanthemumate, a previously recommended attractant. Ethyl 4-methylheptanoate was as attractive as ethyl chrysanthemumate and more attractive than 4-methyloctanoic acid, but further studies are required before it can be classed as an aggregation pheromone. Compared to ethyl 4-methyloctanoate alone, combinations of the three male-produced compounds did not increase attraction, whereas addition of freshly rotting oil palm fruit bunches to pheromone-baited traps significantly enhanced attraction. With increasing dose, captures of *O. rhinoceros* increased, but doses of 6, 9, and 18 mg/day were competitive with 30 mg/day lures. Newly designed vane traps were more effective in capturing beetles than were barrier or pitfall traps. Results of this study indicate that there is potential for using ethyl 4-methyloctanoate in operational programs to control *O. rhinoceros* in oil palm plantations.

*To whom correspondence should be addressed.

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Key Words—Coleoptera, Scarabaeidae, *Oryctes rhinoceros*, coconut rhinoceros beetle, aggregation pheromone, pheromone chirality, ethyl 4-methyloctanoate, ethyl 4-methylheptanoate, 4-methyloctanoic acid.

INTRODUCTION

The coconut rhinoceros beetle, *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae), is one of the most important pests of coconut and oil palms in South and Southeast Asia (Ho and Toh, 1982; Zelazny and Alfiler, 1986). Adult rhinoceros beetles burrow into the growing point of palms and feed on unopened fronds, causing damage to inflorescences and reduction in photosynthetic area, which decreases or delays fruit production (Zelazny, 1979; Liau and Ahmad, 1991, 1993). Prolonged attacks can kill mature palms by defoliation and young palms if the growing point is destroyed. The wounds produced by the beetle provide entry points for lethal diseases and the palm weevils, *Rhynchophorus ferrugineus* Olivier and *R. vulneratus* Panzer (Bedford, 1980; Jacob and Bhumannavar, 1991).

O. rhinoceros breeds in decaying organic matter, such as felled rotting palms, and usually becomes a major problem in newly planted or replanted oil palm plantations. Covering the trunks with a rapidly growing ground cover (Wood, 1968) and/or shredding and burning of the felled trunks are common practices to minimize the build up of *O. rhinoceros* populations (Liau and Ahmad, 1991). Although effective, shredding and burning is very expensive and has been banned in some parts of Southeast Asia (Tajudin et al., 1993) to lower air pollution in this region of 4.5 million hectares of oil palm.

Treatment of breeding sites, such as stumps, with insecticidal drenches and routine application of granular insecticides (e.g., carbofuran) to the leaf axils of young oil palms are recommended (Ho and Toh, 1982). These techniques are currently considered economic (Liau and Ahmad, 1991) but are not very effective and present environmental and health risks. Manual removal of beetles from palms and larvae from decomposing trunks is costly and labor-intensive (Ho and Toh, 1982).

Limited success in managing *O. rhinoceros* populations has been achieved through introduction of the baculovirus, *Rhabdionvirus oryctes* Hüger (Bedford, 1986). Introduction of the baculovirus to the Philippines reduced *O. rhinoceros* populations to 10–20% of prerelease levels, but even low-level populations of *O. rhinoceros* can cause great damage (Zelazny and Alfiler, 1987, 1991). The baculovirus remains effective only if it infects new larval hosts or is repeatedly introduced, and the potential exists for *O. rhinoceros* to develop resistance to the baculovirus after prolonged exposure (Zelazny and Alfiler, 1991). Several compounds, including ethyl chrysanthemumate (EC, rhinolure) have been recommended as lures for trapping *O. rhinoceros* (Barber et al., 1971; Maddison

et al., 1973; Vander Meer et al., 1979), but they are only moderately attractive (Vander Meer et al., 1979; Young, 1986).

O. rhinoceros adults are gregarious. More than one beetle may attack a given palm at the same time while a neighboring tree is unattacked (Gressit, 1953). Aggregation of adults in decaying palm trunks to mate and the occurrence of both single and multiple pairs of adults in the same breeding site (Zelazny and Alfiler, 1991) suggest that *O. rhinoceros* is attracted to host kairomones and employs either an aggregation or sex pheromone or both. Sex pheromones have been identified in several scarabaeids: *Costelytra zealandica* White (Henzell and Lowe, 1970), *Popillia japonica* Newman (Tumlinson et al., 1977), *Kheper Lamarcki* MacLeay (Burger et al., 1983), *Anomala rufocuprea* Motschulsky (Tamaki et al., 1985), *A. cuprea* Hope (Leal, 1991; Leal et al., 1993a), *A. daimiana* Harold (Leal et al., 1993b), *A. schonfeldti* Ohaus (Hasegawa et al., 1993), *Blitopertha orientalis* (Leal, 1993), *A. octiescostata* Burmeister (Leal et al., 1994a), *A. albopilosa sakishimana* Nomura (Leal et al., 1994b), *Holotrichia parallela* Motschulsky (Leal et al., 1993c), *Exomala orientalis* Waterhouse (Leal et al., 1994c), and *A. orientalis* Waterhouse (Zhang et al., 1994). An aggregation pheromone, ethyl 4-methyloctanoate, has recently been reported for *Oryctes monoceros* Olivier (Gries et al., 1994a).

The candidate pheromones revealed for *O. rhinoceros* are 4-methyl alkanoic acids and esters (Figure 1). Because the methyl branch of these compounds is remote from the functional group, syntheses had to be developed that allowed introduction of chirality at remote locations. The candidate pheromones were synthesized through conjugate addition of organocuprates to ethyl acrylate, which provided a shorter route to racemic 4-alkyl substituted ethyl esters than previously reported. Use of readily available enantiomers of citronellol allowed synthesis of both chiral isomers of ethyl 4-methyloctanoate.

We report the structural elucidation, synthesis, and field testing of a male-produced aggregation pheromone and two other male-specific compounds that have the potential to be used in mass trapping of *O. rhinoceros* populations.

METHODS AND MATERIALS

Volatile Analysis and Bioassays

Larvae, pupae, or adults of *O. rhinoceros* were collected at Parungkuda, West Java, Indonesia. Fourteen female and 16 male adults were separately aerated for one week in modified Nalgene desiccators (#5311-0250) containing sugarcane (Oehlschlager et al., 1988, 1992). A water-driven, charcoal-filtered air stream (2 liters/min) was maintained through the chambers and volatiles were captured on Porapak Q held in a glass column (14 cm length × 13 mm OD). In a second series of aerations, 10 female and 10 male adults were aerated

separately. Volatiles were eluted from the Porapak Q with 175 ml of pentane, and the eluant was concentrated by distillation of solvent through a 30-cm Dufton column. The concentration of volatiles was adjusted so that 1 μl equaled 0.6 beetle hours of production.

Porapak Q-captured volatiles from both males and females were analyzed by gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975) (Hewlett Packard 5890A), utilizing a custom-built amplifier with a passive low-pass filter and a cutoff frequency of 10 kHz, and by GC analyses (Hewlett Packard 5830A) and GC–mass spectrometry (GC-MS) (Hewlett Packard 5985B), employing either a fused silica (30 m × 0.25 mm ID) or glass (30 m × 0.5 mm ID) column coated with SP-1000 (Supelco, Mississauga, Ontario).

Bioassays were conducted with a white Plexiglas Y-shaped olfactometer (stem 5 × 5 × 20 cm, arms 5 × 5 × 15 cm) with a clear lid. Test compounds were added with a syringe to filter paper inside a glass cartridge placed in each arm of the olfactometer. Compressed air at 200 ml/min carried the volatiles through each arm towards beetles released singly into the stem of the olfactometer 10 cm from the Y junction. A baffle prevented mixing of the two stimuli in the Y junction. Beetles with intact antennae and walking ability were held 2–3 hr in separate containers without food prior to testing. Prior to each test, the paper lining on the olfactometer floor was replaced, and beetle and position of stimuli were randomly selected. A beetle that did not move forward within 5 min of introduction was classed as a nonresponder, whereas complete entrance of a beetle into one of the arms was recorded as a response. In two experiments, 10 μl of distillate from males (experiment 1) or females (experiment 2) were tested against 10 μl of hexane. In order to determine behavioral activity of two synthetic male-produced compounds, a 612-ng dose of ethyl 4-methyloctanoate, **1**, and ethyl 4-methylheptanoate, **3**, in a 100:2 ratio was tested against 10 μl of hexane (experiment 3).

Instruments and General Synthetic Procedures

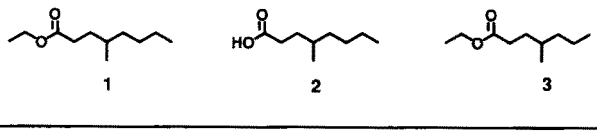
Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker AMX-400 spectrometer at 400.13 and 100.62 MHz for ^{1}H and ^{13}C NMR spectra, respectively. ^{1}H chemical shifts are reported relative to TMS, and ^{13}C chemical shifts are referenced to $CDCl_3$. Gas chromatographic analyses were performed on Hewlett-Packard 5880A and 5890 instruments equipped with a flame ionization detector and a fused silica, DB-1 coated column (15 m × 0.25 mm ID; 0.25 mm film) (J & W Scientific, Folsom, California). Elemental analyses were performed using a Carbo Erba model-1106 Elemental Analyzer. Diethyl ether (Et_2O) and tetrahydrofuran (THF) were freshly distilled from sodium-benzophenone-ketyl, and dichloromethane (CH_2Cl_2) was freshly distilled from CaH_2. Unless otherwise indicated, chemicals obtained from com-

mercial sources were used without further purification. All moisture- and air-sensitive reactions were conducted under argon. Column chromatography refers to flash chromatography using Silica Gel 60 (230–400 mesh, E Merck, Darmstadt, Germany) (Still et al., 1978). Thin-layer chromatography (TLC) was conducted on aluminum-backed plates precoated with Merck Silica Gel 60F-254 as the adsorbent, and visualized by treatment with an acidic solution of 1% $Ce(SO_4)_2$ and 1.5% molybdic acid followed by gentle heating.

Syntheses

(±)-Ethyl 4-methyloctanoate ***(1).*** Compound **1** (97% pure) (Figure 1) was synthesized as previously described (Gries et al., 1994a) or by ethyl ester formation from commercially available 4-methyloctanoic acid, **2** (CTC Organics, Atlanta, Georgia).

(±)-Ethyl 4-methylheptanoate ***(3).*** This compound was prepared by the conjugate addition of 2-pentyl magnesium cuprate to ethyl acrylate (Gries et al., 1994a). Compound **3** (Figure 1) was obtained as a colorless liquid (40% yield, 95% pure). ^{1}H NMR ($CDCl_3$, ppm): 0.88 (6H, m); 1.10 (1H, m); 1.27 (3H, t, J = 8 Hz); 1.30 (2H, m); 1.40 (3H, m); 1.66 (1H, m); 2.30 (2H, m); 4.10



Scheme 1

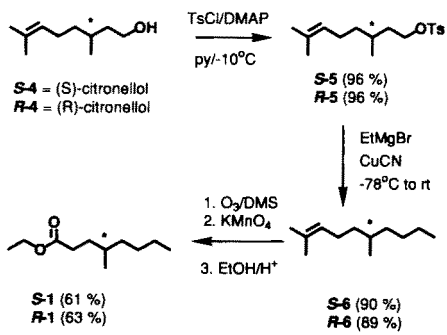


FIG. 1. Compounds **1–3** and scheme for synthesis of (4*S*)- and (4*R*)-ethyl 4-methyloctanoate.

(2H, q, J = 8 Hz); ^{13}C NMR ($CDCl_3$, ppm): 174.16, 60.11, 36.97, 32.17 (2C), 31.95, 31.16, 19.99, 19.23, 14.23. Cl-MS m/z (relative intensity): 172 (M^+, 3), 127 (M^+-OEt, 30), 101 (M^+-CO_2Et, 100).

(3S)-3,7-Dimethyl-6-octenyltosylate ***(S-5).*** Enantiomer ***S*-5** (Figure 1, scheme 1) was prepared according to the procedure of Mori and Harashima (1993b) using (*S*)-citronellol, ***S*-4** (97.5% ee, Aldrich Chemical Co., Milwaukee, Wisconsin). Purification by column chromatography (9:1, pentane/Et_2O as eluant) yielded 5.70 g of ***S*-5,** (96% yield, 98% pure) as a pale yellow oil; ^{1}H NMR ($CDCl_3$, ppm): 0.80 (3 H, d, J = 8.8 Hz); 1.10 (1H, m); 1.20 (1H, m); 1.40 (1H, m); 1.50 (1H, m); 1.52 (1H, m); 1.56 (3H, s); 1.70 (3H, s); 1.90 (2H, m); 2.45 (3H, s), 4.02 (2H, m); 5.0 (1H, t, J = 8.8 Hz); 7.32 (2H, d, J = 8.8 Hz); 7.80 (2H, d, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, ppm): 144.60, 133.41, 131.44, 129.79, 127.88, 124.34, 69.05, 36.72, 35.69, 28.93, 25.66, 25.27, 21.60, 19.06, 17.60; Cl-MS m/z (relative intensity): 310 (M^+, 2), 138 (M^+-OSO_2-C_6H_4-CH_3, 50). ***R*-5** [from (*R*)-citronellol; 96% ee]: 5.61 g, 96% yield (98% pure).

(6R)-2,6-Dimethyl-2-decene ***(R-6).*** This intermediate compound was prepared according to the procedure of Fouquet and Schlosser (1974). CuCN (Aldrich Chemical Co.) was used as received. Column chromatography (pentane) afforded 2.42 g (89% yield, 97% pure) of ***R*-6** as a colorless liquid. ^{1}H NMR ($CDCl_3$, ppm): 0.88 (3H, d, J = 7.3 Hz); 0.90 (3H, t, J = 7.3 Hz); 1.10 (2H, m); 1.30 (7H, m); 1.60 (3H, s); 1.70 (3H, s); 1.90 (2H, m); 5.1 (1H, t, J = 8 Hz); ^{13}C NMR ($CDCl_3$, ppm): 130.83, 125.16, 37.19, 36.68, 32.46, 29.29, 25.62 (2C), 23.01, 19.60, 17.55, 14.05. Cl-MS m/z (relative intensity): 168 (M^+, 40); IR (neat): 2926, 1673, 1458, 1377, 1122, 1094, 984, 826 cm^{-1}. Anal. calcd. for $C_{12}H_{24}$: C, 85.63; H, 14.37. Found: C, 85.69; H, 14.30. ***S*-6:** 2.39 g, 90% yield (98% pure). Anal. calcd. for $C_{12}H_{24}$: C, 85.63, H; 14.37. Found: C, 85.78; H, 14.57.

(4R)-Methyloctanal ***(R-7).*** Compound ***R*-7** was prepared according to the procedure of Mori and Harashima (1993a). Column chromatography (8:2, pentane/Et_2O as eluant, TLC R_f = 0.55) afforded ***R*-7** (1.68 g, 87% yield, 96% pure) as a colorless liquid. ^{1}H NMR ($CDCl_3$, ppm): 0.90 (6H, m); 1.12 (1H, m); 1.30 (5H, m); 1.40 (2H, m); 1.64 (1H, m); 2.64 (2H, m); 9.75 (1H, s); ^{13}C NMR ($CDCl_3$, ppm): 202.87, 41.70, 36.36, 32.38, 29.16, 28.93, 22.91, 19.35, 14.05; Cl-MS m/z (relative intensity): 143 (M^+ + 1, 100); IR (neat): 2927, 2715, 1727, 1465, 1379, 1263, 1133, 1012, 896, 849, 729 cm^{-1}. Anal. calcd. for $C_9H_{18}O$: C, 75.00; H, 12.76. Found: C, 75.09; H, 12.48. ***S*-7:** 1.65 g, 85% yield (98% pure). Anal. calcd. for $C_9H_{18}O$: C, 75.00; H, 12.76. Found: C, 74.98; H, 12.66.

(4R)-4-Methyloctanoic acid ***(R-2).*** This compound was prepared by the oxidation of ***R*-7** (yield 1.78 g) according to the procedure of Furniss et al.

(1989). Compound ***R*-2** was used for the next step without further purification. ^{1}H NMR ($CDCl_3$, ppm): 0.90 (6H, m); 1.12 (1H, m); 1.14 (5H, m); 1.60 (2H, m); 1.80 (1H, m); 2.35 (2H, m), 11.30 (1H, br. s). ***S*-2:** 1.45 g.

(4R)-Ethyl-4-methyloctanoate **(*R*-1).** Enantiomer ***R*-2** was esterified using the Fisher method (Furniss et al., 1989). Column chromatography (9:1, pentane/Et_2O as eluant) gave 1.34 g of ***R*-1** (61% yield based on ***R*-7**, 98% pure). $[\alpha]_D^{20} = -1.67°$ (c = 1.345, $CHCl_3$); ^{1}H NMR ($CDCl_3$, ppm): 0.88 (3H, d, J = 8 Hz); 0.90 (3H, t, J = 8 Hz); 1.10 (1H, m); 1.24 (3H, t, J = 8 Hz); 1.30 (5H, m); 1.40 (2H, m); 1.66 (1H, m); 2.30 (2H, m); 4.10 (2H, q, J = 8 Hz); ^{13}C NMR ($CDCl_3$, ppm): 174.12, 60.14, 36.36, 34.42, 32.21, 31.97, 29.17, 22.94, 19.31, 14.25, 14.06; IR (neat): 2958, 2884, 1737, 1461, 1373, 1261, 1173, 1108, 1032, 932, 855, 779, 732 cm^{-1}. Anal. calcd. for $C_{11}H_{22}O_2$: C, 70.91; H, 11.91. Found: C, 70.99; H, 12.00. ***S*-1:** 1.36 g, 63% yield based on ***S*-7** (98% pure) $[\alpha]_D^{20} = +1.67°$ (c = 1.350, $CHCl_3$). Anal. calcd. for $C_{11}H_{22}O_2$: C, 70.91; H, 11.91. Found: C, 70.82; H, 11.98.

Chiral Determination

Enantiomeric excess of (*R*)- and (*S*)-citronellol was determined through GC analysis (DB-1) as the amides of D-(+)-α-phenylethylamine (Sigma, St. Louis, Missouri) of the corresponding citronellic acids (Sonnet and Gazzillo, 1990).

Analysis of ***S*-1** and ***R*-1** by GC on a fused silica, Cyclodex-B-coated column (30 m × 0.25 mm ID, J & W Scientific) failed to resolve the enantiomers. ^{1}H NMR spectra in the presence of tris[3-(heptanofluoropropylhydroxymethylene)-(−)-camphorato]europium(III) [Eu(hfc)$_3$] in CS_2 (1:1 or 1:2 ratio, ester: shift reagent) (McCreary et al., 1974; Valentine et al., 1976) or the chiral solvating agent (*R*)-2,2,2-trifluoro-1-(9-anthryl)ethanol in CCl_4 (1:1 or 1:2 ratio, ester: anthrylethanol) (Pirkle et al., 1977; Pirkle and Sikkenga, 1977) failed to display diastereoisomeric complexes at different chemical shifts.

Field Experiments

Experiments were conducted in 1- or 2-year-old oil palm plantings at three P.T.P.P. London Sumatra Indonesia estates in North Sumatra, Indonesia. Specific locations are given either in Figures 5–10 below or in the text, if data are not presented in figures. All experiments were set up as randomized complete blocks with intertrap and interblock distances of at least 27 and 54 m, respectively. Traps were checked daily and captured beetles removed.

All compounds, except ***S*-1** and ***R*-1,** were released from heat-sealed, polymer membrane bag devices (Chem Tica International, Costa Rica) at constant rates. Release rates for each compound were determined by placing 10 sealed

devices containing 1 ml of neat compound in a thermostated chamber (25 ± 1°C, 60% relative humidity), allowing 48 hr equilibration, and then weighing (±0.1 mg) each device every two to three days for two weeks (devices releasing 30 mg/day) or four to five weeks (devices releasing <30 mg/day). Variability of release rates between devices was <±5% and over the measurement period was <±10%. Compound **1** was released at 0.9, 3, 6, 9, and 30 mg/day; **2** at 3 and 15 mg/day; **3** at 1 and 30 mg/day; and EC at 15 mg/day. Combinations of devices were used to achieve some release rates. ***S*-1** and ***R*-1** were released at 0.3 mg/day (at 25°C) from 1-mm-ID glass capillary tubes cut 1 cm above the meniscus and placed in capped 400-μl plastic centrifuge tubes with two 3-mm holes 1 cm below the top.

Behavioral Activity of Ethyl 4-methyloctanoate

A three-treatment, 10-replicate experiment (experiment 4) determined activity of the candidate pheromone, **1**. Black pitfall traps (Figure 2) were buried in the ground 1–1.5 m from palms and baited with either decomposing oil palm tissue (1–2 kg of leaf bases or empty fruit bunches), **1** (released at 30 mg per day), or both. Oil palm tissue or a cloth placed in traps baited with **1** only, was treated with 0.3% active ingredient solution of Basudin 60 EC (diazinon, Ciba-

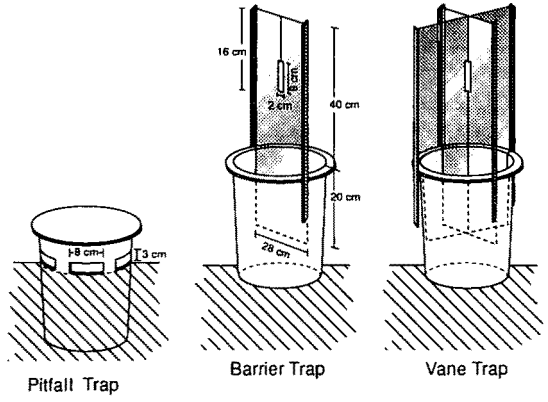


FIG. 2. Trap designs used for capturing *O. rhinoceros*. Pitfall trap: 19-liter black bucket buried in ground to allow beetles to enter through slots below rim; pheromone lure suspended within bucket from plywood lid. Barrier and vane traps: 20-liter white bucket with one or two unpainted sheet metal vanes extending 20 cm into bucket to prevent beetles from flying out; wooden slats on edge of vanes for reinforcement; lures suspended within slot to allow volatile dissemination in all directions.

Geigy). Beetles were captured because of contact with the insecticide-treated substrate or inability to exit the traps.

Trap Design Experiments

A three-treatment, 10-replicate experiment (experiment 5) compared trapping efficacies of pitfall to barrier and vane traps (Figure 2). In experiment 6 ($N = 9$), four types of vane traps were compared: unpainted (standard) vane traps (Figure 2), vane traps fitted with an internal funnel (15 cm opening) to prevent beetle escape, traps with matte black-painted vanes, and traps with both funnels and black-painted vanes (Dolok Estate, February 26–March 11, 1994). In both experiments 5 and 6, traps were baited with lures releasing **1** at 30 mg/day. No insecticide or organic matter was used in any of the traps.

Dose–Response of Ethyl 4-methyloctanoate

In a four-treatment, 18-replicate experiment (experiment 7) with standard vane traps at 36 m intervals, **1** was released at 0, 0.3, 3, or 30 mg/day. In experiment 8 ($N = 15$), standard vane traps were baited with **1** released at 0, 3, 6, 9, or 30 mg/day. In experiment 9 ($N = 12$), standard vane traps were baited with **1** released at 0, 6, 9, 18, or 30 mg/day. To minimize interference between treatments in experiments 8 and 9, intertrap and interblock distances were increased to 54 and ≥ 63 m, respectively.

Chiral Isomers of Ethyl 4-methyloctanoate

A four-treatment, 10-replicate experiment (experiment 10) tested attraction of beetles to racemic **1**, ***S*-1**, ***R*-1**, or a blank control in standard vane traps. ***S***- or ***R*-1** were released at ~ 2 mg/day from eight capillary tubes and racemic **1** was released at ~ 4 mg/day from 16 capillary tubes.

Other Candidate Pheromones and Attractants

Attraction of beetles to three male-produced compounds, **1**, **2**, **3**, and to (±)-ethyl chrysanthemumate (EC, 95% pure, mixture of *cis* and *trans*, Aldrich Chemical Co.) was compared in experiment 11 ($N = 10$). All compounds were released at 30 mg/day. Experiment 12 ($N = 9$) compared attraction of beetles to **1** alone (30 mg/day) and in combination with **3** at ratios of 100:1, 100:10, and 100:100 (Rambong Sialang Estate, April 13–21, 1994). Experiment 13 ($N = 10$) compared attraction of beetles to **1** alone (10 mg/day) or in approximate natural ratios with either or both of **2** (30 mg/day) and **3** (0.1 mg/day) (Rambong Sialang Estate, June 9–17, 1994).

Pheromone–Host Material Interactions

The attraction of beetles to **1** (9 mg/day) alone, freshly milled empty fruit bunches (1/3 to 1/2 bunch per trap), or to both was examined in a three-treatment, 12-replicate experiment (experiment 14).

Statistical Analyses

The proportions of beetles responding to each stimulus in laboratory bioassays were compared by using the normal approximation to the binomial test, and differences between responses of female and male beetles were compared with χ^2 tests (Zar, 1984).

In all field experiments, no significant differences were found in the responses of male and female beetles and so catches were pooled by sex for analysis. Data were transformed by log $(x + 1)$ if they were not normally distributed and were subjected to analysis of variance (general linear modeling) (Minitab, 1989). If replicates were run at different times or locations, data were analyzed for time × treatment or location × treatment interactions. Following ANOVA, multiple pair comparisons were made using Bonferroni t tests. If homoscedasticity was not achieved by transformation, data were analyzed by χ^2 tests (Zar, 1984).

RESULTS

GC and GC-EAD analyses of Porapak Q-trapped volatiles obtained from aerations of either *O. rhinoceros* males or females revealed two abundant male-specific components (Figure 3), of which the early eluting volatile elicited antennal responses by male and female antennae (Figure 4). Retention and mass spectrometric characteristics of these two compounds were identical to ethyl 4-methyloctanoate, **1**, and 4-methyloctanoic acid, **2**. A second EAD-active compound (not visible in Figure 4) had a Kovats retention index (RI = 1379) suggestive of ethyl 4-methylheptanoate, **3**. GC-MS in both electron impact and chemical ionization modes of beetle-produced and authentic **3** confirmed this structural assignment.

In laboratory bioassays (experiments 1 and 2), male-produced volatiles were equally attractive to walking male and female *O. rhinoceros*, but female-produced volatiles were attractive only to males (Table 1). Behavioral activity of synthetic **1** plus **3** was demonstrated in experiment 3 (Table 2) and justified field testing of synthetic candidate pheromones. In field experiments, **1** at 30 mg/day alone or in combination with decomposing palm tissue attracted more beetles than palm tissue alone (Figure 5; experiment 4).

Both vane and barrier traps were superior to the pitfall trap (Figure 2), with

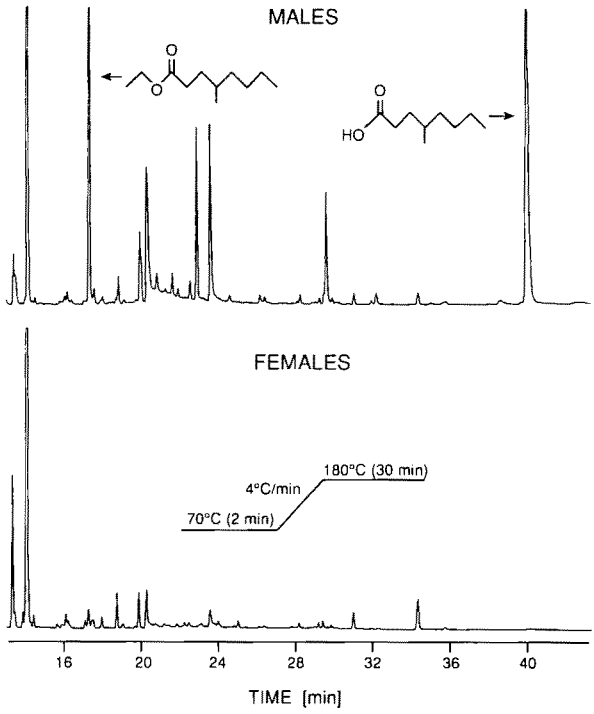


FIG. 3. Gas chromatograms of volatiles from 10 male and 10 female *O. rhinoceros* maintained in aeration chambers for one week with provision of sugarcane as food source. Chromatography: Hewlett Packard 5830A gas chromatograph (GC) equipped with a glass capillary column (30 m × 0.5 mm ID) coated with SP-1000.

vane traps capturing about three times more beetles than pitfall traps (Figure 6; experiment 5). Addition of a funnel to the vane trap and painting vanes black did not alter trap efficacy (experiment 6; ANOVA, $F = 1.19$, $df = 3$, $P = 0.341$).

Catches of *O. rhinoceros* increased with increasing release rates of the pheromone (Figure 7; experiments 7–9). A significant location × dose interaction was observed in experiment 7 ($F = 18.39$, $df = 1$, $P < 0.001$), which arose from the 3 mg/day lure being more attractive relative to the 30 mg/day lure at Rambong Sialang than at Dolok (mean catches in 3 mg/day traps were 64% and 32% of those in 30 mg/day traps, respectively). This result along with the prohibitive cost of 30 mg/day lures, prompted us to examine release rates between 3 and 30 mg/day. In experiments 8 and 9, lures releasing **1** at 6, 9, and 18 mg/day were competitive with 30 mg/day lures (Figure 7).

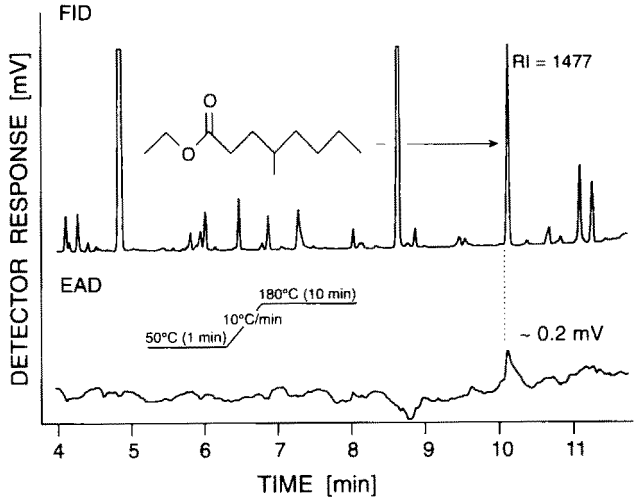


FIG. 4. FID and EAD responses to volatiles obtained from male *O. rhinoceros*. The antennal recording was carried out with an antenna of a female beetle. Chromatography: Hewlett Packard (HP) 5890A gas chromatograph (GC) equipped with a fused silica column (30 m × 0.25 mm ID) coated with SP-1000.

Racemic **1** and ***S*-1** were superior to ***R*-1** in attracting beetles (Figure 8; experiment 10). Racemic **1** was significantly more attractive than **2, 3,** or EC (Figure 9; experiment 11); **3** was significantly more attractive than **2,** but both compounds did not differ in attraction from EC. Traps baited with **1** alone or with **3** in different ratios were equally attractive (experiment 12; ANOVA, $F = 0.49$, $df = 3$, $P = 0.693$), as were traps baited with **1** alone, **1** plus **2,** or **1** plus **2** and **3** (experiment 13; ANOVA, $F = 2.28$, $df = 3$, $P = 0.105$).

Freshly milled oil palm fruit bunches alone were unattractive to rhinoceros beetles, but in combination with **1** they significantly enhanced pheromonal activity (Figure 10; experiment 14).

DISCUSSION

Methyl-branch-substituted pheromones are very common (Mori, 1992). There are several synthetic methods described for the synthesis of 4-methyl alkanoic acids and esters. Compounds **1** and **3** were prepared by Cason et al. (1944) by reaction of ethyl levulinate or levulinic acid with the corresponding Grignard reagent to produce a γ,γ-dialkylbutyrolactone. Treatment of the lactone with $SOCl_2$/ethanol/HCl followed by hydrogenation produced the respec-

TABLE 1. RESPONSES OF MALE AND FEMALE *Oryctes rhinoceros* TESTED INDIVIDUALLY TO PENTANE EXTRACT OF MALE- AND FEMALE-PRODUCED VOLATILES (10 μl = 5.6 BEETLE HOURS) VERSUS 10 μl OF HEXANE CONTROL IN Y-SHAPED OLFACTOMETER

Experiment	Sex	n	Number of responders to stimuli[a]		P^b
			Extract	Control	
1. Female extract *versus* hexane control	Male	20	9	2	0.006
	Female	20	5	8	0.393
2. Male extract *versus* hexane control	Male	15	12	1	0.0001
	Female	17	13	3	0.0014

[a] Responses to female extract in experiment 1 differ between males and females, $\chi^2 = 4.608$, $df = 1$, $P = 0.032$. Responses to male extract in experiment 2 are not significantly different between males and females, $\chi^2 = 0.738$, $df = 1$, $P = 0.39$.
[b] Determined by normal approximation to the binomial test (Zar, 1984).

TABLE 2. RESPONSES OF MALE AND FEMALE *Oryctes rhinoceros* TESTED INDIVIDUALLY TO 612 ng OF 100:2 BLEND OF SYNTHETIC ETHYL 4-METHYLOCTANOATE (**1**) AND ETHYL 4-METHYLHEPTANOATE (**3**) VERSUS 10 μl OF HEXANE CONTROL IN Y-SHAPED OLFACTOMETER (EXPERIMENT 3)

Sex tested	*N*	Number of responders to stimuli[a]		*P*[b]
		Synthetic blend	Hexane control	
Male	29	17	7	0.025
Female	29	19	7	0.008

[a]Responses are not significantly different between males and females, $\chi^2 = 0.031$, $df = 1$, $P = 0.86$.
[b]Determined by normal approximation to the binomial test (Zar, 1984).

tive 4-methyl esters in overall yields of 50–60%. Vasi and Desai (1973) prepared 4-methylheptanoic acid from 2-methylbutyric acid using Arndt-Eistert synthesis. Mrowca (1981) reported the synthesis of carboxylic acids or esters by catalytic carboxylation or alcoxycarbonylation of unsaturated hydrocarbons (**2** was obtained in 86% yield). More recently, Sonnet and Baillargeon (1989) synthe-

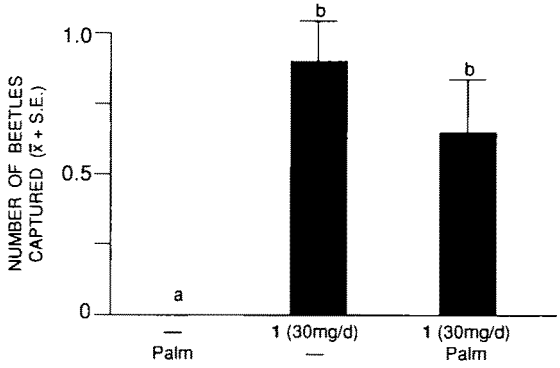


FIG. 5. Attraction of *O. rhinoceros* to ethyl 4-methyloctanoate, **1** (released at 30 mg/day), decaying oil palm tissue, or both together, in pitfall traps at Bah Lias and Rambong Sialang Estates, North Sumatra, Indonesia (October 14–22 and 16–23, 1993, respectively) (experiment 4). Data pooled as no locational differences were found ($\chi^2 = 2.2305$, $df = 1$, $P > 0.10$). Treatment differences for pooled data, $\chi^2 = 13.036$, $df = 2$, $P < 0.01$. Bars with the same letter are not significantly different, pairwise χ^2 tests, $P < 0.05$.

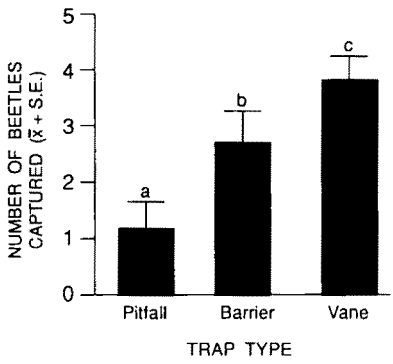


FIG. 6. Efficacy of three insecticide-free trap types for capturing *O. rhinoceros* (February 2–10, 1994), tested at Rambong Sialang Estate (experiment 5). All traps baited with **1** released at 30 mg/day. ANOVA, $F = 6.58$, $df = 2$, $P = 0.007$. Bars with the same letter are not significantly different, Bonferroni t test, $P < 0.05$.

sized 4-methyloctanoic acid, **2**, in 53% overall yield by methylation of the *N*-*t*-butylimine derivative of hexanal by condensation with malonic acid and hydrogenation.

In our study, both **1** and **3** were prepared by conjugate addition of organocuprates to ethyl acrylate (Corey and Boaz, 1985; Matsuzawa et al., 1989; Perlmutter, 1992; Gries et al., 1994a), a shorter synthetic procedure than in earlier reports. The required cuprates were prepared by adding 10 mol% CuCN to a solution of the corresponding Grignard reagent at −40°C. Subsequent addition of trimethylchlorosilane, HMPA, and ethyl acrylate (2:2:1 ratio) in THF or Et_2O produced **1** or **3** in 40–55% yield. Use of CuBr·DMS increased the yield of the conjugate addition by 10–15%. No further attempts to optimize the reaction yields were made because **1** can also be prepared by the esterification of commercially available **2** (CTC Organics, Atlanta, Georgia).

Syntheses of ***R*-2** and ***S*-2**, in high optical purity (95.4%) have been reported by Sonnet and Gazzillo (1990). Accordingly, alkylation of hexanoic acid produced 2-methylhexanoic acid, which was reacted with (*R*)- or (*S*)-α-phenylamine to give diastereoisomeric amides that were separated by crystallization. The optically pure amides were *N*-hydroxylated and reduced to the corresponding 2-methylhexanols, which were oxidized and converted to the methyl 4-methyl-2-octanoates via a Wittig reaction with carbomethoxymethylene triphenylphosphorane. Hydrogenation and saponification afforded ***R*-2** and ***S*-2** in ~10% overall yield (eight steps).

We envisioned a more efficient route using a highly enantiomerically enriched citronellol (Hanessian et al., 1990; Ho, 1992). The citronellane skel-

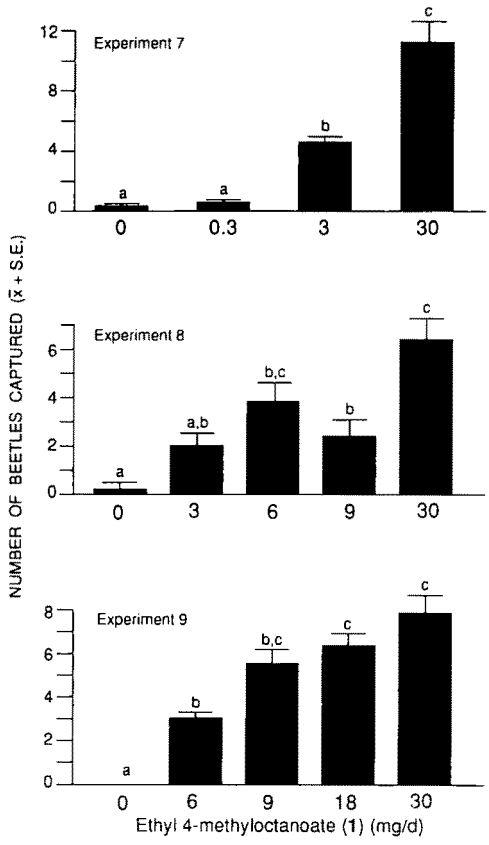


FIG. 7. Attraction of *O. rhinoceros* to **1** released at various rates from standard vane traps. Experiment 7, February 12–26 and February 21–March 4, 1994, Dolok and Rambong Sialang Estates, respectively; ANOVA, $F = 44.89$, $df = 3$, $P < 0.001$. Experiment 8, March 11–17, 1994, Dolok; ANOVA log $(x + 1)$ transformed data, $F = 12.13$, $df = 4$, $P < 0.001$. Experiment 9, May 24–June 8, 1994, Dolok; ANOVA $\log_{10}(x + 1)$ transformed data, $F = 49.84$, $df = 4$, $P < 0.001$. Bars with the same letter are not significantly different, Bonferroni t test, $P < 0.05$. Untransformed means presented.

eton is commonly used in the synthesis of natural products (e.g., Mori et al., 1991; Stork and Nakamura, 1983; Ho, 1992; Mori and Harashima, 1993a,b; Weinges et al., 1993; Mori and Murata, 1994; Paquette et al., 1995). Modifications at both termini of the citronellane skeleton can be performed without perturbing the chiral center (Ho, 1992). The synthesis of ***S*-1** and ***R*-1** (Figure 1, scheme 1) commenced with tosylation of (*R*)- or (*S*)-citronellol. Chain exten-

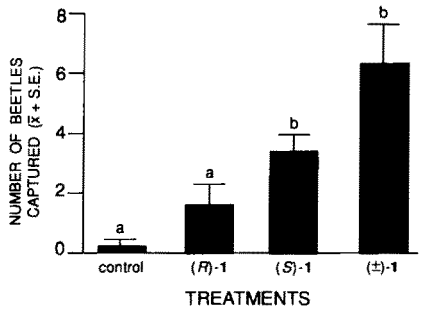


FIG. 8. Attraction of *O. rhinoceros* to standard vane traps containing stereoisomers of **1** at Rambong Sialang Estate (May 23–June 8, 1994) (experiment 10). ANOVA log (x + 1) transformed data, $F = 24.04$, $df = 3$, $P < 0.001$. Bars with the same letter are not significantly different, Bonferroni t test, $P < 0.05$. Untransformed means presented.

sion *via* cuprate displacement of the tosylate, **5** (Fouquet and Schlosser, 1974), produced the corresponding 2,6-dimethyl-2-decenes, **6**, in high yield. Ozonolysis followed by permanganate oxidation and esterification afforded ***S*-1** and ***R*-1** in yields of 45–47% over five steps. Attempts to determine the optical purity of **6**, **7**, ***S*-1**, or ***R*-1** by gas chromatography using a Cyclodex B column and NMR techniques were unsuccessful. Since the above procedures have significant precedent in the literature and involve transformations remote from the stereogenic carbon, we assumed that the chiral purity of ***S*-1** and ***R*-1** was identical to that of citronellol.

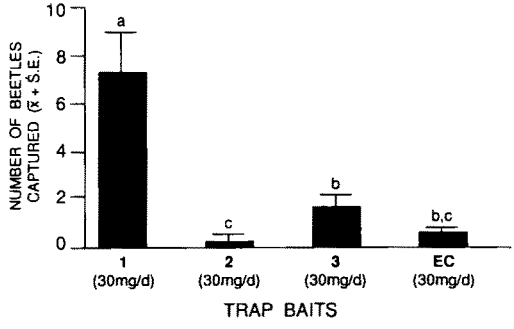


FIG. 9. Attraction of *O. rhinoceros* to **1**, **2**, **3**, and EC in standard vane traps at Rambong Sialang Estate (March 30–April 7, 1994) (experiment 11). ANOVA, log (x + 1) transformed data, $F = 35.77$, $df = 3$, $P < 0.001$. Bars with the same letter are not significantly different, Bonferroni t test, $P < 0.05$. Untransformed means presented.

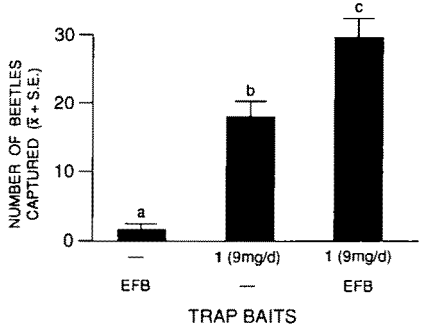


FIG. 10. Attraction of *O. rhinoceros* to standard vane traps containing **1** (released at 9 mg/day), freshly milled empty fruit bunches (EFB), or both together at Rambong Sialang and Dolok Estates (April 26–May 11 and May 3–18, 1994, respectively) (experiment 14). ANOVA, $F = 46.60$, $df = 2$, $P < 0.001$. Bars with the same letter are not significantly different, Bonferroni t test, $P < 0.05$.

Semiochemical communication in *O. rhinoceros* appears to involve both a male-produced aggregation pheromone and a female-produced sex pheromone (Table 1). Because aggregation pheromones have greater potential than sex pheromones for controlling *Oryctes* populations through mass trapping, research was focused on the identification of aggregation pheromones. Compound **1** was confirmed in field experiments as the major male-produced aggregation pheromone of *O. rhinoceros* (Figures 5 and 9). The same compound is a male-produced aggregation pheromone in the African rhinoceros beetle, *O. monoceros* (Gries et al., 1994a). Other geographically or temporally isolated scarabaeid beetles also utilize identical sex pheromones (Leal et al., 1993a,b).

Oryctes spp. respond to vertical silhouettes (Bedford, 1980). Above-ground light traps with vanes are effective in capturing *O. elegans* in the Arabian peninsula. Superior efficiency of vane and barrier traps for capturing *O. rhinoceros* (Figure 6) may mostly be attributed to the vertical silhouettes that are lacking in the pitfall trap. Because response to the vertical silhouette could not be enhanced further by use of nonreflective black vanes and the use of funnels did not improve retention of captured beetles in traps, insecticide-free, unpainted vane traps were adopted as the standard trap for *O. rhinoceros*.

As in *A. octiescostata* (Leal et al., 1994a), increase of pheromone release rate resulted in increasing numbers of captured beetles. Because traps baited with **1** released at 6, 9, 18, or 30 mg/day were similarly attractive, a release rate of 9 mg/day was adopted for operational trapping trials.

In the Japanese beetle, pheromonal attraction is strongly inhibited by the presence of the nonnatural enantiomer of its sex pheromone, (*R*,*Z*)-5-

(1-decenyl)dihydro-2(3H)-furanone (Tumlinson et al., 1977). We therefore investigated the response of *O. rhinoceros* to chiral isomers of **1.** Racemic and ***S*-1** were similarly attractive to *O. rhinoceros* (Figure 8), indicating that ***S*-1** is a naturally produced isomer and ***R*-1** is not repellent. Behavioral activity of ***R*-1** and enhanced attraction of *O. rhinoceros* to racemic **1** rather than ***S*-1** may indicate that both isomers are produced naturally. Accessible racemic **1** can be used operationally.

Synergism between aggregation pheromones and host compounds has recently been demonstrated for *A. octiescostata* (Leal et al., 1994d), and palm weevils, *Rhynchophorus* spp. (Oehlschlager et al., 1992; Gries et al., 1994b; Hallett et al., 1993; Giblin-Davis et al., 1994). Synergistic oil palm volatiles are apparently produced early in the decomposition (fermentation) process because freshly milled fruit bunches but not decomposed palm tissue enhanced pheromone attraction. Investigations are underway to identify palm kairomones and to determine the potential of mass-trapping *O. rhinoceros* in oil palm plantations.

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AN EVALUATION OF REPELLENTS TO SUPPRESS BROWSING BY POSSUMS

A.D. WOOLHOUSE[1,*] and D.R. MORGAN[2]

[1]*Industrial Research Limited*
P.O. Box 31.310, Lower Hutt, New Zealand

[2]*Landcare Research Limited*
P.O. Box 31.011, Christchurch, New Zealand

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Abstract—The efficacy in feeding suppression trials with possums (*Trichosorus vulpecula* Kerr) of six synthetic odorous chemicals uniquely associated with various of the Mustelidae species and of the red fox (*Vulpes vulpes*) has been evaluated with respect to several proprietary repellents. The predator odors, two proprietary repellents, and two other repellent formulations (olive oil in petroleum jelly and a synthetic fermented egg odor cocktail) were applied as 5% w/v solutions in paraffin to the foliage of *Pinus radiata* seedlings and the extent of browse compared with that on seedlings treated with Treepel, an effective egg-based repellent already available commercially. All four of the mustelid predator odors [(∓)-2-*n*-propylthietane, (∓)-3-*n*-propyl-1,2-dithiolane, 2,2-dimethylthietane, and 3,3-dimethyl-1,2-dithiolane] and the volatile compound from fox urine (Δ^3-isopentenyl methyl sulfide) were shown to be more or at least equally effective compared to Treepel. This was true also of one commercial repellent Gori, which contained bitrex and the synthetic fermented egg formulation. The most effective compounds will be evaluated when administered with adhesives and surfactants in the further development of possum repellents with longer field lives.

Key Words—Herbivore repellents, carnivore odors, possum, *Trichosorus vulpecula*, predator-prey interactions.

INTRODUCTION

The damage to both native forests, exotic forest plantations, and agricultural and horicultural crops (Cowan, 1990) that is occurring in New Zealand as a

*To whom correspondence should be addressed.

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result of the introduced Australian brush-tail possum (*Trichosorus vulpecula* Kerr) has created a demand for more effective control measures. Control is further mandated as this animal is also the major vector in the transmission of bovine TB to cattle. Poisoning with sodium monofluoroacetate (1080) or cyanide and trapping are presently effective strategies for the control of possums in uninhabited areas. However, the use of toxins and traps may be inappropriate in situations where people, livestock, or pets are present. There is therefore a demand for alternative strategies of control with nonlethal methods.

We have had a longheld interest in evaluating possum gland secretions as potential sources of compounds (pheromones) that might function uniquely as attractants to possums (Woolhouse et al., 1994) and in evaluating biological fluids (and compounds derived from them) from both sympatric and allopatric carnivorous predators as repellents to possums. The potential for control by repellency, based upon predator avoidance by herbivores, has been demonstrated for a number of herbivorous mammals in recent years (Sullivan et al., 1990a,b; Abbott et al., 1990; Robinson, 1990; Swihart et al., 1991; Pfister et al., 1990; Weldon et al., 1993; Boag and Mltokiewicz, 1994; Epple et al., 1993; for a review of earlier literature see Müller-Schwarze, 1990). Sulfurous metabolites from meat digestion have been suggested as the common factor in predator secretions that elicit aversive behavior among herbivores (Nolte et al., 1994b). Another sulfur-rich formulation, based on egg powder, was effective as a repellent against rabbits (*Oryctolagus cuniculus*) and hares (*Lepus europaeus*) for up to two months (Crozier, 1991) and deterred possums from eating apples in a pen study (Eason and Hickling, 1992).

With the exception of the work reported largely by Sullivan et al. (1990a,b and earlier work), virtually all of the other studies have been carried out with biological fluids (or extracts of them). We have therefore attempted to extend these various findings to the point where active ingredients may be practically applied in wildlife management. We report the results of pen trials on the repellency towards possums of six synthetic mammalian kairomonal compounds uniquely associated with a number of carnivorous mammals and compare these findings to those from other herbivore repellent formulations including three commercial repellents.

METHODS AND MATERIALS

Synthesis. We synthesized six odorous predator compounds (Figure 1) in amounts of 20–50 g, formulated them for field use as 5% w/v solutions in medicinal-grade paraffin oil (to produce a slow release of odor), and dispersed them in the form of a spray, as described by Robinson (1990), for example, in his studies with a synthetic predator odor.

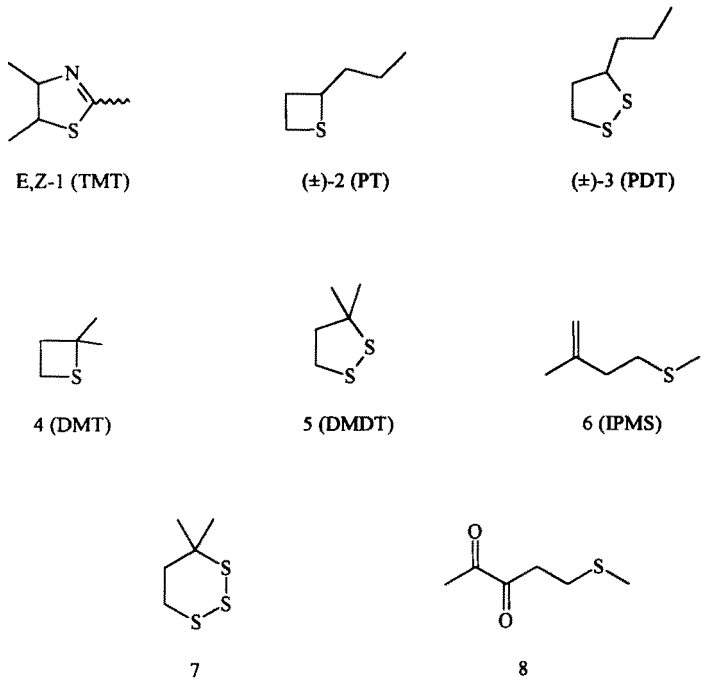

FIG 1. Chemical structures of the compounds referred to in the text.

(*E*,*Z*)-4,5-Dihydro-2,4,5-trimethyl-Δ^3-thiazoline (TMT-1 in Figure 1) is an odorous component of fox (*Vulpes vulpes*) feces that induces fear responses in rats (Vernet-Maury et al., 1984). This compound was synthesized as a mixture of *E* and *Z* isomers by the one-pot condensation of 2-mercaptobutan-2-one (Hromatka and Haberl, 1954) with acetaldimine (prepared in situ from acetaldehyde and ammonia) as outlined by Dubs and Pesaro (1974).

(±)-2-*n*-Propyltheitane (PT-2 in Figure 1) is a volatile compound identified as a major component of the anal sac secretion of the stoat (*Mustela erminea*) (Crump, 1980a) and ferret (*M. putorius* forma *furo*) (Crump, 1980b) that has been shown in field trials to suppress feeding by a number of pest herbivores (see, for example, Sullivan et al., 1988). This compound was synthesized from 3-*n*-propylthiete 1,1-dioxide by the method described by Woolhouse et al. (1993).

(±)-3-*n*-Propyl-1,2-dithiolane (PDT-3 in Figure 1) is also a volatile compound identified as a component of the anal sac secretion of the stoat and of the ferret (Crump, 1980b). It has likewise been shown to field trials to suppress feeding by a number of pest herbivores (Sullivan et al., 1988). This compound

was prepared by the phase transfer-mediated cyclization of (±)-1,3-dibromohexane with disulfide anion, which was based upon the method originally described by Eliel et al. (1975). The dithiolane was obtained pure as a mobile yellow liquid. However, to minimize an apparently slow polymerization, the compound was stored as a 10–20% solution in hexanes.

2,2-Dimethylthietane (DMT-4 in Figure 1) is a highly volatile major component of the anal sac secretion of the mink (*M. vison*) and polecat (*M. putorius*) (Schildknecht et al., 1976). This compound was synthesized by the intramolecular alkali-induced cyclization of 1-chloro-3-mercapto-3-methylbutane as described by Mayer (1974).

3,3-Dimethyl-1,2-dithiolane (DMDT-5 in Figure 1) is a compound found in the feces of the red fox (*Vulpes vulpes*) (Vernet-Maury et al., 1984) and the anal sac secretion of the mink and is obtained as an unstable readily polymerizing oil (Schildknecht et al., 1976). This compound was synthesized by the method of Eliel et al. (1975) but purified to a level of approximately 60% (GC) only with considerable loss of product. GC-MS analysis (Hewlett-Packard MSD, 25-m HP Ultra 2 capillary column operated in the split mode; 100° to 200°C at 10°/min) revealed the presence ($\leq$20%) of a species we believe to be the cyclic trisulfide, 4,4-dimethyl-1,2,3-trithian (**7** in Figure 1), although this has not been authenticated by synthesis.

Δ^3-Isopentenyl methyl sulfide (IPMS-6 in Figure 1) is a urinary constituent of the red fox (*Vulpes vulpes*) (Jorgenson et al., 1978) that has been shown to be effective either alone or in combination with other chemicals in reducing feeding damage by voles (*Microtus montanus, M. pennsylvanicus*) pocket gophers (*Thomomys talpoides*), and red squirrels (*Tamiasciurus hudsonicus*) (see Sullivan et al., 1988, for example). This compound was prepared by methanethiolysis of Δ^3-isopentenyl tosylate by a published method (Wilson et al., 1978) but using DMF rather than HMPA as solvent.

Our attempts to prepare 5-thiomethylpentan-2,3-dione (**8** in Figure 1) by either of the two methods outlined by Wheeler et al. (1975), and not since published in detail, were unsuccessful. This compound, which would represent an unusual member of the carnivore odor arsenal, has been isolated from the anal scent gland of the striped hyena (*Hyaena hyaena*).

The proprietary repellent formulations we tested were Treepel (Aorangi Forestry Services, Waimate), which contains a mixture of egg powder and adhesive (Crozier, 1991); Ro-pel, which contains the bitter compound denatonium saccharide (DS) (Nolte et al., 1994a); and Gori, which contains the bitter compound denatonium benzoate (DB). Two other compounded formulations were tested. These were: (1) a form of synthetic fermented egg (SFE) concocted by mixing the eight predominant odor components used by Bullard et al. (1978) and modified to minimize phytotoxicity before being administered, as a 5%

w/v solution in paraffin oil, and as a 1:1 mixture of petroleum jelly, and (2) olive oil in petroleum jelly (PJO), which we have already shown (unpublished observations) to be highly repellent to rabbits, possibly as a result of the presence of plant defense compounds.

Trial Design. The compounds and preparations were tested in pen trials by spraying them at a rate of 5 ml/seedling directly onto the foliage of *Pinus radiata* seedlings. These were of a uniform size ($\leq$45 cm) to permit a ready assessment of the extent of browse. Three adjacent pens (each 30 $\times$ 5 m) with open gates between them to permit free access were used in all trials. Three groups of six possums were used in rotation to minimize the likelihood of any of them becoming desensitized towards repellents. The animals used a sheltered area with next boxes at one end of each pen during the daytime and were provided with supplementary feed pellets and apples, as well as grass and clover growing in the pens, and a continuous supply of fresh water.

In each of the trials, a row of seedlings was planted in each of the outer pens, one to be treated and one to function as a control, no seedlings were planted in the center pen so that treated and control groups were separated. This arrangement was designed to simulate a field application of repellents to groups of seedlings, where each treated seedling reinforces the effect of the group. At the commencement of the trials, 25 seedlings were planted in each row but in later trials only 15 were planted as they were by this stage approximately 60 cm in height. These measures ensured that feeding pressure on foliage was maintained at a similar level that was deliberately greater than expected in the wild to ensure a rapid aquisition of results. In the first and last trials, untreated seedlings were planted in both end pens to determine whether or not possums demonstrated a preference for feeding in either pen. In the second trial, the repellent action of Treepel was evaluated and confirmed (Table 1) to be repellent to possums. Consequently, this was then used as a positive control in all other trials.

The height of each seedling was measured directly after planting, and the proportion of each seedling left unbrowsed was assessed daily until only about 10% seedling foliage remained in one of the pens. The duration of each trial was about 7–10 days. Trials were started only during fine weather, and rainfall was measured on a daily basis before assessing seedlings. The percentage of each seedling left unbrowsed each day was plotted as a function of time. The mean area under the curve (AUC) for treatments was divided by the mean AUC for controls as a combined measure of treatment effectiveness and durability in relation to Treepel. Values in excess of unity therefore indicate that treatments are more effective than Treepel. The mean AUC's for treatments and controls were also compared by ANOVA to test for significant differences in effectiveness.

TABLE 1. TRIAL DETAILS AND EFFECTIVENESS OF REPELLENTS RELATIVE TO CONTROL

Compounds tested		Mean AUC treatment/mean AUC control	P
Treatment	Control		
Untreated	Untreated	1.08	0.21
Treepel	Untreated	1.45	<0.001
Predator odors[a]			
DMDT	Treepel	2.29	<0.001
PT	Treepel	1.78	<0.001
DMT	Treepel	1.13	0.15
PDT	Treepel	1.03	0.65
PMS	Treepel	1.30	0.006
TMT	Treepel	0.60	<0.001
Proprietary repellents			
Gori (DB)	Treepel	2.23	<0.001
Ropel (DS)	Treepel	0.36	<0.001
Other compounds			
SFE	Treepel	2.23	<0.001
PJO	Treepel	0.50	<0.001
Untreated	Untreated	1.04	0.34

[a]See Methods and Materials for compound identification.

RESULTS AND DISCUSSION

The extent of browsing upon untreated seedlings in each of the two end pens in the trials at the beginning and at the end of the study did not differ significantly (Table 1). Since both test and control compounds applied to seedlings in both pens in most trials proved to be repellent, there was evidently no strong inducement for possums to develop any preference for feeding location.

Three of the predator odor compounds were more effective repellents than Treepel, the most effective being the DMDT, which still remained its activity at the end of the trial when the control seedlings had been almost completely eaten (Figure 2a). Moreover, rain on three days during the trial caused no apparent loss in the repellency of this preparation. Both PT and IPMS were also shown to be more repellent to possums than the control but seedlings treated with PDT and DMT were browsed upon to the same extent as the control-treated plants. The TMT was significantly less repellent than Treepel (Table 1).

These variations in repellent effectiveness over the duration of the trial are unlikely to be solely due to differences in odor strength or volatility: there is no obvious correlation between boiling point, for example, and the apparent order of effectiveness of these predator odors. Treatment effects might, in fact, be

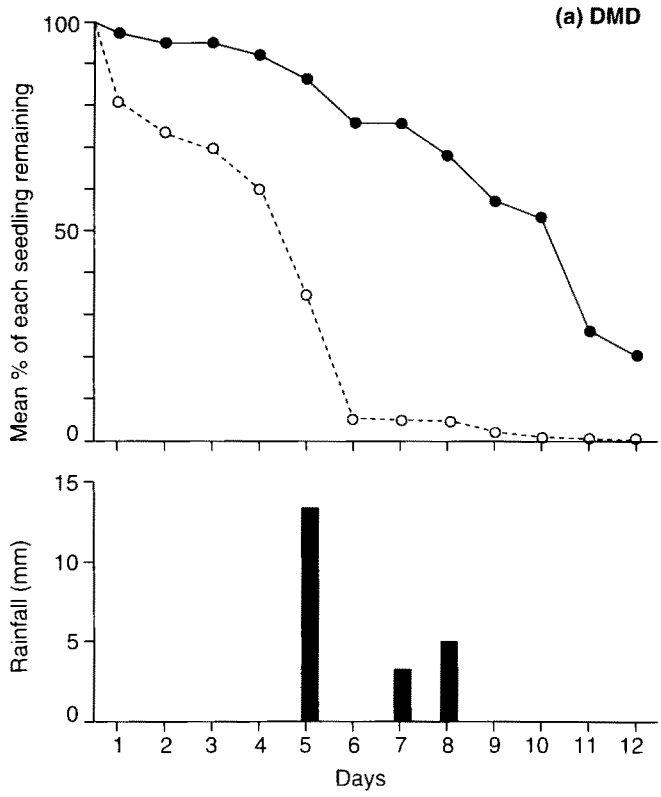

FIG 2. Effectiveness of four repellent compounds (bold line) compared to Treepel (broken line) and daily rainfall.

more dependent upon the sympatric or allopatric nature of the evolution of predator and prey, the duration of the association, and the ability of the prey to learn to avoid predator odors (Swihart et al., 1991). Possums in Australia (where they are endemic) did not evolve sympatrically with foxes and mustelids, but only came into contact with these predators after the predators were introduced by Europeans. Although possums in New Zealand could have learned to avoid mustelids, with which they have been associated for some 200 years, by odor detection, such a response to foxes could not have been learned. This might explain why TMT was the least effective repellent odor in these trials. While there are a few reported instances at an experimental level in which predator odors have no apparent effect upon certain herbivorous prey species (Stoddart, 1976; Gorman, 1984), there is wide support (present study included) for the

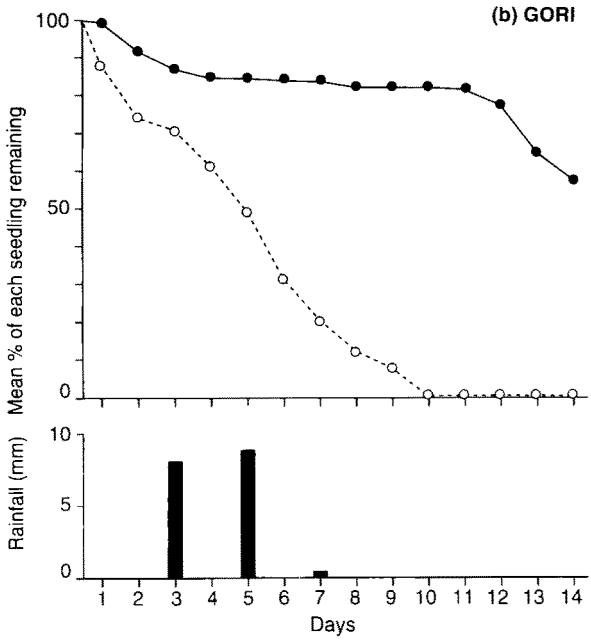

FIG. 2. Continued

suggestion that herbivore avoidance of carnivore odor(s) is inate and generalized rather than being a conditioned response to a specific predator (Stoddart 1982a,b; Dickman and Doncaster, 1984; Gorman, 1984; Robinson, 1990; Kavaliers, 1990; Weldon et al., 1993; Boag and Mlotkiewicz, 1994).

The mechanism of this avoidance behavior is most probably by recognition of the odor of sulfurous by-products of meat digestion. For example, when such sulfurous compounds were removed from coyote urine, it was far less aversive to mountain beaver than whole urine (Nolte et al., 1994b). However, the identity of the active sulfur-containing compounds in coyote urine has not yet been determined. C-S lyase enzymes are known that catalyze fragmentations of naturally occurring *S*-substituted cysteines to produce thiols, and therefrom sulfides and higher ogligosulfides. The apparently unique role of L-cysteine as the only biosynthetic means of introducing sulfur into organic molecules has been noted by Kjaer (1977).

The fate of ingested proteinaceous sulfur is apparently diverse. Whereas mustelid (sloat, ferret, mink) 'odor' compounds (e.g., DMT, DMDT, PT, PDT) or their respective precursors are apprently uniquely present as components of

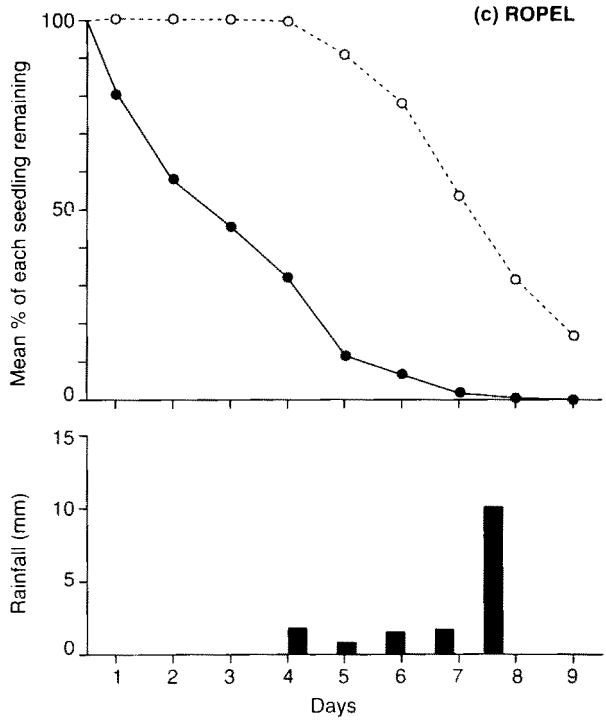

FIG. 2. Continued

anal sac secretions, TMT and IPMS are uniquely associated, the former with fox feces and the latter with fox urine. The many organosulfur compounds associated with skunk (*Mephitis mephitis*) also are apparently uniquely associated with anal sac secretions (Andersen et al., 1982). Similarly, the sulfur-containing aminoacid L-felinine [*S*-(1,1-dimethyl-3-hydroxypropyl)cysteine] is uniquely present as a component of the urine of felids (Datta and Harris, 1953), and 5-thiomethylpentan-2,3-dione is a component of the urine of hyena (*Hyaena hyaena*) (Wheeler et al., 1975). Moreover, L-felinine is believed (but not proven) to be the precursor to a number of interesting sulfur-containing compounds in cat urine (*Felis domesticus*) (Joulain and Laurent, 1989) and in bobcat urine (*Lynx rufus*) (Mattina et al., 1991).

Of the two proprietary repellents evaluated, Gori was more effective than Treepel, whereas Ro-pel was less effective (Table 1, Figures 2b and c). Like DMDT, Gori remained effective after the control-treated seedlings had been eaten, despite rain on two days. Ro-pel was ineffective with possums. Treated

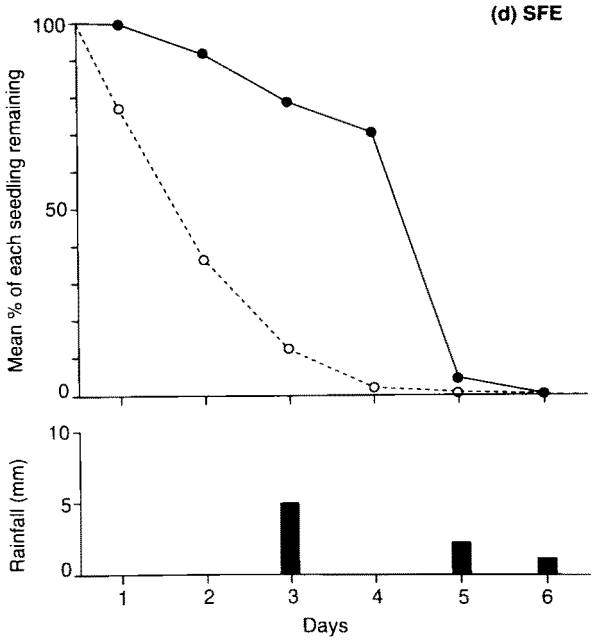

FIG. 2 Continued

seedlings were browsed immediately, while the treated controls were unbrowsed for four days. Both Gori and Ro-pel contain bittering agents. That present in Gori (DB) is much more effective in this formulation than the DS present in Ro-pel. Bitrex is known to be some 50 times more bitter than alkaloids such as brucine and strychnine, which occur naturally in plants and protect them from browsing animals (Payne, 1988). However, herbivores vary in their responses to bittering agents. Guinea pigs (*Cavia porcellus*), for example, are indifferent to DB and DS, and some rodents avoid DS, while others demonstrate a preference for it (Nolte et al., 1994a). While herbivore responses to plant defensive compounds may have developed partly as a result of evolutionary selection (Lindroth, 1988), the lack of uniformity of response to bitter compounds compared with their more generalized response towards sulfurous predator odors suggest that more diverse biochemical pathways have developed in plant chemical defense. Thus, as pointed out by Nolte et al., (1994b), it is unlikely that broadly effective herbivore repellents will be developed using bitter compounds.

Of the other compound tested, SFE was more effective as a repellent than Treepel (Figure 2d), but PJO was much less effective than the control. While the notion is yet untested, the effectiveness of SFE is probably attributable to

the odor profile produced by the four (major) constituents, three unsaturated fatty acids and trimethylamine, which are all found in anal sac secretions of canids (Bullard et al., 1978). The initial activity attributable to PJO might be vitiated by the rapid rancidification typical of triacylglycerides that contain high levels of unsaturated fatty acids.

The use of fear- or avoidance-inducing odors as repellents to minimize seedling predation by herbivores, in general, and possums, in particular, is an important strategy for consideration in any integrated program of pest control. This study has identified several carnivore-derived chemicals that could be further developed as possum repellents. Combinations of these compounds may prove to be even more effective, if, for example, they were used in conjunction with a bittering agent. For example, DB (which is repellent to possums) could be used to reinforce the signal presented by the repellent odor of a compound such as DMDT or that produced with an SFE chemical "cocktail." There is the very real possibility also that, in the wild, the mustelid odors might prove to be even more effective in reducing damage by actually attracting additional predators to the area (Sullivan et al., 1988). Our research will now focus upon the formulation of such combinations with adhesives and surfactants with the express purpose of evaluating habituation effects and of extending the field life of repellent applications to perfect an overall cost-effective means of delivery.

Acknowledgments—This study was funded by the Foundation for Research, Science, and Technology in New Zealand. We wish to acknowledge the assistance of W. Townsend for providing assistance in possum husbandry and pretrialing, and of C. Frampton for statistical advice. A.D.W. acknowledges the forebearance of his colleagues within Industrial Research Limited during the synthesis component of this research effort.

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STRUCTURE-RELATED INSECT ANTIFEEDANT AND GROWTH REGULATING ACTIVITIES OF SOME LIMONOIDS

T.R. GOVINDACHARI,* N.S. NARASIMHAN, G. SURESH, P.D. PARTHO, GEETHA GOPALAKRISHNAN, and G.N. KRISHNA KUMARI

Centre for Agrochemical Research
SPIC Science Foundation
110, Mount Road, Madras 600 032, India

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Abstract—Insect antifeedant and growth-regulating activities of 22 limonoids (both natural and their derivatives) against *Spodoptera litura* were studied to understand the structure-related bioactivities of the limonoids. The C-seco limonoids (azadirachtins A, B, D, H, and I) were the most effective compounds as a group, while the intact limonoids (cedrelone and its derivatives) were the least effective. The cyclohexenone A ring and the α-hydroxy enone group in the B ring appear to be important for antifeedant activity. The presence of a cyclohexenone or 1,2-epoxide in the A ring coupled with an α-hydroxy enone in the B ring correlated well with growth regulatory activity. An acetoxy at C-7 instead of α-hydroxy enone and perhaps the carbonyl at C-16 increase growth regulatory activity. Absence of 14–15 epoxide may not drastically reduce antifeedant activity and growth regulatory activity.

Key Words—Antifeedants, growth regulation, limonoids.

INTRODUCTION

The species of Meliaceae, a family of both herbaceous and arborescent forms, elaborate limonoids (tetranortriterpenoids) of diverse structural types. Over 300 limonoids have been isolated to date (Champagne et al., 1992). These compounds, which are bitter tasting, have attracted much attention due to bioactivities observed in over 70 of them. While it is presumed that limonoids function

*To whom correspondence should be addressed.

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primarily as insect antifeedants, some interfere with insect development and ecdysis. Azadirachtin, a C-seco limonoid, and some of its derivatives possess both antifeedant and insect growth-regulating activities. Azadirachtin at different concentrations, either in pure form or as an active ingredient in mixtures, is active against over 200 insect species (Saxena, 1989). The only group other than the C-seco limonoids known to possess insect growth-regulating (IGR) activity is the intact apo-euphol limonoids, with a 14–15 epoxide and a cyclohexenone (3-oxo-1-ene) A ring, as in cedrelone and anthothecol.

Although these structural features have been assumed to impart IGR activities, simpler compounds, such as nimocinolide and isonimocinolide, which lack the 14–15 epoxide, also have IGR activities (Naqvi, 1987). Studies, however, are restricted only to a few related series of compounds (Blaney et al., 1990), and comparative data on the bioactivities of different limonoids also are limited (e.g., Klocke, 1987). Indeed, the understanding of quantitative structure-related bioactivities in limonoids suffers due to differences in bioassay methods and the choice of insect and larval instars used with different compounds. These limitations indicate the need for a more thorough examination of limonoids, both for antifeedant and for IGR activities.

Cedrelone, an intact apo-euphol type limonoid, isolated from *Cedrela toona* (=*Toona ciliata*) was shown to have IGR activities against some Lepidoptera (Kubo and Klocke, 1986). When administrated orally, topically, or via injection, cedrelone induced mortality, larval growth inhibition, and molt inhibition in *Peridroma saucia* and *Mamestra configurata* but failed to show any appreciable antifeedant activity (Koul and Isman, 1992). As cedrelone can be isolated in large quantity, we made certain synthetic modifications in its structure and tested the compounds against the tobacco cutworm *Spodoptera litura*, in order to understand its structure-related antifeedant and IGR activities. Azadiradione and epoxy-azadiradione (two intact limonoids from *Azadirachta indica*); ocotillone, dymalol, and shoreic acid (protolimonoids from *Dysoxylum malabaricum*); piscidinol C (a protolimonoid from *Walsura piscida* with a 7-membered lactol acetate instead of a furan ring); azadirachtins A, B, D, H, and I (C-seco limonoids from *A. indica*); and limonin (an A-seco limonoid from *Citrus* sp.) were also studied for comparison. The results of this study are presented here.

METHODS AND MATHERIALS

Compounds. Cedrelone was isolated from the powdered wood of *Cedrela toona* by repeated hexane extractions. After concentration in vacuo in a rotary evaporator, cedrelone crystallized. The crystals were captured by filtration, and cedrelone was repeatedly recrystallized from benzene (Hodges et al., 1963). The purity of the sample was confirmed by analytical HPLC (Shimadzu LC 10 AD, Shimpack RP-18 column, 25 cm × 4.6 mm, with 60:40 methyl alcohol–

water (MeOH : H_2O) as eluent, 1 ml/min flow, 215 nm as the detection wavelength). From cedrelone, cedrelone methyl ether, cedrelone acetate, cedrelone epoxide, cedrelone epoxide methyl ether, cedrelone epoxide acetate, dihydrocedrelone, dihydrocedrelone acetate, isocedrelonic acid, and methyl isocedrelonate were prepared (Gopinath et al., 1961; Hodges et al., 1963). The purity of these compounds was checked by analytical TLC on precoated silica plates (Merck) using 20% ethyl acetate (EtOAc) in hexane as the eluent. Azadirachtins A, B, D, H, and I (Govindachari et al., 1991), azadiradione, and epoxy-azadiradione were isolated from neem seed kernel extract by preparative HPLC (Govindachari et al., 1992). Dymalol, shoreic acid and ocotillone were isolated from the leaves of *Dysoxylum malabaricum* (Govindachari et al., 1994a). Piscidinol-C was isolated from the leaves of *Walsura piscida* (Purushothaman et al., 1985; Govindachari et al., 1995). The structures of the compounds used in this study are shown in Figure 1.

Insects. The test insect, *Spodoptera litura*, was reared in the laboratory on *Ricinus communis* (castor) leaves at 25 ± 2°C.

Antifeedant Studies. A dual-choice bioassay was performed for antifeedant tests. Circular discs (180 cm^2) were cut from the leaves of *Ricinus communis*, with the median vein as the marker between the two equal halves. One milliliter of the test compound in acetone (at 50, 10, 5, and 1 μg concentrations/cm^2 area of leaf) was spread using a fine pipet on the right half (90 cm^2 treated). The left half served as the untreated control. In one treatment, 1 ml of acetone alone was spread on the right half, leaving the left half untreated. The latter served to show the solvent effect. For each compound and concentration, five replicates were used. After air drying, each leaf disc was placed inside separate Petri dishes, and five freshly molted third instars (from the same egg batch, approximately similar in weight and size) were placed at the center of the leaf. The Petri dishes were then closed and kept undisturbed. After 24 hr, the insects were removed and the unfed area of both halves of the leaf was measured using a ΔT area measurement meter. The raw data were subjected to analysis of variance and Neuman-Keul means were derived. Using the Neuman-Keul means, percentage of antifeedant activity was calculated using the formula

$$\text{Antifeedant activity (\%)} = 100 - \frac{\text{area fed in treated}}{\text{area fed in untreated control}} \times 100$$

where treated is treated with compound in 1 ml of acetone or treated with 1 ml of acetone only.

Using the Neuman-Keul means, the percentage feeding index (PFI) was also calculated (cf., Luco et al., 1994) using the formula

$$\text{PFI} = \frac{\text{area fed in treated}}{\text{area fed in treated} + \text{area fed in solvent control}} \times 100$$

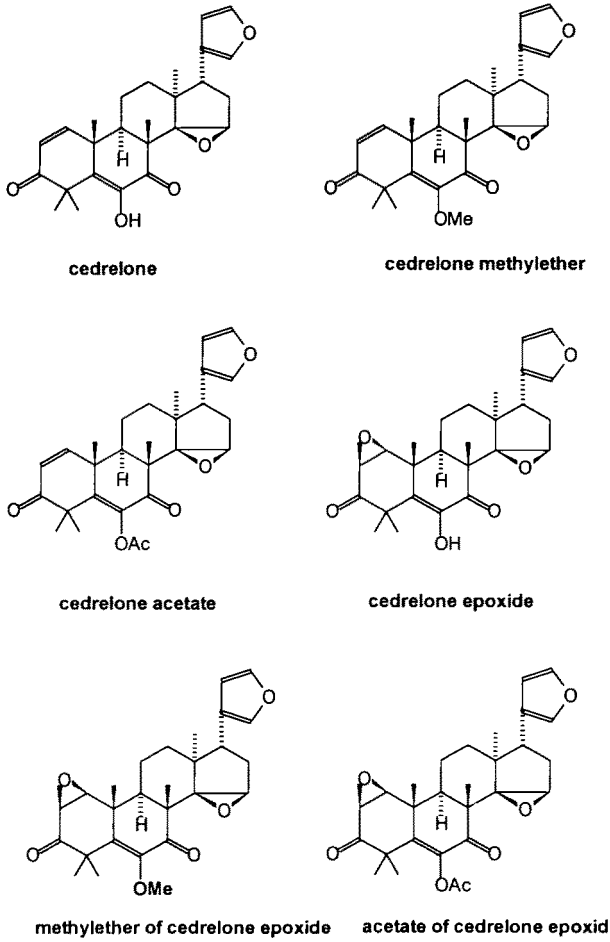


FIG. 1. Limonoids used in the study.

where treated is treated with compound in 1 ml of acetone, and solvent control is treated with 1 ml of acetone only

Insect Growth Regulation Studies (IGR). For insect growth regulatory (IGR) activity, leaf discs (180 cm^2) were spread with 2 ml of the test compound in acetone to give 10 $\mu g/cm^2$ leaf for cedrelone and its derivatives; 1 $\mu g/cm^2$ for azadiradione, epoxy-azadiradione, limonin, piscidinol-C, dymalol, shoreic acid, and ocotillone; and 0.5 $\mu g/cm^2$ for azadirachtins. The differences in concentrations for different compounds were designed to take into account variation in

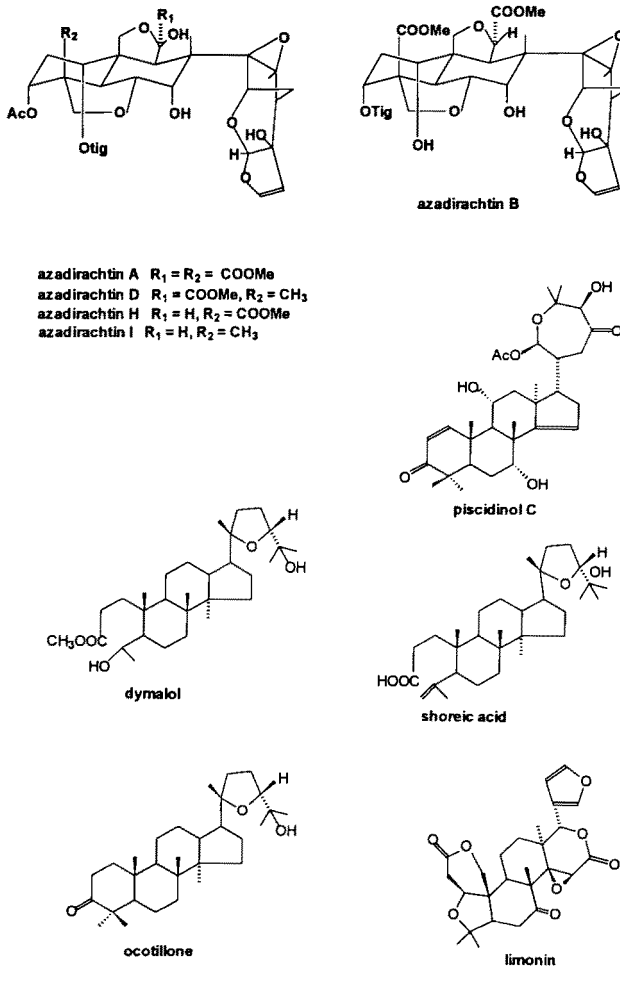
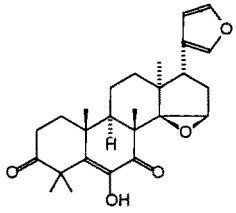
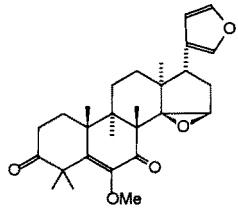


FIG. 1. Continued

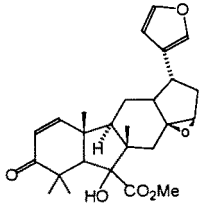
the antifeedant activities of the compounds. The leaves were then air dried and placed in separate Petri dishes. Five replicates were maintained for each compound. Five freshly emerged third instars were introduced into the Petri dish and allowed to feed until they molted into the fourth instar. Fourth instars then were provided with normal diet (*R. communis* leaves) until the end of the experiment. Duration of instars, mortality of larvae and/or pupae, and pupal weight were recorded.



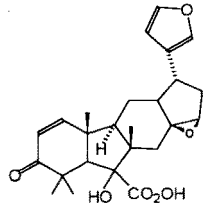
dihydrocedrelone



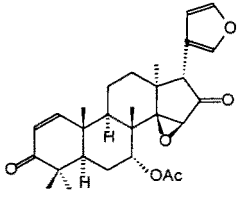
methylether of dihydrocedrelone



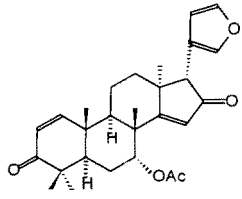
methylisocedrelonate



isocedrelonic acid



epoxy azadiradione

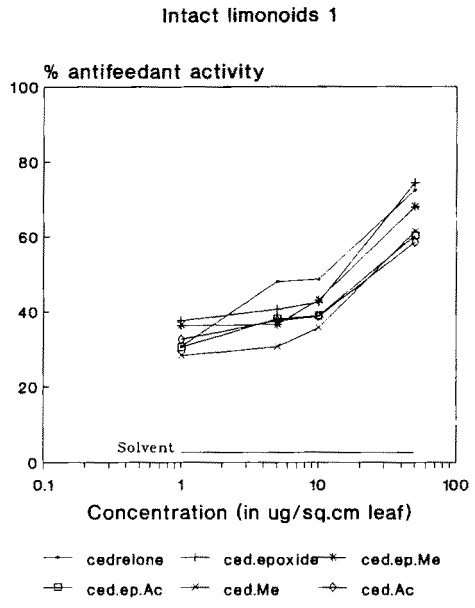


azadiradione

FIG. 1. Continued

RESULTS AND DISCUSSION

Antifeedant Activity. Cedrelone and its derivatives showed appreciable antifeedant activity only at a concentration of 50 $\mu g/cm^2$ of leaf. The degree of antifeedant activity was concentration dependent. Among this group, cedrelone showed maximum antifeedant activity (Figure 2). Comparing the compounds at the highest concentration tested (50 $\mu g/cm^2$), a clear-cut trend could be observed. Compounds in which the 6-OH group is replaced with -OMe or -OAc groups,



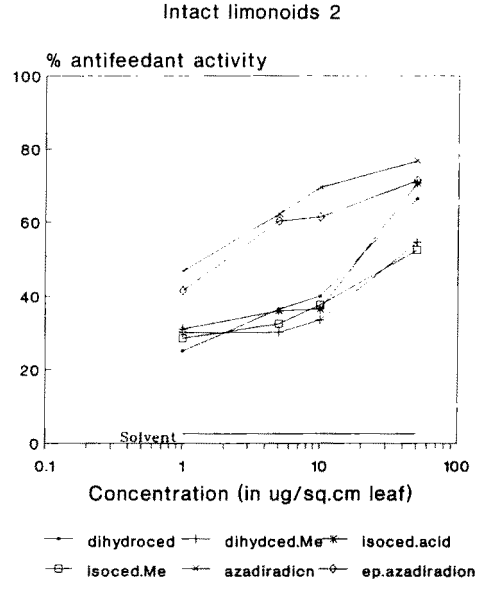
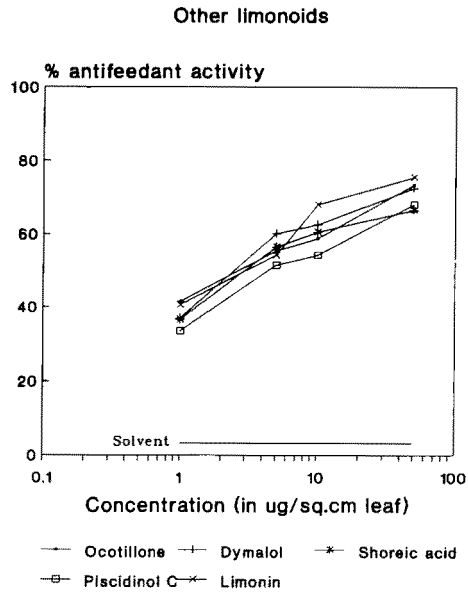


FIG. 2. Percent antifeedant activity of limonoids. Solvent line in graph indicates % antifeedant activity of acetone.



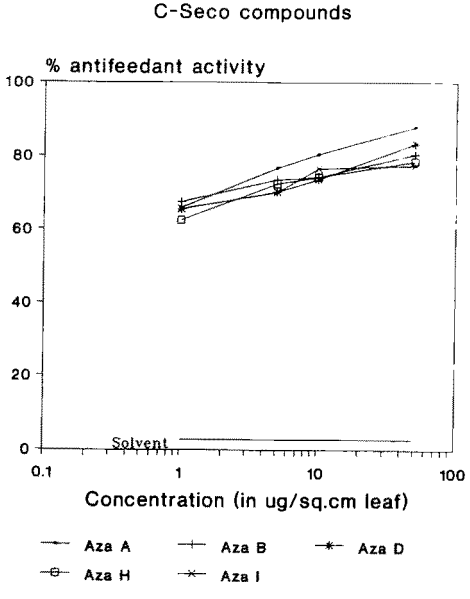


FIG. 2. Continued

irrespective of modifications in the A ring, reduced the antifeedant activity considerably. Presumably this indicated a hydrophilic binding/reactivity site in this part of the molecule. With α-hydroxy enone in ring B intact, introduction of an epoxide at C-1–C-2 position or removal of the C-1–C-2 double bond (e.g., dihydrocedrelone) reduced antifeedant activity. This suggested that the cyclohexenone (3-oxo-1-ene) A ring was important for imparting antifeedant activity.

A comparison of the antifeedant activities of cedrelone and its derivatives with both azadiradione and epoxy-azadiradione was meaningful as all have a cyclohexenone A ring. Both azadiradione and epoxy-azadiradione showed higher antifeedant activity compared to cedrelone and its derivatives, even at 5 $\mu g/cm^2$ area (cedrelone showed comparable antifeedant activity only at 50 $\mu g/cm^2$ area). Differences between azadiradione and epoxy azadiradione antifeedant activity are not statistically significant. Structural comparison between these two compounds indicated that the presence or absence of the C-14–C-15 epoxide may not drastically change the antifeedant activity. The experiment also reaffirmed the point that the presence of the cyclohexenone A ring (a common feature in all these intact limonoids tested) with a C-6 hydroxy group in the B ring was a requirement for antifeedant activity. Modifications of the B ring seem to affect antifeedant activity very much, as exemplified by isocedrelonic acid and its methyl ester.

Ocotillone, dymalol, and shoreic acid showed excellent PFI (percentage feeding index) values comparable to azadiradione, epoxyazadiradione, and limonin (Table 1). Protolimonoids like meliantriol and melianone are known to possess good antifeedant activities (Lavie et al., 1967) as does limonin, an A-seco limonoid (Liu et al., 1990).

Azadirachtins (C-seco compounds) are highly modified structurally and it may be incorrect to compare them with the other compounds tested. Indeed, the azadirachtins stand out as the most effective antifeedants. The PFI values (Table 1) even at 1 $\mu g/cm^2$ were very low, indicating high antifeedant activity. As shown earlier (Govindachari et al., 1994b), a trend towards decreasing antifeedant activity was noticed with the five compounds tested, the order being azadirachtin A > azadirachtin B > azadirachtin D > azadirachtin H > azadirachtin I.

Growth Regulation. The concentrations of each compound used for IGR testing had to be fixed based on its antifeedant activity ranking. The percentage antifeedant activity of each compound at different concentrations was plotted, and the concentration at which less than 50% antifeedant activity was detected was chosen for further testing. Based on this information, the compounds tested were grouped into three categories. Category 1 consisted of cedrelone and its derivatives, where less than 50% antifeedant activity was noticed at concentrations of 10 $\mu g/cm^2$ of leaf. Category 2 consisted of azadiradione, epoxyazadiradione, dymalol, ocotillone, shoreic acid, limonin, and piscidinol-C, where

TABLE 1. PERCENTAGE FEEDING INDEX (PFI) OF *Spodoptera litura* FED ON DIFFERENT LIMONOIDS[a]

Compound	1 $\mu g/cm^2$	5 $\mu g/cm^2$	10 $\mu g/cm^2$	50 $\mu g/cm^2$
Ocotillone	44.5(6.4)	43.2(5.0)	30.8(12.4)	27.5(5.4)
Dymalol	44.4(3.7)	40.5(6.5)	27.5(7.1)	24.9(7.4)
Shorcic acid	48.2(5.4)	44.2(8.2)	25.4(8.4)	24.5(5.6)
Cedrelone	51.5(2.9)	50.5(7.8)	44.9(7.3)	24.2(6.2)
Cedrelone methylether	56.0(6.6)	49.5(8.0)	51.5(5.1)	42.8(4.5)
Cedrelone acetate	55.0(7.5)	54.8(4.4)	43.0(6.8)	38.5(8.2)
Cedrelone epoxide	43.5(5.0)	40.8(12.6)	41.5(8.5)	35.5(6.9)
Cedrelone epoxide-meth.ether	54.0(9.5)	48.4(12.0)	47.6(6.8)	37.1(5.9)
Cedrelone epoxide-acetate	53.2(9.0)	47.6(3.4)	40.8(5.4)	40.9(7.5)
Dihydrocedrelone	50.5(6.3)	42.9(4.5)	29.5(9.8)	32.3(6.8)
Dihydrocedrelone methylether	56.5(10.6)	55.5(5.0)	43.5(4.2)	35.5(8.2)
Isocedrelonic acid	57.4(7.3)	53.5(12.0)	40.2(6.0)	33.6(5.8)
Methylisocedrelonate	54.8(5.0)	47.5(7.5)	41.5(9.0)	25.9(9.3)
Azadiradione	42.5(9.5)	39.0(7.2)	25.9(6.3)	23.0(5.5)
Epoxyazadiradion	48.5(7.8)	38.6(10.5)	35.2(4.2)	27.0(7.3)
Piscidinol C	52.5(12.6)	48.5(6.5)	38.4(8.9)	37.6(7.0)
Limonin	44.6(4.6)	40.3(7.1)	30.9(4.5)	25.2(6.5)
Azadirachtin A	27.5(4.0)	17.9(6.6)	16.5(7.8)	11.5(4.8)
Azadirachtin B	26.7(1.7)	25.0(9.2)	21.6(3.9)	18.0(8.5)
Azadirachtin D	28.9(3.2)	29.5(3.6)	19.0(6.8)	20.9(4.5)
Azadirachtin H	30.5(3.0)	33.4(2.8)	24.4(5.5)	24.0(7.6)
Azadirachtin I	32.2(6.4)	35.6(4.5)	25.0(4.0)	23.5(6.5)

[a]Concentrations per square centimeter of *Ricinus communis* leaf area; data indicate mean of five replicates, each replicate had five individuals of the same age; values in parentheses indicate standard deviation.

less than 50% antifeedant activity was noted at concentrations 1 $\mu g/cm^2$ of leaf. Category 3 consisted of azadirachtins A, B, and D, where more than 50% antifeedant activity was evident even at 1 $\mu g/cm^2$ of leaf. Based on these results, concentrations to be used for growth regulation studies were fixed at 10 $\mu g/cm^2$ leaf area for category 1 compounds, 1 $\mu g/cm^2$ for category 2, and 0.5 $\mu g/cm^2$ for the azadirachtins.

Larval duration during development, as an indication of growth regulation, was studied by feeding third instars with treated food until they molted. In the case of cedrelone and its derivatives, comparison of data indicated that differences were marked only in the fifth instar. The compounds that were effective in extending the fifth instar were cedrelone, cedrelone epoxide, and cedrelone epoxide methyl ether (Table 2). The same compounds also brought about significant reductions in pupal weight and higher pupal mortality (Table 5). This

TABLE 2. DURATION OF DEVELOPMENT OF DIFFERENT INSTARS OF *Spodoptera litura* FED ON CEDRELONE AND ITS DERIVATIVES[a]

Compound	Duration of development (days ± SD)				
	3rd instar	4th instar	5th instar	6th instar	Pupae
Control	3.44 ± 0.62 a	3.2 ± 0.7 a	4.48 ± 0.71	3.2 ± 0.41 a	8.28 ± 0.54 a
Cedrelone	4.54 ± 0.79 c	4.36 ± 1.0 c	6.55 ± 1.19 a, b	3.72 ± 0.82 b	8.55 ± 0.78 a
Cedrelone methylether	3.56 ± 0.87 a, b	3.52 ± 0.86 a, b	5.6 ± 0.86 d	3.25 ± 0.44 a	8.29 ± 0.65 a
Cedrelone acetate	4.0 ± 1.2 d	3.4 ± 0.66 a	6.12 ± 1.12 c	3.42 ± 0.47 a	8.40 ± 0.65 a
Cedrelone epoxide	4.34 ± 0.71 c	4.5 ± 0.91 c	6.9 ± 0.64 b	3.35 ± 0.48 a	8.4 ± 0.68 a
Cedrelone epoxide-methylether	4.45 ± 0.72 c	4.62 ± 0.82 c	7.27 ± 0.88	3.71 ± 0.78 b	8.32 ± 0.43 a
Cedrelone epoxide-acetate	3.8 ± 0.82 a, b	3.72 ± 0.76 b	6.22 ± 0.92 c	3.58 ± 0.67 b	8.47 ± 0.60 a
Dihydrocedrelone	4.28 ± 1.04 d	4.3 ± 1.03 c	6.45 ± 1.29 a, c	3.28 ± 0.85 a	8.42 ± 0.48 a
Dihydrocedrelone methylether	3.56 ± 0.82 a, b	3.36 ± 0.63 a	5.12 ± 0.72	3.20 ± 0.52 a	8.28 ± 0.78 a
Isocedrelonic acid	3.84 ± 0.95 a, b	3.87 ± 1.19 b	6.04 ± 1.50 c	3.31 ± 0.56 a	8.32 ± 0.81 a
Methylisocedrelonate	3.96 ± 1.0 b	3.50 ± 0.74 a, b	5.73 ± 1.17 d	3.35 ± 0.67 a	8.33 ± 0.61 a

[a]Data collected with concentration of compound at 10 $\mu g/cm^2$ leaf area fed in the third instar. In columns, values followed by the same letter are not statistically significant at $P = 0.05$. Data indicate mean of 18–25 individuals.

TABLE 3. DURATION OF DEVELOPMENT OF DIFFERENT INSTARS OF *Spodoptera litura* FED ON SOME LIMONOIDS[a]

Compound	Duration of development (days ± SD)				
	3rd instar	4th instar	5th instar	6th instar	Pupae
Control	3.46 ± 0.5 b	3.60 ± 0.45 d	4.50 ± 0.82 c	3.36 ± 0.78 c	8.31 ± 0.46 a
Azadiradione	4.60 ± 0.75	4.95 ± 0.76 b	7.20 ± 0.6	3.90 ± 0.5 b	8.38 ± 0.51 a
Epoxyazadiradione	4.20 ± 0.86 a	4.50 ± 0.45 c	6.50 ± 0.41 b	3.60 ± 0.87 c	8.40 ± 0.45 a
Ocotillone	3.50 ± 0.82 b	3.82 ± 0.74 d	5.50 ± 0.70 a	3.20 ± 0.66	8.40 ± 0.44 a
Dymalol	3.80 ± 0.90 b	3.80 ± 0.6 d	5.80 ± 0.54	3.60 ± 0.8 c	8.38 ± 0.5 a
Shoreic acid	3.80 ± 1.10 b	3.95 ± 0.84 a	5.30 ± 0.60 a	3.70 ± 0.5 a	8.36 ± 0.54 a
Limonin	4.10 ± 1.45 a	4.71 ± 0.8 c	6.90 ± 0.78 b	3.50 ± 0.6 a, c	8.40 ± 0.4 a
Piscidinol C	3.60 ± 0.8 b	4.0 ± 0.55 a	5.60 ± 0.58 a	4.00 ± 0.4 b	8.36 ± 0.5 a

[a]Data collected with concentration of compound at 1 $\mu g/cm^2$ leaf area fed to the 3rd instar. In columns, values followed by the same letter are not statistically significant at $P = 0.05$. Data indicate mean of 21–25 individuals.

TABLE 4. DURATION OF DEVELOPMENT OF DIFFERENT INSTARS OF *Spodoptera litura* FED ON SOME LIMONOIDS[a]

Compound	Duration of development (days ± SD)				
	3rd instar	4th instar	5th instar	6th instar	Pupae
Control	3.46 ± 0.5	3.60 ± 0.45	4.50 ± 0.82	3.36 ± 0.78	8.31 ± 0.46
Azadirachtin A	4.20 ± 0.86 a	4.50 ± 0.62 a, b	6.80 ± 0.85 a	3.80 ± 0.41 a	8.45 ± 0.52 a
Azadirachtin B	4.00 ± 0.92 a	4.40 ± 0.52 a	6.90 ± 0.82 a	3.70 ± 0.55 a	8.34 ± 0.62 a
Azadirachtin D	3.92 ± 0.43 a	4.40 ± 0.68 a	6.60 ± 0.78 a	3.50 ± 0.6 a	8.42 ± 0.5 a
Azadirachtin H	3.96 ± 0.50 a	4.58 ± 0.72 a, b	6.50 ± 0.70 a	3.58 ± 0.66 a	8.50 ± 0.62 a
Azadirachtin I	3.95 ± 0.70 a	4.75 ± 0.40 b	6.58 ± 0.85 a	3.68 ± 0.78 a	8.42 ± 0.55 a

[a]Data collected with concentration of compound at 0.5 $\mu g/cm^2$ leaf area fed to the 3rd instar. In columns, values followed by the same letter are not statistically significant at $P = 0.05$. Data indicate mean of 21–25 individuals.

TABLE 5. PUPAL WEIGHT AND PERCENTAGE MORTALITY OF *Spodoptera litura* FED ON DIFFERENT LIMONOIDS

Compound	Pupal weight (mg ± SD)	Mortality (%)
Solvent control	438 ± 10.6	0
Ocotillone[a]	412 ± 6.8 h	8
Dymalol[a]	428 ± 10.2	8
Shoreic acid[a]	402 ± 14.2	4
Cedrelone[b]	305 ± 12.4	28
Cedrelone methylether[b]	420 ± 10.0 c	8
Cedrelone acetate[b]	356 ± 7.6 d, f	16
Cedrelone epoxide[b]	316 ± 8.8 b	20
Cedrelone epoxide-meth.ether[b]	295 ± 15.7	12
Cedrelone epoxide-acetate[b]	396 ± 6.4	16
Dihydrocedrelone[b]	356 ± 7.6 d	16
Dihydrocedrelone methylether[b]	415 ± 10.6 c, g, h	4
Isocedrelonic acid[b]	386 ± 16.3	12
Methylisocedrelonate[b]	406 ± 12.8 g	20
Azadiradione[a]	312 ± 14.2 b	12
Epoxyazadiradion[a]	348 ± 15.0 f	12
Piscidinol C[a]	435 ± 9.5 a	12
Limonin[a]	375 ± 8.9	16
Azadirachtin A[c]	318 : 12.5 b	16
Azadirachtin B[c]	329 ± 10.5	8
Azadirachtin D[c]	354 ± 9.6 d	4
Azadirachtin H[c]	362 ± 8.5	12
Azadirachtin I[c]	355 ± 10.2 d, f	12

[a] Data collected using 1 μg of compound per square centimeter of castor leaf. Data indicate 15–20 observations. Values followed by the same letter are not statistically significant at $P = 0.05$.
[b] Data collected using 10 μg/cm^2.
[c] Data collected using 0.5 μg/cm^2.

indicated that these were the most effective compounds in category 1. Interestingly, this correlates very well with the earlier deduction that cyclohexenone or 1,2-epoxide in ring A coupled with an α-hydroxy enone in ring B is of importance for growth regulation. A comparison to azadiradione and epoxyazadiradione, although at 1/10th the concentration, indicated that an acetoxy at C-7 instead of a α-hydroxy enone and, perhaps, the carbonyl at C-16 increases growth regulatory activity considerably, indicating the possible active sites. Comparison between azadiradione and epoxyazadiradione indicated again that a C-14–C-15 epoxide may not drastically affect growth regulatory activity in the intact limonoids. This may explain the IGR activities of nimocinolide and

isonimocinolide (Naqvi, 1987), which have a cyclohexenone A ring with OH at C-6 and an acetoxy group at C-7, but lack the C-14–C-15 epoxide. In category 2, azadiradione and epoxy azadiradione showed remarkable growth regulatory activity by significantly increasing the duration of the feeding stages (third, fourth and fifth instars), decreasing pupal weights considerably and inducing pupal mortality (Table 3, Table 5). While azadiradione has been shown not to possess IGR activity against some Lepidoptera (Kubo and Klocke, 1986), in our experiments it showed good IGR activity at 1/10th the concentration of cedrelone. Limonin also showed growth regulatory activity similar to azadiradione and epoxy-azadiradione.

As in the case of cedrelone and its derivatives, the dammaranes as well as piscidinol C also caused pronounced increased duration only of the fifth instar (Table 3). These compounds also allowed comparatively higher pupal weights and reduced mortality, (Table 5) indicating clearly that protolimonoids and piscidinol C are good antifeedants but are not effective growth regulators.

The azadirachtins as a group were the most effective insect growth regulatory compounds, with all five azadirachtins inducing significantly increased duration in the third, fourth, and fifth instars and decreasing pupal weights (indicating reduced fitness) even at a concentration of 0.5 $\mu g/cm^2$ (Table 4, Table 5).

Among the compounds tested, the only other compounds that can be compared to azadirachtins were azadiradione and epoxyazadiradione, which had both higher antifeedant activity and growth regulatory activity; but only at twice the concentrations of azadirachtins.

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CHEMICAL AND EXPERIENTIAL BASIS FOR REJECTION OF *Tropaeolum majus* BY *Pieris rapae* LARVAE

XIN PEI HUANG and J.A.A. RENWICK*

Boyce Thompson Institute at Cornell University
Tower Road, Ithaca, New York 14853

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Abstract—Rejection of nasturtium, *Tropaeolum majus*, by cabbage-reared larvae of *Pieris rapae* has been explained by the presence of feeding deterrents in the nastrutium foliage. Sensitivity to the deterrents develops as neonate larvae feed on cabbage. The most prominent deterrent compound, which is present in nasturtium at a concentration of 40 mg/100 g fresh leaves, was identified as chlorogenic acid. When neonate larvae were fed on a cabbage leaf treated with high concentrations of deterrent-containing extracts of nasturtium foliage, they remained insensitive to the deterrents, so they accepted nasturtium when transferred as second instars. When neonate larvae were reared on a cabbage leaf treated with 0.1 mg chlorogenic acid, ca. 35% of the second instars accepted nasturtium. Similar dietary exposure of neonates to the subunits of chlorogenic acid, caffeic acid and quinic, acid resulted in much less or no effect on the rejection behavior of second instars. The results suggest that the combined effects of specific chemical constituents of nasturtium can explain the rejection of this plant by larvae of *P. rapae*, but if larvae are continuously exposed to these compounds immediately after hatching, they apparently become habituated to the feeding deterrents. The lack of activity of the subunits of chlorogenic acid suggests that specific structural features are necessary for a dietary constituent to cause such habituation or suppression of sensitivity development.

Key Words—*Pieris rapae*, Lepidoptera, Pieridae, *Tropaeolum majus*, nasturtium, feeding deterrent, chlorogenic acid, habituation.

*To whom correspondence should be addressed.

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INTRODUCTION

Responses of insects to plant constituents are believed to be influenced to a large extent by previous experience. Larvae of some lepidopterans are thought to be "imprinted" on the plant on which they emerge from the eggs, resulting in a preference for that early food source (reviewed by Szentesi and Jermy, 1989). The importance of behavior of newly hatched insects when they encounter a plant has therefore been emphasized (Chapman and Bernays, 1989). This behavior may be crucial for survival of the species when a butterfly or moth oviposits on a novel plant. For example, *Pieris* butterflies lay eggs and their larvae feed only on plants that contain glucosinolates, but not all such plants will support larval development (Chew, 1980). However, if an ovipositing female selects a plant that is generally unsuitable for her progeny, the capability of hatching larvae to survive, through habituation or resistance to feeding deterrents or antibiotics in the plant, may enable them to exploit alternative resources.

Recent studies have shown that cabbage-reared larvae of the white cabbage butterfly, *Pieris rapae* L. refused to feed on nasturtium (*Tropaeolum majus* L.), even though ovipositing butterflies readily accepted the plant and hatching larvae fed and developed normally on it (Renwick and Huang, 1995). Induction of food preference (Szentesi and Jermy, 1989) has been ruled out as a possible mechanism for this rejection behavior, since reverse transfer (from nasturtium to cabbage) resulted in normal feeding. Instead, this appears to be a unique phenomenon of diet-based sensory development or suppression. Chemical fractions from solvent extracts of nasturtium foliage showed strong feeding deterrent activity for cabbage-reared larvae. However, nasturtium-reared larvae were only slightly deterred, and larvae reared on a wheat-germ diet were completely unaffected by these deterrents (Renwick and Huang, 1995). This would suggest that cabbage contains some factor that causes feeding larvae to become sensitive to the deterrents. An alternative explanation is that neonate larvae feeding on nasturtium or the diet become habituated to potentially deterrent compounds. A study addressing questions about the mechanisms controlling acceptance or rejection of nasturtium by the larvae would help us to understand the factors involved in host selection by phytophagous insects.

A previous study showed that *Pieris brassicae* selected cabbage leaves over nasturtium leaves for egg-laying, although the nasturtium contained a slightly higher concentration of glucosinolates (Rothschild, 1987). Recently, nasturtium has been found to contain both oviposition stimulants and deterrents, and a balance between positive and negative chemical input is important in selection of this plant by *P. rapae* and *P. napi oleracea* (Huang and Renwick, 1993). The maor stimulant was identified as glucotropaeolin (Sachdev-Gupta et al., 1992), and a combination of several compounds appeared to be responsible for the deterrent activity (Huang and Renwick, unpubl.). Gupta and Maleyvar (1983)

found that *P. brassicae* larvae consumed significantly less tissue of nasturtium than of *Brassica oleracea* var. *gongylodes*. The growth rates and pupal weights of *P. brassicae* were lowest on nasturtium when compared with Brussels sprouts, Swedish turnip, and rape, but larval development rate and adult longevity of the parasitoid, *Cotesia glomerata*, were greatest for wasps from *P. brassicae* reared on nasturtium (Karowe and Schoonhoven, 1992). The phenolic content of leaves from nasturtium and three other host plant species was correlated with the extent of damage by a leaf miner, *Liriomyza brassicae* (Ipe and Sadaruddin, 1984). Although Ma (1972) observed that *P. brassicae* reared on *Brassicae oleracea* refuse to feed on nasturtium, no further work on the mechanism of rejection was performed. The present study was designed to isolate and identify the feeding deterrents to *P. rapae* larvae in nasturtium and to explain the mechanism for diet-dependent differences in sensitivity of larvae to the deterrents.

METHODS AND MATERIALS

Plants and Insects. Cabbage (*Brassica oleracea* L. var. Golden Acre, 4–6 weeks old) and nasturtium (3–4 weeks old) plants for food, extraction, or bioassays were grown in an air-conditioned greenhouse maintained at ca. 25°C, with supplemental light provided by 400-W multivapor high-intensity discharge lamps. *P. rapae* larvae for feeding deterrent assays were obtained from colonies started from field-collected insects each summer and maintained in the laboratory at ca. 22°C under fluorescent lights providing a photoperiod of 16:8 hr light–dark. Larvae were reared on cabbage plants. Naive neonate larvae were obtained by allowing butterflies to oviposit on a strip of Parafilm (Webb and Shelton, 1988).

Extraction and Fractionation of Plant Materials. Fresh nasturtium foliage was extracted in boiling ethanol for 5 min, cooled, homogenized, and filtered. The ethanolic extract was evaporated to dryness under reduced pressure and lipids were removed with *n*-hexane. The defatted residue was dissolved in water and the aqueous extract partitioned three times with 1-butanol. The hexane extract, the butanol extract, and the postbutanol water extract were concentrated under reduced pressure at ca. 50°C and stored in the refrigerator until used.

The deterrent materials in the butanol extract were subjected to preliminary separation by flash column chromatography using 45 × 2-cm reversed-phase columns packed with 30 g 55- to 105-μm preparative C_{18} silica (Millipore Corporation, Milford, Massachusettes 01757). About 100 gram leaf equivalents (GLE) of extract was loaded onto each column. Five fractions (90 ml each) were collected by sequentially eluting the column with 90 ml each of water and 10%, 20%, 50%, and 100% acetonitrile in water. These fractions were tested for deterrent activity.

Thin-Layer Chromatography. Two thin-layer chromatography systems (TLC) were used to compare the isolated compound with a standard: (A) 5 × 10-cm, 0.25-mm-thick, Whatman K6 silica gel plates, with ethyl acetate–methanol–acetic acid–water (4:1:1:0.5) as the solvent system; and (B) 10 × 10-cm, 0.1-mm-thick, Merck cellulose plates, with 10% acetic acid or water as the solvent system. Plates were examined under long- and shortwave UV light.

HPLC. System 1 was Supelcosil LC-ABZ column (25 × 1 cm), gradient program 0% CH_3CN in 25 mM KH_2PO_4 at 0 min, 10% at 10 min, 30% at 50 min, and 100% at 55 min, 3 ml/min. System 2 was Varian MicroPack MCH-10 semipreparative reversed-phase C_{18} column (50 × 0.8 cm), gradient program 0% CH_3CN in H_2O from 0 to 5 min, and 100% from 10 to 20 min, 3.3 ml/min. System 3 was Phenomenex Bondex10 C_{18} column (30 × 0.78 cm), gradient program 0% CH_3CN in H_2O at 0 min, 20% at 10 min, 25% at 25 min, and 100% at 30 min, 1 ml/min. System 4 was the same column as system 2, gradient program 0% MeOH in 1% acetic acid at 0 min, 15% at 10 min, 30% at 40 min, 50% at 60 min, and 100% at 65 min, 3.3 ml/min. The eluate was monitored at 254 nm for all the HPLC systems.

Bioassays. Feeding deterrent assays were performed as described by Sachdev-Gupta et al. (1993) with some modifications. Twenty microliters of solutions of the test extracts, fractions, or compounds were applied in MeOH, except for the hexane extract, which was applied in ethyl ether, with a Rainin battery-operated motorized pipet onto each side of a cabbage leaf disc (340 mm^2) made with a No. 14 cork borer. For each disc (both sides) 0.01, 0.03 or 0.1 gram leaf equivalents (GLE) of extract or fraction from nasturtium were used. The isolated or standard compounds were used at a dose of 10 ng/mm^2 for each side (6.8 μg/disc). Control discs were treated with the solvent alone. The tests were conducted in ice cream cups (250 ml), each containing a paraffin wax layer at the bottom, which was covered with a moist filter paper. Two discs treated with the same sample and two control discs were alternatively arranged in each cup and supported on No. 3 insect pins. In no-choice tests, either four treated or control discs were placed in each cup. Four early fourth-instar larvae were introduced in the center of the cup, and the cup was covered with a perforated plastic lid. The cups were then placed in a translucent polystyrene box with moist paper towels at the bottom. The covered box was kept in an incubator maintained at 28°C. After 6 hr, the disc areas were measured using a LiCor-3100 area meter. The area consumed was obtained by subtracting the remaining portion of each eaten disc from the average measurement of all discs that showed no sign of feeding.

Nasturtium acceptance assays as described by Renwick and Huang (1995) were employed to determine whether nasturtium constitutents are responsible for the lack of sensitivity of neonate larvae to the deterrents. Test extracts from nasturtium or known compounds were applied to cabbage foliage by spraying

in MeOH solution. Control leaves were sprayed with MeOH alone. Neonate larvae hatching on Parafilm were transferred to the cabbage leaves using a small camel-hair brush. They were allowed to feed until early second instar and were then tested for their acceptance or rejection of nasturtium. The tests were carried out using individual leaves in 250-ml ice cream cups. The leaf petiole was inserted through a hole punched in the bottom of the cup into a beaker of water. One test larva was placed on each leaf, and the cup was covered with a plastic lid. Observations were made after 24 and 48 hr to record whether or not feeding had occurred.

Design and Analysis. For feeding deterrent assays, a replicate consisted of one cup with four larvae, and 6–25 replications were performed for each bioassay. Deterent activities were compared by calculating a feeding deterrent index (FDI) based on the area consumed of treated and control leaf discs: FDI = 100 [(control − treated)/(control + treated)]. The proportion of disc area consumed, treated ÷ (treated + control), was calculated, and the data were subjected to arcsine square root transformation. Differences between treatments and controls were analyzed using a one-sample *t* test on the transformed data, under the null hypothesis that equal feeding occurred on treated and control discs. A Waller-Duncan *K* ratio *t* test on the transformed data was used to assess significance of differences among treatments.

Identification. The deterrent isolated from nasturtium was identified by comparing its retention time on HPLC, R_f on TLC, and its UV, ^{13}C, and ^{1}H NMR spectra with those of an authentic sample. UV spectra were obtained with a Perkin-Elmer Lambda 5 in MeOH. ^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz) spectra in MeOH-d_4 were recorded on a Varian Unity-500 and a Varian VXR-400s, respectively. The chemical shifts were indirectly referenced to tetramethylsilane by using the solvent as an internal reference.

RESULTS

The hexane extract showed no feeding deterrent activity on cabbage-reared fourth instar larvae in a choice bioassay at a dose of 0.01 GLE per cabbage leaf disc (FDI = 8.5; between treatment and control $P > 0.05$). The postbutanol water extract was slightly deterrent at a higher dose of 0.1 GLE/disc (FDI = 29.1; $P < 0.05$). However, the butanol extract was highly deterrent at 0.01 GLE/disc (FDI = 97.8, $P < 0.01$), in the choice bioassay (Renwick and Huang, 1995). This extract deterred larval feeding even in a no-choice feeding bioassay (Figure 1). Six hours after the larvae were introduced into the bioassay cups, little feeding had occurred on the disc treated with 0.1 GLE, and the treatment of 0.01 GLE/disc significantly reduced larval feeding when compared with the control. After 24 hr, only ca. 9% of the leaf discs treated with the extract at

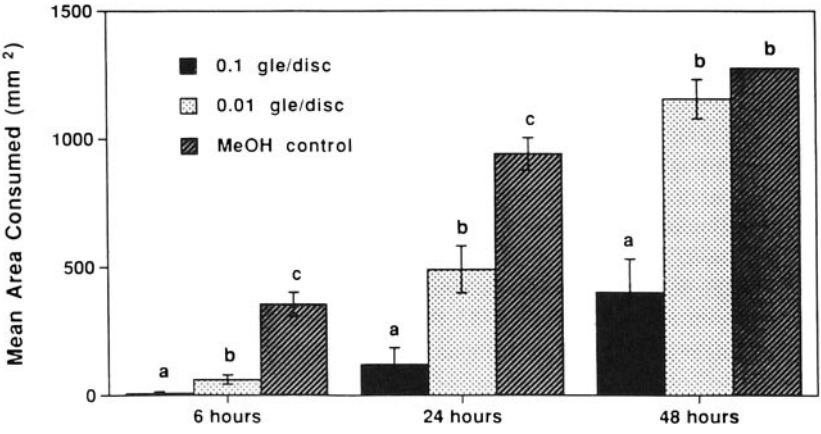

FIG. 1. Feeding by fourth-instar *P. rapae* larvae on cabbage leaf discs treated with the BuOH extract of nasturtium foliage in no-choice assays. Both sides of a leaf disc (340 mm^2) were treated with 0.1 or 0.01 GLE of the extract. Control discs were treated with the solvent (MeOH) alone. Replicated six times. A replication consisted of three bioassay cups, each with four test or control leaf discs and four larvae. Means ($\pm$SE) with the same letters in each time period are not significantly different according to a Waller-Duncan K-ratio t test ($K = 100$).

0.1 GLE/disc was consumed, but ca. 75% of the control discs had been eaten. A significant difference was also found between consumption of control and treated discs at a lower dose (0.01 GLE/disc) (Figure 1). After 48 hr, about one third of the larvae on discs treated with the higher dose (0.1 GLE/disc) were dying due to starvation, and those larvae that did feed were much smaller compared to the control larvae. While there were some leaf fragments remaining from the discs treated with the lower dose of the extract, no control discs were found, and the larvae in control cups were moving rapidly in an apparent search for food. This indicated that all the control discs had been consumed a considerable time before the final measurements were made (48 hr). Therefore, although no statistically significant difference was detected between the control and the treatment with 0.01 GLE/disc, based on the final measurements (Figure 1), control larvae probably would have consumed much more if food had been available. Because the BuOH extract had the most potent deterrent effect, subsequent work was concentrated on this extract.

When the BuOH extract was fractionated into five fractions by open-column chromatography, fractions I, II, and V were not active as feeding deterrents at a dose of 0.03 GLE/disc (Figure 2; between treatment and control, $P > 0.05$ in each case). Fractions III and IV were almost equally deterrent to the larvae,

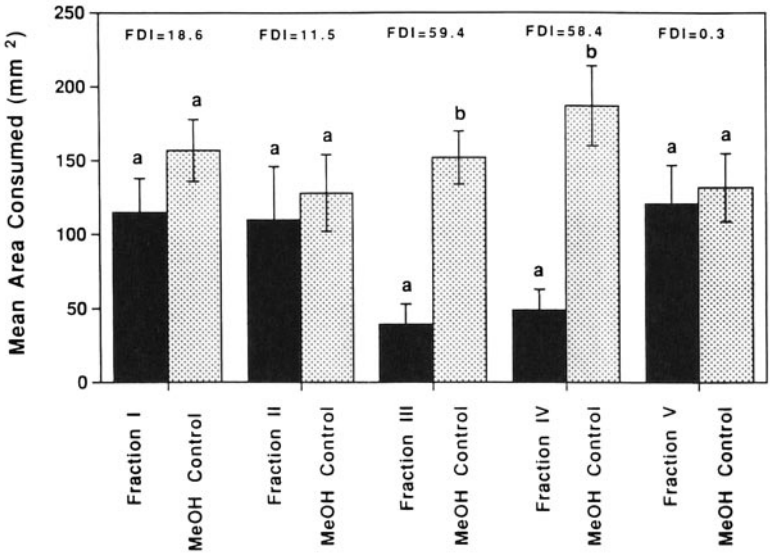

FIG. 2. Feeding by fourth-instar *P. rapae* larvae on cabbage leaf discs treated with five open-column fractions of the BuOH extract of nasturtium foliage in choice assays. Both sides of a leaf disc (340 mm^2) were treated with 0.03 GLE of a fraction. Control discs were treated with the solvent (MeOH) alone. Replications: 7 for fractions I and II, 25 for fraction III, 14 for fraction IV, and 10 for fraction V. A replication consisted of one bioassay cup with two test and two control leaf discs and four larvae. Means ($\pm$SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that feeding occurred evenly on control and treated discs.

giving FDIs of 59.4 ($P < 0.01$) and 58.4 ($P < 0.01$), respectively (Figure 2). The open-column fraction IV was subjected to repeated HPLC separation, and bioassays of the resulting fractions showed that several compounds were responsible for the deterrence. The deterrents in this fraction are not related to compounds detected in III and are under further investigation. However, one prominent component of fraction III, which was absent in IV, appeared to account for most of the activity of this fraction, and the present study focused on the identification of this major deterrent.

Fraction III was separated by HPLC system 1, and three fractions, (III-I, III-II, and III-III) were collected, evaporated under reduced pressure, redissolved in water, and desalted using HPLC system 2. When tested at 0.03 GLE/disc, no deterrent activity was detected in fractions III-I (FDI = -3.9, $P > 0.05$) and III-III (FDI = 5.0, $P > 0.05$) (Figure 3). Only HPLC fraction III-II, which

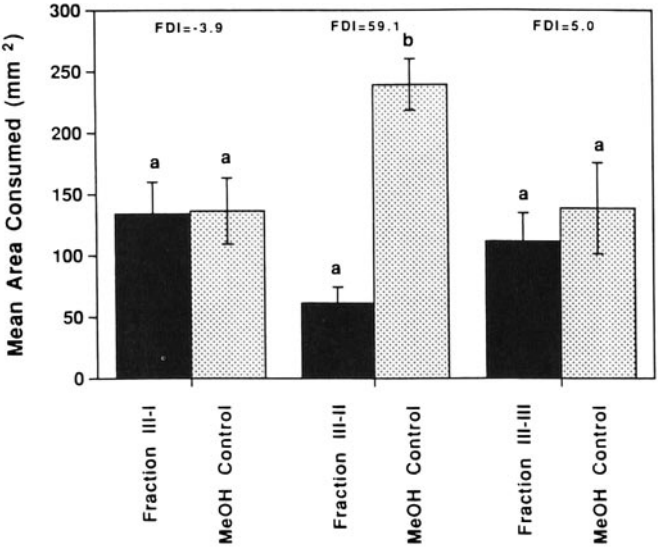

FIG. 3. Feeding by fourth-instar *P. rapae* larvae on cabbage leaf discs treated with three HPLC fractions from open-column fraction III of the BuOH extract in choice assays. Both sides of a leaf disc (340 mm^2) were treated with 0.03 GLE of a HPLC fraction. Control discs were treated with the solvent (MeOH) alone. Replications: 8 for fractions III-I and III-III, and 25 for fraction III-II. A replication consisted of one bioassay cup with two test and two control leaf discs and four larvae. Means ($\pm$SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that feeding occurred evenly on control and treated discs.

contained one prominent compound, was significantly deterrent to the larvae (FDI = 59.1, $P < 0.01$). The major compound in this fraction was purified using HPLC system 3 and named compound **1**. About 40 mg of compound **1** was obtained from a sample of 100 g fresh nasturtium foliage.

The UV spectrum of compound **1** matched that of chlorogenic acid, and preliminary HPLC further suggested that the isolated material could be chlorogenic acid. Additional diagnostic work was conducted to compare the properties of the isolated material with those of the commerically available compound.

The retention times on HPLC were identical for compound **1** and an authentic sample of chlorogenic acid (Sigma Chemical Co.) when systems 1 (21.5 min), 3 (22.0 min), and 4 (37.5 min) were employed. Compound **1** and chlorogenic acid showed the same properties on TLC in systems A (R_f = 0.58) and B (R_f = 0.61 in 10% acetic acid and 0.97 in H_2O). On TLC plates developed

in solvent system A, both compound **1** and chlorogenic acid appeared under UV light as a gray spot on a purple background. They were blue (UV) and bright turquoise (UV + NH_3) on plates using system B. Furthermore, compound **1** and chlorogenic acid gave the same UV absorption pattern: λ_{max} (MeOH) nm 244, 299 (s), 328. These results are consistent with those reported by Feeny et al. (1988) when chlorogenic acid was identified from carrot foliage as an oviposition stimulant for the black swallowtail butterfly. When compound **1** and chlorogenic acid were tested for feeding deterrent activity, they were active at a dose of 10 ng/mm^2 (Figure 4). A sample of compound **1** was isolated and purified with HPLC system 4 for NMR analyses. For comparison, chlorogenic acid was also passed through the same HPLC system and analyzed under the same NMR conditions. The two samples showed identical ^1H and ^{13}C NMR

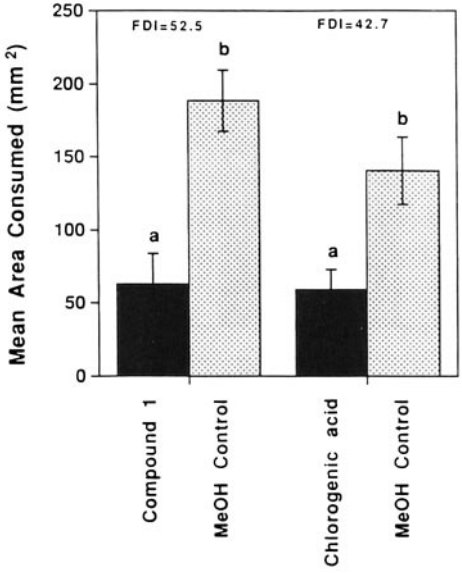

FIG. 4. Feeding by fourth-instar *P. rapae* larvae on cabbage leaf discs treated with chlorogenic acid and compound **1** isolated from open-column fraction III of the BuOH extract of nasturtium foliage in choice assays. Both sides of a leaf disc (340 mm^2) were treated with 10 ng/mm^2 of a sample. Control discs were treated with the solvent (MeOH) alone. Replicated 25 times. A replication consisted of one bioassay cup with two test and two control leaf discs and four larvae. Means ($\pm$SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that feeding occurred evenly on control and treated discs.

profiles. These combined data confirmed the identity of compound **1** as chlorogenic acid.

Figure 5 summarizes the results of nasturtium acceptance assays after exposure of the larvae to the BuOH and the post-BuOH water extracts from nasturtium. Neonate larvae were allowed to feed on cabbage leaves treated with one extract or with a combination of both until they reached early second instar. At 24 and 48 hr after they were transferred to nasturtium leaves, observations were made to record whether or not feeding had occurred. After 48 hr, those larvae that accepted nasturtium usually continued to feed, whereas those that refused were dead or dying. Because most feeding started within 24 hr after transfer and the records made at 24 and 48 hr were similar, only those data from the

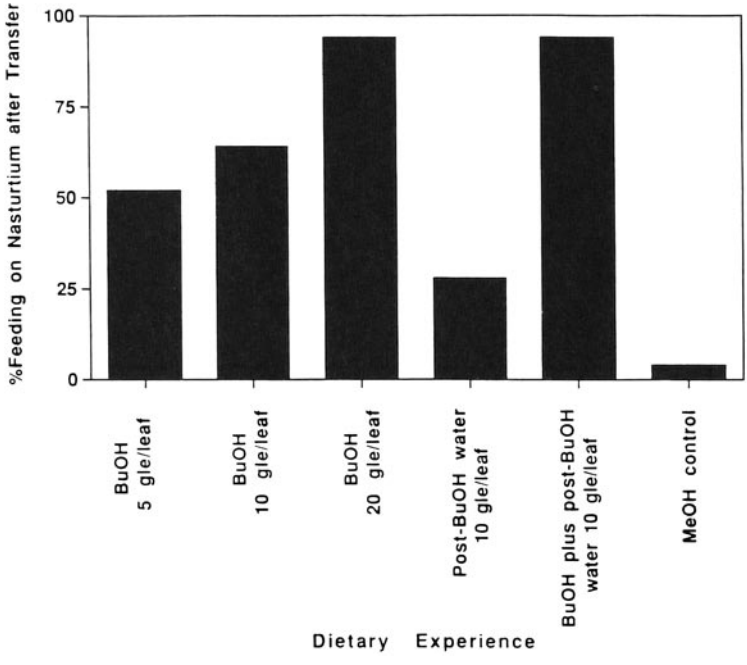

FIG. 5. Feeding by second-instar *P. rapae* larvae on nasturtium. Neonate larvae were reared on cabbage leaves (ca. 120 cm^2) treated with 5, 10, or 20 GLE of the BuOH or the post-BuOH water extracts of nasturtium foliage. Control larvae were reared on cabbage leaves treated with the solvent (MeOH) alone. Individual early second-instar larvae were transferred to nasturtium leaves. Percentage of larvae feeding on nasturtium is shown based on observations made at 48 hr after transfer. Fifty larvae were tested for each treatment.

second observation are presented here. Only two of 50 control larvae fed on nasturtium (Figure 5), but more than 50% of the larvae reared on cabbage leaves treated with the BuOH extract of nasturtium at a dose of 5 GLE/leaf or higher accepted it. The post-BuOH water extract was also active in causing larvae to feed on nasturtium, but the activity was much weaker when compared with the BuOH extract. When the BuOH extract was used at 20 GLE/leaf, almost all larvae accepted nasturtium. Similar results were obtained when a cabbage leaf was treated with a combination of 10 GLE each of the BuOH and the post-BuOH water extracts. The results clearly show that chemical constituents of nasturtium deter cabbage-reared *P. rapae* larvae, but if neonate larvae are exposed to these and/or other compounds, they become habituated to the feeding deterrents.

This idea of habituation is further supported by similar assays using pure chlorogenic acid. When neonate larvae were reared on a cabbage leaf treated with 0.1 mg chlorogenic acid, ca. 35% of the second-instar larvae accepted nasturtium (Figure 6). The percentage of larvae feeding on nasturtium was similar at 1 mg/leaf, but increased when a dose of 10 mg/leaf was used. The two subunits of chlorogenic acid, quinic acid and caffeic acid, were tested under the same conditions. Only four of 100 larvae reared on cabbage leaves treated with quinic acid at a dose of 1 mg/leaf fed on nasturtium. This response was similar to that of the control larvae reared on cabbage leaves treated with the solvent alone (Figure 5). Caffeic acid, however, had some effect, but was less active than chlorogenic acid in causing habituation (Figure 6). Thus, the complete structure of chlorogenic acid appears to be necessary for habituation of *P. rapae* larvae to the feeding deterrents in nasturtium.

DISCUSSION

When *P. rapae* larvae were transferred from cabbage to nasturtium, they refused to feed and eventually starved to death (Renwick and Huang, 1995). The present study shows that nasturtium foliage contains more than one feeding deterrent to cabbage-reared *P. rapae* larvae. In a no-choice situation, the larvae were significantly deterred from feeding on cabbage discs treated with the BuOH extract from nasturtium for at least 48 hr (Figure 1). The results indicate that during this period the larvae do not become habituated to these deterrents. A similar lack of habituation was noted by Jermy et al. (1987) for larvae of a polyphagous strain of *Mamestra brassicae* that was offered nonhost plants that contained a complex of stimuli including deterrents. Nevertheless, habituation has been found to occur in cases of other phytophagous insects (Szentesi and Bernays, 1984). Direct observations of behavior of individual caterpillars of *Pseudaletia unipuncta* revealed that nicotine was deterrent the first time it was

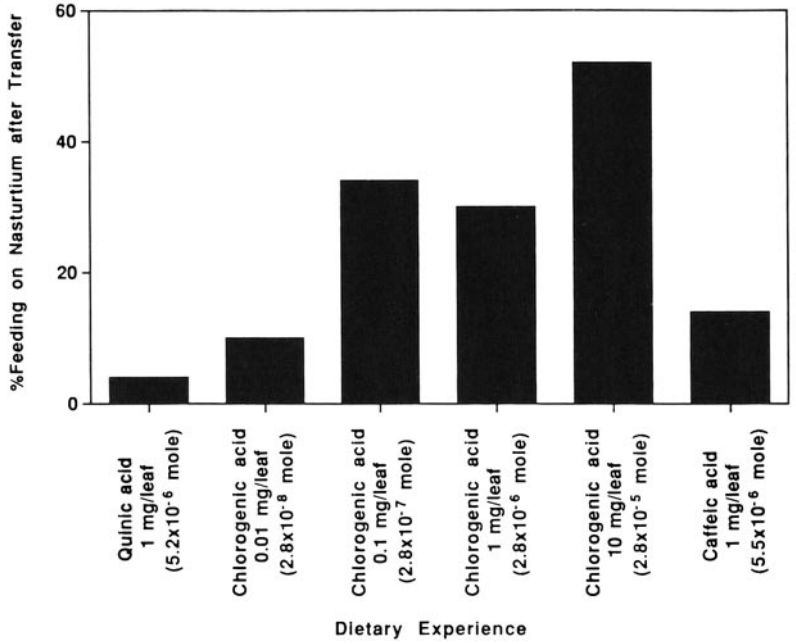

FIG. 6. Feeding by second-instar *P. rapae* larvae on nasturtium. Neonate larvae were reared on cabbage leaves (ca. 120 cm^2) treated with 0.01, 0.1, 1, or 10 mg of quinic acid, chlorogenic acid, or caffeic acid. Control larvae were reared on cabbage leaves treated with the solvent (MeOH) alone. Individual early second-instar larvae were transferred to nasturtium leaves. Percentage of larvae feeding on nasturtium is shown based on observations made at 48 hr after transfer. Fifty larvae were tested for chlorogenic acid at 0.01 mg/leaf, and 100 for all the others.

encountered but not in a 6-hr preference assay (Usher et al., 1988). The results indicated that habituation had developed during this time.

Unlike cabbage-reared larvae, *P. rapae* neonates fed and grew normally on nasturtium (Renwick and Huang, 1995). This could mean that cabbage contains an inducer that triggers sensitivity of larvae to the nasturtium deterrents or that neonates become habituated as they feed on nasturtium constituents. When neonate larvae were reared on a cabbage leaf treated with the deterrent-containing fractions from nasturtium foliage, they remained insensitive to the deterrents, so that they accepted nasturtium when transferred as second instars (Figure 5). It is therefore concluded that the chemical constituents from nasturtium are responsible for the insensitivity of larvae to the deterrents, and the assumption that cabbage contains an inducer is ruled out as a mechanism. The results also

suggest that neonate *P. rapae* can, but larger larvae from cabbage or other host plants (Renwick and Huang, 1995) cannot, become habituated to the nasturtium deterrents. The unique capability of newly hatched *P. rapae* larvae is therefore important for this insect to utilize a marginally acceptable host plant such as nasturtium. Assays using larvae from cabbage leaves treated with the identified feeding deterrent, chlorogenic acid, showed similar feeding as a result of habituation (Figure 6). This implies that the nasturtium constituents responsible for habituation may be those compounds that act as feeding deterrents.

The potential value of feeding deterrents in pest management programs has long been recognized (Bernays, 1983). However, possible habituation of insects to feeding deterrents is a major concern that could limit the practical application of such natural compounds for insect pest control. For example, acceptance of diets containing deterrents has been shown to increase after repeated contact with the active material (Schoonhoven, 1982). When switched from one host plant to another, some larvae initially refused or lowered their consumption rate of the new food (Hanson, 1976), but in no-choice situations they usually accepted the new host after a period of temporary starvation (Grabstein and Scriber, 1982; Scriber, 1982). Here we have been able to show an extreme case of habituation, in which *P. rapae* larvae reared on cabbage leaves treated with nasturtium constituents become habituated to the feeding deterrents and feed on nasturtium, which was otherwise refused.

Chlorogenic acid is found in many higher plants, and its antibacterial, antimutagenic, antitumor, antiviral, and antioxidant activities have been previously reported (reviewed by Harborne and Baxter, 1993). The role this compound plays in insect–plant relationships is of great interest to entomologists and ecologists and intensive investigations have been made on its antibiotic, feeding, or oviposition-deterring or -stimulating effects on phytophagous insects. In a study of chemosensory responses of *P. rapae* larvae using electrophysiological methods, van Loon (1990) found that among five phenolic acids tested, chlorogenic and protocatechuic acids were the strongest stimuli affecting lateral and medial maxillary sensilla styloconica. This chemosensory activity is reflected in the present study where chlorogenic acid is identified as a constituent of nasturtium responsible for deterring feeding by *P. rapae* larvae. Similar results were obtained by Otter (1992), who pointed out that there was a cell sensitive to chlorogenic acid in the medial styloconic sensilla of *Chilo partellus* larvae and that the presence of this cell may account for the positive correlation between total phenol content and resistance of sorghum cultivars to this borer.

Previous studies have shown that chlorogenic acid may play a defensive role that affects host-plant selection by certain phytophagous insects. For example, feeding by *P. brassicae* larvae was deterred by chlorogenic acid (van Loon, 1990). The rejection of *Salix integra*, *S. chaenomeloides*, and *S. koriyanagi* by the leaf beetle, *Lochmaeae capreae cribrata*, was due to the high levels of

chlorogenic acid in these plants, whereas another beetle species, *Plagiodera versicorola distincta*, which accepted these plants, was not deterred by this phenolic compound (Matsuda and Senbo, 1986). de Boer and Hanson (1987) reported a concentration-dependent effect of chlorogenic acid on feeding by *Manduca sexta* larvae. However, despite a strong deterrent effect at high concentrations, it was slightly stimulatory at its natural concentration (1 mM). The authors therefore concluded that chlorogenic acid did not play an important role in food selection by the tobacco hornworm.

The effects of chlorogenic acid on oviposition behavior vary with insect species. This is one of the compounds in carrot foliage responsible for stimulating oviposition by the black swallowtail butterfly (Feeny et al., 1988). Honda (1990) found that chlorogenic acid acted as an excellent synergist for a mixture of compounds that stimulated *Papilio protenor* to oviposit on *Citrus* host plants. On the other hand, ovipositing *P. rapae* was deterred by chlorogenic acid at a 1000 ppm level, based on a bioassay using white paper discs (Traynier, 1987). In a related study using a cabbage plant as the oviposition substrate (Huang and Renwick, unpublished results), however, we found that the chlorogenic acid-containing fraction from HPLC of nasturtium extract had little effect on oviposition. Activity was associated with other components of nasturtium extract separated by open-column chromatography. No oviposition deterrent activity was detected when an authentic sample of chlorogenic acid was tested at 1.6 mg/plant under the same conditions. The inconsistency between Traynier's results and ours might be due to differences in concentration of the compound as well as differences in the oviposition substrate.

Felton et al. (1989) found that chlorogenic acid ingested from tomato (*Lycopersicon esculantum*) foliage and fruit by *Heliothis zea* larvae was oxidized to chlorogenoquinone by polyphenol oxidase in the insect gut. The relative growth rate of the larvae was negatively correlated with polyphenol oxidase activity. This was explained by the fact that the resulting chlorogenoquinone is a highly active electrophilic molecule that binds covalently to amino acids and proteins, thus reducing the digestibility of nutrients from the plant. Antibiotic resistance involving chlorogenic acid was reported for centipedegrass (*Eremochloa ophiuroides*) to *Spodoptera frugiperda* (Wiseman et al., 1990), popcorn (*Zea mays*) to *Helicoverpa zea* (Wiseman et al., 1992), and a wild groundnut (*Arachis paraguariensis*) to *Spodoptera litura* (Stevenson et al., 1993). The growth rate of *Spodoptera exigua* larvae declined when they were fed with dietary proteins treated with chlorogenic acid and polyphenol oxidase (Felton et al., 1992a). The pH status in the digestive system of an insect may have an effect on the binding of chlorogenic acid to proteins (Felton et al., 1992b). When *Bacillus thuringiensis* subsp. *Kurstaki* was incubated with chlorogenic acid and polyphenol oxidase, the bacterium became more toxic to *Heliothis zea* larvae. This suggested the compatibility of hostplant resistance and biological control (Lud-

lum et al., 1991). However, Felton et al. (1987) observed possible incompatibility of this chemically based resistance with viral control of the same insect. Chlorogenic acid significantly inhibited the infectivity of nuclear polyhedrosis viruses on *H. zea*, and the ingestion of this phytochemical significantly prolonged the survival time of virus-infected larvae.

Chlorogenic acid has no deleterious effect on some insects such as *Spodoptera eridania* (Lindroth and Peterson, 1988). Furthermore, a positive correlation was found between the concentrations of chlorogenic acid in different cultivars of carrot and the susceptibility of these plants to carrot fly larval damage (Cole, 1985). Midgut catalase in several lepidopterous pests of tomato plants including *Helicoverpa zea*, *S. exigua*, *Manduca sexta*, and *Heliothis virescens*, in addition to removing toxic hydrogen peroxide, may reduce the oxidation of plant phenolics mediated by plant peroxidases (Felton and Duffey, 1991). Although chlorogenic acid in nasturtium acted as a feeding deterrent to *P. rapae* larvae, it apparently has no significantly harmful impact on this insect. Survivorship, growth rates, and pupal weights were similar for nasturtium-reared and cabbage-reared larvae (Huang and Renwick, unpublished results).

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STRUCTURE, CHIRALITY, AND FIELD TESTING OF A MALE-PRODUCED AGGREGATION PHEROMONE OF ASIAN PALM WEEVIL *Rhynchophorus bilineatus* (Montr.) (Coleoptera: Curculionidae)

A. CAMERON OEHLSCHLAGER,[1,*] R.N.B. PRIOR,[2] ALICE L. PEREZ,[1] REGINE GRIES,[3] GERHARD GRIES,[3] HAROLD D. PIERCE, JR.,[1] and SAM LAUP[2]

[1]*Department of Chemistry, Simon Fraser University Burnaby, British Columbia, Canada, V5A 1S6*

[2]*Papua New Guinea Cocoa and Coconut Research Institute P.O. Box 1846, Rabaul, E. N. B. Papua New Guinea*

[3]*Centre for Pest Management, Department of Biological Sciences Simon Fraser University Burnaby, British Columbia, Canada, V5A 1S6*

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Abstract—4-Methyl-5-nonanol is a male-produced aggregation pheromone of the Asian palm weevil, *Rhynchophorus bilineatus* (Montr.). The pheromone was identified by coupled gas chromatographic-electroantennographic detection (GC-EAD) and coupled GC-mass spectrometric (MS) analyses of male- and female-produced volatiles. Analyses by GC-EAD and GC-MS of weevil-produced and stereoselectively synthesized isomers of 4-methyl-5-nonanol on a Cyclodex B column, which separated isomers with baseline resolution, revealed that only (4*S*,5*S*)-4-methyl-5-nonanol is EAD active and produced by the males. In field experiments in Papua New Guinea, (4*S*,5*S*)-4-methyl-5-nonanol and a racemic mixture of disatereoisomers of it enhanced attraction of male and female weevils to sugarcane-baited traps. (4*S*,5*S*)-4-Methyl-5-nonanol is also an aggregation pheromone of two other Asian palm weevils, *R. ferrugineus* (Oliv.) and *R. vulneratus* (Panz.). The stereoisomeric mixture of 4-methyl-5-nonanol is currently used to manage populations of *R. bilineatus* in Papua New Guinea.

Key Words—Coleoptera, Curculionidae, *Rhynchophorus bilineatus*, aggregation pheromone, pheromone chirality, (4*S*,5*S*)-4-methyl-5-nonanol, coconut palm.

*To whom correspondence should be addressed.

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INTRODUCTION

In Papua New Guinea the palm weevil *Rhynchophorus bilineatus* (Montr.) causes mortality of coconut palms used as shade trees in cocoa plantations. While the weevil may directly attack the palms, they are frequently attracted to and ovipost in feeding tunnels of the New Guinea rhinoceros beetle, *Scopanes australis* (Boisd.). Feeding by weevil larvae often destroys the terminal bud, contributing to the death of the palm (Wattanapongsiri, 1966).

Male-produced aggregation pheromones have been reported for most economically important species of palm weevils (Rochat et al., 1991, 1993a,b; Oehlschlager et al., 1992a; Gries et al., 1993; Hallett et al., 1993a,b; Zagatti et al., 1993; Weissling et al., 1994). This paper reports the structural elucidation, stereochemistry, antennal response to, and behavioral activity of a male-produced aggregation pheromone for *R. bilineatus*.

METHODS AND MATERIALS

Laboratory Analysis. Male and female *R. bilineatus* of mixed age and sex were collected in cocoa plantations with interspersed coconut palms near Rabaul, East New Britain Province, Papua New Guinea. Collections were made in modified 19-liter plastic buckets containing 10–20 pieces of cut sugarcane (10–15 cm). Because weevils may mate within a few days of emergence (Wattanapongsiri, 1966) prior to and after capture in the buckets, it is likely that mainly mated weevils were used in aeration experiments. Twenty-five male and 20 female *R. bilineatus* were aerated separately for 6 to 7 days in a modified Nalgene desiccator containing 0- to 4-day-old sectioned apples. A water aspirator was used to draw charcoal-filtered air through the chamber and insect- and sugarcane-produced volatiles were trapped on Porapak Q. Volatiles were eluted from Porapak Q with pentane, concentrated by distillation (Oehlschlager et al., 1988), and subjected to gas chromatographic analysis (Figure 1). Gas chromatographic–electroantennographic detection (GC-EAD, Figure 2) (Hewlett Packard 5890A) (Arn et al. 1975) and GC-mass spectrometry (MS) (Hewlett Packard 5985B) in both electron impact (EI) and chemical ionization (CI) mode, employing a 30-m × 0.25-mm-ID fused silica column coated with SP-1000 (Supelco Inc., Belafonte, Pennsylvania), revealed 4-methyl-5-nonanol as an antennally active male-produced volatile. For GC-EAD recordings, a weevil antenna was removed from the rostrum and suspended between two glass capillary electrodes with the antennal base inserted into one and the olfactory club impaled by the other electrode. GC-MS-EI in selected ion monitoring mode (SIM) of natural and synthetic chiral isomers of 4-methyl-5-nonanol were further conducted on a fused silica Cyclodex-B-coated column (30 m × 0.25 mm ID, J & W Sci-

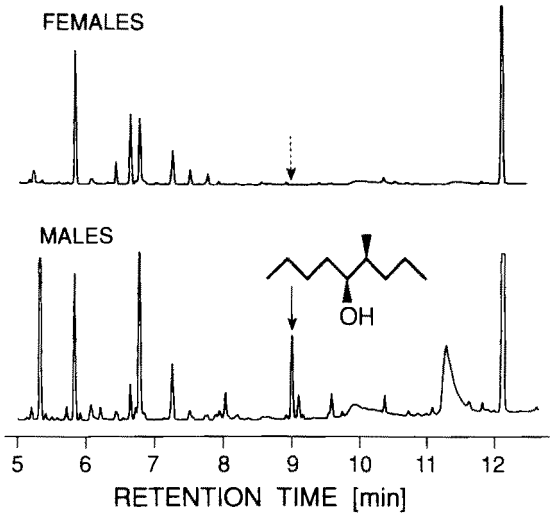

FIG. 1. Gas chromatograms of volatiles obtained from female and male *R. bilineatus* feeding on apples. Chromatography: SP-1000 fused silica column; temperature program: 1 min at 50°C, 10°C/min to 180°C.

entific, Folsom, California; isothermal 100°C) to deduce which stereoisomer was weevil-produced (Figure 3). For GC-MS-EI-SIM, a full-scan mass spectrum of synthetic 4-methyl-5-nonanol, [(±)-**1**], was obtained to select diagnostic ions [*m/z* = 69, (M^+-C_5H_{11}) 87 and (M^+-C_4H_9) 101]. Synthetic (±)-**1**, hexane, and concentrated weevil-produced compound were then injected in split mode and analyzed by scanning for the diagnostic ions. GC-EAD of synthetic chiral isomers of 4-methyl-5-nonanol was also conducted on this column to determine which stereoisomer was antennally active (Figure 4).

Synthesis. Synthetic (±)-**1** was prepared by slow addition of freshly distilled 2-methyl-1-pentanal in ether to butyl lithium in hexane cooled in an ice-water bath. After the usual work-up distillation of the crude product under reduced pressure yielded 67% of (±)-**1** in >95% purity (1.5:1 *syn*:*anti*). The *syn* and *anti* diastereoisomers of (±)-**1** separated on an SP-1000 column. In analogy to previous work (Perez et al., 1994a), *R. bilineatus*-produced **1** was hypothesized to be the *syn* diastereoisomer, which was confirmed by stereoselective synthesis of all isomers of (±)-**1**. Preparation of (4*R*,5*R*)-**1** (97% ee) and (4*S*,5*S*)-**1** (98% ee) was according to methodology previously reported (Perez, et al. 1994a) for the preparation of chiral isomers of 3-methyl-4-octanol and 5-methyl-4-octanol. Details of the preparation of (4*R*,5*R*)-**1** and (4*S*,5*S*)-**1** is described in another paper (Perez et al. 1994b).

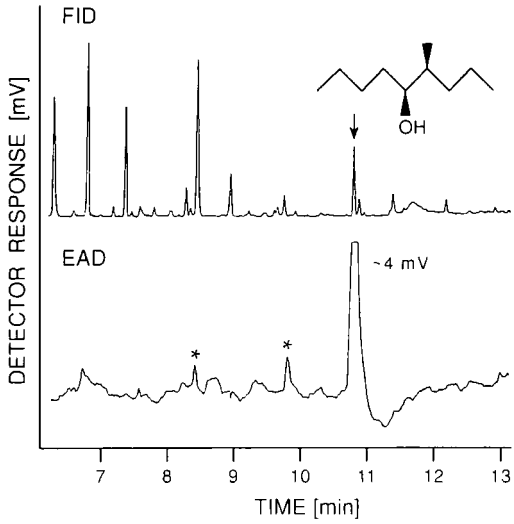

FIG. 2. Flame-ionization (FID) and electroantennographic detector (EAD: female *R. bilineatus* antenna) responses to volatiles obtained from male *R. bilineatus* feeding on apples (*apple-derived components). FID trace: 0.8 weevil hours of an extract containing 4200 weevil hours of volatile production. Column as in Figure 1.

Field Experiments. Field experiments were conducted in coconut-shaded cocoa plantations near Kervera and Vunabang, Papua New Guinea. Modified white 19-liter plastic bucket traps were attached to palms at chest height in randomized blocks with traps at 27-m intervals and blocks 80 m apart (Oehlschlager et al., 1993b). Detergent-laced (0.3%) water (2–3 cm) in the bottom of each trap retained captured weevils. A wire mesh above the water held halved sugarcane stalks. Every three to four days sugarcane was changed and weevils counted.

The first three-treatment experiment tested the attractiveness of (±)-**1** (3 mg/day), sugarcane stalk, and both combined (Figure 5). The second treatment experiment tested attraction of male and female weevils to sugarcane stalk alone and the same combined with (±)-**1** (0.3 mg/day, Figure 6). The third four-treatment experiment tested sugarcane alone and in combination with (±)-**1** released at 0.3, 3.0, or 30 mg/day (Figure 7). The fourth four-treatment experiment tested sugarcane alone and in combination with (±)-**1** (0.4 mg/day), 4*S*,5*S*-**1**, or 4*R*,5*R*-**1** (each stereoisomer released at 0.1 mg/day, Figure 8). Release rates were determined by weight loss of release devices maintained at 25°C. For the low release rate, (±)-**1** was evaporated from three glass capillary tubes (1 mm ID), cut 1 cm above the liquid meniscus and each placed inside

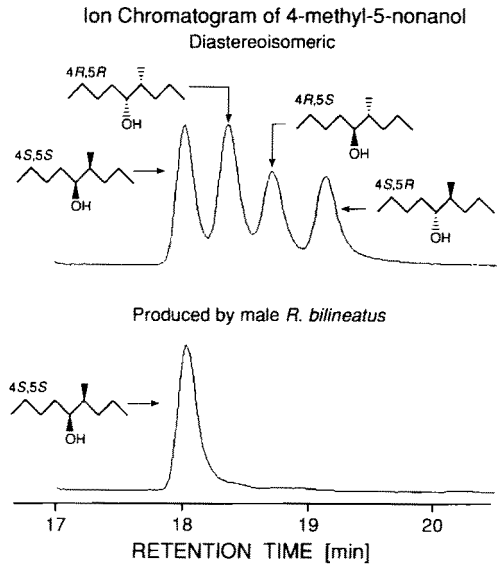

FIG. 3. Selected ion m/z = 69, 87, and 101 chromatogram (Hewlett Packard 5985B GC-MS) of stereoisomeric and weevil-produced **1**. m/z was the parent ion of the full scan EI mass spectrum of $(M^{+}\text{-}C_4H_9)$ 101 or $(M^{+}\text{-}C_5H_{11})$ 87. Analysis on a Cyclodex B column, isothermal 100°C, linear flow velocity of carrier gas 35 cm/sec, split injection and injector temperature 220°C.

capped 300-μl plastic centrifuge tubes with two 2-mm holes near the top. For the medium and high release rates, (±)-**1** was evaporated from 1 or 10 dispensers (heat-sealed plastic bags providing constant release rates under isothermal conditions; ChemTica Internacional, S. A., Apdo. 159-2150, San Jose, Costa Rica).

Statistical Analysis. Assumptions of data normality and homogeneity of variance were tested by graphical assessment of log (variance) versus log (mean), and Bartlett's test, respectively (SAS, 1990). Data were transformed by $(X + 0.5)^{0.5}$ to eliminate heteroscedasicity (Zar, 1984) and were subjected to ANOVA using PROC GLM (SAS) with means compared by Bonferroni's t-test (SAS, 1990).

RESULTS AND DISCUSSION

Analyses of volatiles from males and females revealed several male specific compounds (Figure 1). GC-EAD revealed only one male-specific compound that elicited strong antennal response from male and female weevils (Figure 2).

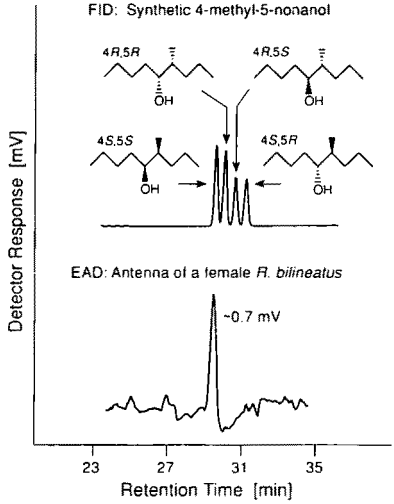

FIG. 4. Flame-ionization (FID) and electroantennographic detector (EAD: female *R. bilineatus* antenna) responses to isomers of 4-methyl-5-nonanol. Column as in Figure 3.

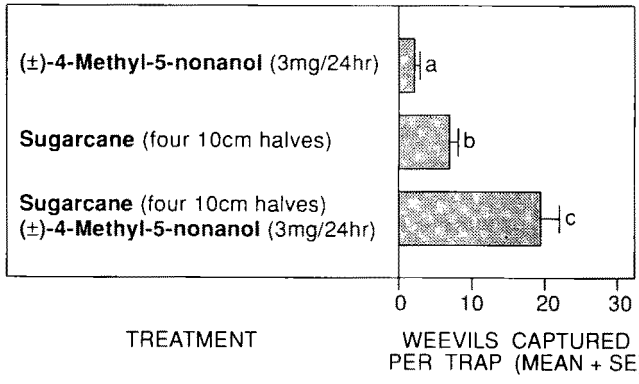

FIG. 5. Mean (+ standard error) captures of *R. bilineatus* in bucket traps baited with sugarcane, (±)-**1**, or both. Experiment ($N = 10$) was conducted in Kervera plantation, November 2–December 4, 1992. Data transformed to approximate homogeneity are presented untransformed. ANOVA, $F = 29.18$; $df = 2,15$; $P < 0.001$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

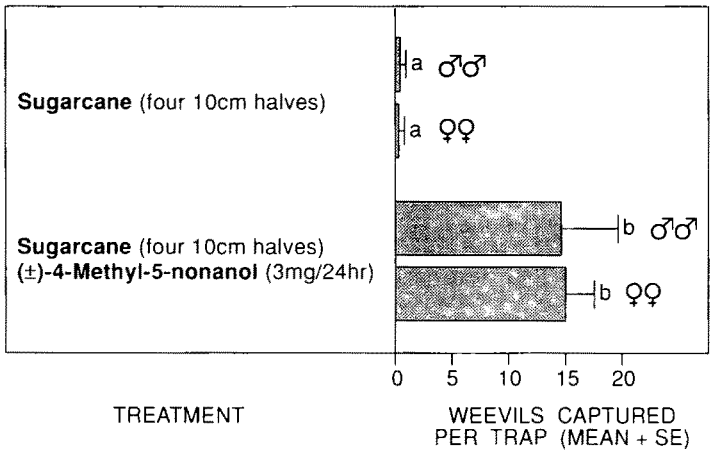

FIG. 6. Mean (+ standard errors) captures of *R. bilineatus* in bucket traps baited with sugarcane or (±)-**1**. Experiment ($N = 4$) was conducted in Vunabang plantation, May 4–14, 1994. Analysis determined there was not a date effect. ANOVA on pooled trap catches for each date, for males $F = 8.84$; $df = 1,6$; $P < 0.02$; for females $F = 35.84$; $df = 1,6$; $P < 0.001$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

Mass spectral and retention characteristics of the EAD-active compound were consistent with 4-methyl-5-nonanol [(±)-**1**], the recently identified aggregation pheromone of *R. ferrugineus* and *R. vulneratus* (Hallett et al., 1993a,b; Perez et al., 1993). This nonanol is also a male-specific volatile produced by *R. palmarum* (Hallett et al., 1993a). Analysis by GC-MS of synthetic chiral isomers of **1** and of **1** produced by male *R. bilineatus* on a Cyclodex B column revealed that 4*S*,5*S*-**1** was produced by male *R. bilineatus* (Figure 3). Analysis of synthetic chiral isomers of **1** by GC-EAD revealed that only 4*S*,5*S*-**1** was antennally active (Figure 4).

The first field experiment revealed that (±)-**1** or sugarcane alone was not very attractive, but in combination they caught many weevils (Figure 5). Equal attraction of males and females to traps containing (±)-**1** and sugarcane demonstrated that **1** constitutes an aggregation pheromone (Figure 6). Synergistic attraction of *R. bilineatus* to traps containing both aggregation pheromone and food is consistent with findings in five other species of the Rhynchophorinae (Chinchilla and Oehlschlager, 1992, Oehlschlager et al., 1992a, 1993a,b; Gries et al., 1993, 1994; Hallett et al., 1993a,b; Perez et al., 1993; Weissling et al., 1994).

A third experiment tested sugarcane alone and in combination with (±)-**1**

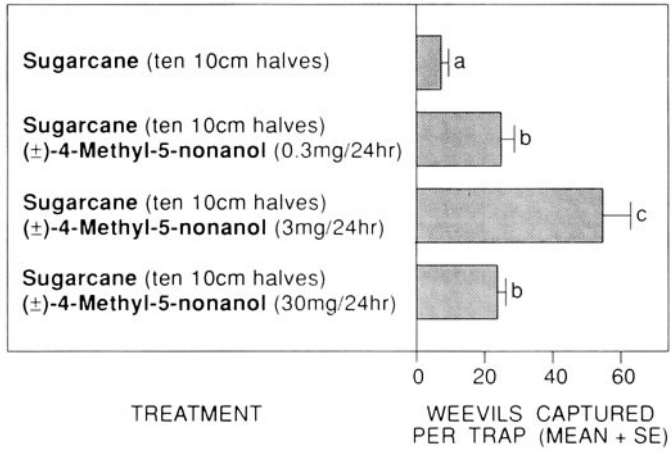

FIG. 7. Mean (+ standard mean error) captures of *R. bilineatus* in bucket traps baited with sugarcane alone or combined with (±)-**1** at three release rates. Experiment (N = 15) conducted at Vanabang plantation, November 25, 1992–January 5, 1993. Data transformed to approximate homogeneity are presented untransformed. ANOVA, $F = 24.67$; $df = 12,67$; $P < 0.0001$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

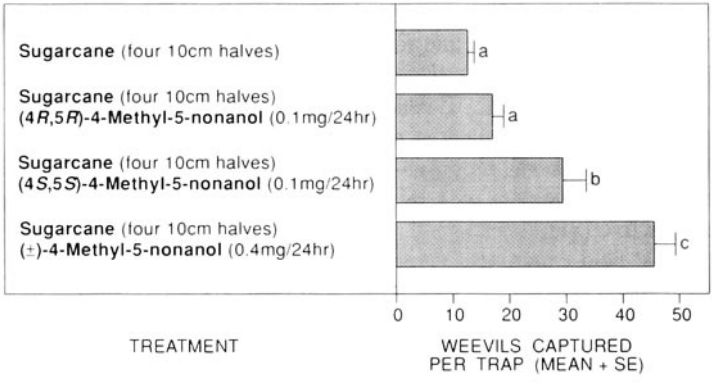

FIG. 8. Mean (+ standard error) captures of *R. bilineatus* in bucket traps baited with sugarcane alone or in combination with (±)-**1**, (4*S*,5*S*)-**1**, or (4*R*,5*R*)-**1**. Experiment (N = 10) at Vimy plantation, January 21–February 22, 1994. Analysis determined there was not a date effect. ANOVA on pooled trap captures for each date gave $F = 22.22$; $df = 3,36$; $P < 0.0005$. Means followed by the same letter are not significantly different (Bonferonni's t test, $P < 0.05$).

released at 0.3, 3.0, or 30 mg/day. Attraction was optimal to those traps from which 3 mg/day of (±)-**1** was released (Figure 7). *R. palmarum* responds equally well to the same three release rates of its pheromone when combined with food, but without food the beetles prefer a 30 mg/day dose (Oehlschlager et al., 1992a, 1993b). In the presence of palm tissue, *R. ferrugineus* is more strongly attracted to traps from which 3 mg/day rather than 0.3 mg/day of (±)-**1** are released (Hallett et al., 1993a,b).

In further field experiments (±)-**1** or 4*S*,5*S*-**1** but not 4*R*,5*R*-**1** enhanced attraction to sugarcane baited traps (Figure 8). The better attractiveness of (±)-**1** than 4*S*,5*S*-**1** is not understood because only the latter is detected in male volatiles (Figure 3) and elicits antennal responses (Figure 4). Interestingly, 4*S*,5*S*-**1** is an aggregation pheromone of *R. ferrugineus* and *R. vulneratus* (Mori, 1993b; Perez et al., 1993). The same stereoisomer of **1** is also produced by male *R. palmarum* (Perez et al., 1993). In Central America, (±)-**1** alone does not enhance attraction of *R. palmarum* to taps containing sugarcane (A.C. Oehlschlager et al., unpublished data).

Production of and antennal response to only one stereoisomer of the aggregation pheromone in *R. bilineatus*, *R. ferrugineus*, and *R. vulneratus* (Perez et al., 1993) is consistent with findings in other Rhynchophorinae. *R. phoenicis* and *R. cruentatus* produce and respond only to (3*S*,4*S*)-3-methyl-4-octanol (Mori et al., 1993a; Perez et al., 1993, 1994a) and (4*S*,5*S*)-5-methyl-4-octanol, respectively (Perez et al., 1994a). Similarly, *R. palmarum* produces and responds to (4*S*,2*E*)-5-methylhepten-4-ol with the antipode being inactive (Oehlschlager et al., 1992a).

The occurrence of 4*S*,5*S*-**1** in the American and three Asian palm weevils supports the hypothesis that the American palm weevil speciated from Asian weevils. This would be consistent with the prevailing theory that palm trees evolved in Southeast Asia, spread through Africa and eventually arrived in the Americas (Harries, 1991). The production of and response solely to 4*S*,5*S*-**1** by *R. ferrugineus*, *R. vulneratus*, and *R. bilineatus* suggest that other mechanisms contribute to reproductive isolation of these weevils. Geographic isolation may allow *R. bilineatus* to use 4*S*,5*S*-**1** as its major pheromone without competition from *R. ferrugineus* or *R. vulneratus*. Sympatric *R. ferrugineus* and *R. vulneratus* are cross-attracted, and males produce the same unique volatiles, suggesting similar communication systems (Hallett et al., 1993a,b). This has raised doubts as to their being separate species (Hallett et al., 1993a,b). Pheromone communication in *R. ferrugineus* and *R. vulneratus* may be different than that of *R. bilineatus* due to the ketone corresponding to **1** that is produced by both the former species (Hallett et al., 1993a) but not by male *R. bilineatus*. The ketone is EAD active in both producing species (Hallett et al., 1993a).

Traps containing (±)-**1**, sugarcane, and detergent-laced water are presently used in Papua New Guinea for management of *R. bilineatus* (R.N.B. Prior and

S. Laup, personal communication). Pheromone-based trapping over one year has proven effective in reducing populations of *R. palmarum* by >80% in commercial oil palm and is used operationally in Central and South America to manage red ring disease vectored by this insect (Oehlschlager et al., 1992b, Chinchilla et al., 1993).

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Book Review

Bioregulators for Crop Protection and Pest Control. Paul A. Hedin (ed.). ACS Symposium Series 557. Developed from a symposium sponsored by the Division of Agrochemicals at the 205th National Meeting of the American Chemical Society, Denver, CO, March 28–April 2, 1993. Washington, D.C.: American Chemical Society, 1994. Hardback $59.95. 221 pages. ISBN 0-9412-2918-X.

Bioregulators as described in the preface to this symposium proceeding include natural and synthetic compounds that are used to regulate any aspect of plant growth and development (excluding those used for killing weeds). The first chapter by L.G. Nickell, "Plant Growth Regulators in Agriculture and Horticulture," provides an introduction to the symposium. He lists the following potential uses for bioregulators; (1) alteration of chemical composition, (2) regulation of dormancy, (3) stimulation of rooting, (4) control of plant or organ size, (5) control of flowering, (6) control of fruit drop, (7) control of fruit set and development, (8) influence of mineral uptake, (9) alteration of maturation, (10) increase resistance to pests or environmental stresses, and (11) prevention of postharvest spoilage.

Following the introductory chapter, the book is divided into three sections. The first section, "Current Applications of Plant Growth Regulators," contains four chapters that give examples of current bioregulator use in agriculture. Chapter two, "New Chemical Approaches for Control of Biennial Bearing of Apples" by M.W. Williams, addresses applied aspects of the use of several chemical thinning agents to prevent excessive fruit set and the subsequent depletion of photosynthate that greatly reduces apple productivity the following year.

In chapter three "Bioregulator-Induced Changes in The Composition of Sugarcane," B.L. Legendre et al. discuss the use of the herbicide glyphosate to improve the sucrose content and other properties of sugarcane.

Chapter four, by P.D. Greeny et al., "Use of Gibberellic Acid to Reduce Citrus Fruit Susceptibility to Fruit Fly," examines the use of gibberellin to prevent senescence of the peel which confers resistance to fruitfly in citrus fruit. This treatment also reduces susceptibility to fruit rot organisms and enhances fruit appearance.

Chapter five, "Bioregulator-Induced Effects on the Allelochemicals and Agronomic Traits of Cotton" by P.A. Hedin and J.C. McCarty, Jr., examines the effect of bioregulators on cotton growth patterns and allelochemical and phytoalexin production. A large number of inorganic and organic substances are

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known to induce the production of defense compounds, and the authors propose that there is potential for using exogenous application of bioregulators to increase pest resistance.

The second section entitled, "Natural Products as Emerging Bioregulators of Crop Protection and Production" contains seven chapters. Chapters in this section are of particular interest to those in chemical ecology inasmuch as they are good examples of commercial applications for this area of research. In chapter six by A. Stierle et al., "Endophytic Fungi of Pacific Yew (*Taxus brevifolia*) as a Source of Taxol, Taxanes and Other Pharmaceuticals," the authors report on the isolation of a fungus associated with the roots of Pacific yew that produces the chemotherapeutic drug taxol, related taxanes, and other antibiotic compounds. They give a detailed description of the chromatographic, radioisotope, and immunoassay techniques use to confirm the ability of the fungus to synthesize taxol.

In chapter seven, "Effects of Neem and Azadirachtin on Aphids and their Natural Enemies," D.T. Lowery and M.B. Isman review their evaluations of the natural compounds from the seed oil of *Azadirachta indica* for selectively controlling aphids.

Chapter eight by P.J. Rice and J.R. Coats, "Structural Requirements for Monoterpenoid Activity against Insects," examines the effect of variations in structure on the topical, fumigant, and ovicidal activity of these compounds against the house fly. Relatively minor differences in basic carbon skeleton, type of functional group, degree of saturation, volatility, and type of derivatization all affect the activity of these compounds. The chapter illustrates the benefit of understanding structure-activity relationships in developing pesticides and bioregulators.

Chapter nine by R.F. Severson et al., "Characterization of Natural Pesticide from *Nicotiana gossei*," describes the isolation and identification of the components of sugar esters in an insectidal extact of the aphid and whitefly resistant species. The relatively high activity of these compounds indicates their potential as biorational insecticides.

Chapter 10 by M.E. Snook et al., "New Flavone C-Glycosides from Corn *Zea mays* for the Control of Corn Earworm (*Heliocoverpa zea*)," describes isolation and characterization of insecticidal compounds other than maysin from silks of earworm resistant corn genotypes.

Chapter 11 "Inhibition of Phytotoxin (Viridiol) Biosynthesis in the Biocontrol Agent *Glyocladium virens*" by R.D. Stipanovic and C.R. Howell, examines the use of cytochrome P-450 inhibiting fungicides to prevent the synthesis of phytotoxins that limit the usefullness of *G. virens* in controlling seedling diseases.

The final chapter in this section, entitled "After Discovery: the Issues of Supply Strategies in the Development of Natural Products," by J.D. McChesney

discusses the problems and advantages encountered in the commercialization of secondary plant compounds for pharmaceuticals and agrochemicals.

The last section, "Mechanisms of Interactions of Natural Bioregulators and Their Hosts," contains four chapters. Chapter 13, "Development of Economical Population Control of Codling Moth (*Cydia pomonella*) by Disrupting Communication with Semiochemicals," by L.M. McDonough examines the feasibility of using semiochemicals to disrupt mating and control insect population. Among the difficulties encountered with this approach were the lack of an adequate control release device and the rapid decomposition of the pheromone.

Chapter 14, "Metabolites of *Pseudomonas corrugata* that Elicit Plant Defense Reactions," by D.L. Gustine et al. provides another example of bioregulator-induced pest resistance in plants. Two peptides from the bacterial pathogen of tomato induced hypersensitive resistance response in nonhost plant species.

Chapter 15, "Mechanisms of *Heliothis virescens* Resistance to Exogenous Ecdysteroids," by M. Zhang and I. Kubo discusses the multiple mechanisms of resistance of tobacoo budworm to these plant defense compounds. Their studies indicate that low gut permeability, occurrence of a unique pathway for metabolism in the gut, and a high rate of metabolite excretion contribute to resistance.

The last chapter, "Characterization of Auxin Transport Proteins Using Phytotropins" by S. Brun et al., provides a good example of the value of inhibitors for elucidating biological mechanisms of action.

As is typical for the ACS Symposium Series, most chapters are compilations of the authors' research. Each chapter is independent and related to other chapters only in their relevance to the symposium topic. This limits the book's usefulness as a text. Due to the limited number of tropics presented at the symposium, this book cannot be considered a comprehensive review of bioregulator use in agriculture. Several important uses of bioregulators were not discussed. However, the authors are experts in their fields, and most chapters will be excellent resources for those interested in their specific topics. Although the chapters vary considerably in content and style, they are generally well written.

Howard F. Harrison
U.S. Department of Agriculture
Charleston, South Carolina

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CAN SOCIAL BEES BE INFLUENCED TO CHOOSE A SPECIFIC FEEDING STATION BY ADDING THE SCENT OF THE STATION TO THE HIVE AIR?

H.B. JAKOBSEN,[1,*] K. KRISTJÁNSSON,[2] B. ROHDE,[2] M. TERKILDSEN[2], and C. E. OLSEN

[1]*Department of Plant Biology, Plant Physiology and Anatomy Laboratory*
[2]*Department of Agricultural Sciences*
[3]*Department of Chemistry*
The Royal Veterinary and Agricultural University
Thorvaldsensvej 40, 1871 Frederiksberg C. Denmark

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Abstract—The behavioral response of honeybees (*Apis mellifera* L.) and bumblebees (*Bombus terrestris* L.) to the flower volatiles 2-ethyl-1-hexanol and myrcene isolated in situ from white clover (*Trifolium repens* L.) and oil seed rape (*Brassica napus oleifera*), respectively, were investigated on a rotating arena with 12 visually identical, but differently scented, feeding stations. When locating a feeding station, neutral in both shape and color, foragers used scent as orientation cue. Introduction of 2-ethyl-1-hexanol to the honeybee hives induced significantly more visits to sites containing this compound. In contrast, introduction of myrcene to the hives did not influence the foraging choices of honeybees significantly. No effect of hive scent composition on the choices made by bumblebees could be detected. "Experienced" bumble bees, i.e., bees with more than five visits to the feeding stations, tended to visit a particular position on the arena without discriminating between the two volatiles. In contrast, honeybees showed no positioning behavior on the arena, using primarily odoriferous stimuli. The observed influences of addition of scents to the hives are discussed in relation to the general knowledge on foraging behavior of social bees and the emission of volatiles from leaves and flowers.

Key Words—*Bombus terrestris*, bumblebee, *Apis mellifera*, honeybee, floral scent, hive scent, foraging choices, foraging behavior, positioning, scent orientation.

*To whom correspondence should be addressed at: Department of Food Science and Technology, Research Center Aarslev, Kirstinebjergvej 12, 5792 Aarslev, Denmark.

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INTRODUCTION

Foraging honeybees (*Apis mellifera* L.) are influenced in their choice of flower by the scents previously experienced in the colony. The scents may originate from the food stores (Wenner et al., 1969), from the cuticula of dancing bees, and from the food samples passed on from the dancing bee to attending bees (Frisch, 1950). This knowledge has been used in early attempts to direct honeybee foragers to certain crops in order to avoid the common phenomenon that the foragers choose a crop other than the one intended. Colonies have been fed inside the hive with sugar solution containing flowers of the crop (Glushkov, 1958; Stapel, 1962; Frisch, 1965), or the bees have been fed outside the hive with a sugar solution either surrounded by flowers from the crop, or they were forced to crawl through flowers of the crop in order to reach the sugar solution (Frisch, 1950). The efficiency of these methods was reported to be highly variable (Frisch, 1965; Free, 1970a), and a more thorough knowledge of the mechanisms influencing the foraging choices based on odoriferous stimuli at the hive is evidently required.

A series of investigations have underlined the influence of the hive scent composition on the foraging behavior of honeybees; Ribbands (1954) demonstrated that foragers, trained to visit feeding stations with a particular scent at a particular time of the day, could be activated to search for it at other times by introducing the scent to the hive air. Free (1969) reported that although the food store scents influenced the foraging choices of honeybees, artificial scent added to the top of the frames showed no detectable effects on the foraging behavior. Further, it has been demonstrated that honeybees exposed to a certain scent, e.g., geraniol, during the first eight days after emergence generally show an increased affinity for this compound (Pham-Delègue et al., 1990a). Similarly, the "inexperienced" solitary bee *Colletes julgidus longiplumosus* has a preference for the species of pollen they received during the larval stage (Dobson, 1987). Finally, in a field study parallel to the present one, we found that application of a defined concentration of natural or artificial white clover (*Trifolium repens* L.) flower fragrance to honeybee hives increased the proportion of foragers returning with white clover pollen (Terkildsen et al., unpublished data). The majority of these studies indicates a potential for influencing the foraging choices of honeybees by changing the composition of scent in the colonies. The influence of hive scent on the foraging behavior of bumblebees (*Bombus* spp.) has received little attention in the literature. Studies by Free (1970b) suggested that hive scent originating from the pollen stores influenced the floral choices made by bumblebee foragers (*Bombus lucorum* L.).

In the present study we make an attempt to interfere in the odoriferous signaling in the hive described above by adding a single floral component to the hives in relatively high concentration in order to influence the foragers to choose

feeding stations scented with the same floral component rather than differently scented stations.

METHODS AND MATERIALS

Insect Material. Two honeybee (*Apis mellifera* L.) colonies (Y and Z) each comprising 1000–1500 workers and a mated queen (supplied by the Danish Institute of Plant and Soil Science, Laboratory of Bee Keeping, Roskilde) were kept in small hives (234 × 175 × 57 mm). All foragers were removed from the colony, leaving only workers with no foraging experience and the queen. Three bumblebee (*Bombus terrestris* L.) colonies (A, B, and C) each with 50–100 workers and a queen (supplied by Koppert B. V., Berkel en Rodenrijs, The Netherlands) were kept in polystyrene boxes (270 × 190 × 160 mm). The bumblebee workers had been raised in captivity and had no foraging experience. Hives of both species were maintained in an incubator at 28°C. Every second day all colonies were fed with a mixture of pollen pellets, originating from a wide range of plant species. The pollen had been collected in pollen traps mounted on honeybee hives and stored at −18°C. All colonies were fed from the same batch of pollen throughout the experiment. When the level of stored sugar in the colonies was evaluated as being deficient, the colonies were offered a sugar solution (80% invert sugar) in a feeder placed on a table 3 m from the hive entrance.

The Arena. The design of the arena was based on that developed by Waller et al. (1973). It consisted of a round grey PVC disc placed on a wooden base (1.25 m diam.) with 12 feeding stations along the rim (Figure 1). At each feeding station a landing disc, with a central hole (6 mm diam.), was located above a

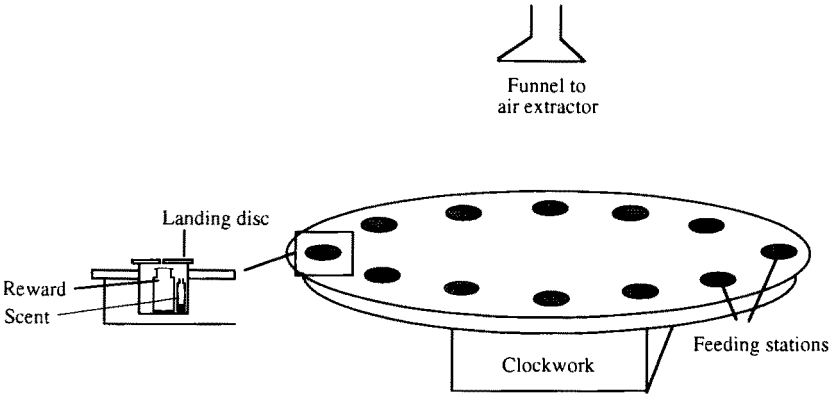

FIG. 1. The rotating arena and a cross section of a feeding station.

100-ml polycarbonate beaker (id: 50 mm ID, 50 mm high). A glass vial (11 ml; 45 mm high, 13 mm ID) containing the reward (80% invert sugar or water) and a 2-ml screw-top glass vial (5 mm ID) (Hewlett Packard) containing the scent were placed inside the beaker (Figure 1). In order to facilitate scent orientation, every second feeding station was unscented and contained water, whereas stations between the water stations contained a sugar reward and were scented alternately with myrcene and 2-ethyl-1-hexanol (Figure 1). The arena was rotated by a clockwork mechanism (12 rotations per hr) to ensure a random location of each feeding station.

Experimental Design. Honeybee colony Y was exposed to three treatments, i.e., initially without scent added to the hive (control), then with myrcene, and finally with 2-ethyl-1-hexanol added to the hive. Colony Z was exposed to the same three treatments, but with a reversed order of scent treatment (control, 2-ethyl-1-hexanol, and finally myrcene). Bumblebee colonies are suitable for participation in the experiments during the relative short interval from the developmental stage with 20–30 workers until the emergence of new males and queens. Consequently, each colony could only be subjected to two treatments as described below. Colony A was initially tested with 2-ethyl-1-hexanol in the hive and subsequently with myrcene. Colony B was tested without addition of scent to the hive, then with 2-ethyl-1-hexanol, and colony C without addition of scent to the hive, followed by conditions with myrcene added. The myrcene and 2-ethyl-1-hexanol vials (0.2 ml scent added, 95–99% purity in each of three 2-ml vials) were placed inside the hives of both species in a netted beaker at least 24 hr prior to test start. Each scent component remained inside the hive for five to seven days, i.e., until a sufficient number of visits at the arena had been recorded.

Each bee participated in one treatment only. Each bumblebee colony participated in the experiments for about one month, i.e., from the stage with 20–30 workers and until the emergence of males and new queens. The honeybee colonies were used for approximately one month. Between nine and 15 bees from each colony were used in each treatment. Colonies were tested in sequence in the period June–November. Visits at the arena were recorded daily between 10 AM and 3 PM. Recordings of bees at the arena took place in a netted flying cage (4 × 12 × 2 m) placed inside a thermoregulated greenhouse (20–25°C) under natural light conditions. Bees were allowed to fly in the cage for a few days prior to participation in the experiments.

Individual bees were marked during first landing at a feeding station in order to be able to follow the foraging pattern of each forager. Honeybees were marked on the abdomen and/or the legs or thorax with combinations of colors (Ceramic Colour, Pepeo, France), whereas bumblebees were marked on the legs only. A visit was regarded as successful if the bee managed to take up sugar solution (or water) at the feeding station.

The relative position of each landing on the arena was noted together with

the scent of the feeding station (i.e., myrcene, 2-ethyl-1-hexanol or water, positions 1–12 (in Figure 2). At most, three bees were permitted to land on the arena at the same time. Each bee was allowed a maximum of eight visits a day. Surplus bees approaching the arena were captured and kept in matchboxes until the recordings of the day were completed. Between two and 10 bees participated each test day, depending on the activity level of the colony. If a bee visited the arena less than five times on its first test day, succeeding visits the following day were included. The arena was removed from the cage after the daily recordings. The hive entrance was closed at the end of the day, and any bees still flying in the cage were transferred to the hive.

Bees of both species normally started to investigate the arena soon after it had been transferred to the cage, landing on the feeding stations within the first 2 hr. However, on some occasions bees hovered above the feeding stations and seemed unwilling to land on the arena. In order to enhance landing, free-flying workers were captured and placed either in the middle of the arena with a sugar droplet or on a separate unscented feeding station containing sugar water. Landing discs were replaced after each bee visit, and all materials were cleaned with an aroma-free detergent (Extran), demineralized water, and ethanol (70%) in order to remove any odor contamination. Handling of all test material was performed with vinyl gloves. To prevent the accumulation of volatiles from the scented feeding stations in the greenhouse air, a vacuum cleaner (Nilfisk GA71, flow 20–25 liters/sec, tube ID: 31 mm, length 1 : 12 m) positioned outside the greenhouse was connected to a funnel [12 cm ID, positioned 65 cm above the center of the arena (Figure 1)].

Scent Sampling. Concentrations of volatiles in the air within the hive and above individual feeding stations were monitored by collecting air samples for 30 min every third day. Samples of the hive air were collected by placing a glass column (4 mm ID, 180 mm long) with 100 mg Porapak Q 50–80 mesh (Waters Inc., Milford, Massachusetts) inserted between two desiloxanized glass-wool plugs in a hole drilled in the side of the hive. Air samples above feeding stations were collected by positioning the Porapak column 5 cm above the landing disc. A vacuum pump carried a constant flow of air (300–600 ml/min) through the columns; flow rates were controlled by a flowmeter. Gas chromatographic quantification of myrcene and 2-ethyl-1-hexanol was performed as described previously (Jakobsen and Olsen, 1994).

Statistics. Visits were statistically grouped into three categories: (1) the first visit made by a bee to the arena ("inexperienced" bees); (2) the first five visits to the arena ("learning" bees); and (3) visits following the initial five visits ("experienced" bees). Only the initial five visits of each bee were used in the statistical analysis on influences of addition of scent to the hives. Visits extending the first five were used to describe the long-term foraging pattern of the individual bee.

The distribution of visits to nonscented, myrcene-scented, and 2-ethyl-1-

hexanol-scented feeding stations was observed for the foragers of each colony prior to introduction of myrcene or 2-ethyl-1-hexanol to the hives. These tests served as controls in the statistical analysis. Visits to a nonscented visiting station were only observed for a minor group of bees that collected water repeatedly from these stations. Therefore they were excluded from the statistics on differences among scented feeding stations.

The null hypothesis that the proportions of foragers choosing feeding stations scented with myrcene and 2-ethyl-1-hexanol, respectively, is the same under conditions with and without scent added to the hive was tested in a 2 × 2 contingency table using the χ^2 distribution. This test was also used in the comparisons of the distribution of visits on the arena during the "learning" phase and the "experienced" phase. Probability values for possible preferences for one of the two scents under control conditions were calculated using common methods under the binomial distribution.

RESULTS

The mean concentrations of volatiles measured directly above the feeding stations were 114 ± 58 ng/liter of air for myrcene and 42 ± 8 ng/liter for 2-ethyl-1-hexanol. Concentrations of the scents in the hives were 21120 ± 1891 ng/liter and 1920 ± 267 ng/liter, respectively. Bees of both species visited only one feeding station per foraging trip and spent 2–3 min taking up the sugar solution.

Honeybees. The two tested scents were equally attractive to honeybees (*Apis mellifera* L.) prior to any addition of scent to the hives (Table 1). After introduction of 2-ethyl-1-hexanol to the hives, a significantly higher proportion of the visits were to feeding stations scented with this compound (70.9%) than under conditions without addition of scent to the hives (43.3%, Table 1). These comparisons were performed using the initial five visits made by each bee. Similarly, high proportions of visits were recorded at stations scented with 2-ethyl-1-hexanol during conditions with this compound added to the hives when only the first visit of each bee was used in the comparisons (Table 1). Introduction of myrcene to the hives did not induce more visits to sites with this compound compared to conditions without addition of scent to the hives (Table 1). A strong correlation was noted between the first and subsequent scent choices on the arena, i.e., honeybees visiting myrcene on the first trip chose myrcene in 66% of the following visits, and bees choosing 2-ethyl-1-hexanol on their first encounter landed on this scent in 69% of the following visits.

An analysis of the visiting pattern for 13 honeybees each visiting the arena approximateiy 25 times showed no preference for visiting feeding stations positioned within a particular area of the test arena (Figure 2A). Eight of the expe-

TABLE 1. EFFECT OF SCENT ADDITION TO HONEYBEE HIVES[a]

Scent added to hive:	Colony number	Initial five visits to arena[a]				First visit to arena			
		Number of visits	% visits to		P	Number of visits	% visits to		P
			Myrcene	2-Ethyl-hex.			Myrcene	2-Ethyl-hex.	
None	1	35	54.3	45.7	NS[b]	7	28.6	71.4	NS[b]
None	2	25	60.0	40.0	NS[b]	5	60.0	40.0	NS[b]
None	1 + 2	60	56.7	43.3	NS[b]	12	41.7	58.3	NS[b]
Myrcene	1	75	56.0	44.0	NS[c]	15	66.6	33.3	NS[c]
Myrcene	2	50	54.0	46.0	NS[c]	10	60.0	40.0	NS[c]
Myrcene	1 + 2	125	55.2	44.8	NS[c]	25	64.0	36.0	NS[c]
2-ethyl-hex.	1	40	25.0	75.0	**[c]	8	50.0	50.0	NS[c]
2-ethyl-hex.	2	45	33.3	66.6	*[c]	9	11.1	88.9	NS[c]
2-ethyl-hex.	1 + 2	85	29.1	70.9	***[c]	17	29.1	70.9	NS[c]

[a] The heading "Initial five visits to arena" refers to the initial five visits made by the bees of each hive participating in a given treatment. These visits are cumulative, whereas only the very first visit made by each bee to the arena is analyzed under the heading "first visit to arena."

[b] Probability under the null hypothesis that the two scents are equally attractive to the bees. Binomial test.

[c] Probability values comparing the distribution of visits prior to addition of scent to the hive with those observed with scent added to the hive. Data were analyzed in a 2 × 2 contingency table.

NS: nonsignificant at the 0.05% level, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

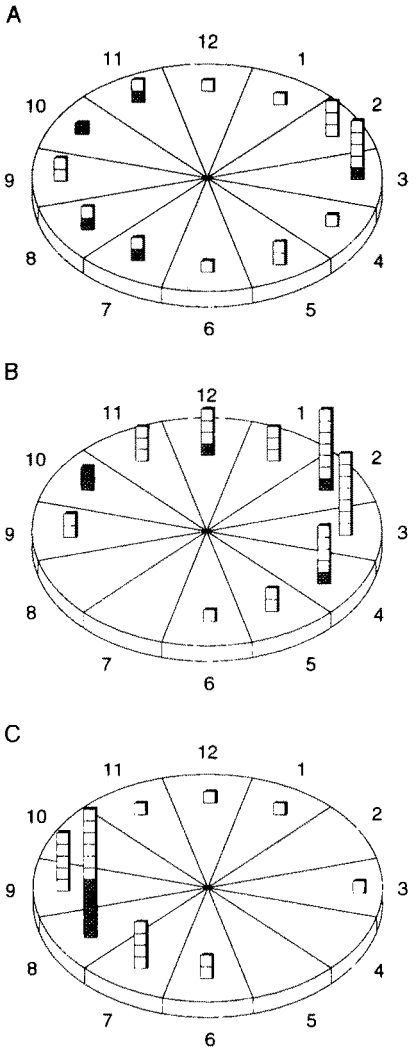

FIG. 2A–C. The three major foraging patterns described by honeybees and bumblebees on the arena. Each cube on the arena represents one visit to a feeding station at the position indicated by numbers along the periphery (e.g., 3 = north, 6 = east, 9 = south, 12 = west). ▨ = first five visits, □ = subsequent visits. (A) A honeybee visiting feeding stations with the same scent every time. The visits are evenly distributed to the 12 different positions on the arena. This pattern was displayed by 92% of the honeybees. (B) Visiting pattern of a bumblebee that did not orient in relation to a particular position during the first five visits, but showed a preference for the 2 and 3 o'clock region during

rienced bees oriented towards a particular scent (although not necessarily the same as the one in the hive) while the remaining five visited both odors equally without showing preference for any of them. A few honeybee foragers collected water repeatedly from the unscented feeding stations.

Bumblebees. Myrcene and 2-ethyl-1-hexanol were equally attractive to bumblebees (*Bombus terrestris* L.) at the arena prior to addition of scent to the hives (Table 2). Addition of 2-ethyl-1-hexanol to the hives did not induce significantly more visits to feeding stations scented with this compound (47.4% before and 57.6% after addition of scent, Table 2). Similarly, introduction of myrcene had no detectable influence on preferences during either the first or subsequent visits to the arena (Table 2). The bumblebees showed a significant constancy towards one of the two components in the first five visits to the arena ($P < 0.001$, Figures 2B and C). This strong correlation between the scent chosen on the first visit and during subsequent visits to the arena was not influenced by addition of scent to the hive ($P > 0.05$). After the first five visits, approximately 75% of all foragers became indifferent to scent type. Furthermore, 91% of these ended up visiting solely a particular position, regardless of the scent emitted from the station (Figure 2B and C). The bumblebees visited primarily sites with odor and sugar water. Some foragers, however, collected water repeatedly and carried it back to the hive (3.4% of all visits, $N = 1008$).

DISCUSSION

The observed similar attractiveness of myrcene and 2-ethyl-1-hexanol to both honeybees and bumble bees on the test arena prior to addition of scent to the hives suggested that a comparison of these two compounds would be appropriate in a test for effects of scent introduction to the hives. The subsequent significant increase in the proportion of honeybees choosing sites scented with 2-ethyl-1-hexanol after introduction of this volatile to the hive air demonstrates that the foraging choices of honeybees can be influenced by introducing odoriferous stimuli to the hive. This conclusion is supported by recent studies showing that introduction of white clover scent to honeybee hives under field conditions incited colonies to collect significantly more white clover pollen than control hives (Terkildsen et al., unpublished data). The added scents were either a mixture of synthetic volatiles or detached white clover flower heads. Two

subsequent visits. This pattern type was displayed by 36% of all the bumblebees. The bee visited only beakers with myrcene during the first eight visits but became gradually indifferent to scent type over time. (C) Bumblebee orienting almost exclusively toward the 8 o'clock region. Pattern displayed by 39% of the bumblebees. This bee was indifferent to scent type from the beginning.

TABLE 2. EFFECTS OF SCENT ADDITION TO BUMBLEBEE HIVES[a]

Scent added to hive	Colony number	Initial five visits to arena: Number of visits	Initial five visits to arena: % visits to Myrcene	Initial five visits to arena: % visits to 2-Ethyl-hex.	Initial five visits to arena: P	First visit to arena: Number of visits	First visit to arena: % visits Myrcene	First visit to arena: % visits 2-Ethyl-hex.	First visit to arena: P
None	2	45	57.7	42.2	NS[b]	9	66.6	33.3	NS[b]
None	3	50	48.0	52.0	NS[b]	12	50.0	50.0	NS[b]
None	2 + 3	95	52.6	47.4	NS[b]	21	57.1	42.9	NS[b]
Myrcene	1	65	63.1	36.9	NS[c]	14	64.3	35.7	NS[c]
Myrcene	3	60	46.7	53.3	NS[c]	12	50.0	50.0	NS[c]
Myrcene	1 + 3	125	55.2	44.8	NS[c]	26	57.7	42.3	NS[c]
2-ethyl-hex.	1	25	32.0	68.0	NS[c]	6	33.3	66.7	NS[c]
2-ethyl-hex.	2	65	47.7	52.3	NS[c]	13	46.2	53.8	NS[c]
2-ethyl-hex.	1 + 2	90	43.3	56.7	NS[c]	19	42.1	57.9	NS[c]

[a]The heading "Initial five visits to arena" refers to the initial five visits made by the bees of each hive participating in a given treatment. These visits are cumulative, whereas only the very first visit made by each bee to the arena is analyzed under the heading "First visit to arena."

[b]Probability under the null hypothesis that the two scents are equally attractive to the bees. Binomial test.

[c]*P* value comparing the distribution of visits prior to addition of scent to the hive with those observed with scent added to the hive. Data were analyzed in a contingency table.

NS: non-significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

possible causes for the lack of response to myrcene application to the hives may be suggested: (1) The concentration of myrcene in the hive was too high (it was approximately 10 times higher than for 2-ethyl-1-hexanol). (2) The information of myrcene in the hive air may not be used by foraging bees in the same way as 2-ethyl-1-hexanol. Menzel (1985) suggested that certain odors are more attractive due to a "prepared search image," i.e., a genetically programmed preference for some orientation cues. Two major observations support this second hypothesis: (1) The scent emitted from the whole plant may be used when locating the plant from a distance, whereas the floral fragrance is used as an orientation cue on close range (Beker et al., 1989). Consequently, volatiles effective in guiding bees to the flower should be emitted from the flowers only (Pellmyr and Thien, 1986). Besides being among the most common floral volatiles (Knudsen et al., 1993), myrcene is also a constituent of green leaves (Jakobsen et al., 1994). It may therefore not be suitable as an orientation cue for pollinators attempting to locate the flower. Previous investigations support the assumption that some of the volatiles comprising the floral fragrance are "excluded," i.e., are not necessarily used as an orientation cue by the bees when identifying the flower of a particular species; Pham-Delègue et al. (1990b, 1991) demonstrated that only a limited fraction of the floral fragrance is needed for the recognition of the total volatile blend in the flower. The compound effective in influencing the foraging choices of honeybees in the present study, 2-ethyl-1-hexanol, has only been detected in the floral headspace of *Theobroma* (Erickson et al., 1987) and *Trifolium repens* L. To our knowledge 2-ethyl-1-hexanol has not been identified in the emissions from leaves. (2) In *Brassica napus* myrcene is emitted from both leaves and flowers, but, in contrast to some of the other floral volatiles, it is emitted in a nonrhythmic manner (Jakobsen et al., 1994). This may be yet another indication that this compound is of secondary importance in the attraction of pollinators to the *Brassica napus* flower. The rhythmic emission of floral volatiles in species with a diurnal or nocturnal maximum of emission has been demonstrated to coincide with the period of activity of the major pollinators (Matile and Altenburger, 1988; Loughrin et al., 1990, 1991; Jakobsen and Olsen, 1994; Hansted et al., 1994; Jakobsen et al., 1994). This correlation suggests that the rhythmically emitted compounds are significant in the attraction of pollinators to the flower. In *T. repens* 2-ethyl-1-hexanol is emitted in a rhythmic manner with a diurnal maximum of emission (Jakobsen and Olsen, 1994), and *T. repens* is pollinated almost entirely by honeybees.

The influence of added scents on the honeybee foraging behavior observed in the present study is in accordance with Ribbands (1954), who reported that honeybees conditioned to visit a feeder scented with either methyl benzoate or benzyl acetate at a particular time of day could be activated to visit the feeder at other times by introducing the feeder scent to the hive air. In contrast, Free (1969) observed that benzyl acetate or methyl heptanone distributed in solution

to the top of the frames of honeybee hives failed to influence the behavior of foragers. However, no information on the concentration levels of the added scents in the hive air was given in any of the investigations. The theories mentioned above on "prepared search images," etc., are not applicable in the experiment by Ribbands (1954) or Free (1969) because the bees in those studies had been conditioned to the scents prior to participation in the hive scent experiments.

The observed lack of response of bumblebees to the added hive scents is in contrast to suggestions by Free (1970b), who compared foraging patterns of bumblebee colonies (*Bombus lucorum* L.) under natural conditions and suggested that hive scents originating from the pollen stores may influence the floral choices made by the bumblebee foragers. The lack of response to hive scents observed in the present study may be partly explained by the positioning behavior discussed below.

The observed strong positive correlation between the scent first encountered at the test arena together with a reward and the scent chosen during subsequent visits is consistent with general theories on associative learning (Menzel, 1993). Changes in foraging behavior through associative learning during the series of visits to the arena showed clearly different patterns for bumblebees and honeybees: After a few visits to the arena, bumblebees associated the reward with a certain position on the arena in most cases, whereas honeybees associated the reward with a particular scent. The observed positioning of bumblebee foragers, in contrast to that of honeybees, may be compared to the foraging strategies of the two species under natural conditions. Honeybees have developed a dance language for recruitment of foragers in order to exploit extensive food sources efficiently (Frisch, 1965, 1974; Michelsen et al., 1987). The message of the dance, however, only communicates the approximate direction and distance to the food source. At the approximate location, several potential sources, i.e., plant species, may be present in the area. The foragers may locate the source by associating the floral scent diffusing from the dancing bee and from the nectar passed on from the dancer with the scents encountered in the area. Consequently, the honeybee forager primarily uses odoriferous orientation only after arriving in the general area of the food source. If the flower odor is used for orientation, then little accurate positioning in relation to the area is required. The significance of a particular odoriferous composition may be reflected in the fact that honeybees normally visit only one flower species per foraging trip. In contrast, bumblebees, which have no known dance language, often follow the same route during trips on successive days, visiting several different species each day (Free, 1970b), and they are not "linked" to a particular scent as are honeybees. This strategy indeed requires some positioning behavior when flying between small patches, i.e., the exact position of the plant is of importance to the bumblebee's strategy. The hypothesis that the foraging choices of bumblebees are based on

the position and scent of the plant rather than the scent stimuli received in the hive is in accordance with studies by Pham et al. (1983). Their study showed that experiences of the individual bee during foraging appears to dominate when it selects among potential food sources, in spite of the communication via indirect trophallaxis among the individuals in the colony. These differences in foraging strategy may be reflected in the positioning behavior observed in bumblebees, which contrast with the odoriferous orientation at the arena observed in honeybees.

Water collection by bumblebees has not been reported previously to our knowledge. These foragers appear to use the odoriferous stimuli of the beakers containing sugar differently from the sugar-collecting bees, i.e., water-collecting foragers avoided scented beakers, thereby locating beakers with water only. The association between hive scent composition and increased preference of honeybees for food sources emitting the same scent is clearly not a simple one. Further investigations on the effects of scents comprising more than one compound, together with tests involving several levels of scent concentrations, are required in order to understand more fully the association between the accumulated floral scents in the hive and the choices made by foraging bees.

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ANTENNAL PERCEPTION OF OILSEED RAPE, *Brassica napus* (Brassicaceae), VOLATILES BY THE CABBAGE SEED WEEVIL *Ceutorhynchus assimilis* (Coleoptera, Curculionidae)

M.M. BLIGHT, J.A. PICKETT, L.J. WADHAMS,* and C.M. WOODCOCK

IACR-Rothamsted
Harpenden, Hertfordshire AL5 2JQ, U.K.

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Abstract—The response of the *Ceutorhynchus assimilis* antenna to volatiles in air entrainment-derived extracts of oilseed rape, *Brassica napus*, was studied using coupled gas chromatography (GC)–electroantennography (EAG) and coupled GC-single cell recording (SCR). By means of these techniques and coupled gas chromatography-mass spectrometry (GC-MS), 25 active compounds were identified, including isoprenoids and compounds derived from fatty acids and amino acids. Some of the latter, the isothiocyanates and goitrin, and probably indole and benzyl cyanide, are catabolites of glucosinolates. The electrophysiological activity of the identified compounds was confirmed by EAG using a physiologically discriminating dose, and by SCR studies. The importance of the combined use of the EAG and SCR techniques was demonstrated, since specific olfactory cells were located for five compounds that did not elicit significant EAG responses. The majority of the olfactory cells from which single cell recordings were obtained showed very high specificity, and in numerous recordings there were consistent pairings of specific cell types.

Key Words—*Ceutorhynchus assimilis*, cabbage seed weevil, Coleoptera, Curculionidae, *Brassica napus*, oilseed rape, volatiles, isothiocyanates, EAG, SCR, GC-EAG, GC-SCR.

*To whom correspondence should be addressed.

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INTRODUCTION

Over the last 20 years, the area grown to oilseed rape, *Brassica napus* (Brassicaceae = Cruciferae), in the United Kingdom has increased approximately 100-fold to a recorded area of 376,700 ha in 1993, i.e., 8% of the total arable crop area. Similar increases have occurred in other temperate European countries (Anon., 1994). One of the more important bud and inflorescence pests of oilseed rape is the cabbage seed weevil, *Ceutorhynchus assimilis* (Payk.) (Curculionidae). The eggs are deposited singly in developing pods and larval feeding on the seeds reduces crop yield in heavy infestations. In addition, punctures in pods made by *C. assimilis* during feeding and egg-laying are used for oviposition by another pest, the pod midge, *Dasineura brassicae* (Cecidomyidae) (Alford et al., 1991).

Host location in phytophagous insects is mediated by a combination of visual and olfactory cues (Visser, 1986). *C. assimilis* is attracted to *B. napus* volatiles (Görnitz, 1953; Free and Williams, 1978; Evans and Allen-Williams, 1989; Bartlet et al., 1993), and a number of the components involved, particularly the isothiocyanates (NCS), volatile catabolites of the glucosinolates that are characteristic of the Brassicaceae, have been identified and their antennal perception studied using both electroantennograph (EAG) (Kozlowski, 1984; Evans and Allen-Williams, 1992) and single cell recording (SCR) techniques (Blight et al., 1989, 1992). Although excellent field-trapping results have been obtained with synthetic isothiocyanates (Blight et al., 1992; Smart et al., 1993), the complex mixture of volatiles emitted by current varieties of *B. napus* contains a preponderance of secondary metabolites not derived from glucosinolates (Tollsten and Bergström, 1988; Blight et al., 1992; Evans and Allen-Williams, 1992) and many of these may also influence orientation of *C. assimilis* to the host plant.

We now report a detailed study in which electrophysiological techniques directly coupled to high-resolution capillary column gas chromatography (GC) were used to identify *B. napus* volatiles that interact with the seed weevil antenna. The activities of these volatiles, and some related compounds were then examined using both the EAG and SCR techniques.

METHODS AND MATERIALS

Plant Volatiles. Volatiles were collected from flowering shoots of *B. napus* using a dynamic headspace (air-entrainment) technique described previously (Blight, 1990). Cut flowering stems, bearing a few leaves, of the single low *B. napus* cv. Willi were contained in water in conical flasks placed in a glass culture vessel (20 liters). Volatiles were drawn from the vessel, using purified

air, onto a tube of the adsorbent Porapak Q (4 g). At the conclusion of each air entrainment, which lasted 7–10 days, the volatiles were eluted from the Porapak with pentane and the extract was concentrated by fractional distillation of the solvent using a column of glass helices. After final concentration under nitrogen, the extracts (100–500 μl) were stored at $-20°C$ in microvials for immediate use or sealed in glass ampoules.

Insects. C. assimilis were collected from crops of flowering *B. napus* and sexed, under carbon dioxide, according to Bonnemaison (1957). The sexes were maintained separately on flowering racemes of glasshouse-grown *B. napus* (cv. Topas) contained in jars of water that were enclosed in polyester mesh bags. The cultures were kept in a controlled environment cabinet at 15°C:9°C, 16L:8D.

Chemicals. Pentane and hexane (both HPLC grade, Fisons) were purified as in Blight (1990). Most chemicals were obtained from commercial sources (Aldrich Chemical Co. Ltd., Fluka Chemie AG, Lancaster Synthesis Ltd., Bedoukian Research Inc.), but (*E,E*)-α-farnesene was extracted from apple skins with ether (Murray, 1969) and purified by elution from Florisil. 3-Butenyl and 4-pentenyl NCS and all the cyanides (Table 3 below) except 2-phenylethyl cyanide (purchased from Aldrich) were synthesized in the laboratory by standard methods (Dawson et al., 1993). α-Terpinolene was a gift from Professor F. Camps, University of Barcelona. Most substances were of purity $\geq 98\%$, except for 4-pentenyl NCS, linalool, and benzthiazole (each 97%), allyl NCS (94%), and phenylacetaldehyde (90+%). α-Terpinene and myrcene were ca. 85% pure. Standard solutions (10^{-5} to 10^{-10}g/10 μl) of all compounds were prepared in purified hexane for electrophysiological studies.

Gas Chromatography (GC). Air entrainment volatiles were separated on a 50 m × 0.32 mm ID methyl silicone bonded-phase fused silica capillary column (HP-1) fitted in a Hewlett Packard 5880A gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID). The carrier gas was hydrogen and the oven temperature was maintained at 40°C for 5 min and then programmed at 5°/min to 150°C, then at 10°/min to 250°C.

Gas Chromatography–Mass Spectrometry (GC-MS). A capillary column (50 m × 0.32 mm ID HP-1) fitted in a Hewlett Packard 5890 GC was directly coupled to the MS and integrated data system (70-250 VG Analytical). Ionization was by electron impact at 70 eV, 230°C. The GC was maintained at 30°C for 5 min and then programmed at 5°/min to 180°C. Tentative identifications made by GC-MS were confirmed by comparison of the mass spectral data with those of authentic samples and by peak enhancement when coinjected with authentic compounds using GC, as above (Pickett, 1990).

Electrophysiology: EAG. Recordings from whole antennae were made using Ag–AgCl glass electrodes filled with saline solution (composition as in Maddrell, 1969, but without the glucose). Antennae were excised and suspended

between the two electrodes. The signals generated by the antenna were passed through a high-impedance amplifier (Syntech UN-03b) and displayed on an oscilloscope. The output from the oscilloscope was monitored on a chart recorder. EAG responses were normalized with respect to the response elicited by 10^{-6} g of (Z)-3-hexen-1-ol presented after every sixth stimulation. Compounds were tested against five individual antennae and results were analyzed using Student's *t* test.

Electrophysiology: SCR. Recordings from individual olfactory cells on the antenna were made with tungsten microelectrodes (Boeckh, 1962). The indifferent electrode was placed in the antennal scape, and the recording electrode was brought into contact with the surface of the antenna until impulses were recorded. Signals were amplified (Syntech UN-03b), displayed on an oscilloscope, and stored on magnetic tape. Permanent copies of the action potentials generated by the olfactory cells were obtained by standard methods (Wadhams et al., 1982). Impulse frequency was determined as the number of impulses elicited during the first 1 sec after stimulation. Compounds were presented twice to each preparation at intervals of 2–10 min, the exact interval being determined by the concentration of the previous stimulus.

Stimulus Delivery. For both the EAG and SCR techniques, the stimulus (1 sec duration) was delivered into a purified airstream (1 liter/min) flowing continuously over the preparation. The delivery system, which employed a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). Samples (10 μl) of the standard solutions of test compounds were applied to filter paper strips, and the solvent was allowed to evaporate (30 sec) before the paper strip was placed in the cartridge. The control stimulus was hexane (10 μl). Fresh cartridges were prepared immediately prior to each stimulation.

GC-EAG and GC-SCR. The GC-EAG and GC-SCR systems, in which the antennal preparation is directly coupled to the capillary column of the gas chromatograph, have been described previously (Wadhams, 1990). Separation of the air entrainment samples was achieved on an AI 93 gas chromatograph equipped with a cold on-column injector and a FID. The carrier gas was hydrogen and the column (50 m × 0.32 mm ID HP-1) was maintained at 40°C for 1 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. Simultaneous records of the FID and antennal responses were obtained by detecting with a level discriminator and plotting by means of a voltage/frequency convertor.

RESULTS

On GC analysis, volatiles from intact flowering shoots of *B. napus* were shown to comprise highly complex mixtures (Figure 1), as expected from pre-

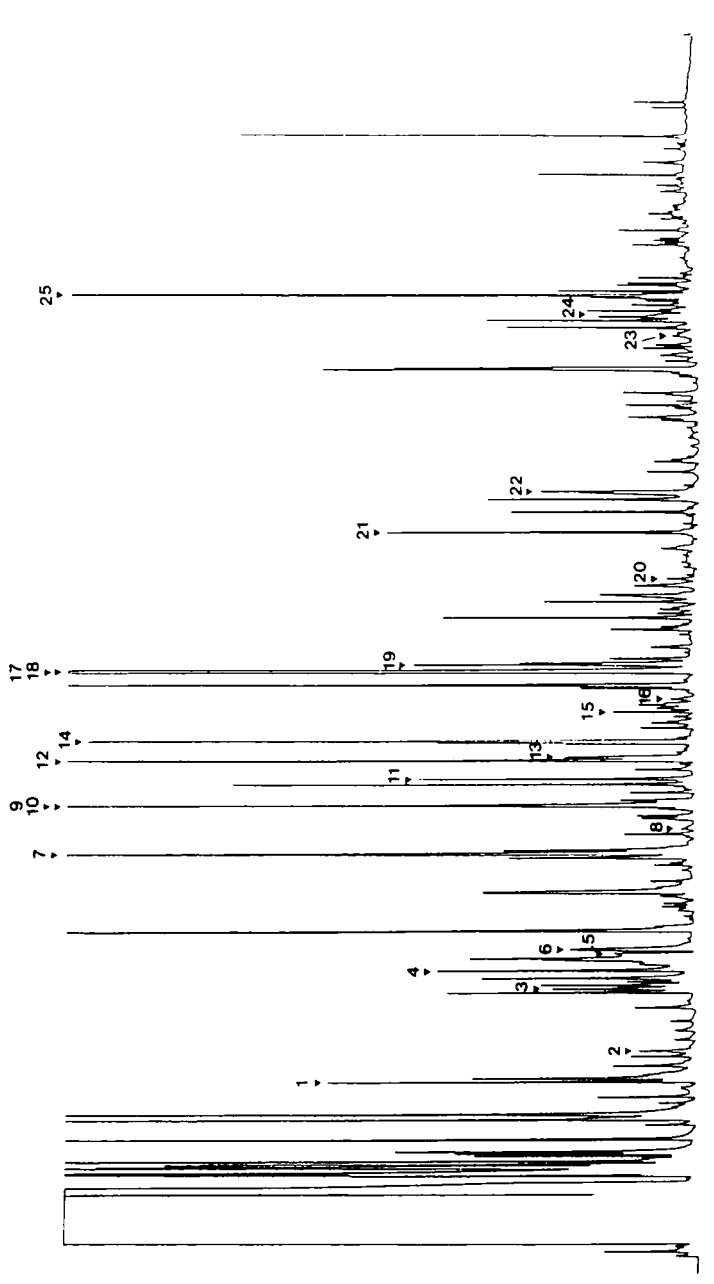

FIG. 1. Gas chromatogram of an extract of volatiles from *Brassica napus* cv. Willi. Numbered peaks (see Table 1) showed electrophysiological activity.

vious studies (Tollsten and Bergström, 1988; Evans and Allen-Williams, 1992). Biologically active compounds in these samples were located using coupled GC-electrophysiological techniques, initially GC-EAG and then GC-SCR (Tables 1 and 2). A typical GC-SCR trace is shown in Figure 2.

The electrophysiological activity of the identified compounds was confirmed by EAG recordings at the 10^{-6} g level, using authentic samples. Most of the active compounds (Tables 1 and 2) were ubiquitous plant volatiles such

TABLE 1. *Ceutorhynchus assimilis*: EAG RESPONSES TO VOLATILES RELEASED BY *Brassica napus*, EXPRESSED AS PERCENTAGE OF RESPONSE TO (*Z*)-3-HEXEN-1-OL

Type	Peak no. (see Figure 1)	Compound (10^{-6} g)	Females (± SE)	Males (± SE)
Amino acid derivatives	7	benzaldehyde	3 ± 2 NS	13 ± 3**
	12	benzyl alcohol	31 ± 3***	64 ± 7***
	13	phenylacetaldehyde	10 ± 2*	46 ± 5***
	16	2-methoxyphenol (guaiacol)	20 ± 3***	83 ± 19**
	17	2-phenylethanol	85 ± 1***	104 ± 11***
	19	benzyl cyanide	32 ± 6**	117 ± 17**
	20	methyl salicylate	123 ± 10***	159 ± 25***
	21	4-methoxybenzaldehyde (*p*-anisaldehyde)	35 ± 5***	40 ± 7***
	22	indole	28 ± 6**	43 ± 5***
	24	goitrin	21 ± 2**	13 ± 6 NS
Isothiocyanates	5	allyl NCS	3 ± 1 NS	16 ± 4 NS
	8	3-butenyl NCS	4 ± 1 NS	18 ± 5 NS
	15	4-pentenyl NCS	11 ± 4 NS	24 ± 6 NS
	23	2-phenylethyl NCS	78 ± 12***	150 ± 19***
Isoprenoids	9	sabinene	2 ± 2 NS	21 ± 6*
	14	1,8-cineole	10 ± 1 NS	20 ± 5 NS
	18	(±)-linalool	30 ± 7*	38 ± 6**
	25	(*E*,*E*)-α-farnesene	154 ± 4***	24 ± 6**
Fatty acid derivatives	1	1-pentanol	101 ± 3***	110 ± 9***
	2	hexanal	20 ± 2**	42 ± 2***
	3	(*E*)-2-hexenal	4 ± 1 NS	25 ± 5***
	4	(*Z*)-3-hexen-1-ol[a]	100	100
	6	1-hexanol	127 ± 16***	79 ± 16**
	10	1-octen-3-ol	25 ± 5**	38 ± 5*
	11	(*Z*)-3-hexenyl acetate	23 ± 9 NS	22 ± 9 NS

[a]Female response 0.22 mV ± 0.025; male response 0.28 mV ± 0.045 (± SE). *, **, ***: significantly different from control at $P = 0.05$, 0.01, and 0.001 respectively. NS = not significantly different from control at $P = 0.05$ (control = hexane, 10 μl).

TABLE 2. *Ceutorhynchus assimilis*: COMPOUNDS FOR WHICH SPECIFIC OLFACTORY CELLS HAVE BEEN LOCATED

Compound	Cells (*N*)
Isothiocyanates[a] (3-butenyl, 4-pentenyl, 2-phenylethyl)	60
1-Octen-3-ol	29
Methyl salicylate[a]	25
2-Phenylethanol[b]	13
Oxygenated monoterpenes (verbenone, verbenol)	9
1,8-Cineole[b]	8
(±)-Linalool	7
(*Z*)-3-Hexenyl acetate[c]	7
(*Z*)-3-Hexen-1-ol[c]	4
Benzyl alcohol	2
(*E*)-2-Hexenal	1
Sabinene	1

[a,b,c] Compounds followed by the same letter are detected by cells frequently occurring in pairs.

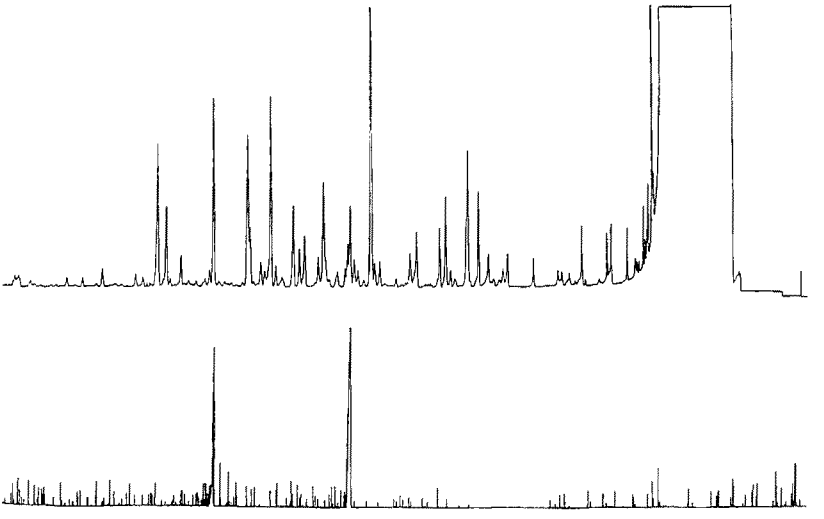

FIG. 2. Coupled gas chromatography–single cell recording (GC-SCR) from *Ceutorhynchus assimilis*. Upper trace: GC of volatiles from insect-damaged *Brassica napus* cv. Willi; lower trace: SCR showing response to 3-butenyl and 4-pentenyl isothiocyanates (small spikes are spontaneous cell activity).

as isoprenoids and compounds derived from fatty acids and amino acids. The isothiocyanates and goitrin, and probably the other nitrogen-containing compounds, indole and benzyl cyanide, are catabolites of glucosinolates derived originally from amino acids. Four of the compounds, 3-butenyl and 4-pentenyl

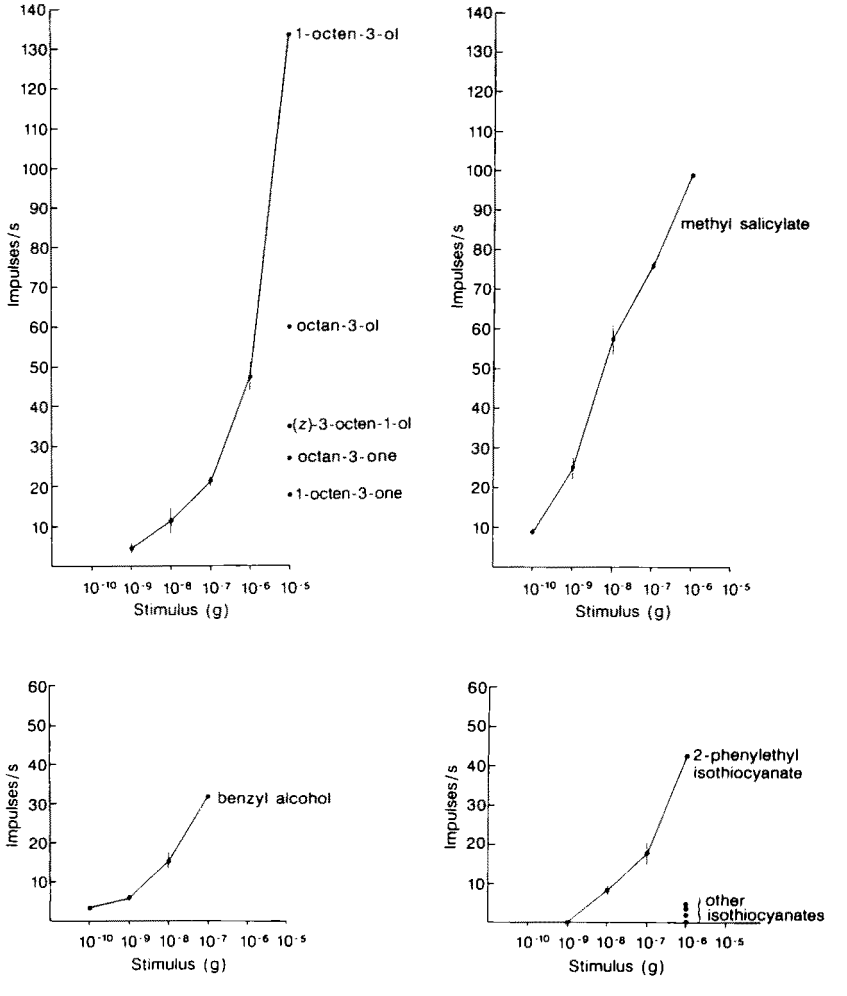

FIG. 3. Dose-response of *Ceutorhynchus assimilis* olfactory cells to *Brassica napus* volatiles. (Other isothiocyanates = allyl, butyl, pentyl, propyl, 3-butenyl, 4-pentenyl). Each point is the mean of two stimulations (bars = SE).

NCS, 1,8-cineole and (*Z*)-3-hexenyl acetate, did not elicit significant EAG responses, but nevertheless, specific olfactory cells were located for them using the SCR technique (Table 2, Figures 2 and 3).

Some additional compounds, including the known *B. napus* volatiles 2-aminobenzaldehyde, β-ionone, myrcene, dimethyldisulfide and dimethyltrisulfide, and six cyanides, which are glucosinolate catabolites found in macerated tissues of *Brassica* spp. (Tollsten and Bergström, 1988), were tested against females but elicited no EAG activity at physiologically discriminating levels (Table 3) and did not stimulate specific olfactory cells in these studies.

A total of 166 responding cells were recorded from the antenna of *C. assimilis*, most of which showed considerable specificity in their response pro-

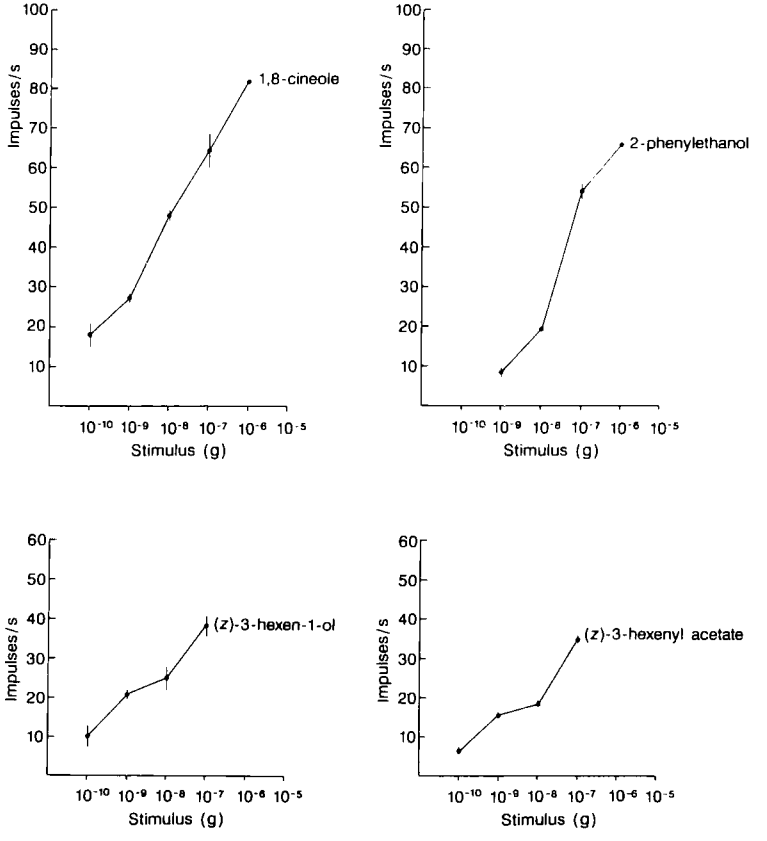

FIG. 3. Continued.

TABLE 3. *Ceutorhynchus assimilis* FEMALES: VOLATILES RELEASED FROM *Brassica* SPP. SHOWING NO SIGNIFICANT ELECTROPHYSIOLOGICAL ACTIVITY AT 10^{-6} GRAMS

Type	Compound
Aromatic	2-aminobenzaldehyde
	benzthiazole
Isoprenoids	β-ionone
	myrcene
	α-terpinene
Cyanides	2-propenyl cyanide
	4-pentenyl cyanide
	2-hydroxy-3-butenyl cyanide
	4-methylthiobutyl cyanide
	3-butenyl cyanide
	2-phenylethyl cyanide
Sulfides	dimethyldisulfide
	dimethyltrisulfide

files. Typical dose-response data and the numbers of the various cell types are shown in Figure 3 and Table 2, respectively. Approximately one third of the cells from which recordings were made responded specifically to 3-butenyl, 4-pentenyl and/or 2-phenylethyl NCS.

In a number of instances, recordings showed consistent pairings of certain cell types (Table 2, Figure 4). In most cases, the amplitudes of the two cells could be discriminated and the relative amplitudes of these cell pairs remained consistent between recordings. Thus, four of the seven recordings containing cells responding specifically and at low stimulus concentrations to (*Z*)-3-hexenyl acetate were associated with cells responding to (*Z*)-3-hexen-1-ol. In this instance, the large amplitude cell responded to the acetate while the smaller amplitude cell responded to the alcohol. Similar cell pairings were found with specific cells for methyl salicylate (small amplitude)/isothiocyanates (large amplitude) and for 1,8-cineole (large amplitude)/2-phenylethanol (small amplitude) (Figure 4).

DISCUSSION

The biologically active components of a mixture of *B. napus* volatiles, at concentrations appropriate to those encountered by insects in the field, have been identified by coupled GC–electrophysiology and subsequent GC-MS. However, coupled GC-EAG alone did not prove exhaustive in locating all the activity

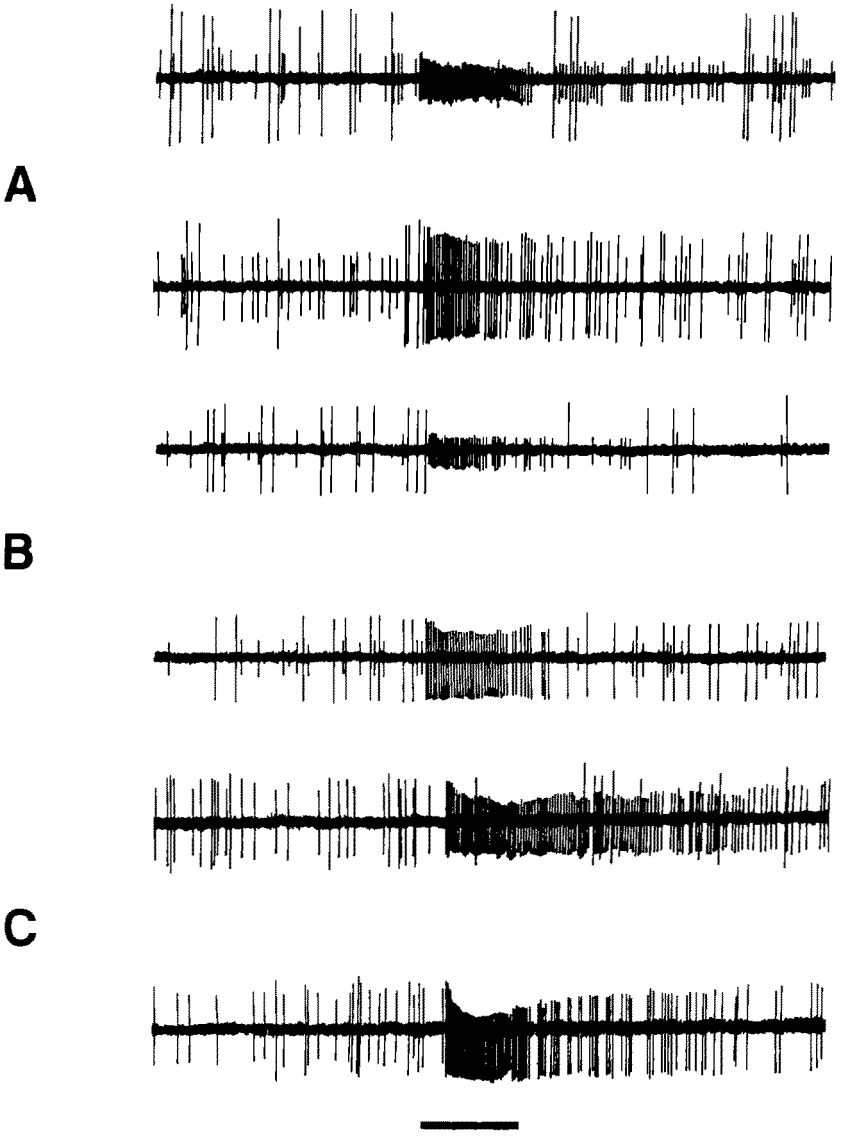

FIG. 4. Cell pairings occurring on *Ceutorhynchus assimilis* antenna: response to *Brassica napus* volatiles applied at 10^{-7} g. (A) Upper trace, methyl salicylate; lower trace, 2-phenylethyl isothiocyanate. (B) Upper trace, (*Z*)-3-hexen-1-ol; lower trace, (*Z*)-3-hexenyl acetate. (C) Upper trace, 2-phenylethanol; lower trace, 1,8-cineole. Bar = 1 sec stimulation.

in the samples in that some components, e.g., (*Z*)-3-hexenyl acetate and 1,8-cineole, elicited only weak EAG responses but showed significant SCR activity. This illustrates the importance of the combined use of these techniques.

The biological activity of the compounds identified from coupled GC–electrophysiology was confirmed initially by EAG at the 10^{-6} g level, which was used as a physiologically discriminating dose reflecting the situation occurring naturally. Previous studies using the same delivery system have shown that, at this level, stimulus concentrations at the antenna for compounds with molecular weights ranging from 120 to 200 are of the order of 10^{11} to 10^{12} molecules/ml of air (Wadhams et al., 1982). Such stimulus concentrations are close to the maximum level of volatiles produced from many crop plants.

Of the 25 electrophysiologically active compounds identified during this study, benzyl alcohol, methyl salicylate, 2-methoxyphenol (guaiacol), and 1-octen-3-ol have not been reported previously among volatiles from *Brassica* spp. Differences in the volatile profiles observed by different authors are undoubtedly due, in part, to variations between cultivars, but are also due to differences in sampling methods. Indeed, use of a coupled thermal desorption–GC-MS technique gave an entirely different profile of volatiles (Robertson et al., 1993), and (*E*,*E*)-α-farnesene, which was found as a major component in this and other studies (Tollsten and Bergström, 1988; Blight et al., 1992; Evans and Allen-Williams, 1992; Jakobsen et al., 1994), was not detected, probably because of its thermal instability.

Differences were observed in the EAG response given by males and females for a number of compounds (Table 1), suggesting a sexual dimorphism that is not apparent from the antennal morphology (Isidoro and Solinas, 1992). In the majority of cases, males showed an enhanced response compared to females and appeared to be more sensitive to the green leaf aldehydes hexanal and (*E*)-2-hexenal. Females were relatively more sensitive to (*E*,*E*)-α-farnesene. This is a dominant component of *B. napus* flower volatiles, and further studies are required to determine the behavioral significance of the differential response. It is interesting to note that (*E*,*E*)-α-farnesene is also one of the key compounds mediating mixture recognition of *B. napus* flower volatiles by foraging honeybees (Pham-Delègue et al., 1992).

A more detailed study on the perception of the electrophysiologically active compounds in *B. napus* air-entrainment samples was carried out using SCR techniques. From coupled GC-SCR analysis, where the antenna is stimulated with a wide range of compounds, and from dose–response data, it is evident that the majority of the olfactory cells have very high compound or functional group specificity, e.g., for the 1-octen-3-ol and 2-phenylethyl NCS cell types (see Figure 3A). Only in the case of cells responding to the oxygenated monoterpenes such as verbenol and verbenone is there any evidence for a gen-

eralist receptor system. These compounds have not been detected among *B. napus* volatiles but occur among volatiles from *B. nigra* (Tollsten and Bergström, 1988). However, the possible generalist nature of this receptor may simply reflect our inability in this study to identify the appropriate key compound for these cells.

Two types of olfactory sensilla are present on the antenna of *C. assimilis* (Isidoro and Solinas, 1992). The more numerous peglike sensilla basiconica are innervated by two sensory neurons, while the hairlike sensilla basiconica are singly innervated. The density of olfactory sensilla on the antenna precludes recording from predetermined olfactory structures with tungsten electrodes. However, the occurrence of numerous recordings showing consistent pairings of specific cell types indicates that these cell pairs are associated with the same peglike basiconic sensillum. The presence of paired receptor systems suggests that specific ratios of these compounds are important in the chemical ecology of this insect.

In general, there was a good correlation between the EAG amplitudes obtained and the proportion of cells responding to the active compounds, although cells responding to (*E*,*E*)-α-farnesene have not yet been found. Only weak EAG responses were obtained with the alkenyl isothiocyanates, but 2-phenylethyl NCS elicited significant EAG responses from both male and female *C. assimilis* antennae (Table 1). Significant EAG responses to alkenyl isothiocyanates have been obtained in previous studies but only at unnaturally high stimulus concentrations (Kozlowski, 1984; Evans and Allen-Williams, 1992). Our SCR studies have shown that perception of isothiocyanates by *C. assimilis* is mediated by both specialist and generalist isothiocyanate cell types, which constitute by far the greatest proportion of receptors on the antenna (Blight et al., 1989). None of these cells responded strongly at natural stimulus concentrations to allyl NCS.

Attraction of *C. assimilis* to mixtures of isothiocyanates has been demonstrated in a linear track olfactometer (Bartlet et al., 1993) and in the field (Blight et al., 1992) and suggests that these compounds play a key role in host-plant recognition by this insect. The behavioral function of 2-phenylethyl NCS requires further investigation, since *C. assimilis* did not respond to this substance when presented alone in the olfactometer, although responses were obtained to both 3-butenyl and 4-pentenyl NCS (Bartlet et al., 1993). However, the presence of cells that respond specifically to 2-phenylethyl NCS (see Figure 3A) does suggest that the compound is involved in insect–plant interactions.

Until relatively recently, agricultural ecosystems incorporating brassicaceous plants comprised a variety of species including *B. oleracea* (cabbage, cauliflower), *B. rapa* (= *campestris*) (turnip rape), *B. nigra* (black mustard), and *Sinapis alba* (white mustard). *B. napus* (swede rape), which originated from crosses between *B. rapa* and *B. oleracea* (U.N., 1935), was at first a relatively

minor component of such ecosystems. The chemical compositions of these species, particularly the glucosinolate content, differ from those of contemporary cultivars of *B. napus* (Fenwick et al., 1983; Heaney and Fenwick, 1987) and, as a result, the volatiles originally influencing host-plant recognition by pests would have been different from those now produced by *B. napus*. Thus, as populations of *C. assimilis* in the U.K. adapted to the large relative area of oilseed rape, the detection of 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS by specific olfactory cells would have become of paramount importance. However, modern double low cultivars of *B. napus* have a lower glucosinolate content and produce only trace amounts of isothiocyanates. The large number of other electrophysiologically active components in the volatiles of present-day *B. napus* suggests that, in the future, these may become more important than glucosinolate catabolites as signals for orientation to the host plant.

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ISOLATION, PURIFICATION, AND CHARACTERIZATION OF NEW *Daphnia*-TOXIC COMPOUND FROM AXENIC *Microcystis flos-aquae* STRAIN PCC7806

DIRK JUNGMANN[1]

Max-Planck-Institute of Limnology
August-Thienemann-Str. 2
24306 Ploen, Germany

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Abstract—A new compound, that is toxic to *Daphnia*, called the *Daphnia*-toxic compound (DTC), was isolated and purified from water extracts of *Microcystis* PCC7806. An established *Daphnia*-bioassay was used as a detection system for the DTC in purified fractions. Temperature and pH stability were used to separate DTC from unstable compounds by changing the pH while the temperature was kept at 90°C. This was followed by ultracentrifugation at 100,000*g*. A molecular cutoff filtration technique was used to separate compounds with a molecular weight >100,000 Da and to reduce the volume of the fraction that was >500 Da. Evaporation in a vacuum led to a fraction that had a suitable volume for further characterization procedures. DTC was resistant to degradation by two bacteria that were isolated from a laboratory culture. DTC was not inactivated by incubation with protease K or pronase E. A strong anion-exchange resin at the stationary phase of the FPLC equipment was used to purify and concentrate the DTC. The molecular weight determined by gel filtration was between 1.0 and 3.1 kDa. A UV spectrum from this purified fraction exhibited absorption maxima at 220, 260, and 360 nm. The ecological and analytical relevance of the DTC is compared with microcystins.

Key Words—*Daphnia*, *Microcystis*, DTC, chemical interaction, toxicity.

[1]Present address: Dresden University of Technology, Institute of Hydrobiology, 01062 Dresden, Germany.

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INTRODUCTION

Freshwater cyanobacteria are known to produce different toxins, as described in detail by Carmichael (1992). Toxic blooms are commonly associated with the cyanobacterium *Microcystis* spp., which produces cyclic hepatotoxic heptapeptides named microcystins (cyanoginosins). There is no apparent evolutionary adaptive value for cyanobacteria to produce compounds toxic to land animals, but the relevance of secondary metabolites of *Microcystis* to their interaction with aquatic organisms has been investigated. Lampert (1981a,b, 1982) examined the interactions between *Microcystis* and *Daphnia* and found that *Microcystis* was toxic to *Daphnia* and inhibited the filtering rate. Jungmann et al. (1991) separated the compounds responsible for these interactions and found that toxicity to *Daphnia* and filtering inhibition are caused by chemically different compounds. DeMott et al. (1991), who investigated toxic effects of purified microcystin-LR and extracts from *Microcystis* PCC7820 to copepods and daphnids, found significant effects at high concentrations. Jungmann (1992), however, found a fraction from *Microcystis* PCC7806 water extracts that was more active than two known microcystins. Reinikainen et al. (1993) concluded that microcystin concentrations in their experiments were low and the reason for toxicity of *Microcystis* PCC7820 was to be found in other compounds. Jungmann and Benndorf (1994) investigated a *Daphnia*-toxic compound (abbreviated DTC) isolated from the natural and laboratory samples of *Microcystis*.

In this paper I report evidence that acute toxicity of *Microcystis* to *Daphnia* is caused by a compound other than microcystin. This work was conducted to characterize DTC, which is a new compound or compounds with biological activity isolated from *Microcystis* PCC7806. It is toxic to daphnids and is a possible source of interference in bioassays established for microcystin detection. Ecologists should take these results into considerations of *Microcystis*–*Daphnia* interactions and biomanipulation (Shapiro et al., 1975) in nutrient-rich lakes.

METHODS AND MATERIALS

Origin and Cultivation of Organisms. Axenic *Microcystis* PCC7806 was originally supplied by R. Rippka, Pasteur Culture Collection (Paris) and tested for sterility by plating on DEV-agar (Merck No. 11471). Culture conditions and harvesting procedure were described earlier (Jungmann, 1992). *Daphnia pulicaria* was cultured in filtered lake water (0.45 μm, Schleicher & Schuell) taken from the Schöhsee (Lampert, 1982) as previously described by Jungmann et al. (1991). *Microcystis* PCC7806 produces considerable amounts of microcystin-LR and 3-desmethylmicrocystin-LR (Jungmann, 1992; Martin et al., 1990).

Detection of DTC by Daphnia *Bioassay.* Toxicities of different *Microcystis* cell extract fractions were tested against *Daphnia* in 2-ml Eppendorf tubes (polyethylene) filled with 2 ml filtered lake water and a 4-day-old daphnid. The procedure for detection of DTC by *Daphnia* bioassay (a two-day survivorship test) was described in detail by Jungmann and Benndorf (1994). Statistically significant differences between fractions were calculated with two-by-two-tables based on χ^2 test with 51 individuals for each treatment ($P < 0.05$). Animals incubated without food or extract (starved animals) were used as controls. The *Microcystis* extract is expressed as dry weight of lyophilized cell material in a tested volume (milligrams per milliliter).

Extraction Procedure. Lyophilized *Microcystis* was suspended in double-deionized water (−18 MΩ/cm conductivity) to give a concentration of 10 mg/ml at room temperature and sonicated for 15 min. During extraction, the pH was adjusted to pH 10 with 0.1 N NaOH, and the suspension was stirred over night (12 ± 1 hr). The procedure was terminated after readjusting the pH to 10 followed by centrifugation (25,000*g*, 4°C, 30 min, Sorval RC-5B). The pH of the resulting supernatant was lowered to 4 with 0.1 N HCl, the extract was stirred for 15 min, and centrifuged at 100,000*g* (30 min, 4°C, Centrikon T-1075, Kontron). The supernatant contained the toxin (Jungmann and Benndorf, 1994) and was used for further purification steps.

Influence of Temperature on DTC Activity. The supernatant from ultracentrifugation was placed in polyethylene tubes and stored at −18°C up to 10 days. The activity of the DTC was tested after thawing at room temperature. Toxicity to *Daphnia* was determined at a concentration corresponding to 347 μg lyophilized *Microcystis* cell material per milliliter of water. It was necessary to suspend this in 1 ml water to get a DTC concentration in the extract toxic to daphnids. This corresponded to the earlier calculated LC_{50} for extracts from *Microcystis* PCC7806 (Jungmann and Benndorf, 1994). After heating the supernatant for 15 min to 50, 70, and 90°C as well as autoclaving (20 min, 0.21 MPa), toxicity was determined with the *Daphnia* bioassay. The autoclaving procedure was also conducted with double-deionized water to test whether the procedure by itself had any effect on sample toxicity.

Purification of DTC. In further experiments the extraction procedure was modified to incorporate the following steps: (1) extraction at pH 10 overnight (12 hr), (2) centrifugation at 25,000*g*, (3) adjustment of the pH of the supernatant to 4 and heating for 15 min to 90°C, and (4) ultracentrifugation at 100,000*g*. The resulting supernatant from ultracentrifugation was passed through a 100,000-Da ultrafilter membrane (Amicon Diaflo YM-100, 0 62 mm) using a membrane-ultrafilter cell (Amicon series 8000, Witten, Germany). The filter was activated by passing through 50 ml double-deionized water with slight pressure (nitrogen, 0.03 MPa). The sample was passed through by pressure

(nitrogen, 0.05 MPa) with stirring. The fraction > 100,000 Da was resuspended stepwise from the membrane filter (50 mM CH_3COONH_4) until the original volume was reached. The filtered fraction (< 100,000 Da, adjusted to pH 7 with 0.1 M NaOH) and the resuspended fraction (> 100,000 Da) were tested for toxicity to *Daphnia* at a concentration of 347 μg/ml. The next purification step was carried out with same equipment and conditions but using a 500-Da ultrafilter membrane (YC-05, 0 62 mm). The fraction > 500 Da was stepwise resuspended (50 mM CH_3COONH_4 adjusted to pH 7 with 0.1 M NaOH) in 50% of the original volume. Toxicity of the fraction < 500 Da was tested at 347 μg/ml and the > 500-Da fraction at 174 μg/ml.

The volume of the fraction > 500 Da was reduced in vacuum in a glass flask to 10% of the original volume. The glass flask was then stepwise washed with double-deionized water equal to 10% of the original volume and both fractions were pooled. The reduction of the original volume was thus 80%, and *Daphnia* toxicity was tested with 50 μg/ml. This partially purified fraction was stored in polyethylene caps at −18°C until it was used, but not longer than 15 days.

Stability of DTC to Bacterial Attack. Microbiological decomposition of DTC was tested with bacteria isolated by filtration (0.8 μm, cellulose acetate, Nalgene) from the nonaxenic *Microcystis* strain NRC-1 (Gorham, 1964). The two isolated bacteria were characterized with the bioassay of api 20 E (Bio Merieux SA, Marcy l'Etoil, France). One bacterium was identified as a gram negative, motile, staff-shaped organism without spores belonging to the genus *Pseudomonas*. The genus of the second bacterium was not identified, but it was cocci-shaped, gram positive, nonmovable, and without spores.

An unsterile sample of the fraction stored at −18°C was thawed at room temperature and tested immediately and again after 48 hr for toxicity to *Daphnia*. The effect of sterile filtration with 0.2-μm mesh size (Gelman Sciences) was tested with double-deionized water as a control. Then 500 μl of the bacteria-containing culture filtrate was incubated for three days in 50 ml CPS medium (Taylor, 1940; modified by Collins and Willoughly, 1962). A 1-ml sample of partially purified extract was thawed, 200 μl of glucose stock solution (0.5 g/liter) added, and it was incubated with 500 μl of the pregrown bacteria and a sterile filtered medium from a pregrown bacterial culture. The growth of the bacteria in the samples was tested by plating on 4% CPS–agar before testing for toxicity.

Stability of DTC to Proteolytic Enzymes. To test for the presence of peptide bonds, the DTC partially purified sample was incubated with two proteases. A stock solution of pronase E (Merck, Darmstadt, Germany) was made in 40 mM KH_2PO_4/K_2HPO_4, pH 7, 2 mM $CaCl_2$ buffer with an enzyme concentration of 1 μg/μl. A stock solution of protease K (Merck) was made in 50 mM Tris HCl

buffer, pH 8, with a concentration of 1 μg/μl. Pronase E (300 μl) or protease K (300 μl) and 200 μl $CaCl_2$ (200 mM) were added to a 1-ml partially purified sample of DTC and incubated in a temperature-controlled waterbath for 6 hr at 30 ± 2°C. Samples treated with pronase E or protease K were tested for toxicity to *Daphnia* at a concentration of 100 μg/ml. The control was buffer solution with and without enzymes.

Purification with Anion-Exchange Resin. The partially purified extract was concentrated and purified using a strong anion-exchange resin (Mono Q HR 5/5, Pharmacia, Germany) and FPLC equipment (two P500 pumps, LCC 500 controller, 2-ml sample loop, UV M 280 nm detector, fraction collector Frac-100, Rec 482 recorder, Pharmacia). The mobile phase was a gradient of A: 50 mM NH_4CH_3COO, pH 8 (adjusted with 0.1 M NaOH); and B: 75 mM CH_3COOH, 225 mM NaCl. The flow was 1 ml/min and the controller program was as follows: Solvent composition: 0–8 min 100% A; 9–12 min the proportion of B rose from 0 to 100%; 9–45 min 100% B. Sample: From 0 to 8 min, 2-ml samples were loaded twice. Six fractions were collected at 2-min intervals between 8 and 20 min, leading to the 2-ml sample amount. After 45 min the separation procedure was terminated and the column was equilibrated to 100% A. The pH of all collected fractions was monitored by pH indication paper (Macherey & Nagel). If the pH was below 5, NaOH (0.1 M) was added until the pH was between 5 and 6. All sample fractions were tested for DTC activity with a concentration of 200 μg/ml in the *Daphnia* bioassay.

Determination of Molecular Weight of DTC. The above-described FPLC equipment was used to estimate the molecular weight of the DTC. However, the stationary phase was a HiLoad 16/60 column filled with Sephacryl S-100 HR resin (Pharmacia, Germany). The mobile phase was a 100 mM NH_4CH_3COO buffer with a flow of 1 ml/min. The column was calibrated with a kit from Pharmacia containing blue dextran 2000, chymotrypsin A (25 kDa), ovalbumin (43 kDa), and albumin (67 kDa). The total volume of the column was determined with acetone and the K_{av} values were calculated with the equation:

$$K_{av} = V_e - V_0/V_t - V_0$$

in which K_{av} is the distribution coefficient; V_e the elution volume of the collected fraction, V_0 the dead volume of the gel; and V_t the total volume of the gel. The fraction sampler collected 10-ml fractions.

Wavelength Spectra of Fraction Containing DTC. The UV-visible absorption spectrum of DTC in the *Daphnia*-toxic fraction F7 from gel filtration was determined by a UV-VIS photometer (Uvikon 930, Kontron) between 200 and 700 nm using 1-cm quartz cuvettes.

Protein Determination. The protein concentration in fractions was deter-

mined by the Bio-Rad Protein Assay (Bio-Rad, Muenchen). The concentration of dye complex was measured with a photometer (Uvikon 930, Kontron) at 595 nm wavelength in quartz cuvettes. Albumin (Pharmacia) was used as standard. All chemicals were of analytical grade from Merck unless otherwise noted.

RESULTS

Treatment of water extracts with temperatures ranging from −18°C to 121°C did not affect the toxicity of DTC to *Daphnia* (Figure 1). After centrifugation, the toxic supernatant was passed through two different membrane ultrafilters. The supernatant from the ultracentrifugation was the positive toxicity control for the following procedures. The fraction with a molecular weight < 100,000 Da showed significant toxicity (Figure 2), and its toxicity was significantly higher than the toxicity of the fraction with a molecular weight > 100,000 Da.

The fraction < 500 Da showed no toxicity, whereas the fraction with a molecular weight > 500 Da was toxic. Thus, DTC appears to have a molecular weight between 500 and 100,000 Da.

The DTC was resistant to degradation by two isolated bacteria during a 48-hr test (Figure 3). Sterile filtration had no detectable influence on DTC activity.

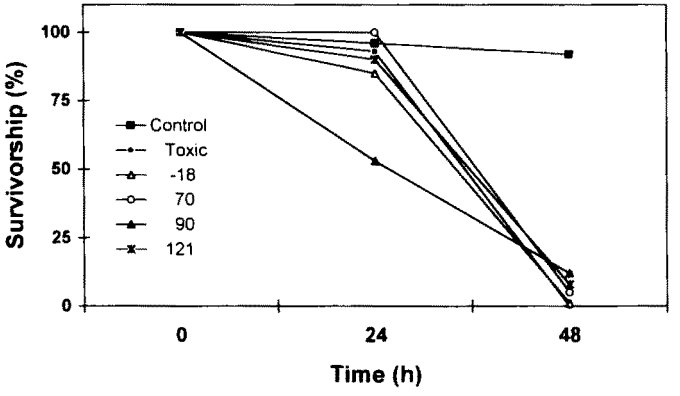

FIG. 1. Survivorship of *D. pulicaria* during incubation with different temperature treated fractions of partially purified *Microcystis* PCC7806 extracts. Control: starved animals; Toxic: positive control (the toxin in fraction of the previous purification step); −18: sample frozen for 10 days at −18°C; 70, 90: fractions heated for 15 min; 121: autoclaved sample; $P < 0.05$.

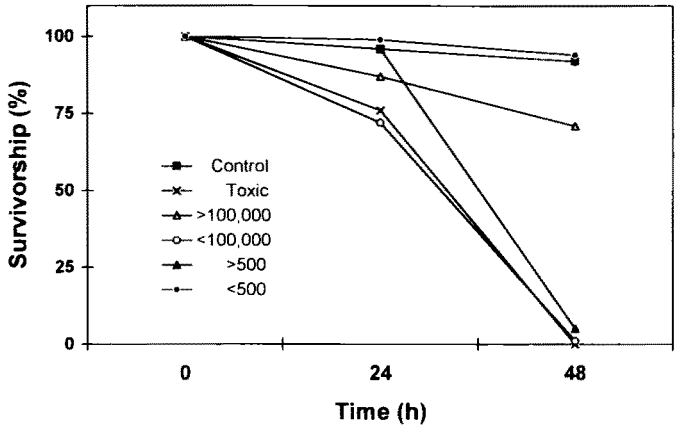

FIG. 2. Survivorship of *D. pulicaria* during incubation with fractions obtained by membrane ultrafiltration of partially purified *Microcystis* PCC7806 extract. Control: starved animals; Toxic: positive control (the toxin in fraction of the previous purification step); fractions of molecular-dependent membrane ultrafiltration are given in Daltons; $P < 0.05$.

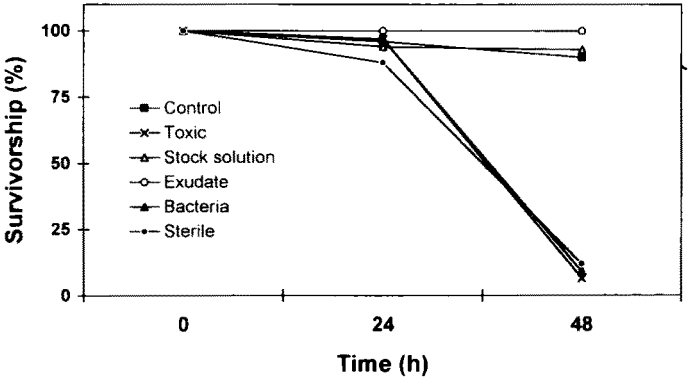

FIG. 3. Survivorship of *D. pulicaria* during incubation with different fractions from bacterial degradation experiments of partially purified *Microcystis* PCC7806 extract. Control: starved animals; Toxic: positive control (the toxin in fraction of the previous purification step); Exudate: exudates from cultured bacteria; Bacteria: fraction after bacterial degradation; Sterile: sterile filtered sample; $N = 51$, $P < 0.05$.

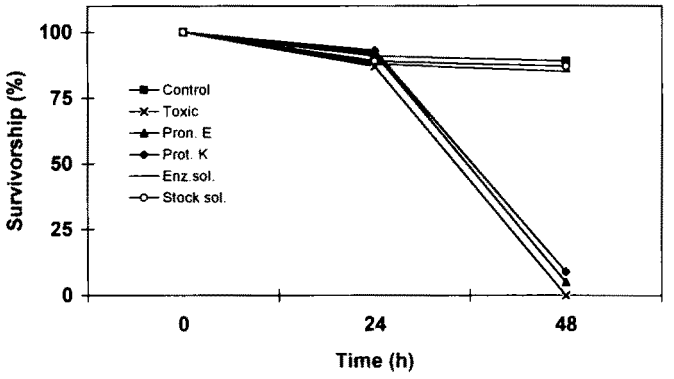

FIG. 4. Survivorship of *D. pulicaria* during incubation with samples exposed to enzymatic degradation of partially purified *Microcystis* PCC7806 extract. Control: starved animals; Toxic: positive control (the toxin in previous fraction); Pron E: pronase E; Prot. K: protease K; Enz.sol.: enzymes without toxin containing sample; Stock sol: stock solutions; $N = 51$; $P < 0.05$.

Figure 4 shows the results of experiments with two nonspecific proteases. The stock solutions as well as the two enzymes had no toxic effect on daphnids or on their survival rates. The activity of DTC was not influenced by an incubation for 6 hr with each protease.

Table 1 shows the toxicity to *Daphnia* of different fractions after separation by an anion-exchange resin. No toxicity was found in fractions sampled from 0 to 8 ml and 20 to 45 ml (data not shown). The 12 to 14-ml fraction (Table 1) was the only sample that had toxic action, and it was used for further purification.

Gel filtration of the toxic fraction indicates a molecular weight between 1.0 and 3.2 kDa for the toxic substance (F7, Table 2). Toxicity of all other fractions was not different from the control except fraction F1, which showed a molecular

TABLE 1. SURVIVORSHIP OF *D. pulicaria* DURING INCUBATION WITH DIFFERENT FRACTIONS OF ANION-EXCHANGE SEPARATION[a]

Time (hr)	Fraction retention time (min)					
	8–10	10–12	12–14	14–16	16–18	18–20
0	100	100	100	100	100	100
24	98	100	86	96	90	100
48	90	98	0	90	86	92

[a]Time: exposure of *Daphnia* to test solution, and survivorship (%). $N = 51$; $P < 0.05$.

TABLE 2. SURVIVORSHIP OF *D. pulicaria* DURING INCUBATION WITH DIFFERENT FRACTIONS FROM GEL FILTRATION[a]

	F1	F2	F3	F4	F5	F6	F7
M_r (kDa)	100–67	67–40.5	40.5–22	22–11.9	11.9–6.2	6.2–3.2	3.2–1
Prot. (μg/ml)	11.55	ND	17.92	17.03	9.21	7.53	6.35
Prot. purity (%)	2.9	ND	4.5	4.3	2.4	1.9	1.6
Survivors (%)	52	92	76	96	100	96	0

[a]F1–F7: molecular weight-dependent fractions; M_r (kDa): molecular weight expressed as kilodaltons; Prot.: protein concentration (ND = not detected). Prot. purity: purity of fractions in comparison with protein concentration of the pH 10 extract. Survivors: relative survivorship of daphnids after 48 hr of incubation. $N = 51$, $P < 0.05$.

weight between 67 and 100 kDa. The 67 to 100-kDa fraction was less toxic in the assay than the fraction with molecular weight (1.0–3.2 kDa). Table 2 shows the survival of *Daphnia* after incubation with each of seven fractions from gel filtration. Protein concentrations in the fractions were between 6.4 and 17.9 μg/ml. In comparison with the protein concentration of crude pH 10 extract (396.2 μg/ml), the purified fractions contained 1.6–4.5% of the originally extracted proteins. Figure 5, a wavelength scan of fraction 7, shows three absorbance maxima at 200, 260, and 340 nm, respectively.

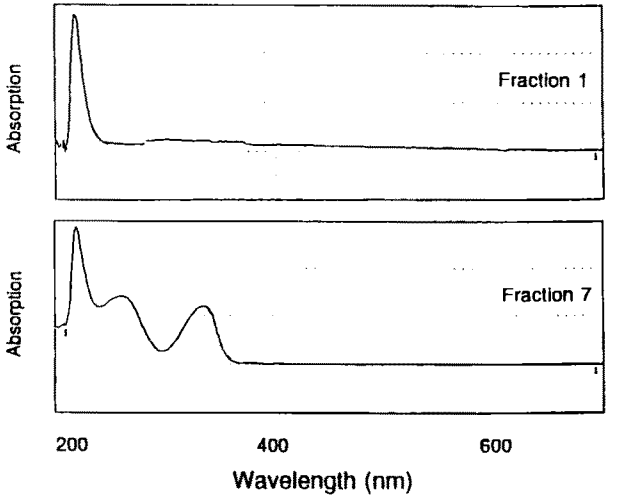

FIG. 5. Wavelength spectrum of the fraction resulting from gel filtration of the partially purified water extract. Spectrum was taken from fraction 7, which contains the DTC. Absorbance maxima were found at 220, 260, and 340 nm.

DISCUSSION

The toxic compound(s) in the extract from *Mycrocystis* described herein is probably not a micocystin, since known microcystins from *Microcystis* spp. are not responsible for acute toxicity to *Daphnia* (Jungmann, 1992; Reinikainen et al., 1993; Jungmann and Benndorf, 1994). Weckesser and Martin (1990) found that microcystins were stable at 160°C, and Martin et al. (1993) examined cyanopeptolins, which are stable at 100°C. These temperature stabilities are comparable with that found for DTC. Temperature stability of the *Microcystis* toxin was used to advantage in purification of the extract by heating it to 90°C and then separating precipitated heat-sensitive compounds by centrifugation. Crude extract or purified fractions of DTC were stable when stored at −18°C. These results are in contrast to those from Peñaloza et al. (1991), who extracted and purified a compound from natural *Microcystis* samples that was toxic to *Daphnia magna* but was inactivated by boiling for 5 min. Possible explanations for these contradictory results could be that *Microcystis* can synthesize a variety of different toxins or that field-collected samples might contain other contaminants that are toxic to zooplankton.

Fractionation by molecular cutoff filtration separated large compounds, reduced the original volume by 50%, and gave an indication of the range of molecular weight for the DTC. Bacteria and fungi typically degrade complex compounds in lake ecosystems (Odum, 1983), but DTC was resistant to degradation by two bacteria tested. Investigations with bacteria or fungi from natural sources with *Microcystis* may reveal organisms with degradation activity. Kiviranta et al. (1991) found that microcystin-LR persisted for two to three months in cultures enriched with water and bacteria from a Finnish river, but Watanabe et al. (1992) observed the decomposition of microcystin-LR and microcystin-YR within two months after being released into a nonaxenic culture medium. Jones and Orr (1994) observed decomposition of microcystin-LR within one day and concluded that fauna and chemical composition of the environment were major influences on the decomposition process.

DTC was resistant to hydrolysis by pronase E and protease K, both of which cut peptide bonds unspecifically. This may mean the absence of peptide bonds in DTC or the presence of some unusual amino acid composition of DTC. Separation by strong anion-exchange resin was successful for the purification and concentration of DTC and indicates toxic compounds with a negative charge. Molecular cutoff filtration showed that DTC has a molecular weight between 500 and 100,000 Da. Two fractions from gel filtration showed activity in the *Daphnia* bioassay. Fraction 7 was more toxic to *Daphnia* than fraction 1 ($P > 0.05$). This leads to the conclusion that fraction 7 contains the DTC. However, it is not clear whether another *Daphnia*-toxic compound is responsible for the minor toxicity in fraction 1 or whether this is a result of the technique used.

The DTC in fraction 7 has a molecular weight between 1 and 3.2 kDa. This molecular weight is close to the molecular weight of microcystins (Carmichael, 1992). However, other results (Jungmann, 1992; Jungmann and Benndorf, 1994; Reinikainen et al., 1993) showed that toxicity of *Microcystis* to *Daphnia* is independent of microcystins. The molecular weight of the compound toxic to *Daphnia* isolated by Peñaloza et al. (1991) is similar to the DTC in fraction 7. However, the compound isolated by Peñaloza et al. (1991) was not stable at high temperatures, in contrast to DTC in this report.

Determination of absorbance maxima of fraction 7 (Table 2) suggests a method that may be useful for detecting and measuring DTC in future work. It is not clear at present, however, if the toxic principle(s) show these maxima or if that absorption might be due to other compounds in the fraction.

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QUADRUPOLE STORAGE MASS SPECTROMETRY OF MONO- AND DIMETHYLALKANES

ROBERT E. DOOLITTLE,* ADRON T. PROVEAUX, HANS T. ALBORN, and ROBERT R. HEATH

Insect Attractants, Behavior, and Basic Biology Research Laboratory
Agricultural Research Service, U.S. Department of Agriculture
Gainesville, Florida 32604

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Abstract—Monomethyl and dimethylalkanes with one, two, three, four, five, and seven methylene groups separating the methyl branches were synthesized and analyzed by magnetic sector and quadrupole storage (ion trap) mass spectrometry. The spectra produced by the magnetic sector instrument were in good agreement with previously reported data, whereas the ion trap spectrometer produced ions resulting from cleavages adjacent to the branching points, markedly different than those from the magnetic sector instrument. Fragmentation patterns show that the ion trap mass spectrometer can be used to characterize branched alkanes in nanogram and subnanogram quantities.

Key Words—insect hydrocarbons, monomethyl alkanes, dimethylalkanes, mass spectra ion trap, magnetic sector.

INTRODUCTION

The determination of the structure of methyl-branched alkanes in nanogram amounts or less is important in investigations designed to elucidate the chemistry of interactions of insects with other insects and their environment, and especially when the cuticular waxes of individual insects are analyzed for chemotaxonomic purposes (Jackson and Blomquist, 1976; Lockey, 1978, 1980; Jacob, 1979). Hydrocarbons found on the cuticle of insects can serve as chemical messengers

*To whom correspondence should be addressed.

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(Blomquist and Dillwith, 1985); they also can protect insects from desiccation (Hadley, 1994; Lockey, 1976). 13-Methylhentriacontane, found in the corn earworm *Helicoverpa zea* (Boddie), triggers a short-range host-seeking response in *Microplitis croceipes* (Cresson), which is a parasitic wasp (Jones et al., 1971). Dimethyl-substituted alkanes have been identified in cuticular extracts of gypsy moth, *Lymantria dispar* (L.) pupae that elicit host recognition and oviposition behavior by the parasitoid *Brachymeria intermedia* (Nees) (Alborn et al., in press).

During the course of identification of hydrocarbons from insects, we recorded their mass spectra on a quadrupole storage (ion trap) mass spectrometer. Since this methodology generally displays enhanced sensitivity and thus a lower detection limit than conventional quadrupole spectrometers (and the instruments are of significantly lower cost) (March and Hughes, 1989), it is being applied widely in the identification of nanogram and smaller quantities of biologically active chemicals from biological and environmental sources. Fragment ions in the electron impact mass spectra of monomethyl-branched alkanes from conventional magnetic sector instruments that are characteristic and diagnostic for the position of the branching point were observed by Biemann (1962) and Spiteller (1966). Fragmentation of the molecular ion occurs on either side of the branch with charge retention on the substituted carbon atom producing a stable carbocation ($C_nH_{2n+1}^+$) or rearrangement and cleavage resulting in the production of a $C_nH_{2n}^+$ ion that is prominent and sometimes more intense than the $C_nH_{2n+1}^+$ ion. This phenomenon was initially observed by Biemann and Spiteller and subsequently thoroughly investigated in a definitive study of dimethyl-branched alkanes by McCarthy et al. (1968) with subsequent publications by Nelson et al. (1972) and Pomonis et al. (1978, 1980), in which this type of cleavage was demonstrated and documented.

Simultaneously, the parent ions produce ions of even mass ($C_nH_{2n}^+$) that do not contain the other branch. These ions will generally be dominant when they contain seven to nine carbon atoms (McCarthy et al., 1968) and when the mass ratio of the neutral particle (NP) produced concomitantly with these fragment ions (FI) is high (NP/FI > 1) (Nelson et al., 1972). When the fragment ions contain more than nine carbon atoms, the ratio of the even to odd ion intensities decreases in magnitude unless there is a coincidental increase in the mass of the neutral particle. The fragment ions containing the remaining branch do not exhibit a marked doublet of odd and even peaks, but the abundance of these ions can be significant (Pomonis et al., 1980; Nelson and Sukkestad, 1970, 1975). Since the mass spectrometry of methyl-branched alkanes using a quadrupole storage (ion trap) spectrometer has not been reported, we present here spectra from a variety of mono- and dimethylbranched alkanes and compare their spectra with those obtained from a magnetic sector instrument.

METHODS AND MATERIALS

Mass Spectrometry. The magnetic sector mass spectrometer built by Gunnar Stenhagen at the Department of Chemical Ecology, Göteborg Universitet, Göteborg, Sweden, is a large-radius (300-mm) magnetic sector (90°) instrument built for high-sensitivity GC-MS work, and it consisted of equipment from several manufacturers. The high-vacuum system and the analyzer were constructed in Göteborg. The ion source housing was evacuated by a diffusion pump (Edwards Diffstak 160-700) and was largely isolated from the analyzer vacuum system by the ion source exit slit. The analyzer was evacuated with a second diffusion pump (Edwards EO4) and the detector with a turbomolecular pump (Pfeiffer 250). The forevacuum system consisted of two independent forevacuum pumps (Edwards 112). The first pump backed the ion source diffusion pump. The second forevacuum backed the analyzer and the detector. The ion source temperature was 200°C and was operated at 70 eV ionizing energy. Samples were introduced via a direct inlet probe as dilute solutions (2–4 ng/μl) in hexane, which was evaporated by the forepump.

The quadrupole storage mass spectrometer (ion trap) was a Finnigan MAT ITS 40 Magnum operated at 70 eV ionizing energy in the electron impact mode. The spectrometer was interfaced to a Varian 3400 gas chromatograph equipped with either a 30-m × 0.25-mm (J&W Scientific, Inc.) nondeactivated fused silica capillary tube or a 30-m × 0.25-mm DB-5ms (J&W Scientific, Inc.) fused silica capillary column with a 0.1-μm film thickness. Both the column and tube were operated at a linear flow velocity of 18 cm/sec, and the samples were introduced as solutions (2–4 ng/μl, injections) in hexane directly on column via an SPI injector. The SPI injector is an on-column septum programmable injector that is programmed from 60°C to 300°C at 150°C/min during a column programmed run. The column was programmed during an analytical run as follows: the temperature was held at 60° for 1 min and then programmed at 20°C/min to a maximum temperature of 285°C. The source temperature and acquisition (ACQ) tuning parameters were varied in order to study the effect of these parameters on the ion abundances for 13-methylhentriacontane (entry B, Table 1).

Syntheses. The hydrocarbons (designated as entry A, B, C, etc., in Tables 1–5 and Figures 5–7 below) were synthesized by a variety of methods depending upon their structure. The monomethylalkanes A, B, and C were prepared by a Wittig olefination procedure involving the preparation of a 1-methylalkyltriphenylphosphonium salt followed by reaction with the appropriate aldehyde as described by Sonnet (1976), with subsequent reduction of the olefinic product to the requisite hydrocarbon.

The hydrocarbon with one methylene group between the methyl branches, 11,13-dimethyltritriacontane (D), was prepared from α-methyldodecanoic acid

TABLE 1. INTENSITIES OF CHARACTERISTIC IONS PRODUCED BY MONOMETHYLALKANES

Compound	Entry	m/z[a]	a	a-1	a-2	a-3	b	b-1	b-2	b-3	g	g-1	g-2	g-3	h	h-1	h-2	h-3	b Chain length X	b Chain length Y
9-Methylheptacosane	A	m/z	141	140	139	138	281	280	279	278	253	252	251	250	113	112	111	110		
Ion trap		INT.	3.7	11.3	6.7	6.2	2.5	3.3	1.3	1.0	1.6	3.2	1.9	1.0	5.2	6.5	18.1	6.7	8	18
Magnetic		INT.	28.5	48.9	5.1	2.3	26.1	14.5	0.9	0.5	2.6	4.3	0.6	0.0	18.6	9.4	17.6	3.5		
13-Methylhentriacontane	B	m/z	197	196	195	194	281	280	279	278	253	252	251	250	169	168	167	166		
Ion trap		INT.	14.9	25.8	6.2	10.7	13.8	16.4	2.4	2.6	9.8	14.6	4.7	4.7	11.7	12.1	9.8	14.8	12	20
Magnetic		INT.	34.7	41.7	3.0	1.5	40.1	28.5	1.6	1.1	4.6	7.6	1.2	0.5	13.9	8.4	3.9	1.7		
16-Methylhexatriacontane	C	m/z	239	238	237	236	309	308	307	306	281	280	279	278	211	210	209	208		
Ion trap		INT.	2.3	3.1	0.5	3.9	4.3	5.2	1.0	0.9	27.6	0.0	0.5	0.9	15.7	7.5	52.5	0.0	15	20
Magnetic		INT.	39.2	51.3	2.9	1.3	48.2	29.1	2.3	1.1	3.8	8.3	1.0	0.5	8.4	10.5	2.7	2.0		

[a] Data are *m/z* abundances as percentage of base peak which was *m/z* 57 for each spectrometer.
[b] *X* and *Y* are the number of carbon atoms on either side of the methyl branch.

via reduction to the alcohol, oxidation to the aldehyde, and Wittig condensation with 1-methyldocosyltriphenylphosphonium bromide followed by reduction of the olefin. 11,14-Dimethyltetratriacontane (E) was prepared via the reaction of the same phosphonium salt as for D with 3-methyltridecanal prepared from 4-hydroxy-2-butanone by conversion to the tetrahydropyranyl (THP) ether, condensation with decyltriphenylphosphonium bromide, hydrogenation and cleavage of the THP ether, and oxidation of the resultant alcohol to the requisite aldehyde. 15,18-Dimethyltritriacontane (F) was prepared from 19-methyl-16-tritriacontanone via methylenation with methylene triphenylphosphorane followed by hydrogenation. The hydrocarbons with three methylene groups between the methyl branches (G–P) were prepared from 5-hydroxy-2-pentanone THP and the appropriate 1-methylalkyltriphenylphosphonium salts according to Sonnet (1976). The remaining dimethylalkanes (Q–U) were prepared from thiophene by procedures and modifications of procedures developed by Pomonis et al. (1989).

RESULTS AND DISCUSSION

Inspection of the mass spectra in Tables 1 and 2 shows clearly the differences in the ion trap and magnetic sector instruments in regard to the production of diagnostic ions from cleavages at branching points. In addition, the ion trap produces even lower abundances of molecular ions than the magnetic sector instrument. The ion trap does not, at least in the present work, appear to have an advantage of enhanced sensitivity over the magnetic sector instrument for the detection of very small amounts of hydrocarbons. The reference peak for the calculation of relative abundances is m/z 57 for all spectra in both spectrometers.

When the spectra were recorded using the standard "normal" values for the source temperature and acquisition (ACQ) parameters [source temperature $\approx$ 200°C (ACQ) segment tune parameters (SC) = 100, and radio frequency time factor (RF) = 125], very low abundances were observed for all ions. Since a study had been ongoing in our laboratory delineating the effects of these parameters on the abundances of ions produced from other types of organic molecules, and since Phinney (1993) had reported the effect of source temperature on ion abundance, these parameters were varied for 13-methylhentriacontane and the results recorded.

It is clear from the data in Figures 1 and 2 that the source temperature and ACQ tuning parameters have marked effects on the abundances of ions produced from hydrocarbons in the ion trap. We studied the effect of variation of the source temperature on the intensities of ion b-1 (m/z 280) and h-3 (m/z 166) for 13-methylhentriacontane (B), and the data are presented in Figure 1. We studied the effect of changing the acquisition (ACQ) tuning parameters on the intensities

TABLE 2. INTENSITIES OF CHARACTERISTIC IONS PRODUCED BY DIMETHYLALKANES

Compound	Entry	m/z^a	N^b	a	a-1	a-2	a-3	b	b-1	b-2	b-3	c	c-1	c-2	c-3	d	d-1	d-2	d-3	Chain lengths' X	Chain lengths' Y
11,13-Dimethyltritriacontane	D	m/z	1	169	168	167	166	309	308	307	306	211	210	209	208	351	350	348	349		
Ion trap		INT.		12.8	15.0	24.3	31.0	4.0	4.9	9.4	13.3	14.9	7.7	15.0	6.7	8.0	2.6	0.5	0.4	10	20
Magnetic		INT.		25.2	28.4			14.8	9.8			39.2	13.9			28.9	8.6				
11,14-Dimethyltetratriacontane	E	m/z	2	169	168	167	166	309	308	307	306	225	224	223	222	365	364	363	362		
Ion trap		INT.		5.4	1.8	5.0	24.6	5.0	5.5	8.2	19.8	10.1	2.7	4.5	2.9	4.9	1.8	0.3	0.7	10	20
Magnetic		INT.		24.0	34.4			18.3	10.0			44.4	8.5			28.5	4.0				
15,18-Dimethyltritriacontane	F	m/z	2	225	224	223	222	239	238	237	236	281	280	279	278	295	294	293	292		
Ion trap		INT.		7.2	6.8	9.4	26.5	4.8	6.1	8.6	27.1	17.8	6.2	0.5	1.9	12.7	4.7	0.0	1.1	14	15
Magnetic		INT.		17.9	23.6			26.2	33.9			36.5	8.0			33.4	7.0				
7,11-Dimethylhentriacontane	G	m/z	3	113	112	111	110	309	308	307	306	183	182	181	180	379	378	377	376		
Ion trap		INT.		47.7	54.0	80.2	51.6	5.9	5.2	7.0	15.4	12.6	10.0	5.3	6.1	4.7	1.2	0.2	0.3	6	20
Magnetic		INT.		44.2	51.7			20.7	12.6			45.7	14.0			28.3	4.3				
9,13-Dimethylhentriacontane	H	m/z	3	141	140	139	138	281	280	279	278	211	210	209	208	351	350	349	348		
Ion trap		INT.		8.3	11.3	22.2	33.0	6.8	4.3	7.0	17.5	6.4	4.8	12.9	7.1	3.2	0.9	0.6	0.6	8	18
Magnetic		INT.		38.4	45.3			24.3	18.1			51.5	13.2			36.9	8.0				
9,13-Dimethyltritriacontane	I	m/z	3	141	140	139	138	309	308	307	306	211	210	209	208	379	378	377	376		
Ion trap		INT.		8.3	12.8	20.1	36.5	3.3	2.8	6.0	18.4	6.8	6.0	14.2	9.4	1.5	1.3	0.4	0.1	8	20
Magnetic		INT.		19.1	29.1			21.3	10.7			34.7	8.9			31.6	5.5				
9,13-Dimethylpentatriacontane	J	m/z	3	141	140	139	138	337	336	335	334	211	210	209	208	407	406	405	404		
Ion trap		INT.		14.9	17.8	23.6	37.0	3.5	3.1	6.5	17.6	28.0	17.4	43.8	14.5	11.4	4.1	1.1	0.9	8	22
Magnetic		INT.		30.9	44.8			18.8	7.5			43.8	10.5			26.2	5.0				
11,15--Dimethylhentriacontane	K	m/z	3	169	168	167	166	253	252	251	250	239	238	237	236	323	322	321	320		
Ion trap		INT.		7.1	7.0	11.3	29.2	9.3	11.0	12.2	24.1	3.9	2.7	1.6	3.0	5.2	2.6	1.2	1.1	10	16
Magnetic		INT.		40.6	52.6			33.8	42.2			57.8	17.5			55.9	11.5				
11,15-Dimethyltritriacontane	L	m/z	3	169	168	167	166	281	280	279	278	239	238	237	236	351	350	349	348		
Ion trap		INT.		6.6	8.8	10.2	27.4	7.9	5.7	6.2	18.1	4.1	3.3	1.9	2.9	3.6	1.9	1.0	0.6	10	18
Magnetic		INT.		23.9	35.0			24.1	17.5			39.2	12.0			37.2	7.1				

11,15-Dimethyltetratriacontane	M	*m/z*	3	169	168	167	166	295	294	293	292	239	238	237	236	365	364	363	362		
Ion trap		INT.		5.9	8.9	10.3	24.5	3.7	3.0	6.3	15.3	3.0	2.6	0.6	2.6	2.6	1.6	0.2	0.1	10	19
Magnetic		INT		19.0	24.6			17.1	12.3			32.4	10.4			27.4	7.2				
11,15-Dimethylpentatriacontane	N	*m/z*	3	169	168	167	166	309	308	307	306	239	238	237	236	379	378	377	376		
Ion trap		INT.		10.4	12.1	14.7	31.4	5.1	5.9	9.4	23.3	5.9	3.3	2.9	5.1	4.7	2.5	1.0	1.2	10	20
Magnetic		INT.		18.0	26.6			17.5	9.4			29.2	7.7			24.4	4.9				
11,15-Dimethylheptatriacontane	O	*m/z*	3	169	168	167	166	337	336	335	334	239	238	237	236	407	406	405	404		
Ion trap		INT.		5.7	5.4	6.9	23.3	0.6	0.8	3.7	14.1	0.8	0.6	0.8	1.9	5.6	3.4	1.5	1.6	10	22
Magnetic		INT.		53.9	57.8			34.6	25.1			64.9	21.5			54.4	14.3				
13,17-Dimethylheptatriacontane	P	*m/z*	3	197	196	195	194	309	308	307	306	267	266	265	264	379	378	377	376		
Ion trap		INT.		5.8	7.5	11.6	32.2	5.0	6.1	10.0	27.0	12.7	6.9	5.4	3.9	3.9	2.2	0.9	0.7	12	20
Magnetic		INT.		17.7	23.6			15.6	10.4			29.5	8.5			22.9	5.6				
11,16-Dimethylhexatriacontane	Q	*m/z*	4	169	168	167	166	309	308	307	306	253	252	251	250	393	392	391	390		
Ion trap		INT.		6.8	7.6	11.0	25.1	3.8	3.3	7.9	21.8	4.4	4.2	4.7	2.4	1.3	0.4	0.1	0.1	10	20
Magnetic		INT.		30.7	36.7			23.5	12.4			38.3	10.2			27.3	5.9				
7,13-Dimethyltritriacontane	R	*m/z*	5	113	112	111	110	309	308	307	306	211	210	209	208	407	406	405	404		
Ion trap		INT.		22.2	36.3	62.7	52.5	5.3	5.2	9.4	18.5	8.8	6.7	12.1	10.0	15.0	6.7	4.5	2.4	6	20
Magnetic		INT.		30.4	40.3			18.1	8.6			26.6	8.0			20.1	4.0				
9,15-Dimethyltritriacontane	S	*m/z*	5	141	140	139	138	281	280	279	278	239	238	237	236	379	378	377	376		
Ion trap		INT.		14.4	32.0	35.1	52.0	11.2	9.3	11.5	29.0	6.0	4.0	2.2	4.5	3.4	2.0	0.8	0.6	8	16
Magnetic		INT.		20.5	30.0			18.5	14.0			22.2	9.0			24.8	6.5				
11,17-Dimethylheptatriacontane	T	*m/z*	5	169	168	167	166	309	308	307	306	267	266	265	264	407	406	405	404		
Ion trap		INT.		8.1	10.9	14.2	31.9	5.4	6.6	9.1	23.9	6.6	6.3	5.7	4.0	18.0	4.0	2.3	2.2	10	20
Magnetic		INT.		19.3	36.5			7.2	14.2			19.4	9.1			10.5	4.4				
7,15-Dimethyltritriacontane	U	*m/z*	7	113	112	111	110	281	280	279	278	239	238	237	236	407	406	405	404		
Ion trap		INT.		20.7	42.7	98.4	58.0	9.7	4.3	8.1	21.4	3.5	2.1	1.7	4.2	10.1	6.1	1.7	1.3	6	18
Magnetic		INT.		19.8	28.5			18.1	11.4			20.1	9.4			20.2	4.9				

[a] Data are *m/z* abundances as percentage of base peak which was *m/z* 57 each spectrometer.
[b] *N* is equal to the number of methylene groups ($-CH_2-$) between the methyl branches.
[c] *X* and *Y* are the number of carbon atoms on either side of the methyl branches.

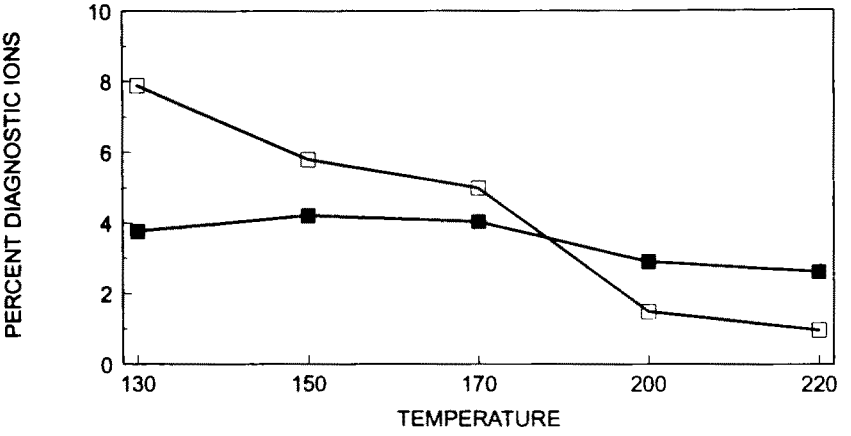


FIG. 1. Diagnostic ions *m/z* 166 (h-3; - ■ -) and 280 (b-1; - □ -) vs. ion source temperature for 13-methylhentriacontane (entry B).

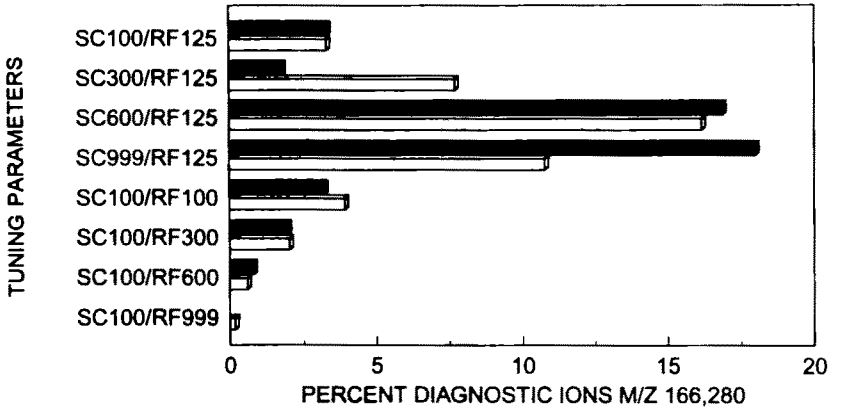


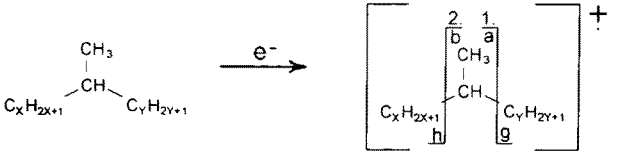
FIG. 2. Acquisition tuning parameters (segment tune parameters, SC; radio frequency tune factor, RF) vs. diagnostic ions at *m/z* 166 (h-3; ■) and 280 (b-1; □) for 13-methylhentriacontane (entry B). SC represents the ionization time determined by the automatic gain control prescan pulse. The values of 100–999 are arbitrary values on a scale and do not represent 100% through 999%. RF is the radio frequency level number given as a percent of the RF voltage (storage voltage) applied to the ring electrode.

of these same two ions, and the data are presented in Figure 2. The (ACQ) SC values are on an arbitrary scale that represents the segment ionization pulse lengths as a percent of the calculated ionization pulse time determined by the automatic gain control prescan pulse. By changing this value, we were able to increase the lengths of the ionization pulse times in three of the four segments of the mass scale. The four segments are 10–99, 100–249, 250–449, and 450–650 amu. We increased the ionization time for the three highest atomic mass unit segments and did so uniformly for all three segments, although the capability of using a different ionization time for each amu segment is available.

RF represents the radio frequency level number as a percent for the RF applied to the ring electrode. This RF voltage is used to focus the ions in the center of the ion trap, and thus, it is referred to as the storage voltage. It creates the field that temporarily stores ions until they are made unstable by changing the voltage and are ejected from the trap into the electron multiplier for detection. The fixed RF voltage sets a cutoff of mass, and ions below this value are ejected from the trap prior to ionization. This has the effect of limiting the ionization of residual ions and increases sample ion concentration, which increases spectral sensitivity.

The data presented in Figures 1 and 2 show clearly that the source temperature and ionization pulse time SC parameters have the greatest influences on the abundances of the ions from hydrocarbons. The RF variation had very little effect on ion abundances.

The monomethylalkanes exhibited a markedly different fragmentation pattern than the dimethylalkanes in the two spectrometers, producing the ions listed in Figure 3. The results from the magnetic sector instrument were in good agreement with those reported (McCarthy et al., 1968; Nelson and Sukkestad, 1970), with monomethyl-branched compounds cleaving at the branches with production of pairs of peaks representing ions a and b from cleavages 1 and 2.



Cleavage	Produced Ions
1.	a, a-1, a-2, a-3, g, g-1, g-2, g-3
2.	b, b-1, b-2, b-3, h, h-1, h-2, h-3

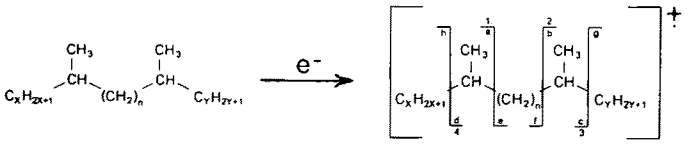
FIG. 3. Characteristic ions produced by fragmentation of monomethylalkanes.

respectively. The ratio of the substituted even-fragment-ion intensity (a-1 or b-1) to the intensity of the fragment ions a or b is greater than 1, i.e., the even ion dominates in the pair. The ion abundances for three monomethyl compounds, 9-methylheptacosane, 13-methylhentriacontane, and 16-methylhexatriacontane, are presented in Table 1.

The same compounds produced markedly different spectra in the ion trap (Table 1). The abundances of ions in which the charge was retained on the unsubstituted carbon atom (ions g and h of Figure 3) were clearly evident in these spectra, and ions with one, two, or even three fewer hydrogen atoms, i.e., g-1, g-2, g-3 and h-1, h-2, h-3, were significant, especially in the case of 13-methylhentriacontane (entry B, Table 1).

The abundances of the ions produced from the dimethyl-branched alkanes (Figure 4) in the magnetic sector and the ion trap instruments are listed in Table 2. Examination of the data shows that there are dramatic differences between the spectra produced by the two spectrometers. The abundances for the magnetic sector instrument are in agreement with published reports (McCarthy et al., 1968; Nelson and Sukkestad, 1970). The fragment ions containing a substituted carbon gave high abundances (ions a, b, c, and d). The molecular ions produced significant amounts (sometimes dominant) of even ions only when no second branch was present (ions a and b from cleavages 1 and 2). Since ions e, f, g, and h were usually produced in low abundances they were not included in Table 2. An exception to this is 11,16-dimethylhexatriacontane (Q) containing four methylene groups between the branches. It produced ions e and f (from cleavages 1 and 2) at *m/z* 365 and 225 in significant abundances. This special case has been reported (Pomonis et al., 1978), and these ions are clearly present in the spectrum of Q (Figure 5, magnetic sector).

Examination of the abundances produced by the dimethyl-branched alkanes in the ion trap reveals that, analogous to the magnetic sector instrument, only ions a, b, c, and d were prominent. As with the monomethyl compounds,



Cleavage	Produced Ions
1.	a, a-1, a-2, a-3
2.	b, b-1, b-2, b-3
3.	c, c-1, c-2, c-3
4.	d, d-1, d-2, d-3

FIG. 4. Characteristic ions produced by fragmentation of dimethylalkanes.

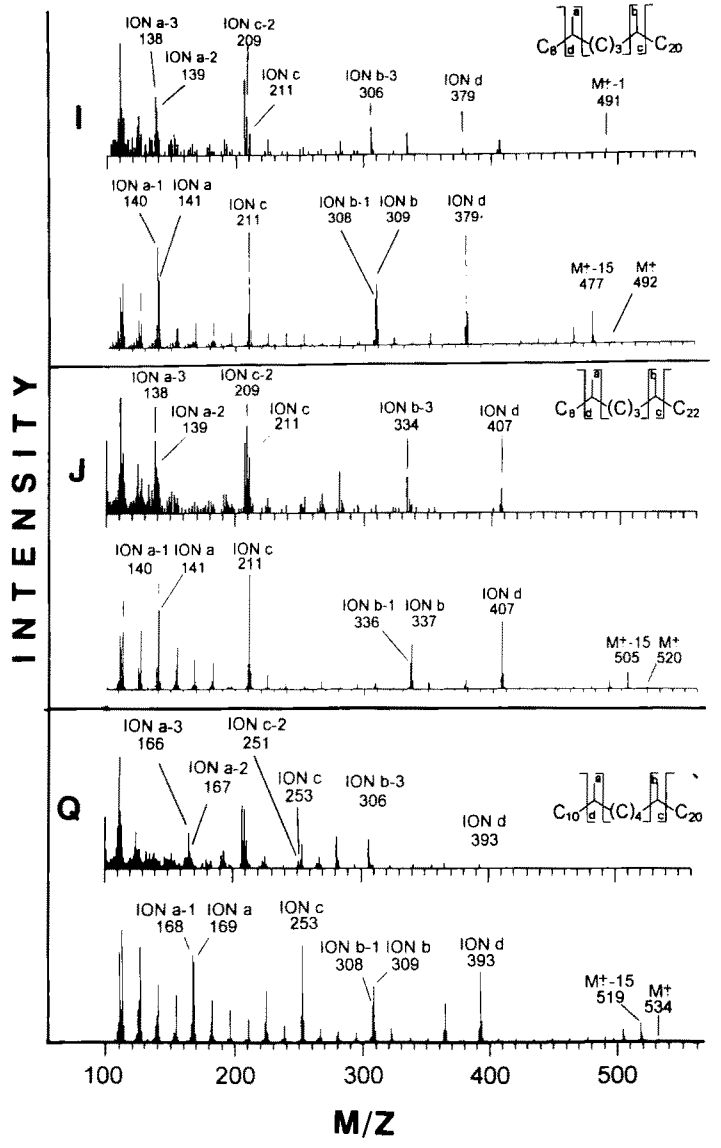


FIG. 5. Ion trap (top) and magnetic sector (bottom) mass spectra pairs of 9,13-dimethyltritriacontane (I), 9,13-dimethylpentatriacontane (J), and 11,16-dimethylhexatriacontane (Q). Abundances are relative to *m/z* 57.

production of ions with one, two, or three fewer hydrogen atoms was highly significant, with ions a-3 and b-3 predominant. Production of ions c-3 and d-3 was minor. 11,16-Dimethylhexatriacontane (Q) gave quite different results with the ion trap; ions at *m/z* 365 and 225 could not be seen in the ion trap spectrum (Figure 5). Conditions, i.e., source temperature and ACQ parameters, that gave optimum results for the monomethyl-branched alkanes were found to give the optimum results for the dimethyl-branched alkanes as well (low source temperature and SC 600, RF 125).

The ratios of the even to odd ion abundances for all the hydrocarbons in the magnetic sector instrument are presented in Table 3. These data are in general agreement (with two exceptions) with results reported earlier (McCarthy et al., 1968). The monomethyl-branched alkanes produce an a-1/a intensity ratio larger than 1.0 when the neutral particle (NP) to fragment ion (FI) ratio (NP/FIa as expressed in carbon numbers) is relatively large and less than 1 when the neutral particle to fragment ion (NP/FIb ratio) is relatively small. The data of Table 3 for the dimethyl-branched alkanes are at variance with these observations in the two entries F and K that have ratios of the NP to FIb of 0.82 and 0.56, respectively. Ratios of b-1/b for F and K are 1.29 and 1.25, respectively. Entry P has a NP/FIb ratio of 0.55 (approximately equal to entry K) and a ratio of 0.67 for b-1/b. We do not have an explanation for these discrepancies.

Our data confirm observations (McCarthy et al., 1968) that the ions containing the second branch (c and d) do not produce an even-to-odd ion ratio larger than 1.0 for any values of FI size or NP value. The data in Table 3 confirm the conclusions reached by Pomonis (1978), i.e., that when the number of methylene groups between the methyl branches is 0 or 1, it is difficult to predict the ratio of the intensities of the even and odd ion types (i.e., entry D had a ratio of a-1/a greater than 1). The data in Table 3 also confirm the observation that when the number of intervening methylene groups is two or greater, the even-to-odd ion ratios for ions a and a-1 (the smaller unbranched ions) will be greater than 1.

The eight even ion/odd ion ratios, a-1/a, a-1/a-2, a-3/a, a-3/a-2, b-1/b, b-1/b-2, b-3/b, and b-3/b-2, that can be produced for the substituted unbranched fragment ions a and b were calculated from the ion trap data and are presented in Tables 4 and 5, respectively. In general, ratios were fairly consistent within each group of hydrocarbons (either mono- or dimethyl-branched) with the ratios of a-1/a-2 and b-1/b-2 being the most consistent. The monomethylalkanes produced ratios greater than 1 and the dimethylalkanes gave ratios less than 1. The a-3/a and b-3/b ratios were consistent for the dimethylalkanes, with the a-3/a-2 and b-3/b-2 ratios consistently larger than 1, except for G, R, and U where the *m/z* values were low. The ion trap and magnetic sector spectra for 13-methylhentriacontane (B) and for each value of the number of methylene units separating the methyl branches are presented in Figures 5–7. The background

TABLE 3. INTENSITY RATIOS OF CHARACTERISTIC IONS FROM CLEAVAGES 1 AND 2 IN FIGURE 1 AND 1, 2, 3, AND 4 IN FIGURE 4[a]

Compound	Entry	N[b]	m/z (a-1/a)	Intensity ratio (a-1/a)	m/z (b-1/b)	Intensity ratio (b-1/b)	m/z (c-1/c)	Intensity ratio (c-1/c)	m/z (d-1/d)	Intensity ratio (d-1/d)	Fragment ions[c] a	Fragment ions[c] b	NP/FI Ratios[d] a	NP/FI Ratios[d] b
9-Methylheptacosane	A	NA	140/141	1.71	280/281	0.55					10	20	1.80	0.40
13-Methylhentriacontane	B	NA	196/197	1.20	280/281	0.71					14	22	1.43	0.55
16-Methylhexatriacontane	C	NA	238/239	1.31	308/309	0.60					17	22	1.18	0.68
11,13-Dimethyltritriacontane	D	1	168/169	1.13	308/309	0.66	210/211	0.35	350/351	0.30	12	22	1.67	0.46
11,14-Dimethyltetratriacontane	E	2	168/169	1.46	308/309	0.55	224/225	0.19	364/365	0.14	12	22	1.67	0.46
15,18-Dimethyltritriacontane	F	2	224/225	1.31	238/239	1.29	280/281	0.22	294/295	0.21	16	17	1.25	0.82
7,11-Dimethylhentriacontane	G	3	112/113	1.17	308/309	0.61	182/183	0.31	378/379	0.15	8	22	2.50	0.27
9,13-Dimethylhentriacontane	H	3	140/141	1.18	280/281	0.74	210/211	0.26	350/351	0.22	10	20	1.80	0.40
9,13-Dimethyltritriacontane	I	3	140/141	1.52	308/309	0.50	210/211	0.26	378/379	0.18	10	22	2.00	0.36
9,13-Dimethylpentatriacontane	J	3	140/141	1.45	336/337	0.40	210/211	0.24	406/407	0.19	10	24	2.20	0.33
11,15-Dimethylhentriacontane	K	3	168/169	1.30	252/253	1.25	238/239	0.30	322/323	0.21	12	18	1.33	0.56
11,15-Dimethyltritriacontane	L	3	168/169	1.47	280/281	0.73	238/239	0.31	350/351	0.19	12	20	1.50	0.50
11,15-Dimethyltetratriacontane	M	3	168/169	1.29	294/295	0.72	238/239	0.32	364/365	0.63	12	21	1.58	0.48
11,15-Dimethylpentatriacontane	N	3	168/169	1.47	308/309	0.54	238/239	0.26	378/379	0.20	12	22	1.67	0.45
11,15-Dimethylheptatriacontane	O	3	168/169	1.07	336/337	0.73	238/239	0.33	406/407	0.26	12	24	1.83	0.45
13,17-Dimethylheptatriacontane	P	3	196/197	1.33	308/309	0.67	266/267	0.29	378/379	0.24	14	22	1.43	0.55
11,16-Dimethylhexatriacontane	Q	4	168/169	1.19	308/309	0.53	252/253	0.27	392/393	0.21	12	22	1.67	0.45
7,13-Dimethyltritriacontane	R	5	112/113	1.33	308/309	0.48	210/211	0.30	406/407	0.20	8	22	2.50	0.36
9,15-Dimethyltritriacontane	S	5	140/141	1.47	280/281	0.76	238/239	0.40	378/379	0.26	10	20	1.80	0.40
11,17-Dimethylheptatriacontane	T	5	168/169	1.32	308/309	0.71	266/267	0.34	406/407	0.28	12	22	1.67	0.45
7,15-Dimethyltritriacontane	U	7	112/113	1.44	280/281	0.63	238/239	0.47	406/407	0.24	8	20	2.25	0.30

[a] Spectra recorded on magnetic sector instrument.
[b] *N* is equal to the number of methylene (-CH_2-) between the methyl branches.
[c] Size of fragment ions a and b (in total number of carbon atoms) from cleavages 1 and 2, Figures 1 and 4.
[d] NP = the neutral particles produced when fragment ions a (FIa) and b (FIb) are formed. This column is the ratio of NP mass/FIa or FIb mass.

TABLE 4. COMPARISON OF INTENSITY RATIOS OF CHARACTERISTIC IONS FROM CLEAVAGE 1 IN FIGURES 1 AND 4[a]

Compound	Entry	N[b]	m/z (a-1/a)	Intensity ratio (a-1/a)	m/z (a-1/a-2)	Intensity Ratio (a-1/a-2)	m/z (a-3/a)	Intensity ratio (a-3/a)	m/z (a-3/a-2)	Intensity ratio (a-3/a-2)	Fragment ion a'	NP/Fla ratio[d]
9-Methylheptacosane	A	NA	140/141	3.15	140/139	1.69	138/141	1.68	138/139	0.92	10	1.80
13-Methylhentriacontane	B	NA	196/197	1.73	196/195	4.17	194/197	0.72	194/195	1.73	14	1.43
16-Methylhexatriacontane	C	NA	238/239	1.54	238/237	6.25	236/239	1.74	236/237	1.64	17	1.18
11,13-Dimethyltritriacontane	D	1	168/169	1.17	168/167	0.62	166/169	2.43	166/167	1.27	12	1.67
11,14-Dimethyltetratriacontane	E	2	168/169	0.34	168/167	0.36	166/169	4.56	166/167	4.88	12	1.67
15,18-Dimethyltritriacontane	F	2	224/225	0.94	224/223	0.73	222/225	3.67	222/223	2.83	16	1.25
7,11-Dimethylhentriacontane	G	3	112/113	1.13	112/111	0.67	110/113	1.08	110/111	0.65	8	2.50
9,13-Dimethylhentriacontane	H	3	140/141	1.36	140/139	0.51	138/141	3.98	138/139	1.49	10	1.80
9,13-Dimethyltritriacontane	I	3	140/141	1.54	140/139	0.64	138/141	4.40	138/139	1.82	10	2.00
9,13-Dimethylpentatriacontane	J	3	140/141	1.19	140/139	0.75	138/141	2.08	138/139	1.57	10	2.20
11,15-Dimethylhentriacontane	K	3	168/169	0.98	168/167	0.62	166/169	4.12	166/167	2.59	12	1.33
11,15-Dimethyltritriacontane	L	3	168/169	1.34	168/167	0.86	166/169	4.16	166/167	2.68	12	1.50
11,15-Dimethyltetratriacontane	M	3	168/169	1.51	168/167	0.87	166/169	4.13	166/167	2.34	12	1.58
11,15-Dimethylpentatriacontane	N	3	168/169	1.17	168/167	0.83	166/169	3.03	166/167	2.13	12	1.67
11,15-Dimethylheptatriacontane	O	3	168/169	0.95	168/167	0.79	166/169	4.09	166/167	3.38	12	1.83
13,17-Dimethylheptatriacontane	P	3	196/197	1.29	196/195	0.65	194/197	5.55	194/195	2.78	14	1.43
11,16-Dimethylhexatriacontane	Q	4	168/169	1.12	168/167	0.69	166/169	3.71	166/167	2.28	12	1.67
7,13-Dimethyltritriacontane	R	5	112/113	1.63	112/111	0.58	110/113	2.36	110/111	0.84	8	2.50
9,15-Dimethyltritriacontane	S	5	140/141	2.22	140/139	0.91	138/141	3.61	138/139	1.48	10	1.80
11,17-Dimethylheptatriacontane	T	5	168/169	1.36	168/167	0.77	166/169	3.96	166/167	2.25	12	1.67
7,15-Dimethyltritriacontane	U	7	112/113	2.06	112/111	0.43	110/113	2.80	110/111	0.59	8	2.25

[a] Spectra recorded on ITS40 ion trap instrument.
[b] *N* is equal to the number of methylene groups (-CH_2-) between the methyl branches.
[c] Size of fragment ion a (in total number of carbon atoms) produced in cleavage 1, Figure 1.
[d] NP = the neutral particle produced when fragment ion a (Fla) is formed. This columnn is the ratio of NP mass/Fl a mass.

TABLE 5. COMPARISON OF INTENSITY RATIOS OF CHARACTERISTIC IONS FROM CLEAVAGE 2 IN FIGURES 1 AND 4[a]

Compound	Entry	N[b]	m/z (b-1/b)	Intensity ratio (b-1/b)	m/z (b-1/b-2)	Intensity Ratio (b-1/b-2)	m/z (b-3/b)	Intensity ratio (b-3/b)	m/z (b-3/b-2)	Intensity ratio (b-3/b-2)	Fragment ion a'	NP/Fla ratio[d]
9-Methylheptacosane	A	NA	280/281	1.37	280/279	2.63	278/281	0.42	278/279	0.83	20	0.40
13-Methylhentriacontane	B	NA	280/281	1.18	280/279	6.67	278/281	0.19	278/279	1.06	22	0.55
16-Methylhexatriacontane	C	NA	308/309	1.21	308/307	5.56	306/309	0.21	306/307	1.50	22	0.68
11,13-Dimethyltritriacontane	D	1	308/309	1.22	308/307	0.53	306/309	3.28	306/307	1.42	22	0.46
11,14-Dimethyltetratriacontane	E	2	308/309	1.10	308/307	0.67	306/309	3.93	306/307	2.40	22	0.46
15,18-Dimethyltritriacontane	F	2	238/239	1.28	238/237	0.71	236/239	5.70	236/237	3.17	17	0.82
7,11-Dimethylhentriacontane	G	3	308/309	0.87	308/307	0.75	306/309	2.59	306/307	2.21	22	0.27
9,13-Dimethylhentriacontane	H	3	280/281	0.63	280/279	0.61	278/281	2.57	278/279	2.51	20	0.40
9,13-Dimethyltritriacontane	I	3	308/309	0.84	308/307	0.47	306/309	5.55	306/307	3.08	22	0.36
9,13-Dimethylpentatriacontane	J	3	336/337	0.86	336/335	0.47	334/337	4.99	334/335	2.73	24	0.33
11,15-Dimethylhentriacontane	K	3	252/253	1.18	252/251	0.90	250/253	2.61	250/251	1.98	18	0.55
11,15-Dimethyltritriacontane	L	3	280/281	0.72	280/279	0.92	278/281	2.30	278/279	2.93	20	0.50
11,15-Dimethyltetratriacontane	M	3	294/295	0.81	294/293	0.48	292/295	4.12	292/293	2.45	21	0.48
11,15-Dimethylpentatriacontane	N	3	308/309	1.16	308/307	0.64	306/309	4.54	306/307	2.49	22	0.45
11,15-Dimethylheptatriacontane	O	3	336/337	1.36	336/335	0.24	334/337	25.72	334/335	3.82	24	0.45
13,17-Dimethylheptatriacontane	P	3	308/309	1.20	308/307	0.08	306/309	5.35	306/307	2.69	22	0.56
11,15-Dimethylhexatriacontane	Q	4	308/309	0.86	308/307	0.42	306/309	5.70	306/307	2.77	22	0.45
7,13-Dimethyltritriacontane	R	5	308/309	0.99	308/307	0.55	306/309	3.52	306/307	1.96	22	0.36
9,15-Dimethyltritriacontane	S	5	280/281	0.83	280/279	0.81	278/281	2.60	278/279	2.54	20	0.40
11,17-Dimethylheptatriacontane	T	5	308/309	1.21	308/307	0.72	306/309	4.42	306/307	2.63	22	0.45
7,15-Dimethyltritriacontane	U	7	280/281	0.44	280/279	0.53	278/281	2.19	278/279	2.64	20	0.30

[a]Spectra recorded on ITS40 ion trap instrument.
[b]*N* is equal to the number of methylene groups (-CH_2-) between the methyl branches.
[c]Size of fragment ion b (in total number of carbon atoms) produced in cleavage 2, Figure 4.
[d]NP = the neutral particle when fragment ion b (Flb) is formed. This column is the ratio of NP mass/Flb mass.

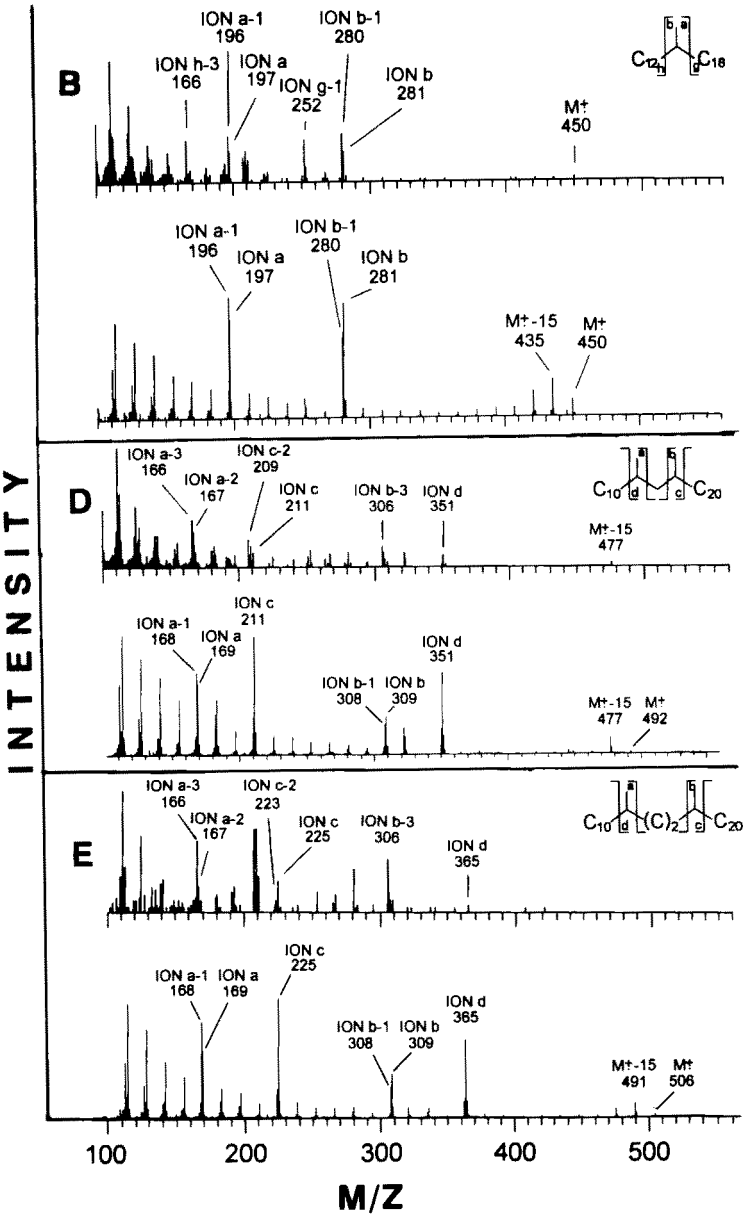


FIG. 6. Ion trap (top) and magnetic sector (bottom) mass spectra pairs of 13-methylhentriacontane (B), 11,13-dimethyltritriacontane (D), and 11,14-dimethyltetratriacontane (E). Abundances are relative to *m/z* 57.

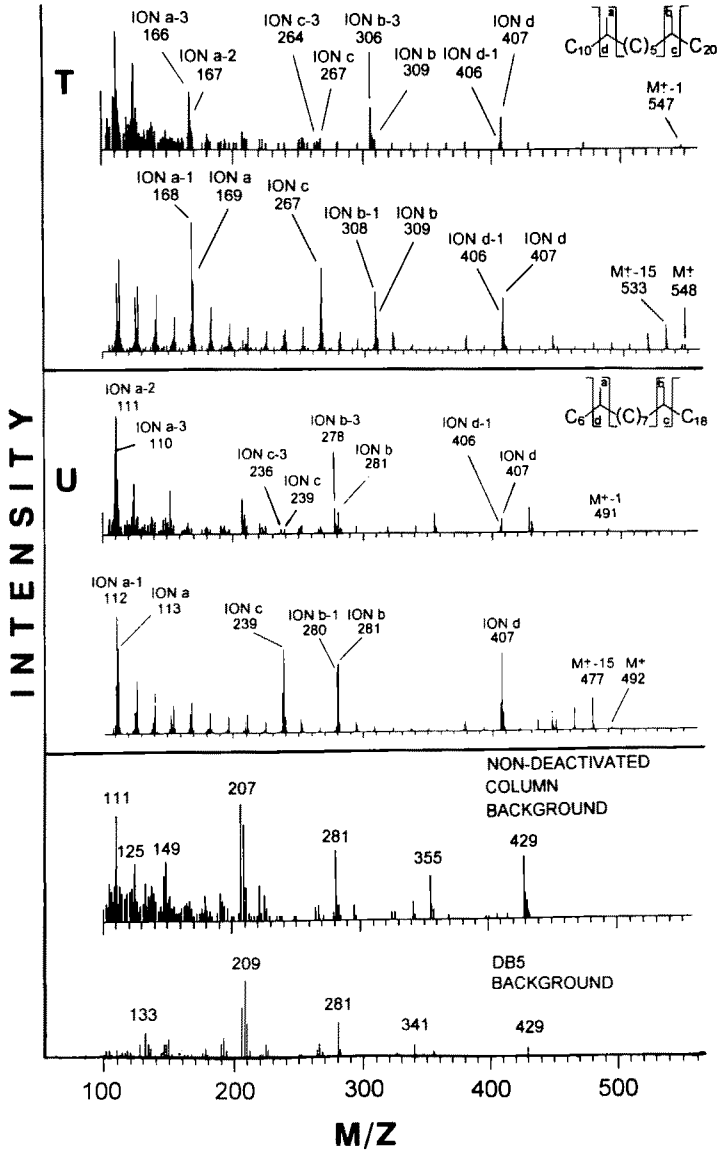


FIG. 7. Ion trap (top) and magnetic sector (bottom) mass spectra pairs of 11,17-dimethylheptatriacontane (T), 7,15-dimethyltritriacontane (U), and background magnetic sector mass spectra from non-deactivated and DB-5 columns. Abundances are relative to m/z 57.

spectra for the deactivated capillary tube and the DB-5ms column are included for comparison purposes. Analysis of the spectra recorded from the varying amounts of 11,16-dimethylhexatriacontane showed that the ratios of ion abundances, such as a-1/a-3, etc., were not dependent upon the amount of sample introduced into the ion trap spectrometer.

CONCLUSIONS

The mass spectra produced by the magnetic sector instrument from the series of hydrocarbons studied are consistent with those reported in the literature. The data collected from the quadrupole storage spectrometer (ion trap) clearly demonstrate that it can be used to identify mono- and dimethyl-branched alkanes in nanogram quantities, providing conditions in the spectrometer are adjusted for this application. The atomic mass unit segments did not vary in size from their standard values, and the highest three segments were subjected to the same ionization pulse length. Since these are two more parameters that can be changed once the *m/z* values for ions resulting from cleavages at branching points are known or suspected for a hydrocarbon, changes in these parameters might result in higher abundances for diagnostic ions. We would encourage other researchers interested in this area to pursue such investigations. At present, we do not know why the changes in the source temperature and ACQ parameters affected diagnostic ion abundances, but the subject is under investigation. It is also important to be aware that the *m/z* values and distribution of fragment ions in the ion trap will differ markedly from the patterns produced by magnetic sector instruments. Scientists attempting to use the ion trap for the identification of branched hydrocarbons will also have to be aware that molecular ion intensities will be weak and sensitivities not as good as is normally found for other structural types of organic molecules. The spectra presented in Figures 5–7 illustrate clearly the differences to be expected when the spectra of monomethyl- and dimethylalkanes from magnetic sector and ion trap spectrometers are compared. These data can aid scientists in the identification of branched-chain hydrocarbons using ion trap spectrometers.

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HOST-SPECIFIC RECOGNITION KAIROMONE FOR THE PARASITOID *Microplitis croceipes* (CRESSON)

H.T. ALBORN,[1,2] W.J. LEWIS,[3] and J.H. TUMLINSON[1,*]

[1]*Insect Attractants, Behavior, and Basic Biology Research Laboratory Agricultural Research Service, U.S. Department of Agriculture Gainesville, Florida 32604*

[3]*Insect Biology and Population Management Research Laboratory Agricultural Research Service, U.S. Department of Agriculture Tifton, Georgia 31793*

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Abstract—A water-extractable host recognition kairomone in frass of corn earworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), host larvae stimulates antennation by females of the parasitoid *Microplitis croceipes* Cresson (Braconidae: Hymenoptera). In addition, when the wasps contact water extracts of host frass they will subsequently fly in a flight tunnel to odor associated with the extract. Contact with water extracts of cowpea leaves or with water extracts of frass from larvae of nonhost beet armyworm, fall armyworm, or cabbage looper that were fed cowpea leaves does not stimulate antennation; nor do wasps fly to associated odors after contact with these substances. However, contact with the water extract of host frass in association with hexane extract of cowpea-fed nonhost frass will induce the wasps to subsequently fly to the hexane extract of the nonhost frass when it is used as an odor source in a flight tunnel. Thus the host-specific kairomone by which *M. croceipes* recognizes the frass of its host is extractable with water. This substance plays a crucial role in the foraging behavior of this parasitoid by allowing it to recognize host frass and to learn to search for odors originating from plants on which the host is feeding.

Key Words—Associative learning, recognition kairomone, host specific, *Microplitis croceipes*, corn earworm, *Helicoverpa zea*.

*To whom correspondence should be addressed.

[2]Present address: Department of Chemical Ecology, University of Göteborg, Carl Skottsbergs gata 22, S-413 19 Göteborg, Sweden.

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INTRODUCTION

The oligophagous wasp *Microplitis croceipes* (Cresson) attacks only *Heliothis* and *Helicoverpa* larvae (Lewis, 1970), which are polyphagous and severe pests on several important crops such as corn and cotton. *M. croceipes* is considered a candidate for biological control of these pest insects (Lewis, 1970), but its use on a practical basis is limited because of a lack of a reliable method for employing it as a control agent. In an effort to develop more effective methods of biological control of insect pests with natural enemies, we have been studying the foraging behavior of insect parasitoids. Artificially reared parasitoids must be able to locate and to recognize their host insects in the field. We must also be able to retain native or released parasitoids in a target crop and keep them searching for hosts.

Semiochemicals associated with the host or the host habitat play a major role in location and recognition of hosts by many parasitoids (Vinson, 1976, 1984; Turlings et al., 1990, 1992). However, it has been shown that females of *M. croceipes*, as well as the closely related *M. demolitor* (Wilkinson), reared in the laboratory on host larvae fed artificial diet do not respond to host-related cues released by a natural plant–host complex, unless first given a brief encounter with plant-fed hosts or their products (e.g., frass) (Drost et al., 1986; Hérard et al., 1988; Eller et al., 1988). An actual encounter with a host larva is not necessary. After experiencing host frass only, or a combination of water and hexane extracts of the frass, female *M. croceipes* wasps flew in a wind tunnel as often to a volatile source consisting of hexane extract of the frass as to the frass itself. However, after experiencing only hexane extract of frass, the wasps showed a very low flight response, which was not significantly different from the response by inexperienced wasps (Lewis and Tumlinson, 1988). Response after experiencing only water extracts of frass was also very low. The conclusion was that host frass contained recognition kairomones. When the kairomones are perceived by female wasps in conjunction with host-related volatiles, such as those released from host frass hexane extract, the likelihood of subsequent upwind anemotactic flight and eventual location of a source releasing the same volatiles is increased. Although antennation of host frass and the subsequent encounter with recognition kairomones will initiate host searching behavior in mated female wasps, this is not simple innate behavior. Lewis and Tumlinson (1988) showed that female wasps also are able to learn to recognize, and subsequently to fly to, novel volatiles, such as vanilla, presented in combination with the host frass. Thus, associative learning is a very important part of the host recognition process.

While antennating host frass on a plant, a naive female wasp (without a

previous experience) will be able to associate volatiles released by the frass as well as volatiles released by the damaged plant. Eller et al. (1992) showed that host frass is an important emitter of volatile attractants. More recently, it has been shown that, at least for long distance attraction, volatiles emitted by the damaged host plant are more important (McCall et al., 1993). If given several experiences with host insects feeding on one plant species and nonhost insects feeding on another plant species, the wasps preferentially respond to volatiles being released by damaged plants of the species on which the host insects were feeding (McCall et al., 1993). Again, an encounter with host larvae does not seem to be necessary for this association. However, if the wasps are given an experience with nonhost frass, no association will be made, and the wasps will not respond to any volatile cues. When *M. croceipes* females were experienced on host frass, they flew almost as well to a hexane extract of nonhost frass (*Trichoplusia ni* Hübner) as to host frass hexane extract, but showed no flight response to either of the hexane extracts after experience on nonhost frass (Lewis et al., 1991).

All these results point to very host-specific recognition kairomones present in host frass or possibly the presence of deterring components in frass from nonhost insect larvae. In either case, the antennation of frass and the subsequent association of host-related cues seems to be an extremely important part of the foraging strategy of *M. croceipes* wasps.

Jones et al. (1971) showed that *Helicoverpa* (*Heliothis*) *zea* (Boddie) frass contained hydrocarbons that were attractive to *M. croceipes* females at close range and induced extensive antennation by the females. The most active component of these hydrocarbons was 13-methylhentriacontane. Lewis et al. (1991) showed that addition of this component to hexane and water extracts of host frass on which wasps were experienced resulted in an increased flight response in the wind tunnel to host frass hexane extract. The question then arose as to whether there might not be other factors like 13-methylhentriacontane present in the hexane extract of host frass that serve as important recognition kairomones, in addition to the factor present in the water extract. The possibility also existed that the water-soluble component might be present in nonhost frass, and the lack of response by female wasps after experiencing nonhost frass might be because some hexane-soluble component of host frass is not present in nonhost frass. To answer these questions we decided to investigate the host specificity of water/hexane-soluble kairomones by preparing the same type of extracts of frass from the host—corn earworm, CEW (*Helicoverpa zea*)—and from three nonhosts—cabbage looper, CL (*Trichoplusia ni*); beet armyworm, BAW (*Spodoptera exigua* Hübner); and fall armyworm, FAW (*Spodoptera frugiperda* J.E. Smith); and to bioassay these in different combinations. To test the possibility

that the recognition kairomone might be of plant origin, we also bioassayed water extracts of the host plant, prepared in the same way as the frass extract, in combination with host frass hexane extract.

METHODS AND MATERIALS

Plants and Insects

Pink-eye purple hull cowpeas, *Vigna unguniculata*, were grown in a green house in trays (35 × 50 cm) with 50 seedlings per tray. Peat moss-based potting soil was used with no additional fertilizer. Fully developed young leaves from 7- to 14-day-old plants were used for rearing of larvae and for leaf extraction.

Larvae of all moth species reared for three to four days after hatching on a pinto bean-based artificial diet prepared according to the method of King and Leppla (1984) were transferred to fresh leaves in polystyrene trays with individual compartments. Frass produced the first 24 hr after the transfer contained artificial diet and was discarded. After that the larvae were daily transferred to new trays with fresh cut leaves and the frass collected.

M. croceipes parasitoids were reared on *H. zea* larvae according to the procedures of Lewis and Burton (1970). The wasps were held in 30 × 30 × 20-cm cages at 26°C, 70% relative humidity, under a 14 L : 10D-hr photoperiod. Males were removed two days after emergence and 2- to 4-day-old females were used in all experiments.

Extraction of Frass

Frass produced in the trays by larvae feeding on leaves was collected, taking care not to acquire bits of leaves, weighed, and stored at −60°C in glass vials with Teflon-lined screw caps. Collection continued until the larvae were about 10 days old. For each species of larvae, all frass collected was pooled and extracted with hexane (B&J GC[2], Burdick and Jackson, Muskegon, Michigan) and water (B&J HPLC grade). The frass was first extracted with hexane overnight at 4°C while being continuously stirred (2 ml hexane/g frass). The hexane was decanted and the frass extracted for another 24 hr with an equal amount of new hexane. Both extracts were combined and the concentration adjusted to 2 mg frass equivalents per 10 μl by adding hexane. The extract was stored at −60°C until used in bioassays. To prevent interference with the subsequent water extraction, the hexane still left in the frass was eliminated by placing it under vacuum (200 mm Hg) for approximately 1 hr. The frass was then extracted overnight with water at 4°C while being continuously stirred (2 ml water/g frass). The thick slurry was centrifuged at 4000*g* for 30 min, and

the supernatant was decanted and saved. The water extraction was repeated a second time, and both water extracts were combined and the concentration adjusted to 2 mg frass equivalents/10 μl by adding more water. The water extract was then filtered through a 0.45-μm HV-type membrane filter (Millipore, Bedford Massachusetts) followed by sterile filtering through a 0.22-μm GV-type sterile filter (Millipore) directly into sterile glass containers, which immediately were capped and stored at −60°C.

When used in the experiments below, the extracts were thawed and the water extracts diluted with HPLC grade water to a concentration of 1.25 mg frass equivalents/10 μl. The hexane extracts were diluted with GC[2] hexane to 1 mg frass equivalents/10 μl.

Leaf Extraction

Young, fully developed leaves taken from 14-day-old cowpea plants were ground in a mortar and extracted with water in the same way as the frass (2 ml/g leaf material). The final concentration of the water extract was adjusted in the same way as above for storing and bioassaying.

Learning Experience

Prior to use in bioassays, female wasps were conditioned by giving them a contact experience with a combination of the water extract and the hexane extract to be used as a volatile source in the flight tunnel. Water extract (5 mg frass equivalents in 40 μl) was pipetted onto Whatman No. 40 filter paper in a Petri dish. After the filter paper absorbed the water, 10 μl (1 mg frass equivalents) of the hexane extract was added on top of the water extract and the hexane allowed to evaporate for a few minutes. Then, one wasp at a time was placed on the filter paper with the extracts, and her antennae were gently pushed toward the surface with tweezers. The wasp was allowed to remain on the filter paper for 60 sec, and the duration of antennation after initial contact was recorded. Within a minute following this experience, the wasp was released in the flight tunnel. Normally six to nine female wasps could be conditioned and tested with each Petri dish application during a 30-min time period.

Flight Tunnel Bioassays

All behavioral bioassays were conducted as described by Lewis et al. (1991) in the flight tunnel described by Drost et al. (1986). Wasps were bioassayed 3 hr daily starting 2 hr into the photophase (9–12 AM). Flight responses were tested at 26–28°C, with a wind speed of 56 cm/sec and a light intensity of 2500 lux. The hexane extract to be used as an attractant source (30 μl at a concen-

tration of 1 mg frass equivalents/10 μl) was pipetted onto a triangular (9.5 × 9.5 × 6.5 cm) piece of Whatman No. 1 filter paper suspended from the tip of a disposable pipet placed in an inverted position at the center of the upwind end of the flight tunnel. The females were released from a 4-dram shell vial 80 cm downwind and directly in line with the odorant source. They were allowed to walk to the rim of the vial and their behaviors were observed and recorded for a maximum of 5 min after the vial had been placed in the flight tunnel. Females either dispersed (i.e., flew to the sides, top, or bottom of the tunnel) or responded with anemotactic flight. A positive response consisted of a complete flight resulting in landing on the target source.

Experiment 1. The wasps were experienced with water extract of either *H. zea* frass, host plant (cowpea) leaves, or HPLC-grade water in combination with hexane extract of *H. zea* frass. Hexane extract of *H. zea* frass was used as the attractant in the wind tunnel.

Experiment 2. Wasps were given an experience with water and hexane extracts of frass from each of the four insect species used: the nonhost BAW, FAW, and CL insects and the host CEW insect. In each case the hexane extract used in the experience was also used as the attractant source in the wind tunnel.

Experiment 3. Wasps were experienced with water extract of CEW frass in combination with hexane extract from CEW, BAW, FAW, or CL. The same hexane extract that was used in conditioning a female was also used as the attractant source when she was released in the flight tunnel.

Experiment 4. Wasps were experienced with hexane extract from CEW frass combined in separate trials with water extract from each of the four insect species. Hexane extract of CEW frass was also used as the attractant source in the wind tunnel.

RESULTS AND DISCUSSION

Experiment 1. All 36 wasps experienced to hexane extract of CEW frass in combination with CEW frass water extract subsequently flew to the hexane extract in the wind tunnel (Figure 1). Only one of 36 wasps flew after experiencing the host frass hexane extract together with host plant water extract. No wasps flew in the wind tunnel after experiencing the host frass hexane extract and a water control. These results, in agreement with previous reports (Lewis and Tumlinson, 1988; Lewis et al., 1991), indicate that a water-extractable component of host frass is required, in combination with components of the hexane extract of the same frass, to condition the wasps to subsequently fly to the odor of the hexane extract. Since hexane extract of CEW frass should contain the antennation activator 13-methylhentriacontane (Jones et al., 1971), these results also indicate that this compound alone is not sufficient to evoke associ-

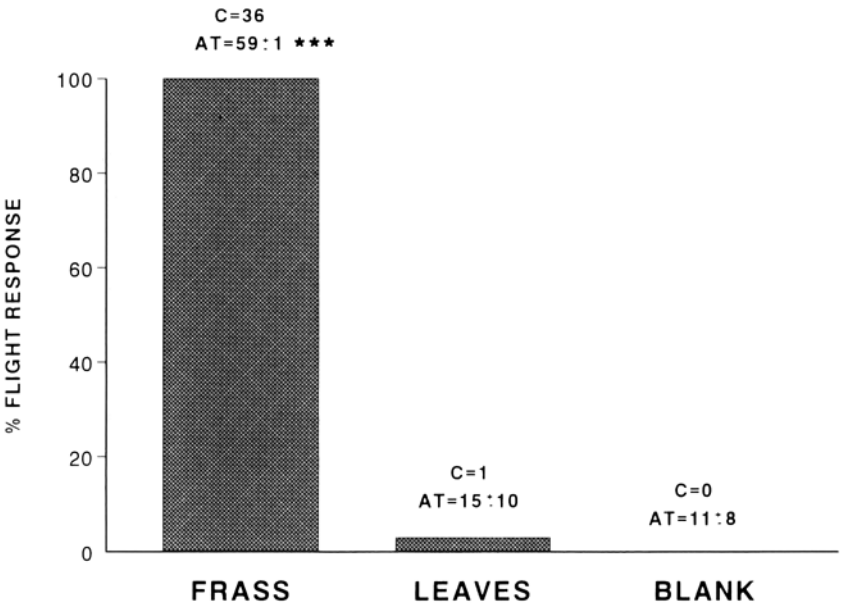

FIG. 1. Percent of female wasps flying to CEW frass hexane extract odorant source in a flight tunnel after an antennation experience with: FRASS—frass water and hexane extracts; LEAVES—cowpea water extract and frass hexane extract; and BLANK—water only and frass hexane extract. A total of 36 wasps were tested with each treatment and C is the number of insects making a complete flight to the target in the wind tunnel. AT is the mean antennation time $\pm$ SD per wasp for each treatment during the 60 sec of preflight experience. ***indicates a significantly higher antennation time (least significant difference test at $P < 0.005$)

ative learning. Furthermore, the results of this experiment indicate that the water-extractable kairomone is not a plant constituent that passes through the insect gut unchanged and acts in combination with larval components (like 13-methylhentriacontane) and plant volatiles (present in the frass hexane extract) to induce the wasps to respond to the odor of the hexane extract.

Experiment 2. No wasps flew to hexane extracts of frass from nonhosts after experiencing the hexane extract in combination with the water extract of frass from the same species. Of 21 wasps tested, 16 flew to the hexane extract of host frass after experiencing it together with host frass water extract (Figure 2). Thus, *M. croceipes* female wasps have a very strong preference for host insect larval frass, and they are capable of distinguishing hosts from nonhosts

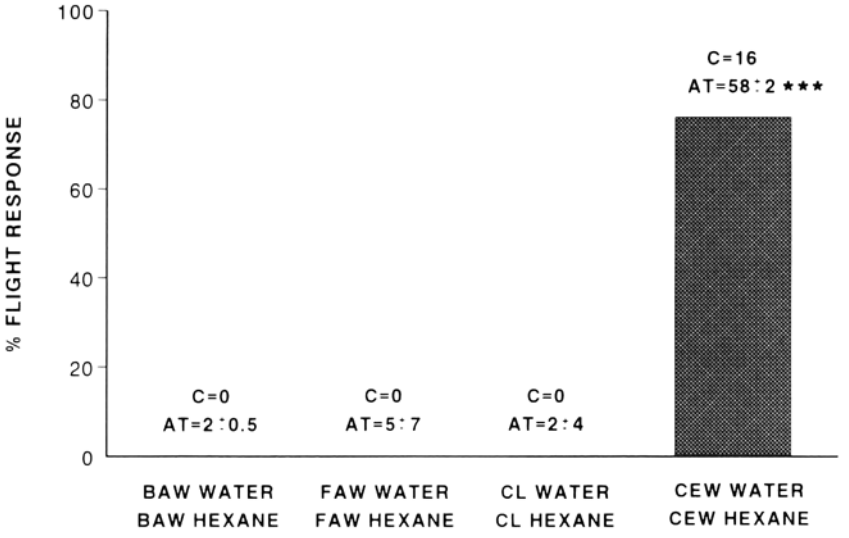

FIG. 2. Percent of female wasps flying to hexane extract of frass of BAW, FAW, CL, or CEW as an odorant source in the flight tunnel after an antennation experience with hexane and water extracts of frass from each insect species. A total of 21 wasps were used/treatment, and C is the number of insects making complete flights in the wind tunnel. AT is the mean antennation time ± SD per wasp for each treatment during the 60 sec of preflight experiment. ***indicates a significantly higher antennation time (least significant difference test at $P < 0.005$)

based on kairomones present in the frass. These results confirm and extend those of Lewis et al. (1991).

Experiment 3. The results of experiments 1 and 2 do not eliminate the possibility that kairomones crucial to learning in *M. croceipes* are present not only in the water extract but also in hexane extract of CEW frass. However, in experiment 3 all treatments showed activity, indicating that the important and host-specific factor responsible for associative learning of various odor sources is located in the water extract only (Figure 3). Despite that, the response to the CEW–CEW combination was significantly higher (least significant difference test at $P < 0.05$) than to any of the other treatments. This suggests that some important, but not crucial, factor is present in host frass hexane extract. Alternatively, a deterring component could be present in hexane extract of nonhost insect frass.

Experiment 4. When water extract of frass from each species was combined with hexane extract from CEW, the wasps responded in the wind tunnel to the

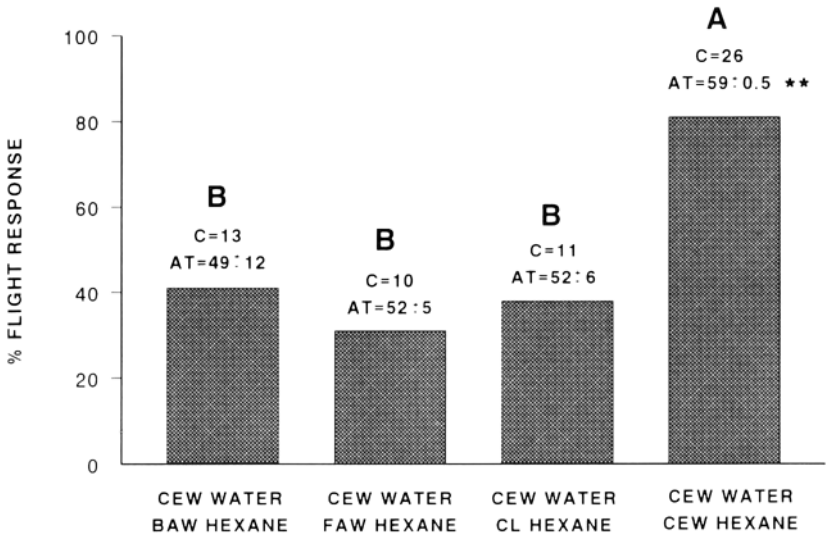

FIG. 3. Percent female wasps flying to hexane extract of frass of BAW, FAW, CL, or CEW as an odorant source in the flight tunnel after experiencing the same hexane extract in combination with CEW water extract. Four replications of eight wasps were used per treatment, and bars with different letters indicate significant difference using a least significant difference test ($P < 0.05$). C is the total number of the 32 insects per treatment that made a complete flight in the wind tunnel. AT is the mean antennation time ± SD per wasp for each treatment during the 60 sec of preflight experience. **indicates a significantly higher antennation time (least significant difference test at $P < 0.01$).

CEW hexane extract only when experienced with the CEW–CEW combination (Figure 4). If the water-soluble kairomone had also been present in frass from another species, but with the activity covered by a deterring factor present in the hexane extract of nonhosts, other treatments should have shown some activity. If the water-soluble kairomone also is present in the water extract from nonhost frass, but must be perceived in combination with 13-methylhentriacontane, then again other treatments should have shown activity.

Antennation Time. In experiment 1 (Figure 1) all treatments contained CEW frass hexane extract and thus the same amount of 13-methylhentriacontane. Despite that, the mean antennation times were very different (least significant difference test at $P < 0.01$). Most of the wasps given the host CEW–CEW combination of extracts antennated continuously for the entire minute, while wasps that experienced the plant water extract or water blank, in combination with CEW frass hexane extract, gave up antennation after a few seconds.

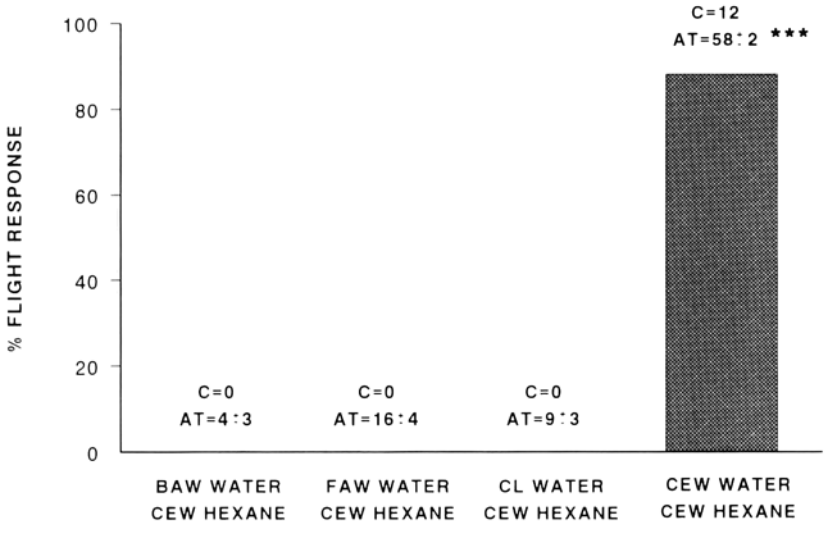

FIG. 4. Percent of female wasps flying to CEW frass hexane extract after experiencing CEW frass hexane extract in combination with BAW, FAW, CL, or CEW frass water extract. A total of 14 insects were used per treatment, and C is the number of insects making complete flights in the wind tunnel. AT is the mean antennation time ± SD per wasp for each treatment during the 60 sec of preflight experience. ***indicates a significantly higher antennation time (least significant difference test at $P < 0.005$).

In experiment 2, again, only wasps that experienced the host frass water + hexane extract combination showed sustained antennation (Figure 2). The same results were also obtained in experiment 4 (Figure 4). However, in experiment 3, in which all treatments contained CEW frass water extract, the difference in antennation time was less prominent. Although the wasps experiencing the CEW water and hexane combination antennated the target significantly longer than wasps given other combinations (least significant difference test at $P < 0.05$), all wasps antennated for 40 sec or more (Figure 3). Thus, the presence of 13-methylhentriacontane in frass (or a frass extract) is not enough to induce sustained antennation. The water-soluble recognition kairomone must also be present.

The antennation of host frass is an important component of the foraging strategy of *M. croceipes* wasps. When female wasps encounter and antennate host insect frass, they learn to associate volatile components or odors of the frass with the presence of a host recognition signal and will be attracted to these odors subsequently (Lewis and Tumlinson, 1988). Furthermore, contact with host frass appears to have a priming function (Eller et al., 1992) and is sufficient

to evoke host-searching behavior in these wasps even if a host larvae is not encountered. Manifestly, the wasps must be able to distinguish host frass from nonhost frass, as earlier results have indicated (Drost et al., 1986; Hérard et al., 1988; Eller et al., 1988). From the results of this investigation we can conclude that the crucial host recognition kairomone for *M. croceipes* females is present only in water extract of host frass. 13-Methylhentriacontane plays an important role as an antennation activator, and its importance has already been well documented (Jones et al., 1971; Lewis et al., 1991). However, this component is not host specific and occurs in frass from other lepidopteran larvae (Jones et al., 1971). Therefore, it could hardly function as a host-specific recognition kairomone. The results in experiment 3, where a significantly higher percentage of the wasps flew to the CEW frass hexane extract than to the nonhost frass hexane extract, might be explained by lower or less optimal concentrations of this and similar antennation-activating hydrocarbons in the nonhost frass hexane extract, but the kairomone present in CEW water extract was strong enough to induce a good flight response in all treatments.

This investigation has also eliminated the plant as a direct source for the host recognition kairomone. The origin of a precursor to the recognition kairomone might still be the host plant, but in that case only *Heliothis* and *Helicoverpa* larvae make this component into a reliable host-recognition kairomone for the parasitoid *Microplitis croceipes*.

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CHEMICAL ANALYSIS OF STERNAL GLAND SECRETION OF PAPER WASP *Polistes dominulus* (CHRIST) AND ITS SOCIAL PARASITE *Polistes sulcifer* (ZIMMERMANN) (HYMENOPTERA: VESPIDAE)

FRANCESCA R. DANI,[1,*] E. DAVID MORGAN,[2] and STEFANO TURILLAZZI[1]

[1]*Dipartimento di Biologia Animale e Genetica*
Università di Firenze
Via Romana 17, 50125 Firenze, Italia

[2]*Department of Chemistry, Keele University*
Staffordshire ST5 5BG, England

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Abstract—The secretion of the tegumental glands of the fifth and sixth gastral sternites of *P. dominulus* and of its social parasite *P. sulcifer* was analyzed by gas chromatography–mass spectrometry. Seven long-chain carboxylic acids have been identified; hexadecanoic, octadecadienoic and octadecenoic acids are the major components of the gland secretion of *P. dominulus* and octadecenoic acid the main compounds of *P. sulcifer* sternal gland secretion. The same carboxylic acids have been found on the nest pedicel.

Key Words—Vespidae, Polistinae, *Polistes*, sternal glands, long-chain carboxylic acids.

INTRODUCTION

In the independently founding polistine wasps (*Polistes*, *Mischocyttarus*, *Belonogaster*, *Parapolybia*, and many species of *Ropalidia*) the nest, consisting of a single comb of cells, is suspended from the substrate by a narrow pedicel or by a small number of pedicels (Jeanne, 1975; Wenzel, 1991). As the pedicel constitutes the only access to the nest for invertebrate pedestrian predators (among which ants have been shown to exert the highest predation pressure), this struc-

*To whom correspondence should be addressed.

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ture is advantageous for defending the immature brood contained in the nest. The effectiveness of the pedicel in the nest defense is increased by the wasps' application of a repellent substance by rubbing the pedicel with the abdomen (Jeanne, 1970; Marino Piccioli and Pardi, 1970; Corn, 1972; Hermann and Dirks, 1974; Turillazzi and Ugolini, 1979; Post and Jeanne, 1981; Kojima, 1983a,b; Raposo Filho, 1989; Keeping, 1990).

Gland cells situated on the gastral sternites, especially those on the anterior part of the two last sternites (see Downing, 1991; Jeanne, 1995), have been indicated as the source of the repellent substances. Nevertheless, some authors have hypothesized that the sternal glands have a role in the chemical communication of dominance between nestmates (West-Eberhard, 1982; Downing and Jeanne, 1983), and that the fifth and sixth gastral sternite glands may differ in their function (Post and Jeanne, 1980).

In the three species of obligate and permanent social parasites among *Polistes* (*P. sulcifer*, *P. semenowi*, *P. atrimandibularis*), females invade nests of other *Polistes* species and rely on the host workers for rearing their brood (Weyrauch, 1937). Soon after the invasion of the nest, the parasite intensively strokes the nest comb with the abdomen, often near the cell opening. Some hours after the invasion this behavior is no longer performed (Turillazzi et al., 1990; Cervo et al., 1990). Indeed, during their cohabitation with the host, parasites rub the pedicel only rarely (Cervo, 1990). It has been hypothesized that the sternal glands of these social parasites may produce pheromones that control the host colony (Turillazzi et al., 1990; Borgioli and Delfino, 1994).

Despite speculation about the role of the sternal glands, chemical analyses have been carried out only on the sixth gastral sternite glands of two new world species of *Polistes*, namely *P. fuscatus* (Post et al., 1984) and *P. annularis* (Espelie and Hermann, 1990). Methyl hexadecanoate has been identified in the secretion of the first species, while hexadecanoic and octadecenoic acids have been found in the second one.

The aim of this work has been the analysis of the fifth and sixth gastral sternite gland secretions of foundresses and workers of the European species *P. dominulus*. We also analyzed the fifth and sixth gastral sternite gland secretions of *P. sulcifer*, one of the social parasites of *P. dominulus*; we wanted to determine if the sternal glands of this parasite produce a secretion similar to that of the host species.

METHODS AND MATERIALS

Polistes dominulus nests and foundresses were collected near Florence, Italy, during the month of May before the emergence of the first workers (number of nests = 4; number of foundresses = 2.5 ± 0.58 (mean ± SD), number

of cells = 33.7 ± 2.63, number of pupae = 7 ± 3.56) and again during the month of June, when some workers had already emerged (number of nest = 2; number of cells = 48.5 ± 4.95). *P. sulcifer* females ($N = 3$) that had invaded the nests of *P. dominulus* some days earlier were collected at the beginning of summer on the pre-Alps near Verona in northern Italy. Once in the laboratory, nests and animals were frozen and maintained at −20°C. All samples were chemically analyzed at the Chemistry Department of Keele University.

The sternal glands were separated from cuticle under a binocular microscope using thin forceps and placed in glass capillaries (2 × 20 mm) sealed at one end (Morgan and Wadhams, 1972; Morgan, 1990). The samples prepared are reported in Table 1.

As preliminary gas chromatography–mass spectrometry analysis of sternal glands had shown traces of carboxylic acids in the samples (but not the presence of methyl esters), acids were derivatizated to methyl esters to make them more suitable for gas chromatography. An ethereal solution of diazomethane (Arndt, 1943) (20 μl) was inserted into each capillary with a microliter syringe. After the solution made good contact with the tissue, the excess was evaporated with a stream of nitrogen and the capillary resealed in a small flame.

The analyses were carried out by combined gas chromatography–mass spectrometry (GC-MS) on a Hewlett Packard 5890 gas chromatograph connected to a 5970 Mass Selective Detector. The GC-MS unit was controlled by a HP59970C ChemStation. The gas chromatograph was equipped with a fused silica capillary column (12 m × 0.2 mm), coated with a 0.33-μm-thick film of immobilized dimethylsiloxane. Helium was used as carrier gas. The mass spectrometer was set to scan m/z from 30 to 500.

TABLE 1. SAMPLES ANALYSED[a]

Number of capillaries	Content of each capillary
5	Sixth sternal glands of one foundress of *P. dominulus*
3	Fifth sternal glands of one foundress of *P. dominulus*
1	Sixth sternal glands of three workers of *P. dominulus*
2	Sixth sternal glands of one female of *P. sulcifer*
2	Fifth sternal glands of one female of *P. sulcifer*
2	3 mg of material taken near the cell openings of one preemergence nest (two different nests were used)
2	3 mg of pedicel material from one preemergence nest (two different nests were used)

[a]Each capillary represents one analysis.

A sealed capillary tube was placed in the injection area of the gas chromatograph (Morgan and Wadhams, 1972; Morgan, 1990), which was heated to 220°C, and the capillary crushed after 2 min, at which time chromatography commenced. The initial oven temperature of 100°C was increased by 7°C/min to 290°C. Substances were identified by their mass spectra and retention times compared with standard samples of the same esters.

The amount of each methyl ester was calculated by comparing the area of its peak with that given by a known amount of methyl octanoate. As two of the methyl esters (methyl octadecadienoate and methyl octadecenoate) elute in one peak (see below), we used the proportion between the area given by two diagnostic ions (*m/z* 294 and *m/z* 296, respectively) for calculating the amounts of these compounds. For samples containing the sixth sternal glands of *P. dominulus* foundresses, using this method, it was possible to estimate the amount of the two esters in only three of the five samples.

RESULTS

Polistes dominulus. The following carboxylic acids were identified (as their methyl esters) in the fifth and sixth sternite glands of the foundresses and in the sixth sternal glands of workers: dodecanoic (lauric), tetradecanoic (myristic), hexadecenoic (palmitoleic), hexadecanoic (palmitic), octadecadienoic (linoleic), octadecenoic (oleic), and octadecanoic (stearic) acids. Methyl octadecadienoate and methyl octadecenoate eluted as one peak (Figure 1A). The average total quantity of carboxylic acids found in *P. dominulus* foundress fifth and sixth sternal glands were 1.9 ± 1.9 μg and 5.3 ± 3.7 μg, respectively, while an average of 6.6 μg was found in the sixth sternal glands of the workers. In many samples, methyl dodecanoate and methyl tetradecanoate were only present in traces; sometimes also traces of an unidentified hydroxymethyl ester were found. As esters were not found in nonderivatizated samples, we are certain that these methyl esters indicate the presence in the tissues of the corresponding carboxylic acids.

The amounts of the seven acids found in the fifth and sixth sternal glands of *P. dominulus* foundresses and in the sixth sternal glands of workers are reported in Table 2. Octadecenoic acid was the main component extracted and identified for all three groups of samples. Octadecadienoic and hexadecanoic acids were present in similar percentages in the sixth sternite glands of workers and foundresses, while the quantity of octadecadienoic acid was higher than that of hexadecanoic acid in the fifth sternal glands of the foundresses.

The same acids have been found on the nest carton. The total amount of carboxylic acids found on 3 mg of pedicel material is on average 5.5 μg, while an average of 2.0 μg was present in the same amount of nest bottom material.

P. sulcifer. Four acids found in *P. dominulus* were present in the sternal glands of *P. sulcifer* (Figure 1B). The average quantity of carboxylic acids found in the sixth sternite glands was 1.5 ± 1.2 μg while only 157 ± 79.2 ng was found on average in the glands of the fifth sternite. Table 2 shows the average quantity per female and the average percentage of each acid in the fifth and sixth sternal glands.

DISCUSSION

Our work confirms that, as already found by Espelie and Hermann (1990) in *P. annularis*, the sternal glands of *P. dominulus* secrete carboxylic acids. In *P. dominulus* we found five acids in addition to those identified by Espelie and Hermann (1990).

The presence of the same carboxylic acids in the sixth and fifth sternite glands of *P. dominulus* foundresses indicate that, as suggested (Hermann and Dirks, 1974; Turillazzi, 1979), both glands may have the same function. The sixth sternite glands of *P. dominulus* workers contain the same carboxylic acids. Four of the same carboxylic acids are also present in the sixth and fifth sternite glands of the social parasite *P. sulcifer*. It seems likely that the function of the fifth and sixth sternite gland is similar for both foundress and workers of *P. dominulus* and for females of the social parasite *P. sulcifer*. It does not seem that in the parasite *P. sulcifer* the sternal glands have assumed a different function with respect to nonparasite species.

It is noteworthy that these acids are commonly found in the lipids of insects and higher animals, but the proportion of unsaturated acids (hexadecenoic, octadecadienoic, and octadecenoic) is much higher than in other animal lipids (Gunstone et al., 1986). Approximately 70% of the acids in the fifth sternal glands of *P. dominulus* foundresses are unsaturated, while 77% of the acids in the sixth sternal glands of foundresses and 64% from workers are unsaturated. While there is less total secretion in the glands of *P. sulcifer*, they are 88% unsaturated in the fifth sternal glands and 80% unsaturated in the sixth sternal glands.

The higher amount of carboxylic acids in the pedicel with respect to the comb material indicates that more of the carboxylic acids produced by the sternal glands are applied to the pedicel than to the comb. It must be stressed that the pedicel is enlarged by the wasps during nest growth and in our analyses we probably measured acids in the internal layers of the pedicel as well as on its surface. Bioassays are needed in order to determine if the carboxylic acids have a repellent effect against ants. Unlike our results and those by Espelie and Hermann (1990), Post and coworkers (1984) found methyl esters in the sternal glands of *P. fuscatus* females; the reasons for this difference are unclear, and it would be interesting to repeat the analyses of the glandular secretion of this

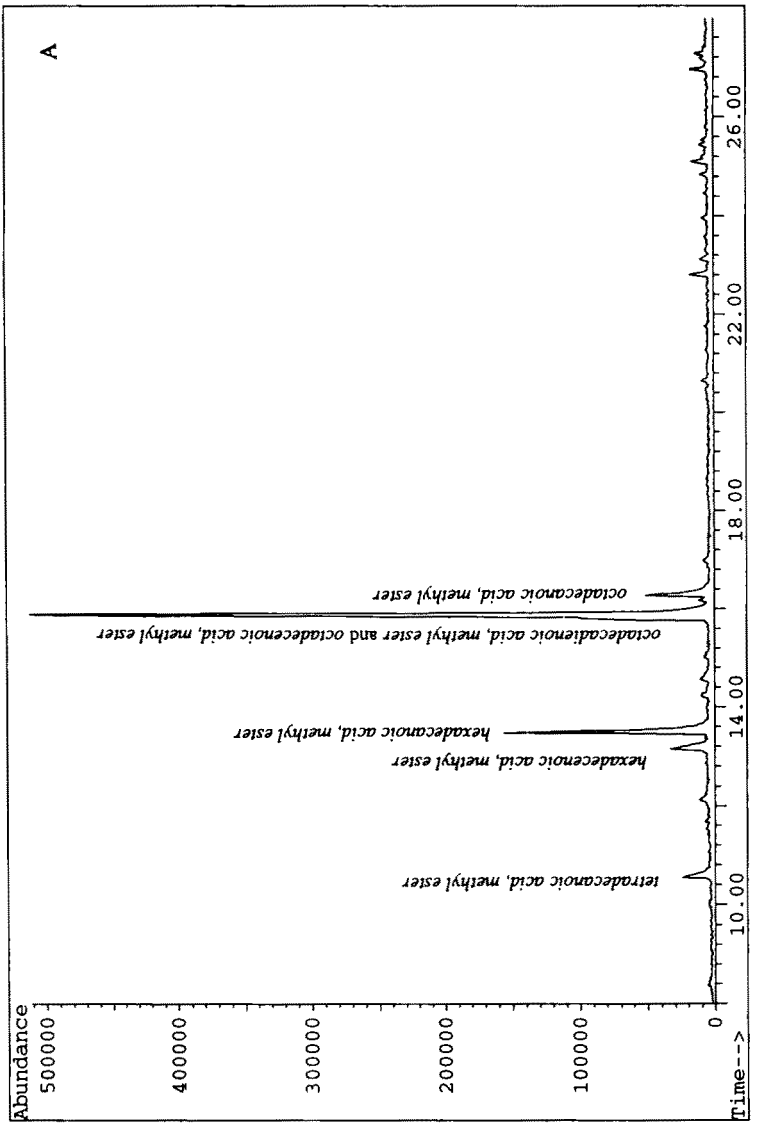

FIG. 1. Total ion chromatogram of the fifth sternite glands of one *P. dominulus* foundress (A) and of the sixth sternite glands of one *P. sulcifer* female. (B).

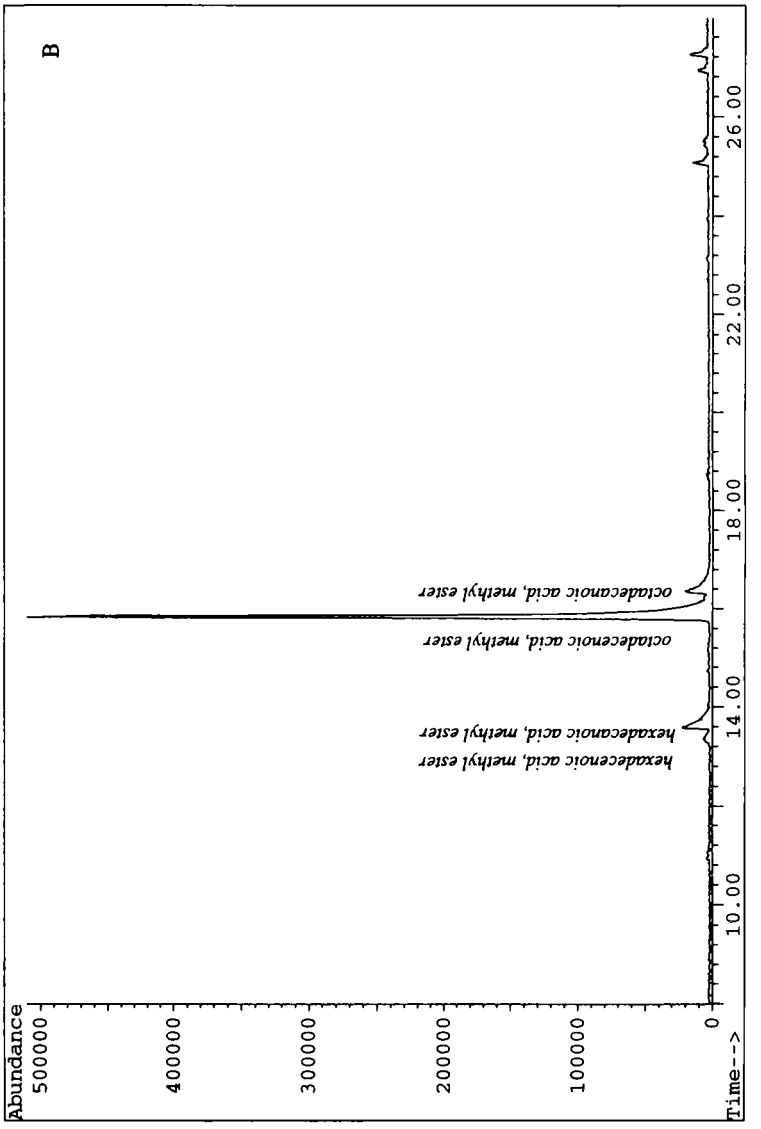

FIG. 1. Continued.

TABLE 2. COMPOSITION OF STERNAL GLAND SECRETION OF *Polistes dominulus* FOUNDRESSES AND WORKERS AND OF *P. sulcifer* FEMALES[a]

	Polistes dominulus								*Polistes sulcifer*			
	Sixth sternite glands of foundresses			Fifth sternite glands of foundresses			Sixth sternite glands of workers		Sixth sternite glands of females		Fifth sternite glands of females	
	Average			Average			Average		Average		Average	
Acid	ng	% of total	SD of %	ng	% of total	SD of %	ng	% of total	ng	% of total	ng	% of total
Dodecanoic	26 (N = 5)	0.59	1.33	77 (N = 3)	0.80	1.40	195	2.96	0 (N = 2)	0	0 (N = 2)	0
Tetradecanoic	58 (N = 5)	1.30	2.91	260 (N = 3)	4.81	1.33	393	5.96	0 (N = 2)	0	0 (N = 2)	0
Hexadecenoic	43 (N = 5)	1.04	1.55	260 (N = 3)	4.79	0.20	271	4.11	12 (N = 2)	0.51	0 (N = 2)	0
Hexadecanoic	293 (N = 5)	13.91	10.29	859 (N = 3)	16.54	1.00	1410	21.40	126 (N = 2)	10.94	14 (N = 2)	6.54
Octadecadienoic	485 (N = 3)[b]	11.75	11.73	1547 (N = 3)	22.16	15.86	1405	21.33	0 (N = 2)	0	0 (N = 2)	0
Octadecenoic	1652 (N = 3)[b]	64.05	22.68	1785 (N = 3)	42.22	19.73	2523	38.32	1288 (N = 2)	80.06	132 (N = 2)	88.08
Octadecanoic	116 (N = 5)	8.93	6.14	545 (N = 3)	8.65	3.45	390	5.92	112 (N = 2)	7.86	11 (N = 2)	5.37

[a]For the sixth and fifth sternite glands of *P. dominulus* foundresses, average quantity per individual, average of the percentages and standard deviation of the percentages of each carboxylic acids are given. For the workers, for which only one sample containing the sixth sternite glands of three individuals has been analyzed, the average quantity per workers and the percentage of each acids are given. For the sixth and fifth sternite glands of *P. sulcifer*, average quantity per female and average of the percentages are given for the carboxylic acids.

[b]Only in three samples of the sixth sternite glands was it possible to calculate the quantity of oleic and linoleic acids on the basis of the diagnostic ions (see materials and methods.)

species by means of GC-MS. The production of ant repellents seems to be a common feature of all independently founding Polistinae (Kojima, 1992), and although the sternal gland secretions have been implicated in ant repellency, the chemical nature of the secretion from the sternal glands has been investigated only in the genus *Polistes*. Indeed, nothing is known about the chemistry of the secretion of these glands in the swarm founding Polistinae, where they are thought to be involved in the production of trail pheromones used during swarming (Jeanne et al., 1983). In the subfamily Vespinae, the function of the sternal glands is uncertain (Landolt and Akre, 1979) and only recently Martin (1992) has shown that the fifth and sixth gastral sternite glands of two species of *Vespa* secrete ant repellent substances. Future comparative studies await further data on the chemical nature of the secretion of these glands.

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ALLOCATION OF PEDERIN DURING LIFETIME OF *Paederus* ROVE BEETLES (COLEOPTERA: STAPHYLINIDAE): EVIDENCE FOR POLYMORPHISM OF HEMOLYMPH TOXIN

RUPERT L.L. KELLNER* and KONRAD DETTNER

Lehrstuhl für Tierökologie II
Universität Bayreuth
D-95440 Bayreuth, Germany

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Abstract—Pederin, a hemolymph toxin found in the rove beetle genus *Paederus*, is quantified in individual specimens of *P. fuscipes* and *P. riparius*. Whereas males always contain only small quantities of the toxin (0.1–1.5 μg), females are not so restricted (0.2–20.5 μg) and in most cases possess roughly 10-fold that in males. There are, however, females containing as little pederin as the males, and so two clearly separate groups of females can be discerned. These two groups hint at two types of females defined by the eggs they lay. About 85% of the females, i.e., those containing much of the substance, transfer pederin into every egg and thus are denoted (+)-females. The remaining 15%, the (−)-females, contain at best small amounts of the toxin, which is transferred in minute quantities into the first eggs while the subsequent ones lack pederin. With respect to the pederin content of their eggs, there is no overlap between these two types of females. If fed with pederin, (−)-females can transfer it into the eggs like (+)-females. After hatching the larvae store pederin when present. Larvae are not able to biosynthesize the toxin on their own, but storage of that received in the egg is very efficient, and the difference between larvae with and without pederin is preserved until imaginal eclosion. In (+)-females, pederin increases, probably reflecting a biosynthetic capacity, but in males and presumptive (−)-females the amount of pederin never exceeds the quantity transferred by parental (+)-females. Consequently, males and (−)-females probably are unable to biosynthesize pederin. This polymorphism of females may have a genetic basis. Individuals of all stages nevertheless sequester pederin if it is supplied with the diet.

*To whom correspondence should be addressed.

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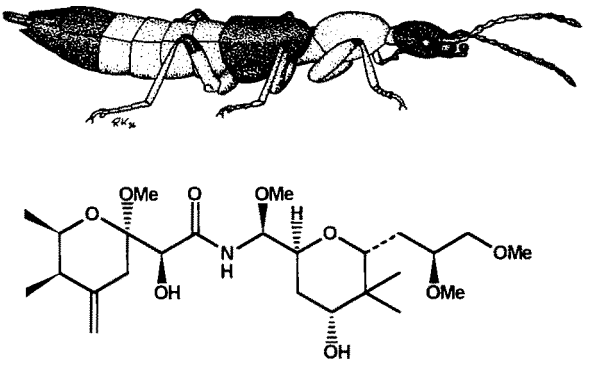
Key Words—Pederin, toxin, *Paederus fuscipes*, *Paederus riparius*, polymorphism, sequestration, biosynthesis, Staphylinidae, Coleoptera.

INTRODUCTION

Rove beetles of the genus *Paederus* are notorious for having a toxin that causes lesions on human skin. This kind of dermatitis was first described by Vorderman (1901), who noted its similarity to the blistering caused by cantharidin that was already known from *Lytta vesicatoria* and other meloids. Although Netolitzky (1919), by isolating crystals and carefully observing the formation of the blisters, discovered the difference between cantharidin and the toxin of *Paederus*, this distinction was not unanimously accepted until Pavan and Bo (1953), working with *P. fuscipes* again obtained and examined crystals of the substance that they named pederin. Subsequently, the molecular structure of this new compound (Figure 1) was elucidated by Italian and Japanese groups (Cardani et al., 1965b; Matsumoto et al., 1968). Among the trace constituents of *P. fuscipes* extracts, two analogs of pederin were described and named pseudopederin (Quilico et al., 1961) and pederone (Cardani et al., 1967), respectively. Apart from toxic peptides, these amides still are the most complex toxins found in beetles (Dettner, 1993).

Pederin has been chemically identified in only six different species but most of the *Paederus* studied so far have the same vesicant properties (Frank and Kanamitsu, 1987), indicating a wider distribution of the amide in this genus. The toxin is dispersed throughout the body of adults and is located in the



Pederin

FIG. 1. Female of *Paederus riparius* (length about 8 mm) and formula of pederin, a toxin contained in the hemolymph of the beetle.

hemolymph (Pawlowsky and Stein, 1927; Ito, 1934; Pavan, 1963). Since *Paederus* beetles do not reflexly bleed as meloids do, they have to be injured in order to release the amide contained in their hemolymph (Pavan and Valcurone Dazzini, 1971). Pederin is highly toxic to various eukaryotic cells, in which it blocks protein synthesis (Pavan, 1975). If injected intraperitoneally, comparatively small quantities of pederin suffice for killing mammals such as mice (LD_{50} = 0.14 mg/kg, Pavan and Valcurone Dazzini, 1971). Because of its cytotoxicity, pederin is regarded as a defensive compound (Blum, 1981).

Little is known about the pederin content of individuals because in former investigations pederin was evaluated after extracting a great number of specimens: Since 1 kg of *P. fuscipes* yields 0.25 g pederin (Quilico et al., 1961), one individual beetle weighing 4 mg might be expected to contain 1 μg of pederin (Pavan, 1963). Cardani et al. (1965a) stressed finding considerable variation of the pederin content of individual specimens by biological tests, but gave no details of this variation other than a broad outline. Pavan (1975) reported that adults of *P. fuscipes* contain 1 μg pederin, with females having up to 10 times that amount. Eggs, larvae, and pupae of *P. fuscipes* are known to contain the toxin, but there is no information on pederin content of these stages, and they were only tested for their ability to produce the dermatitis (Kurosa, 1958; Pavan, 1975).

Assessing the severity of dermatitis apparently has been the only method to quantify the pederin content of individuals. Research by Théodoridès (1950) has shown, however, that this method is not really reliable, as he had to postulate immunization in order to explain failure of skin reactions. Therefore, a chemical method had to be found to determine trace amounts of pederin present in single individuals. After developing a procedure that took advantage of the known properties of pederin (e.g., Pavan and Bo, 1953), we present quantitative data here for the first time concerning the amount of pederin present in adults and all developmental stages. These data enable a preliminary answer to the question as to which stadium might synthesize this unusual compound.

METHODS AND MATERIALS

Beetles. Adult *Paederus fuscipes* Curtis 1826 and *P. riparius* (Linné 1758) were collected from several sites in northeastern Bavaria, Germany. In order to obtain eggs, pairs of beetles were kept in plastic boxes (10 × 10 × 5.5 cm) containing a layer of plaster, some flowerpot fragments, and a small dish with moistened cotton. They were fed flightless *Drosophila melanogaster* and honey–water. The cages were placed in a climate chamber at 20°C with 15 hr of photophase, where females laid one to five eggs per day for a period of several weeks. Daily or every other day the eggs were isolated and kept singly in small

Petri dishes (3.5 cm diameter) on moist absorbent paper. The two larval stages (L1 and L2) were fed twice a day with frozen *Drosophila* flies until they pupated under the absorbent paper. Newly emerged adults were transferred to large Petri dishes (9 cm diameter) and fed *ad libitum* with living *Drosophila* flies. Breeding trials with these laboratory-reared adults were not successful because females only rarely laid eggs. Samples of adults and all developmental stages were preserved at −25 or −40°C for pederin analysis.

Extraction. Each specimen was ground in a small volume of water (eggs and L1 in 10 μl; L2, pupae, and adults in 30 μl demineralized H_2O). Extraction of the suspension proceeded with 100 μl ethyl acetate and was repeated three times. All three extracts were combined and the solvent removed in a centrifugal evaporator (Jouan RC 10-10). The following cleanup procedure was necessary only for extracts from L2, pupae, and adults: The dry sample was again dissolved in 50 μl water and extracted once with 100 μl hexane in order to eliminate most of the accumulated lipids, whereas pederin is left in the watery phase. This was dried in the centrifugal evaporator and, like the unmodified extracts from eggs and L1, it could then be analyzed.

High-Performance Thin-Layer Chromatography (HPTLC). Extracts of single specimens as well as known amounts of authentic pederin (from D. Ghiringhelli) were dissolved in 10 μl ethyl acetate and applied to the starting line on HPTLC plates (0.2 mm silica gel 60 on glass plates, 10 × 10 cm, Merck). The plates were developed in ethyl acetate in a vapor-saturated tank (Cardani et al., 1966). After evaporation of the solvent, they were stained in a dipping solution of anisaldehyde (4-methoxybenzaldehyde) and sulfuric acid in acetic acid (1 : 2 : 100 v/v/v) and heated for 2 min on a hot-plate (Desaga Thermoplate) at 90°C (Jork et al., 1989). Pederin ≥0.05 μg gives lilac spots at R_f = 0.22 after this procedure. This method is suited for detecting the analogs of pederin, pseudopederin and pederone, but they were not found in the extracts of either species. This study therefore treats only pederin, which certainly is the main if not the only toxic compound of *Paederus*. Images of fluorescent screen light transmitted through the plates were analyzed with a computer program (BASys 1D, Biotec-Fischer GmbH, Reiskirchen). Pederin spot intensities were transformed into peak areas, which then could be compared quantitatively within one plate. Known amounts of authentic pederin on several lanes of every plate were used to calculate a regression line in order to estimate the unknown amounts of samples on the plate. The amounts of pederin applied to calibration lanes were chosen to represent the expected range of samples. In most cases 0.5, 1.0, and 1.5 μg pederin were used to determine the regression line, but other combinations, such as 0.5, 1.0, and 2.0 μg or more than three standards, were also used. As the regression of spot intensity on pederin amount was linear only up to about 2 μg pederin, extracts with suspected higher amounts of pederin were

analyzed in diluted portions. Thus, in the first instance 1/10 of the extract (in 10 μl ethyl acetate as above, i.e., a 10^{-1} dilution) was applied to a silica plate and, if the resulting measurement still exceeded 2 μg, 1/20 of the original sample (i.e., a $5 \cdot 10^{-2}$ dilution) was used in a second determination. If little pederin was found in the first measurement, 9/10 of the original sample could be applied in the second trial to quantify the small amount of pederin. Repeated quantifications of the same samples on different plates show that, as a rule, the error due to the method is about 0.1 μg, but much better results with two identical decimal places were also obtained. Using this method, it was, for the first time, possible to quantify chemically the amount of pederin present in individual specimens of *Paederus*.

Feeding with Pederin. Selected specimens were given access to conspecific eggs, whose pederin load was known according to other eggs laid by the same female. The number of eggs eaten was counted and, as the egg content was always ingested completely, the amount of pederin received could be summed.

Data Analysis. Statistical tests were chosen according to the requirements described by Sachs (1984). They were performed using the statistical software packages CSS (StatSoft Inc., Tulsa, Oklahoma; Release 2.1) and SPSS (SPSS Inc., Chicago, Illinois; Version 3.1). One-way ANOVA was applied to pederin data of the eggs of 26 *P. riparius* females that transferred pederin to their offspring (treated as groups) in order to reveal variation between females. To adjust the different amounts of pederin that distinct females transfer into their eggs, the differences between the actual value and the mean amount of pederin transferred by the respective *P. riparius* female were calculated individually for eggs, L1, L2, pupae, and young adults and analyzed using the H test of Kruskal and Wallis. In case of nonlinear correlation, the curve fitting option of SigmaPlot (Jandel Scientific, Corte Madera, California; Version 5.0) was applied.

RESULTS

Pederin in Adults. Pederin could be identified in extracts of single specimens of *Paederus fuscipes*, the species from which Pavan and Bo (1953) had first extracted and named the toxin. Pederin was also found in *P. riparius*, whose vesicant action had not been analyzed chemically. All adults, which were preserved just after collection from the field, had at least small amounts (0.1 μg) of the amide, but the sexes differ greatly in the maximum amount determined. Males had only 1.2 (*P. fuscipes*) to 1.5 μg pederin (*P. riparius*), whereas females had up to 20.5 and 16.8 μg, respectively. In both species, females thus have significantly more pederin than males (Table 1, Kolmogorov-Smirnov two-sample test: *P. fuscipes:* $D = 1.00$, $P < 0.001$; *P. riparius:* $D = 0.85$, $P <$

0.001). Within sexes there are no discernible differences between the two species (Kolmogorov-Smirnov test not significant, $\alpha = 0.05$), although *P. riparius* is about 1.36 times the weight of *P. fuscipes*.

Remarkably, the amount of pederin detected in *P. riparius* females is not evenly distributed within the range measured (0.2–16.8 μg). Most of the females ($N = 28$) had more than 6.6 μg pederin, and the others ($N = 5$) had less than 1.2 μg with only one female in between (4.5 μg pederin, cf. Figure 3a below, open bars). The females do not belong to a uniform population, but can be subdivided into a smaller group (about 15% of the females) with little pederin, like the males, and a larger group (the remaining 85%) containing much more of the amide. These two groups are not linked to a seasonal rising and falling of the pederin content of females but coexist throughout the year. The few females with little pederin cause the overall mean of *P. riparius* females (Table 1) to be somewhat biased. Within the smaller number of *P. fuscipes* analyzed after collecting in spring, there was no female with little pederin, with all containing at least 9.5 μg of the amide.

Pederin Transferred into Eggs. The eggs of *P. riparius* ($N = 212$) are composed of two clearly defined groups (Figure 2), namely eggs with 0.4–2.1 μg pederin ($N = 172$) and others almost devoid of pederin ($N = 40$). As the female from which the eggs were derived is known in every case, these two groups evidently are not related to the time of laying (early or late), the volume or age of the eggs, or the number of eggs laid per female. The females studied can, however, be classified into two different types, here denoted (+)-females and (−)-females according to their ability to endow all their eggs with pederin or not (Figure 3b). In (−)-females, only the first few eggs laid may contain fairly small amounts of pederin (<0.1 μg). These two types of females proven by the eggs they lay are equally found in *P. fuscipes*. Quantitatively analyzed eggs of (+)-females lie in the range of 0.7–1.8 μg pederin, $N = 13$. As the distinction is very clear-cut (Figure 2), it is sufficient to analyze one egg laid by a female in order to classify her type. According to these determinations three of 15 *P. fuscipes* females (20%) and four of 37 *P. riparius* females (11%) that laid eggs in the laboratory belonged to the (−)-female group. These per-

TABLE 1. PEDERIN CONTENT OF ADULTS OF TWO *Paederus* SPECIES

	P. fuscipes		*P. riparius*	
Sex	*N*	μg ± SE	*N*	μg ± SE
Male	8	0.40 ± 0.140	13	0.67 ± 0.106
Female	10	14.84 ± 1.229	34	9.27 ± 0.821

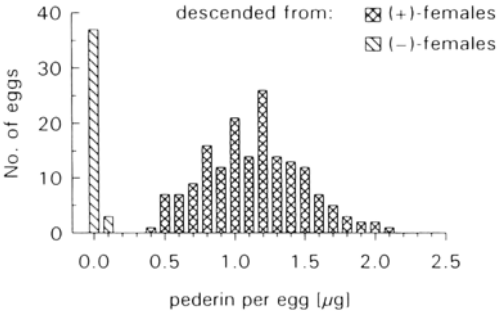


FIG. 2. Amount of pederin in the eggs of *Paederus riparius* laid by two types of females.

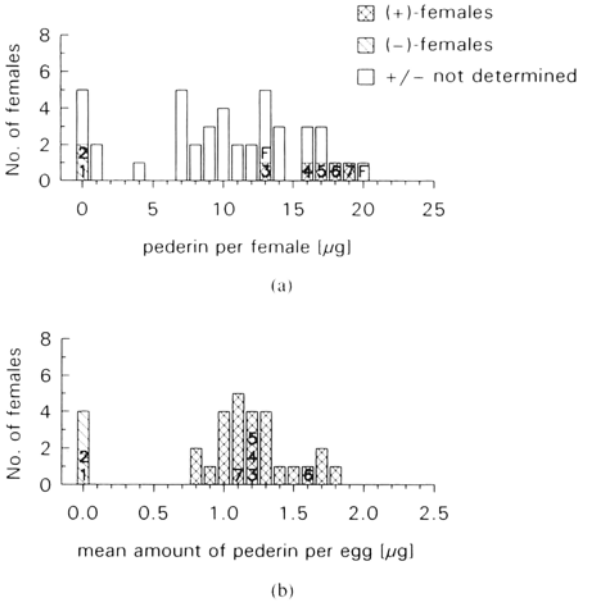


FIG. 3. Pederin content of *Paederus riparius* females and their eggs. (a) Whole bodies of females collected from the field were analyzed immediately ($N = 34$) or after an egg-laying period in the laboratory ($N = 9$). Two females failed to lay eggs (F); females 1–7 laid eggs. (b) Females were assigned to the two types, (+)-females and (−)-females, by the amount of pederin they transferred to their eggs. All females from which at least four eggs were analyzed are considered. Numbers refer to individual females in a, b and Table 2.

TABLE 2. PEDERIN CONTENT OF SELECTED *Paederus* FEMALES AFTER THEY CEASED LAYING EGGS AND AMOUNT OF PEDERIN TRANSFERRED INTO THEIR EGGS

Species	Female			Pederin per egg	
	No.	Type	μg	*N*	μg ± SE
P. riparius	1	(−)	0.0	10	0.00 ± 0.000
	2	(−)	0.0	16	0.04 ± 0.026
	3	(+)	12.7	7	1.16 ± 0.078
	4	(+)	16.2	7	1.17 ± 0.097
	5	(+)	16.9	8	1.16 ± 0.033
	6	(+)	18.1	7	1.56 ± 0.104
	7	(+)	18.8	13	1.13 ± 0.087
P. fuscipes	8	(−)	0.0	1	0.00 ± nd
	9	(+)	16.0	5	1.02 ± 0.038

centages correspond quite well to the ratio of the two groups of *P. riparius* females found from whole-body extracts. (+)-Females of both species contained a lot of pederin after they had ceased laying eggs (*P. fuscipes*: $\bar{X} \pm SE = 13.7 \pm 1.33$ μg, $N = 6$; *P. riparius*: $\bar{X} \pm SE = 16.5 \pm 1.06$ μg, $N = 5$), whereas (−)-females did not (Table 2). Consequently, those *P. riparius* females that had elevated amounts of pederin in their bodies but were not kept to lay eggs presumably were (+)-females (Figure 3a).

In order to determine whether a shortage of pederin in (−)-females may be the cause for eggs being laid without the amide, a (−)-female of *P. riparius* that had already laid 34 eggs was fed 10 eggs with pederin (about 11 μg) descended from a conspecific (+)-female (female 7 in Table 2). The next two eggs from this (−)-female, which were obtained on consecutive days three days after the pederin feeding, contained appreciable amounts of pederin, 0.7–0.8 μg each (Figure 4). This level equals that of (+)-females that transfer only limited quantities of pederin into their eggs. The amount of pederin transferred by the (−)-female dwindled until depletion of the resource (Figure 4). After her last egg (egg 61) the (−)-female had no pederin left in her body, and approximately 3.3 μg had been transferred into the eggs. Another (−)-female *P. fuscipes* that did not lay eggs after being fed with pederin contained about half of the pederin ingested five months later.

Pederin during Development of Offspring. The amount of pederin found in L1 matches that of the eggs and nearly nothing is left in the eggshell (only a trace in 200 eggshells of *P. riparius*). The same is true for L2 and exuviae. Furthermore, the descendants of the two types of females can be discerned in all developmental stages (Figure 5). Larvae, pupae, and young adults thus con-

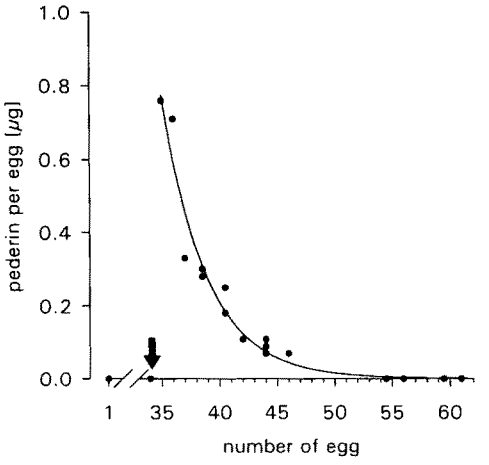


FIG. 4. Fluctuation of the amount of pederin transferred by a (−)-female of *Paederus riparius* to her eggs after feeding once with pederin (arrow). The curve shown [$P(x) = 1.005e^{-0.261x}$, x = (egg number −34)] is derived from the equation of a first-order process.

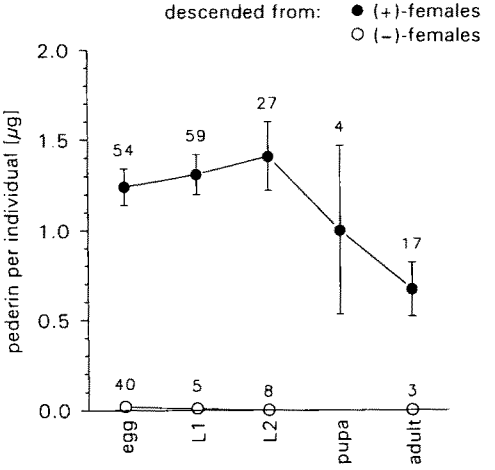


FIG. 5. Pederin in descendants of (+)-females and (−)-females of *Paederus riparius* [$\bar{X}$ with 95% confidence interval, which is too small to be visible in the offspring of (−)-females; N above] in all developmental stages and young adults (at most 1 week old). To reduce the excessive number of eggs analyzed of (+)-females, these are represented only when the developing larva was not yet visible through the chorion.

tain pederin if they are descended from (+)-females. In contrast, the offspring of (−)-females have no pederin and clearly cannot compensate for this deficiency by *de novo* biosynthesis. The possibility exists that larvae descended from (+)-females might synthesize pederin on their own, which would be obscured because (+)-females (*P. riparius* only) endow their eggs with significantly different amounts of pederin (ANOVA: $F = 6.81$, $df = 25, 145$, $P < 0.0001$). Analysis of the data with the H test, however, did not reveal any differences among eggs, L1, and L2 ($H = 4.36$, $df = 2$, $P = 0.113$), while significant differences arise if pupae and young adults are considered as well ($H = 38.71$, $df = 4$, $P < 0.0001$). The larvae do not increase their pederin content, whereas the decrease in pupae and adults (Figure 5) is confirmed.

Pederin in Maturing Adults. Shortly after eclosion most adults have less pederin than originally received with the egg. In all males and some of the females of *P. fuscipes* and *P. riparius*, the quantity of pederin does not increase over time. Only in some females is pederin accumulated, reaching 19.4 μg in *P. fuscipes* and 15.5 μg in *P. riparius* (Figure 6). These quantities are close to those of females collected from the field. After living 8.5 months, a female of *P. riparius* laid eggs that contained 1.38 ± 0.120 μg pederin per egg ($\bar{X}$ ± SE, $N = 5$) and was thus a (+)-female. With respect to the offspring of (+)-females only, the next generation of females is split into (+)-females that accumulate

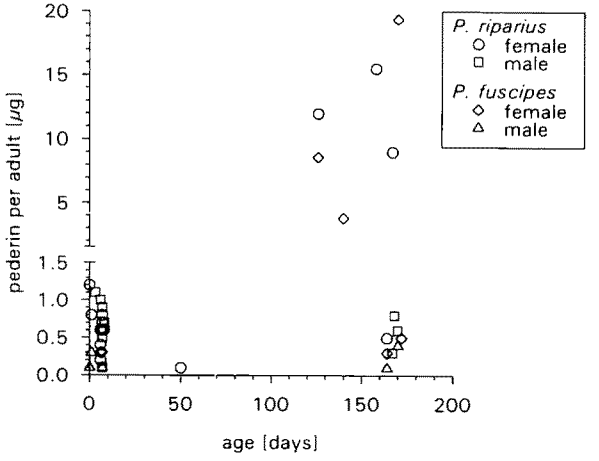


FIG. 6. The amount of pederin in *Paederus fuscipes* and *P. riparius* after imaginal eclosion. All specimens are descended from (+)-females and had not reproduced prior to analysis.

pederin and transfer it into their eggs and presumptive (−)-females that obviously are not able to biosynthesize that substance (Figure 6). Females analyzed after more than 100 days (*P. fuscipes*: $N = 5$, *P. riparius*: $N = 4$) are each descended from different (+)-females. Unfortunately, we did not obtain enough females descended from (−)-female to keep them several months in order to quantify their pederin content at an advanced age.

Pederin Ingested. If specimens are allowed to feed on conspecifics containing pederin, adults as well as larvae sequester the amide. Storage of the toxin is accomplished very efficiently, so that the amount of pederin found in (−)-females that do not lay eggs and in males is still elevated considerably several months after the feeding. The intake of adults collected from the field cannot be analyzed quantitatively, however, because the pederin content at the starting point of the feeding is not known well enough. In feeding trials conducted during rearing of *P. riparius* the pederin content of larvae, pupae, and adults was greatly increased, depending on the amount of pederin ingested during the larval stages (Figure 7). Freshly hatched males and females thus can have an amount of pederin that matches that of older (+)-females. The slope of the regression line in Figure 7 shows that on average 60% of the maternal and ingested amount of pederin could be recovered after up to two molts.

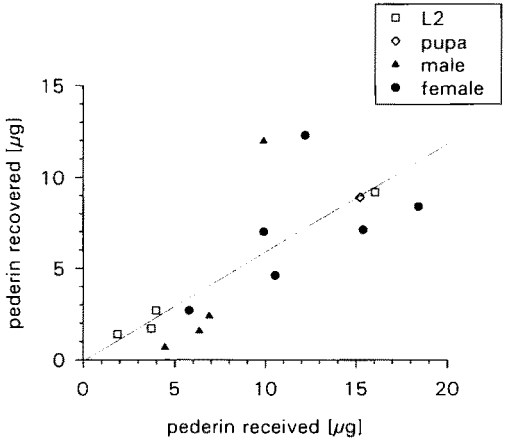


FIG. 7. Total pederin recovered from various stadia of *Paederus riparius* (adults at most 1 week old) that were fed with pederin (usually as L2) during larval development (regression line: $y = -0.08 + 0.60x$). The quantity received is the sum of maternally transferred and ingested pederin.

DISCUSSION

Using the microanalytical technique developed in the course of this study, it was possible for the first time to quantify the amount of pederin present in individual specimens of *Paederus*. Data obtained by this method are only partly identical with those summarized by Pavan (1975). Males of both *P. fuscipes* and *P. riparius* possess relatively little pederin, as expected, but females containing as much as 15–20 μg pederin are not exceptional. At least 85% of the females have much more of the amide than males, and a higher pederin content of females is the rule rather than an exception. More important, however, is the finding that females can be classified into (+)-females and (−)-females. This differentiation is suggested by their pederin content, but only becomes clear if the pederin content of the eggs laid is considered. The two types of females might be confused if only adults were studied. This kind of entanglement may be the reason for the vague descriptions in the literature.

Offspring of (+)-females and (−)-females are different with regard to their pederin content throughout all developmental stages. When individuals are kept apart so that they cannot prey on each other, no accumulation of the amide could be determined in larvae and pupae descended from both types of females, or in males descended from (+)-females. Although biosynthetic capabilities for pederin might be obscured by unknown losses balancing the gain, it would be quite astonishing that larval growth is not reflected in an increase of the actual amount of pederin, whether retarded or not, if larvae were able to produce the amide. Pupae are about 18 times heavier than eggs, and growth is accompanied with a marked dilution of pederin that is not compensated. Larvae are able to sequester pederin if it is contained in the diet. (+)-Females are the source of pederin present in the offspring. This is corroborated by some females reared in the laboratory that were able to increase their pederin content well before laying eggs. They clearly biosynthesized the substance on their own. They were analyzed four to five months after eclosion, but the first eggs were obtained four months later, indicating that the increase of pederin is not linked to egg production. Biosynthesis must occur during the egg-laying period, however, because most females lay 50 eggs but do not contain the amount of pederin needed for all of it at the same time. (+)-Females are not depleted of pederin in course of laying eggs; (−)-females, however, are not able to synthesize the substance and thus cannot produce eggs with pederin. Small amounts transferred into their first eggs may be derived from a maternal donation or from conspecifics eaten in the field. Likewise, transfer from the gut to the ovaries is not hindered if they have access to extraneous sources of pederin during the egg laying period. Only then are (−)-females able to lay eggs with appreciable amounts of pederin, so that the distinction between (+)- and (−)-females is disrupted temporarily.

The amount of pederin in whole-body extracts of females collected from the field might not be related in every case to the two types of females. On the one hand, females with little pederin include (−)-females and also young (+)-females that have not accumulated much of the toxin. In this study this effect was minimized by collecting most of the females analyzed during spring, when there are no young females because the adults hibernate (Horion, 1965). Females containing much pederin might include (−)-females that had eaten conspecifics with pederin and had not lost the pederin through egg-laying. Cannibalism probably is not common in the field, and the pederin content of females appropriately gives evidence of a pederin polymorphism with regard to the two types of females.

The polymorphism of females might be explained by genetic differences between (+)-females and (−)-females. The biosynthesis of pederin is not known in detail but is classified rudimentarily as a kind of polyketide synthesis (Cardani et al., 1973). If part of the population is heterozygous with regard to the genes necessary for synthesis of pederin, these may recombine to give homozygous specimens without the genes in question. The presumptive (−)-females that are descended from (+)-females but do not accumulate pederin during several months of adulthood suggest this possibility. Polymorphism for cyanogenesis in *Trifolium repens*, for example, is known to be controlled by two genes (Jones, 1972), and plants with and without the ability to release hydrogen cyanide (HCN) coexist within the same field (Dirzo and Harper, 1982).

The capability for synthesizing pederin is apparently confined to the (+)-females of *Paederus*. Cantharidin, the toxin that was formerly confounded with pederin, is synthesized by female as well as male and larval oedemerid beetles (Holz et al., 1994), whereas in meloid beetles only the larvae and males produce the substance. Meloid females get cantharidin from their mates and then transfer it into their eggs (Sierra et al., 1976; Carrel et al., 1993). The biosynthesis of a defensive chemical may occur in all stages or in only some of them, and the substance may be transferred intraspecifically. Offspring without pederin, however, may not be as fit for life as those with the toxin because all adults collected from the field had some pederin. Pederin may deter significant arthropod predators from larvae containing the toxin (Kellner and Dettner, unpublished data). For adults pederin may not be as necessary as for the soft-bodied offspring. Males do not synthesize the toxin and eat conspecifics only rarely because their pederin content was always low. The pederin content of young adults of both sexes was clearly lower than that of the larvae. Adults thus might reduce their amount of pederin either by decomposition or by discharge.

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STRUCTURE–ACTIVITY RELATIONSHIPS OF CYCLOPENTANE ANALOGS OF JASMONIC ACID FOR INDUCED RESPONSES OF CANOLA SEEDLINGS, *Brassica napus* L.

ROBERT BODNARYK[1,*] and TERUHIKO YOSHIHARA[2]

[1]*Agriculture and Agri-Food Canada*
Winnipeg Research Centre
Winnipeg, Manitoba, R3T 2M9 Canada
[2]*Department of Bioscience and Chemistry*
Faculty of Agriculture, Hokkaido University
Sapporo 060 Japan

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Abstract—Jasmonic acid (JA) has potent activity in enhancing cotyledon toughness and stimulating the biosynthesis of 3-indolymethyl glucosinolate in seedlings of canola, *Brassica napus* L. Structure–activity relationships among cyclopentane analogs of JA revealed that maximum activity in both systems was achieved when an acetyl side chain (or a methylated acetyl side chain) occurred at the C-1 ring position, an *n*-pentenyl side chain at the C-2 ring position, and a keto group at the C-3 ring position. Although coronatine and coronafacic acid both possess a cyclopentane ring with a keto group at the C-3 position, only coronatine was active in *B. napus* seedlings. Coronatine, a chlorosis-inducing toxin essential to the infectivity of pathovars of *Pseudomonas syringae*, acted as a complete molecular mimic of JA and had the same stimulatory effect on specific indole glucosinolates in *Brassica* species, thereby casting doubt on the hypothesis that indole glucosinolates serve in bacterial pathogen defense. Similarities and differences for structural requirements for activity among several diverse physiological systems affected by jasmonates likely reflect species-, tissue-, and developmental-specific differences.

Key Words—Jasmonate, *Brassica napus*, canola, toughness, glucosinolate, structure–activity, analogue, defense.

*To whom correspondence should be addressed.

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INTRODUCTION

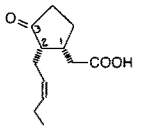
Wounding or feeding by flea beetles, *Phyllotreta cruciferae* Goeze, induces many striking changes in the physicochemical properties of the seedlings of canola, *Brassica napus* L. (Bodnaryk, 1992, 1994; Bodnaryk and Rymerson, 1994). Some of the changes, such as increased cotyledon toughness, may serve to defend the seedling against subsequent insect attacks. Other effects of wounding, such as the stimulation of indole glucosinolate biosynthesis, may be involved in defense against invading pathogens, a hypothesis that has not been adequately tested (see review by Bennett and Wallsgrove, 1994).

Jasmonates mimic many of the effects of wounding and insect feeding in *B. napus* seedlings (Bodnaryk, 1994; Bodnaryk and Rymerson, 1994) and may act as natural mediators of signal transduction during wounding and pathogen infection (Farmer and Ryan, 1990, 1992; Farmer et al., 1992; Gundlach et al., 1992; Enyedi et al., 1992; Creelman et al., 1992; Albrecht et al., 1993). Structural features of jasmonates that are required for biological activity are of interest because the various actions of jasmonates may be mediated by specific receptor(s). Chemical moieties of jasmonates important in the induction of expression of plant defense genes have been determined for protease inhibitor genes in potato (Ishikawa et al., 1994) and tomato (Feys et al., 1994). In this research, we have used various cyclopentane compounds related to jasmonic acid to investigate structure–activity relationships for two induced responses in seedlings of *B. napus*, namely the stimulation of indole glucosinolate biosynthesis (Bodnaryk, 1994) and the enhancement of cotyledon toughness (Bodnaryk and Rymerson, 1994).

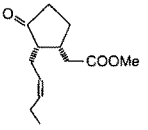
METHODS AND MATERIALS

Plants. Seed of the canolas *B. napus* cv. Legend and *B. rapa* cv. Parkland and the mustards *B. juncea* cv. Cutlass and *Sinapis alba* cv. Gisilba was obtained from commerical suppliers. The seeds were planted in a mixture of sunshine potting mix, Vermiculite, and Perlite (2 : 1 : 1) in 175-ml styrofoam coffee cups with a 0.5-cm hole punched in the bottom and subirrigated with Hoagland's nutrient medium. Seedlings received 240 μmol/sec/m^2 of photosynthetically active radiation from wide-spectrum fluorescent tubes in a plant growth room maintained at 20 $\pm$ 1°C, 60–70% relative humidity, and 18L : 6D photoperiod. Experiments used 6-day-old seedlings randomly assigned to control and treatment groups, which were analysed 24hr after-treatment.

Treatment of Seedlings. The jasmonates and cyclopentane analogs in Figure 1 were first dissolved in methanol and then diluted 50-fold with 0.01% Triton X-100 to a final concentration of 0.5 mM. Cotyledons were treated topically



Jasmonic acid



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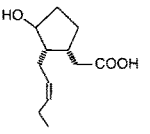
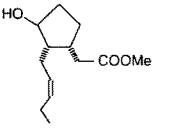
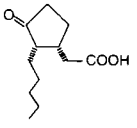


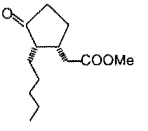
Cucurbic acid



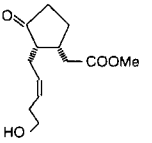
Methyl Cucurbic acid



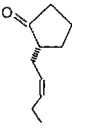
Dihydrojasmonic acid



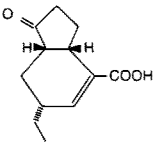
Methyldihydrojasmonic acid



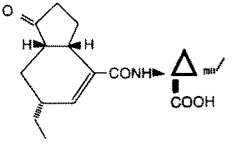
Methyl tuberonic acid



2-[(z)-2-pentenyl]-2-cyclopentene-1-one



Coronofacic acid



Coronatine

FIG. 1. Structure of jasmonic acid and some cyclopentane analogs tested for their ability to alter cotyledon toughness and glucosinolate biosynthesis in seedlings of canola, *B. napus*.

with a single dose of 5 μl applied to the periphery of the upper surface of a cotyledon. Controls received 5 μl of the methanol–0.01% Triton X-100 solvent alone.

Extraction and Quantitation of Glucosinolates. Cotyledons (2 g per replicate; four to six replicates) were cut from seedlings and extracted in boiling 80% methanol. Glucosinolates in partially purified extracts were bound to a column of DEAE-Sephadex A-25, desulfated with arysulfatase (Sigma), and eluted from the column for separation by HPLC on a 250-mm LiChrosphere 100 RP-18 (10 μm) column (Merck) with monitoring at 227 nm. Glucosinolates were quantified by comparison of peak areas with an internal standard of *o*-nitrophenyl-β-D-galactopyranoside (Sigma) (Bodnaryk and Palaniswamy, 1990; Bodnaryk, 1992).

Toughness. Relative cotyledon toughness in treated and untreated seedlings was expressed as the weight in grams needed to drive a polished stainless steel rod 3 mm in diameter through the larger of the two cotyledons, as measured in a block punchameter (Bodnaryk and Rymerson, 1994). A minimum of 15 seedlings were used for each treatment.

Chemicals. Allyl glucosinolate was from Sigma. Other glucosinolates (*p*-hydroxybenzyl, 3-indolylmethyl, 4-hydroxy-3-indolymethyl) were isolated from natural sources (Hanley et al., 1983). Coronatine and coronafacic acid were a gift from Robin Mitchell, The Horticulture and Food Research Institute of New Zealand, Auckland, New Zealand. The remaining jasmonates and cyclopentanes were as described by Ishikawa et al. (1994).

RESULTS AND DISCUSSION

Jasmonic acid (Figure 1) enhanced cotyledon toughness and stimulated the biosynthesis of 3-indolylmethyl glucosinolate in cotyledons of *B. napus* (Table 1). The acetyl side chain at the C-1 position of the cyclopentane ring of jasmonic acid seems essential for activity since its elimination in 2-[(*Z*)-2-pentenyl]-2-cyclopentene-1-one (Figure 1) abolished activity in both assays (Table 1). The acetyl side chain, however, can be methylated (methyl jasmonate, Figure 1) without any significant loss of activity (Table 1). The double bond in the *n*-pentenyl group at the C-2 position of the cyclopentane ring of jasmonic acid is important for activity, since its elimination in dihydrojasmonic acid and methyl

TABLE 1. EFFECT OF TREATING SEEDINGS OF CANOLA, *Brassica napus*, WITH JASMONIC ACID AND VARIOUS CYCLOPENTANE ANALOGS ON COTYLEDON TOUGHNESS AND 3-INDOLYLMETHYL GLUCOSINOLATE CONCENTRATION

Treatment	Cotyledon response	
	Toughness g (N = 15)	3-indolylmethyl glucosinolate (nmol/g wet wt)
Untreated	72.4 ± 4.8a[a]	200 ± 15a
Solvent only	70.9 ± 5.4a	187 ± 12a
Jasmonic acid	95.6 ± 9.7b	1304 ± 350b
Methyl jasmonic acid	92.1 ± 10.0b	1378 ± 309b
Dihydrojasmonic acid	80.7 ± 9.2c	389 ± 13c
Methyl dihydrojasmonic acid	78.8 ± 9.4c	434 ± 38c
Cucurbic acid	85.9 ± 9.0c	437 ± 34c
Methyl cucurbic acid	74.8 ± 6.5a	468 ± 19c
Methyl tuberonic acid	78.9 ± 6.3c	378 ± 93c
2-[(Z)-2-pentenyl]-2-cyclopentene-1-one	71.5 ± 6.6a	159 ± 35a
Coronafacic acid	75.9 ± 11.5a	332 ± 82c
Coronatine	90.4 ± 8.1b	1447 ± 293b

[a]Values are means ± SD obtained 24 hr after treatment. Means in the same column followed by the same letter do not differ (ANOVA, Student-Newman-Keuls test, $P < 0.05$).

dihydrojasmonic acid significantly reduced activities in both assays. Lengthening the *n*-pentenyl group with a hydroxy group in methyl tuberonic acid also significantly reduced activity in both assays. The keto group at the C-3 position of the cyclopentane ring was important for activity, since its replacement by a hydroxy group in cucurbic acid and methyl cucurbic acid significantly reduced activities in both assays. Coronatine, which has some structural resemblance to jasmonic acid in that both have a cyclopentane ring with a keto group at C-3, was as potent as jasmonic acid in both assays. Coronafacic acid, which lacks the conjugated amino acid of coronatine, had greatly reduced activity in stimulating 3-indolylmethyl glucosinolate biosynthesis and no activity in enhancing cotyledon toughness.

Coronatine acted as a molecular mimic of jasmonic acid and had the same effect on specific glucosinolates in the canola and mustard species (Table 2). Like jasmonic acid, coronatine stimulated the accumulation of 3-indolylmethyl glucosinolate, but not 4-hydroxy-3-indolylmethyl glucosinolate in *B. napus*; both stimulated the accumulation of 4-hydroxy-3-indolylmethyl glucosinolate, but not 3-indolylmethyl glucosinolate in *B. rapa*. Coronatine and JA stimulated the accumulation of 4-hydroxy-3-indolylmethyl glucosinolate and 3-indolylmethyl glucosinolate in *B. juncea*, and neither had any effect on the aromatic

TABLE 2. EFFECT OF TREATING SEEDLINGS OF MUSTARDS, *Brassica juncea* AND *Sinapis alba*, AND CANOLAS, *B. napus* AND *B. rapa*, WITH JASMONIC ACID OR CORONATINE ON CONCENTRATION OF ALIPHATIC, AROMATIC, OR INDOLE GLUCOSINOLATES IN THEIR COTYLEDONS

Glucosinolate (common name) (nmol/g wet wt)	Treatment		
	Control	Jasmonic Acid	Coronatine
Allyl glucosinolate (sinigrin)			
B. juncea cv. Cutlass	340 ± 40a[a]	366 ± 71a	554 ± 49b
p-Hydroxybenzyl glucosinolate (sinalbin)			
S. alba cv. Gisilba	1458 ± 78a	1485 ± 176a	1500 ± 248a
3-Indolylmethyl glucosinolate (glucobrassicin)			
B. napus cv. Legend	191 ± 2.2a	1373 ± 294b	1428 ± 350b
B. rapa cv. Parkland	24 ± 1.3a	23 ± 4.7a	29 ± 12a
B. juncea cv. Cutlass	9.5 ± 1.9a	24 ± 10b	144 ± 63c
4-Hydroxy-3-indolylmethyl glucosinolate (4-hydroxyglucobrassicin)			
B. napus cv. Legend	24 ± 6.5a	28 ± 5.1a	31 ± 11a
B. rapa cv. Parkland	18 ± 5.2a	155 ± 28b	358 ± 56c
B. juncea cv. Cutlass	5.0 ± 1.0a	9.8 ± 1.4b	18.6 ± 4.2c

[a] Values are means ± SD obtained 24 hr after treatment. Means in the same row followed by the same letter do not differ (ANOVA, Student-Newman-Keuls test, $P < 0.05$).

glucosinolate, sinalbin, in *S. alba*. Unlike jasmonic acid, which had no effect on the aliphatic glucosinolate sinigrin in *B. juncea*, coronatine had a relatively weak stimulatory effect (1.6×) on sinigrin (Table 2). Although coronatine appeared to act more powerfully than jasmonic acid on the indole glucosinolates (Table 2), our jasmonic acid was an unidentified mixture of isomers (only one of which is known to be active) and quantitative comparisons therefore are tenuous.

Similarities and differences exist for structural requirements for activity among several physiological systems affected by jasmonates (Yamane et al., 1980; Sembdner and Parthier, 1993; Ishikawa et al., 1994) and may reflect species-, tissue-, and developmental-specific differences. Similar requirements for chemical groups at the C-1, C-2, and C-3 positions of the cyclopentane ring of jasmonic acid were observed for enhancing cotyledon toughness and stimulating biosynthesis of 3-indolylmethyl glucosinolate in *B. napus* and for inducing the expression of proteinase inhibitor genes of potato (Ishikawa et al., 1994). Maximal activity in both systems was achieved when an acetyl side chain (or a methylated acetyl side chain) occurred at the C-1 ring position, an *n*-pentenyl side chain at the C-2 ring position, and a keto group at the C-3 ring position. Although coronatine and coronafacic acid both possess a cyclopentane ring with a keto group at the C-3 position, only coronatine is active in enhancing cotyledon toughness and stimulating biosynthesis of 3-indolylmethyl glucosinolate in *B. napus*, in stimulating hypertrophy of potato tuber tissue (Shiraishi et al., 1979), and causing chlorosis of bean leaves (Mitchell and Young, 1978). On the other hand, both coronatine and coronafacic acid are active in inducing systemic proteinase inhibitor activity in tomato, in stimulating anthocyanin and protein accumulation, and in inhibiting root growth in the cruciferous weed *Arabidopsis thaliana* (Feys et al., 1994).

The function of indole glucosinolates in seedlings and young plants in *Brassica* species is not well understood. Indole glucosinolate biosynthesis in the cotyledons of *B. napus* is nutritionally and developmentally controlled (Bodnaryk and Palaniswamy, 1990) and is strongly stimulated by flea beetle feeding, wounding, or jasmonates (Bodnaryk, 1992, 1994). However, indole glucosinolates have no antiinsect properties (Larsen et al., 1985; Weber et al., 1986; Bodnaryk and Palaniswamy, 1990; Birch et al., 1992), and it has been suggested that indole glucosinolates (Butcher et al., 1974; Rausch et al., 1983; Koritsas et al., 1989; Schnug and Ceynowa, 1990; Doughty et al., 1991; Stobbs et al., 1991; Spak et al., 1993a) and indole phytoalexins (Conn et al., 1988; Dahiya and Rimmer, 1988; Rouxel et al., 1990, 1991; Jejelowo et al., 1991; Browne et al., 1991; Tsuji et al., 1992) may participate in pathogen defense. It is difficult to reconcile participation of indole glucosinolates in pathogen defense with our finding that coronatine, a chlorosis-inducing toxin essential to the infectivity of pathovars of *Pseudomonas syringae* (Ichihara et al., 1977; Mitchell and Young,

1978; Mitchell et al., 1983; Bender et al., 1987; Greulich et al., 1993; Feys et al., 1994; Weiler et al., 1994), stimulates the biosynthesis of indole glucosinolates. Recent studies also cast doubt on the hypothesis that glucosinolates, at least in *B. napus*, participate in viral (Spak et al., 1993b) or bacterial pathogen defenses (Rawlinson et al., 1989; Mithen and Magrath, 1992; Nashaat and Rawlinson, 1994). The partial and complete mimics of jasmonic acid described here may be of use in probing and redefining indole glucosinolate function in crucifers.

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NEUTRAL COMPOUNDS FROM MALE CASTOREUM OF NORTH AMERICAN BEAVER, *Castor canadensis*

RONG TANG,[1] FRANCIS X. WEBSTER,[1] and DIETLAND MÜLLER-SCHWARZE[2,*]

[1]*Department of Chemistry*
[2]*Department of Biology*
State University of New York
College of Environmental Science and Forestry
Syracuse, New York 13210

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Abstract—North American beavers (*Castor canadensis*) mark their territories with castoreum, the strong-smelling paste in their castor sacs. In their own territories, beavers respond with scent marking to experimental scent marks that consist of strange castoreum (or selected components). In part, the unique odor of castoreum is due to large amounts of phenolic compounds and neutral compounds. Purified neutral compounds were analyzed by GC, GC-MS, and NMR; identities of the neutral compounds were confirmed by comparing the properties of authentic compounds with those of the isolated compounds. We identified 13 neutral compounds that had not been reported before for castoreum. Most of these are oxygen-containing monoterpenes. Of the nine neutral compounds reported by Lederer (1949), only three are confirmed in our analysis; the other six neutral compounds are either absent or are not volatile enough to be detected by our methods. Eight compounds—6-methyl-1-heptanol, 4,6-dimethyl-1-heptanol, isopinocamphone, pinocamphone, two linalool oxides, and their acetates—were synthesized for structure identification and bioassays.

Key Words—beaver, *Castor canadensis*, castoreum, neutral compounds, monoterpenes, co-injection, fractionation, identification, synthesis, territory marking.

INTRODUCTION

Castoreum is a strong-smelling brown paste contained in the paired castor sacs of both the Old and New World beaver (*Castor fiber* and *Castor canadensis*,

*To whom correspondence should be addressed.

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respectively). Distinguished from the castor sacs are a pair of anal glands, termed "oil sacs" by trappers. Castoreum consists of phenolic, neutral, basic, and acidic components.

As a result of several field studies in recent years, the biological role of castoreum in the social life of beaver is now better understood (Hodgdon, 1978; Bollinger, 1980; Müller-Schwarze and Heckman, 1980; Svendsen, 1980; Houlihan, 1989; Schulte et al., 1994). Beaver deposit castoreum and anal gland secretion on mud piles that they build at the banks of their ponds. The scent marks applied to these "scent mounds" are territorial signals (Houlihan, 1989). Furthermore, unoccupied beaver habitat is less likely to be colonized if it is scented with artificial scent mounds (Welsh and Müller-Schwarze, 1989).

Castoreum has been bioassayed in behavioral experiments several times. Resident beaver respond to experimental scent mounds by approaching, going on land, sniffing the mound, pawing it, and often marking the sample (Hodgdon, 1978; Bollinger, 1980; Müller-Schwarze and Heckman, 1980; Svendsen, 1980). Castoreum fractions also elicit these behavioral activities (Müller-Schwarze et al., 1986; Svendsen and Huntsman, 1988), but less so with progressive fractionation. The activity disappears completely well before the level of single compounds is reached (Müller-Schwarze et al., 1986).

Because bioassays of fractions led to a level of fractionation with no demonstrable biological activity, single compounds known to be constituents of castoreum and their mixtures were tested in subsequent experiments (Houlihan, 1989; Müller-Schwarze and Houlihan, 1990; Schulte et al., 1994). Several compounds were active, albeit at a very low level. The activity of mixtures was intermediate between castoreum and single compounds (Müller-Schwarze and Houlihan, 1990; Müller-Schwarz, 1992, Schulte et al., 1994).

This chemical study was prompted by these and other ongoing behavior investigations. It is imperative to know the composition of castoreum from the specific study populations and to bioassay any compounds hitherto unknown for castoreum.

The composition of castoreum has been investigated several times. Walbaum and Rosenthal (1927) first identified some phenolic and neutral compounds from castoreum; the neutral compounds were acetophenone, borneol, and benzyl alcohol. Later, Stevens (1943) separated some neutral red oil from dried beaver castors called American musk, but he did not report any further isolation work. Lederer (1946, 1949) identified nine neutral compounds from castoreum: benzyl alcohol, cholesterol, β-cholestanol, mannitol, *cis*-5-hydroxytetrahydroionol, borneol, acetophenone, 4-methoxyacetophenone, and cholesteryl oleate. Valenta et al. (1960) isolated *cis*-cyclohexane-1,2-diol from beaver castoreum. Maurer and Ohloff (1976) isolated nitrogen containing compounds from castoreum. Newly found phenolic components were reported in Tang et al. (1993).

The anal gland contains chemically very different compounds. Many C_{15}–C_{22} carboxylic acids and their waxy esters from the anal gland secretion were characterized by Grønneberg (1978) and Grønneberg and Lie (1984) using thin layer chromatography (TLC), gas chromatography (GC), and mass spectroscopy (MS). No further work concerning neutral compounds has been reported since.

We have reinvestigated the neutral compounds extracted from male castoreum. The compounds were isolated by packed column GC fractionation and analyzed by capillary GC, gas chromatography–mass spectrometry (GC-MS), capillary GC coinjection, nuclear magnetic resonance (NMR) spectrometry and by comparing the spectra of the unknown components with those of synthesized compounds. We identified 16 neutral compounds, 13 of which had not been known from castoreum.

METHODS AND MATERIALS

Chemicals. The solvents used for extraction and for flash column chromatography were reagent grade. Solvents used for capillary GC were HPLC grade. Anhydrous tetrahydrofuran (THF) was dried over 4 Å molecular sieves. Ethylene oxide was purchased from Eastman company, dried over sodium, and distilled before use. Linalool was purchased from Kodak. All other reagents were purchased from Aldrich Chemical Company.

Instrumentation. Analytical capillary GC was performed on a Varian 3700 gas chromatograph equipped with a splitless injector, fused silica column, and a flame ionization detector (FID). Standard capillary GC conditions for all identification work used the following temperature program: 40°C (3 min), 3°C/min, 210°C (30 min). Carrier and makeup gas for capillary GC work was nitrogen. Capillary GC analyses were carried out on one of two columns: (A) DB-1, 30 m × 0.25 mm; (b), FFAP column, 50 m × 0.25 mm.

Capillary GC coinjection was conducted by taking 0.5 ml of a fraction (5–10 ng/μl) and adding 1 μg of a standard to it; 2 μl of this mixture were injected onto the capillary GC. The unknown compounds were coinjected with the same standards on one or two capillary GC columns (one polar and one nonpolar), except for nojigiko alcohol and junenol because they are not commercially available and their syntheses are beyond the scope of this investigation.

Semipreparative-scale packed-column GC fractionations were performed on a Varian 2700 aerograph instrument. All GC fractions were collected in glass capillary tubes (3 mm × 30 cm) using a Brownlee-Silverstein thermal gradient collector (Brownlee and Silverstein, 1968). The purity of the isolated fractions was checked by capillary GC on column A. The pure fractions were then char-

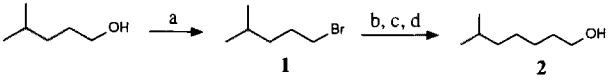
acterized by GC-MS and NMR. The following packed columns were used to purify the crude extract: column C, OV-101 (5%) on Chromasorb W, 3 m × 10 mm OD; column D, Carbowax 20 M (4%) on Chromasorb G, 6 m × 6 mm, temperature program: 100°C (0 min), 4°C/min, 230°C (5 min).

Mass spectra were obtained using a Finnigan 4500 automated gas chromatograph/EI-CI mass spectrometer system (GC-MS). All spectra were obtained at 70 eV. Proton NMR (^{1}H NMR) spectra were obtained using a Bruker AMX-300 (300 MHz). All spectra were run in $CDCl^3$ using the residual $CHCl_3$ protons as an internal reference. Carbon nuclear magnetic spectra (^{13}C NMR) were obtained using the Bruker AMX-300 instrument at 75.5 MHz in $CDCl_3$. All chemical shifts are given in ppm relative to tetramethylsilane.

Isolation and Identification. Sixteen frozen male castor sacs (970 g) were blended in a Waring Laboratory Blender for 2 min in methylene chloride (500 ml). The homogenate was filtered through a large Büchner funnel, and the solvent was removed by fractional distillation through a Vigreux column (17.5 cm) to prevent loss of the low boiling point fraction. The castoreum was treated with dilute sulfuric acid (200 ml of 2 N) and extracted with ether (3 × 200 ml) to isolate the neutral, acidic, and phenolic materials. The ether extract was washed with saturated sodium bicarbonate (3 × 130 ml) to remove the acidic compounds. The ether solution was then treated with enough sodium hydroxide to bring the pH to 12 (ca. 200 ml of 20%) in order to remove phenolic materials. The remaining ether solution was washed completely with brine, dried over anhydrous sodium sulfate, filtered, and the solvent removed by fractional distillation.

Synthetic Work: All compounds that were identified and not commercially available were synthesized except for nojigiko alcohol and junenol. Enough material was made not only to confirm our identification, but also for field bioassays with beaver.

6-Methyl-1-heptanol (2). 4-Methyl-1-pentanol (5.11 g, 50 mmol) and pyridine (0.5 g, 6 mmol) were dissolved in methylene chloride (8 ml), and freshly distilled phosphorus tribromide (13.5 g, 50 mmol) in methylene chloride (8 ml) was added dropwise over 30 min with stirring at −20°C under a nitrogen atmosphere (Scheme 1). After the addition was completed, the mixture was stirred for another hour at 0°C. The reaction mixture was treated with water



1 2

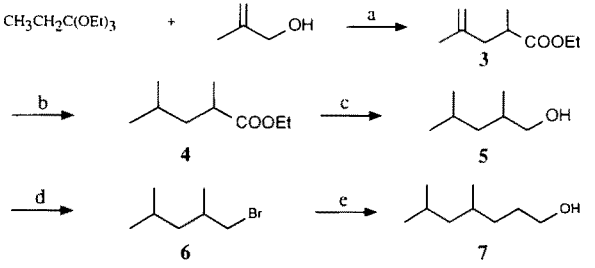
(a) PBr_3-pyridine, 1.5 hours, -20-0°C; (b) Mg, THF;
(c) ethylene oxide, -20°C; (d) HCl-H_2O.

SCHEME 1.

(5 ml), saturated sodium bicarbonate and brine, and dried over anhydrous sodium sulfate. The crude 1-bromo-4-methylpentane was purified by distillation (70.0–71.5°C/53 mm Hg) to yield **1** 2.30 g (28%).

Active magnesium powder (322 mg, 13.2 mmol) was placed in anhydrous THF (8 ml), and 1-bromo-4-methylpentane (2.20 g, 13.3 mmol) in anhydrous THF (16 ml) was slowly added. The reaction mixture was stirred for 4 hr at room temperature under nitrogen. Anhydrous cuprous iodide (250 mg, 1.3 mmol) was added after cooling to −10°C. Ethylene oxide (3 g, 68 mmol) was added through a tube with its end about 10 mm above the surface of the liquid (Dreger, 1941); the temperature was maintained at −20°C overnight. Water (3 ml) was added to the mixture, and the mixture was filtered through a Büchner funnel. The residue was rinsed with hexane (4 × 5 ml), and the combined organic phase was washed with dilute hydrochloric acid (3 N) and brine and dried over anhydrous sodium sulfate. The crude alcohol was purified by distillation (152–159°C) to yield 1.64 g (95%) of (2) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.67–3.63 (t, 2H, CH_2OH), 2.61 (s, 1H, OH), 1.58–1.48 (m, 3H, CH, CH_2), 1.38–1.29 (m, 4H, CH_2CH_2), 1.27–1.13 (m, 2H, CH_2), 0.86 (d, 6H, $CHMe_2$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 63.0, 38.9, 32.7, 27.9, 27.1, 25.9, 22.6.

4,6-Dimethylheptanol (7). Unsaturated ester (**3**) was prepared by Claisen rearrangement (Johnson et al., 1970) (Scheme 2). 2-Methyl-2-propen-1-ol



(a) CH_3CH_2COOH, heat; (b) H_2, Pd-C; (c) $LiAlH_4$;
(d) TsCl; LiBr-acetone; (e) Mg, THF; ethylene oxide, -20°C.

SCHEME 2.

(5.95g, 83 mmol) was added to six equivalents ethyl orthopropionate containing a few drops of propionic acid. The ethanol produced in the reaction was continuously removed by fractional distillation. The crude ester was purified by distillation to yield 12.0 g (93%) of compound **3**. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 4.68 (d, 2H, $=CH_2$), 4.08 (q, 2H, OCH_2CH_3), 2.69–2.30 (m, 2H,

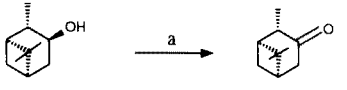
CH_2), 2.01 (q, 1H, CH), 1.67 (s, 3H, CH_3), 1.21 (t, 3H, OCH_2CH_3), 1.11 (d, 3H, CH_3). Catalytic hydrogenation (Augustine, 1965) of compound **3** in absolute ethanol over Pd-C at room temperature yielded 11.3 g (86%) of 2,4-dimethylvalerate **4**. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 4.08 (q, 2H, OCH_2CH_3), 2.49 (q, 2H, CH_2), 1.64–1.51 (m, 2H, 2CH), 1.21 (t, 3H, OCH_2CH_3), 1.17 (d, 3H, CH_3), 0.88 (d, 3H, CH_3), 0.87 (d, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 177.2, 60.0, 43.1, 37.7, 25.8, 22.3, 17.7, 14.3. The saturated ester **4** was added to 0.6 equivalent $LiAlH_4$ suspended in dry ether (Moffett, 1963). After stirring for 2 hr at room temperature, the mixture was quenched with H_2O–15% NaOH–H_2O. The precipitate was filtered and rinsed with ether. The combined organic solution was washed with brine and dried over anhydrous sodium sulfate. After removal of the solvent, the crude product was distilled to yield 7.37 g (89%) of 2,4-dimethyl-1-pentanol. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.51–3.44 (m, 2H, OCH_2), 1.74 (bs, 1H, OH), 1.75–1.56 (m, 1H, CH), 1.23–1.13 (m, 1H, CH), 1.04–0.95 (m, 2H, CH_2), 0.88 (d, 3H, CH_3), 0.87 (d, 6H, $2CH_3$). ^{13}C NMR (75.5 MHz, $CDCL_3$): δ (ppm) 68.6, 42.3, 33.5, 25.4, 23.9, 22.1, 17.0.

The alcohol (**5**) (7.37 g, 63 mmol) was added to 1.3 equivalent toluenesufonyl chloride in CH_2Cl_2 and pyridine at 0°C (Marvel and Sekera, 1955). The mixture was kept in a refrigerator overnight. The crystals were filtered and rinsed with hexane. The combined organic phase was washed with cold water, 2 N HCl, saturated $NaHCO_3$, and brine and dried over anhydrous sodium sulfate. After removal of solvent, the tosylate was added to three equivalents of LiBr in acetone and stirred overnight at room temperature (Wilt et al., 1970). After removal of the solvent, the syrup was extracted with hexane, and the organic phase was washed with water and brine, and dried over anhydrous sodium sulfate. After removal of the solvent, the crude product was purified by distillation to yield 10.1 g (94%), of 1-bromo-2,4-dimethylpentane (**6**) as a colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.41–3.24 (m, 2H, $BrCH_2$), 1.96–1.78 (m, 1H, CH), 1.73–1.54 (m, 1H, CH), 1.33–1.22 (m, 1H, CH_2), 1.15–1.01 (m, 1H, CH_2), 0.97 (d, 3H, CH_3), 0.88 (d, 3H, CH_3), 0.87 (d, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 44.3, 41.8, 32.9, 25.2, 23.0, 22.3, 18.8.

Active magnesium powder (312 mg, 12.8 mmol) was placed into anhydrous THF (7 ml), and 1-bromo-2,4-dimethylpentane (2.01 g, 11.2 mmol) in anhydrous THF (14 ml) was slowly added to it. The reaction mixture was stirred for 4 hr at room temperature under nitrogen. The solution was cooled to −10°C and anhydrous cuprous iodide (200 mg, 1.2 mmol) was added. Ethylene oxide (0.57 g, 13.3 mmol) was added through a tube whose end was about 10 mm above the surface of the liquid; the temperature was maintained at −20°C overnight. Water (3 ml) was added to the mixture, and the solution was filtered through a Büchner funnel. The residue was rinsed with hexane (4 × 5 ml), and

the combined organic phase was washed with dilute hydrochloric acid (3 N) and brine and dried over anhydrous sodium sulfate. The crude alcohol was purified by distillation (146–150°C) to yield 1.25 g (87%) of **7** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.63 (t, 2H, CH_2OH), 1.69–1.42 (m, 4H, 2CH, CH_2), 1.39–1.27 (m, 2H, CH_2), 1.20–1.07 (m, 2H, CH_2), 1.06–0.89 (s, 1H, OH), 0.89–0.83 (sextet, 9H, $3CH_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 63.5, 46.7, 33.3, 30.3, 30.2, 25.3, 23.4, 22.3, 19.7.

Isopinocamphone (8). Pyridinium chlorochromate (18 g, 84 mmol was dissolved in methylene chloride (100 ml), and (1*S*, 2*S*, 3*S*, 5*R*)-(+)-isopinocampheol (4.63 g, 30 mmol) in methylene chloride (15 ml) was added at room temperature (Corey and Suggs, 1975). After the addition was completed, the reaction mixture was stirred for 2 hr and diluted with anhydrous ether (60 ml).



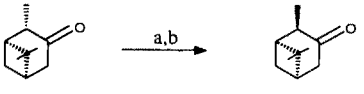
8

(a) PCC-CH_2Cl_2

SCHEME 3.

The solution was decanted, and the black residue was washed twice with ether. The combined organic solution was filtered through a short column packed with Florisil. The solvent was removed by rotary evaporator. The residual oil was purified by distillation (59.5–60.5°C/1.75 mm Hg) to yield 2.98 g (65%) of **8** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 2.56–2.46 (m, 2H, CHC=O, CH_2C=O), 2.37–2.28 (m, 2H, 2CH), 2.05–1.86 (m, CH_2), 1.19 (s, 3H, CH_3), 1.08 (d, 3H, CH_3), 0.76 (s, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 214, 51.2, 45.0, 44.8, 39.0, 34.4, 27.5, 21.8, 16.7.

Pinocamphone (9). Sodium (0.20 g, 8.7 mmol) was dissolved in absolute ethanol (5 ml), and the solution was cooled to 0°C; isopinocamphone (**8**) (1 g, 6.5 mmol) was then added quickly (Scheme 4). The reaction mixture was stirred overnight and neutralized with dilute hydrochloric acid (3 N). The crude product



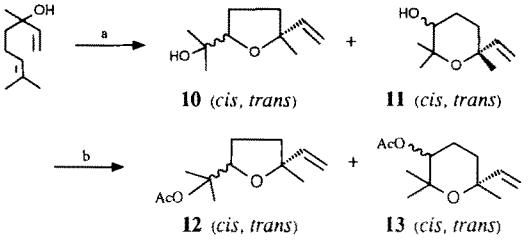
8 **9**

(a) NaOEt-EtOH; (b) H_2O-HCl

SCHEME 4.

(**9**) and starting material (**8**) were taken up in ether, and the organic phase was washed with brine and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by separation by flash chromatography (hexane–EtOAc, 10:1) yielded 0.39 g (40%) of **9** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 2.63–2.57 (m, 2H, $CH_2C{=}O$), 2.45–2.29 (m, 3H, $CH_2C{=}O$, CH), 2.10–1.84 (m, 2H, CH_2), 1.28 (s, 3H, CH_3, H-9), 1.05 (d, 3H, CH_3, H-10), 0.84 (s, 3H, CH_3, H-9), ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 216, 51.2, 46.3, 44.3, 39.3, 38.8, 29.5, 26.4, 20.0, 15.5.

*Oxides of linalool (**10** and **11**) and their acetates (**12** and **13**).* Our synthesis (Scheme 5) was based on that of Klein et al. (1964) for the oxidation of linalool.



(a) MCPBA-CH_2Cl_2; (b) CH_3COCl-DMAP-pyridine-CH_2Cl_2.

SCHEME 5.

m-Chloroperbenzoic acid (MCPBA, 4.3 g, 25 mmol) was dissolved in CH_2Cl_2 (30 ml) and cooled to 0°C; linalool (3.1 g, 20 mmol) was added dropwise over 30 min with stirring at 0°C. After the addition was completed, the mixture was stirred for 5 hr at 0°C, then stirred overnight at room temperature. The mixture was filtered through a Büchner funnel, and the filtrate was treated with aqueous NaOH (10%, 3 × 10 ml), washed with brine (3 × 10 ml), and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by flash chromatography (hexane–EtOAc, 10:1) yielded 1.61 g (47%) of **10** and 0.36 g (11%) of **11**. Separation of isomers of **10** by packed column gas chromatography (column D) yielded *cis*- and *trans*-isomers of linalool oxide. *trans*-Linalool oxide: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 5.87–5.76 (four d, 1H, =CH), 5.16–5.09 (d t, 1H, $=CH_2$), 4.95–4.90 (d t, 1H, $=CH_2$), 3.76–3.70 (t, 1H, CHOH), 2.27 (s, 1H, OH), 1.88–1.61 (m, 4H, CH_2), 1.25 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.07 (s, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 143.7, 112.0, 86.1, 83.0, 72.0, 37.7, 27.0, 26.3, 25.8, 24.1. *cis*-Linalool oxide: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 5.99–5.89 (q, 1H, =CH), 5.19–5.12 (d d, 1H, $=CH_2$), 5.01–4.95 (d d, 1H, $=CH_2$), 3.83 (t, 1H, CHOH), 2.10 (s, 1H, OH), 1.92–1.71 (m, 4H, CH_2), 1.28 (s, 3H, CH_3), 1.20 (s, 3H,

CH_3), 1.10 (s, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 144.9, 112.0, 85.6, 82.8, 71.9, 37.7, 27.3, 26.4, 25.9, 24.2. Mixture **11** was not separated but was directly converted to its acetate.

Freshly distilled acetyl chloride (117.8 mg, 1.50 mmol) was added to pyridine (240 mg, 3 mmol) and 4-dimethylaminopyridine (DMAP, 5 mg, 0.04 mmol) in 1.5 ml CH_2Cl_2. The solution was cooled to −20°C and oxide **10** or **11** (120 mg, 0.71 mmol in 0.5 ml CH_2Cl_2) was added. The reaction mixture was kept at 0°C for 3 hr and at room temperature overnight. The mixture was cooled to −20°C, the supernatant decanted, and the precipitate washed with 0.5 ml CH_2Cl_2. The combined organic phases were treated with water (1 ml), hydrochloric acid (3 N 2 × 0.5 ml), and brine (2 × 0.5 ml) and dried over anhydrous sodium sulfate. The crude ester was purified by gas chromatography (packed column D) to yield 80 mg (53%) of acetate **12** and **13**. ^1H NMR of **12**: δ (ppm) 4.11-4.03 [q, 1H, −O−CH−C(Me)$_2$−OAc]. ^1H NMR of **13**: δ (ppm) 4.62-4.57 [q, 1H, −O−C(Me)$_2$−CHOAc].

RESULTS

Our reinvestigation of the neutral compounds in beaver castoreum led us to identify the 16 neutral compounds listed in Tables 1 and 2. Of these 16 compounds, only three had previously been reported as constituents of castoreum. Table 1 lists the characterization methods and structures of the oxygen-containing monoterpenes. Of these nine monoterpenes, only borneol was previously known as a constituent of castoreum (Walbaum and Rosenthal, 1927). Table 2 lists the characterization methods and structures of other neutral compounds. Among these seven compounds, acetophenone and benzyl alcohol had been reported as constituents of castoreum before (Walbaum and Rosenthal, 1927; Lederer, 1946, 1949). The remaining five—two aromatic compounds, two branched aliphatic alcohols, and a sesquiterpene—were found in castoreum for the first time.

Table 3 gives the average amounts (in milligrams) of the various neutral compounds found in one beaver castor sacs (gland), termed one gland equivalent (GE). The quantity refers to the amount of each individual neutral compound per castor gland. The determination of GE is as follows: The most abundant neutral compound, borneol, was isolated and weighed directly. The gland equivalents of the remaining neutral compounds were obtained by comparing the GC peak areas of each compound to that of borneol.

Of the nine neutral compounds reported by Lederer as being constituents of castoreum, only three were found in this study: benzyl alcohol, acetophenone, and borneol. The other six neutral compounds reported by Lederer, but not found in our neutral fractions are cholesterol, β-cholestanol, mannitol, choles-

TABLE 1. NEUTRAL COMPOUNDS IN CASTOREUM OF BEAVER (*Castor canadensis*): OXYGEN-CONTAINING MONOTERPENES

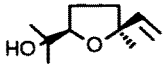
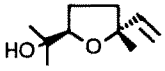
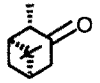
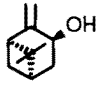
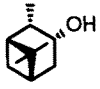
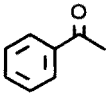
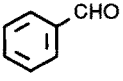
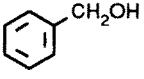
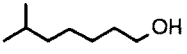
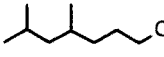
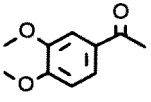
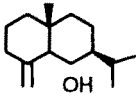
Name	Structure	Co-injection		Isolated and confirmed by						
				GC-MS		NMR				
		DB-1	FFAP	CI	EI	^{1}H	^{13}C	COSY	Hetcor	Synthesis
cis-Linalool oxide		X	X	X	X	X	X			X
trans-Linalool oxide		X	X							X
Isopinocamphone		X	X	X	X	X	X			X
trans-Pinocarveol		X	X	X	X	X	X			
Pinocamphone		X	X	X	X	X	X			X
Nojigiko alcohol				X	X	X	X	X	X	
Verbenone		X	X	X	X	X	X		X	
(1*R*)-Myrtenol		X	X	X	X	X	X			
Borneol		X	X	X	X	X	X			

TABLE 2. NEUTRAL COMPOUNDS IN CASTOREUM OF BEAVER (*Castor canadensis*): OTHERS

		Coinjection		Isolated and confirmed by						
				GC-MS		NMR				
Name	Structure	DB-1	FFAP	CI	EI	^{1}H	^{13}C	COSY	Hetcor	Synthesis
Acetophenone		X		X	X	X	X			
Benzaldehyde		X	X	X	X	X				
Benzyl alcohol		X	X	X	X	X				
6-Methyl 1-heptanol		X	X	X	X	X	X			X
4,6-Dimethyl 1-Heptanol		X		X	X	X	X			X
3,4-Dimethoxy Acetophenone		X	X	X	X	X	X			
Junenol				X	X	X	X	X	X	

terol oleate, *cis*-5-hydroxytetrahydronol, and 4-methoxyacetophenone. Except for 4-methoxyacetophenone, these compounds may not be volatile enough to be detected by our methods. The other possibility is that Lederer appears to have used a different species of beaver (*Castor fiber*), but his papers are not clear on this point.

Eight compounds—6-methyl-1-heptanol, 4,6-dimethyl-1-heptanol, iso-

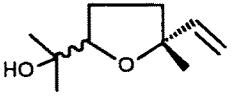
TABLE 3. AVERAGE AMOUNTS OF NEUTRAL COMPOUNDS FOUND IN ONE BEAVER CASTOR SAC

Name	Gland equivalent (mg)
Acetophenone	0.18
Benzaldehyde	0.65
Benzyl alcohol	2.01
6-Methyl-1 heptanol	0.35
4,6-Dimethyl-1-heptanol	0.53
3,4-Dimethoxyacetophenone	1.20
Junenol	1.03
trans-Linalool oxide	0.45
cis-Linalool oxide	0.46
Isopinocamphone	0.35
trans-Pinocarveol	1.52
Pinocamphone	0.42
Nojigiko alcohol	1.19
Verbenone	3.33
(1*R*)-Myrtenol	0.29
Borneol	16.9

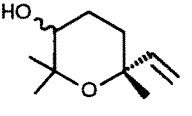
pinocamphone, pinocamphone, and two linalool oxides and their acetates—were synthesized as described under Methods and Materials.

DISCUSSION

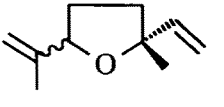
Many of the neutral castoreum constituents occur in plants or in excretions of mammals. Linalool oxides have been isolated from *Cryptocaria aschersoniana* oil of southern Brazil (Naves et al., 1963). Even though they have been used as flavor and perfume agents for some time, the first definite structure was not established until 1963 (Felix et al., 1963). They first oxidized (±)-linalool with monoperoxyphthalic acid yielding two pairs of diastereometric oxides, $C_{10}H_{18}O_2$ (**9**: *cis*, *trans* and **10**: *cis*, *trans*).



9 (*cis*, *trans*)



10 (*cis*, *trans*)



13 (*cis*, *trans*)

Furans **9** are the major products. The structures of **9** (*cis*, *trans*) were deduced by conversion of **9** to its benzoate, which was pyrolyzed at 180–220°C to give a mixture of two doubly unsaturated ethers (**13**). IR analysis showed the presence of a vinyl group and a methylene double bond; ^{1}H NMR analysis gives a multiplet at δ (ppm) 4.2–4.3, which is a proton in the environment of C=C−CH−O. Therefore, Felix et al. named these tetrahydrofuran derivatives "linalool oxide," which was accepted by the *Handbook of Terpenoids* (Dev et al., 1982) and many others. However, there are some differences in the ^{1}H NMR data of our isolated compounds with those reported by Felix et al. This may be due to different instruments or solvent effects. Pure **9** (*cis*, *trans*) and **10** (*cis*, *trans*) are not commercially available. To determine whether our isolated compounds are furan-type or pyran-type compounds, a modified method was employed to convert linalool into its oxides, which were separated into two mixtures, **9** and **10**, by flash chromatography. These two mixtures, **9** and **10**, were converted to acetates **11** and **12**, respectively. Comparing the ^{1}H NMR data of the alcohols and their esters, the peak at δ (ppm) 3.83–3.78 (t) in alcohol **9** shifts to δ (ppm) 4.10–4.03 (q) in ester **11**, while the peak at δ (ppm) 3.44–3.38 (t) in alcohol **10** shifts to δ (ppm) 4.62–4.54 (q) in ester **12**. The former small difference is caused by a field effect of the OAc group; the latter big difference is caused by a scalar effect of the OAc group (Duddeck and Dietrich, 1989). In summary, this experiment gives another proof that **9** (*cis*, *trans*) are tetrahydrofuran derivatives, and **10** (*cis*, *trans*) are tetrahydropyran derivatives.

A further separation of **9** (*cis*, *trans*) on column D gave pure *cis*- and *trans*-linalool oxides, whose ^{1}H NMR data and ^{13}C NMR data coincide with our isolated compounds. In the *cis*-isomer, the proton attached to the C=C was deshielded by the *cis*-OH group and its chemical shift is further downfield than that of the *trans*-isomer.

Isopinocamphone is a major constituent of the essential oil from the mint, *Mentha aquatica* L. found in southern Europe (Shimizu et al., 1966). The compound was prepared from isopinocampheol by oxidation with CrO_3 (Zweifel and Brown, 1964). In our method, we employed the more effective reagent pyridium chlorochromate. The chemical shifts of three characterizing methyl groups (C_8, C_9, and C_{10}) (Erskin and Knight, 1960) are in good agreement with our isolated and synthetic samples.

(−)-Pinocamphone can constitute up to 45% of the oil of *Hyssopus officinialis* L. (Guenther, 1949). Pinocamphone was synthesized from α-pinene by treatment with perphthalic acid and acidic alumina (Nigam and Levi, 1968). The epimerization of isopinocamphone to pinocamphone was employed in our work (Zweifel and Brown, 1964). The equilibrium favors pinocamphone. The reported ^{1}H NMR spectrum (Erskin and Knight, 1960) is identical with our isolated and synthetic compounds.

trans-Pinocarveol has been found in Spanish eucalyptus, *E. globulus* (Schmidt, 1944). The ^{1}H NMR spectrum of the synthetic compound (Jashi et al., 1968; Heikman et al., 1968) is the same as that from our isolated fraction.

Nojigiku alcohol and its acetate are major components of the essential oil of *Chrysanthemum japonense* (Uchio, 1977). The ^{1}H NMR spectrum (Uchio, 1977) is identical with our isolated fraction, and the ^{13}C NMR data (Darby et al., 1978) agree with our sample.

Verbenone (**14**) occurs in small amounts in *Verbena* and various other species (Kerschbaum, 1900). The ^{1}H NMR spectrum of synthetic verbenone (Bates and Thalacker, 1988) is identical with that of our isolated sample.

Both (+)- and (−)-myrtenol have been isolated from the essential oil of *Cyperus africalatus* L. in West Africa by vacuum fractionation (Couchman et al., 1964). The ^{1}H NMR of myrtenol (Heikman et al., 1968) is nearly the same as that from our isolated fraction. The only difference is that the peak of CH_2OH is reported at 3.89 ppm (a mutiplet in Heikman's paper), and in our spectrum it is at 3.98 ppm, doublet (J = 1.7 Hz). This split is caused by long-range olefinic proton coupling.

No natural source of the two branched alcohols was found in the literature. The first synthesis of 6-methyl-1-heptanol was reported by Levene and Allen (1916). They treated 4-methylpentyl iodide with malonic ester and reduced the resulting ester with Na-EtOH to give the above alcohol. This method requires more steps than ours. The ^{1}H NMR and ^{13}C NMR data of the synthesized 6-methyl-1-heptanol agree with authetic data (Sadtlar, 1980, 1981). The synthesis of 4,6-dimethyl-1-heptanol has also been reported (S. Julia, et al., 1961, M. Julia, et al., 1966). In Julia's work (1961), the starting material, 1,1-diethoxy-1-cyclopropylethane, was converted to 1,3-dimethyl 1-cyclopropyl-1-butanol in six steps. The homoallylic rearrangement was accomplished by treatment of the alcohol with HBr. The resulting unsaturated bromide was converted to 4,6-dimethyl-1-heptanol by catalytic hydrogenation. In our work, the starting material, 2,4-dimethylpentyl bromide, was prepared from triethyl orthopropionate and 2-methylallyl alcohol via Claisen rearrangement and three subsequent steps. Our synthetic route is short and the yield is good.

Junenol is an eudesmane class sesquiterpene. A dextrorotatory alcohol, junenol was first isolated from the essential oil of juniper berries (Herout et al., 1954). The first total synthesis of (±)-junenol was completed by Schwarz et al. (1972). From our isolated fraction, we acquired ^{1}H NMR, ^{13}C NMR, HETCOR, and HH COSY (Duddeck and Dietrich, 1989). From the NMR data, the structure of our isolated compound was deduced as junenol. The ^{1}H NMR of the isolated compound [(300 MHz, CDC_3): δ (ppm) 4.96 (d, 1H), 4.66 (d, 1H), 3.65 (t, 1H), 2.35–1.25 (m, 14H), 0.96 (d, 3H), 0.87 (s, 3H), 0.73 (s, 3H)] agrees with Schwartz's data (Schwartz et al., 1972). The ^{13}C NMR data are not

available in the literature. The ^{13}C NMR (75.7 MHz, $CDCl_3$) of our sample: δ (ppm) 148.3, 106.3, 67.2, 58.3, 49.8, 41.9, 40.4, 37.9, 37.6, 26.8, 24.0, 21.1, 18.4, 17.5, 16.1.

A number of the described compounds are known as detoxification products in the urine of various herbivores and also of carnivores. For instance, in rabbits *trans*-pinocarveol is derived from β-pinene and myrtenol is a metabolite of α-pinene. In rabbits and brushtail possums, verbenol is formed from α-pinene. Acetophenone occurs in the urine of dogs and cats that have been fed cinnamic acid. Benzyl alcohol is excreted by dogs and cats that had been given benzyl acetate. In rabbits, borneol is a metabolite of camphor. Benzaldehyde was found in rat hepatocytes as a metabolite of benzylamine. These numerous studies are summarized by Scheline (1991). The beaver appears to sequester metabolites of secondary plant compounds in its castor sac.

Our purpose for identifying the neutral constituents from beaver castoreum was to determine what role these and other compounds, such as phenolics and basics from beaver scent marks, play in chemical communication. Beaver possibly use at least some of the neutral compounds for intraspecific communication via their scent marks. Several studies have compared beaver responses to whole castoreum and to its components. The neutral fraction released responses from beaver in experiments by Svendsen and Huntsman (1988). While some of the phenolic castoreum constituents have been shown to elicit responses from beaver (Müller-Schwarze and Houlihan, 1991, Schulte et al., 1994), only one neutral compound, borneol, has been bioassayed individually so far (Schulte et al., 1994). A mixture of 12 neutrals reported here released slight to moderate responses from beaver. The 12 tested neutral compounds were borneol, benzaldehyde, benzyl alcohol, 6-methyl-1-heptanol, *cis*- and *trans*-linalool oxide, 4,6-dimethylheptane-1-ol, *trans*-pinocarveol, isopinocamphone, verbenone, (1*R*)-myrtenol, and 3,4-dimethoxyacetophenone. A fivefold higher concentration of the same mixture rendered it more active. Finally, a mixture of these 12 neutrals and 14 phenolics proved to be almost as active as whole castoreum (Schulte et al., 1994). The beaver appear to recycle catabolites and detoxification products by storing them in their castor sacs for scent marking when needed. This added benefit may offset the cost of detoxification.

Having identified a series of neutral compounds, we can now analyze individual scent mounds and castoreum from specific individuals on a routine basis. The chemical identifications reported here have opened the door for selecting and manipulating specific compounds in castoreum. In our experiments, borneol released scent-marking responses (Schulte et al. 1994). Castoreum composition profiles may be indicative of individuals, sex, age, colony membership, diet, population, or species. Knowledge of the precise pattern of compounds will permit analysis of genotypic and environmental influences on castoreum composition.

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AGGREGATION PHEROMONE OF *Carpophilus dimidiatus* (F.) (COLEOPTERA: NITIDULIDAE) AND RESPONSES TO *Carpophilus* PHEROMONES IN SOUTH CAROLINA[1]

ROBERT J. BARTELT,[2,*] DAVID K. WEAVER,[3,4] and RICHARD T. ARBOGAST[3,4]

[2]*Bioactive Constituents Unit, USDA Agricultural Research Service*
National Center for Agricultural Utilization Research
1815 N. University Street, Peoria, Illinois 61604

[3]*USDA Agricultural Research Service*
Stored-Product Insects Research and Development Laboratory
3401 Edwin Street, Savannah, Georgia 31405

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Abstract—The major component of the male-produced aggregation pheromone of *Carpophilus dimidiatus* (F.) is (3*E*, 5*E*, 7*E*, 9*E*)-6,8-diethyl-4-methyl-3,5,7,9-dodecatetraene. It attracts beetles of both sexes in the field and is synergized by odors from fermenting bread dough; mean trap catches for the tetraene alone, tetraene plus dough, dough alone, and control were 24.5, 48.3, 0.02, and 0.00, respectively. In the laboratory, individual males produced 0.58 μg ± 0.35 μg (SD) of the tetraene per day, but males in groups of 10–50 produced <2% as much per beetle. A second male-specific compound, (3*E*, 5*E*, 7*E*, 9*E*)-5,7-diethyl-9-methyl-3,5,7,9-tridecatetraene, was also identified from *C. dimidiatus* and is about 5% as abundant as the major pheromone component. *Carpophilus* flight activity was monitored for one year in South Carolina corn fields with the pheromones for *C. dimidiatus*, *C. freemani* Dobson, *C. mutilatus* Erichson, *C. hemipterus* (L.), *C. lugubris* Murray, and *C. obsoletus* Erichson, all in combination with bread dough. The first four of these species accounted for 18, 70, 5.7, and 0.03%, respectively, of the total *Carpophilus* trapped, but no *C. lugubris* or *C. obsoletus* were captured. Captures of *C. freemani* were as high as 11,400/trap/week. Species specificity

*To whom correspondence should be addressed.

[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the names by USDA implies no approval of the product to the exclusion of others that may also be suitable.

[4]Present address: USDA Agricultural Research Service, Insect Attractants, Behavior, and Basic Biology Research Laboratory, P.O. Box 14565, Gainesville, Florida 32604.

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for the first four pheromones was high, except that a synthetic impurity in the *C. dimidiatus* pheromone was somewhat attractive to *C. freemani* and *C. mutilatus*. Three other species captured, *C. antiquus* Melsheimer, *C. marginellus* Motschulsky, and *C. humeralis* (F.), accounted for 0.005, 5.0, and 1.3% of the total catch, respectively. *C. antiquus* was attracted primarily to the pheromone of *C. dimidiatus*, but *C. marginellus* and *C. humeralis* responded to most of the test pheromones. There were two major periods of *Carpophilus* flight activity: February through June and September through November.

Key Words—*Carpophilus* spp., *C. dimidiatus*, Coleoptera, Nitidulidae, pheromone, hydrocarbon, tetraene, trap, corn.

INTRODUCTION

The corn sap beetle, *Carpophilus dimidiatus* (F.), is a small (2 to 3-mm) dark brown nitidulid that occurs in tropical, subtropical, and warmer temperate regions throughout the world. In the United States it ranges north to North Carolina and northern California (Connell, 1991). It attacks a wide variety of agricultural commodities such as corn, peanuts, cacao, and various spices, either in storage or in the field (Connell, 1975). In the early literature, the name *C. dimidiatus* was often applied to all five members of a complex of very similar, sibling species occurring in North America. Dobson (1954, 1956) and Connell (1991) provided keys and descriptions that allowed *C. freemani* Dobson, *C. mutilatus* Erichson, *C. fumatus* Boheman, and *C. pilosellus* Motschulsky to be distinguished from *C. dimidiatus*. *C. dimidiatus*. Two of the sibling species, *C. freemani* and *C. mutilatus*, are very common in corn fields in South Carolina.

A goal of our project has been to understand pheromone chemistry and biology in nitidulid beetles so that synthetic pheromones can become tools for monitoring beetle populations and for preventing beetle-related damage (e.g., direct feeding injury or transmission of harmful microorganisms). Male-produced aggregation pheromones have been reported previously for a number of species, including *C. freemani* and *C. mutilatus* (Bartelt et al., 1990b, 1993a), but *C. dimidiatus* was unstudied in this way. We report here the identification of a male-produced pheromone from *C. dimidiatus* and verification of its activity under field conditions. We also present baseline information about the responses of *C. dimidiatus* and other *Carpophilus* species to various *Carpophilus* pheromones in South Carolina cornfields throughout the year.

METHODS AND MATERIALS

Pheromone Identification for C. dimidiatus. A culture of beetles was started from individuals captured 17 km southeast of Bamberg, Bamberg County, South Carolina. The beetles were reared on a wheat-germ and brewer's yeast diet as

described previously for *C. antiquus* Melsheimer (Bartelt et al., 1993b; Dowd and Weber, 1991).

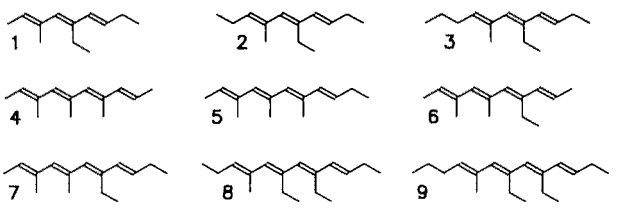
Bartelt et al. (1990a) described the system for collecting volatiles from feeding beetles. Beetle-produced and diet volatiles were collected on filters of Super-Q porous polymer (Alltech Associates, Deerfield, Illinois) and were eluted from the filters every one to three days with hexane. Initial collections were from groups of about 10 males or females on the artificial diet. Later collections were made from groups of 50 males or females and from individual males. Beetles were placed in volatile collectors within one week of emergence. All were in the volatile collectors for at least two weeks; one individual male was monitored for 3.5 months. Nonadecane (2.5 μg) was added to each collection as an internal standard for quantitation by GC.

Male- and female-derived collections were compared by capillary gas chromatography (DB-1 column, 15-m length, 1-μm film thickness, 0.25-mm ID; temperature programmed from 50°C to 250°C at 10°C/min), and GC retention indices relative to *n*-alkanes were calculated for sex-specific compounds. (Any sex-specific compounds would be considered as likely pheromone components.) The Hewlett Packard 5890 gas chromatograph was equipped with splitless injector, flame ionization detector, and Hewlett Packard 3396A integrator. Electron impact mass spectra were obtained for the detected sex-specific compounds on a Hewlett-Packard 5970 Mass Selective Detector with capillary GC inlet.

When it became evident that *C. dimidiatus* followed the pattern existing in other *Carpophilus* species (i.e., that the only detectable sex-specific compounds were hydrocarbons emitted by males), the male-derived hydrocarbons were analyzed further for any related, minor components because multiple component pheromones occur in other *Carpophilus species*. The 58 volatile collections from individual males were combined, and the constituents were separated by polarity on silica gel (Bartelt et al., 1990a). The hexane (hydrocarbon) fraction from this sample was examined in detail by GC and mass spectrometry for compounds analogous to the major, male-specific hydrocarbon.

Synthetic Compounds. The synthetic hydrocarbons used in this study are shown in Figure 1: (2*E*, 4*E*, 6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene (**1**), (3*E*, 5*E*, 7*E*)-6-ethyl-4-methyl-3,5,7-decatriene (**2**), (3*E*, 5*E*, 7*E*)-5-ethyl-7-methyl-3,5,7-undecatriene (**3**), (2*E*, 4*E*, 6*E*, 8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (**4**), (2*E*, 4*E*, 6*E*, 8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (**5**), (2*E*, 4*E*, 6*E*, 8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-decatetraene (**6**), (2*E*, 4*E*, 6*E*, 8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene (**7**), (3*E*, 5*E*, 7*E*, 9*E*)-6,8-diethyl-4-methyl-3,5,7,9-dodecatetraene (**8**), and (3*E*, 5*E*, 7*E*, 9*E*)-5,7-diethyl-9-methyl-3,5,7,9-tridecatetraene (**9**). Compounds **1**–**8** were synthesized previously and were available for the present study (Bartelt et al., 1990b,c, 1993b).

Compound **9** was prepared and purified in the same way as **8** (Bartelt et al., 1993b) except that (*E*)-2-methyl-2-hexenal, an intermediate in the synthesis of **3** (Bartelt et al., 1993a), was used as the starting material instead of (*E*)-2-



SEPTUM TYPE	COMPOUND							
	1	2	3	4	5	6	7	8
DIM		7:*						100
FRE	100:						4	
MUT		7:	100					
HEM				100:	31:	11:	8	
LUG					10:		100	
OBS					100			

FIG. 1. Structures of compounds involved in this study and assigned structure numbers (top). Composition of pheromone baits for six *Carpophilus* species (bottom). The abbreviations DIM, FRE, MUT, HEM, LUG, and OBS refer to septa for *C. dimidiatus*, *C. freemani*, *C. mutilatus*, *C. hemipterus*, *C. lugubris*, and *C. obsoletus*, respectively. Compositions are relative, by weight, with the most abundant (or only) component given as 100. For DIM, total weight of pheromone was 1.2 mg/septum; for all other baits, the total was 500 μg/septum. Compound **9** was not used in field baits. Asterisk: compound **2** in the DIM bait was an artifact of synthesis and was not intentionally added.

methyl-2-pentenal. The mass spectrum of **9** is given in Figure 2, and the following NMR proton spectrum was obtained (400 MHz, Bruker ARX-400 spectrometer, deuterobenzene solvent): δ 0.94, 3H, t, J = 7.4; δ 1.02, 3H, t, J = 7.4; δ 1.12, 3H, t, J = 7.5; δ 1.22, 3H, t, J = 7.5; δ 1.40, 2H, sext, J = 7.3; δ 1.79, 3H, br s; δ 2.05–2.15, 4H, apparent distorted sext (actually δ 2.08, 2H, q, J = 7.4; and δ 2.11, 2H, quin, J = 7.4); δ 2.42, 2H, q, J = 7.5; δ 2.60, 2H, q, J = 7.5; δ 5.58, 1H, br t, J = 7.3; δ 5.76, 1H, dt, J = 15.7, 6.6; δ 5.95, 1H, br s; δ 6.11, 1H, br s; δ 6.13, 1H, d, J = 15.7 (half obscured by δ 6.11 signal). Proton assignments are given in Figure 2 and are consistent with previously analyzed all-*E* tetraenes and trienes having partial structures in common with **9** (Bartelt et al., 1990b, 1992, 1993b). The identification and synthesis of compound **9** were not completed in time for field testing.

Baits for Field Tests. The compositions of the six pheromone types are presented in Figure 1 (Bartelt et al., 1990b, 1991, 1992, 1993a; Petroski et al.,

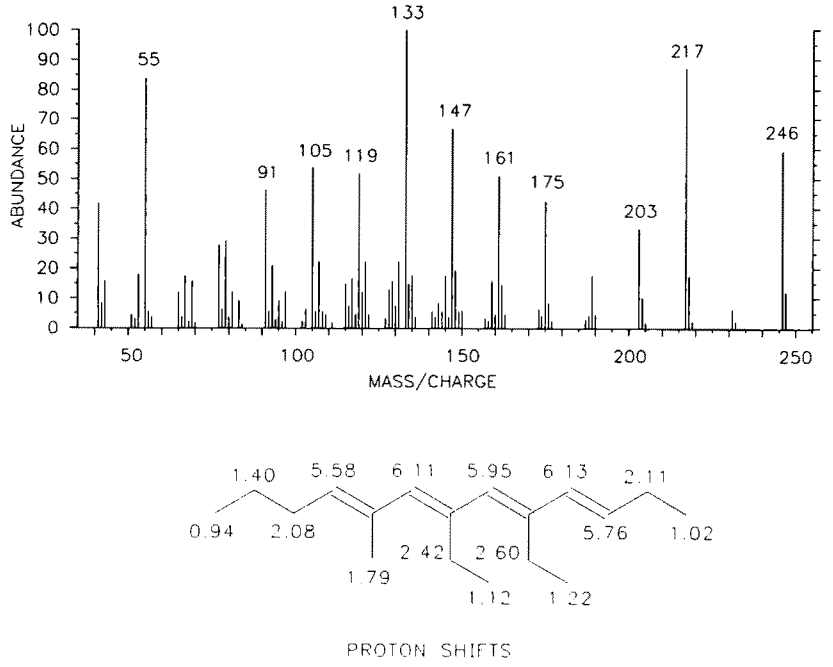


FIG. 2. Mass spectrum and assigned NMR proton shifts for tetraene **9.** Shifts are in parts per million from tetramethylsilane.

1994). Proportions were chosen so that emissions from septa would be as similar in composition as possible to those from beetles. For all species except *C. dimidiatus*, a total of 500 μg of all-*E* isomers was used per trap bait. The pheromone for *C. dimidiatus* was used at the rate of 1.2 mg/bait; this higher value was as in an earlier study (Bartelt et al., 1993b) and was chosen to compensate for the relatively low volatility of **8.** Each trap bait was prepared by adding the synthetic pheromone in 10 μl hexane, followed by 300 μl methylene chloride, to a rubber septum. After the chemicals had soaked in, the septa were stored in tightly closed bottles in a freezer until used.

The *C. dimidiatus* pheromone was intended to include only compound **8,** but compound **2** was also present due to an incomplete Wittig-Horner reaction during synthesis (Bartelt et al., 1993b). Compound **2** was removed from a sample of this synthetic mixture by reverse-phase HPLC (Serva Biochemicals 5-cm × 25-cm C_{18} column, 5-μm particle size, distributed by Crescent Chemical Co., Hauppauge, New York); the solvent was acetonitrile, and effluent was monitored with a refractive-index detector. Injection size was 100 mg; com-

pound **2** eluted 580–670 ml after injection, and compound **8** at 780–950 ml. The HPLC fractions were diluted with water and extracted with hexane to recover the hydrocarbons. Additional septa were prepared with the purified **8.**

Otherwise, technical grade synthetic components were used for all field baits (60–90%). Except for **2** as a contaminant of synthetic **8,** none of the synthetic compounds contained detectable levels of any other component shown in Figure 1. The impurities were almost exclusively *Z* isomers unavoidably produced during synthesis and could not be removed on the scale required for field baits. Bartelt et al. (1992) found that *Z* isomers of the major component of the *C. hemipterus* (L.) pheromone were inert or slightly attractive but not repellent.

Field Bioassays. The field experiments were conducted at two sites in South Carolina. The first (33°11′ north latitude, 80°55′ west longitude) was 17 km southeast of Bamberg, in Bamberg County, and the other (30°23′ north latitude, 81°18′ west longitude) was at Blackville, in Barnwell County. The traps (wind-oriented funnel traps; Dowd et al., 1992) were always placed at the edges of corn fields at the interface with native vegetation. The traps were attached to tree branches and suspended about 1.5 m above the ground. The experimental design was a randomized block. Traps within a block were separated by ca. 10 m. Each week, captured beetles were removed from the traps, fermenting whole-wheat bread dough (usually present as a coattractant) was replaced, and the traps were rerandomized within the blocks. Pheromone septa were replaced every two weeks. Trap catches were frozen to kill any remaining live beetles and were sent to NCAUR at Peoria for separation and counting by species (and in experiment 1, by sex). Species were identified primarily by using the characteristics described by Okumura and Savage (1974) and Connell (1991). Species identifications were confirmed by the USDA Systematic Entomology Laboratory at Beltsville, Maryland. The field bioassays consisted of three experiments.

Experiment 1 tested for the activity of the putative pheromone of *C. dimidiatus*, both with and without a food-related coattractant. The four treatments were synthetic *C. dimidiatus* pheromone (with impurity **2** present, Figure 1), the pheromone in combination with fermenting whole-wheat bread dough (15 ml/trap), bread dough alone, and unbaited controls. The trap design prevented incoming beetles from contacting any trap baits; the two components of combination baits (septum and dough) were physically separated from each other. The treatments were arranged in six blocks, four at the Bamberg County site and two at the Barnwell County site. The study ran for six weeks, from March 18 to April 29, 1993.

Experiment 2 was a year-long survey for *Carpophilus* flight activity at the study sites (May 5, 1993, to May 4, 1994). There were seven treatments; six were the synthetic pheromones for *C. dimidiatus* (with impurity **2**), *C. freemani*,

C. mutilatus, *C. hemipterus*, *C. lugubris* Murray, and *C. obsoletus* Erichson (Figure 1), each combined with bread dough, and the seventh treatment was the bread-dough control. The study was arranged in three blocks, two in Bamberg County and one in Barnwell County.

Experiment 3 explored the previously observed cross attraction of *C. freemani* and *C. mutilatus* to the pheromone of *C. dimidiatus* (with impurity **2**) and of *C. freemani* to the pheromone of *C. mutilatus*. The experiment was established by adding three additional treatments to all three blocks of experiment 2 during a 10-week period (October 13–December 22, 1993). These treatments were: compound **8** (1.2 mg) from which impurity **2** had been chromatographically removed plus dough; compound **2** (500 μg) plus dough; and compound **3** (500 μg) plus dough. Catches for these treatments were to be compared with those for four treatments of experiment 2: the pheromones for *C. dimidiatus* (containing **8** and impurity **2**), *C. freemani*, and *C. mutilatus* plus dough and the dough control.

For all field experiments, trap catches were transformed to the log $(x + 1)$ scale to stabilize variance and subjected to analysis of variance. Separation of means was by the least significant difference (LSD) method, and the probability level was 0.05.

Weather and Corn Phenology. The daily maximum and minimum temperatures and daily precipitation amounts at Bamberg during the field studies were obtained from the National Oceanic and Atmospheric Administration. The town of Bamberg is located about halfway between the two field sites. The times of certain events and stages for the corn crop were recorded: planting, silking, milk stage, dough stage, and harvest.

RESULTS

Pheromone of C. dimidiatus. Volatile collections from males on artificial diet consistently contained a compound (GC retention index = 1620) that was always lacking in collections from females on diet. The compound eluted from silica gel with hexane, indicating it had the polarity of a hydrocarbon. The compound was identical to synthetic compound **8** by both GC retention and mass spectrum. Compound **8** was previously identified as the aggregation pheromone of *C. antiquus* (Bartelt et al., 1993b).

The amount of compound **8** emitted from *C. dimidiatus* depended greatly on the number of males present in the collection flask. Individual males emitted a mean of 0.58 μg/day (±0.35 SD, $N = 58$ observations), but the mean was only 0.012 μg/male/day (±0.014 SD, $N = 8$) in collections from groups of 10–13 males and only 0.0011 μg/male/day (±0.0009 SD, $N = 15$) in collections from 50 males. Regardless of beetle density, emission began within two to five

days of placing the beetles into the volatile collectors. One individual was monitored for 3.5 months, and compound **8** was still being produced (0.5 μg/day) when the study was terminated.

Whenever tetraene **8** was prominent in the gas chromatogram of a volatile collection, a second male-specific peak was also observed (retention index = 1725; peak area 4.9% as large as that for **8** in the combined volatile collections from individuals). By mass spectrometry, the molecular weight was 246, which was consistent with an 18-carbon tetraene. Study of retention indices of various hydrocarbon analogs (Bartelt et al., 1990b, 1992) suggested that the new compound had structure **9**. Compound **9** was synthesized and was identical to the beetle-derived compound both in mass spectrum and GC retention.

Experiment 1. Synthetic pheromone for *C. dimidiatus* was highly attractive to this species in the field (Table 1). The activity of the pheromone was increased about two-fold by the presence of fermenting bread dough, but dough by itself was not attractive. The unbaited control caught no *C. dimidiatus*. Overall, 21,681 *C. dimidiatus* were captured.

Five other species responded and were significantly more attracted to the combination of *C. dimidiatus* pheromone plus dough than to dough by itself or to the unbaited control (Table 1). For *C. antiquus*, the combination and pheromone alone were equally attractive, but for *C. freemani*, *C. mutilatus*, *C. marginellus* Motschulsky, and *C. humeralis* (F.), synergism was evident, and the combination was clearly more attractive than the pheromone alone. Overall

TABLE 1. FIELD RESPONSES TO SYNTHETIC PHEROMONE FOR *C. dimidiatus* WITH AND WITHOUT COATTRACTANT, MARCH 18 TO APRIL 29, 1993

Species	Mean trap catch (N = 36)[a]				Percent females (%)[c]
	Pheromone + dough[b]	Pheromone	Dough	Control	
C. dimidiatus	48.3 a	24.5 b	0.02 c	0.00 c	69
C. antiquus	0.39 a	0.40 a	0.00 b	0.00 b	51
C. freemani	47.9 a	8.00 b	0.08 c	0.00 c	64
C. mutilatus	10.1 a	0.47 b	0.33 bc	0.00 c	73
C. marginellus	2.39 a	0.96 b	0.04 c	0.02 c	46
C. humeralis	0.15 a	0.02 ab	0.00 b	0.00 b	38

[a] In each row, means not accompanied by the same letter are significantly different (LSD, 0.05 level). Means were calculated in the analysis scale [log(x + 1)] but were converted back to the numerical scale for presentation.

[b] Synthetic pheromone for *C. dimidiatus* is shown in Figure 1 (DIM) and contains impurity **2**; dough is fermenting whole-wheat bread dough.

[c] Percentages are based on totals over all treatments.

totals for the latter five species were 59, 4764, 966, 383, and 13, respectively. Both sexes of all species were trapped.

Experiment 2. The overall effectiveness of the six synthetic pheromones is summarized in Figure 3. *C. dimidiatus*, *C. freemani*, *C. mutilatus*, and *C. hemipterus* responded significantly better to their pheromones (plus bread dough) than to the dough controls. The overall trap totals for these species during the one-year study were 41,500; 164,000; 13,300; and 75, respectively. The final two species for which pheromones were put out, *C. lugubris* and *C. obsoletus*, were not detected.

As in the first experiment, *C. antiquus* was also captured; nine of the 11 specimens were in traps baited with the *C. dimidiatus* pheromone, and the *C. lugubris* and *C. obsoletus* pheromones each caught one. In addition, *C. marginellus* and *C. humeralis*, for which pheromones are not yet known, were caught consistently (Figure 3); their overall totals were 11,700 and 2960, respectively. *C. marginellus* responded best to the *C. lugubris* and *C. freemani* pheromones, but only for the *C. mutilatus* pheromone was the response not significantly higher than the dough control. *C. humeralis* responded significantly to all six pheromones, but these responses were relatively moderate (in the range of only two to five times higher than the control).

Various degrees of cross-attraction occurred for *C. dimidiatus*, *C. freemani*, *C. mutilatus*, and *C. hemipterus* and their pheromones (Figure 3). Cross-attraction was least for *C. dimidiatus*, and the mean trap catch to an interspecific bait never exceeded 5% of that to its own pheromone. For *C. freemani*, cross-attraction to the *C. dimidiatus* and *C. mutilatus* pheromones was about 22% of the response to its own pheromone, but cross-attraction to the other pheromones was only 4–5%. For *C. mutilatus*, cross-attraction was greatest to the pheromone of *C. dimidiatus* (17% of the response to the pheromone of *C. mutilatus*). *C. hemipterus* was not significantly cross-attracted to the pheromones of the other three species but responded clearly to those of *C. lugubris* and *C. obsoletus*. The small numbers of *C. hemipterus* captured during the study made it difficult to assess relative attractiveness of baits.

Responses to Pheromones over Time. Trap catches for all six species were relatively high during autumn (September through November) and, for all species except *C. humeralis*, again during late winter and spring (late February through mid-June) (Figure 4). Few beetles were captured during the coldest part of winter (mid-December to mid-February) or during summer, when the corn crop was developing and ripening. Data for temperature, rainfall, and corn phenology are included in Figure 4 for reference. Harvest times for the Bamberg and Barnwell County fields (plots 1 and 2, respectively, Figure 4) differed by six weeks, but other events and stages for the two fields were synchronized to within a week.

Experiment 3. The synthetic pheromone for *C. dimidiatus*, which contained

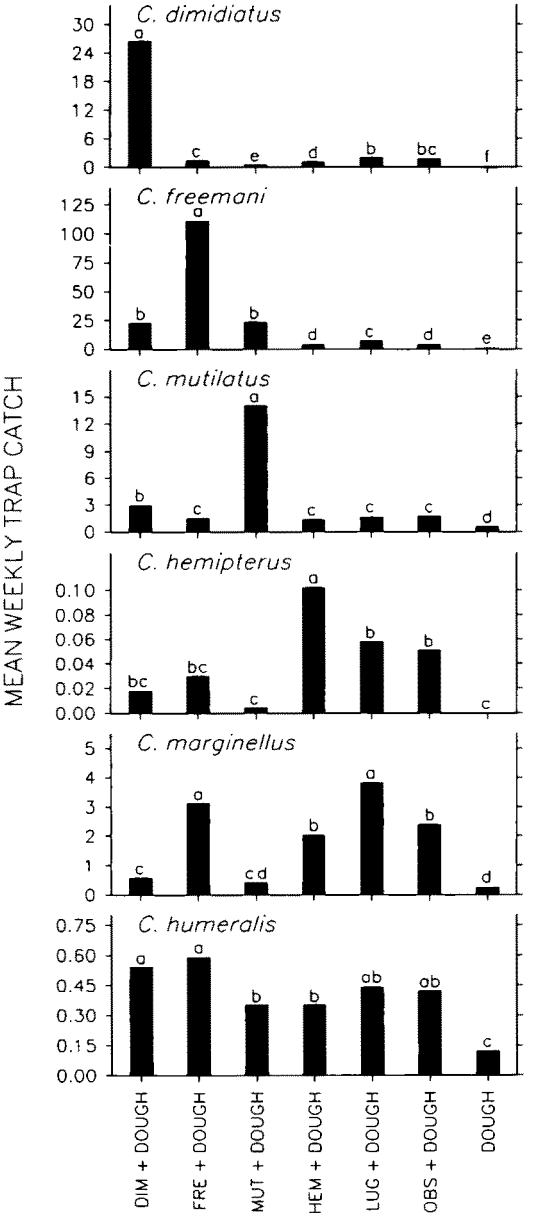


FIG. 3. Mean weekly captures for six *Carpophilus* species by traps baited with synthetic pheromones plus fermenting dough (N = 156). The study period was May 5, 1993, to May 4, 1994. Within each panel, means accompanied by the same letter were not significantly different (LSD, 0.05 level).

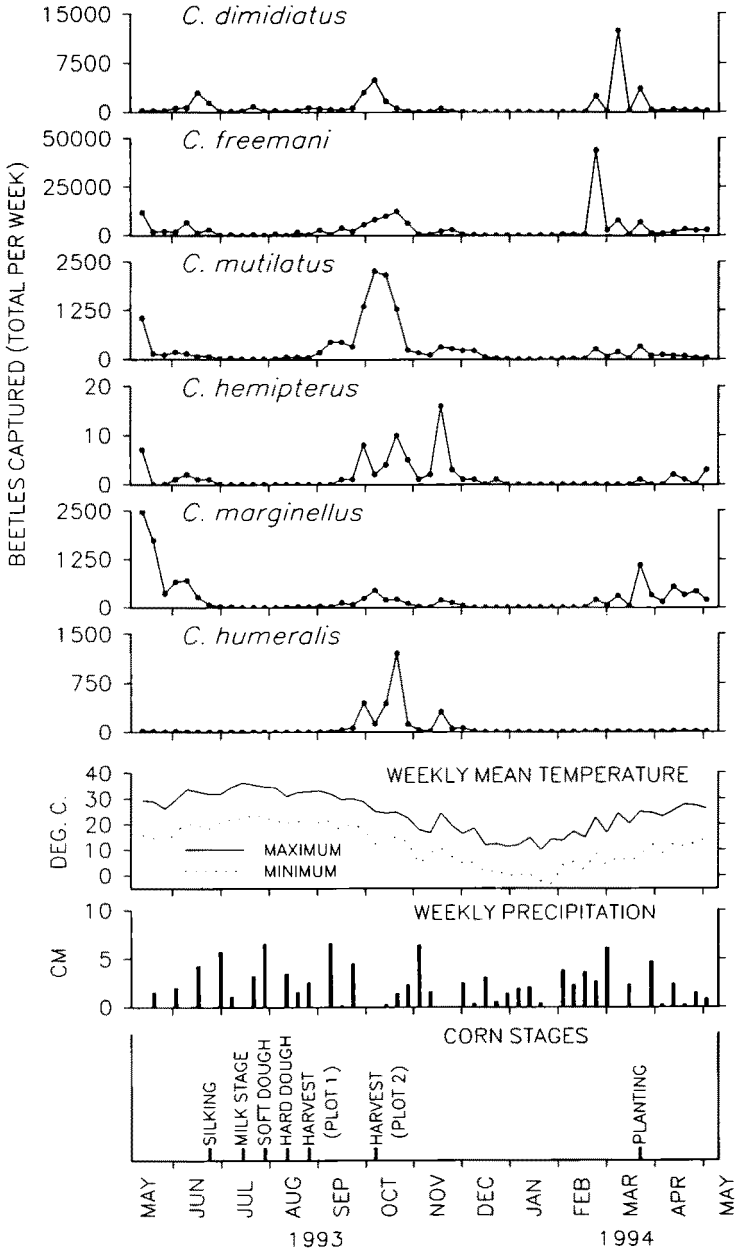


FIG. 4. Weekly trap catches for six *Carpophilus* species, totaled over all treatments, and data for temperature, precipitation, and corn phenology.

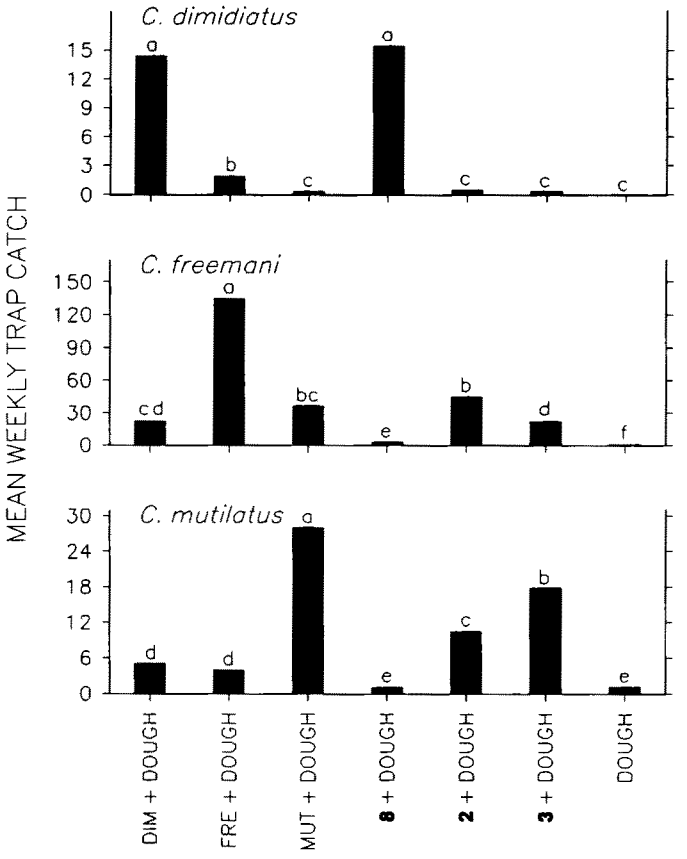


FIG. 5 Field responses of the three most abundant *Carpophilus* species to their synthetic pheromones and to various single components ($N = 30$). Study period was October 13 to December 22, 1994. Within each panel, means accompanied by the same letter were not significantly different (LSD, 0.05 level).

compound **8** and impurity **2**, and chromatographically purified compound **8** were equivalent for attracting *C. dimidiatus* in the field (Figure 5). However, responses of *C. freemani* and *C. mutilatus* to the *C. dimidiatus* pheromone decreased markedly when impurity **2** was removed. Correspondingly, compound **2** was active by itself for both *C. freemani* and for *C. mutilatus* but not for *C. dimidiatus*. *C. mutilatus* responded clearly to the separate components of its pheromone (**2** and **3**), but the standard synthetic blend, which contains both components, was most attractive. For *C. freemani*, compound **3** was less active

than **2** but still attracted significantly more beetles than the control. Neither **2** nor **3** was added to the synthetic bait for *C. freemani*; see Discussion.

DISCUSSION

Pheromone of C. dimidiatus. The major pheromone component of *C. dimidiatus* is concluded to be (3*E*, 5*E*, 7*E*, 9*E*)-6,8-diethyl-4-methyl-3,5,7,9-dodecatetraene (compound **8**). The same compound was previously identified as the pheromone of *C. antiquus* (Bartelt et al. 1993b). The compound by itself was highly attractive in the field, but trap catches nearly doubled when fermenting whole-wheat bread dough was added to the trap as a coattractant; the dough was not attractive by itself. It is typical for *Carpophilus* pheromones to be synergized by volatiles from a food source such as fermenting dough or fruit (Bartelt et al., 1994; Blumberg et al., 1993). The major volatiles from fermenting bread dough were reported by Lin and Phelan (1991).

A second male-specific compound, (3*E*, 5*E*, 7*E*, 9*E*)-5,7-dimethyl-9-methyl-3,5,7,9-tridecatetraene (**9**), is about 5% as abundant as **8** in male-derived volatiles and may serve as an additional pheromone component. Compound **9** is a novel structure. Synthetic material was not available before the field trials ended, and its activity remains unmeasured.

The pheromone emission rate per male decreased greatly as the number of males in the pheromone collector increased. A similar reduction was reported for *C. davidsoni* Dobson (Bartelt and James, 1994) and *C. obsoletus* (Petroski et al., 1994). In nature, such a reduction would help to limit the size of forming aggregations; otherwise, the largest aggregations would be most attractive to flying beetles and would continue to grow still larger. The mechanism controlling emission rate is unstudied.

Relationships among C. dimidiatus, C. freemani, *and* C. mutilatus. *C. dimidiatus* and its two sibling species, *C. freemani* and *C. mutilatus*, were the nitidulids captured in greatest numbers, and each demonstrated a great deal of specificity for its own pheromone. The differences in pheromone chemistry must contribute to their successful coexistence in the same habitat. The significant cross-attraction of *C. freemani* and *C. mutilatus* to the synthetic *C. dimidiatus* pheromone noted in experiments 1 and 2 was primarily an artifact of synthesis; when compound **2**, which *C. dimidiatus* does not make, was removed from the *C. dimidiatus* pheromone for experiment 3, captures of *C. freemani* and *C. mutilatus* were reduced essentially to control levels.

That *C. mutilatus* would respond to **2** was expected because its aggregation pheromone contains **2** (Bartelt et al., 1993a); however, compound **2** was less attractive than an equal weight of compound **3**, the major pheromone component of *C. mutilatus*, and both compounds alone were less attractive than an equal

weight of the appropriate mixture of compounds **2** and **3** (Figure 5). A greater response of *C. mutilatus* to a mixture than to individual components was not noted in earlier wind-tunnel tests (Bartelt et al., 1993a).

Compound **2** is also a minor component of the pheromone of *C. freemani* (Bartelt et al., 1990b). It did not enhance the attractiveness of the main component **1**, in earlier wind-tunnel tests (Bartelt et al., 1990b); therefore, **2** has been left out of the "standard" field bait. Compound **3** also occurs to a very minor extent in emissions from *C. freemani*. It was not active in initial wind-tunnel tests (Bartelt et al., 1990b), but it was slightly active in the field (Figure 5). The modest attraction of *C. freemani* to the baits for *C. mutilatus* apparently has a biological basis, but the *C. mutilatus* baits are still far less attractive than the baits prepared specifically for *C. freemani* (containing **1** and **7**). The present results also suggest that an even more potent bait for *C. freemani* could be developed if more of the known minor components were included.

Other Relationships among Species. *C. dimidiatus* and *C. antiquus* share the same pheromone chemistry (except for possible minor components), but conflicts in chemical communication for these species appear to be avoided primarily by geographic separation. *C. antiquus* has a more northern distribution than *C. dimidiatus* (Connell, 1991; Bartelt, et al., 1993b and references therein). South Carolina may represent a transition zone, south of which only *C. dimidiatus* would be found.

C. dimidiatus did not exhibit the obvious cross-attraction to the *C. lugubris* pheromone that was previously demonstrated for *C. antiquus* (Bartelt et al., 1993b; Williams et al., 1995). In the midwestern United States, *C. antiquus* appears to use the *C. lugubris* pheromone as a kairomone, locating suitable host sites (e.g., corn ears) previously colonized by *C. lugubris*. There was no indication that *C. dimidiatus* exploits another species in this way.

The absence of *C. lugubris* in this study was surprising because it is common in the eastern states, being the most abundant *Carpophilus* species in Delaware and having been reported in the eastern states from New Jersey south to South Carolina (Connell, 1956). The species occurs widely in North, Central, and South America (Williams et al., 1983).

C. hemipterus was caught in very low numbers, but cross-attraction to the pheromones of *C. lugubris* and *C. obsoletus* was especially clear. This is not surprising because these pheromones contain one or both of compounds **5** and **7**, which are active minor components in the *C. hemipterus* pheromone (Bartelt et al., 1992). *C. marginellus*, for which a pheromone is not yet known, also responded very clearly to all baits that contained tetraenes **5** or **7**, or both.

C. humeralis, for which a pheromone is also unknown, responded significantly to all of the pheromones, whether they were tetraenes or trienes. While a preference for a small subset of the synthetic compounds was evident for *C. marginellus*, such was not the case for *C. humeralis*.

Flight Activity Throughout the Year. Most of the beetles were captured during periods when food resources were scarce. As the weather warmed in late winter, adults dispersed from their overwintering sites and probably emerged from pupation in the soil as well (Connell, 1956). Moderate flight activity (and presumably, reproduction at various ephemeral feeding sites) continued through the spring until the developing corn crop, probably the richest nitidulid food source in the area, had reached the silking stage and become infestable. Examination of developing ears revealed that the beetles were present in the corn and that reproduction was occurring, but the traps detected little further flight activity until the corn was ready to harvest. The autumn flight probably represented adults reemerging from the dried corn crop as well as their progeny that had developed on the corn and were maturing.

The species were generally similar in their trap-catch trends over time except for *C. humeralis*, which was captured commonly only during September–November (Figure 4). It is unknown whether the population of this species was large only in autumn (e.g., from immigration) or if the responsiveness to the traps changed during the year.

Most trap catches occurred when the daily maximum temperature was in the range of 20–30°C (Figure 4). Other factors besides temperature, such as condition of food supply, must be involved in regulating overall flight activity, but it does appear that rising temperatures after relatively cool periods are able to trigger and synchronize beetle flights. The final trap catch in February and the second and fourth catches in March were relatively large, and this heavy flight activity was correlated with weekly mean maximum temperatures that were 5–8°C higher than for the prior or intervening weeks (Figure 4). A similar peak, apparently related to temperature, occurred for the third trap check of November. There was no obvious correlation between flight activity and rainfall.

The 1993 growing season was poor for South Carolina corn farmers. Despite frequent rains from June to harvest, precipitation for April and May was spotty and below normal, resulting in stunted corn plants and low yields. It is unknown whether nitidulid flight patterns would be different in more normal years.

Comparison with Other Field Studies. The responses to the *Carpophilus* pheromones observed in this study were generally very similar to those in Australia (James et al., 1993), Israel (Blumberg et al., 1993), Ohio (Williams et al., 1995), and California (Bartelt et al., 1994), to the extent that the same pheromones were tested and that the responding species were present in meaningful numbers. The pheromones have become useful tools for revealing the *Carpophilus* fauna and monitoring its seasonal activity in a variety of habitats.

Nitidulids and Aflatoxin. Although the feeding damage caused by nitidulids in field corn is of little concern, nitidulids have been implicated as vectors of *Aspergillus flavus* Link: Fr., the fungus that produces aflatoxin (Lussenhop and Wicklow, 1990; Wicklow, 1991). Aflatoxin has been a recurrent problem in

corn, particularly in the southeastern United States. The nitidulid pheromones would be useful in the study of beetle-mediated movement of *A. flavus* inoculum. Furthermore, it was recently suggested that feral beetles could be manipulated to disseminate biocompetitive microrganisms into corn that would suppress *A. flavus* development (Vega et al., 1995). The concept depends on pheromone-baited autoinoculators. Now that pheromones are known for the major indigenous *Carpophilus* species in the Southeast, the utility of this approach for reducing aflatoxin levels in this region can be explored.

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PHLOEM TRANSPORT OF ANTIRRHINOSIDE, AN IRIDOID GLYCOSIDE, IN *Asarina scandens* (SCROPHULARIACEAE)

ESTHER GOWAN,[1] BETTY A. LEWIS,[2] and ROBERT TURGEON[1,*]

[1]*Section of Plant Biology*
[2]*Division of Nutritional Sciences*
Cornell University
Ithaca, New York 14853

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Abstract—Iridoid glycosides, terpene-derived compounds found in many plant families, protect the plant against generalist and nonadapted specialist insect herbivores, fungi, and bacteria. Antirrhinoside, a common iridoid glycoside in the tribe Antirrhineae (Scrophulariaceae), was rapidly labeled when mature leaves of *Asarina scandens* were exposed to $^{14}CO_2$. Antirrhinoside was translocated in the phloem along with sucrose. Radiolabeled antirrhinoside appeared in the petiole of the labeled leaf within 20 min of the beginning of the labeling period. Antirrhinoside was also found in phloem sap obtained by the EDTA method.

Key Words—Antirrhinoside, *Asarina scandens*, iridoid glycoside, phloem, Scrophulariaceae, translocation.

INTRODUCTION

Plants synthesize a wide array of compounds to protect them against herbivores and pathogens. If any of the components of the chemical defense system are translocated, the plant gains a potential defense against phloem-feeding organisms and also gains the ability to coordinate the synthesis and use of protective resources in various organs. Note, for example, the systemic nature of signals in acquired resistance to virus, bacteria, and fungi (Ryals et al., 1994) and to insects (Narvaez-Vasquez et al., 1994).

*To whom correspondence should be addressed.

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An ideal candidate class of phloem-mobile defensive chemicals is the group of terpene-derived compounds known as iridoid glycosides (Bowers, 1991). There are almost 600 known iridoid glycosides in at least 57 families of plants (Bowers, 1991). Some of these compounds, which can comprise almost one quarter of the dry weight of certain leaves (Bowers, 1991; Boros et al., 1991), are among the most bitter substances known and are effective in protecting many plant species against generalist and nonadapted specialist insect herbivores. They may also inhibit seed germination and seedling growth (Adam et al., 1979) and act as toxins to both fungi (van der Sluis et al., 1983) and microbes (Kubo et al., 1985). Many insects sequester iridoid glycosides that they obtain from plants in order to defend themselves against potential predators (Bowers, 1991; Bowers and Farley, 1990).

Iridoid glycosides are synthesized via the mevalonic acid pathway (Inouye and Uesato, 1986). Many are potentially phloem-mobile because they are of low molecular weight, water soluble, and nonreducing, as are other phloem-mobile substances. We report here that antirrhinoside, a common iridoid glycoside found throughout the tribe Antirrhineae in the family Scrophulariaceae (Kooiman, 1970) and sequestered by aposematic moth larvae (Boros et al., 1991) is translocated in *Asarina scandens*.

METHODS AND MATERIALS

Plant Material. Seeds of *Asarina scandens* (Cav.) Penn. were obtained from Thompson and Morgan (Jackson, New Jersey). Plants were grown in a greenhouse in artificial soil (Metro-Mix 350; E.C. Geiger, Harleyville, Pennsylvania) and used for experimentation when approximately 3 months old (Turgeon et al., 1993). Daylight in the greenhouse was extended to 16 h with fluorescent lamps.

Analytical Methods. Mature leaf blade tissue was frozen and ground in liquid N_2 and extracted at 50°C in methanol–chloroform–water (12:5:3, v/v/v) (Haissig and Dickson, 1979). Water was then added to obtain biphasic partitioning of the extract (3 parts water per 5 parts extract). Following centrifugation (2,000*g*, 10 min), the separated aqueous phase was passed through ion-exchange membranes [Bio-Rex AG50W cation membrane (H^+ form) and AG1 anion membrane (carbonate form)] (Bio-Rad, Richmond, California) (Turgeon and Gowan, 1992), filtered through a 0.22-μm nylon spin filter, and separated by HPLC using a Waters (Milford, Massachusetts) model 510 pump, a Waters SugarPak 1 column held at 90°C, and a Waters model 410 differential refractometer. Water was used as the mobile phase. A large peak migrating at 10.5 min was isolated by pooling three 0.5-min fractions and dried down at 70°C under a stream of N_2 gas. When rechromatographed by the same methods, 96% of the pooled material ran as a single peak.

Two-dimensional TLC on plant extracts and purified compounds was performed using silica gel GHL plates (Analtech, Newark, Delaware), pretreated by dipping in a solution of 0.03 M boric acid in 60% ethanol (v/v), and activated at 95–110°C for 1 h. The first development was in chloroform–acetic acid–water (6:7:1, v/v/v) and the second in isopropanol–water (5:1, v/v).

NMR spectroscopy of the purified material was performed in a 5-mm tube in D_2O on a Varian VXR-400S with acetone as internal reference. The ^{13}C spectrum was obtained in the proton-decoupled mode. Carbon-13 chemical shifts were corrected to 61.55 ppm for C-6 of the glucosyl residue.

Radiolabeling Methods. Plants were brought into a laboratory fumehood illuminated by a water-filtered incandescent 1000-W metal-halide lamp providing approximately 400 μmol photons/m^2/sec (photosynthetically active radiation) at plant level. Approximately 1 hr later a mature leaf blade was enclosed in a plastic bag and $^{14}CO_2$ (1.5 MBq), generated in the barrel of a syringe by addition of excess 80% lactic acid to [^{14}C]Na_2CO_3 (6.6×10^5 MBq/mmol), was injected into the bag without delay. The petiole of the labeled leaf was covered with aluminum foil to prevent accidental photosynthetic incorporation of leaked gas. After a 5-min exposure to $^{14}CO_2$, the bag was removed and, following a chase period in room atmosphere, the leaf blade and petiole were quickly removed and separately plunged into liquid N_2. Tissue was extracted and analyzed by TLC as described above. Radiolabeled spots were detected with X-ray film, scraped from the plate, and quantified by scintillation counting.

Phloem Exudate. Ten leaves were cut at the base of the petiole, in the middle of the day, and immediately recut under water. The leaves were then placed in a single group in a small tube so that the petiole bases were immersed in 0.2 ml of a solution of 20 mM EDTA in 20 mM PIPES buffer (pH 6.8). The leaves were then placed, in their tube, in a humid chamber in complete darkness at room temperature. The EDTA solution was entirely removed for analysis and replaced every hour for 4 hr. The solutions were purified by ion-exchange chromatography and the neutral fraction analyzed by HPLC as described above.

RESULTS

Identification of Antirrhinoside. In extracts of *A. scandens* leaf blade tissue we detected an HPLC peak that eluted at 10.5 min from the SugarPak column, while sucrose eluted at 7.8 min. There was considerably more of this material (9.5 mg/g fresh weight) than sucrose (1.4 mg/g fresh weight) or any other component of the neutral fraction. This substance was present in all five species of the Antirrhineae that we previously studied (Turgeon et al., 1993). It co-chromatographed with several monosaccharide standards on the SugarPak column but tested negatively as a reducing compound with Benedict's solution or 2,2′-bicinchoninate (Sturgeon, 1990). On TLC plates it produced an intense

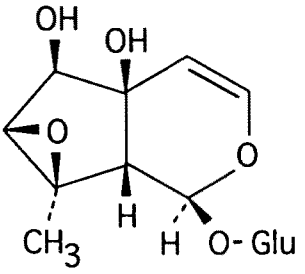

FIG. 1. Structure of antirrhinoside.

dark brown to black color with Seliwanoff's reagent (Zweig and Sherma, 1972) or vanillin-H_2SO_4 reagent (Touchstone and Dobbins, 1983) and a blue fluorescence with *p*-anisidine phosphate reagent (Kooiman, 1970). Glucose was produced upon hydrolysis with 2 N trifluoroacetic acid (room temperature for 2 hr).

The compound was identified as antirrhinoside (Figure 1) by ^1H and ^{13}C NMR. Chemical shifts and coupling constrants for protons of the aglycone and the anomeric carbon (H1′) of the glucosyl unit agreed closely with reported values for antirrhinoside (Scarpati et al., 1968). Carbon-13 NMR data confirmed the identity of antirrhinoside. The chemical shifts for all carbons in the aglycone and glucosyl unit were in close agreement with published values (Bianco et al., 1981).

Translocation Experiments. Antirrhinoside in the lamina became labeled, although not as quickly as sucrose, following exposure of the leaf to $^{14}CO_2$ (Figure 2). With increasing time the amount of labeled antirrhinoside increased in both absolute terms and in proportion to sucrose so that within an hour the label in antirrhinoside was approximately one quarter that of sucrose. To determine if antirrhinoside is translocated, the petioles of the same leaves were extracted. Radiolabeled sucrose and antirrhinoside were first found in the petioles in measurable quantities 20 min after exposure of the leaves to $^{14}CO_2$, and the amount of these radiolabeled compounds increased rapidly over the next 40 min (Figure 2). The proportion of labeled antirrhinoside in the petiole increased in the same way that it did in the lamina.

It is important to note that radioactive antirrhinoside was already present in the translocate when label was first detected in the petiole. This rules out the possibility that labeled antirrhinoside is synthesized in the petiole from transported sucrose, rather than being translocated to the petiole from the leaf blade. At any given time point, the ratio of labeled antirrhinoside to labeled sucrose was lower in the petiole than in the leaf blade. This lag in the labeling pattern

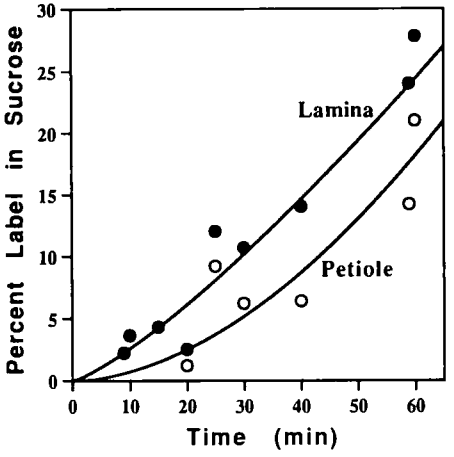

FIG. 2. Labeling of antirrhinoside in the lamina and petiole of *Asarina scandens* leaves following exposure of the leaf blade to $^{14}CO_2$. The amount of label in antirrhinoside is expressed in a percentage of that in sucrose.

of the compound in the petiole in comparison to the blade is to be expected, since the composition of translocate entering the petiole is a reflection of the labeling pattern in the mesophyll cells at an earlier point in time.

While labeling experiments demonstrated that antirrhinoside is transported, the experiments did not reveal the concentration of this compound in the phloem sap. Samples of sap, obtained by the EDTA method (King and Zeevart, 1974), were composed mainly of sucrose with smaller amounts of raffinose and stachyose, as previously shown (Turgeon et al., 1993), and also contained antirrhinoside (Figure 3), as well as monosaccharide. The amount of antirrhinoside was approximately 7% that of sucrose. If it is assumed that the concentration of sucrose in the phloem is in the range of 1 M (Winter et al., 1992), the concentration of antirrhinoside would be approximately 70 mM.

DISCUSSION

The data reported here indicate that antirrhinoside is translocated from source leaves to other parts of the plant in the phloem of *Asarina scandens*. Very little work appears to have been done on the transport of iridoid glycosides. Aucubin is transferred from *Penstemon teucrioides* to the parasitic plant *Castilleja integra* (Stermitz et al., 1993) but the route of transport has not been determined. An aphid, *Acyrthosiphon nipponicus*, sequesters paederoside, an

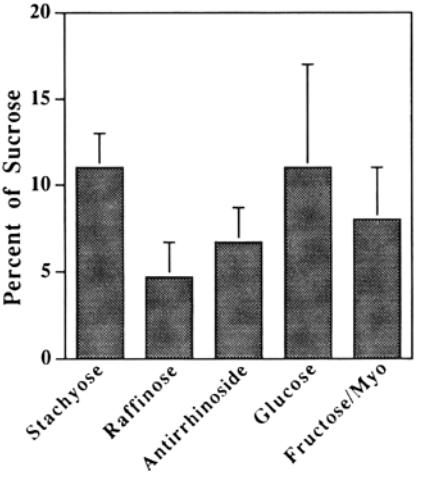

FIG. 3. Sugars in the phloem sap obtained from leaves of *Asarina scandens* by the EDTA method and measured by HPLC. The amount of each compound is expressed as a percentage of the amount of sucrose in the sap. Fructose and myoinositol coeluted. Bars = SE (N = 4).

iridoid glycoside that protects it from ladybird beetles (Nishida and Fukami, 1989); since aphids are known to feed in sieve elements, it is reasonable to conclude that this iridoid is also phloem mobile. Considered together the results indicate that phloem transport of iridoid glycosides may be a common occurrence.

The labeling of antirrhinoside after exposure of the leaf to $^{14}CO_2$ follows a complex pattern, increasing in the leaf blade and lamina over time in proportion to sucrose. While there is more antirrhinoside than sucrose in the leaf blade, there appears to be a higher proportion of sucrose in the transport system, as determined by the EDTA exudation method. These data are consistent with the occurrence of two pools of antirrhinoside in the leaf blade, a large nonmobile pool, presumably in the vacuoles, and a high-specific-activity transport pool that is the source of antirrhinoside in the phloem. It should be noted, however, that the EDTA procedure on which these conclusions are partially based provides only a rough estimate of phloem sap composition (Girousse et al., 1991). While it is clear from our labeling experiments that antirrhinoside is transported, the data we report here on the composition of the phloem sap should be considered tentative until they are confirmed by other methods, such as collection of sap from aphid stylets.

On the basis of the information at hand it is difficult to know whether the

amount of antirrhinoside in the phloem would be high enough to deter phloem-feeding organisms such as aphids. First, we have been unable to determine the concentration of transported antirrhinoside with complete confidence, as discussed above. Second, deterrence tests are usually conducted with dry diets, so it is not possible to compare those results with our own. However, the fact that antirrhinoside is present in the phloem in easily detectable quantities indicates that such deterrence may be possible. Furthermore, if imported antirrhinoside is stored in sink tissues, it will accumulate to high levels over time; thus the transported compound could be an important factor in protecting growing regions of the plant from herbivores.

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ROLE OF VOLATILE INFOCHEMICALS EMITTED BY FECES OF LARVAE IN HOST-SEARCHING BEHAVIOR OF PARASITOID *Cotesia rubecula* (HYMENOPTERA: BRACONIDAE): A BEHAVIORAL AND CHEMICAL STUDY

NICKY G. AGELOPOULOS,[1] MARCEL DICKE,[1,*] and MAARTEN A. POSTHUMUS[2]

[1]*Department of Entomology, Wageningen Agricultural University P.O. Box 8031, NL-6700 EH Wageningen, The Netherlands*

[2]*Department of Organic Chemistry, Wageningen Agricultural University Dreijenplein 8, NL-6703 HB Wageningen, The Netherlands*

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Abstract—The role of volatile infochemicals emitted by feces of larvae in the host-searching behavior of the parasitoid *Cotesia rubecula* was evaluated during single- and dual-choice tests inside a wind tunnel. The following treatments were tested: feces produced by second and fourth instars of *Pieris rapae* (preferred host), second instars of *P. brassicae* (inferior host), second instars of *P. napi* (nonhost), and wet feces of second instars of *P. rapae*. During a single-choice situation females of *C. rubecula* oriented to all types of feces tested. When a preference was to be made, *C. rubecula* preferred feces of second instars of *P. rapae* over that of fourth, feces of *P. rapae* over that of *P. brassicae*, feces of *P. napi* over that of *P. brassicae*, and wet over normal host feces. No preference was exhibited between feces of second instars of *P. napi* and that of second instars of *P. rapae*. The relative importance of infochemicals from host feces versus plant damage caused by host larvae to the searching behavior of *C. rubecula* was also evaluated. Plant damage was more important to the searching females than host feces when feces were present in specific concentrations in relation to damage. The volatiles released by normal and wet feces of second instars of *P. rapae*, wet feces of fourth instars of *P. rapae*, and normal and wet feces of *P. brassicae* were collected and identified. Overall, 85 chemical compounds were recorded belonging to the following chemical groups: alcohols, ketones, aldehydes, esters, isothio-

*To whom correspondence should be addressed.

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cyanates, sulfides, nitriles, furanoids, terpenoids and pyridines. The blend of chemicals emitted by feces of different instars of *P. rapae* and different species of *Pieris* exhibited an instar and species specificity in both quantity and quality. Wetting of normal feces increased the amount of volatile chemicals released, and it was also responsible for the appearance of new compounds. The role of feces of larvae in the host-seeking behavior of *C. rubecula* is discussed.

Key Words—Volatile infochemicals, kairomone, host feces, parasitoid, *Cotesia rubecula*, host-searching behavior, *Brassica oleracea gemmifera*, *Pieris rapae*, *Pieris brassicae*, *Pieris napi*, Lepidoptera.

INTRODUCTION

During their search for hosts, parasitoids attacking larvae of Lepidoptera are known to utilize volatile chemicals emitted by plants infested by their hosts. Some of the chemicals emitted by host-infested plants may be released by the plant itself and others may originate from activities related to the biology of larvae, e.g., feces, silk, etc. (for an overview see Vinson, 1976, 1984; Vet and Dicke, 1992). Some of the volatiles emitted by the host plant are produced independently of the presence of larvae, and others may appear only during or after feeding of larvae has occurred (Whitman and Eller, 1990; Turlings et al., 1990, 1991, Turlings and Tumlinson, 1992; Agelopoulos and Keller, 1994c; Blaakmeer et al., 1994; Mattiacci et al., 1994; Potting et al. 1995, Takabayashi et al., 1995). Plant compounds induced by larval feeding are extensively used by female parasitoids during location of host-infested plants (Loke et al., 1983; Nadel and van Alphen, 1987; Steinberg et al., 1993; Turlings et al., 1990; Agelopoulos and Keller, 1994b; Geervliet et al., 1994, Takabayashi et al., 1995). Volatiles emitted by feces of larvae may also be involved in orientation to host-infested plants since many species of parasitoids are known to be attracted to feces of their host larvae (Lecomte and Thibout, 1986, 1993; Eller et al., 1988a,b; Elzen et al., 1987; Ding et al., 1989; Ramachandran et al., 1991; Turlings et al., 1991; Cortesero et al., 1993; Steinberg et al., 1993; Agelopoulos and Keller, 1994a; Geervliet et al., 1994). The aim of this study was to investigate the role of host feces in orientation to host-infested plants by the parasitoid *Cotesia rubecula*.

Cotesia rubecula Marshall (Hymenoptera: Braconidae) is a larval endoparasitoid of *Pieris rapae*. *Pieris rapae* is the preferred host of *C. rubecula*, and all host stages can be attacked. Other host species have also been reported (Shenefelt, 1972). For example, it is known that females of a Dutch population of *C. rubecula* develop most successfully in *P. rapae* although they do oviposit and develop less successfully in *Pieris brassicae* as well (Geervliet and Brodeur, 1992). Although the parasitoid accepts *Pieris napi* as a host, the parasitoid cannot successfully develop in this species (Geervliet and Brodeur, 1992). Dur-

ing their search for hosts, females of *C. rubecula* are known to be attracted from a distance to host-infested plants (Nealis, 1986, 1990; Keller, 1990), and their searching behavior is influenced by experience (Kaiser and Cardé, 1992; Geervliet et al., 1993). Detailed studies on the role and identity of plant volatiles involved in long-distance orientation to host-infested plants have been conducted for two different populations of *C. rubecula*: an Australian population (Agelopoulos and Keller, 1994a–c) and a Dutch one (Geervliet et al., 1993, 1994, 1995; Blaakmeer et al., 1994). For both populations the same holds true: females of *C. rubecula* rely mainly on plant volatiles related to damage to locate host-infested plants. They are able to distinguish from a distance between plants damaged by larvae of Lepidoptera and those damaged by mechanical means, but they are not able to discriminate between host plants damaged by larvae of different species of Lepidoptera. However, in a more complex situation where plants infested by *P. rapae* are simultaneously present with plants infested by *P. brassicae*, females of *C. rubecula* preferably land on plants infested by *P. rapae* (Wiskerke and Vet, 1994). Since the blends of volatiles released by cabbage plants damaged by different Lepidoptera is not species specific (Blaakmeer et al. 1994), the question still remains as to which component of the plant-herbivore complex provides species-specific information to the searching females. Studies on other sources of volatiles related to infestation that may be involved in orientation to host-infested plants have revealed that females of *C. rubecula* are not attracted to host larvae (Kaiser and Cardé, 1992; Agelopoulos and Keller, 1994a), but they are attracted to feces of the host (Agelopoulos and Keller, 1994a; Geervliet et al., 1994). In the chemical study by Agelopoulos and Keller (1994c), there is evidence of species specificity related to volatiles emitted by feces of larvae, but the question still remains as to whether feces are the component that provides species-specific information to the searching females.

In this study we explored further the role of feces in attracting *C. rubecula* to its host. By conducting single- and dual-choice tests and by using feces of different host instars and feces of three closely related species of *Pieris*, we tested whether the information released by feces and utilized by *C. rubecula* was: (1) host-instar specific, (2) species specific, (3) dose dependent, and (4) affected by feces moisture. The relative importance of volatiles released by feces, compared to those released by plant damage was also evaluated. Finally, we sought to identify the volatiles released by feces of different instars of *P. rapae*, feces of *P. rapae* with different moisture levels, and feces of *P. brassicae*.

METHODS AND MATERIALS

Plants and Insects. Brussels sprouts plants, *Brassica oleracea gemmifera* cv. Titurel, were grown under greenhouse conditions (20–30°C, 50–80% rel-

ative humidity, 16L:8D). *Pieris rapae* and *P. brassicae* (Lepidoptera: Pieridae) were reared on Brussels sprouts plants inside a climate-controlled room (20–22°C, 50–70% relative humidity, 16L:8D), and *P. napi* was reared on Brussels sprouts plants under greenhouse conditions (20–30°C, 50–80% relative humidity, 16L:8D). The parasitoid *C. rubecula* was reared on *P. rapae* at 20–22°C, 50–70% relative humidity, 16L:D8. Upon emergence, mated females of *C. rubecula* were released into a gauze cage with honey and water. Two- to 3-day-old "experienced" females were used for the bioassays. Experience of females was obtained by introducing inside the cage a leaf of Brussels sprouts bearing feeding damage and feces of the host. The leaf remained there for 22 hr and was removed 4 hr prior to testing.

Bioassays. All tests were performed inside a wind tunnel under the following conditions: 25°C, 45–60% relative humidity, and wind speed 50 cm/sec (description of wind tunnel by Geervliet et al., 1994). One female, held in a vial that was placed in vertical position, was introduced into the wind tunnel 1 m away from the stimulus. The observation started when the plug of cotton wool restricting the insect inside the vial was removed. A maximum of six tries was given to insects that failed during earlier attempts. "Attraction to stimulus" was defined as a complete flight of the insect inside the odor plume and successful location (landing) of the stimulus. Insects that flew half way to the stimulus were given a second try. To reduce the effect of day-to-day variation in response, the same test was conducted on at least three different days in different weeks. Feces were collected by placing Petri dishes under infested leaves for a period of 24 hr (25°C, 50–60% relative humidity).

Single-Choice Tests. The response of females of *C. rubecula* to a single stimulus was observed for the following: feces of second instars of *P. rapae*, feces of fourth instars of *P. rapae*, feces of second instars of *P. brassicae*, and feces of second instars of *P. napi*. For each bioassay, 65 mg of feces was used and placed on a flat surface at the upwind end of the wind tunnel. Five, 10, and 20 mg of feces of late second instars of *P. rapae* were used to record dose-dependent responses. The three different doses were tested on the same day.

Dual-Choice Tests. The landing preference of *C. rubecula* was tested for the following: (1) feces of late second instars of *P. rapae* vs. feces of early fourth instars of *P. rapae* (second vs. fourth *P. rapae*), (2) feces of late second instars of *P. rapae* vs. wet feces of late second instars of *P. rapae* (second *P. rapae* vs. wet second *P. rapae*), (3) feces of late second instars of *P. rapae* vs. feces of late second instars of *P. brassicae* (*P. rapae* vs. *P. brassicae*), (4) feces of late second instars of *P. rapae* vs. feces of late second instars of *P. napi* (*P. rapae* vs. *P. napi*), and (5) feces of late second instars of *P. brassicae* vs. feces of late second instars of *P. napi* (*P. brassicae* vs. *P. napi*). "Wet" feces is defined as feces wetted prior to testing with three drops of water. "Normal" feces is rather dry. In each case, 65 mg of feces was used.

The two stimuli were placed on separate flat surfaces located 5 cm apart and 1 m away from the release point of insects. To avoid bias caused by the left or right position of stimuli inside the wind tunnel, the stimuli were alternated after every fifth observation. To evaluate the relative significance of feces over plant damage in leading *C. rubecula* to its host, the following were tested: (1) a leaf carrying feeding damage of 10 instars of *P. rapae* vs. a leaf carrying feeding damage of 10 instars of *P. rapae* plus 10 mg of feces of *P. rapae* (D_{10} vs. $D_{10}F_{10mg}$), (2) a leaf carrying feeding damage of 10 instars of *P. rapae* vs. a leaf carrying feeding damage of 10 instars of *P. rapae* plus 65 mg of feces of *P. rapae* (D_{10} vs. $D_{10}F_{65mg}$), (3) a leaf carrying feeding damage caused by 20 instars of *P. rapae* vs. a leaf carrying feeding damage of 10 instars of *P. rapae* plus 65 mg of feces of *P. rapae* (D_{20} vs. $D_{10}F_{65mg}$). For all tests, damage and feces were produced by late second instars of *P. rapae*. Damage on the leaves was produced over a period of 24 hr. Prior to testing, larvae and larval by-products were removed. In the cases where leaf and feces were used together, the feces were located on a flat surface next to the leaf. To avoid bias caused by left or right position of stimuli inside the wind tunnel, the stimuli were alternated after every fifth observation. Production of feces by second and fourth instars of *P. rapae* was accomplished by placing 20 late second instars and 20 early fourth instars of *P. rapae* individually on single leaves held in vials with water where they were left to feed for 24 hr. The feces produced by each larva were collected and weighed.

Statistics. The chi-square test for goodness-of-fit was used to determine preference for one of the stimuli tested during dual choice situation (distribution of expected values 50:50).

Collection of Volatiles from Larval Feces and Chemical Analysis. Feces used for identification of volatile components were collected by placing Petri dishes under infested leaves for a period of 24 hr (25°C, 50–60% relative humidity). Volatiles from feces were collected by placing feces in a glass flask (50 ml), which was closed with a glass top having a ground glass connection and an inlet and outlet glass tube. Purified air was passed through the inlet tube with an air flow of 90 ml/min. Volatile chemicals emitted by feces were transported with the aid of airflow to the outlet tube, where they were trapped on 90 mg of Tenax-TA. The Tenax-TA was placed inside a glass tube connected immediately to the outlet tube (Dicke et al., 1990). Thermodesorption was the method used to release the volatiles trapped on Tenax-TA (description of method by Dicke et al., 1990). The samples were analyzed by using a gas chromatograph–mass spectrometer (Finnigan MAT 95). The GC column used was Supelcowax 10 (60 m × 0.25 mm ID; 0.25-μm film thickness), and the temperature conditions were 3 min at 40°C, increased by 3°C/min to 140°C, and after that increased by 6°C/min to 270°C. The mass spectrometer was operated in the 70-eV electron impact ionization mode. Control samples (clean air) were also

analyzed to ascertain which chemicals were specific to feces. Identification of compounds was based on the commercial spectra libraries NIST and WILEY and on our own collection of spectra of natural compounds. Volatiles released by 250 mg of feces produced by late second instars of *P. rapae* (samples named as normal) were collected for a period of 2.5 hr. At the end of that collection period, the same feces were wetted with nine drops of distilled water and volatiles were collected for a further 2.5 hr (samples named as wet). The same procedure was followed for 250 mg of feces produced by late second instars of *P. brassicae*. Volatiles were also collected from 250 mg of feces produced by early fourth instars of *P. rapae*. The samples for that particular treatment were all wetted with nine drops of distilled water. Three samples were collected for each treatment.

RESULTS

Bioassays. During a single-choice situation, females of *C. rubecula* oriented to and landed on all types of feces tested irrespective of instar or species that had produced them (Table 1). *C. rubecula* was able to locate the source of volatiles even when very small amounts (e.g., 5 mg of feces) of host feces were used (Table 2). A higher degree of attraction was observed for 20 mg of feces over that of 5 and 10 mg. However, the attraction of females to the different doses tested was not significantly different (contingency table, $P = 0.07$). *C. rubecula* preferred feces of *P. rapae* over that of *P. brassicae*, but it did not show preference for feces of *P. rapae* when tested against that of *P. napi*. Feces of *P. napi* were also preferred over that of *P. brassicae* (Table 3). Preference was also exhibited for feces of second instars over feces of fourth instars of *P. rapae* (Table 3). The preference of females for leaves damaged by the host over leaves damaged by the host that also carried feces of the host depended on the amount of feces present on the leaves (Table 4). When leaves carrying

TABLE 1. ORIENTATION OF FEMALES OF *C. rubecula* TO 65 mg OF FECES PRODUCED BY LARVAE OF DIFFERENT INSTARS AND DIFFERENT SPECIES OF *Pieris* ($N = 30$) IN SINGLE-CHOICE EXPERIMENTS

Source of feces	Attraction	No attraction
Second instars of *P. rapae*	24	6
Fourth instars of *P. rapae*	29	1
Second instars of *P. brassicae*	23	7
Second instars of *P. napi*	18	12

TABLE 2. DOSE-RELATED RESPONSES OF FEMALES OF *C. rubecula* TO FECES OF SECOND INSTARS OF *P. rapae* ($N = 30$)

Amount of feces (mg)	Attraction	No attraction
20	25 (83%)	5
10	19 (63%)	11
5	17 (56%)	13

damage caused by 10 second instars of *P. rapae* were tested against leaves carrying the same amount of damage plus 10 mg of feces of *P. rapae*, the females did not show preference for any of the two stimuli. When the 10 mg of feces was replaced with 65 mg, the females showed a preference for the latter. Leaves bearing damage caused by 20 second instars of *P. rapae* were preferred over leaves carrying feeding damage of 10 second instars of the host plus 65 mg of host feces.

A 10-fold increase in production of feces was observed from second (0.62 ± 0.03 m/larva/24 h, mean ± SEM) to fourth instars of *P. rapae* (5.87 ± 0.17 m/larva/24 h).

TABLE 3. PREFERENCE OF *C. rubecula* FOR FECES OF DIFFERENT SPECIES OF *Pieris*, DIFFERENT INSTARS OF *P. rapae*, AND NORMAL VERSUS WET FECES OF *P. rapae*[a]

Choice test	Attraction		No attraction
Second vs. fourth *P. rapae* ($N = 66$)	2nd	4th	
	29	12*	25
Second *P. rapae* vs. wet second *P. rapae* ($N = 34$)	*frass*	*wet frass*	
	5	22*	7
Second *P. rapae* vs. second *P. brassicae* ($N = 40$)	*P. rapae*	*P. brassicae*	
	23	8*	9
Second *P. rapae* vs. second *P. napi* ($N = 75$)	*P. rapae*	*P. napi*	
	15	17 NS	43
Second *P. brassicae* vs. second *P. napi* ($N = 49$)	*P. brassicae*	*P. napi*	
	8	22*	19

[a] NS = not significantly different, *significantly different $P \leq 0.05$.

TABLE 4. PREFERENCE OF FEMALES OF *C. rubecula* TO PLANT DAMAGE CAUSED BY HOST LARVAE OVER HOST FECES

Choice test	Attraction		No attraction
D_{10} vs. $D_{10} + F_{10mg}$ ($N = 46$)	D_{10}	$D_{10} + F_{10mg}$	
	19	16 NS	11
D_{10} vs. $D_{10} + F_{65mg}$ ($N = 32$)	D_{10}	$D_{10} + F_{65mg}$	
	9	22*	1
D_{20} vs. $D_{10} + F_{65mg}$ ($N = 35$)	D_{20}	$D_{10} + F_{65mg}$	
	26	7*	2

"D_{10}, D_{20} = plant damage caused by 10 and 20 second instars of *P. rapae* respectively, *F* = feces, D + F = damage and feces together, NS = not significantly different, *significantly different $P \leq 0.05$).

Chemical Analysis. Overall, 85 volatile chemical compounds were identified from the various treatments tested belonging to the following chemical groups: alcohols, ketones, aldehydes, esters, isothiocyanates, sulfides, nitriles, furanoids, terpenoids, and pyridines (Table 5). The blends of volatiles released by feces of larvae of different species of *Pieris*, different instars of *P. rapae*, and by feces affected by moisture are different in both quantity and quality (Table 5; Figure 1). The average amount of the identified compounds is represented in Table 5 by units of peak area. Depending on the chemical nature of the compound, one unit of peak area is equal to 0.3–0.5 ng. Wet feces of second instars of *P. rapae* and *P. brassicae* emitted the highest amounts of volatiles (total peak area, Table 5). Wet feces of fourth instars of *P. rapae* emitted much less and normal feces of second instars of *P. rapae* and *P. brassicae* emitted the lowest amount of chemicals.

Most of the compounds detected were present in all treatments tested, but some compounds or classes of compounds were present in one treatment only (Table 6). For example, nitriles were solely, and pyridines mainly, present in feces of fourth instars of *P. rapae*. Feces of *P. brassicae* emitted more aldehydes, alcohols, and ketones in comparison with that of *P. rapae*, and feces of second instars of *P. rapae* exhibited the least qualitative specificity but showed a quantitative specificity for a number of compounds. For example, compounds such as 2-methyl-1-propanol, 1-penten-3-ol, 1-penten-3-one, 3-methylbutanal, pentanal, and β-cyclocitral appeared in high concentration in that treatment (Table 5, Figure 2).

TABLE 5. VOLATILE COMPOUNDS EMITTED BY FECES OF SECOND AND FOURTH INSTARS OF *P. rapae* AND SECOND INSTARS *P. brassicae*[a]

	Compounds	*P. rapae*			*P. brassicae*	
		Second instars, normal ($N = 3$)	Second instars, wet ($N = 3$)	Fourth instars, wet ($N = 3$)	Second instars, normal ($N = 3$)	Second instars, wet ($N = 3$)
Alcohols						
1.	methanethiol		$2.0 \pm 2.0^{+}$	$0.2 \pm 0.2^{+}$		$0.8 \pm 0.8^{+}$
2.	methanol					$44 \pm 23^{++}$
3.	2-propanol*					$0.5 \pm 0.3^{++}$
4.	ethanol*				$0.6 \pm 0.3^{++}$	$1.4 \pm 0.3^{++}$
5.	2-methyl-1-propanol*	$9.3 \pm 5.6^{+++}$	$54 \pm 45^{+++}$	$14 \pm 10^{+++}$	$1.6 \pm 0.7^{+++}$	$21 \pm 13^{+++}$
6.	3-pentanol*			$9.7 \pm 7.8^{++}$		$0.7 \pm 0.7^{+}$
7.	1-penten-3-ol*	$5.0 \pm 2.1^{+++}$	$141 \pm 17^{+++}$	$8.5 \pm 3.9^{+++}$	$3.1 \pm 2.8^{+++}$	$83 \pm 43^{+++}$
8.	3-methyl-2-pentanol			$0.9 \pm 0.5^{++}$		
9.	3-methyl-1-butanol*			$59 \pm 49^{+++}$		$0.2 \pm 0.2^{+}$
10.	2-methyl-1-butanol			$0.9 \pm 0.5^{++}$		
11.	1-pentanol*	$1.3 \pm 0.3^{+++}$	$30.7 \pm 6.3^{+++}$	$23 \pm 14^{+++}$	$0.83 \pm 0.60^{++}$	$16.7 \pm 8.6^{++}$
12.	(*E*)-2-penten-1-ol		$5.7 \pm 0.7^{+++}$	$8.0 \pm 8.0^{+}$		$4.3 \pm 2.3^{++}$
13.	(*Z*)-2-penten-1-ol*	$2.5 \pm 1.8^{+++}$	$48.0 \pm 3.6^{+++}$	$7.0 \pm 6.0^{+++}$	$3.0 \pm 2.5^{++}$	$40 \pm 20^{+++}$
14.	1-hexanol*		$4.7 \pm 1.5^{+++}$	$6.0 \pm 1.5^{+++}$		$3.2 \pm 1.5^{+++}$
15.	(*Z*)-3-hexen-1-ol*	$8.3 \pm 2.6^{+++}$	$137 \pm 50^{+++}$	$95 \pm 48^{+++}$	$7.3 \pm 7.3^{+}$	$110 \pm 83^{+++}$
16.	(*E*)-2-hexen-1-ol*			$4.5 \pm 4.2^{++}$		
17.	1-octen-3-ol*		$8.0 \pm 2.1^{+++}$	$1.1 \pm 0.5^{+++}$		$5.0 \pm 2.6^{++}$
18.	1-heptanol		$1.7 \pm 0.3^{+++}$	$1.2 \pm 0.4^{+++}$		
19.	2-methylthioethanol			$6.4 \pm 5.8^{+++}$		
20.	1-octanol	$2.3 \pm 1.3^{+++}$	$5.7 \pm 1.2^{+++}$	$2.0 \pm 0.6^{+++}$	$1.6 \pm 0.5^{+++}$	$3.4 \pm 1.6^{+++}$

TABLE 5. Continued

	Compounds	*P. rapae*			*P. brassicae*	
		Second instars, normal ($N = 3$)	Second instars, wet ($N = 3$)	Fourth instars, wet ($N = 3$)	Second instars, normal ($N = 3$)	Second instars, wet ($N = 3$)
Ketones						
21.	2-butanone				2.3 ± 1.9$^{+\ +}$	36 ± 35$^{+\ +}$
22.	3-methyl-2-butanone					0.8 ± 0.4$^{+\ +}$
23.	2-pentanone	1.6 ± 0.4$^{+\ \ +}$	17.3 ± 5.9$^{+\ +\ +}$	0.6 ± 0.3$^{+\ +}$	2.5 ± 1.0$^{+\ +\ +}$	31 ± 27$^{+\ +\ +}$
24.	2,3-butanedione	0.8 ± 0.2$^{+\ \ +}$	36.5 ± 5.3$^{+\ +\ +}$	19.0 ± 7.1$^{+\ +\ +}$	0.8 ± 0.5$^{+\ +}$	35 ± 18$^{+\ +\ +}$
25.	3-methyl-2-pentanone*		7.1 ± 2.9$^{+\ \ +}$	2.5 ± 1.8$^{+\ +}$		2.3 ± 1.2$^{+\ +}$
26.	1-penten-3-one*		53.7 ± 2.7$^{+\ \ +}$	2.3 ± 1.4$^{+\ +\ +}$	0.3 ± 0.3^{+}	33 ± 16$^{+\ +\ +}$
27.	3-hexanone					1.5 ± 0.8$^{+\ +}$
28.	2,3-pentanedione*		11.7 ± 0.9$^{+\ \ +}$	1.8 ± 1.1$^{+\ \ +}$		5.4 ± 2.9$^{+\ +}$
29.	3-penten-2-one* *isomer*		9.3 ± 1.8$^{+\ \ +}$			3.8 ± 1.9$^{+\ +\ +}$
30.	2-heptanone		3.7 ± 2.7$^{+\ \ +}$	0.5 ± 0.3$^{+\ +}$		0.9 ± 0.8$^{+\ +}$
31.	6-methyl-2-heptanone					2.3 ± 1.5$^{+\ +}$
32.	2,4-pentanedione		10.0 ± 10.0$^{+\ +}$	8.3 ± 8.3^{+}		
33.	3-hydroxy-2-butanone			29 ± 15$^{+\ +\ +}$	4.7 ± 4.7^{+}	3.3 ± 3.3^{+}
34.	2,2,6-trimethylcyclohexanone		8.3 ± 0.7$^{+\ +\ +}$			4.7 ± 2.3$^{+\ +}$
35.	6-methyl-5-hepten-2-one	1.0 ± 0.5$^{+\ \ +}$	14.0 ± 2.0$^{+\ \ +}$	1.2 ± 0.4$^{+\ +\ +}$	1.4 ± 0.8$^{+\ +\ +}$	11.1 ± 5.6$^{+\ +\ +}$
36.	4-hydroxy-4-methyl-2-pentanone	1.7 ± 1.7^{+}	0.3 ± 0.2$^{+\ +}$	0.7 ± 0.6$^{+\ +}$		
37.	2,5 hexanedione	1.8 ± 1.6$^{+\ +}$	2.3 ± 1.2$^{+\ +}$	1.3 ± 1.3^{+}		
38.	3-nonen-2-one *isomer*					1.2 ± 0.6$^{+\ +}$
39.	3,5-dimethyl-2-cyclohexen-1-one		8.0 ± 6.6$^{+\ +}$			
40.	3,3,5-trimethyl-2-cyclohexen-1-one		11 ± 0$^{+\ +\ +}$	1.0 ± 0.6$^{+\ +\ +}$		
41.	3,5 octadien-2-one		8.7 ± 0.3$^{+\ +\ +}$		0.5 ± 0.5^{+}	3.7 ± 1.9^{+}
42.	dihydrofuranone					3.0 ± 1.7$^{+\ +}$
Aldehydes						
43.	acetaldehyde					9.7 ± 7.8$^{+\ +}$
44.	propanal		2.2 ± 1.0$^{+\ \ +}$	0.10 ± 0.05$^{+\ +}$		7.7 ± 6.7$^{+\ +}$
45.	2-methylpropanal		6.9 ± 2.5$^{+\ \ +}$	3.2 ± 1.4$^{+\ +\ +}$		7.3 ± 5.9$^{+\ +}$

46.	2-methylbutanal		39.7 ± 6.3^{+++}	26 ± 13^{+++}	0.4 ± 0.2^{++}	38 ± 12^{+++}
47.	3-methylbutanal		105.7 ± 8.5^{+++}	80 ± 27^{+++}	0.4 ± 0.2^{++}	58 ± 28^{+++}
48.	pentanal	0.8 ± 0.2^{+++}	36.5 ± 5.3^{+++}	3.4 ± 1.2^{+++}	1.3 ± 0.7^{+++}	11.9 ± 5.5^{+++}
49.	2-butenal		8.3 ± 1.9^{+++}			4.3 ± 2.2^{++}
50.	(*Z*)-2-pentenal*		3.7 ± 0.9^{+++}			4.3 ± 2.3^{++}
51.	(*E*)-2-pentenal*		57.0 ± 7.6^{+++}	1.4 ± 0.6^{+++}	0.2 ± 0.2^{+}	40 ± 22^{+++}
52.	heptanal	0.7 ± 0.3^{+++}	6.3 ± 0.9^{+++}	0.90 ± 0.05^{+++}	1.0 ± 0.2^{+++}	8.8 ± 5.5^{+++}
53.	(*Z*)-2-hexenal*		1.3 ± 1.3^{+}			6.0 ± 6.0^{+}
54.	(*E*)-2-hexenal*	2.3 ± 1.5^{++}	174 ± 20^{+++}	20 ± 12^{+++}	7.0 ± 6.5^{++}	191 ± 114^{+++}
55.	4-heptenal *isomer*					3.3 ± 1.7^{++}
56.	methylthioacetaldehyde	1.0 ± 1.0^{++}	29.3 ± 8.3^{+++}	4.9 ± 4.1^{++}	1.8 ± 0.7^{+++}	94 ± 60^{+++}
57.	octanal*	2.3 ± 0.3^{+++}	5.6 ± 1.9^{+++}	6.7 ± 6.2^{++}	3.3 ± 1.9^{+++}	6.8 ± 3.2^{+++}
58.	(*E*)-2-heptenal		7.3 ± 2.4^{+++}			4.3 ± 2.2^{++}
59.	2-octenal *isomer*					2.3 ± 1.2^{++}
60.	2,4-heptadienal *isomer*		34.0 ± 2.5^{+++}	2.0 ± 2.0^{+}	0.3 ± 0.3^{+}	22 ± 10^{+++}
61.	2,4-heptadienal *isomer*		45.7 ± 7.2^{+++}	5.8 ± 5.1^{++}		27 ± 15^{++}
62.	2-nonenal *isomer*				0.6 ± 0.6^{+}	1.8 ± 1.8^{+}
63.	phenylacetaldehyde					2.3 ± 0.9^{+++}
Esters						
64.	(Z)3-hexen-1-ol isovalerate*		1.2 ± 0.2^{+++}			
Isothiocyanates (ITC)						
65.	methyl ITC*	1.0 ± 0.5^{+++}	15.6 ± 6.7^{+++}	5.3 ± 2.3^{+++}	0.6 ± 0.2^{+++}	32 ± 18^{+++}
66.	but-3-en-1-yl ITC			2.7 ± 1.3^{+++}		
67.	allyl ITC		3.3 ± 3.3^{+}	12 ± 10^{+++}		1.6 ± 1.2^{++}
Sulfides						
68.	dimethyl disulfide*	5.0 ± 0.6^{+++}	303 ± 84^{+++}	113 ± 45^{+++}	3.9 ± 1.3^{+++}	267 ± 194^{+++}
69.	methylvinyldisulfide		2.7 ± 0.9^{+++}			9.3 ± 4.8^{++}
70.	dimethyl trisulfide*	8.0 ± 3.6^{+++}	745 ± 327^{+++}	57 ± 25^{+++}	5.8 ± 2.6^{+++}	831 ± 588^{+++}
71.	methyl(methylthio)methyl disulfide		11.3 ± 5.8^{+++}	0.2 ± 0.2^{+}		15.7 ± 12.3^{+++}
72.	2,4-dithiopentane			0.7 ± 0.3^{+++}		
Nitriles						
73.	2,4-pentadienenitrile *isomer*			4.1 ± 2.5^{+++}		
74.	2,4-pentadienenitrile *isomer*			7.3 ± 3.8^{+++}		
75.	4-methylthiobutanenitrile			9.3 ± 5.3^{+++}		

TABLE 5. Continued

	Compounds	*P. rapae*			*P. brassicae*	
		Second instars, normal (N = 3)	Second instars, wet (N = 3)	Fourth instars, wet (N = 3)	Second instars, normal (N = 3)	Second instars, wet (N = 3)
Furanoids						
76.	2,4-dimethylfuran		1.3 ± 0.7''			
77.	2-ethylfuran*					0.5 ± 0.3''
Terpenoids						
78.	linalool		1.2 ± 0.4'''			0.9 ± 0.7''
79.	safranal		4.0 ± 0.6'''			
80.	β-cyclocitral	2.1 ± 0.5'''	114.0 ± 9.2'''	10.1 ± 6.6'''	3.4 ± 2.4'''	65 ± 34'''
Pyridines						
81.	2,4-dimethylpyridine			4.3 ± 2.3''		
82.	2,4,6-trimethylpyridine	0.2 ± 0.2'	13.7 ± 7.8'''	29 ± 24'''	0.3 ± 0.3'	2.1 ± 1.5''
83.	2-ethyl-4,6-dimethylpyridine			0.7 ± 0.6''		
Unknown						
84.	Unknown 1		14.3 ± 1.5'''	1.2 ± 0.9''	0.3 ± 0.3'	9.1 ± 4.5'''
85.	Unknown 2	2.5 ± 1.3'''	196 ± 18'''	14.7 ± 9.8'''	2.4 ± 1.1'''	125 ± 70'''
Total peak area (mean ± SE)		62 ± 9	2633 ± 917	746 ± 483	64 ± 56	2515 ± 1949

"The number after each compound represents the average peak area (±SE) in samples of the same treatment. Depending on the chemical nature of compounds, one unit of peak area is approximately equal to 0.3–0.5 ng [N = number of samples analysed in each treatment; + = presence of compound in one sample; ++ = present in 2 samples; +++ = present in all 3 samples; * = presence of compound in the volatile blend released by Brussels sprouts infested by *P. brassicae* (Mattiacci et al., 1994), *isomer* = the identity of the geometric isomer of the compound is unknown].

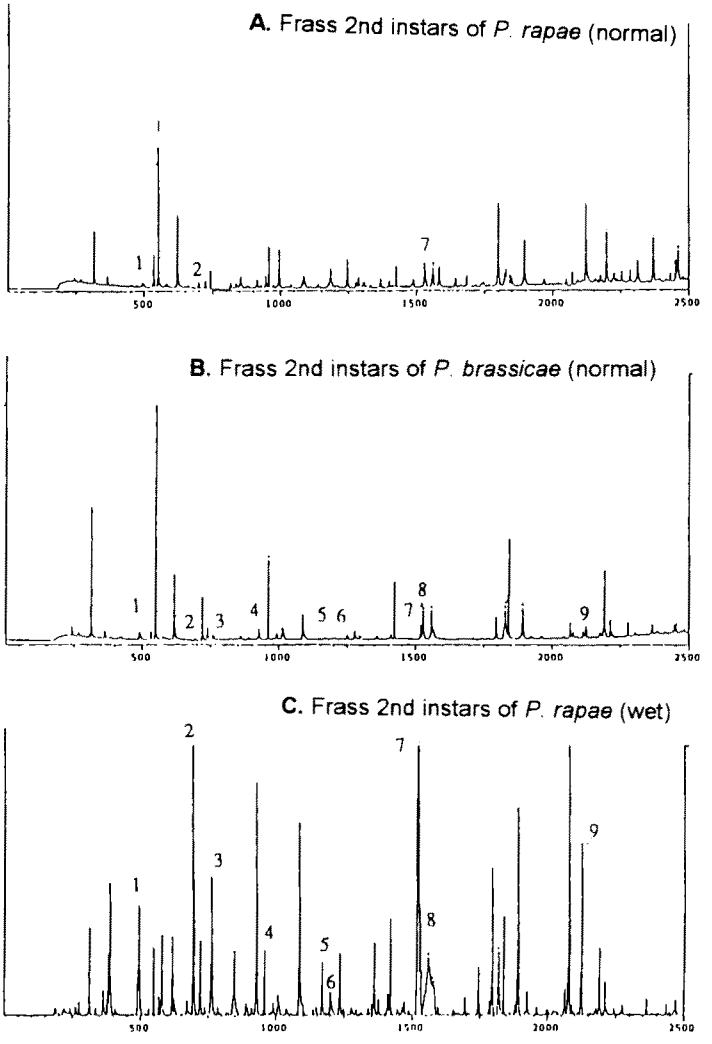

FIG. 1. Chromatograms of volatiles emitted by feces of second instars of *P. rapae* [(A) normal, (C) wet], second instars of *P. brassicae* [(B) normal, (D) wet], and fourth instars of *P. rapae* [(E) wet]. Each chromatogram represents only one of the three samples analysed in each treatment. 1 = 2-pentanone, 2 = dimethyl disulfide, 3 = 2-methyl propanol, 4 = 1-penten-3-ol, 5 = 1-pentanol, 6 = methylthioacetaldehyde, 7 = dimethyl trisulfide, 8 = (*Z*)-3-hexen-1-ol, 9 = β-cyclocitral.

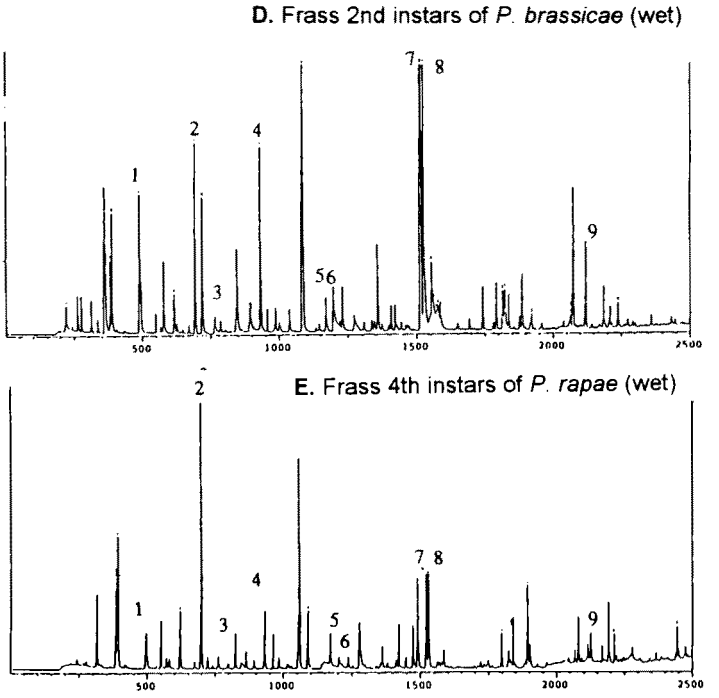

FIG. 1. Continued.

Wetting of samples not only increased the amount of compounds already present in normal samples but was also responsible for the appearance of new compounds. At present, it is not known if the appearance of new compounds is strictly related to reactions initiated by the presence of water or if those compounds were also present in normal feces but in undetectable amounts. The detection limit of the GC-MS used is approximately 0.1 ng.

Variation in the presence and concentration of some compounds between samples of the same treatment was recorded. Figure 3 shows the spectra of the unidentified compounds, unknown 1 and unknown 2.

DISCUSSION

Feces of Larvae and Their Influence on Host-Searching Behavior of Parasitoids. The host-seeking behavior of some parasitoid species is known to be influenced by feces of larvae from a distance (Lecomte and Thibout, 1986, 1993; Eller et al., 1988a,b; Elzen et al., 1987; Ding et al., 1989; Ramachandran

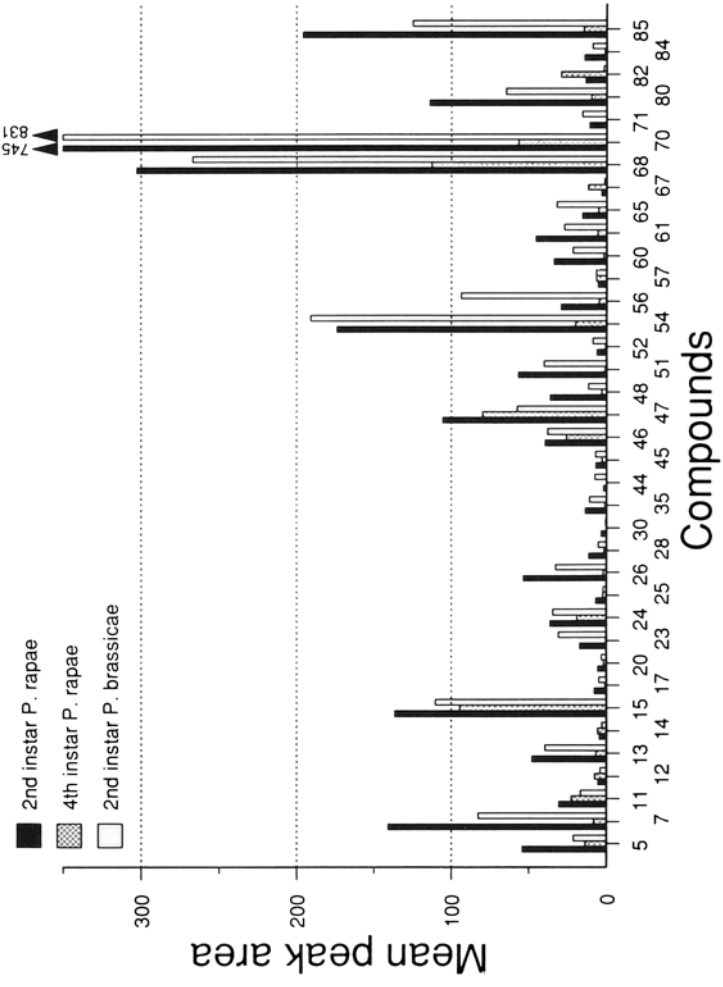

FIG. 2. Relative concentration (mean peak area) of compounds common in the treatments: second instar *P. rapae* (wet feces of second instars of *P. rapae*), fourth instar *P. rapae* (wet feces of fourth instars of *P. rapae*), second instar *P. brassicae* (wet feces of second instars of *P. brassicae*). Compound numbers are the same as in Table 5.

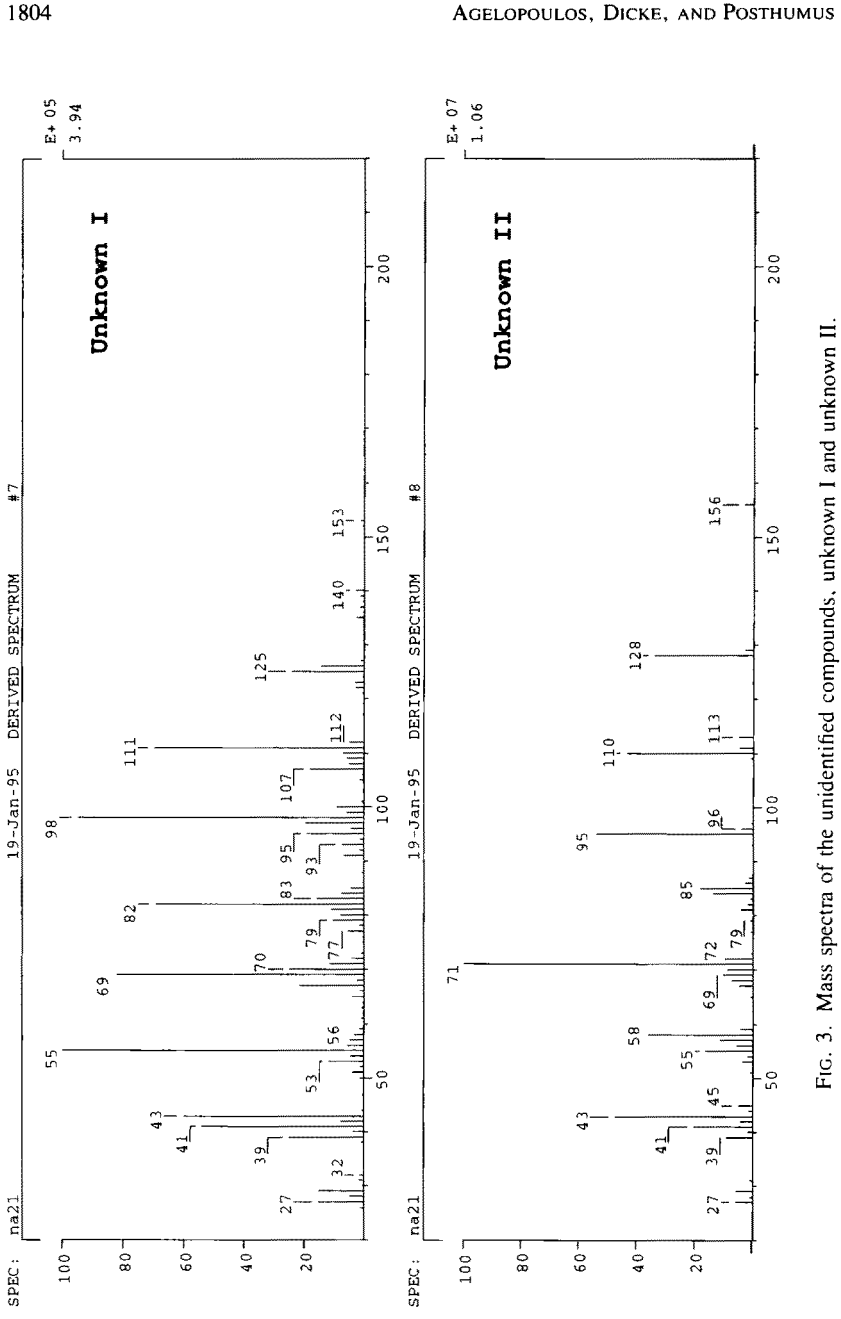

FIG. 3. Mass spectra of the unidentified compounds, unknown I and unknown II.

TABLE 6. COMPOUNDS PRESENT ONLY IN ONE OF THE FOLLOWING CLASSES: FECES OF SECOND INSTARS OF *P. rapae* (WET OR NORMAL), FECES OF FOURTH INSTARS OF *P. rapae*, AND FECES OF SECOND INSTARS OF *P. brassicae* (WET OR NORMAL)

Second instars of *P. rapae*	Fourth instars of *P. rapae*	Second instars of *P. brassicae*
(*Z*)-3-Hexen-1-ol isovalerate (wet)[a]	3-Methyl-2-pentanol	Methanol (wet)
2,4-Dimethylfuran (wet)	2-Methyl-1-butanol	2-Propanol (wet)
Safranal (wet)	(*E*)-2-Hexen-1-ol	Ethanol (normal, wet)[a]
	2-Methylthioethanol	2-Butanone (normal, wet)
	But-3-en-1-yl isothiocyanate	3-Methyl-2-butanone (wet)
	2,4-Dithiopentane	3-Hexanone (wet)
	2,4-Pentadienenitrile *isomer*	3-Nonen-2-one *isomer* (wet)
	2,4-Pentadienenitrile *isomer*	Dihydrofuranone (wet)
	4-Methylthiobutanenitrile	Acetaldehyde (wet)
	2,4-Dimethylpyridine	4-Heptenal *isomer* (wet)
	2-Ethyl-4,6-dimethylpyridine	2-Octenal *isomer* (normal, wet)
		2-Nonenal *isomer* (normal, wet)
		Phenylacetaldehyde (wet)
		2-Ethylfuran (wet)[a]

[a] Presence of compound in the volatile blend released by Brussels sprouts plants infested by *P. brassicae* (Mattiacci et al., 1994).

et al., 1991; Turlings et al., 1991; Cortesero et al., 1993; Steinberg et al., 1993; Agelopoulos and Keller, 1994a; Geervliet et al., 1994) and upon contact (Lewis and Jones, 1971; Sato, 1979; Nordlund and Sauls, 1981; van Leerdam et al., 1985; Nordlund and Lewis, 1985; Ding et al., 1989; Fukushima et al., 1989; Takabayashi and Takahashi, 1989; Mattiacci and Dicke, 1995). Factors related to production of feces, such as species of larvae (Lewis and Jones, 1971; Thibout et al., 1993; Agelopoulos and Keller, 1994a; present study), species of plant that larvae feed on (Nordlund and Sauls, 1981; Ramachandran et al., 1991), developmental stage of larvae (Mattiacci and Dicke, 1995; present study), and moisture content of feces (Sato 1979; van Leerdam et al., 1985; present study) are known to influence the behavior of female parasitoids from a distance and upon contact.

The variation in percentage of attraction of females during the various tests conducted here is not uncommon for the population of *C. rubecula* kept in our laboratory. Females of that population exhibit an average attraction of 50% to host-infested plants, varying from 20% to 95%, depending on the day of testing (Geervliet et al., 1994, 1995; N.G. Agelopoulos personal observations). Variation in attraction to host-infested plants has also been observed for a Dutch population of *C. glomerata* (Steinberg et al., 1993). The reason behind the observed variation in the responses of *C. rubecula* is not known, although

decreasing barometric pressure has been shown to be one of the factors affecting the response of females of *C. glomerata* (Steinberg et al., 1993). Because of the day-to-day variation in percentage of wasps that fly upwind to the odor source, the data for the different odor sources in Table 1 (different experimental days per treatment) cannot be compared to draw conclusions on the relative attraction towards different feces. This can only be done with the data in Table 3.

Plant Origin of Chemicals in Feces. Indications of the plant origin of chemicals present in feces of larvae have been abundant in studies related to behavior of parasitoids. The differential responses of parasitoids to feces produced after feeding of host larvae on different plants (van Leerdam et al., 1985), the nonattraction of parasitoids to feces produced after feeding of larvae on artificial diet (Nordlund and Lewis, 1985; Ding et al., 1989; Ramachandran et al., 1991), and the attraction of parasitoids to feces of nonhost larvae feeding on hostplants (Lewis and Jones, 1971; Thibout et al., 1993; Agelopoulos and Keller, 1994a) are indicative of the involvement of the plant in the chemical composition of feces. Identification of volatiles released by feces of different species of larvae has revealed the plant origin of those compounds (Auger et al., 1989; Ramachandran et al., 1991; Agelopoulos and Keller, 1994c). Our chemical study further supports the plant origin of compounds emitted by feces, since most of the volatiles emitted by feces of *P. rapae* and *P. brassicae* have previously been reported in cruciferous plants (Bailey et al., 1961; Buttery et al., 1976; Wallbank and Wheatley, 1976; Cole, 1976, 1980a,b; Tollsten and Bergström, 1988; Evans and Allen-Williams, 1992; Blaakmeer et al., 1994; Mattiacci et al., 1994). The presence of some of the volatiles emitted by feces may be related to breakdown processes of plant material after digestion by larvae and the presence of others may be the end product of processes related to microorganisms inhabiting the feces (Thibout et al., 1993).

Agelopoulos and Keller (1994c) identified volatiles from "normal" frass of *P. rapae* larvae on a *capitata* variety of cabbage and recorded eight compounds, five of which are also recorded here. One of these five compounds is identified here as methylthioacetaldehyde on the basis of mass spectrum and chromatographic behavior and has the same mass spectrum as the compound labeled as methyl propyl sulfide by Agelopoulos and Keller (1994c). The spectrum has small, but significant differences from that of methyl propyl sulfide. Our analyses show that moistening of the feces greatly increases the amount of chemicals recorded.

Specificity Related to Volatiles Emitted by Feces of Different Species and Instars of Larvae. The chemical profile emitted by feces of *P. rapae* and *P. brassicae* produced after feeding of larvae on Brussels sprouts plants is species specific in both quantity and quality. Species specificity related to the blend of chemicals released by feces of larvae has also been observed for

P. rapae and *Plutella xylostella* (Lepidoptera: Yponomeutidae) (Agelopoulos and Keller, 1994c). An instar specificity, in both quality and quantity, is also exhibited in the blend of chemicals emitted by feces of second and fourth instars of *P. rapae*. The quantitative differences consist of a higher emission of chemicals in feces of second instars than of fourth instars. This may be due to higher evaporation from the feces of second instars. Pellets of feces of second instars are much smaller than those of fourth instars, and consequently the number of pellets in 250 mg of feces of second instars is greater than that in the same amount of feces of fourth instars; this increases the surface area of evaporation in feces of second instars. If higher evaporation causes the greater emission of chemicals from feces of second instars, the quantitative differences between the two will be eliminated as the amount of feces of fourth instars increases. This is possible since fourth instars produce 10 times more feces than second instars. The species and instar specificity reported in feces may be due to different digestion processes of larvae and to different microorganisms inhabiting the feces of larvae.

Effects of Volatiles Released by Feces of Larvae in Host-Searching Behavior of C. rubecula. Females of *C. rubecula* are attracted to both infested plants and feces of larvae. Many volatiles emitted by feces of *P. rapae* and *P. brassicae* are also present in the blend of chemicals emitted by Brussels sprouts plants infested by either of the two species of larvae (Blaakmeer et al., 1994; Mattiacci et al., 1994). Nevertheless, some groups of chemicals appear only in one of the two infested plants or feces. For example, esters are a predominant group of chemicals related to plant damage caused by larvae and are hardly represented in feces. The same holds true for terpenoids, which are mainly emitted by infested or uninfested Brussels sprouts plants. On the other hand, pyridines are only present in feces and are totally absent in the volatiles released by plants. The question arises as to whether the chemicals utilized by *C. rubecula* to orient to feces are chemicals common to both infested plants and feces or a combination of common and specific chemicals.

The results of our bioassays showed that during landing leaves carrying large amounts of plant damage were preferred over those carrying less damage and small amounts of feces. Although landing manifests the final preference of females, it does not convey information about the different sources of information employed from a distance or while flying before landing. Specific chemicals in feces may be utilized from a distance in discriminating between plants infested by different species of *Pieris* or instars of the host. Upon landing, plant chemicals related to plant damage may be more important, because plant damage relates to the position of the larvae while feces rarely do. Fecal pellets rarely are present on the leaf of production; in most cases they fall, contaminating the lower parts of the plant and the surrounding ground. The position of feces on or around the plant can be influenced by factors such as the orientation and

structure of leaves and plant, hygienic behavior of the larvae (Usher, 1984), factors shaking the plant (wind, storms, passing animals), the size of fecal pellet (larger pellets tend to roll more easily than smaller ones), and evaporation. When fecal pellets are produced, they are more sticky than when dry.

C. rubecula also favored wet over normal feces of its host. Moisture is one of the factors increasing the release of volatile compounds from feces, and high humidity related to climatic or microclimatic conditions (e.g., dense foliage), morning dew, or rain may increase the volatile emission of feces in favor of the parasitoid.

Although at present we have a good knowledge of the identity of volatiles that may be involved in orientation to host-infested plants, much more work has to be done to understand which of those chemicals are the most reliable indicators of host presence and to understand how females of *C. rubecula* cope with the great variability associated with the chemical information available in every moment of their search for hosts.

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IDENTIFICATION OF SEX ATTRACTANT PHEROMONE COMPONENTS OF THE TUSSOCK MOTH, *Euproctis taiwana* (SHIRAKI) (LEPIDOPTERA: LYMANTRIIDAE)

TETSUYA YASUDA,* SADAO WAKAMURA, and NORIO ARAKAKI

Department of Insect Physiology and Behavior
National Institute of Sericultural and Entomological Science,
Tsukuba, Ibaraki 305, Japan

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Abstract—Two compounds were isolated and identified from abdominal tips of the female tussock moth, *Euproctis taiwana* (Shiraki), by a combination of gas chromatography–electroantennographic detection, coupled gas chromatography–mass spectrometric analysis, microreaction, and synthesis. These compounds were (*Z*)-16-methyl-9-heptadecenyl isobutyrate (74.8%) and 16-methylheptadecyl isobutyrate (25.2%). Their total amount was determined to be ca. 29.8 ng/female. Field bioassays showed that (*Z*)-16-methyl-9-heptadecenyl isobutyrate is a sex attractant for the male moths. These chemical compounds had not previously been found as the sex pheromone components in the genus *Euproctis*.

Key Words—Tussock moth, *Euproctis taiwana*, sex attractant pheromone, (*Z*)-16-methyl-9-heptadecenyl isobutyrate, 16-methylheptadecyl isobutyrate.

INTRODUCTION

The tussock moth, *Euproctis taiwana* (Shiraki), distributed in the Okinawan region of Japan and Taiwan, is polyphagous and a serious pest of a variety of crops. These include guava, mango (Azuma, 1968), rose (Wang, 1982a), gladiolus (Wang, 1982b), loquat (Miyatake et al., 1990), kidney beans, tea, cabbage, and corn. Furthermore, *Euproctis* larvae are covered with bristles that can lead to severe dermatitis when they contact human skin (Ogata, 1958; Su, 1981).

*To whom correspondence should be addressed.

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In the genus *Euproctis*, sex pheromones have been identified for *E. chrysorrhoea* (Leonhardt et al., 1991), *E. psudoconspersa* (Wakamura et al., 1994), *E. similis similis* (Yasuda et al., 1994), and *E. similis xanthocampa* (Tan et al., 1984), but no common component has been found in the sex pheromones of these species of *Euproctis*.

This paper describes the isolation and identification of the female sex attractant pheromone of *E. taiwana*.

METHODS AND MATERIALS

Insects. In May 1993, *Euproctis taiwana* larvae were collected from fields of cabbage and kidney beans, Naha City, Okinawa Prefecture, Japan. The larvae were reared on an artificial diet at 25°C under a 16L:8D photoperiod.

Preparation of Pheromone Extracts. Abdominal tips were excised from 2-day-old virgin females 1 hr into photophase and extracted with hexane for 30 min at room temperature. The extracts were filtered through a piece of absorbent cotton, accumulated, and stored below −30°C until used.

Column Chromatography. For separation of the extracts, column chromatography on Florisil was carried out (Carroll, 1961). Components were successively eluted with hexane and 5%, 10%, and 50% ether in hexane.

For separation of geometric isomers, column chromatography on silica gel impregnated with silver nitrate ($AgNO_3$: 16.7%) ($AgNO_3$–SiO_2 chromatography) was used. Components were successively eluted with hexane and 1%, 2%, 3%, 4%, 5%, 7%, and 10% ether in hexane.

Gas Chromatograph–Electroantennographic Detector (GC-EAD). The fractions from Florisil chromatography were analyzed by a GC-EAD system (Struble and Arn, 1984). Gas chromatography was performed with a Hewlett-Packard HP 5890 Series II gas chromatograph with a DB-WAX column (polyethylene glycol 20M, 30 m × 0.25 mm ID × 0.25-μm film thickness) and a flame ionization detector (FID) and electroantennographic detector (EAD). FID and EAD responses were simultaneously recorded. The column temperature was set at 50°C for 1 min, programmed at 20°C/min to 150°C, and then programmed at 5°C/min to 220°C and held for 10 min. Helium was the carrier gas.

Gas Chromatograph–Mass Spectrometer (GC-MS). GC-MS analyses were conducted with a JEOL JMS-SX102A mass spectrometer combined with a Hewlett-Packard HP 5890 Series II gas chromatograph using the following columns: DB-1 (100% dimethyl polysiloxane, 30 m × 0.25 mm ID × 0.25-μm film thickness), HP-5 (5% diphenyl–95% dimethyl polysiloxane, 30 m × 0.25 mm ID × 0.33-μm film thickness), DB-WAX and DB-23 (50% cyanopropyl polysiloxane, 30 m × 0.25 mm ID × 0.25-μm film thickness). The column temperature conditions were the same as those in the GC-EAD system. Helium was

the carrier gas. High-resolution mass spectra (HRMS) were obtained by the same system.

Chemical Reactions. The monounsaturated components were hydrogenated with diimide to determine the acid moieties (Yamaoka et al., 1976). The diimide reduction products were analyzed by the GC-MS system. Esters of 1-octadecanol were used as authentic standards.

Microozonolysis was used to determine the double-bond positions of alcohol moieties (Beroza and Bierl, 1967). Oxygen with ozone was blown into a hexane solution of the sample below −50°C for 1 min, and it was then immediately injected into the GC-MS system.

Naturally occurring esters were hydrolyzed in 1% KOH in ethanol at 50°C for 22 hr. After evaporation of the solvent, the residue was extracted with hexane and poured on a silica gel column. Compounds were successively eluted with hexane and 5%, 10%, 25%, and 50% ether in hexane. A part of the 25%-ether-in-hexane fraction, which contained alcohols, was concentrated and chlorinated with methanesulfonyl chloride and dimethylformamide at 65°C for 16 hr (Evans et al., 1968). The reaction mixture was then placed directly onto a silica gel column, and the chloride derivatives were eluted with hexane and then injected into the GC-MS system with the DB-WAX column.

Synthesis. 8-Methyl-1-nonyne was synthesized from 1-bromo-3-methylbutane and 2-propyn-1-ol following the methodology of Naoshima et al. (1990). (*Z*)-16-Methyl-9-heptadecen-1-ol (16Me-Z9-17:OH) was synthesized from 8-methyl-1-nonyne and 8-bromo-1-octanol following the methodology of Hendry et al. (1975). 16-Methyl-1-heptadecanol (16Me-17:OH) was prepared from methyl 16-methylheptadecanoate by reduction with lithium aluminum hydride. Corresponding esters were obtained from 16Me-Z9-17:OH and 16Me-17:OH reacted with acid anhydride. (*Z*)-16-Methyl-9-heptadecenyl isobutyrate (16Me-Z9-17:iBu) was purified by $AgNO_3$–SiO_2 chromatography.

Field Bioassay. Field tests were conducted at the Okinawa Prefectural Agricultural Experiment Station, Naha City, using sticky traps (SE trap, Sankei Chem. Co.). Traps were placed 0.5–1.5 m above ground at intervals greater than 5 m. Hexane solutions of test samples were impregnated into a piece of filter paper (2 × 5 cm, Advantec No. 2) and placed into the trap. The reference trap was baited with three 3-day-old virgin females. Trap data ($x + 0.5$) were subjected to square-root transformation before analysis of variance, and Tukey's test was used for comparing means where the variance analysis (general linear models procedure) indicated a significant F value (SAS Institute, 1988).

RESULTS

The accumulated extract of 262 virgin females was fractionated with the Florisil column. Each fraction was checked by the GC-EAD analysis at 1 female equivalent. The 5%-ether-in-hexane fraction showed two EAD-active peaks

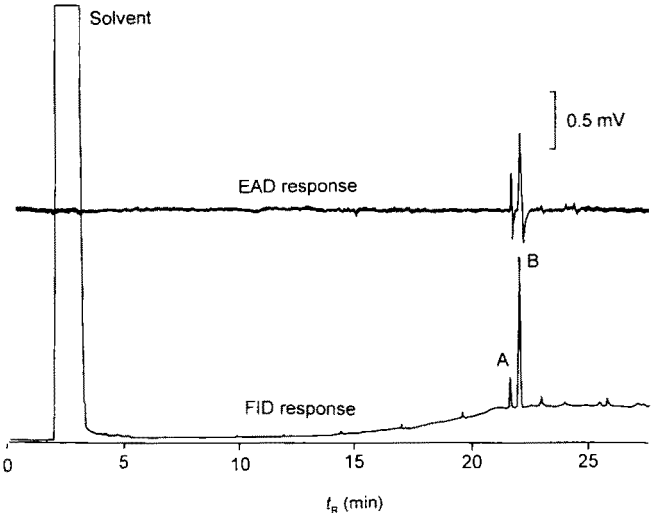

FIG. 1. GC-EAD profile of female gland extract of *Euproctis taiwana*. One female equivalent of 5% ether-in-hexane fraction from Florisil column was injected. GC: DB-WAX, operated at 50°C for 2 min, programmed at 20°C/min to 150°C, and then programmed at 5°C/min to 220°C, where it was kept for 10 min.

(Figure 1, peaks A and B). The ratio of the areas of these two compounds was 1:3, and the total amount was estimated to be ca. 29.8 ng/female by GC analysis. GC-MS analysis with the DB-WAX column of the 5% fraction led to an estimate that compound A was an isomer of an 18-carbon saturated alcohol ester of a 4-carbon carboxylic acid, which showed the following characteristic fragment ions: *m/z* 89 ($C_3H_7CO_2$ + 2H, base peak), 252 (M−88, 29%) and 340(M^+, 32%) (HRMS found: 340.3338, calcd. for $C_{22}H_{44}O_2$: 340.3341). Compound B was also estimated to be an isomer of an 18-carbon monounsaturated alcohol ester of a 4-carbon carboxylic acid, which showed the following characteristic fragment ions: *m/z* 82 (base peak), 89 ($C_3H_7CO_2$ + 2H, 44%), 250 (M−88, 58%) and 338 (M^+, 2%) (HRMS found: 338.3126, calcd. for $C_{22}H_{42}O_2$: 338.3185).

The 5% fraction was further chromatographed by $AgNO_3$–SiO_2 chromatography to confirm the degree of unsaturation of the components. Compound A was eluted in 2%-ether-in-hexane fraction and compound B was eluted in the 4%-, 5%-, and 7%-ether-in-hexane fractions.

One peak was detected in the products of diimide reduction of compound B, the 5% fraction of $AgNO_3$–SiO_2 chromatography, by GC-MS analysis with the DB-WAX column. This compound was shown to be identical to compound

A by cochromatography with the 2% fraction from $AgNO_3$-SiO_2 chromatography and by GC-MS analysis. Furthermore, the product of diimide reduction of compound B showed a clear EAD response.

The retention time of compound A on DB-1 column was, however, lower than on those of authentic esters possessing a straight chain of the corresponding number of carbon atoms in the alcohol moiety. This indicated that the compound had alkyl branch(es) in the alcohol moiety. The alkyl chloride derived from the ester in the 2% fraction from $AgNO_3$-SiO_2 chromatography after hydrolysis and subsequent chlorination was analyzed with the HP-5 column. One peak was observed at 16.83 min. The mass spectrum of this compound showed a molecular ion at *m/z* 288 (15%) and an M+2 ion at *m/z* 290 (5%), which indicated that this compound contained 18 carbon atoms and one chlorine atom, that is, it was derived from the alcohol moiety of compound A. Diagnostic fragment ions were observed at *m/z* 245 (M−43, 74%) and 273 (M−15, 34%), which were accompanied by the isotopic ions at *m/z* 247 (23%) and 275 (11%), respectively. This suggested the presence of one methyl branch at the position 16 of the 17-carbon chain. This was confirmed by GC-MS analysis of authentic 1-chloro-16-methylheptadecane. The mass spectrum and retention time of compound A were identical to those of synthetic 16-methylheptadecyl isobutyrate (16Me-17:iBu) in subsequent GC-MS analysis, but did not fit with those of synthetic 16-methylheptadecyl *n*-butyrate. Compound A was, therefore, identified as 16Me-17:iBu.

GC-MS analysis with the DB-WAX column of reductive ozonolysis products of the 5% fraction from $AgNO_3$-SiO_2 chromatography showed two major compounds: a 9-carbon aldehyde and 8-formyloctyl isobutyrate. These indicated the double bond at carbon 9 in the 18-carbon alcohol moiety of compound B. Furthermore, the alkyl chlorides derived from the products of diimide reduction of compound B showed properties identical to those of compound A by GC-MS analysis. Compound B was estimated to be 16-methyl-9-heptadecenyl isobutyrate on the basis of these microreactions.

The synthetic compounds were analyzed by GC-MS with the DB-23 column. The retention time of compound B fit well with synthetic (8)-16-methyl-9-heptadecenyl isobutyrate (16Me-Z9-17:iBu), and (*E*)-16-methyl-9-heptadecenyl isobutyrate did not fit with compound B. The mass spectrum of synthetic 16Me-Z9-17:iBu was identical to that of compound B. Therefore, compound B was identified as 16Me-Z9-17:iBu.

The mixture of the two synthetic esters at the natural ratio was analyzed by the GC-EAD. This showed two clear EAD responses to the synthetic mixture, which were similar to those occurring when the 5% fraction from Florisil chromatography was injected.

Field tests were conducted to determine the activity of synthetic 16Me-Z9-17:iBu (Table 1). More *E. taiwana* males were caught with 16Me-Z9-17:iBu

TABLE 1. TRAP CATCHES OF MALE *Euproctis taiwana* WITH (Z)-16-METHYL-9-HEPTADECENYL ISOBUTYRATE (16ME-Z9-17:IBU) (OCTOBER 18–20, 1994, NAHA)[a]

16Me-Z9-17:iBu (μg/filter paper)	No. of males caught/trap/night (mean ± SE)[b]
0	0.0 ± 0.0 a
30	0.2 ± 0.2 a
100	0.8 ± 0.6 a
300	0.7 ± 0.7 a
1000	0.3 ± 0.3 a
3VF[c]	0.0 ± 0.0 a

[a]SE sticky traps were used.
[b]Values indicated are means and standard errors with three traps for two nights. Data were transformed to $\sqrt{(x + 0.5)}$ and submitted to analysis of variance. The means followed by the same letter in the same column are not significantly different at 5% level by Tukey's test.
[c]Three 3-day-old virgin females.

alone than with three 3-day-old females, but the difference was not statistically significant. The male catch increased when the dose was increased from 30 μg to 100 μg. These results suggest that this is the optimum dose level for male attraction.

Field tests were also conducted to determine the activity of synthetic 16Me-17:iBu, 16Me-Z9-17:iBu, or their blends (Table 2). From June 22 to July 4, males were caught with three 3-day-old females and also with 300 μg of 16Me-Z9-17:iBu alone. No significant increase or decrease was observed when 10, 100, or 200 μg of 16Me-17:iBu was added to 300 μg of 16Me-Z9-17:iBu. From October 20 to 22, no males were caught with 100 μg of 16Me-17:iBu alone, although many males were caught with 300 μg of 16Me-Z9-17:iBu alone or a blend of 300 μg of 16Me-Z9-17:iBu and 100 μg of 16Me-17:iBu.

DISCUSSION

Two compounds were identified as possible sex pheromone components in *Euproctis taiwana*. The major component was characterized as 16Me-Z9-17:iBu and the minor one as 16Me-17:iBu. These two components have not previously been reported as natural products. Field bioassays showed that 16Me-Z9-17:iBu is essential to attract male *E. taiwana* moths. Synergistic effects on male attraction by 16Me-17:iBu were not apparent in the field tests (Table 2), although 16Me-17:iBu was EAG-active. Further studies are necessary to check the behavioral activity of the minor components and to develop an effective synthetic

TABLE 2. TRAP CATCHES OF MALE *Euproctis taiwana* WITH (Z)-16-METHYL-9-HEPTADECENYL ISOBUTYRATE (16ME-Z9-17 : iBU), 16-METHYLHEPTADECYL ISOBUTYRATE (16ME-17 : iBU) OR THEIR BLENDS (1994, NAHA)[a]

Chemicals (μg/filter paper)		No. of males caught/trap (mean ± SE)[b]		
16Me-Z9-17 : iBu	16Me-17 : iBu	June 20–22	June 22–July 4	October 20–22
0	0			0.0 ± 0.0 a
10	3	0.0 ± 0.0 a		
30	10	0.2 ± 0.2 a		
100	30	0.2 ± 0.2 a		
300	0		3.7 ± 1.5 a	1.5 ± 0.8 a
300	10		3.3 ± 1.5 a	
300	100	3.7 ± 1.5 b	1.0 ± 0.0 a	1.0 ± 0.5 a
300	200		3.3 ± 3.3 a	
0	100			0.0 ± 0.0 a
3VF[c]		0.3 ± 0.2 ab	2.5 ± 0.0 a	0.0 ± 0.0 a

[a] SE sticky traps were used.
[b] Values indicated are means and standard errors with three traps. Data were transformed to $\sqrt{(x + 0.5)}$ and submitted to analysis of variance. The means followed by the same letter in the same column are not significantly different at 5% level by Tukey's test.
[c] Three 3-day-old virgin females.

TABLE 3. SEX PHEROMONE COMPONENTS IN GENUS *Euproctis*

Species	Components	Ratio (%)	Reference
Euproctis chrysorrhoea	(Z,Z,Z,Z)-7,13,16,19-docosatetraenyl isobutyrate	100	Leonhardt et al. (1991)
Euproctis psudoconspersa	10,14-dimethylpentadecyl isobutyrate	94.3	Wakamura et al. (1994)
	14-methylpentadecyl isobutyrate	5.7	
Euproctis similis similis	(Z)-7-octadecenyl 2′-methylbutyrate	53.5	Yasuda et al. (1994)
	(Z)-7-octadecenyl isovalerate	32.1	
	(Z)-7-octadecenyl isobutyrate	2.7	
	(Z)-7-octadecenyl *n*-butyrate	2.7	
	(Z)-9-octadecenyl 2′-methylbutyrate	4.3	
	(Z)-9-octadecenyl isovalerate	4.8	
Euproctis similis xanthocampa	(Z)-7-octadecenyl isovalerate	92.0	Tan et al. (1984)
	6-octadecenyl isovalerate	1.4	
	6-octadecenyl *n*-butyrate	1.6	
	9-octadecenyl isovalerate	5.0	
Euproctis taiwana	(Z)-16-methyl-9-heptadecenyl isobutyrate	74.8	This paper
	16-methylheptadecyl isobutyrate	25.2	

lure for monitoring field populations and/or use as a tool in integrated pest management strategy.

In the genus *Euproctis*, sex pheromones have already been identified for three species and one subspecies (Table 3). As volatile sex pheromones, these chemical compounds have relatively high molecular weights. The alcohol moieties of the esters identified from *Euproctis* include 16-, 17-, 18- or 22-carbon alcohols that includes saturated or unsaturated and straight or methyl-branched chains. The acid moieties of the esters are derived from 4- or 5-carbon organic acids.

In many closely related species of Lepidoptera, the sex pheromones include one or more common components (Arn et al., 1992). For example, 2-methylheptadecane is one of the sex pheromone components in the genus *Holomelina* (Arctiidae) and (*Z,E*)-9,12-tetradecadienyl acetate has been identified as a common component of sex pheromones in many species of the genus *Spodoptera* (Noctuidae). However, no such common compound has been found in the genus *Euproctis*.

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ELECTROANTENNOGRAPHIC AND COUPLED GAS CHROMATOGRAPHIC-ELECTROANTENNOGRAPHIC RESPONSES OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*, TO MALE-PRODUCED VOLATILES AND MANGO ODOR

ALLARD A. COSSÉ,[1,*] JULIE L. TODD,[1] JOCELYN G. MILLAR, LEE ANN MARTÍNEZ,[2] and THOMAS C. BAKER[1]

Department of Entomology, University of California, Riverside, California 92521

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Abstract—We have identified five compounds from the headspace of calling male Mediterranean fruit flies (medfly), *Ceratitis capitata* (Wiedemann), and three compounds from the headspace of ripe mango (*Mangifera indica* L). using coupled gas chromatographic-electroantennographic (GC-EAG) recordings, coupled gas chromatographic-mass spectrometric (GC-MS) analysis, and electroantennographic (EAG) assays of standards. The male-produced volatiles eliciting responses from female antennae were ethyl-(*E*)-3-octenoate, geranyl acetate, (*E,E*)-α-farnesene, linalool, and indole. An EAG dose-response test of linalool enantiomers and indole with female medfly antennae showed relatively strong EAG activities, but no significant difference between (*R*)-(−)-linalool and (*S*)-(+)-linalool. The three mango volatiles were identified as (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene. In addition, a strong antennal response was recorded from a contaminant, α-copaene, present in a commercial sample of β-caryophyllene. The EAG response amplitudes from both male and female antennae to the above three mango volatiles were significantly greater than to a hexanol control. For both male and female medfly antennae, the greatest EAG responses were elicited by β-caryophyllene followed by ethyl octanoate. The mean EAG responses of female antennae to β-caryophyllene and (1*S*)-(−)-β-pinene were significantly greater than those of male antennae.

*To whom correspondence should be addressed.
[1] Present address: Department of Entomology, Iowa State University, Ames, Iowa 50011.
[2] Present address: Department of Biology, University of Southern Colorado, Pueblo, Colorado 81001.

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Key Words—Diptera, Tephritidae, Mediterranean fruit fly, *Ceratitis capitata*, pheromone, attractants, mango volatiles, *Mangifera indica*, headspace analysis, gas chromatography-electroantennography.

INTRODUCTION

The medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is a notorious pest because of its broad host range, attacking over 250 different types of fruit and vegetables, and its importance to international quarantine (Hagen et al., 1981). The most widely used lure in trapping programs for medfly detection is trimedlure, a synthetic compound primarily attractive to male medflies (Beroza et al., 1961). The lack of an effective female attractant has prompted several studies to examine the male sex pheromone and host odors as lures for attracting female flies. Several researchers have reported on the isolation, identification, or attractancy of male-produced volatiles from calling male medflies, resulting in a total of 56 identified compounds (R.R. Baker et al., 1985; P.S. Baker et al., 1990; Jang et al., 1989, 1994; Heath et al., 1991; Landolt et al., 1992a; Flath et al., 1993). The focus of these studies has been mainly on the attractiveness of the most abundant male odor components. A three-component blend was shown to have limited attraction for medfly females in wind-tunnel bioassays and in field tests (Heath et al., 1991; Landolt et al., 1992a). Recently, Jang et al. (1994) reported on the attractiveness of a five-component blend in wind-tunnel experiments, including the three components tested by Heath et al. (1991) and Landolt et al. (1992a); they found that this blend was still less attractive than the natural male pheromone. These results indicate that improvements in the synthetic male pheromone blend may be possible. Analysis of volatile compounds with coupled gas chromatographic–electroantennographic (GC-EAG) assays may help eliminate compounds with no biological activity and suggest compounds for behavioral studies. Furthermore, host odor volatile analysis by GC-EAG assay could identify compounds that, in combination with male pheromone, may further enhance female attraction to male pheromone, as was shown with the papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker (Landolt et al., 1992b).

By using GC-EAG and EAG recordings, we have examined the peripheral olfactory selectivity of female medflies to volatile compounds from males and from ripe mango in order to identify potentially attractive novel compounds. Volatiles of mango were analyzed because this fruit is a preferred host of several fruit fly species and the identity of many volatiles of intact mango have been determined (Engel and Tressl, 1983; MacLeod and Pieris, 1984; MacLeod and Snyder, 1985; Bartley and Schwede, 1987; Bartley, 1988; MacLeod et al., 1988). Our study represents the first use of the GC-EAG technique with medfly

and allowed us to pinpoint compounds present in trace amounts that may have gone undetected by using GC analysis and EAG recordings as separate techniques.

METHODS AND MATERIALS

Insects. Pupae were obtained from a medfly rearing facility operated by USDA-APHIS in Petapa, Guatemala. Upon arrival at the UCR Insect Quarantine Facility, the dyed (Day Glo Yellow) male and female pupae were placed in separate cages and held under natural light conditions. Adults flies used for the EAG and GC-EAG studies were fed honey and tested 3–30 days after emergence.

Collection of Male-Produced Volatiles. Extracts of volatiles produced by aeration of calling males were provided by R.R. Heath at the Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA-ARS, Gainesville, Florida, who used collection procedures previously described (Heath et al., 1991). Volatiles were collected from wild male medflies 5–10 days old in groups of 8–12 flies for 2-hr periods throughout the photophase. The males had access to a yeast hydrolysate food source, which was replaced with sugar water 24 hr prior to the collection of the volatiles. The male-produced volatiles were collected in glass tubes (4 cm long × 4 mm ID) containing 25 mg of Super-Q (Alltech Assoc. Inc., Deerfield, Illinois) placed between two stainless steel frits. The Super-Q traps were eluted with 200 μl of methylene chloride. Each sample was concentrated to ca. 50 μl under nitrogen prior to GC-EAG analysis.

Collection of Mango Fruit Volatiles. Ripe mangos (cv. Tommy Atkins) were obtained from a local supermarket and washed with water before being analyzed. A piece of mango peel with pulp attached (ca. 0.5 × 0.5 × 0.5 cm) was placed in a 25-ml fritted sparger, which was connected to a Tekmar 2000 purge and trap concentrator (Tekmar, Cincinnati, Ohio). The conditions for collecting volatiles on a Tenax trap were as follows: 5 min purge at room temperature (He, 20 ml/min), 5 min dry purge, and 4 min desorb at 180°C. The Tekmar 2000 was directly interfaced to the GC-EAG setup.

Chemical Analyses. One-microliter aliquots of the collected male-produced volatile extract were injected in splitless mode onto 30-m × 0.25-mm-ID fused silica capillary gas chromatographic columns, coated either with DB-5 or DB-1 (J & W Scientific, Folsom, California) or Ultra-2 (Hewlett-Packard Company, Palo Alto, California; equivalent to DB-5) for analyses by GC-EAG and GC–mass spectrometry (GC-MS). Column conditions were as follows: He carrier gas flow of 1.5 ml/min, injector temperature 200°C, oven temperature program 1 min delay on inlet purge, 2 min at 40°C, then 20°C/min to 220°C.

Mango volatiles were analyzed by interfacing the GC-EAG and GC-MS

with the Tekmar 2000 purge and trap collector, using the same columns with the following column conditions: He carrier gas flow of 1.5 ml/min, injector temperature 200°C, oven temperature program, 4 min at 0°C, 4 min at 40°C, 25°C/min to 220°C (GC-EAG); and injector temperature 250°C, oven temperature program, 4 min at 0°C, 10°C/min to 250°C (GC-MS).

GC-MS analyses were performed using a Hewlett-Packard 5890 gas chromatograph with a direct interface to a Hewlett-Packard 5970 mass selective detector (electron impact, 70 eV).

Electroantennogram Responses. Simultaneous GC-EAG and EAG analyses were performed as described by Baker et al. (1991) using a Varian model 3740 GC (Varian Associates, Sugarland, Texas). EAG recordings were made by placing a saline-filled (Beadle-Ephrussi Ringer) glass electrode in the back of an excised medfly head. The recording electrode was inserted into the tip of one of the antennae. To examine antennal sensitivity to the identified "minor" male-produced volatiles and to mango odor, EAGs, not coupled to GC, were recorded for a series of commercial or synthetic compounds. Serial dilutions of the tested compounds were made in redistilled HPLC-grade hexane such that the tested compounds were applied to filter-paper strips (0.5 × 3.0 cm, Whatman No. 1) in 10 μl of solvent. The filter-paper strips were placed inside Pasteur pipets (15 cm long). Control puffs of blank (filter paper plus solvent) and air (empty pipet) controls showed relatively low EAG activities, which were difficult to distinguish from the background activity. For this reason and to compensate for possible deterioration of the preparation, each 2-ml puff of test compound was preceded by a standard control compound, hexanol (10-μg dose). EAG amplitudes were standardized according to the responses to hexanol by dividing the amplitude of the EAG generated from the test compounds by that of hexanol. Within a particular series of test compounds, presentation of the test compounds was randomized. EAG data were subjected to ANOVA and mean responses were compared using a t test.

Chemicals. Racemic linalool, ethyl octanoate, indole, geranyl acetate, and (1*S*)-(−)-β-pinene were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin) and were >98% pure (label information and GC analysis). (*R*)-(−)-Linalool and β-caryophyllene were purchased from K & K Laboratories (Cleveland, Ohio). The β-caryophyllene sample contained 1% α-copaene based on GC-MS comparisons with authentic samples of α-copaene isolated from cubeb oil (Millar et al., 1986) and 10% α-humulene based on GC-MS comparisons with commercially available material (Fluka Chemical Company, Ronkonkoma, New York). (*S*)-(+)-Linalool was synthesized according to Landolt et al. (1994) and ethyl-(*E*)-3-octenoate was prepared as described by Heath et al. (1991). (*E*,*E*)-α-Farnesene was obtained from R.R. Heath, USDA, Gainesville, Florida.

RESULTS

Analysis of Volatiles Collected from Calling Males. A total of 30 combined GC-EAG analyses of the volatiles collected from calling male medflies by aeration was obtained using 30 different female medfly antennae. Only those GC peaks that consistently revealed EAG activity were targeted for further analysis. The combined GC-EAG analysis consistently revealed five GC peaks on both DB-1 and DB-5 columns with corresponding EAG activity with female medfly antennae (Figure 1, compounds I, II, III, IV, and V). The retention times on both columns and GC-MS spectra of compounds I–V corresponded precisely to those of five compounds known to be produced by male medfly: linalool, ethyl-(*E*)-3-octenoate, indole, geranyl acetate, and (*E*,*E*)-α-farnesene, respectively. Moreover, combined GC-EAG recordings on both DB-1 and DB-5 columns demonstrated that linalool, ethyl-(*E*)-3-octenoate, indole, geranyl acetate, and (*E*,*E*)-α-farnesene had retention times identical to compound I–V, respectively, from the collected male volatiles and were EAG active.

Standardized EAG dose responses revealed that 100 ng and higher dosages of indole and (*S*)-(+)-linalool, and 1 μg and higher dosages of (*R*)-(−)-linalool elicited stronger EAG responses in female antennae than hexanol (Figure 2). Indole (10 μg) generated responses 2.1 times (±0.4 SD) as great as those elicited by hexanol, (*S*)-(+)-linalool (10 μg) 1.5 times (±0.5 SD) as great, and (*R*)-(−)-linalool (10 μg) 1.4 times as great (±0.4 SD). The 10-μg dosages of indole generated significantly stronger responses than either (*R*)-(−)-linalool or (*S*)-(+)-linalool ($t = 2.61$, $P < 0.05$, $t = 2.15$, $P < 0.05$, respectively), and the 100-ng dosage of indole generated significantly stronger responses than (*R*)-(−)-linalool ($t = 5.12$, $P < 0.05$). However, there were no significant differences in the EAG responses to (*R*)-(−)-linalool and (*S*)-(+)-linalool for the dosages tested. The mean EAG response of female medfly antennae to hexanol was 0.4 mV (±0.5 SD, $N = 9$).

Analysis of Mango Volatiles. Analyses of trapped mango volatiles by coupled GC-EAG on both DB-1 and DB-5 columns revealed three compounds that consistently elicited strong antennal responses from female medflies (Figure 3). These compounds were identified as β-pinene, ethyl octanoate, and β-caryophyllene, respectively, by comparison of GC-MS retention times and mass spectra with those of authentic compounds. Moreover, combined GC-EAG recordings of (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene on the same two capillary columns demonstrated that these retention times were identical to compounds VI, VII, and VIII, respectively, from the collected mango volatiles and that all three compounds were EAG active (Figure 4). Closer examination of the GC-EAG responses obtained with a mixture of commercially available compounds identified from mango revealed an additional minor peak with EAG

activity labeled with a question mark in Figure 4. Mass spectral and retention time analyses of the β-caryophyllene sample revealed the presence of β-caryophyllene, α-copaene, and α-humulene. The identities of the latter two compounds were confirmed by comparison of retention times and mass spectra with authentic standards. Combined GC-EAG analysis of β-caryophyllene consistently revealed that α-copaene elicited strong EAG responses in addition to the

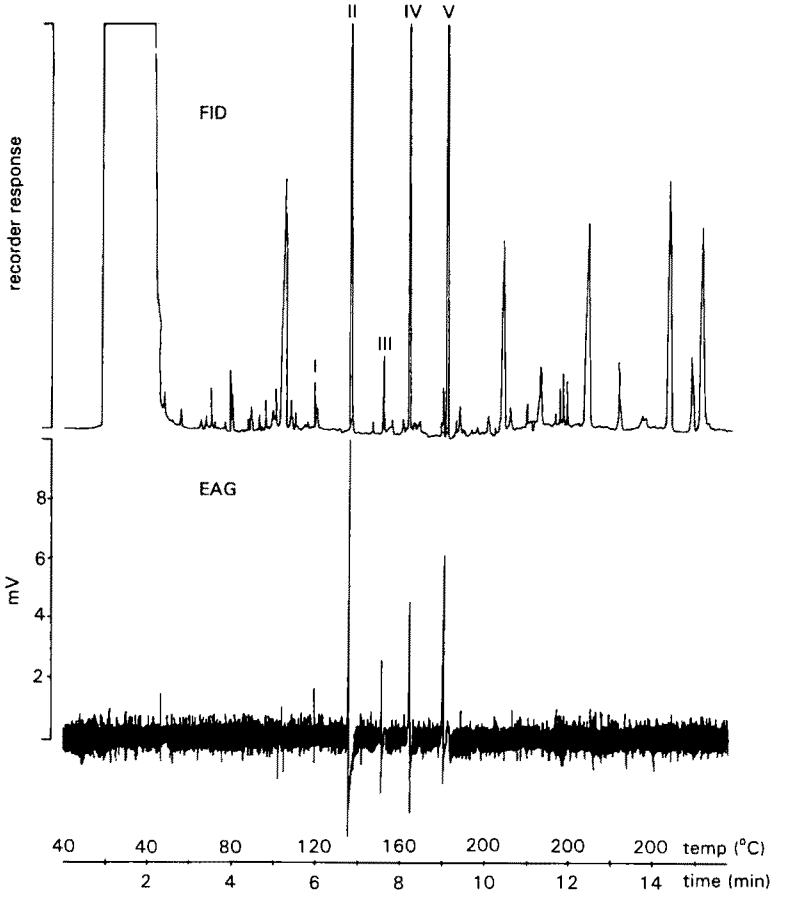

FIG. 1. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of trapped volatiles of calling male medflies (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled I, II, III, IV, and V (see text for details).

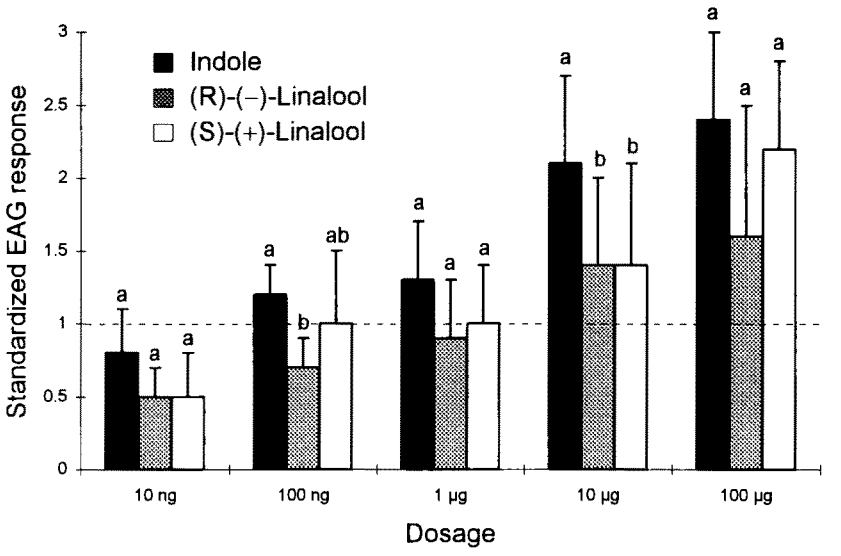

FIG. 2. EAG dose responses of female medfly antennae to selected known volatile compounds released by calling males. Stimuli were commercial or synthetic standards. Standard deviations of mean responses are presented as error bars. Dotted line indicates the mean response to the hexanol standard (10 μg). Dosage means with the same letter are not significantly different by Student's t test at $P < 0.05$ ($N = 9$).

EAG activity of β-caryophyllene, while α-humulene showed no activity on female antennae (Figure 5). No α-copaene was detected in the trapped mango volatiles. The following compounds were also identified by retention time and mass spectral matches with standards, but showed no GC-EAG activity: 1-butanol, ethyl propanoate, 3-methyl butanol, ethyl butanoate, ethyl-(*E*)-2-butenoate, α-pinene, *o*-cymene, myrcene, ocimene, *p*-cymene, limonene, terpinolene, and α-humulene.

Standardized EAG response measurements with 100-ng dosages of (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene showed greater EAG responses elicited by ethyl octanoate and β-caryophyllene than by hexanol for both male and female antennae (Figure 6). Average responses to (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene of female antennae were 0.78 $\pm$ 0.04 SD, 1.88 $\pm$ 0.25 SD, and 4.68 $\pm$ 0.70 SD, respectively, while average responses from male antennae were 0.41 $\pm$ 0.08 SD, 1.90 $\pm$ 0.28 SD, and 3.84 $\pm$ 0.42 SD, respectively. The mean EAG responses of female medflies to β-caryophyllene and (1*S*)-(−)-β-pinene were significantly greater than those of male flies ($t = 3.27$, $P < 0.01$, $t = 12.86$, $P < 0.01$, respectively). The

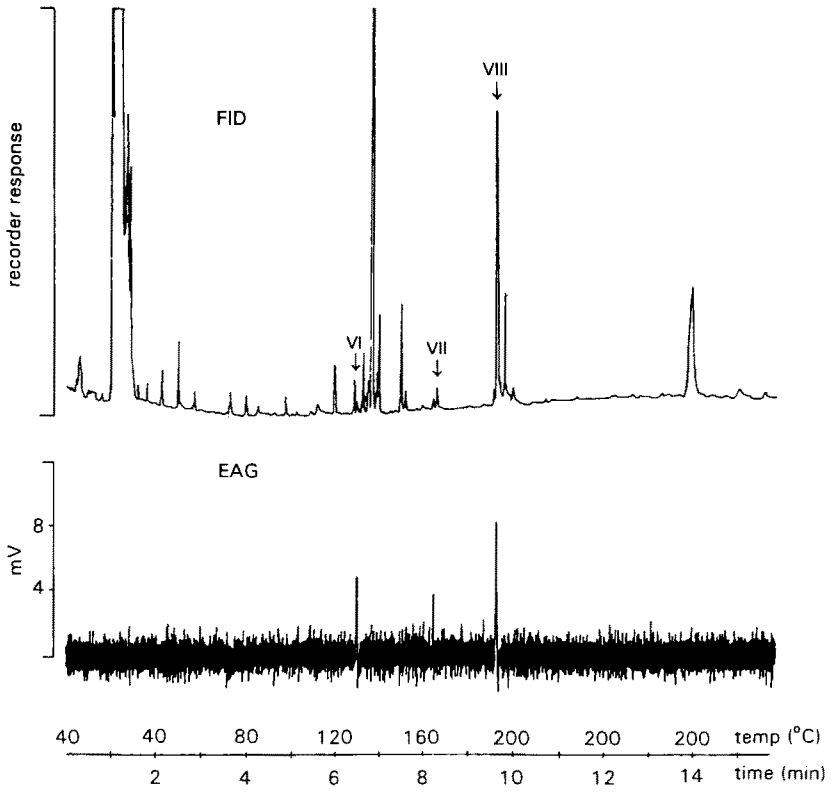

FIG. 3. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of trapped volatiles of ripe mango (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled VI, VII, and VIII (see text for details).

presence of a 1% α-copaene contamination in the 100-ng dosage of β-caryophyllene would have elicited at most a 40% increase in the EAG response to that of β-caryophyllene alone (see Figure 5). The mean EAG responses of female and male medfly antennae for hexanol were 0.3 mV ($\pm$0.3 SD, $N = 9$) and 0.3 mV ($\pm$0.2 SD, $N = 11$), respectively.

DISCUSSION

The goal of this research was to provide some focus regarding the many compounds that have been identified from volatile emissions of calling male

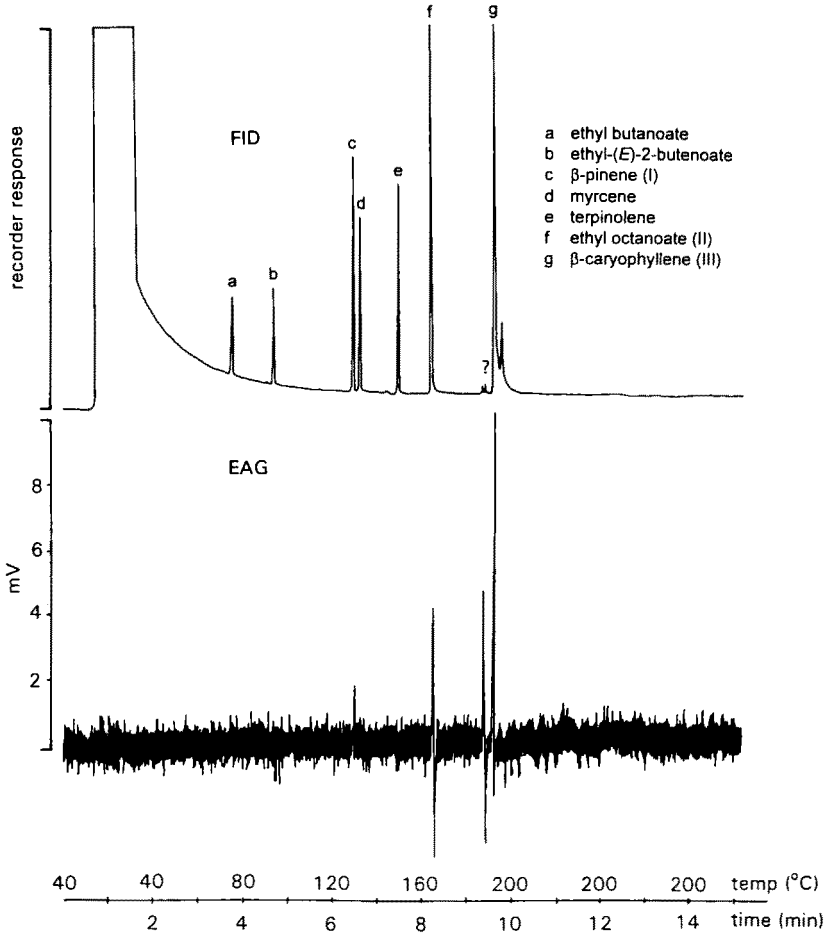

FIG. 4. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of a mixture of commercial and synthetic compounds identified from ripe mango (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled VI, VII, VIII, and ? (see text for details).

medflies and from host fruits. In particular, we wanted to see if some heretofore overlooked minor or trace compounds in these volatile emissions might stand out with regard to EAG amplitude and indicate that behavioral testing should be performed with those compounds added to the blend if such testing had not previously been done.

The coupled GC-EAG provides a real-time plot of the sequential responses

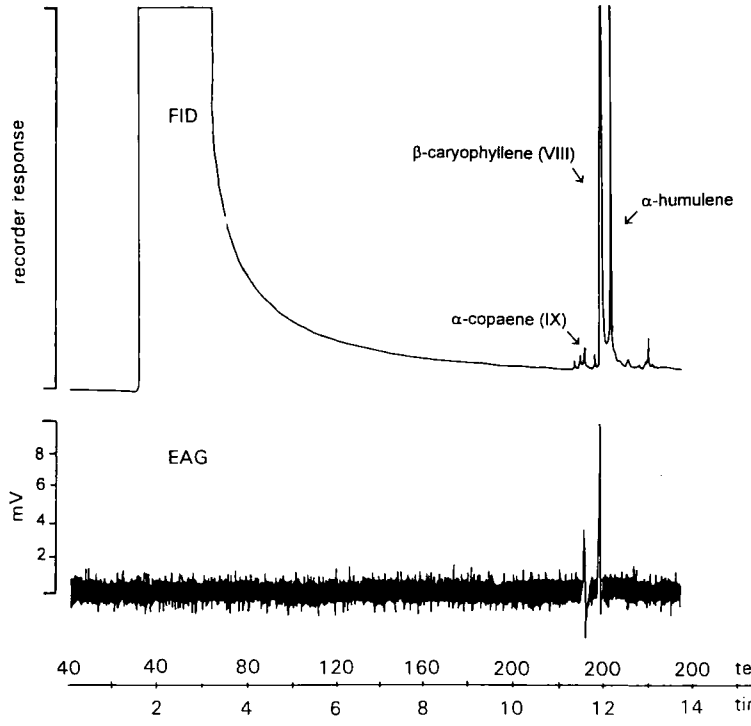

FIG. 5. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of a mixture of α-copaene, β-caryophyllene, and α-humulene (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled VIII and IX (see text for details).

of a single antenna to every compound in a natural extract. Thus, a single run provides comparative antennal response data for each compound and unequivocally highlights the compounds in the extract to which the antennae are most sensitive. Furthermore, possible problems of being misled by strong responses to trace contaminants in a synthetic sample that is puffed over the antenna are eliminated because the sample is separated into its component compounds, which are then passed over the antenna one at a time as they elute off the GC column. This technique requires that the preparations stay extremely stable over the course of the GC run in order to avoid spurious peaks from occurring and confounding the analysis. We were able to achieve this stability and sensitivity for our medfly preparations. This study, to the best of our knowledge, represents the first-ever medfly GC-EAGs.

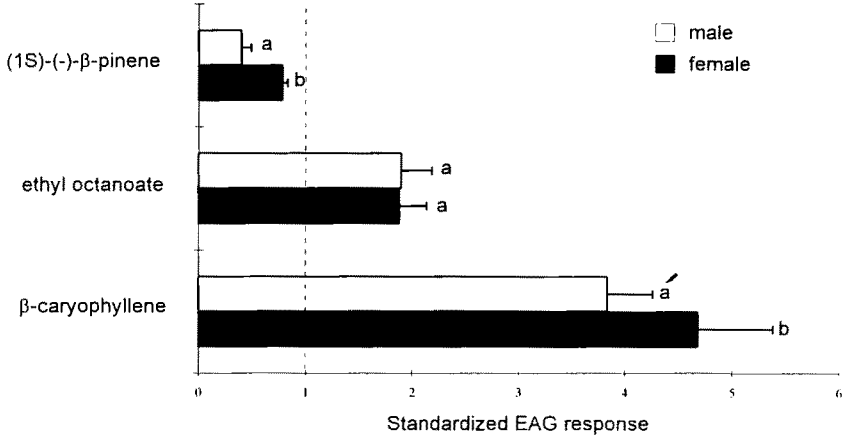

FIG. 6. EAG responses of male and female medfly antennae to commercial or synthetic compounds (100 ng) identified from ripe mango. Standard deviations of mean responses are presented as error bars. Dotted line indicates the mean response to the hexanol standard (10 μg). Male and female response means from the same compound with the same letter are not significantly different by Student's t test at $P < 0.05$ ($N = 10$).

Our approach of combined GC-EAG analyses demonstrated that female medfly antennae selectively responded to only five of the many compounds that were present in the headspace of calling male medflies. We identified these compounds as ethyl-(*E*)-3-octenoate, geranyl acetate, (*E*,*E*)-α-farnesene, linalool, and indole by GC-MS analyses. All five compounds had been previously identified (Baker et al., 1985; Jang et al., 1989; Heath et al., 1991; Flath et al., 1993). Ethyl-(*E*)-3-octenoate, geranyl acetate, and (*E*,*E*)-α-farnesene represent three of the five most abundant male odor components (Jang et al., 1989; Heath et al., 1991; Flath et al., 1993), and linalool and indole have been previously quantified as "intermediate" and "trace" components, respectively, based on reconstructed gas chromatograms (Figure 1 and Jang et al., 1989; Flath et al., 1993). Jang et al. (1989), working with medflies from Hawaii, isolated and identified 56 compounds from the odor of calling males and, by using EAGs, ranked the response to linalool in the top half of all the compounds tested, whereas the response to indole was ranked as one of the two lowest responses recorded. In contrast, our EAG dose–response tests showed indole equaling or exceeding the responses elicited by either (*R*)-(−)-linalool or (*S*)-(+)-linalool. However, due to differences in methodology, no accurate comparison can be made between our results and those of Jang et al. (1989).

Several studies have reported on the field attractancy of female medfly to the identified compounds, individually or as mixtures, with the exception of

indole. Recently, Jang et al. (1994) reported on flight behavior of females in a wind tunnel to a synthetic blend of ethyl-(*E*)-3-octenoate, geranyl acetate, (*E*,*E*)-α-farnesene, ethyl acetate, and 1-pyrroline, as well as to the odor of calling males, and they found that the natural male pheromone was still the most active blend for female attraction. Indole and linalool were not included in these tests. Initial laboratory assay results showed a significant increase in attraction of a previously tested three-component sex pheromone blend when indole was added (R.R. Heath, personal communication).

Our results with mango volatiles have revealed potential new attractants. Of the previously reported 54 volatile compounds identified from mango (Bartley, 1988), we identified 16 compounds in the headspace of mango pieces. Only three compounds consistently evoked responses in combined GC-EAG analyses with female antennae. Our GC-MS analyses identified the compounds as β-pinene, ethyl octanoate, and β-caryophyllene. In addition, the results from our studies showed that the known attractant, α-copaene, a contaminant in our commercial sample of β-caryophyllene, was strongly EAG active, as might be expected from its behavioral activity (Flath et al., 1994a,b). β-Caryophyllene has been reported as a medfly attractant (Beroza and Green, 1963), but Warthen and McInnis (1989) were unable to reproduce these results and suggested that the β-caryophyllene samples tested were attractive because of the presence of α-copaene as an impurity. This conclusion possibly should be reexamined in light of our results showing highly selective GC-EAG responses to both α-copaene and β-caryophyllene by female antennae. The behavioral activity of ethyl octanoate either alone or as part of a blend has not been tested. Jang et al. (1989) did not find ethyl octanoate in medfly emissions, but they did test it as an analog of ethyl-(*E*)-3-octenoate and ranked it as giving an intermediate EAG response on both male and female medfly antennae. Ethyl octanoate does function as one component of a four-component host-fruit blend that attracts male and female Mexican fruit flies, *Anastrepha ludens* Loew (Robacker et al., 1992). Its structure is similar to that of the medfly sex phermone component, ethyl-(*E*)-3-octenoate.

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ROLES OF CUTICULAR HYDROCARBONS IN INTRA- AND INTERSPECIFIC RECOGNITION BEHAVIOR OF TWO RHINOTERMITIDAE SPECIES

SHOZO TAKAHASHI* and AHDIN GASSA

Pesticide Research Institute, Faculty of Agriculture
Kyoto University, Kyoto 606, Japan

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Abstract—Soldiers of two termite species, *Reticulitermes speratus* and *Coptotermes formosanus*, showed aggressive behavior toward workers of other species. Soldiers always exhibited aggressive behavior to a conspecific worker treated with heterospecific cuticular hydrocarbons. A bioassay using live workers to test contact chemical cues was developed.

Key Words—Specific recognition, aggressive behavior, contact chemical cues, Rhinotermitidae, cuticular hydrocarbons.

INTRODUCTION

Cuticular hydrocarbons in a number of social insects have been analyzed in recent years to determine their chemotaxonomic, physiological, and semiochemical roles (Howard, 1993). Termites show a species-specific profile of hydrocarbons from the cuticle, although the composition seems to differ among families (Haverty et al., 1990). In Rhinotermitidae, each sympatric species has a qualitatively unique hydrocarbon composition. The hydrocarbons function as critical ecological cues, and there are populational and seasonal differences within species (Bagnères et al., 1991; Su and Haverty, 1991). It was postulated that the hydrocarbons from the North American termites *Reticulitermes flavipes* (Kollar) and *Zootermopsis anguisticollis* (Hagen) were being used as species- and caste-

*To whom correspondence should be addressed.

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recognition cues. A role for cuticular hydrocarbons as species-recognition cues was reported (Howard et al., 1982b) for termites in which aggressive behavior was shown towards a critical-point-dried (CPD) specimen treated with heterospecific hydrocarbons. Application of cuticular hydrocarbon mixtures to CPD specimens elicited the same behavioral response as that directed towards live heterospecifics, but the biting response occurred at relatively low frequency due to the lack of movement by the specimens. Various behavioral tests were made with living or dead individuals to clarify the role of cuticular hydrocarbons in interspecific recognition of two species of termites, *Reticulitermes* (*lucifugus*) *grassei* and *R.* (*l.*) *banyulensis*. These termites show a high degree of aggressiveness towards each other, and dead (CPD) termite lures covered with an extract of nonpolar lipids from *R.* (*l.*) *grassei* provoked the strongest aggression in *R.* (*l.*) *banyulensis* workers (Bagnères et al., 1991). Agonistic behavior was observed in an arena test when groups of *C. formosanus* from the same colony or a different colony were paired (Su and Haverty, 1991). Among six colonies each from Florida and Hawaii, that were tested, three Florida colonies were not attacked aggressively by any other colony. Thirteen of 36 pairings of colonies from Hawaii resulted in aggressive responses. Comparison of cuticular hydrocarbon patterns of the colonies showed no apparent relation between cuticular hydrocarbon composition and agonistic behavior in the *C. formosanus* populations (Su and Haverty, 1991).

Coptotermes formosanus Shiraki inhabits the Pacific coastal area of the eastern and western parts of Honshu, Shikoku, and southern Japan. *Reticulitermes speratus* (Kolbe) is widely distributed all over Japan except for the northeastern part of Hokkaido. In a small arena test, 100% of the soldiers tested showed biting behavior toward a heterospecific worker. The soldiers never attacked freeze-killed heterospecific workers in this test, which suggested the need to use live workers when testing the role of cuticular hydrocarbons in species recognition (Gassa and Takahashi, 1995). When a soldier encountered a conspecific worker coated with powdered material or liquid paraffin, it recognized the worker not as a conspecific, but as a different species and attacked it with open mandibles, biting the worker to death (Gassa et al., 1994). Coating of the worker body surface with foreign hydrocarbons apparently imbued them with a kairomonal activity that elicited biting from conspecific soldiers (Gassa et al., 1994). In order to understand more clearly this interspecific aggression, behavioral assays with live termites coated with the cuticular wax from workers of the two species were conducted. In this paper we report a bioassay method confirming that cuticular hydrocarbons have a major function in intra- and interspecific recognition in termites. This bioassay seems applicable to many termite species as an aid in identifying nest and caste recognition cues.

METHODS AND MATERIALS

Insects. Workers and soldiers of *C. formosanus* were obtained from a stock culture in the insectory of the Wood Research Institute, Kyoto University, Uji. The colony of *R. speratus* was collected from the Botanical Garden of the Faculty of Science, Kyoto University, Kyoto. Groups of both species were maintained in a plastic container in 25 ± 2°C in the Pesticide Research Institute laboratory.

Extraction of Cuticular Wax. Fifty workers (or soldiers) of each termite species were immobilized by cooling and placed in a filter funnel plugged with a piece of cotton. The insects were washed with hexane three times, and the hexane solution was concentrated in vacuo after drying over anhydrous sodium sulfate. The extract was then placed on a silica gel column and hydrocarbons were eluted with hexane.

Preparation of Cuticular Wax Test Mixtures. To solvent evaporated and dried cuticular hydrocarbons from the 50 workers was added 50 μl of 2% aqueous Triton X-100 solution, and the mixture was warmed (at about 40°C) and shaken in an ultrasonic cleaner (Branson B-92) for 30 min to give an opaque liquid.

Observation of Termite Behavior. Observations were made at 25 ± 2°C, and humidity was not adjusted. A soldier and a worker of the same species were released into a Petri dish (30 mm diameter, 12 mm high) with a filter paper. Combinations of a solider and a worker from the two species, *C. formosanus* and *R. speratus*, were then made for behavioral observations.

One worker equivalent of the cuticular hydrocarbons (as a suspension or as a solution in 2% Triton X-100) was topically applied to the dorsal surface of a worker resting on a cooled Petri dish, and the dish and worker were then kept at room temperature for 15 min. After drying, each treated worker was transferred to an observation Petri dish (arena), and a conspecific soldier or a soldier of the other species was introduced. Antennal contact, opening of the mandibles, and biting by the soldier during a 1-hr test period were recorded. The survival time of the treated worker was noted. All behaviors were recorded on video tape.

Analysis of Cuticular Wax Extract. The cuticular hydrocarbons were analyzed by gas–liquid chromatography (GLC) with a Shimadzu GC-14A gas chromatograph with a 15-m × 0.2-mm-ID HP-1 column. All GC analyses were done with temperature programming from 120°C to 320°C at 8°C/min, with a 2-min initial hold period. Helium was the carrier gas with a head pressure of 1.0 kg/cm^2. Retention times were compared to those of *n*-alkane standards.

Gas chromatograph–mass spectroscopy (GC-MS) analyses were performed on a Shimadzu GC-14A operated in splitless mode, with an injection temperature

of 300°C, a 25-m × 0.28-mm DB-1 capillary column, temperature programmed from 200°C to 300°C at 5°C/min. A Shimadzu QP-1000EX mass spectrometer was used with an ion source temperature of 250°C and interface temperature of 300°C for EI-MS (70 eV). CI-MS was obtained using isobutane.

RESULTS

A conspecific soldier and worker in the arena showed antennal scissors movement several times at their first encounter. *C. formosanus* workers and soldiers in the arena survived without food or water about 24.7 hr and 32.2 hr, respectively (Table 1). When a soldier and a heterospecific worker were placed in the arena, the soldier quickly made antennal contact with the worker and simultaneously opened its mandibles. Soldiers of both species killed heterospecific workers within a short time after mandible opening and biting (Table 1). When a soldier was placed in the arena with both a conspecific and a heterospecific worker, the soldier selectively killed only the heterospecific worker. However, no soldier ever demonstrated aggressive behavior toward a freeze-killed worker, whether conspecific or heterospecific. These findings confirmed that a bioassay to test chemical cues for species recognition behavior by soldiers must be conducted with live workers.

Soldiers showed a strong aggressive response to conspecific workers treated with one worker equivalent of heterospecific hydrocarbons prepared from the cuticular wax of 50 workers suspended in Triton X-100 (Tables 2 and 3). Soldiers showed only antennal scissors movements toward heterospecific workers treated with a suspension of hydrocarbons from the soldier's conspecific work-

TABLE 1. INDUCTION OF AGGRESSIVE BEHAVIOR IN SOLDIERS OF *C. formosanus* (*C. f.*) AND *R. speratus* (*R.s.*) TOWARD HETEROSPECIFIC WORKERS (MEAN ± SE)[a]

	C. formosanus soldier	*R. speratus* soldier
Time before attack on worker (min)	0.13 ± 0.03	0.96 ± 0.49
Number of mandible openings/(1-hr test period)	6.00 ± 0.53	14.10 ± 1.50
Number of bitings of worker/(1-hr test period)	14.90 ± 1.69	12.10 ± 1.79
Survival time of worker (min)	7.23 ± 1.37	33.39 ± 11.72
Survival time of worker	1642.95 ± 40.59[b]	1486.32 ± 19.08[c]
with conspecific soldier (min) (control)	2135.40 ± 52.67	1933.03 ± 25.01

[a] Average of 10 replications. Intact individuals were used in each test in an arena of a petri dish (30 mm in diameter, 12 mm high)

[b] *R.s.* worker (above); *R.s.* soldier (below).

[c] *C.f.* worker (above); *C.f.* soldier (below).

TABLE 2. INDUCTION OF AGGRESSIVE BEHAVIOR IN *C. formosanus* SOLDIERS TO CONSPECIFIC WORKER TREATED WITH *R. speratus* CUTICULAR HYDROCARBONS (MEAN ± SE)[a]

	Response of *C. formosanus* soldier	
	C. formosanus worker treated with *R. speratus* HC[b]	*C. formosanus* worker treated with 2% Triton X-100[c]
Time before attack on worker (min)	0.32 ± 0.05	0.00 ± 0.00
Number of mandible openings/(1-hr test period)	3.50 ± 0.92	0.00 ± 0.00
Number of bitings of worker/(1-hr test period)	8.30 ± 1.71	0.00 ± 0.00
Survival time of worker (min)	83.60 ± 9.19	1493.59 ± 36.90

[a] Average of 10 replications. Intact individuals were used in each test.
[b] 1 μl topical application of suspension (2% Triton X-100 + HC = 1 worker equivalent/μl).
[c] 1 μl topical application of 2% Triton X-100 (control).

ers. In the latter case, the soldiers only rarely displayed mandible opening or biting (Table 4).

Hydrocarbons in cuticular wax extracts from workers of the two species were clearly different in composition. *R. speratus* hydrocarbons had carbon numbers from 23 to 26 (Table 5), whereas those from *C. formosanus* showed 25–31 carbons (Table 6). Comparison of the hydrocarbon composition of the Japanese *C. formosanus* colony with colonies from the United States showed

TABLE 3. INDUCTION OF AGGRESSIVE BEHAVIOR IN *R. speratus* SOLDIERS TO CONSPECIFIC WORKERS TREATED WITH *C. formosanus* CUTICULAR HYDROCARBONS (MEAN ± SE)[a]

	R. speratus soldier	
	R. speratus worker treated with *C. formosanus* HC[b]	*R. speratus* worker treated with 2% Triton X-100[c]
Time before attack on worker (min)	0.62 ± 0.11	0.00 ± 0.00
Number of mandible openings/(1-hr test period)	1.70 ± 0.21	0.00 ± 0.00
Number of bitings of worker/(1-hr test period)	5.30 ± 0.53	0.00 ± 0.00
Survival time of worker (min)	119.14 ± 15.03	1351.20 ± 17.35

[a] Average of 10 replications. Intact individuals were used in each test.
[b] 1 μl topical application of suspension (2% Triton X-100 + HC = 1 worker equivalent/μl).
[c] 1 μl topical application of 2% Triton X-100 (control).

TABLE 4. BEHAVIOR OF *C. formosanus* SOLDIERS OR *R. speratus* SOLDIERS TOWARD HETEROSPECIFIC WORKERS TREATED WITH CONSPECIFIC CUTICULAR HYDROCARBONS OF SOLDIER (MEAN ± SE)[a]

	Response of *C. formosanus* soldier	Response of *R. speratus* soldier
	R. speratus worker treated with *C. formosanus* HC[b]	*C. formosanus* worker treated with *R. speratus* HC[b]
Time before antennal contact (min)	0.65 ± 0.15	0.73 ± 0.17
Number of mandible openings/(1-hr test period)	0.20 ± 0.13	0.00 ± 0.00
Number of bitings of worker/(1-hr test period)	0.60 ± 0.60	0.00 ± 0.00
Survival time of worker (min)	872.28 ± 46.05	840.42 ± 44.58

[a] Average of 10 replications. Intact individuals were used in each test.
[b] 1 μl topical application of suspension (2% Triton X-100 + HC = 1 worker equivalent/μl).

slight differences in composition but similarities in the large amount of mixed, internal methyl-branched heptacosanes (Haverty et al., 1990).

DISCUSSION

A bioassay for intra- and interspecies recognition behavior by termite soldiers has been needed for many years by researchers involved in the chemical

TABLE 5. PERCENT COMPOSITION OF CUTICULAR HYDROCARBONS FROM WORKERS OF *R. speratus*

Component	Total carbon number	Diagnostic MS ions	Composition (%)
n-C_{23}	23	324	10.35
2-Me-C_{23}	24	323, 338	1.90
3-Me-C_{23}	24	281, 338	5.20
2-Me-C_{24}	25	309, 337, 352	15.44
n-C_{25}	25	352	28.80
x-Me-C_{25}	26	71, 85, 141, 169, 183, 211, 225, 295, 366	8.09
2-Me-C_{25}	26	323, 351, 366	7.46
3-Me-C_{25}	26	337, 351, 366	16.56
n-C_{26}	26	366	6.20

TABLE 6. PERCENT COMPOSITION OF CUTICULAR HYDROCARBONS FROM WORKERS OF *C. formosanus*

Component	Total carbon number	Diagnostic MS ions	Composition (%)
n-C_{25}	25	352	1.42
x-Me-C_{25}	26	281, 366	1.18
2-Me-C_{25}	26	323, 351, 366	10.33
3-Me-C_{25}	26	337, 351, 366	1.53
n-C_{26}	26	366	1.48
x-Me-C_{26}	27	281, 380	1.80
2-Me-C_{26}	27	337, 365, 380	4.71
n-C_{27}	27	380	6.48
9-, 11-, 13-Me-C_{27}	28	211, 239, 267, 394	27.95
2-Me-C_{27}	28	351, 379, 394	14.41
3-Me-C_{27}	28	365, 394	7.89
n-C_{28}	28	394	3.76
9-, 11-, 13-Me-C_{28}	29	267, 281, 408	3.21
9-, 11-, 13-Me-C_{29}	30	127, 211, 408	0.90
13, 15-DiMe-C_{29}	31	197, 225, 239, 267, 421, 436	11.40
2-Me-C_{30}	31	393, 436	1.45

ecology of social insects (Howard, 1993). The effect of moving of surrogate insects was shown in defense behavior of *Nasutitermes exitiosus* (Hill) soldiers. The device consisted of a small ferromagnetic bar that was placed on the floor of the arena on a magnetic stirrer. The soldiers approached the moving bar and sprayed a defensive substance but not the bar ceased twirling (Beaton et al., 1973; Eisner et al., 1976). Dead termites or various inert dummies treated with hydrocarbons have never elicited a response behavior in soldiers (Howard, 1993). Critical-point-dried (CPD) termites only infrequently elicited the biting response in soldiers (Howard et al., 1982b). Our test with live termite workers covered with heterospecific hydrocarbons elicited aggressive behavior in the conspecific soldiers 100% of the time, and the aggressive action strongly affected the survival time of the workers. Soldiers showed no aggressive behavior toward a heterospecific worker covered with added conspecific hydrocarbons of the soldier. Apparently, recognition of the conspecific hydrocarbons on the worker prevented such behavior. A similiar situation has been found in semiochemical studies on inquilines of termites. Many inquilines possess a cuticular hydrocarbon profile qualitatively identical to that of its host termites. The similarity to its termite host in composition of cuticular hydrocarbons seems to be a factor for success of the inquiline in living in close association with its host (Howard et al., 1980; 1982a).

Ethological studies on the role of chemical cues, called "chemical ethology" by Takahashi (1993), has been aided greatly by advances in chemical technology and in the discovery, identification, and synthesis of many semiochemicals. Bioassays, even if inherently limited, are absolutely critical in studies in semiochemical cues in all animals. Limited behavioral tests, however, have made it more difficult to understand the role of cuticular wax in species recognition of termites. Recent bioassays with dead, critical-point-dried specimens, and the use of dummies treated with heterospecific hydrocarbons to elicit aggression failed to show the level of agression elicited by live alien termites. Termite soldiers aggressively respond to moving objects similar to them in size but chemically or physically different in body composition (Gassa et al., 1994). Bioassays using live termites designed to determine the role of cuticular hydrocarbons indicate the success of this approach, and similar bioassays will likely be utilized in many chemical perception tests of social insects.

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EVALUATION OF ORGANIC INFUSIONS AND SYNTHETIC COMPOUNDS MEDIATING OVIPOSITION IN *Aedes albopictus* AND *Aedes aegypti* (Diptera: Culicidae)

SANDRA A. ALLAN* and DANIEL L. KLINE

United States Department of Agriculture
Agriculture Research Service
Medical and Veterinary Entomology Research Laboratory
P.O. Box 14565, Gainesville, Florida 32604

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Abstract—Oviposition responses of gravid *Aedes albopictus* and *Ae. aegypti* were evaluated to natural organic infusions (hay infusion, larval rearing water, and field-collected larval water) as well as compounds isolated from hay infusion (3-methylindole, 4-methylphenol, 4-ethylphenol, indole, and phenol) known to elicit oviposition in *Culex* mosquitoes. In laboratory bioassays, significant oviposition responses were obtained from *Ae. albopictus*, but not from *Ae. aegypti*, to dilutions of hay infusion and field water. Oviposition responses of both species were moderate to the synthetic compounds tested in the laboratory (0.01–100 μg/liter). Only 3-methylindole (0.1 μg/liter) and 4-ethylphenol (1.0 μg/liter) elicited significantly more oviposition by *Ae. albopictus* than did well water. Of the synthetic compounds tested with *Ae. aegypti*, only phenol (1.0 μg/liter) and 4-ethylphenol (0.1 μg/liter) elicited significantly more oviposition than did well water. Significant repellency or oviposition deterrence for both species occurred in response to at least one high concentration of most of the compounds tested. In field cage evaluations, oviposition responses by *Ae. albopictus* were strongest to larval water and field water, moderate to hay infusion and 3-methylindole (100 μg/liter), and low to well water. A mixture of five synthetic compounds mimicking hay infusion was no more effective than 3-methylindole alone. For *Ae. aegypti*, oviposition responses were greatest to larval water and least to 3-methylindole. In an olfactometer, gravid females of both species oriented more to field water than to well water and only *Ae. albopictus* oriented more to larval water or hay infusion than well water. In general, gravid *Ae. albopictus* responded more strongly to oviposition stimuli than did *Ae. aegypti*.

*To whom correspondence should be addressed.

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INTRODUCTION

The selection of oviposition sites by many mosquito species is greatly influenced by chemical stimuli (Bentley and Day, 1989). Chemical factors mediating oviposition may result from the previous presence of larvae or pupae (reviewed in Bentley and Day, 1989) or from eggs, which in several *Culex* species contain an apical droplet of pheromone (Laurence and Pickett, 1985; Starratt and Osgood, 1973). In addition, water from natural breeding sites such as ponds (Gjullin et al., 1965) or infusions of organic material (such as hay or grass infusions) are attractive to gravid female mosquitoes (Ritchie, 1984; Millar et al., 1992). Different chemical cues may elicit oviposition behaviours beginning with the initial orientation towards a potential oviposition site and culminating in the deposition of eggs.

A wide range of mosquito species, including *Aedes albopictus* (Hein, 1976; Holck et al., 1988), *Ae. aegypti* (Gjullin et al., 1965; Chadee, 1993), *Ae. triseriatus* (Holck et al., 1988), *Culex nigripalpus* (Ritchie, 1984), *Cx. pipiens* (Madder et al., 1980; Reiter, 1986), *Cx. quinquefasciatus* (Gjullen et al., 1965; Reisen and Meyer, 1990; Millar et al., 1992), *Cx. restuans* (Madder et al., 1980; Reiter, 1986), and *Cx. tarsalis* (Reisen and Meyer, 1990) have been reported to be attracted to hay and grass infusions for oviposition. For this reason, infusions have been used to enhance collections in CDC light traps (Reiter et al., 1991), gravid female traps (Reisen and Meyer, 1990; Reiter, 1983), and ovitraps (Holck et al., 1988; Kitron et al., 1989).

Recently Millar et al. (1992) isolated and identified five compounds (3-methylindole, 4-methylphenol, 4-ethylphenol, phenol, and indole) from fermented Bermuda grass infusions that strongly stimulated oviposition by *Cx. quinquefasciatus*, both individually and in a blend of similar concentrations as isolated from the grass infusion. The five-compound blend and 3-methylindole alone were equally attractive to ovipositing *Cx. quinquefasciatus* in laboratory bioassays. In addition, 3-methylindole also mediated oviposition of *Cx. tarsalis* and *Cx. stigmatosoma* under field conditions (Beehler et al., 1994). It is not known if these compounds have similar effects on the oviposition behavior of other mosquito species known to be attracted to grass or hay infusions.

The objectives of our study were to: (1) evaluate oviposition responses of *Ae. albopictus* and *Ae. aegypti* to various organic infusions and solutions; and (2) to evaluate the effect of the recently identified components from Bermuda hay infusion (Millar et al., 1992) on their oviposition.

METHODS AND MATERIALS

Aedes albopictus and *Ae. aegypti* were reared and maintained at 27 ± 2°C, 70–80% relative humidity, and a 16L:8D-hr photoperiod. Larvae were reared by conventional methods using standardized density and nutrition (1000 immatures/3 liters of water and fed on a 1:1 mixture of liver powder and brewer's yeast). Female mosquitoes were blood-fed four to five days after emergence on defibrinated beef blood (*Ae. aegypti*) or chicks (*Ae. albopictus*). Blood-fed mosquitoes were held four to five days at 30 ± 1°C under fluorescent lighting with a 14L:10D photoperiod including 1 hr of simulated dawn and dusk period (provided by a 15-W incandescent bulb). A 10% sucrose solution was provided continuously to mosquitoes prior to bioassays.

Laboratory Bioassays. Bioassay cages were cylindrical cardboard containers (25.4 cm high × 24.7 cm diam.) with clear plastic lids. A 2-cm-diam. plugged access hole in the side of the container allowed introduction of mosquitoes into the cage. Disposable 70-ml plastic cups were painted black and filled with either 60 ml of well water (nonchlorinated water used to rear mosquitoes as controls) or the treatment preparations. One control and one treatment cup were placed in each bioassay cage ca. 6 cm apart, and a strip of seed germination paper (2 × 5 cm) (Steinley et al., 1991) was placed against the inside of each cup. A single gravid female was added to each cage at least 1 hr prior to the scotophase and mosquitoes were removed after 22 hr. Bioassays were conducted using individual females to reduce the potential influence of previously laid eggs on oviposition site preference (Chadee et al., 1990). Assays were run overnight in an environmental chamber at 30 ± 1°C with 14L:10D photoperiod with 1 hr of simulated dawn and dusk. They were scored by counting the numbers of eggs on the germination paper from the control and treatment cups.

Organic infusions used for bioassays included hay infusion (prepared after Reiter, 1986), larval rearing water, and water collected from a field site known to contain populations of *Ae. albopictus*. Preparation of the hay infusion involved adding 450 g Bermuda grass hay, 5 g brewer's yeast, and 20 g lactalbumin hydrolysate to 75 liters of well water, held at ambient temperatures (25–30°C) for 12 days. Two preparations of infusion were made and each was frozen in ca. 2-liter aliquots until needed. Hay infusion was diluted with well water as needed to produce dilutions of 10%, 25%, 50%, and 100% for testing in bioassays. Larval rearing water that had contained immature mosquitoes was obtained from the mosquito colony and stored at 10°C for a maximum of two days prior to use. Field-collected water originated from a natural oviposition site (an open container with oak leaves) that contained a continuous population of immature *Ae. albopictus*. Water was collected directly from the source prior to bioassays.

Field water was diluted with well water to produce dilutions of 5%, 25%, 75%, and 100% for testing in bioassays.

Synthetic compounds previously identified as components of hay infusion (3-methylindole, indole, phenol, 4-methylphenol, 4-ethylphenol) (Sigma Chemical Co.) (Millar et al., 1992) were prepared in hexane and added to well water in 10 μl of solvent. Concentrations of test compounds ranged from 0.01 to 100 μg/liter, and reflected concentrations typical of hay infusions (Millar et al., 1992). Control cups were treated with 10 μl hexane. All treatments consisted of 20–30 replicates.

Oviposition activity was expressed using the oviposition activity index (OAI) of Kramer and Mulla (1979), which is calculated as follows:

$$OAI = (N_t - N_c)/(N_t + N_c)$$

where N_t = number of eggs deposited in treatment cups and N_c = the total number of eggs deposited in control cups. Responses using this system range from 1 (attraction) to −1 (repellency) with zero denoting no preference. In this context, attraction refers to the deposition of eggs as the end result of a complex sequence of behaviors including orientation towards the source and stimulation to oviposit. These behaviors were not differentiated under the current bioassay conditions and only the endpoint of the pre- and oviposition behaviors (i.e., oviposition) was used to determine attraction or repellency. Treatment means were subjected to ANOVA to determine if significant differences were present ($P < 0.05$). Mean numbers of eggs laid under each treatment were compared by chi-square analysis to the number of eggs. Statistical comparisons were made by chi-square analysis between the mean numbers of eggs deposited in treatment cups (that were paired with well water control cups) compared to the expected number of eggs if oviposition was random between the treatment and control.

Field Bioassays. Bioassays were conducted in a screened field cage (ca. 5 × 5 × 5 m). Large potted plants were placed along the perimeter and in the center of the cage to provide resting sites for mosquitoes. Black glass oviposition jars (500 ml), each with 400 ml of water or solution and a strip of seed germination paper (4.5 × 9.6 cm) fastened with a paper clip to the inside upper edge of the jar, were used to evaluate oviposition responses. Jars were spaced evenly around the interior of the cage about 0.5 m from the cage wall. Each day, one jar containing each treatment was randomly assigned to one of five positions (experiment 1) or four positions (experiment 2) in the cage and treatments rotated in position from one day to the next to avoid positional bias. Each day, 50 gravid females were released from a central point in the cage in mid-afternoon. Treatments placed in the cage in mid-afternoon and oviposition strips with eggs were collected 24 hr later. Temperature and climatic conditions were

noted for each day. The numbers of eggs present on seed germination paper from each day were counted to determine oviposition responses.

Two experiments were conducted for each species. For experiment 1, treatments consisted of well water (nonchlorinated), a 25% solution of hay infusion, 3-methylindole (100 μg/liter), larval rearing water, and field water. Immediately before the test, hay infusion and 3-methylindole solutions were prepared in well water and field water and larval rearing water were obtained. Aliquots of thawed hay infusion and rearing water were kept at 10°C for up to two days. Treatments were repeated for 15 days (replicates).

For experiment 2, treatments consisted of 25% hay infusion, 3-methylindole (100 μg/liter), well water, and a mixture of synthetic compounds combined in concentrations similar to those found in hay infusion as described by Millar et al. (1992). This mixture consisted of phenol (0.27 mg/liter), 4-methylphenol (1.99 mg/liter), 4-ethylphenol (0.07 mg/liter), indole (0.01 mg/liter), and 3-methylindole (0.25 ng/liter). Synthetic compounds and mixtures were added to jars 10–15 min prior to placement in the field cage. Treatments were repeated for 10 days (replicates).

For each day, the percentage of eggs deposited in response to each treatment was determined. Means for each treatment were transformed to arcsine values and subjected to ANOVA ($P < 0.05$) to determine if significant differences were present in each experiment. Differences between means were determined using Tukey's studentized t test ($P < 0.05$). Data are presented as untransformed means.

Olfactometer Bioassays. To determine if organic infusions elicited an upwind orientation response, gravid *Ae. albopictus* and *Ae. aegypti* were tested in a dual-port olfactometer similar to that described by Schreck et al. (1967), but consisting of three stacked chambers (35 cm high × 90 cm long × 48 cm wide). Only one chamber at a time was used for assays. Outside air was conditioned prior to entry through the choice ports, the mosquito trap, and the olfactometer by passing through a series of charcoal filters and then heated and humidified. Conditions in the olfactometers were 27°C, 80% relative humidity with an air flow of 1 liter/sec. One hour before initiation of tests (ca. 14:00 hr EST), females were aspirated into the olfactometer chamber and allowed to acclimate. For each of the 10 replicates, 50 gravid females were used. Treatments were placed into two test ports upwind of the traps and olfactometer chamber. For each olfactometer test, one port contained well water and the other contained a test solution. Treatments (field water, larval water, and 50% hay infusion) were presented in glass Petri dishes that were cleaned and handled with gloves to minimize contamination and were alternated between ports from one day to the next. After 24 hr, the relative numbers caught in the treatment and control traps upwind of each port were compared. Treatments were added to each port and tests initiated at 15:00 hr EST.

Means were transformed to arcsine values and differences between means of treatments and controls determined by ANOVA ($P < 0.05$). Data are presented as untransformed means.

RESULTS

Oviposition responses of *Ae. albopictus* females differed significantly across the range of concentrations of hay infusion (ANOVA; $F = 6.58$; $df = 3,76$; $P < 0.001$) and field water tested (ANOVA; $F = 3.94$; $df = 3,76$; $P < 0.01$) (Figure 1). Only the 25% dilution of hay infusion elicited significantly more oviposition than expected (if oviposition was random between the paired treatments and well water controls) (chi square, $P < 0.001$). In response to 10% and 100% hay infusion, significantly lower levels of oviposition occurred (chi square, $P < 0.001$). Oviposition responses were strongly in response to solutions of 25% field water and 75% field water (chi square, $P < 0.001$). Oviposition responses to 100% field water were significantly lower than expected (chi square, $P < 0.001$).

Oviposition responses of *Ae. aegypti* did not differ across the same range of concentrations of hay infusion (ANOVA; $F = 0.94$; $df = 3,76$; $P = 0.42$) or field water (ANOVA, $F = 0.54$; $df = 3,76$; $P = 0.65$) (Figure 1). In general, OAI values for hay infusion were negative with significantly lower oviposition than expected in response to a 25% solution of hay infusion (chi square, $P < 0.001$). OAI values were moderately positive in response to field water with significantly greater oviposition than expected with solutions of 75% and 100% field water (chi square, $P < 0.001$).

Responses of gravid female *Ae. albopictus* to the synthetic compounds tested were moderate. Oviposition responses differed significantly across the range of concentrations of 3-methylindole (ANOVA; $F = 2.48$; $df = 4,145$; $P < 0.05$) and phenol (ANOVA; $F = 2.45$; $df = 4,95$; $P < 0.05$) but not indole (ANOVA; $F = 0.83$; $df = 4,95$; $P = 0.50$), 4-ethylphenol (ANOVA, $F = 0.55$, $df = 4,145$; $P = 0.69$) and 4-methylphenol (ANOVA; $F = 0.64$; $df = 4, 95$; $P = 0.63$) (Figure 2). Of the treatments with positive OAI values, only one concentration of 4-methylphenol (0.01 μg/liter) (chi square, $P < 0.001$) and 3-methylindole (0.1 μg/liter) (chi-square, $P < 0.05$) elicited significantly more oviposition than expected. Significantly fewer eggs were deposited in treatment cups than expected for 10 μg/liter (chi square, $P < 0.05$) and 100 μg/liter (chi square, $P < 0.001$) of 3-methylindole, 0.01 μg/liter (chi square, $P < 0.001$) and 100 μg/liter (chi square, $P < 0.001$) of phenol, 0.01 μg/liter (chi square, $P < 0.001$) of 4-ethylphenol and 100 μg/liter (chi square, $P < 0.001$) of indole.

Oviposition responses to synthetic compounds by *Ae. aegypti* females were

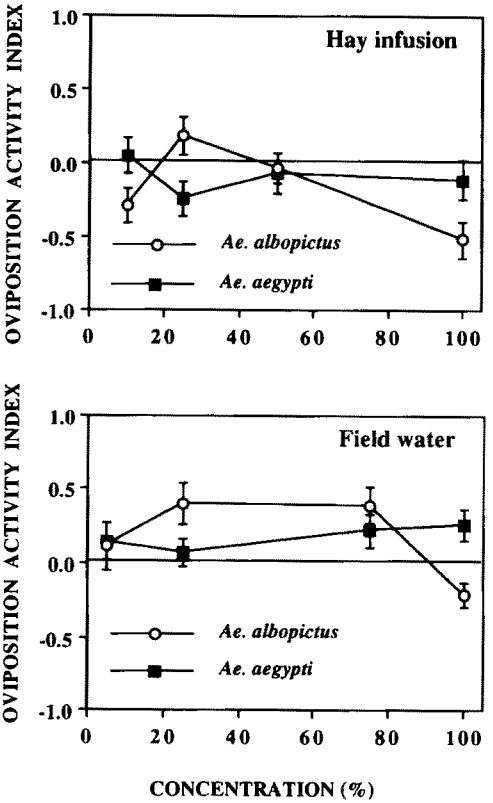

FIG. 1. Oviposition responses of gravid *Ae. albopictus* and *Ae. aegypti* to organic infusions in a laboratory bioassay. Data are presented as means (±SE) of oviposition activity indices (OAI) from 20 replicates. Positive OAI values indicated attractiveness and negative values repellency. *Significantly different at $P < 0.05$; **Significantly different at $P < 0.001$.

also moderate and only increased significantly across the range of concentrations of 4-ethylphenol (ANOVA; $F = 3.82$; df = 4,95; $P < 0.01$) and phenol (ANOVA; $F = 6.68$; df = 4,95; $P < 0.001$) but not 3-methylindole (ANOVA, $F = 0.943$; df = 4,95; $P = 0.44$), indole (ANOVA, $F = 1.19$, df = 2,57; $P = 0.31$), or 4-methylphenol (ANOVA; $F = 0.59$; df = 4,95; $P = 0.27$) (Figure 2). Of the treatments with positive OAI values, only 1.0 μg/liter of phenol and 0.1 μg/liter of 4-ethylphenol were significantly attractive (chi square, $P < 0.001$). Significantly lower oviposition than expected was noted for 3-methylindole (0.1 μg/liter) (chi square, $P < 0.001$), phenol (0.01 μg/liter, 100 μg/liter)

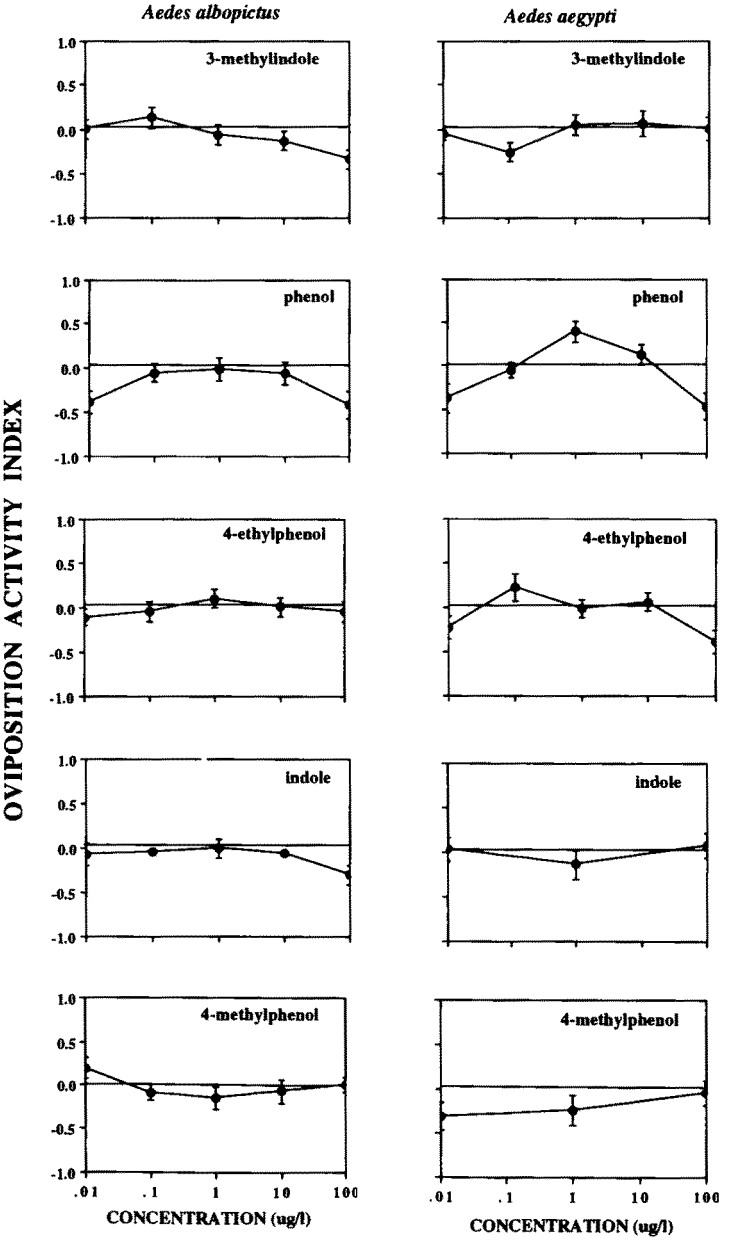
Aedes albopictus
Aedes aegypti
3-methylindole
phenol
4-ethylphenol
indole
4-methylphenol
1.0
0.5
0.0
-0.5
-1.0
OVIPOSITION ACTIVITY INDEX
.01
.1
1
10
100
CONCENTRATION (ug/l)

TABLE 1. OVIPOSITIONAL RESPONSES IN FIELD CAGES OF GRAVID *Ae. albopictus* AND *Ae. aegypti* TO OVITRAPS BAITED WITH ORGANIC INFUSIONS, 3-METHYLINDOLE, OR A MIXTURE OF SYNTHETIC COMPOUNDS REPRESENTING MAJOR COMPONENTS OF HAY INFUSION

Treatment	*Aedes albopictus*		*Aedes aegypti*	
	% of eggs[a]	*N*	% of eggs	*N*
Experiment 1				
Well water	3.5 ± 1.2 a	10	16.0 ± 4.2 ab	10
Hay infusion	20.4 ± 4.6 bc	10	15.9 ± 2.4 ab	10
3-Methylindole (100 mg/liter)	14.9 ± 3.5 bc	10	13.5 ± 3.6 b	10
Larval water	34.9 ± 6.6 b	10	28.9 ± 3.3 a	10
Field water	31.4 ± 5.7 bc	10	25.5 ± 4.6 ab	10
Experiment 2				
Well water	18.2 ± 2.8 a	15	16.2 ± 0.8 a	10
3-Methylindole (100 mg/liter)	24.1 ± 3.2 a	15	21.1 ± 2.1 ab	10
Mixture	21.1 ± 2.4 a	15	29.1 ± 4.0 b	10
Hay infusion	36.1 ± 3.9 b	15	30.8 ± 4.1 b	10

[a] Mean ± SE of 10–15 replicates of 50 mosquitoes. Means in the same column and experiment followed different letter are significantly different (Tukey's studentized *t* test, $P < 0.05$).

(chi square, $P < 0.001$), 4-ethyl phenol (0.01 μg/liter, 100 μg/liter) (chi square, $P < 0.001$), and 4-methyl phenol (0.01 μg/liter, 1.0 μg/liter) (chi square, $P < 0.001$).

In field cages, ovipositional responses by gravid *Ae. albopictus* differed significantly between well water, hay infusion, 3-methylindole (100 μg/liter), larval water, and field water (ANOVA; $F = 9.23$; $df = 4,45$, $P < 0.001$) (Table 1). In comparison to well water, significantly more eggs were deposited in response to hay infusion, 3-methylindole, larval water, and field water. Ovipositional responses to larval water were greater than to 3-methylindole but similar to hay infusion and field water. Ovipositional responses by gravid *Ae. aegypti* were also significantly different between treatments in experiment 1 (ANOVA; $F = 3.35$; $df = 4,45$; $P < 0.01$) (Table 1). Ovipositional responses to ovitraps with larval water were significantly greater than those with 3-meth-

FIG. 2. Oviposition responses of gravid *Ae. albopictus* and *Ae. aegypti* to concentrations of synthetic chemical compounds. Data are presented as means (±SE) of oviposition activity indices (OAI) from 20 to 30 replicates. Positive OAI values indicated attractiveness and negative values repellency. *Significantly different at $P < 0.05$; **Significantly different at $P < 0.001$.

TABLE 2. RESPONSES OF GRAVID *Ae. albopictus* AND *Ae. aegypti* FEMALES TO FIELD WATER, LARVAL WATER AND HAY INFUSION (50%) COMPARED TO WELL WATER IN DUAL-PORT OLFACTOMETER

	% responding[a]	
	Ae. albopictus	*Ae. aegypti*
Field water	83.9 ± 6.3**	63.8 ± 8.7*
Well water	17.0 ± 6.2	35.9 ± 8.6
Laval water	74.5 ± 6.6**	54.5 ± 5.9
Well water	18.2 ± 5.1	40.2 ± 6.4
Hay infusion	69.4 ± 8.7*	59.9 ± 5.5
Well water	30.5 ± 9.2	40.1 ± 5.5

[a]Mean ± SE of 10 replicates of 50 mosquitoes. Paired comparisons of means of well water and paired treatment significantly different by ANOVA and *F* statistics, $*P < 0.05$, $**P < 0.001$.

ylindole. No differences were present in ovipositional response between well water, hay infusion, larval water, and field water, or between well water, hay infusion, 3-methylindole, and field water.

Oviposition responses of *Ae. albopictus* in experiment 2 differed significantly between treatments (ANOVA; $F = 5.04$, $df = 3, 56$; $P < 0.01$) (Table 1). Ovipositional responses to well water, 3-methylindole, and the mixture of compounds were similar and significantly lower than to hay infusion. Ovipositional responses of *Ae. aegypti* also differed significantly between treatments (ANOVA, $F = 4.79$, $df = 3, 36$; $P < 0.05$). Ovipositional responses to well water and 3-methylindole were similar. Responses to hay infusion and the mixture of synthetic compounds were significantly greater than to well water, but not significantly different from 3-methylindole.

Significantly more gravid *Ae. albopictus* and *Ae. aegypti* were collected in the olfactometer port containing field water than in the one containing well water (Table 2). The preference for field water over well water appeared stronger for *Ae. albopictus* (4.9-fold increase) than for *Ae. aegypti* (1.8-fold increase). Significantly more gravid *Ae. albopictus* were collected in the ports with larval water and hay infusion than in the ports with well water. Gravid *Ae. aegypti*, however, were collected equally in olfactometer ports with well water and either of these solutions.

DISCUSSION

Organic infusions such as hay infusion, larval rearing water, and field water clearly elicited strong oviposition responses from gravid *Ae. albopictus*, whereas

oviposition by *Ae. aegypti* was moderate to larval rearing water and field water and low in response to hay infusion even over a wide range of concentrations. These results are in agreement with previous reports that gravid *Ae. albopictus* oviposit readily in grass or hay infusions (Gubler, 1971; Hein, 1976; Holck et al., 1988) and in water containing immatures (Gubler, 1971). Our data support previous reports of the low response of gravid *Ae. aegypti* to grass or hay infusions (Hazard et al., 1967; Holck et al., 1988; Chadee, 1993), and the lack of orientation of gravid *Ae. aegypti* to hay infusion in an olfactometer is consistent with previous reports (Hazard et al., 1967). In contrast, Reiter et al. (1991) reported enhanced oviposition by *Ae. aegypti* in ovitraps with hay infusion. Responses of both species to organic infusions were lower than those reported for *Cx. quinquefasciatus* (Millar et al. 1992), and this may reflect that these *Aedes* species characteristically deposit eggs from a single gonotrophic cycle in several sites and that both species readily oviposit in clean water (Gubler, 1971; Holck et al., 1988). Organic infusions such as those tested in our study consist of complex mixtures of compounds that vary over time. At least some of the oviposition attractants from such organic infusions are products of bacterial degradation (Hazard et al., 1967; Ikeshoji et al., 1979; Benzon and Apperson, 1988). Larval water and field water, both of which contained mosquito larvae, elicited more oviposition by both *Aedes* species than hay infusion, and the production of more volatiles by these infusions related to attraction of females and/or stimulation of oviposition could possibly be related to the presence of larvae.

Compounds isolated and identified from Bermuda grass infusions known to elicit oviposition from *Cx. quinquefasciatus* (Millar et al., 1992) clearly play a lesser role in mediation of oviposition for both *Ae. albopictus* and *Ae. aegypti*. In *Cx. quinquefasciatus*, a species generally associated with polluted water, oviposition responses to 3-methylindole were strong across the entire range of concentrations tested (0.01–100 μg/liter), with up to 3.5× more egg rafts deposited in treatment cups compared to control cups (Millar et al., 1992). In our study, oviposition responses of female *Ae. albopictus* were significantly greater than to well water at only one concentration of 3-methylindole (0.1 μg/liter) and one of 4-methylphenol (0.01 μg/liter). Gravid *Ae. aegypti* did not appear to differentiate between well water and 3-methylindole at any dose for selection of oviposition sites. Millar et al. (1992) also reported moderate oviposition responses by gravid *Cx. quinquefasciatus* to one concentration of 4-ethylphenol (10 μg/liter) and two concentrations of indole (10 and 100 μg/liter). However, the latter responses were possibly due to trace amounts of 3-methylindole present. In contrast, 3-methylindole did not increase oviposition by either *Ae. albopictus* or *Ae. aegypti*. *Ae. aegypti* also exhibited a moderate increase in oviposition to 4-ethylphenol and phenol, but only at one of the concentrations tested. Exposure of gravid females to several concentrations of the synthetic compounds and organic infusions clearly resulted in lower oviposition responses than to well

water controls. These decreased responses may reflect repellency at these concentrations. The presence of the compounds identified by Millar et al. (1992) in concentrations found in this study to elicit low oviposition responses may contribute to the apparent repellent/deterrent effects to these species. Results from the field cage and olfactometer studies clearly indicate that larval water and field water contain attractants and/or stimulants for gravid females of both *Aedes* species. More studies are needed to isolate and identify compounds from these sources and to evaluate the behavioral roles of these compounds.

Compounds of infusions that attract gravid *Ae. albopictus* and *Ae. aegypti* females to potential oviposition sites have considerable potential for both increasing the sensitivity for monitoring populations of these species and for the potential delivery of pathogens or pesticides into larval populations (Schlein and Pener, 1990; Itoh et al., 1994). Surveillance of *Ae. albopictus* and *Ae. aegypti* is difficult, as adults are not readily attracted to light traps (Service, 1977; Hawley, 1988) and larval sampling is time-consuming. Ovitraps are often used for population monitoring and surveillance (Chadee et al., 1988; McHugh and Hanny, 1990), and the use of oviposition attractants/stimulants to increase the sensitivity of these traps as well as gravid female traps (Reiter, 1983; Freier and Francy, 1991) has considerable potential. Of particular importance for this application are infusions or compounds that both elicit long-range orientation of gravid females to potential oviposition sites and stimulate oviposition once at the oviposition site. The use of infusions such as hay infusions in conjunction with ovitraps or gravid females traps may, however, bias trap collections towards *Ae. albopictus*, as our results indicated a greater attraction of *Ae. albopictus* than *Ae. aegypti* to hay infusion. This is currently being examined under field conditions.

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ATTRACTANTS FROM *Staphylococcus aureus* CULTURES FOR MEXICAN FRUIT FLY, *Anastrepha ludens*

D.C. ROBACKER[1,*] and R.A. FLATH[2]

[1]*Crop Quality and Fruit Insects Research, ARS, USDA*
2301 South International Blvd., Weslaco, Texas 78596
[2]*Plant Protection Research, ARS, USDA*
800 Buchanan, Albany, California 94710

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Abstract—Volatile chemicals from tryptic soy broth cultures of *Staphylococcus aureus* that attract sugar-fed, protein-hungry adult Mexican fruit flies were identified. Chemicals identified from the headspace above the filtrate of the bacterial cultures were ammonia, trimethylamine, isoamylamine, 2-methylbutylamine, 2,5-dimethylpyrazine, and acetic acid. Each chemical attracted flies. A mixture of the chemicals in the same concentrations as were found in the bacterial filtrate was 89% as effective in attracting flies as the bacterial filtrate in laboratory bioassays. Additional chemicals were identified from various concentrated or pH altered preparations made from the filtrate. Many of these chemicals also attracted flies. One of these chemicals, dimethylamine, was the most effective chemical identified. The use of solid-phase microextraction for volatile collection and of thick-film (5-μm) capillary GC columns was essential to the success of this work.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, bacteria, amines, solid-phase microextraction.

INTRODUCTION

Associations of bacteria with fruit flies have been known for nearly a century (Petri, 1910) but remain little understood to this day. Popular views during the first half of this century were that bacteria and fruit flies engaged in obligate symbioses (Stammer, 1929; Hellmuth, 1956; Boush and Matsumura, 1967; Miyazaki et al., 1968). Later studies suggested that bacteria serve as a protein

*To whom correspondence should be addressed.

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source for fruit flies (Drew and Lloyd, 1989; Robacker and Moreno, 1995). Neither of these views is entirely satisfactory nor entirely disproven, and the possibility of other explanations must also be acknowledged. Whatever the explanation, associations of bacteria with fruit flies seem nearly ubiquitous. As examples, Stammer (1929) isolated bacteria from 37 species of fruit flies and Hellmuth (1956) from 43 species. Fitt and O'Brien (1985) isolated at least 20 species of bacteria from four *Dacus* species, and Martinez et al. (1994) isolated at least 11 species of bacteria from *A. ludens* alone.

Some understanding of the nature of fruit fly–bacteria associations has been gained by studies showing that bacteria or their metabolic products attract fruit flies in many cases. Several studies have shown that inoculation of protein baits with bacteria increased the attractiveness of the baits (Gow, 1954; Bateman and Morton, 1981; Drew and Fay, 1988). Others have shown that bacteria or their cultures alone, or combined with fruit volatiles, make good trap baits (MacCollom et al., 1992; 1994; Martinez et al., 1994) or attract fruit flies in laboratory bioassays (Jang and Nishijima, 1990; Robacker et al., 1991; MacCollom et al., 1992).

Papers in which attractants from bacterial preparations have been assessed generally reported little progress beyond identification and evaluation of ammonia (Gow, 1954; Drew and Fay, 1988). Most who have worked with bacterial attractants agree that additional chemicals probably are involved. Drew (1987) tested several components of bacterial odor and demonstrated that one of these, 2-butanone, was significantly more attractive to *Bactrocera tryoni* Froggatt than cuelure, the standard attractant for this fly. The attractiveness of this chemical was attributed to its structural similarity to cuelure rather than any bacteria–fruit fly relationship. Two other components, butanol and isopentanol, were not attractive.

Recent work by Robacker et al. (1993) showed that standard insect attractant volatile collection methods were unsuitable for collecting attractive compounds from a highly attractive bacterial fermentation. Specifically, volatile collections of bacterial odors on adsorbents typically used in insect attractant research such as Porapak Q, Tenax, and Super Q were not attractive to Mexican fruit flies. The work also demonstrated that the attractants were water soluble and could not be partitioned into organic solvents by solvent–solvent extraction. The conclusion of the work was that the most attractive chemicals probably were low-molecular-weight amines or other compounds containing ionizable nitrogen. It also appeared that some attractants did not have properties of amines and possibly were carboxylic acids or other water-soluble organic molecules. These findings may help explain the lack of progress in identification of bacteria-produced chemicals attractive to fruit flies since most reported studies used standard volatile collection methods.

The current reseach is a continuation of the work reported by Robacker et al. (1993) to identify the volatile chemicals produced by tryptic soy broth cultures of *Staphylococcus aureus* (strain RGM-1) originally isolated from the mouthparts of a laboratory-colony, adult Mexican fruit fly (Robacker et al., 1991). Solid-phase microextraction technology developed especially for analysis of trace organics in aqueous solution (Belardi and Pawliszyn, 1989; Zhang and Pawliszyn, 1993) was used to collect volatiles from the headspace above the filtrate of RGM-1 cultures and various concentrated or pH-altered preparations made from the filtrate. Trapped chemicals were identified and tested for attractiveness to Mexican fruit flies.

METHODS AND MATERIALS

Insects and Test Conditions. Flies used to test attractiveness of bacterial preparations and chemicals identified from the preparations were from a culture that had been maintained on laboratory diet for about 400 generations with no wild-fly introductions. Mixed-sex groups of 180–200 flies were kept in 473-ml cardboard cartons with screen tops until used in tests. Flies were tested when 5–12 days old. Flies were deprived of protein as adults but were fed sucrose and water up until the time of attractiveness testing. Recent tests have shown that protein-deprived flies are maximally attracted to bacterial odor when 5–7 days old and remain highly responsive through 15 days of age (Robacker and Garcia, 1993). All tests were conducted in the laboratory between 08:30 and 15:30 hr under a combination of fluorescent and natural light. Laboratory conditions were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 06:30 to 19:30 hr.

Bacterial Preparations. S. aureus strain RGM-1 (Robacker et al., 1991) was grown in tryptic soy broth (Difco Laboratories, Detroit, Michigan) in a shaker for 144 hr at 30°C. Bacterial culture was centrifuged at 10,000 rpm for 20 min and separated into pellet and supernatant. The pellet was discarded because previous experiments indicated that the pellet, which contained most of the bacterial cells, was less attractive than tryptic soy broth (Robacker et al., 1993). Supernatant was filtered through 0.45-μm type HA aqueous filters (Millipore Corporation, Bedford, Massachusetts) followed by 0.22-μm type GV aqueous/organic filters (Millipore). Filtration was conducted to remove remaining bacterial cells. Robacker et al. (1993) demonstrated that the attractants were dissolved in the filtrate.

RGM-1 filtrate was concentrated and otherwise manipulated to enhance collection and identification of volatiles. RGM-1 concentrate was prepared by lowering pH of filtrate to 4.0 with 10 N HCl and concentrating it 12-fold by

rotary evaporation (Rotavapor RE-111, Buchi-Brinkman Instruments, Inc., Westbury, New York). The evaporation was aided by heating the filtrate while pulling a vacuum on the system. RGM-1 salt was prepared as described previously (Robacker et al., 1993), with a few differences. Briefly, RGM-1 filtrate was put into a two-neck 500-ml flask and heated gently while steam was used to sweep the headspace from the flask into a water-cooled condenser. Steam was generated from vigorous boiling of Milli-Q (Millipore)-grade water in a second two-neck 500-ml flask. The procedure was carried out until 400 ml of steam distillate was collected per 100 ml of original RGM-1 filtrate. The pH of the steam distillate was lowered to 4.0 with HCl, and the water was removed by rotary evaporation as described for the RGM-1 concentrate. The resulting dry powder was termed RGM-1 salt.

Volatile Collections and Chemical Identifications. Chemicals in the headspace above RGM-1 filtrate, RGM-1 filtrate adjusted to pH 11 with aqueous NaOH, RGM-1 concentrate at pH 4 and also pH 11 after adjustment with NaOH, and RGM-1 salt partially dissolved in a few drops of saturated aqueous NaOH were collected and identified. Volatiles were collected by solid-phase microextraction (SPME) with a 100-μm polydimethylsiloxane-coated fiber (Supelco, Inc., Bellefonte, Pennsylvania). The fiber was inserted through a septum into the headspace above 1 ml of bacterial preparation in a 4-ml vial for 5 min to 24 hr. Sampling time varied inversely with the concentration of chemicals in the preparation.

Volatiles also were collected on ORBO 52 silica gel tubes (Supelco) connected to an outlet port in the volatile collection apparatus developed by Heath and Manukian (1992). One half milliliter of saturated NaOH was added to 0.5 ml of RGM-1 concentrate in a 4-ml glass vial to produce free amines. The vial was mounted inside the apparatus such that the inlet end of the silica tube was inside the lip of the vial. Air was pulled through the system for 17 hr at 0.5 liters/min. The silica gel was removed from the tube and extracted by sonication in a 4-ml glass vial with 0.5 ml of methanol for 0.5 hr followed by a rinse with a second 0.5 ml of methanol. The two methanol aliquots were combined and filtered through 0.45-μm type HV syringe filters (Millipore).

GC analyses were conducted on DB-1 and DB-5 fused-silica capillary columns (J & W Scientific, Folsom, California). The DB-1 column (60 m, 0.32 mm ID, 5 μm film) was used for most analyses because it appreciably retained but did not irreversibly adsorb nanogram-level quantities of ammonia and C_1 to C_5 aliphatic amines, providing baseline resolution of most of these compounds. The DB-5 column (30 m, 0.53 mm ID, 5 μm film) was used to separate a few amines that coeluted on the DB-1 column and to confirm identifications that were based on retention time matching to standards on the DB-1 column. Preliminary work with films of less than 5 μm demonstrated that the thick films (5 μm) were responsible both for the lack of irreversible adsorption and appreciable

retentions of the chemicals discussed above and also improved peak symmetry allowing for better resolution of components with similar retention times.

GC analyses were conducted with a Shimadzu GC-9A (Shimadzu Scientific Instruments, Inc. Columbia, Maryland) with flame ionization and flame thermionic (model FTD-9) detectors. The FTD was used to establish the presence of C—N bonds and thus confirm characterization of many of the bacteria volatiles as amines, to provide highly sensitive detection of trace quantities of several of the compounds and to calculate FID/FTD response ratios of unknowns for comparison with ratios of standards as another identification method.

Volatiles were thermally desorbed from the SPME fiber in the split/splitless injection port at 210°C. Splitless injection was conducted with the splitter closed 0.5 min. Analyses were at 100°C. ORBO 52 extracts were concentrated 10-fold under nitrogen assisted by low heating. Splitless injection was also used for 1 μl samples of the concentrated ORBO 52 extracts. Analyses of ORBO 52 extracts were conducted with an initial temperature at 100°C for 5 min then 4°C/min to 210°C. For all analyses, carrier gas was helium at a linear velocity of 20 cm/sec.

GC-MS analyses were conducted with a Finnigan MAT 4500 GC-MS data system (Finnigan MAT, San Jose, California) operated in the electron-impact ionization mode (emission current = −0.30 mA, electron energy = 70 eV, multiplier voltage = −1150 V). Mass spectral data were acquired over a mass range of 33–350 amu, using a 1-sec scan rate. Volatile injection was the same as described above except the injector temperature was 220°C and the SPME fiber was exposed in the injector for 2 sec. The column-oven temperature was programmed from 50 to 200°C at 4°/min. Carrier gas was helium at 28 cm/sec.

Cage-Top Bioassays. Various RGM-1 preparations and chemicals identified from the preparations were tested for attractiveness using cage-top bioassays. Tests of bacterial preparations were conducted because volatiles were identified from many of these preparations, and we wanted to compare attractiveness of preparations based on similarity of their volatiles.

Bioassays were conducted by placing four filter paper triangles (3 cm/side), two containing test samples and two containing appropriate controls, near the corners on the top of an insect cage (30 cm/side, aluminum-screened). The numbers of flies beneath each filter paper were counted once each minute for 10 min. The two papers containing test chemical were positioned diagonally from each other on two corners of the cage top, and the two papers containing controls were positioned diagonally from each other on the other two corners. The filter papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction and not contact chemoreception was solely responsible for the response of the flies. One carton of 180–200 flies was used in each bioassay cage.

For bioassays of RGM-1 preparations, 10-μl test quantities were put on two papers and 10 μl of water were put on two papers as controls. The ORBO 52 collections were bioassayed similarly except methanol was used as the control. Ten replications were conducted for each RGM-1 preparation and for the ORBO 52 collection.

Chemicals identified from various RGM-1 preparations were obtained (Table 1) and dissolved in water or methanol for attractiveness testing. Chemicals dissolved in water were: dimethylamine HCl; trimethylamine HCl; ethylamine; propylamine; isopropylamine; isobutylamine; isoamylamine; 2-ethyl-5-methylpyrazine; and acetic acid. One drop of saturated NaOH was added to solutions of dimethylamine HCl and trimethylamine HCl to produce free amines.

TABLE 1. CHEMICAL NAMES, SOURCES, AND PURITIES OF CHEMICALS USED AS GC STANDARDS AND/OR TESTED IN CAGE-TOP BIOASSAYS

Chemical	Source[a]	Purity (%)[b]
Ammonium bicarbonate	Sigma	99
Ammonium carbonate	Aldrich	unknown[c]
Methylamine HCl	Sigma	99
Dimethylamine HCl	Sigma	>95
Trimethylamine HCl	Sigma	99
Ethylamine (70% in water)	Sigma	>99
Diethylamine	Aldrich	98
Propylamine	Aldrich	99
Isopropylamine	Aldrich	99
Butylamine	Sigma	99
Isobutylamine	Aldrich	99
sec-Butylamine	Aldrich	99
tert-Butylamine	Aldrich	98
Isoamylamine	Aldrich	99
2-Methylbutylamine	Aldrich	>97
2,5-Dimethylpyrazine	Aldrich	99
2-Ethyl-5-methylpyrazine	Pyrazine Specialties	50[d]
Acetamide	Aldrich	>99
Propionamide	Aldrich	97
2-Methylpropionamide	Aldrich	99
Acetic acid	Mallinckrodt	>99
Methyl disulfide	Aldrich	99

[a] Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; Sigma Chemical Company, St. Louis, Missouri; Pyrazine Specialties, Inc., Atlanta, Georgia; Mallinckrodt Specialty Chemicals Co., Paris, Kentucky.
[b] Determined from label information and GC analysis.
[c] Mixture with ammonium bicarbonate and ammonium carbamate.
[d] Approximately equal mixture with 2-ethyl-6-methylpryazine.

The other chemicals were dissolved in methanol. Four concentrations of each chemical were prepared and subsequently four quantities (10 ng, 100 ng, 1 μg, and 10 μg of most chemicals; 100 ng, 1 μg, 10 μg, 100 μg of amides) of each chemical were tested, each in 10 μl of solution. The control for each chemical was 10 μl of water or methanol for chemicals dissolved in water or methanol, respectively. Ten replications were conducted for each of the four concentrations of all of the chemicals, except trimethylamine and 2,5-dimethylpryazine for which 20 replications were conducted.

Chemicals identified from headspace above RGM-1 filtrate that were attractive to flies in cage-top bioassays were mixed together in aqueous solution in the same concentrations as were found in RGM-1 filtrate. Concentrations of the chemicals in RGM-1 filtrate were determined by comparing SPME collections in headspace above known concentrations of mixtures of standards, with SPME collections in RGM-1 headspace. The pH of the standards solution was adjusted to 7.9 with aqueous NaOH to equal the pH of unaltered RGM-1 filtrate. A trial-and-error approach was used in which standards were mixed together in a vial in various concentrations that were adjusted until FID peak heights on the DB-1 column matched those obtained from a vial containing RGM-1 filtrate. Concentrations in the final synthetic mixture that matched RGM-1 were: ammonium carbonate, 2.0 mg/ml; trimethylamine HCl, 4.0 μg/ml; isoamylamine, 1.6 μg/ml; 2-methylbutylamine, 0.3 μg/ml; 2,5-dimethylpyrazine, 5.0 μg/ml; and acetic acid, 250 μg/ml. Sources of chemicals used in the mixture are shown in Table 1. The final synthetic mixture was tested in cage-top bioassays using water as the control. Test quantities were 10 μl. Twenty-eight replications were conducted. Separate bioassays of RGM-1 filtrate ($N = 28$) were conducted concurrently using flies from the same cohort.

Statistical Analysis. Responses of flies to the various bacterial preparations were compared with each other in a randomized complete block experiment. This was done by subtracting total counts at solvent-blank papers from total counts at treatment papers to obtain differences that were then subjected to analysis of variance. Means were compared by Fisher's protected least significant difference method. Total counts of flies at filter papers containing test samples were compared to counts at solvent-blank papers by paired t tests in all experiments. Finally, responses of flies to the synthetic mixture of RGM-1 chemicals were compared with responses to RGM-1 filtrate by a t test of independent samples. Data for this test were differences between total counts at treatment and solvent-blank papers for each replication.

RESULTS AND DISCUSSION

All bacterial preparations were more attractive to flies than water in cage-top bioassays (Table 2). RGM-1 filtrate was the most attractive preparation.

TABLE 2. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING WATER OR VARIOUS BACTERIAL PREPARATIONS USING CAGE-TOP BIOASSAYS

	Preparation[a]	Water
RGM-1 filtrate	141.7 a	14.3
RGM-1 filtrate pH 11	108.2 b	14.5
RGM-1 concentrate pH 4	29.9 c	18.5
RGM-1 concentrate pH 11	99.2 b	17.4
RGM-1 salt pH 11	113.5 b	17.1
ORBO 52 volatiles collection	36.4 c	20.1

[a] Mean responses to all bacterial preparations were significantly greater than responses to water by paired *t*-tests ($P < 0.05$); mean responses to bacterial preparations followed by the same letter were not significantly different from each other by LSD ($P < 0.05$).

Most of the results in Table 2 are similar to findings reported in Robacker et al. (1993). One preparation not tested by Robacker et al. (1993) was the ORBO 52 volatile collection. Here we show that at least some of the material responsible for attractiveness can be trapped on ORBO 52 silica gel tubes, although the attractiveness was low compared with that of RGM-1 filtrate. Robacker et al. (1993) reported that attractants evolving from concentrated RGM-1 filtrate could not be trapped on Porapak Q, Super Q, or Tenax GC.

Chemicals identified from headspace of RGM-1 filtrate at pH 7.9 (unaltered) are shown in Table 3. Calculated concentrations (mean ± SE; $N = 3$) in RGM-1 filtrate were: ammonia, 710 ± 41 μg/ml; trimethylamine, 2.5 ± 0.1 μg/ml; isoamylamine, 1.6 ± 0.7 μg/ml; 2-methylbutylamine, 0.3 ± 0.05 μg/ml; 2,5-dimethylpyrazine, 5.0 ± 0.1 μg/ml; and acetic acid, 250 ± 84 μg/ml.

All six chemicals found in the headspace above the RGM-1 filtrate attracted Mexican fruit flies. Data for trimethylamine, isoamylamine, 2-methylbutylamine, 2,5-dimethylpyrazine, and acetic acid are shown in Table 4. Ammonia was tested previously, and the data were reported in Robacker and Warfield (1993). Like most of the chemicals, ammonia was most attractive at the higher concentrations. Ammonia, isoamylamine, 2-methylbutylamine, and acetic acid appear to be nearly equally attractive according to ratios of counts at chemicals (10 μg test quantities) to counts at water. Trimethylamine and 2,5-dimethylpyrazine were much less attractive than the others. Trimethylamine was unusual in that it was attractive only at the lower concentrations.

The synthetic mixture of the chemicals found in RGM-1 filtrate was much more attractive than water but significantly less attractive than RGM-1 filtrate (Table 5). Nevertheless, the response to the synthetic mixture was 89% as high as the response to RGM-1 filtrate, indicating that the synthetic mixture was a fairly good match to the RGM-1 filtrate. Reasons for the slightly lower response

TABLE 3. CHEMICALS IDENTIFIED FROM VARIOUS BACTERIAL PREPARATIONS

	Chemicals Identified and Methods of Identification[a]
RGM-1 filtrate (pH not altered from 7.9)	ammonia, 2-methylbutylamine by DB-1 RT, FID/FTD ratios; trimethylamine, isoamylamine, 2,5-dimethylpyrazine, acetic acid by MS, DB-1 RT, FID/FTD ratios
RGM-1 filtrate (pH 11)	ammonia, trimethylamine, isoamylamine, 2-methylbutylamine by DB-1 RT, FTD detection; 2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine by MS
RGM-1 concentrate (pH 4)	acetic acid by MS
RGM-1 concentrate (pH 11)	ammonia, 2-methylbutylamine by DB-1 RT, FTD detection; trimethylamine, isoamylamine by MS, DB-1 RT, FTD detection; acetic acid, methyl disulfide by MS
RGM-1 salt (NaOH added)	ammonia, methylamine, dimethylamine, trimethylamine ethylamine, diethylamine, propylamine, isopropylamine, butylamine, isobutylamine, *sec*-butylamine, *tert*-butylamine, isoamylamine, 2-methylbutylamine by both DB-1 and DB-5 RT, FID/FTD ratios
ORBO 52	acetamide, propionamide, 2-methylpropionamide, 3-methylbutyramide by MS

[a] MS = mass spectrometry using DB-1 column; RT = retention time comparison with standards on DB-1 and/or DB-5 columns; FID/FTD ratios = comparison of response by flame ionization vs flame thermionic detectors

to the synthetic mixture are unknown. Possible reasons are: (1) one or more attractants in RGM-1 filtrate was not identified and consequently left out of the synthetic mixture; (2) contaminants in the synthetic mixture from the commercially obtained chemicals were repellent; and/or (3) concentrations of chemicals in the synthetic mixture were not identical to concentrations in RGM-1 filtrate.

The synthetic mixture did not have the same odor as RGM-1 filtrate, as judged by human olfaction. The Porapak Q and similar volatile collections tested in earlier work (Robacker et al., 1993) smelled very similar to RGM-1 filtrate. Nevertheless, the Porapak Q volatile collections did not attract Mexican fruit flies, whereas the synthetic mixture used in the present study was highly attractive.

Chemicals identified from RGM-1 filtrate adjusted to pH 11 were ammonia, trimethylamine, isoamylamine, 2-methylbutylamine, 2,5-dimethylpyrazine, and 2-ethyl-5-methylpyrazine (Table 3). Only acetic acid was identified from RGM-1 concentrate at pH 4. No other carboxylic acids were detected. Chemicals identified from RGM-1 concentrate adjusted to pH 11 were ammonia, trimethylamine, isoamylamine, 2-methylbutylamine, acetic acid, and methyl disulfide.

TABLE 4. MEAN COUNTS OF *A. ludens* AT SOLVENT BLANKS OR TEST CHEMICALS IDENTIFIED FROM VARIOUS BACTERIAL PREPARATIONS USING CAGE-TOP BIOASSAYS

	10 ng		100 ng		1 μg		10 μg	
	Test	Solvent	Test	Solvent	Test	Solvent	Test	solvent
Dimethylamine[a]	13.3	11.5	15.4	14.0	41.3[b]	12.8	77.0[b]	12.3
Trimethylamine[a]	19.2[b]	13.1	22.1[b]	16.0	19.1	16.6	19.3	17.8
Ethylamine	13.1	13.1	12.5	12.6	44.8[b]	15.2	43.8[b]	13.4
Diethylamine	14.8	14.8	14.3	15.0	20.3[b]	13.2	29.1[b]	11.1
Propylamine	13.0	13.3	15.8	10.4	27.3[b]	14.2	28.8[b]	12.3
Isopropylamine	12.9	13.9	12.4	10.0	25.5[b]	10.9	44.5[b]	12.0
Butylamine	12.8	10.7	16.4	13.5	32.3[b]	13.2	29.2[b]	15.2
Isobutylamine	10.7	10.2	14.1	11.2	28.8[b]	12.8	29.6[b]	12.1
sec-Butylamine	13.2	13.7	11.7	12.7	39.8[b]	12.7	29.6[b]	11.9
tert-Butylamine	17.5	17.7	20.4	16.3	20.2	17.7	25.8[b]	17.8
Isoamylamine	11.9	13.6	13.6	14.5	21.9[b]	10.7	43.4[b]	15.9
2-Methylbutylamine	20.6	16.1	20.8	21.4	23.8	23.1	36.6[b]	20.8
2,5-Dimethylpyrazine	14.3	15.4	16.0	15.6	18.9[b]	13.6	13.0	14.2
2-Ethyl-5-methylpyrazine	24.1	21.8	22.2	21.0	13.0[b]	18.9	8.9[b]	22.5
Acetic acid	15.9	10.1	14.5	12.2	21.8[b]	10.8	47.1[b]	14.1
Methyl disulfide	21.2	19.9	22.8	20.4	15.1	19.0	21.4	18.8

[a]Tested as their respective HCl salts at pH > 10.
[b]The mean response to a chemical was significantly different from the response to water by paired *t*-test ($P < 0.05$).

TABLE 5. MEAN COUNTS (±SE) OF *A. ludens* AT FILTER PAPERS CONTAINING SYNTHETIC MIXTURE OF CHEMICALS IDENTIFIED FROM RGM-1 HEADSPACE COMPARED WITH COUNTS AT PAPERS CONTAINING RGM-1 FILTRATE OR WATER USING CAGE-TOP BIOASSAYS

	Test preparation	Water
Synthetic mixture	135.9 ± 4.8[a]	20.5 ± 1.2
RGM-1 filtrate	152.9 ± 5.5	19.7 ± 1.2

[a] The mean response to the synthetic mixture was significantly greater than the response to water by a paired t test ($P < 0.01$), and significantly lower than the response to RGM-1 filtrate by a t test ($P < 0.05$)

Neither 2-ethyl-5-methylpyrazine nor methyl disulfide was attractive to flies (Table 4). 2-Ethyl-5-methylpyrazine was slightly repellent at the two highest test quantities. Methyl disulfide previously was identified from volatiles of the bacterium *Proteus* by Hayward et al. (1977), but it had not been tested as a fruit fly attractant.

Many additional chemicals were identified from the headspace above the RGM-1 salt partially dissolved in NaOH (Table 3). All of the chemicals were attractive to flies, mostly at the higher test quantities (Table 4). Dimethylamine was the most attractive chemical identified from any of the bacterial preparations according to ratios of counts at chemicals to counts at water. Attractiveness of methylamine was about equal to ammonia in experiments reported previously (Robacker and Warfield, 1993).

The only chemicals identified from ORBO 52 collections were acetamide, propionamide, 2-methylpropionamide, and 3-methylbutyramide (Table 3). Of the three that were tested, none attracted the flies (Table 6). These chemicals

TABLE 6. MEAN COUNTS OF *A. ludens* AT SOLVENT BLANKS OR TEST CHEMICALS IDENTIFIED FROM ORBO 52 COLLECTION OF VOLATILES FROM RGM-1 CONCENTRATE AT pH > 10 USING CAGE-TOP BIOASSAYS[a]

	100 ng		1 μg		10 μg		100 μg	
	Test	Solvent	Test	Solvent	Test	Solvent	Test	Solvent
Acetamide	12.2	10.3	14.3	14.0	13.0	15.2	13.9	12.0
Propionamide	9.5	9.1	10.0	9.0	11.3	9.3	9.9	10.9
2-Methylpropionamide	12.0	13.0	12.5	13.3	14.2	15.1	10.2	11.0

[a] 3-Methylbutyramide also was identified from ORBO 52 collection, but was not available for testing. None of the chemicals were more attractive than blanks.

were tested at higher concentrations than the others because of their lower volatilities. Other chemicals that may have been present in the ORBO 52 extract and which may have accounted for its attractiveness (Table 2) probably were lost when it was concentrated for GC-MS analysis.

Not all of the chemicals in the various preparations were identified. The emphasis of the investigation was to identify chemicals that were water soluble and contained ionizable nitrogen, because chemical characterization studies had indicated that the chemicals most attractive to *A. ludens* fit this profile (Robacker et al., 1993). Most of the unidentified chemicals were minor components. However, some relatively large FID peaks present in the headspace of the RGM-1 salt were not identified because they were not detectable by FTD. One exception was a major unidentified peak in the headspace of the RGM-1 salt that produced a large FTD/FID ratio, indicating that it contained C-N. This peak had a retention time that matched that of 1,2-dimethypropylamine on the DB-1 column but not on the DB-5 column.

Another important point is that some of the chemicals identified in this study may have been artifacts of the harsh procedures used to concentrate the volatiles. The strong acids and bases used to manipulate pH may have catalyzed various hydrolyses and condensations involving water, alcohols, carboxylic acids, amines, etc., present in the original solution. Furthermore, headspace sweeping with steam was assisted with heating, and no attempt was made to remove oxygen from the system. Thus, numerous products not originally present in the RGM-1 filtrate may have formed. This probably occured the most in the RGM-1 salt, the product that was produced using the most rigorous treatment. Interestingly, all of the chemicals identified from RGM-1 salt headspace were attractive to the flies. Perhaps many of these same chemicals also form in natural fermentations where heat, water, and oxygen are also present.

The chemicals identified from the headspace of unaltered RGM-1 filtrate are considered natural products of bacterial metabolism of the tryptic soy broth. Therefore, only these chemicals were quantified and used to construct a synthetic mixture to mimic bacterial odor. The other bacterial preparations were not reproduced for attractiveness testing because they may have contained chemicals that were not present in the original culture. Quite possibly, many of the same chemicals identified in the various other preparations, including RGM-1 salt, may have been present in the RGM-1 filtrate but at concentrations too low to detect by our methods.

This is the first investigation to identify volatile chemicals produced by a bacterial culture attractive to an insect species, test the attractiveness of the chemicals to the insect, construct a mixture of the chemicals at the same concentrations as found in the bacterial preparation, and test the attractiveness of the mixture relative to the attractiveness of the bacterial preparation. As it has been shown that preparations made from bacterial fermentations can be highly

attractive to fruit flies in the field (Martinez et al., 1994), it is hoped that results of this work can contribute to development of better attractants for the Mexican fruit fly and perhaps other species of Tephritidae. However, it should be noted that the study was not entirely successful, as the synthetic mixture was only 89% as attractive as the bacterial filtrate.

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SYNERGISM OF AN INSECT SEX PHEROMONE SPECIALIST NEURON: IMPLICATIONS FOR COMPONENT IDENTIFICATION AND RECEPTOR INTERACTIONS

M.S. MAYER* and R.E. DOOLITTLE

Insect Attractants, Behavior, and Basic Biology Research Laboratory
Agricultural Research Service, U.S. Department of Agriculture
Gainesville, Florida 32604

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Abstract—Extracellular recordings show that the response to the sex pheromone component, (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) by the HS(a) antennal olfactory specialist neuron of the cabbage looper, *Trichoplusia ni* (Hübner), is synergized by two synthetic sex pheromone analogs and two components of the female sex pheromone. Complementary behavioral measures of upwind flight and copulatory responses to mixtures of *Z*7-12:Ac with the analogs and sex pheromone components in a wind tunnel produced behavioral evidence consistent with the neuron's electrophysiological responses. The phenomenon of receptor neuron synergism impacts several areas and provides a note of caution for common practices of identifying secondary sex pheromone components only from field traps and/or wind tunnel tests.

Key Words—Cabbage looper, electrophysiology, antenna, single cell responses, behavior.

INTRODUCTION

Chemical components of female moth sex signals are detected by olfactory receptor neurons housed within characteristic, often sexually dimorphic, sensilla located on the antenna. These receptor neurons are specialized to detect low, physiologically relevant, airborne concentrations of primarily one of the compounds that comprise the female signal. At elevated stimulus concentrations, however, even one of the most highly specialized neurons, the bombykol spe-

*To whom correspondence should be addressed.

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cialist of *Bombyx mori* (L.), will respond to other compounds (Priesner, 1969). This is typical also of the HS(a) specialist of the cabbage looper moth, *Trichoplusia ni* (Hübner), which detects the behaviorally most important component of its female chemical signal, (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac) (Mayer, 1993). As a consequence of this type of stimulus-strength-dependent specificity, it seems reasonable that the admixture of a sex pheromone component with a less active compound would elicit an additive response if both compounds are present at concentrations exceeding their individual thresholds.

There are no reports of specialist neuron responses to mixtures of pheromone components or a particular component with other compounds at high stimulus intensities. At physiologically relevant airborne concentrations, Mayer (1993) showed that mixtures of pheromone components did not modify the response of the HS(a) specialist to *Z*7–12:Ac, but he did not assay these or other compounds at elevated stimulus intensities. What we examine in this report, inter alia, is whether or not a binary mixture stimulus, in which one of the components is *Z*7–12:Ac, will elicit a mixed response from the HS(a) specialist neuron.

This examination was prompted by our interest in the report of Burger et al. (1990) that 7-vinyldecyl acetate (7-VD) reduced trap captures of male false codling moths, *Cryptophlebia leucotreta* (Meyrick), when incorporated into an otherwise effective sex pheromone bait. Comparison of the structure (Figure 1) of one of the two false codling moth sex pheromone components, (*Z*)-8-dodecenyl acetate (*Z*8–12:Ac), with 7-VD shows that the length of the carbon chain between the double bond and the acetate function is the same in both molecules. In addition, the length of the carbon chain beyond the point of attachment of the double bond and the overall molecular size is the same. With these parameters in mind, we undertook the synthesis and behavioral and neurophysiological evaluation of molecules that would have a similar relationship to *Z*7–12:Ac and, therefore, might be expected to stimulate the HS(a) neuron. We chose 6-vinyldecyl acetate (6-VD, Figure 1) and 10-vinyltetradecyl acetate (10-VT, Figure 1) as candidates. In the case of 6-VD, the relationship to *Z*7–12:Ac is analogous to the relationship between 7-VD and *Z*8–12:Ac of the false codling moth. Indeed, one can make the reasonable claim that because the vinyl group has no stereochemical restrictions, 7-VD can be spatially compared with the other false codling moth sex pheromone component, (*E*)-8-dodecenyl acetate (*E*8–12:Ac), as well. Inclusion of 10-VT in the study enabled the evaluation of the combined effect on the HS(a) neuron of *Z*7–12:Ac and a molecule that had a longer carbon chain between the acetate function and the double bond than *Z*7–12:Ac but retained the same four-carbon chain on the other side of the double bond. We tested 6-VD and 10-VT alone and in binary mixtures with *Z*7–12:Ac and found that these mixtures synergized both the response of the HS(a) neuron and upwind flight responses in a wind tunnel.

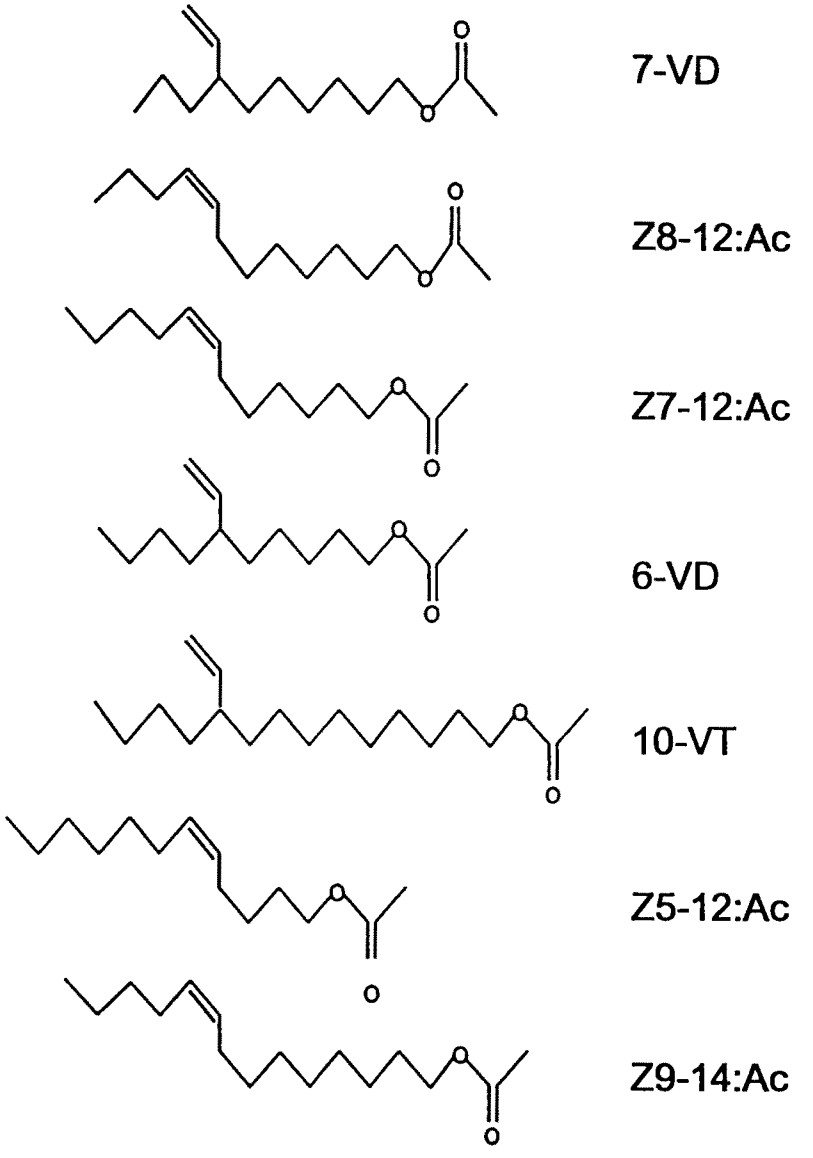

FIG. 1. Chemical structures of the compounds discussed in the text and/or used as odor stimuli in the electrophysiological and behavioral experiments: 7-vinyldecyl acetate, 7-VD; (Z)-8-dodecenyl acetate, Z8-12:Ac; (Z)-7-dodecenyl acetate, Z7-12:Ac; 6-vinyldecyl acetate, 6-VD; 10-vinyltetradecyl acetate, 10-VT; (Z)-5-dodecenyl acetate, Z5-12:Ac; and (Z)-9-tetradecenyl acetate, Z9-14:Ac.

Until now, however, synergism has not been observed in moth sex pheromone specialist neurons. Synergism, in the sense that we use it for these studies, is the resultant response of the specialist neuron to the cooperative action of two or more discrete chemicals that is greater than the sum of the responses to the individual compounds. From this juncture, we gave thought to the possibility that synergism might be the means by which female volatile emission components that lack specialists are able to influence behavior.

Two components that are considered to be part of the female sex signal were chosen for assay for the following reasons. One component, Z9–14 : Ac (Figure 1), has been shown to be detected by a specialist neuron (Mayer and Mankin, 1990), although its effect on behavior is marginal (Linn et al., 1984; Mayer and McLaughlin, 1991, 1993). The other component, Z5–12 : Ac (Figure 1), was chosen because no specialist yet has been observed for its detection, but some preliminary assays in our wind tunnel at high stimulus intensities showed that it elicited some upwind flight behavior. Both of the compounds were examined alone and mixed with Z7–12 : Ac. The mixtures synergized both the neural response and upwind flight responses in a wind tunnel. One component, Z5–12 : Ac, synergized the response at near physiological airborne concentration levels, but Z9–14 : Ac did not. The implications of these results are discussed with respect to pheromone component identification, the effect of synergism on some aspects of behavior, and briefly, the potential use of synergists in field applications.

METHODS AND MATERIALS

Insects. The moths were reared on a semisynthetic medium described in Guy et al. (1985). Males and females were separated during the pupal stage. After emergence, 40–60 males were maintained in 25 × 25 × 25-cm Plexiglas and fiberglass screen cages until assayed at 3–4 days of age. Two to four small plastic cups filled with a nutrient solution of 10% sugar-water on cotton were provided for each cage. The cages were maintained in cabinets at 25–27°C, 70–80% relative humidity on a 14 : 10 light-dark photoperiod.

Chemicals. 6-VD and 10-VT were synthesized at this laboratory and were 98% pure by capillary column gas chromatographic (GC) analysis. Details of their synthesis will be the subject of a future report. The (*Z*)-7-dodecen-1-ol acetate (Z7–12 : Ac) was a synthetic sample purified by HPLC and silicic acid column chromatographic procedures. It contained impurities totaling less than 0.1% of (*E*)-7-dodecen-1-ol acetate (*E*7–12 : Ac) or other compounds as determined by GC analysis. The sex pheromone components were analyzed by GC and were provided by R.R. Heath of this laboratory. Z5–12 : Ac was from Aldrich Chemical Co. (Milwaukee, Wisconsin) and was greater than 96% pure;

Z9–14:Ac was from Sigma Chemical Co. (St. Louis, Missouri) and was 96% pure. Based on GC comparisons with authentic standards, none of the compounds contained significant amounts of high boiling point inclusions that could affect the emission rate.

Electrophysiology. Action potentials were recorded from HS(a) specialists within sensilla by inserting electrolytically sharpened tungsten electrodes into the base of individual antennal sensilla (O'Connell, 1975; O'Connell et al., 1983; Grant et al., 1989). The spikes were amplified by a Grass P-16 AC-coupled preamplifier and digitized, sorted, counted, verified, and stored digitally (Mankin et al., 1987).

Pheromone Dispensers. All compounds and mixtures in both electrophysiological and behavioral assays were evaporated from calibrated glass dispensers (Mayer et al., 1987; Mayer, 1993). The dispensers were connected to two different glass devices that control the stimulus duration for the electrophysiological and behavioral assays. Chemical mixtures were released from the same dispenser. Slightly different airborne concentrations result from mixtures of 12- and 14-carbon acetates because there is about a 10-fold evaporation rate difference (Mayer, 1993).

The principles of the stimulus control device have been described by Grant et al. (1989) and Mayer (1993). The stimulus control device controls the stimulus and provides a constant 1200 ml/min of air to isolate the antenna from ambient laboratory air. A dosed dispenser is constantly purged with air at 200 ml/min. A bypass vacuum valve enables the stimulus to be injected countercurrently into the isolating airflow. Thus, a measured amount of pheromone from the dispenser is injected into the isolating airflow for a final airflow dilution of 1400 ml/min. The final airborne concentration can be calculated easily from the measured pheromone emission rate and the metered air flow.

Control of the stimulus for behavioral assays is by a glass Y tube modified from that of Mayer and McLaughlin (1993) as described below. The Y tube was fitted with a glass diverter port that was fused at a point after the junction of the two female ground-glass joints and a longer nozzle. The diverter port was attached to a valve-controlled vacuum. One treated and one untreated dispenser, both purged constantly with air at 200 ml/min were inserted into the joints. During periods when the stimulus was off, the stimulus-laden air from the constantly purging dispensers was vented into the vacuum line. Upon activation of the valve, the stimulus was diverted into the center of the wind tunnel. To end the assay, the valve to the vacuum was opened, and the stimulus was vented into the vacuum. The final airflow dilution of the stimulus in this system was 400 ml/min.

Wind Tunnel. Assays were conducted in a 183-cm-long, 60-cm-ID cylindrical Plexiglas tunnel. The input air was conditioned to 21–23°C, 50–70% relative humidity by an air handling unit (American Air Filter Inc., Louisville,

Kentucky) that brought in outside air, passed it through the laboratory's primary heating and cooling coils (96,000 BTU/hr and 129,000 BTU/hr capacity, respectively), filtered the conditioned air in turn through a 90% Varicel filter and then an Astrocel 95% filter (capacity 1500 cfm), and passed the conditioned air into the tunnel. The airspeed in the tunnel was maintained at 15–20 cm/sec. The air in the tunnel was exhausted through the roof of the building.

A white background with 37-mm-wide black strips 12 cm apart lay about 10 cm below the floor of the tunnel. Fiberglass sheeting diffused the light from an array of incandescent bulbs at the ceiling, controlled by a rheostat, to illuminate the tunnel uniformly. The light intensity at the center of the tunnel was maintained at 0.3 lux. The principle of this tunnel is the same as for other tunnels described by Baker and Linn (1984) and Miller and Roelofs (1978), except that it uses air drawn from the laboratory air supply. The plume produced from a dispenser loaded with tissue papers treated with NH_4OH and HCl was "turbulent" as defined by Mafra-Neto and Cardé (1994).

Behavior. For behavioral assays individual male moths were released from a holding cage centered downwind from the stimulus source. The behaviors observed were typical of moths flying upwind to a pheromone source and were characterized by a combination of opportunistic optomotor-chemoanemotaxis and/or a program of self-steered counterturning (Baker and Haynes, 1987). The final, most distinctive, and perhaps the most definitive of sex pheromone-stimulated intrinsic behaviors released by Z7–12:Ac are those just prior to and during copulation. Typically, this behavior occurs at the stimulus source and is characterized by ventrad abdominal thrusts, extrusion of claspers, and the eversion of both the lateral and terminal abdominal hairpencils.

Relationship of Deployed Stimulus Intensities to a Female. As discussed in Mayer (1993), it is necessary to reference the stimulus intensity to that of a female to both ensure the selectivity of the HS(a) specialist and maintain the environmental relevance of the stimulus. A female's instantaneous emission of Z7–12:Ac averages about 10 ng/min (Bjostad et al., 1980). More recent time-averaged measures support an average emission rate of about 4 ng/min (Haynes and Hunt, 1990; Hunt et al., 1990; Hunt and Haynes, 1990). On a logarithmic scale such a difference is within half an order of magnitude of our estimate. Each of the other components of the sex pheromone, (Z)-5-dodecenyl acetate (Z5–12:Ac), (Z)-7-tetradecenyl acetate (Z7–14:Ac), (Z)-9-tetradecenyl acetate (Z9–14:Ac), dodecenyl acetate (12:Ac), and Δ11-dodecenyl acetate (11–12:Ac), are considered to reflect their ratios to Z7–12:Ac as determined by Bjostad et al. (1984) from time-averaged collections of airborne volatiles: 0.0935, 0.0105, 0.0046, 0.0719, and 0.0278, respectively, although some differences may exist (Haynes and Hunt, 1990; Dunkelblum and Mazor, 1994).

Thus, if 10 ng/min of Z7–12:Ac were emitted into the dispenser used for the neurophysiological experiments, the airborne concentration at the antenna

of each of these components due to dilution by 1400 cm^3/min air (200 cm^3/min from the dispenser + 1200 cm^3/min from the stimulus delivery system) would average, as follows: Z7-12:Ac, 3.2×10^{-11} M; Z5-12:Ac, 2.9×10^{-12} M; Z7-14:Ac, 2.9×10^{-13} M; Z9-14:Ac, 1.3×10^{-13} M; 12:Ac, 2.3×10^{-12} M; and Δ11-12:Ac, 8.8×10^{-13} M. Correspondingly, the airborne concentration of each of these components at the nozzle of the dispenser in the wind-tunnel experiments due to dilution by 400 cm^3/min air (200 cm^3/min from one treated and one untreated dispenser) would be: Z7-12:Ac, 1.1×10^{-10} M; Z5-12:Ac, 1.0×10^{-11} M; Z7-14:Ac, 1.0×10^{-12} M; Z9-14:Ac, 4.5×10^{-13} M; 12:Ac, 7.9×10^{-12} M; and Δ11-12:Ac, 3.1×10^{-12} M. There may be further dilution by a factor of about 8 within the plume of the wind tunnel (Mayer and McLaughlin, 1991).

Estimation of Airborne Stimulus Concentration. Details of the estimation of the airborne stimulus concentration of the odorants deployed in these studies are in Mayer et al., (1987) and Mayer (1993). In essence, the estimate of the airborne concentration of the various compounds derives from gas chromatographic, radiometric, and electrophysiological measures of the emission rate of Z7-12:Ac from the same glass dispensers used in these assays (Mayer et al., 1987). From the established Z7-12:Ac emission rate and additional gas chromatographic measures of emission rates at high dispenser dosages, Mayer (1993) estimated the airborne concentration of the other cabbage looper sex pheromone components by extrapolation. The emission rate of 6-VD was assumed to be the same as that for 14-carbon compounds. There are no measures for the emission rate of an 18-carbon compound from the dispenser used here, and consequently the emission rate for 10-VT was judged to be about 10 times less than the emission rate measured of (*Z*)-11-hexadecenal (Mayer et al., 1987).

RESULTS

The single most behaviorally active sex pheromone component, Z7-12:Ac, elicits reliable responses from the HS(a) specialist at airborne concentrations at or lower than about 2×10^{-13} M (Table 1) (Mayer, 1993). Among the eight HS(a) specialists assayed, this concentration of Z7-12:Ac elicited an average of 8.7 ± 2.6 (SEM) action potentials (=spikes)/sec. Five other HS(a) specialists in the test series responded with an average of 40.7 ± 10.9 spikes/sec to 2.1×10^{-11} M Z7-12:Ac. The same eight neurons that were exposed to Z7-12:Ac alone also responded reliably to 6-VD alone at an airborne concentration of $\sim 1 \times 10^{-10}$ M (Table 1). They did not respond reliably to 10-VT alone at either level tested.

The response of the HS(a) specialists to admixtures of Z7-12:Ac with either 6-VD or 10-VT was greater than the sum of the responses elicited by

TABLE 1. RESPONSE OF HS(a) ANTENNAL SEX PHEROMONE SPECIALIST RECEPTOR NEURONS TO INDICATED STIMULI

Airborne synergen concentration (M)[a]	Average spikes/sec HS(a) neurons[a] with airborne Z7-12:Ac concentration (M)		
	0	1.9×10^{-13}	2.1×10^{-11}
None	0.3 ± 0.1 (9)[b] a[c]	8.7 ± 2.6 (8)a	40.7 ± 10.9 (5)[d]
6-Vinyldecyl acetate			
$\sim 2 \times 10^{-12}$	4.4 ± 3.9 (8)a	17.1 ± 4.7 (8)a	
$\sim 1 \times 10^{-11}$	0.6 ± 0.3 (8)a	35.1 ± 8.7 (7)b	
$\sim 2 \times 10^{-11}$	1.9 ± 0.7 (8)a	42.7 ± 13.6 (6)b	
$\sim 1 \times 10^{-10}$	23.6 ± 8.9 (8)b	73.5 ± 37.1 (5)b	
10-Vinyltetradecyl acetate			
$\sim 3 \times 10^{-13}$	3.7 ± 2.8 (8)a	47.9 ± 12.8 (8)b	
$\sim 3 \times 10^{-12}$	6.0 ± 2.6 (7)a	70.4 ± 23.9 (4)b	

[a] The emission rate and airborne stimulus concentration of 6-VD is estimated from the emission of Z11-16:Al because of their similarity in molecular weight; concentration of 10-VT stimulus is estimated as 10× less than the emission of Z11-16:Al (14).

[b] Number of neurons measured.

[c] Different letters within a column indicate means that differ from the response to an untreated dispenser or 1.9×10^{-13} M Z7-12:Ac alone at the $P < 0.01$ level of significance (Student's t test).

[d] Mixtures of the synergens with 2.1×10^{-11} M Z7-12:Ac were not measured because the resultant responses were overdriven spike trains characterized by significantly decremented spikes followed by an abnormally elevated spontaneous activity.

either of the two stimuli alone, thus fulfilling the definition of synergism (Table 1; Figure 2). Moreover, the response was proportional to the $\log_{10}$ M concentration of 6-VD added to constant levels of Z7-12:Ac. The four values for 6-VD mixtures fit the regression equation, log spikes/sec = 5.6 + 0.02 (log M) ($r^2 = 0.987$; SE = 0.204; $P < 0.004$).

The HS(a) specialist neurons also respond reliably to 1.6×10^{-10} M Z9-14:Ac and 2.0×10^{-10} M Z5-12:Ac alone (Figure 3). These stimulus intensities exceed the level emitted by females, and the response was expected (Mayer, 1993). These two pheromone components also synergized the response to 2.0×10^{-13} M Z7-12:Ac (Figure 3). The stimulus levels of these two compounds necessary to synergize the response to Z7-12:Ac exceed by two to four orders of magnitude the level emitted by females. Further work is necessary to demonstrate whether or not these compounds might synergize the response at physiological levels.

To ascertain both that the synergism observed in the receptor neurons is reflected in behavior and to measure how closely the proportion of sex phero-

mone-elicited behaviors reflect the responses of the HS(a) specialists, flight behaviors and copulatory responses were measured in a wind tunnel at nearly the same stimulus levels that were used in the electrophysiological measures (Table 2). The three concentrations of Z7–12 : Ac used all elicited submaximal upwind flight behaviors. In support of the electrophysiological measured synergism, the proportion of males flying upwind and copulating increased with increase in the amount of Z7–12 : Ac at constant levels of all four synergists. Additionally, the flight response also generally increased proportionally with the increase in amount of the four synergists at constant levels of Z7–12 : Ac.

DISCUSSION AND CONCLUSIONS

The data obtained by both electrophysiological and behavioral experiments are consistent with and support the conclusion that the HS(a) specialist response to Z7–12 : Ac is synergized by two synthetic pheromone analogs and at least two sex pheromone components. There are several facets to specialist synergism that impact such diverse areas as receptor interactions, behavior, and the identification and practical field use of sex pheromones that are significant and merit further comment.

Receptor Interactions. The receptor neuron's response begins when pheromone molecule(s) penetrate minute sensillar pores (Slifer et al., 1959; Schneider et al., 1964; Kaissling and Priesner, 1970) and bind to a carrier protein (Riddiford, 1970; Vogt and Riddiford, 1981; Steinbrecht et al., 1992; Laue et al., 1994) and/or inositol-1,4,5-triphosphate-coupled receptors in the dendritic membrane of the receptor neuron (Zufall and Hatt, 1991; Yoshii et al., 1992; Wegener et al., 1993; Stengl, 1994; and Schleicher et al., 1994). The stimulus-induced cascade of molecular events ultimately leads to action potentials that convey information directly to a macroglomerulus in the deutocerebral lobe of the brain (Boeckh et al., 1976; Hildebrand et al., 1980; Christensen et al., 1991). Based on what is now known about receptor interactions, we consider that synergism most likely reflects a simultaneous reaction of Z7–12 : Ac and the synergen with a receptor macromolecule. There appears to be a relative lack of specificity required of the synergen's structure in the *T. ni* receptor because the double bond can be located on a side chain.

Nevertheless, there is not a total lack of synergen specificity because some synergens elicit a greater response than others. This is demonstrated by the quantitative differences in responses elicited by mixtures of Z7–12 : Ac and 6-VD or 10-VT. While both of these synthetic analogs synergized the response, 10-VT elicits about the same response as 6-VD but at about 100 times lower airborne concentration (Table 1). Structurally, the 10-VT molecule has four more methylene groups between the double bond and the acetate group than the

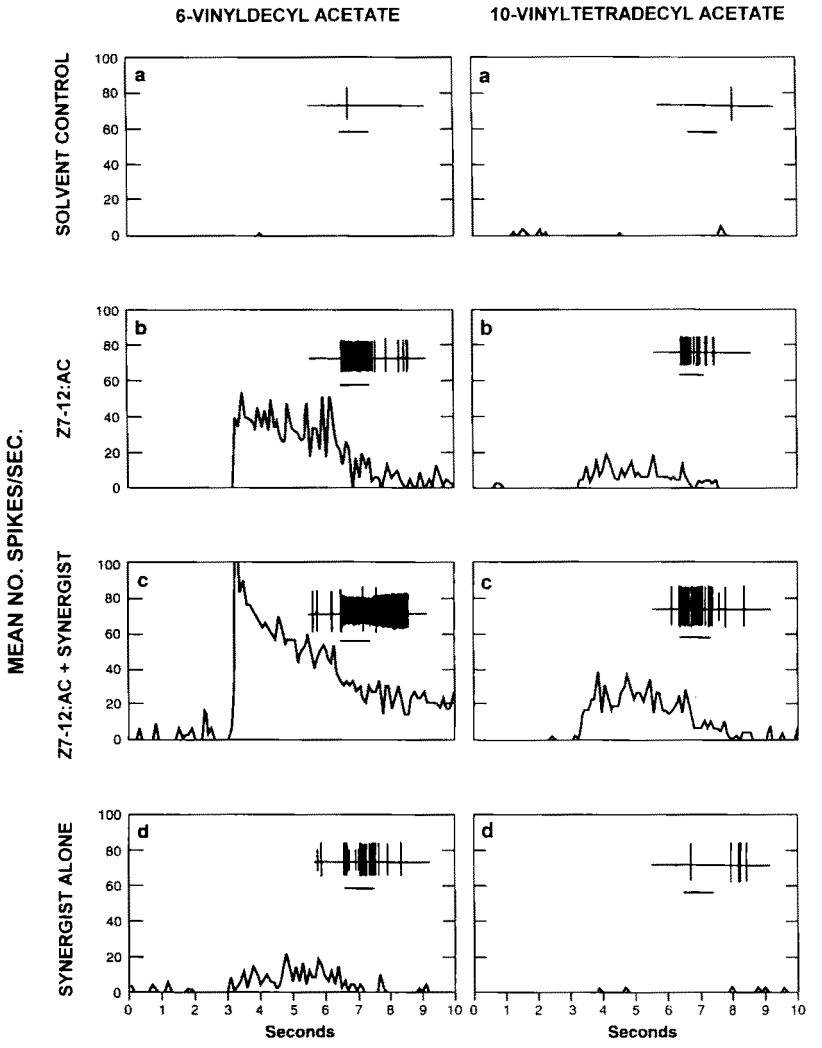

FIG. 2. Synergism of HS(a) specialists by 6-VD and 10-VT. Reading vertically, each of the four large panels shows the average of five consecutive stimuli of: (a) solvent control; (b) Z7-12:Ac alone; (c) admixture of Z7-12:Ac with 6-VD (left panel) or 10-VT (right panel); (d) 6-VD or 10-VT alone. The inset is a digitally reconstructed spike train from the recording that was nearest that of the average. The stimulus began at sec 3 and ended at sec 6, corresponding to the bar under the spike trains in the insets. The randomly occurring small spike in some of the recordings is the HS(b) neuron that responds to (Z)-7-dodecenol. The airborne concentration of Z7-12:Ac alone and mixed

6-VD molecule. We speculate that the increased chain length of 10-VT enables it to assume a greater number of active conformations that facilitate the synergistic molecular interaction at a hydrophilic receptor site (Kafka and Neuwirth, 1976; Bestmann and Vostrowsky, 1982; Jönsson et al., 1991, 1992).

Synergism and Behavior. The behavioral assays that corroborate the synergism of the electrophysiological responses to 6-VD and 10-VT were performed at airborne concentrations within the wind tunnel that were nearly the same as those of the electrophysiological assays (Table 2). The behavioral assays to corroborate Z5-12:Ac and Z9-14:Ac synergism were performed at airborne concentrations above that expected downwind of a female (see Methods and Materials and Table 2). While these two components may utilize other specialist or generalist neuron channels to transfer information to the brain, none yet have been observed at normally encountered stimulus levels. Clearly, however, the synergism of the HS(a) neurons is confirmed by the synergism of behavioral responses. Furthermore, because the moth behavior so reliably reflects primarily the response of HS(a) neurons, these data also support the contention that the cabbage looper's sex pheromone-elicited behavioral upwind flight sequence is predominantly a response to the single component, Z7-12:Ac (Mayer and McLaughlin, 1991, 1993). Consequently, the moth's behavior should increase or decrease as the response of HS(a) neurons increases or decreases as considered below.

Synergism—Behavioral Increase. Because the amount of behavioral response is proportional to the increased HS(a) neural response, significant and sometimes unwanted effects can result from synergized responses. Our results may have bearing upon the common practice of using field trap assays to decide which of several analytically determined compounds may constitute a conspecific sex pheromone signal. Assays of additional putative components at elevated dosages may result in greater amounts of behavior, but the increase might be due to synergism rather than the addition of an intrinsic component part of the sex signal. The likelihood of synergism would increase with increasing dosages similar to that shown in Table 2. Thus, electrophysiological assay methods may be necessary ultimately to conclude whether or not an additional compound constitutes a part of the conspecific sex signal.

Synergism—Behavoral Reduction. Elevated pheromone bait levels in field traps also may reduce trap captures through synergism. Trap captures or wind-tunnel responses typically increase with increasing dosages, reach an asymptote,

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with 6-VD was 2.0×10^{-13} M; that of 6-VD was 1.6×10^{-10} M. Because of the enhanced response magnitude, the concentration of Z7-12:Ac was reduced to 1.8×10^{-14} M in mixtures with 10-VT; the 10-VT concentration was less than $\sim 6 \times 10^{-13}$ M.

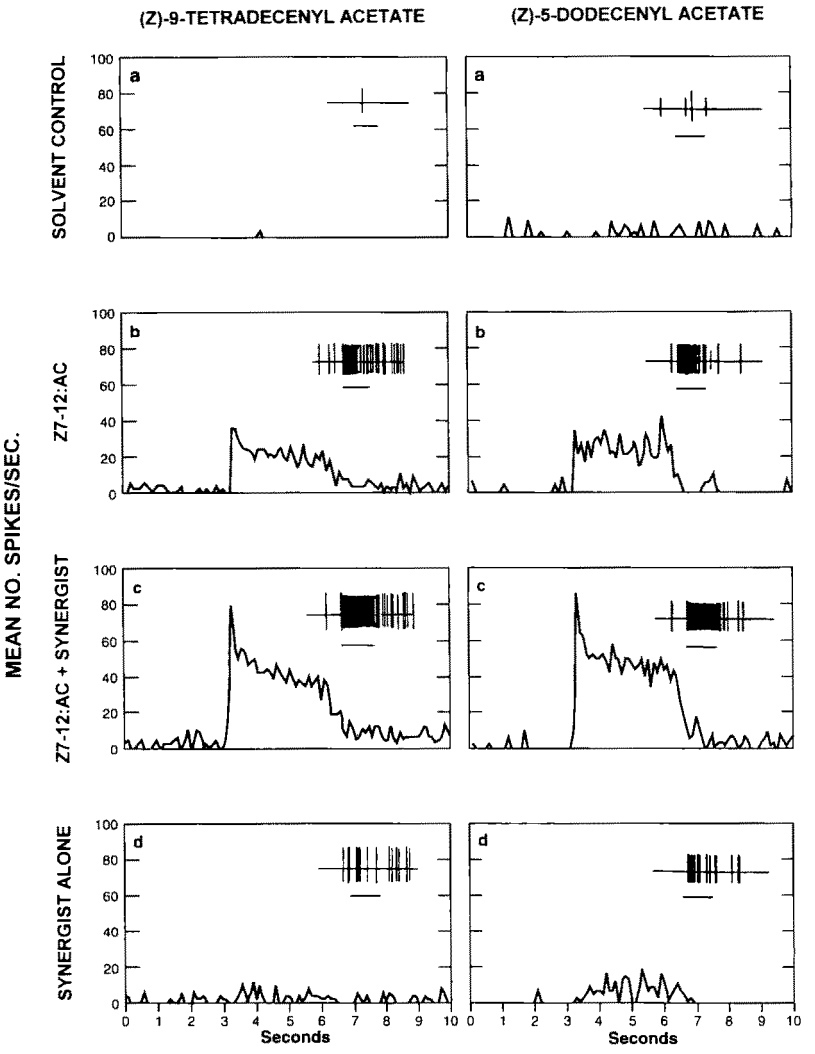

FIG. 3. Synergism of HS(a) specialists by Z9-14 : Ac and Z5-12 : Ac. Reading vertically, each of the four large panels is the average of five consecutive stimuli of: (a) solvent control; (b) Z7-12 : Ac alone; (c) admixture of Z7-12 : Ac with either Z9-14 : Ac (left panel) or Z5-12 : Ac (right panel); (d) Z9-14 : Ac or Z5-12 : Ac alone. The inset is a digitally reconstructed spike train from the recording that was nearest that of the average. The stimulus began at sec 3 and ended at sec 6, corresponding to the bar under the spike trains in the insets. The randomly occurring small spike in some of the recordings is the HS(b) neuron that responds to (Z)-7-dodecenol. The airborne concentration Z7-12 : Ac was 2.0×10^{-13} M, Z9-14 was 1.6×10^{-10} M, and Z5-12 : Ac was 2.0×10^{-10} M.

TABLE 2. PERCENTAGE OF MALE COPULATORY RESPONSES FOLLOWING UPWIND FLIGHTS ELICITED BY MIXTURES OF Z7-12:Ac AND TWO FEMALE SEX PHEROMONE COMPONENTS AND TWO SYNTHETIC ANALOGS IN WIND-TUNNEL ASSAYS

Airborne synergen concentration (M)[a]	Percentage attempted copulations with airborne Z7-12:Ac concentration (M)			
	0	6.2×10^{-14}	6.6×10^{-13}	7.4×10^{-12}
None	0	0.05[b]	0.06	0.08
(Z)-5-Dodecenyl acetate[c]				
7.0×10^{-12}	0 (20)[d]	20.0 (30)	12.0 (30)	10.0 (10)
1.0×10^{-9}	32.0 (25)	36.0 (25)	68.0 (25)	84.0 (25)
(Z)-9-Tetradecenyl acetate[e]				
3.7×10^{-11}	0 (25)	0 (20)	0 (20)	16.7 (30)
5.0×10^{-10}	0 (25)	16.7 (30)	43.3 (30)	70.0 (30)
2.4×10^{-9}	0 (30)	42.9 (35)	50.0 (40)	82.9 (35)
6-Vinyldecyl acetate[f]				
$\sim 1 \times 10^{-11}$	0 (15)	0 (15)	7.0 (15)	33.3 (15)
$\sim 7 \times 10^{-11}$	0 (30)	13.3 (30)	30.0 (20)	60.0 (20)
$\sim 1 \times 10^{-10}$	0 (25)	44.0 (25)	36.7 (20)	56.7 (30)
10-Vinyltetradecyl acetate[g]				
$\sim 9 \times 10^{-13}$	[h]	0 (20)	20.0 (30)	[h]
$\sim 4 \times 10^{-13}$	0 (35)	24.4 (45)	15.6 (45)	36.0 (25)
$\sim 9 \times 10^{-12}$	0 (25)	28.0 (25)	76.0 (25)	92.0 (25)

[a] Under the assay conditions at the dispenser nozzle the airborne concentrations from a typical female should average: Z5-12:Ac, 1.0×10^{-11} M; Z9-14:Ac, 4.5×10^{-13} M (see text).

[b] The expected probability of copulation at the three airborne concentrations of Z7-12:Ac alone was based on logistic analysis of the flights of 525 moths (Proc Probit; logistic model, SAS Inc., Cary, North Carolina); 0.50 probability of copulation is expected at an average airborne concentration of 1.3×10^{-10} M. The airborne Z7-12:Ac concentration at the dispenser nozzle from a calling female emitting 10 ng/min under the same conditions in the wind tunnel would be 1.1×10^{-10} M.

[c] Numbers in parentheses are the number of males assayed.

[d] Average airborne emission concentration of 6-vinyldecyl acetate was computed using a previous calibration for (Z)-14-tetradecenyl acetate (see text). Dispenser dosages were 1, 3.16, and 10 μg.

[e] Airborne emission concentration was estimated as 10× less than the emission of equivalent dosages of (Z)-11-hexadecenal (see text). Dispenser dosages were 1, 3.16, and 10 μg.

[f] Not tested.

and diminish as the stimulus level is raised further (Mayer, 1973; Löfstedt et al., 1985; McDonough et al., 1993; Haniotakis and Pittara, 1994 among others). When a synergist is added to an already elevated pheromone stimulus level the combination could elicit a specialist receptor overdischarge of action potentials. In insects such overdischarges characteristically are observed at airborne concentration levels considerably above those found downwind from a female, usually exceed 100 spikes/sec, and display a decrementation of spike heights.

This overdischarge could lead either to neuronal adaptation, central habituation, and/or a perceptual change in the organoleptic signal quality.

There is a second alternative that might reduce the behavior of the false codling moth and other species such as the European corn borer, *Ostrinia nubilalis* (Hübner) (Kochansky et al., 1975) that encode ratios of two components. The European corn borer detects the pheromonal signal pair through two specialized receptor neuron lines (Hansson et al., 1987). If one of these specialized neuronal lines were to be synergized and the other not, the perceptual ratio of the two components would be different. Such modification of the perceptual ratio could modify or eliminate certain behaviors. It is not clear which of these, or perhaps other alternative mechanisms, apply to the false codling moth studies of Burger et al. (1990).

Synergism—Is Z5-12:Ac a Sex Pheromone Component? One or more components in the volatile secretion of the female could function as a part of the conspecific sexual signal by synergizing the response of the HS(a) specialists to Z7-12:Ac. Because all five acetates found in the volatile secretion of female cabbage loopers (Bjostad et al., 1984) [Z7-12:OH is found in Israel cabbage loopers (Dunkleblum and Mazor, 1994)] may synergize the HS(a) specialist at high airborne concentrations, it is crucial to assay at levels found downwind from the female. The component Z5-12:Ac alone was able to elicit a 0.32 probability of upwind flight and copulatory behavior, but only at 1.0×10^{-9} M, about 100× its concentration in the female's volatile secretion at about 1.0×10^{-11} M (Table 2). It was ineffective alone in eliciting upwind flight and copulation at 7.0×10^{-12} M. However, in mixtures with Z7-12:Ac it elicited greater than expected flight and copulatory behavior at two stimulus levels assayed than could be accounted for by Z7-12:Ac alone. By contrast, the other cabbage looper sex pheromone component, Z9-14:Ac, also synergized the HS(a) response and increased behavioral responses, but only at airborne concentrations about 1000× higher than would be found downwind of a female. We consider these data to support the hypothesis that Z5-12:Ac may function as a component of the sex signal by synergizing the HS(a) response to Z7-12:Ac, rather than by being detected by a specialist neuron ensemble.

Synergism—Practical Significance. Synergists could enhance the effectiveness of mating disruptants, reduce the amount of an expensive compound, and/or extend the life of labile compounds.

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Erratum

The author M.B. Dicklor should be added to author names and the following change made in the text.

B.M. Zuckerman, M.B. Dicklor, M. Matheny, and N. Acosta

Control of Plant-Parasitic Nematodes by a Nematicidal Strain of *Aspergillus niger*.

In Volume 20, Number 1, on p. 39, the sentence, "Second, CF was dialyzed against Spectrophor membrane tubing (6000–8000 molecular weight cutoff), and the fraction remaining within the tubing evaluated for nematicidal activity as described below," should be deleted and replaced with the following: "Nine-centimeter Petri plates containing PDA were overlayed with sterile dialysis membrane (Spectrophor, 6000–8000 molecular weight cutoff) and PD-42 allowed to grow on the membrane for four days at 22°C in the dark. PD-42 and the membrane were removed under sterile conditions and a drop of *C. elegans* or freshly hatched larvae of *M. incognita* added. Plates were observed at 10-min intervals until namatode activity ceased."

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CYCLIC CHANGES IN VOLATILE CONSTITUENTS OF BOVINE VAGINAL SECRETIONS

WEIDONG MA, B.A. CLEMENT, and W.R. KLEMM*

Department of Veterinary Anatomy and Public Health
Texas A&M University
College Station, Texas 77843-4458

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Abstract—Headspace gas chromatography was used to examine the volatile components of bovine vaginal secretions in three consecutive estrous cycles from each of four cows. Results indicated that there was an interesting peak with a retention of 2.7 min. It showed a successive rise and fall zero to three days before estrus. Gas chromatographic evidence and mass spectral data confirmed that this peak was acetaldehyde. Another unidentified compound with a retention time of 27 min was found to be unique to proestrus. It occurred two to three days prior to estrus, but was absent at any other time of the cycle.

Key Words—Cow, vaginal secretions, estrus, pheromones, acetaldehyde, gas chromatography.

INTRODUCTION

In many vertebrate species the estrous female attracts the male and informs the male of its receptive state by means of chemical signals deriving from vaginal discharge (Albone, 1984). The amount and consistency of bovine vaginal secretions vary with different stages of estrous cycle (Cole, 1930; Hammond, 1972). Vaginal secretions generally become more abundant and flow out from the vulva at estrus. (We recognize that not all components of vaginal secretions originate in the vagina. Significant contributions come from cervical glands and vestibular glands). Under field conditions, bulls show overt behavioral responses such as sniffing, licking, and repeated flehmen in response to chemical cues from the

*To whom correspondence should be addressed.

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vulvar region and vaginal secretions (Hart et al., 1946; Jacobs et al., 1980; Hradecky et al., 1983; Klemm et al., 1987).

Behavioral studies have demonstrated that vaginal secretions from estrous cows contain estrous pheromones. Paleologou (1977) indicated that a volatile odor in the vaginal secretions of estrous cows was a source of sexual attraction for bulls. When placed in glass dishes and presented to a bull, vaginal secretions elicited sniffing, but no interest was shown in secretions from metestrous or diestrous cows. When vaginal secretions from estrous cows were rubbed on a dummy cow, bulls would mount the dummy, but did not mount a dummy smeared with secretions from diestrous cows. Dogs could even be trained to differentiate the smell of vaginal swabs between estrous and diestrous cows (Kiddy et al., 1978). The trained dogs were used to confirm that there was a distinctive odor in the urogenital tract of cows, which appeared a few days before estrus and disappeared or became less potent immediately after estrus (Kiddy and Mitchell, 1981). Estrus-related odors were apparently strongest in bovine vaginal secretions, but were also present in other body fluids such as milk and blood (Kiddy et al., 1984). Nishimura et al. (1991) reported that vaginal secretions from heifers at estrus stimulated sexual activity and mounting behaviors when the secretions were applied to the hindquarters of the donor heifer during diestrus.

All the evidence above suggests that vaginal secretions from estrous cows contain biochemical markers of proestrus or estrus. Clearly an understanding of the chemical nature of such reproductive signals employed by cows could have great scientific interest, and in some cases considerable economic importance. However, although the above references show that estrous pheromones from bovine vaginal secretions have been well-characterized biologically, chemical knowledge remains slight. Active compounds responsible for the sexual attraction have not been identified.

In a previous study in our laboratory, cow blood acetaldehyde was found to show a successive rise and fall just prior to estrus (Klemm et al., 1994). The present investigation had two purposes: (1) to examine whether acetaldehyde is present in bovine vaginal secretions and determine its variations with the stages of the estrous cycle; and (2) to monitor changes of other volatiles across the estrous cycle.

METHODS AND MATERIALS

Animals. Four Holstein dairy cows (308, 87086, 89124, and 88544) were used for this study. All animals were healthy and had normal estrous cycles. In order to synchronize estrous cycles, cows were given injections of $PGF_2\alpha$. After these treatments, cows were monitored for three natural estrous cycles.

Sample Collection. Vaginal secretions were collected with tampons. Before use, tampons were soaked with a chloroform–methanol solution (85 : 15) for 48 hr and followed with a diethyl ether soak for another 48 hr. The washed tampons were dried in air for 3 hr and then dried in the oven (100°C) for 1 hr. The cleaned tampons were stored in sealed containers until ready for use. Before collection, perineum and vulvar regions were washed with water and dried with paper towels. A tampon was gently inserted into the vagina by hand and left there for 30 min. Then the tampon was removed and placed into a screw-capped bottle (teflon-sealed). Samples were put on ice until they could be stored in a −20°C freezer. Samples were collected daily around the time of estrus (three to four days before and one to two days after estrus) and every three or four other days during diestrus. The stages of the estrous cycle were estimated by rectal palpation of ovarian structure and behavioral observations. Samples were collected from each cow for three consecutive estrous cycles.

Analytical Protocol. Prior to analysis, each sample was thawed at room temperature. Then the capped bottle containing the sample was preheated at 70°C in a water bath for 20 min. One milliliter of headspace was taken from the closed bottle with a hot (70°C) gas-tight syringe and injected immediately into the gas chromatograph (GC) Gas chromatography was carried out on a Hewlett-Packard model 5890 GC fitted with a cross-linked methyl silicone microbore capillary column (DB-1, nonpolar, 30 m × 0.5 mm × 0.53 μm film thickness, J&W Scientific, Folsom, California) and an FID. The oven temperature was kept at 30°C for 10 min and then programmed to 110°C at a flow rate of 4°C/min. The injector was set at 150°C and the detector at 200°C. Helium was used as the carrier gas at a rate of 4 ml/min. Compressed air and hydrogen were set at flow rates of 400 and 20 ml/min, respectively. Peak areas were calculated with the PC software "ChromPerfect" (Justice Innovations, Palo Alto, California).

Identification of Acetaldehyde. Acetaldehyde was initially identified by comparing the retention time of the sample with that of authentic acetaldehyde in solution and by its coelution with the putative acetaldehyde peak in the sample. To further confirm the identification, combined GC-MS was performed. Previous experiments in our laboratory showed that direct headspace samples were not appropriate for GC-MS analysis (Klemm et al., 1994). Because the headspace samples of body fluids contained much water, carbon dioxide, nitrogen and oxygen, mass spectral analysis could not unequivocally identify small molecules such as acetaldehyde. Furthermore, the GC-MS system can be easily overloaded and shut down by the large amounts of water and carbon dioxide. In the present study, organic solvent extraction was used to isolate volatiles for GC-MS analysis. Tampons were soaked with 100 ml ice-cold distilled water. Extracts were obtained by a simultaneous steam distillation and extraction method

(Maarse and Kepner, 1970) using diethyl ether (HPLC grade) as the organic solvent. GC-MS analysis was performed on a Hewlett-Packard model 5970C quadrupole mass spectrometer coupled to a Hewlett-Packard model 5890 GC with a microbore capillary column (Hewlett-Packard Inc., Avondale, Pennsylvania). The spectra were obtained by electron ionization at 70 eV. Identification of acetaldehyde was made by matching the mass spectrum with the library spectral data as well as with the data of authentic acetaldehyde.

RESULTS

A peak that eluted at about 2.7 min occurred in all samples across estrous cycles. This peak had the same retention time as that of authentic acetaldehyde (Figure 1). When samples were spiked with acetaldehyde, the peak coeluted with it, suggesting that this peak may represent acetaldehyde.

GC-MS analysis of the ether extract indicated that there was a peak that eluted at 2.7 min. The spectrum of this peak had significant ions at *m/e* 44, 43, 29, and 15. This is consistent with the spectrum expected for acetaldehyde. To further verify this identity, a comparison was made with an authentic sample of freshly distilled acetaldehyde dissolved in diethyl ether. It was found that the retention time and fragmentation pattern of the known acetaldehyde were identical to that of the 2.7-min peak.

Acetaldehyde levels changed with the stages of the estrous cycle. They showed an increase and decrease zero to three days prior to estrus (Figure 2). This result is similar to that obtained from bovine blood samples (Klemm et al., 1994). Figure 3 illustrates the relative changes of acetaldehyde for three consecutive cycles from each of four cows. In every cycle, there was a relative increase in acetaldehyde. The peak concentration occurred one to three days prior to the day of estrus.

Apart from acetaldehyde, seven other peaks were found in the early elution (0–8 min, Figure 4), but they changed intensities irregularly across the estrous cycles. These peaks seemed to have no close correlation with the estrous cycle. However, there was an interesting late-eluting component with a retention time of about 27 min (Figure 4). It was always present two to three days before estrus and disappeared suddenly thereafter. This peak had similar size in all cows at each cycle. Unfortunately, this peak was not clearly recognized in the profile of the ether extract, therefore we were not able to identify it at this time.

DISCUSSION

Acetaldehyde was found in bovine vaginal secretions. It showed a successive rise and fall in concentration just before estrus. This phenomenon coincides

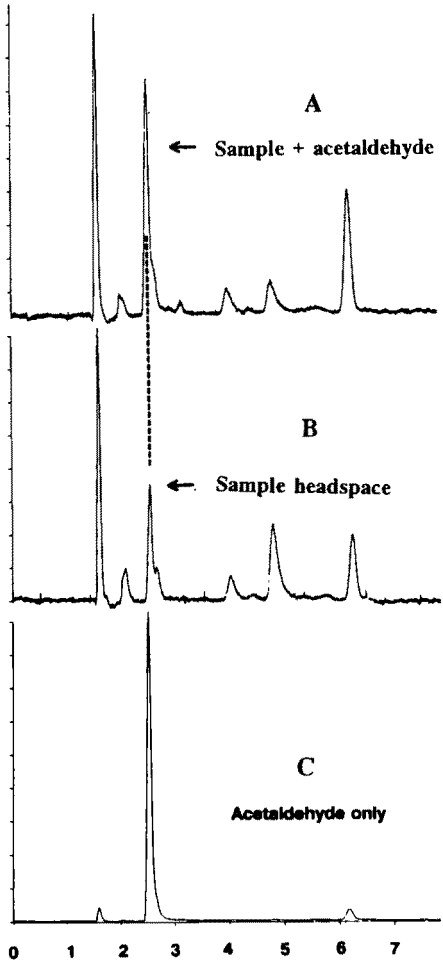

FIG. 1. Headspace gas chromatograms from vaginal secretions showing coelution of acetaldehyde standard at the same retention time as the 2.7 min peak. (A) Sample was spiked with authentic acetaldehyde. (B) Sample headspace. (C) Headspace from authentic acetaldehyde solution.

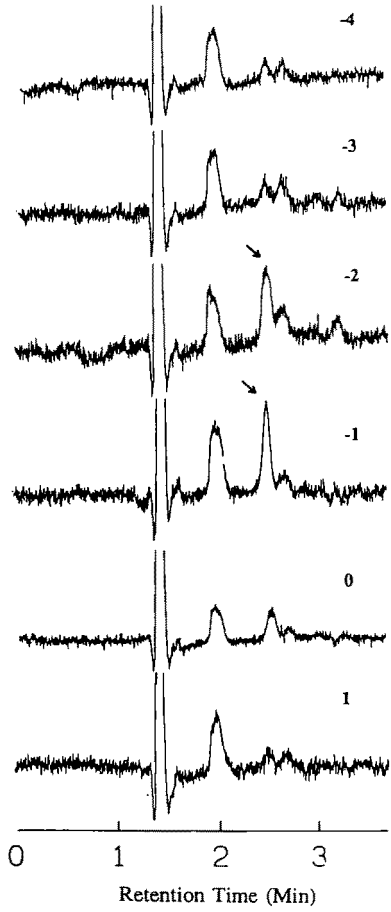

FIG. 2. Headspace chromatograms from vaginal secretions just before estrus. The acetaldehyde peak (arrow) increased two days before estrus (−2) and decreased thereafter. Day 0 was the day of estrus.

with the occurrence of estrus-related odors. We originally supposed that acetaldehyde might serve as a sex pheromone for attracting bulls during the estrous period. Acetaldehyde is highly volatile at room temperature and pressure. Pure acetaldehyde possesses a pungent irritating odor, but at dilute concentrations it gives a pleasant fruity aroma and is a necessary component for a balanced flavor in many dairy products (Vedamuthu, 1982). These characteristics could enable

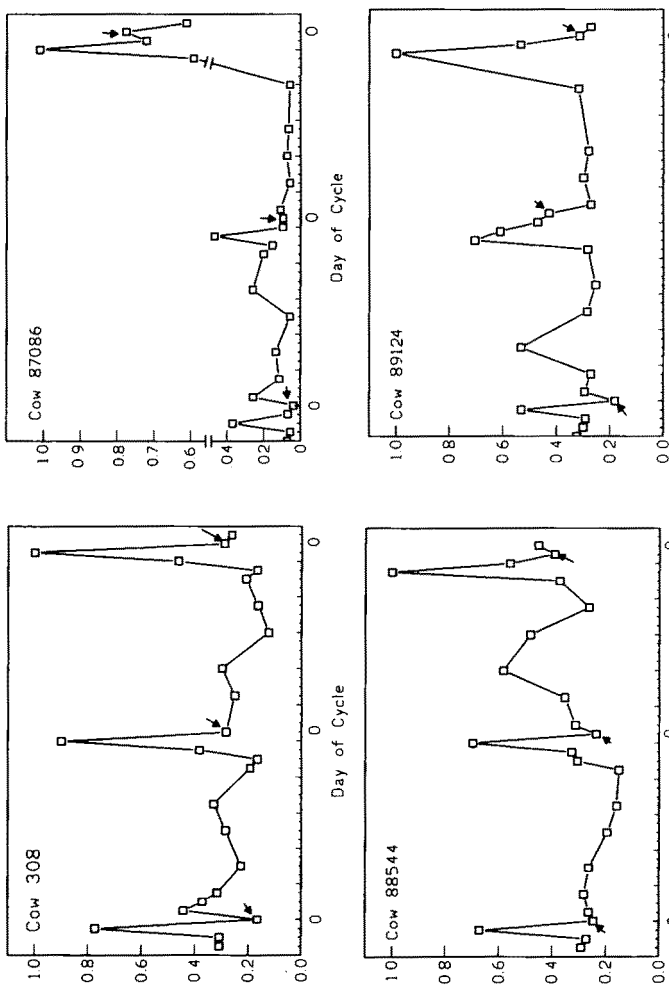

FIG. 3. Observations of acetaldehyde variations for three consecutive estrous cycles from each of four cows. The x axis indicates the day of cycle. Day 0 (arrows on the curve) was the day of heat. The y axis indicates the normalized data of peak area (detector response) of acetaldehyde.

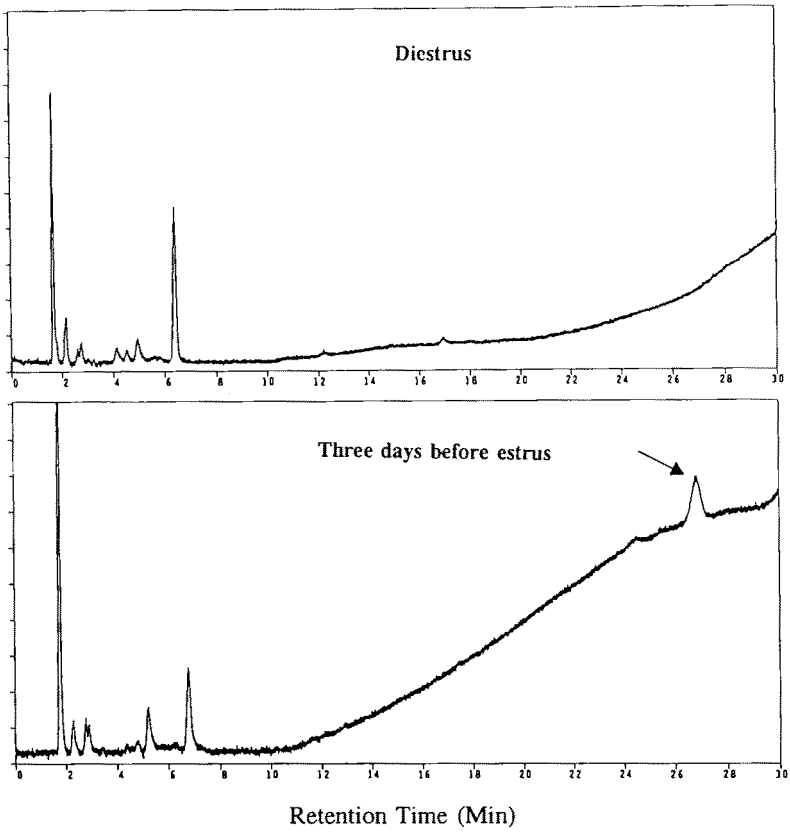

FIG. 4. Chromatograms from different samples of the same cow showing that a peak (arrow) with a retention time of 27 min was unique to proestrus. Top: from a diestrous sample. Bottom: from a proestrous sample (three days before estrus).

acetaldehyde to be a pheromone. In fact, acetaldehyde was found to be a very powerful attractant for a parasitoid (Jones, 1981). However, in a bull bioassay study (Prescicce et al., 1993), acetaldehyde at a wide range of concentrations (1–100 ppm) failed to elicit appreciable responses in bulls. Although these behavioral tests were negative, we still cannot conclude that acetaldehyde is not an estrous pheromone. There are at least three possibilities:

1) Beauchamp et al. (1976) pointed out that mammalian chemical signals were likely to be coded in the patterns of compounds in a mixture rather than in individual components. A pheromonal action may derive from highly complex mixtures of chemicals in which synergistic or antagonistic effects could occur

among the components. Sometimes the behavioral response disappears completely when chemical mixtures are broken into individual components (Müller-Schwarze et al., 1986). Such a phenomenon was found in hamsters (O'Connell et al., 1978). Some compounds (aliphatic acids and alcohols) from estrous hamsters had no effects on either attraction or copulatory behavior when given alone but were effective when given in combination. Therefore, in bovine vaginal secretions, a mixture of compounds including acetaldehyde may be responsible for sexual attraction to bulls.

2) Behavioral responses often depend not only on chemical stimuli, but also on nonchemical cues. Many responses occur only in the proper physiological, social, and temporal contexts. For instance, receptors for some pheromones may be available only at a certain period of time. Only under these proper conditions is it possible to choose a significant compound for behavioral tests.

3) The taste of acetaldehyde could be a meaningful signal in bull courtship behavior, during which bulls lick the vulva and exhibit associated flehmen responses (Rivard and Klemm, 1992).

Whether acetaldehyde is a pheromone or not, it seems to be a good estrous marker. This finding may have real economic importance. In the modern dairy industry, cows are routinely bred by artificial insemination. Detection of estrus is necessary to obtain satisfactory conception rates. A basic problem facing producers, however, is the failure to detect estrus. In most dairy operations, detection of estrus is only about 50% effective (Williamson et al., 1972; Esslemont and Bryant, 1974). As a biochemical approach to this problem, it should be possible to use acetaldehyde as a marker to develop a biosensor system for estrus detection. For instance, one might be able to develop a chemically treated paper to monitor acetaldehyde change, whereby dairymen could test for estrus by simply dipping a strip of the paper into the body fluids. Acetaldehyde could be detected by other methods such as electric sensors. Additional work along these lines is being carried out in our laboratory in collaboration with a dairy cooperative.

The vagina is rich in blood vessels in the regions of lamina propria that underly the epithelium (Cormack, 1987). The lumen of the vagina receives a transudate of blood plasma through the intercellular channels of the epithelium. Since blood acetaldehyde in cows also showed a successive rise and fall three days before estrus (Klemm et al., 1994), acetaldehyde in the vaginal secretions may come directly from the bloodstream. The vagina also receives secretions from cervical glands and vestibular glands, although neither of these have been directly tested for acetaldehyde. Alternatively, acetaldehyde may be the result of vaginal microbial activity. Some microbial species can utilize and transform mammalian secretions and excretions, and in this process, mammalian chemical signals may be generated (Albone et al., 1977). Some studies indicate that certain bacteria (e.g., lactic acid bacteria) contain enzymes that can catalyze the

formation of acetaldehyde from a wide variety of substrates such as protein, glucose, amino acids, nucleic acids, and ethanol (Gonzalez et al., 1994). The accumulation of acetaldehyde in vaginal secretions before estrus may be caused by a high production rate that is regulated by the availability of substrates and the activities of enzymes, both of which are associated with the female's physiological state. Vaginal tissues, which are rich in glycogen (Albone, 1984), are very sensitive to circulating estrogen levels. As ovulation approaches, the high level of circulating estrogen stimulates the breakdown of glycogen and other materials into glucose, thus supplying microorganisms with increased nutrients. The increased glucose from the turnover of glycogen may be responsible for the high level of acetaldehyde at proestrus.

Some evidence indicated that estrogen was directly involved in the process of acetaldehyde formation. Birth control pills seem to elevate breath acetaldehyde levels (Jeavons and Zeiner, 1984). Alcohol dehydrogenase catalyzes the formation of acetaldehyde from ethanol. In some animals gene expression and activity of this enzyme can be induced by estrogen (Qulali et al., 1991; Qulali and Crab, 1992; Hiremath et al., 1992). If this is true for cows, the acetaldehyde increase before estrus may be regulated by the high circulating estrogen level before ovulation. Elevated acetaldehyde levels might also be caused by a deficiency or inhibition at estrus of aldehyde dehydrogenase, the enzyme that metabolizes acetaldehyde into acetate.

The 27-min peak is a potentially important compound. It was specific to proestrus and did not occur during the diestrus. Plans are under way to extract the vaginal secretions with an appropriate method so that the peak can be identified by GC-MS.

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BEHAVIORAL CORRELATES FOR MINOR VOLATILE COMPOUNDS FROM STINK BUGS (HETEROPTERA: PENTATOMIDAE)

J.R. ALDRICH,[1,*] M.C. ROSI,[2] and F. BIN[2]

[1]*Insect Chemical Ecology Laboratory*
USDA-ARS, Bldg 007
Agricultural Research Center-West
Beltsville, Maryland 20705

[2]*Agricultural Entomology Institute*
Perugia University
Borgo XX Giugno, 06100 Perugia, Italy

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Abstract—Many terrestrial Heteroptera have small, but functional, dorsal abdominal glands as adults. The chemistry, and associated intra- and interspecific behavior, for dorsal abdominal gland secretions from 10 species representing four genera of Pentatomidae was investigated. Eighteen volatile compounds were identified in species-specific blends from the dorsal abdominal gland secretions of *Euschistus*, *Acrosternum*, and *Eurydema* adults, including aliphatic, aromatic, and terpenoid constituents. Evidence from bioassays is presented that parasitic Tachinidae (Diptera) and Scelionidae (Hymenoptera) use these secretions as kairomones. A field experiment was performed to test the hypothesis that minor volatiles increase the specificity of the main pheromone component from Nearctic *Euschistus* species, methyl (2*E*,4*Z*)-decadienoate. However, significantly fewer individuals of *E. tristigmus* were captured in traps baited with the complete blend for this species than in traps baited with methyl (2*E*,4*Z*)-decadienoate alone. Thus, at the concentrations tested, these kinds of dorsal abdominal gland secretions may be epideictic, promoting spacing in the natural habitat.

Key Words—Parasitoid, Scelionidae, Tachinidae, *Nezara*, *Euschistus*, *Eurydema*, *Acrosternum*, *Trissolcus*, pheromone, kairomone.

*To whom correspondence should be addressed.

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INTRODUCTION

Methyl (2*E*,4*Z*)-decadienoate is the major male-specific volatile in several Nearctic *Euschistus* stink bugs (Aldrich et al., 1991a). In fact, traps baited with this fragrant, pear-like compound often simultaneously attract males, females, and nymphs of three species, and their tachinid fly parasitoids. Although a few minor components were identified from aerations of *Euschistus* males, field tests of lures including a minor component along with methyl (2*E*,4*Z*)-decadienoate did not alter attraction (e.g., decanoic acid for *E. tristigmus*; geranylacetone for *E. servus*) (Aldrich et al., 1991a). Therefore, it seemed that volatiles contributing to reproductive isolation of *Euschistus* species remained to be discovered.

An observation of the South American stink bug, *Acrosternum aseadum* (J.R.A., unpublished data), provided a clue to one possible source of biologically active, minor pheromone constituents in pentatomids. Dissection of a wild *A. aseadum* adult revealed two tachinid macroeggs underneath the bug's wings, with an egg perfectly positioned on each opening of the paired dorsal abdominal glands. Many terrestrial Heteroptera have small, but functional, dorsal abdominal glands as adults (Lucchi and Solinas, 1990; Pavis, 1987; Staddon, 1979). The chemistry of secretions from these adult glands is known for a few, mostly unrelated, species (Aldrich et al., 1978, 1990; Evans et al., 1990; Farine et al., 1992a,b; Staddon, 1990; Staddon et al., 1987). Behavioral roles for dorsal abdominal gland secretions are virtually unknown (Aldrich, 1995; Staddon, 1986). Female tachinids commonly use heteropteran pheromones as host-finding kairomones (Aldrich, 1988, 1995), so the apparent orientation of ovipositing tachinids toward the dorsal glands of *A. aseadum* suggested that secretion from these glands has a pheromonal role.

Here we report on our comparative investigations of the chemistry, and associated intra- and interspecific behavior, for dorsal abdominal gland secretions from 10 species representing four genera of Pentatomidae.

METHODS AND MATERIALS

Insects. Euschistus servus, *E. tristigmus*, and *E. politus* adults were caught in traps baited with methyl (2*E*,4*Z*)-decadienoate at the Beltsville Agricultural Research Center in 1989. *Acrosternum hilare* and *A. pennsylvanicum* were collected on the Research Center grounds in May and June, 1989 and 1990, from empress trees (Scrophulariaceae: *Paulownia tomentosa*), and bracken fern (Filicopsida: Dennstaedtiaceae: *Pteridium aquilinum*), respectively. A few individuals of *E. variolarius* were collected in the same area from woolly mullein, *Verbascum thapsus* (Scrophulariaceae), in August 1993. *Acrosternum aseadum* adults were collected on soybean in Brasília, Brazil, in August 1991. *Eurydema*

ventrale and *E. oleraceum* were found on various wild crucifers in and around Perugia and Colfiorito, Italy, July through August 1994. Experiments involving *Nezara viridula* (Pentatomidae) and its scelionid egg parasitoid, *Trissolcus basalis*, used the Italian strains of both these insects. *Nezara* was reared on sunflower seeds (*Helianthus annuus*), green beans (*Phaseolus vulgaris*), and water. *Trissolcus basalis* adults were maintained in glass tubes supplied daily with Safavi diet (Safavi, 1968). Host eggs were exposed to parasitoids for 24 hr, removed, and placed in another tube for incubation. Adult parasitoids emerged in 11–13 days (Mattiacci et al., 1993). *Nezara* and *Trissolcus* were maintained at 24 ± 1°C, 70 ± 5% relative humidity, and a 16L:8D photoperiod, and the other pentatomids were maintained under conditions similar to those for *N. viridula* for up to a month prior to dissection.

Chemical Analyses. Dorsal abdominal glands were excised from CO_2-anesthetized bugs submerged in tap water and extracted in ca. 200 μl of either hexane or CH_2Cl_2 (usually glands from 5–10 adults of each sex were pooled per sample). Samples were analyzed on DB-1 or DB-5 columns (0.25 μm film, 30 m × 0.25 mm ID) in a Varian 3500 GC with helium as carrier (50 cm/sec), a temperature program from 50°C for 2 min to 235°C at 15°/min, with a flame ionization detector. Data were recorded using the Varian GC Star Workstation software on a Gateway 2000 386/25 computer. Gas chromatogram (GC) traces presented in the figures begin at 2.5 min (at left) so as to exclude the solvent peak, and peaks were automatically normalized to the most abundant peak. Electron impact–mass spectra (EI-MS) were obtained using Hewlett Packard 5971 GC-MS instruments at 70 eV, with a HP-5 column (instrument in USA: 0.11 μm film; 25 m × 0.2 mm ID; instrument in Italy: 0.25 μm film; 30 m × 0.25 mm ID), programmed from 50°C for 2 min to 250°C at 15°/min. 4-Heptanol (Aldrich Chemical Co., Milwaukee, Wisconsin) was added to some *Euschistus* samples as an internal standard.

Identifications based on mass spectrometry were verified by comparison and coinjection with the following standards: hexanal, hexanoic acid, octanal, phenylacetaldehyde, acetophenone, nonanal, 2-phenylethanol, 1-nonanol, octanoic acid, decanal, citral (geranial + neral) (Aldrich Chemical Co.); (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*E*)-2-hexenoic acid, (*E*)-2-octenal, (*E*)-2-decenal (Bedoukian Research Inc., Danbury, Connecticut). The preparation of 4-nonenal was accomplished using a three-step procedure based on dihydropyran, which was first stereoselectively converted to (*E*)-4-nonen-1-ol by reaction with butyllithium (Pattison and Dear, 1963). The alcohol was acetylated, and the (*E*)-4-nonenyl acetate was isomerized to a 67:33 ratio of *Z* and *E* isomers, respectively, using sequential bromochlorination–dehalogenation (Sonnet and Oliver, 1976). Finally, alkaline hydrolysis and pyridinium chlorochromate oxidation afforded the corresponding mixture of (*Z*)- and (*E*)-4-nonenal.

TABLE 1. COMPOUNDS IDENTIFIED FROM DORSAL ABDOMINAL GLAND SECRETIONS OF ADULT *Euschistus*, *Acrosternum*, AND *Eurydema* SPECIES[a]

No.	Compound	Composition (%)[b]							
		Eu.p.	*Eu.t.*	*Eu.s.*	*A.h.*	*A.p.*	*A.a.*	*Er.v.*	*Er.o.*
1	hexanal	29*	34*	1*	17				20
2	(*E*)-2-hexenal	33*	31*	73*	28	79	5	12	22
3	(*E*)-2-hexenol					10			
4	hexanol	17*	4*						3
5	hexanoic acid		4*		19				8
6	octanal		5*						3
7	(*E*)-2-hexenoic acid		1	11*	22	4			2
8	phenylacetaldehyde	4*	2	4*					
9	(*E*)-2-octenal		1	1*		2		24	13
10	acetophenone	2*							
11	(*Z*)-4-nonenal						48		
12	nonanal	3*						20	4
13	2-phenylethanol	8*							
14	nonanol							19	
15	octanoic acid		1*						
16	decanal		5						
17	(*E*)-2-decenal			1*				3	
18	geranial		4*	2*		1			
% of total volatiles		96	92	93	86	96	53	78	75
No. males/No. females		3/7	10/10	7/2	14/12	7/11	1/4	7/0	8/1

[a] *Eu.t.* = *Euschistus tristigmus*, *Eu.p.* = *E. politus*, *Eu.s.* = *E. servus*; *A.h.* = *Acrosternum hilare*, *A.p.* = *A. pennsylvanicum*, *A.a.* = *A. aseadum*; *Er.v.* = *Eurydema ventrale*, *Er.o.* = *E. oleraceum*.

[b] Percentages are based on GC peak areas; compounds with * were combined for each *Euschistus* sp. for the pheromone field test.

Field Pheromone Test. Components marked with an asterisk in Table 1 (seven for each *Euschistus* sp. investigated) were combined proportionally using microliter volumes equal to the percent abundances of each compound multiplied by the density of the compound. The total volume of each species-specific blend was then combined with a volume of methyl (2*E*,4*Z*)-decadienoate (Bedoukian Research Inc.) equal to twice the total volume of dorsal abdominal gland components for each species, and this mixture was diluted 50% with heptane. One treatment consisted of only methyl (2*E*,4*Z*)-decadienoate diluted 50% with heptane. In the initial analysis of *E. tristigmus*, phenylacetaldehyde (8) and decanal (16) were not detected at concentrations as high as in later analyses; therefore, these components were not included in the synthetic blend used for the pheromone field test.

Traps were made of transparent cylindrical containers having either white or transparent lids (20.2 × 19.7 cm; Tri-State Molded Plastics, Inc., Dixon, Kentucky) by cutting two holes (9 cm diameter) in opposite sides, and covering each hole with an inwardly projecting screen funnel. The containers were turned upside-down, so that the normal top of the container became the trap bottom, small drainage holes were made in the bottom, and the bottoms were held in place by two elastic cords hooked into each screen funnel. Traps (alternating treatments) were hung from branches about 1.7 m from the ground, at least 50 m apart, bordering oak-dominated deciduous forest at the Beltsville Agricultural Research Center-East, Prince George's County, Maryland. Traps were monitored daily and rebaited every two to three days with 10 μl of pheromone solution from July 28 through September 15, 1992. Five traps were deployed per treatment, plus five unbaited control traps.

Egg Parasitoid Bioassays. Earlier research (Mattiacci et al., 1993) showed that *Trissolcus basalis* females could be induced to attempt oviposition in glass beads coated with extracts of host eggs. This bioassay was employed here to determine if the dorsal abdominal secretion of *Nezara viridula* influenced the acceptance of host eggs. *Trissolcus basalis* females used for bioassays were kept with males for 24 hr to allow mating, then placed singly in small vials with a drop of honey solution, and used for bioassays when they were 3–4 days old. *Nezara viridula* females were segregated from males when they were ≤1 day old and held for at least a week prior to dissection. Dorsal abdominal glands were removed from *N. viridula* females as described above with as small a piece of attached cuticle as possible, and taking care to not cause the bugs to emit metathoracic scent gland secretion. The glands from 20 virgin females were pooled per sample in 100 μl hexane. Samples were divided in half and, to one half, 1 μl of paraffin oil solution (50 μg/μl hexane; Aldrich Chemical Co.) was added. Control treatments consisted of hexane and 1 μg paraffin oil/μl hexane.

A flat-bottomed depression slide (15 mm diameter × 3 mm deep) covered with a glass cover slip served as the bioassay arena. The ends of the depression slide were cut off so that four arenas could be arranged in a compact square for video recording. Clean 1.0-mm-diameter glass beads (the size of host eggs) served as artificial eggs. The glass beads were coated with a control solution or an extract solution by rolling three beads in 10 μl of a solution in a small watch glass until the hexane evaporated. The three coated beads were held firm by pressing them onto a 1-cm^2 piece of parafilm (American National Can, Greenwich, Connecticut), and the beads on parafilm squares were placed in a bioassay arena. Wasps were released singly into an arena, and four arenas were run simultaneously, one for each treatment. The following behaviors were recorded: (1) walking (without contacting glass beads), (2) encounter (touching or walking over glass beads), (3) examination (antennation of glass beads), and (4) acceptance (attempting to oviposit in glass beads). Female wasps that did not contact

the glass beads within 5 min were excluded and, if acceptance did not occur after 7 min from the first encounter, the wasp was scored as rejecting the beads and the bioassays was terminated. Otherwise, bioassays were allowed to proceed for 10 min. Each set of beads was used to test three wasps and then replaced with freshly coated beads. Bioassays were conducted at room conditions (20–25°C, 55–65% relative humidity), with overhead fluorescent lighting and were recorded on video tape.

Data Analysis. Field data were analyzed by ANOVA (General Linear Models, SAS Institute, 1985) after square-root transformation, and reported values are back-transformed for easier interpretation. Laboratory bioassay data were scored using. "The Observer 2.0" video recorder software package (Noldus, 1991) and then analyzed by ANOVA.

RESULTS

Chemistry. Extracts from *Euschistus* spp. contained 208, 183, and 42 ng/adult of volatile compounds for *E. politus*, *E. tristigmus*, and *E. servus*, respectively, and the glands of *Acrosternum* and *Eurydema* adults appeared comparable in size. Eighteen volatile compounds were identified from the dorsal abdominal gland secretions of *Euschistus*, *Acrosternum*, and *Eurydema* adults, including aliphatic, aromatic, and terpenoid constituents (Table 1). The secretions of males and females were not obviously sexually dimorphic but, within each genus, the dorsal abdominal gland secretions were qualitatively and quantitatively species-specific (Figures 1–4).

Six-carbon compounds (1, 2, and 4) were most abundant among the three *Euschistus* spp. common in the study area, but these components were distinctively exhibited (Figure 1). Other components unique to a species further characterized these *Euschistus* secretions, such as 2-phenylethanol (13) for *E. politus*, and octanal (6) for *E. tristigmus*. Although only two males of *E. variolarius* were sampled (insufficient for EI-MS), the gas chromatogram of their dorsal gland extract indicates that the blend from this species is strikingly different from its closest relative, *E. servus* (Figure 2) (McPherson, 1982).

The Nearctic *Acrosternum* species produce similar dorsal abdominal gland secretions (Figure 3, top two traces); however, hexanal (1) is absent in the secretion of *A. pennsylvanicum*, whereas in *A. hilare* this compound is prominent in the secretion. In addition, the secretion of *A. hilare* appears to be more acidic than those of the other two *Acrosternum* spp. investigated. Secretion from *A. aseadum* is chemically novel, being dominated by (*Z*)-4-nonenal (11) (Figure 3, bottom trace).

Finally, the dorsal abdominal gland secretions of *Eurydema ventrale* and *E. oleraceum* are also species-specific, despite the coexistence of certain con-

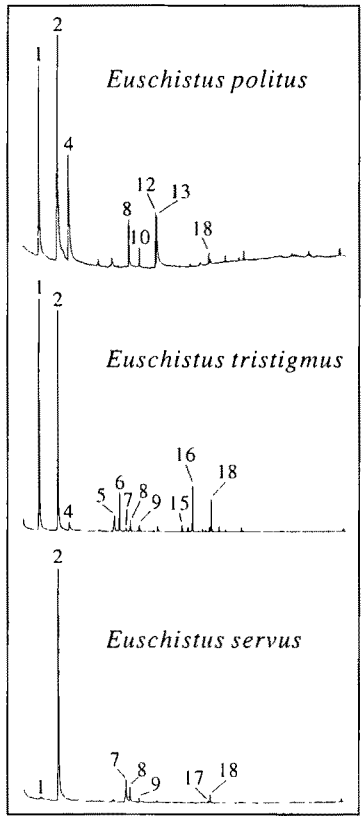

FIG. 1. Gas chromatograms of adult *Euschistus* species dorsal abdominal gland secretion (*E. politus*, 3 males and 7 females; *E. tristigmus*, 10 males and 10 females; *E. servus*, 7 males and 2 females). Numbers in this and other figures correspond to compounds listed in Table 1.

stituents (Figure 4). Substantial differences between these *Eurydema* secretions are the presence of hexanal (1) in *E. oleraceum*, and the occurrence of nonanol (14) in *E. ventrale*. Additionally, the unidentified, late-eluting component of the *E. ventrale* secretion (probably a sesquiterpene hydrocarbon based on EI-MS) was not detected in the secretion of *E. oleraceum*.

Phereomone Field Test. A total of 386 *E. tristigmus*, 33 *E. politus*, 15 *E. servus*, and 21 *Gymnosoma par* (Diptera: Tachinidae) were caught in pheromone-baited traps from July 28 through September 15, 1992. Only one male *E. tristigmus* was caught in unbaited control traps. For all three *Euschistus* spp.,

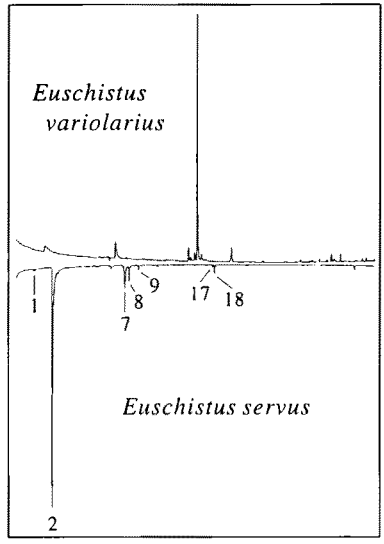

FIG. 2. Comparison of dorsal abdominal gland secretion of *Euschistus variolarius* (2 males) to that of *E. servus* (7 males and 2 males).

equivalent numbers of males, females, and second- to fifth-stage nymphs were captured. Only females of the tachinid parasitoid, *G. par*, were captured (Table 2).

The results of field attractancy are shown in Table 2; attraction to methyl (2*E*,4*Z*)-decadienoate alone (treatment E), and attraction to this ester combined with synthetic blends (treatments EP, ES, and ET) mimicking dorsal abdominal gland secretions of the three *Euschistus* spp. in the study area were tested. No treatment affect was detected for *E. politus*, *E. servus*, or *G. par*. However, significantly fewer adults and nymphs or *E. tristigmus* were caught in traps baited with its specific lure (ET) than in traps baited solely with methyl (2*E*,4*Z*)-decadienoate (E) or with the ES lure. Capture of *E. tristigmus* individuals was also lower in ET-baited traps than EP-baited traps, but this difference was not statistically significant ($P = 0.089$). Analysis of the data for *E. tristigmus* by sex and nymphal catch (Table 2) showed that all stages of *E. tristigmus* generally exhibited reduced attraction to ET-baited traps relative to traps baited with other treatments.

Tachinid Parasitism of Eurydema. A total of 27 wild *E. oleraceum* adults were carefully examined for the presence of tachinid eggs, including 17 females and 10 males. All the tachinid eggs observed were fastened onto the abdominal

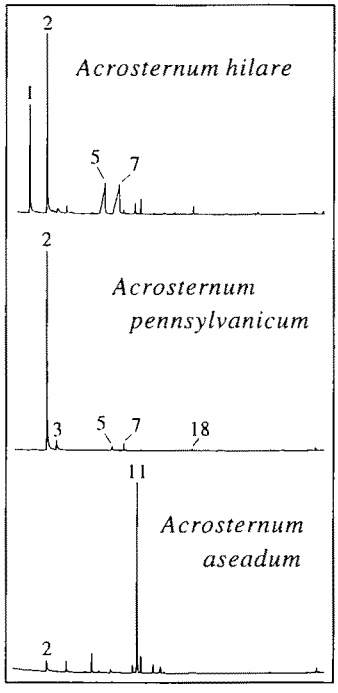

FIG 3. Gas chromatograms of adult *Acrosternum* species dorsal abdominal gland secretion (*A. hilare*, 14 males and 12 females; *A. pennsylvanicum*, 7 males and 11 females; *A. aseadum*, 1 male and 4 females).

tergum of the bugs near the openings of the dorsal abdominal glands underneath the wings; six females had one egg each, one male had three eggs, and one male had one egg. One female *E. ventrale* was observed to have a tachinid egg on her tergum near a dorsal abdominal gland ostiole. No tachinid eggs were observed anywhere else on the body for either *Eurydema* species.

Egg Parasitoid Bioassays. Trissolcus basalis females were significantly more likely to attempt oviposition in glass beads coated with extract of the dorsal abdominal glands from *N. viridula* females than in beads treated with solvent alone (Table 3). The addition of paraffin oil as a fixative for volatile compounds did not affect the behavior of *T. basalis* females. In addition to increasing acceptance of an artificial egg, the treatment of glass beads with dorsal abdominal gland extract also significantly increased the encounter time of the wasps toward beads ($F = 12.26$, 1 *df*, $P < 0.0001$).

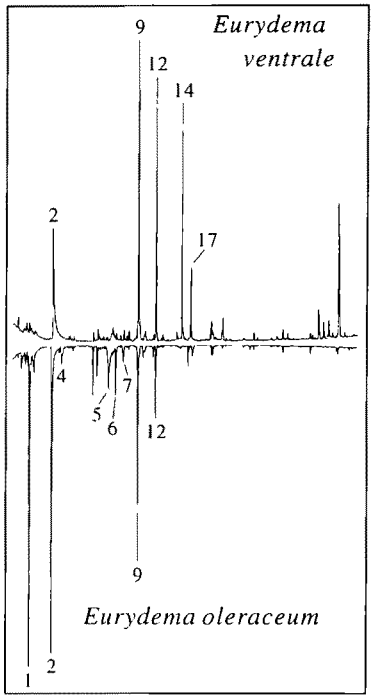

FIG. 4. Gas chromatograms of adult *Eurydema* species dorsal abdominal gland secretion (*E. ventrale*, 7 males; *E. oleraceum*, 8 males and 1 female).

DISCUSSION

In some stink bugs and assassin bugs, males release attractant pheromones from dorsal abdominal glands that are tremendously enlarged compared to those of females (Aldrich et al., 1991b; James et al., 1994a,b). However, in most heteropteran adults where these glands remain active, they are tiny and not noticeably sexually dimorphic (Pavis, 1987; Staddon, 1979). Curiously, the only other occurrence in insects of the most esoteric dorsal gland compound we identified, (Z)-4-nonenal from *Acrosternum aseadum*, is the presumed sex pheromone of an African scutellerid bug produced in the hypertrophied dorsal abdominal glands of males (Gough et al., 1986). In fact, all of the compounds now being reported have been found previously in Heteroptera (Aldrich, 1988), and most are known pheromone constituents of other insects (Mayer and McLaughlin, 1991). Nonetheless, the chemical analyses reported here, along with similar analyses for two *Elasmostethus* species (Acanthosomatidae) (Evans

TABLE 2. *Euschistus* SPECIES AND TACHINID FLY PARASITOID CAUGHT IN FIELD TRAPS BAITED WITH SYNTHETIC PHEROMONE BLENDS

	Euschistus spp. (adult + nymph)[b]			*E. tristigmus*			Tachinid[c]
Treatment[a]	*Eu.t.*	*Eu.p.*	*Eu.s.*	Male	Female	Nymph	*G.par*
E	0.718a	0.040a	0.013a	0.73a	0.98ab	0.30ab	0.038a
EP	0.631ab	0.040a	0.027a	0.19b	0.70b	0.66a	0.006a
ES	0.823a	0.040a	0.033a	1.02a	1.39a	0.31ab	0.013a
ET	0.423b	0.094a	0.027a	0.25b	0.49b	0.16b	0.028a

[a]E = Methyl (2*E*,4*Z*)-decadienoate; EP, ES, and ET = methyl (2*E*,4*Z*)-decadienoate plus the dorsal abdominal gland components marked with an asterisk in Table 1 for *E. politus*, *E. servus*, and *E. tristigmus*, respectively; 1 *E. tristigmus* male caught in controls; details in text.
[b]Values are means per trap per day; species abbreviations as in Table 1; means in a column followed by a common letter are not significantly different ($P < 0.05$, ANOVA).
[c]Only females of *Gymnosoma par* were captured ($N = 21$).

et al., 1990), reveal that these pentatomoid secretions are normally species-specific.

Tachinid parasitoids of *A. aseadum* and *Eurydema* species seem to prefer to oviposit underneath the wings of hosts. Similarly, Eger (1981) found over 90% of the tachinid eggs on *Brochymena* spp. (Pentatomidae) hidden underneath the wings. This parasitoid behavior is circumstantial evidence that secretions from diminutive dorsal abdominal glands are exploited as kairomones. Alternative explanations for this type of oviposition are that the flies cannot stick their eggs to the powdery wax on *Brochymena* adults (Aldrich 1988; Leston, 1953) or that concealing eggs protects them from adverse environmental con-

TABLE 3. ACCEPTANCE OF COATED GLASS BEADS BY FEMALE *Trissolcus basalis* FOR ATTEMPTED OVIPOSITION

Treatment[a]	Replicates	Acceptance (%)[b]
DAG	33	88 a
DAG + paraffin	31	90 a
Hexane	30	27 b
Hexane + paraffin	30	37 b

[a]DAG = dorsal abdominal gland extract; in treatments getting paraffin, the paraffin concentration = 1 μg/μl hexane; details in text.
[b]Percentages followed by a common letter are not significantly different ($P < 0.05$, ANOVA).

ditions (Eger, 1981; Giangiuliani et al., 1991). There is even a report that (*E*)-2-hexenol from the dorsal abdominal glands of adults of one scutellerid species may be toxic to tachinid eggs (Hamilton et al., 1985). Nevertheless, in the past the ovipositional host sex preferences of tachinids foreshadowed discoveries of heteropteran pheromones (e.g., Todd and Lewis, 1976). Insectary workers in Brazil recognized (*Z*)-4-nonenal as the distinctive "colony odor" of *A. aseadum* (J.R.A. personal communication), and it seems plausible that tachinid flies are able to do the same.

Our demonstration that dorsal abdominal gland extract from *N. viridula* females elicits acceptance by *Trissolcus basalis* is added evidence that parasitoids use dorsal abdominal gland odors as kairomones. Earlier research on this egg parasitoid–host association showed that the female wasps orient at long range to the defensive secretion of *N. viridula* (Borges and Aldrich, 1994; Mattiacci et al., 1993) and, upon contact, they recognize host eggs by the glue used to fasten eggs to the substrate and to each other (Bin et al., 1993). Evidently, dorsal abdominal gland volatiles constitute intermediate-range kairomones in the hierarchy of cues leading to parasitism (Aldrich et al., 1984; Bin et al., 1988).

Scents from the small, nondimorphic dorsal abdominal glands of pentatomid bugs apparently betray their presence to parasitoids, raising the question: what is the true biological role for these secretions (Blum, 1974)? Field experiment results seem to refute the hypothesis that minor volatiles increase the specificity of methyl (2*E*,4*Z*)-decadienoate. The surprising discovery that fewer individuals of *E. tristigmus* were captured in traps baited with the lure more completely mimicking its own scent verifies that dorsal abdominal gland secretions play a pheromonal role, but the nature of this communication is more complicated than anticipated. At the concentrations tested, these kinds of dorsal abdominal gland secretions may be epideictic, promoting spacing in the natural habitat (Haynes and Birch, 1985), *Euschistus tristigmus* is the most abundant *Euschistus* species in the study area, and it could well be adaptive for individuals to avoid overpopulated host plants. Still, it is distinctly possible that secretions from the tiny dorsal abdominal glands of adult bugs both attract and repel conspecifics, depending on concentration. In ants, the most social of all insects, it is normal for the same exocrine compounds to recruit workers at low concentration, and elicit "fight or flight" at high concentration (Hölldobler and Wilson, 1990). Indeed, the only compound found in all of the heteropteran secretions examined in the present study, (*E*)-2-hexenal, performs just such a role in some ants (Blum et al., 1969).

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STRUCTURE–ACTIVITY RELATIONSHIP OBSERVATIONS FOR EUROPEAN CORN BORER MOTH PHEROMONE AND FLUORO ANALOGS VIA COMPUTER MOLECULAR MODELING

J.D. WARTHEN,* J.A. KLUN, M. SCHWARZ, and N. WAKABAYASHI

United States Department of Agriculture, Agricultural Research Service
Insect Chemical Ecology Laboratory, BARC-W
Beltsville, Maryland 20705

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Abstract—Structure–activity relationship (SAR) observations were made for the Z-type European corn borer moth pheromone, (Z)-11-tetradecen-1-ol acetate, and a series of analogs with fluorination in the alcohol portion of the molecule. The attractiveness of these analogs and the pheromone was compared to the electrostatic potential map of the molecular mechanics (MM) minimized lowest energy conformation for each compound. A critical range of electrostatic potential on the protons of the double-bond appears to be essential for optimal acceptor fit and attractiveness.

Key Words—Molecular modeling, SAR, Lepidoptera, Pyralidae, *Ostrinia nubilalis*, European corn borer moth, sex pheromone, attractant.

INTRODUCTION

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is distributed over the greater part of Europe and parts of Asia. It has been present in North America since 1917 and is a major pest of corn (Metcalf and Flint, 1939).

The sex pheromone of the ECB moth was demonstrated by Sparks (1963) and confirmed by Klun (1968). It was subsequently identified as (Z)-11-tetradecen-1-ol acetate (1) (Figure 1, Table 1) (Klun and Brindley, 1970; Klun and Robinson, 1971). It was also discovered that there are two subpopulations of

*To whom correspondence should be addressed.

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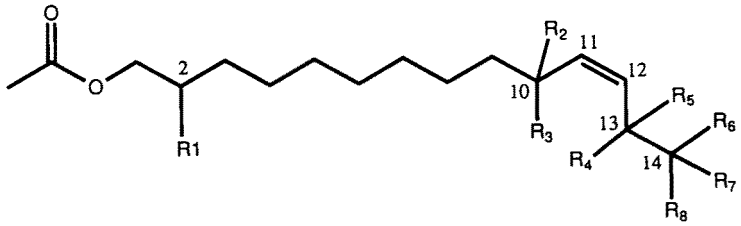

FIG. 1. European corn borer moth pheromone and fluoro analogs: (1) $R_1=R_2=R_3=R_4=R_5=R_6=R_7=R_8=H$, (*Z*)-11-tetradecen-1-ol acetate (*Z*-type pheromone); (5) $R_1=F$, $R_2=R_3=R_4=R_5=R_6=R_7=R_8=H$, (±)-(*Z*)-2-fluoro-11-tetradecen-1-ol acetate; (6) $R_8=F$, $R_1=R_2=R_3=R_4=R_5=R_6=R_7=H$, (*Z*)-14-fluoro-11-tetradecen-1-ol acetate; (7) $R_6=R_7=R_8=F$, $R_1=R_2=R_3=R_4=R_5=H$, (*Z*)-14,14,14-trifluoro-11-tetradecen-1-ol acetate; (8) $R_4=R_5=R_6=R_7=R_8=F$, $R_1=R_2=R_3=H$, (*Z*)-13,13,14,14,14-pentafluoro-11-tetradecen-1-ol acetate; (9) $R_2=R_3=F$, $R_1=R_4=R_5=R_6=R_7=R_8=H$, (*Z*)-10,10-difluoro-11-tetradecen-1-ol acetate; (10) $R_4=R_5=F$, $R_1=R_2=R_3=R_6=R_7=R_8=H$, (*Z*)-13,13-difluoro-11-tetradecen-1-ol acetate.

ECB, a *Z*-type and an *E*-type, that respond to a 97:3 ratio of (*Z*:*E*)- and a 3:97 ratio of (*Z*:*E*)-11-tetradecen-1-ol acetate, respectively (Klun et al., 1973; Cardé et al., 1975.; Klun and Cooperators, 1975; Klun and Junk, 1977; Klun and Maini, 1979).

Structure–activity relationships (SAR) for the ECB were first investigated by Klun and Robinson (1971) and then by Chapman et al. (1978a,b), the latter of whom found that (−)-(*S*)-9-(2-cyclopenten-1-yl)nonan-1-ol acetate (2) (Table 1) was as effective in eliciting precopulatory behavior as the natural sex pheromone (1) (refers to the *Z*-type subpopulation and hereafter in this paper); a chiral acceptor for the chiral pheromone was suggested. Further studies were carried out by Schwarz et al. (1989, 1990) to provide clues for spatial and functional group requirements for activity and to define a picture of the acceptor site. Flight-tunnel activity was induced only by those compounds that had a total chain length of 13–14 carbons and in which branching at carbon-13 was limited to one methyl group or extended as a cyclopropyl group. Two analogs were attractive: (*Z*)-13-methyl-11-tetradecen-1-ol acetate (3) and (*Z*)-12-cyclopropyl-11-dodecen-1-ol acetate (4) (Table 1).

More recently, Klun et al. (1994) investigated selectively fluorinated analogs of the sex pheromone (1) (Figure 1, Table 1) to study pheromone metabolism. Because the fluorine atom is only 10% larger than the hydrogen atom, substitution for hydrogen would not cause a significant molecular volume or surface area change (Wenqi et al., 1993), but would give valuable information

TABLE 1. ELECTROSTATIC POTENTIAL[a] MAP EXTREMES AND OTHER MOLECULAR MEASUREMENTS OF ECB MOTH PHEROMONE AND ANALOGS

Compound	Behavioral activity	Electrostatic potential: Global Max (location)	Global Min (location)	C-11-H Max (local level)	C-12-H Max (local level)	MM (kcal/mol)	Mol vol (Å^3)[b]	Mol area (Å^2)[b]
1 (*Z*)-11-14:OAc	ECB pheromone	41.5136 (OAc end)	−79.5104 (OAc end)	12.1390 (7th)	12.3695 (6th)	6.2311	222.857	235.764
2 (−)-(*S*)-9-(2-Cyclopenten-1-yl)-9:OAc	+	41.6024 (OAc end)	−82.2160 (OAc end)	12.0869 (7th)	13.0133 (6th)	14.2303	219.159	231.515
3 (*Z*)-13-Methyl-11-14:OAc	+	42.9473 (OAc end)	−81.6013 (OAc end)	12.3364 (8th)	12.5131 (7th)	4.7849	239.787	257.641
4 (*Z*)-12-Cyclopropyl-11-12:OAc	+	44.8737 (OAc end)	−80.5578 (OAc end)	12.3906 (7th)	14.3686 (6th)	149.6240	234.978	249.387
5 (±)-(*Z*)-2-Fluoro-11-14:OAc	+	60.6823 (OAc end)	−78.4387 (OAc end)	12.5277 (6th)	12.8378 (5th)	10.9170	230.910	245.603
6 (*Z*)-14-Fluoro-11-14:OAc	+	46.2870[c] (F end)	−81.2198 (OAc end)	13.9653 (8th)	12.9436 (7th)	6.3285	229.468	240.947
7 (*Z*)-14,14,14-Trifluoro-11-14:OAc	+	74.4944[d] (F end)	−80.7433 (OAc end)	18.7420 (7th)	19.2798 (6th)	6.2663	236.526	250.529
8 (*Z*)-13,13,14,14,14-Pentafluoro-11-14:OAc	−	135.2650[e] (F end)	−82.7152 (OAc end)	25.1848 (5th)	Located near global max.[f]	29.4135	245.947	257.671
9 (*Z*)-10,10-Difluoro-11-14:OAc	−	50.0028[g] (F end)	−78.8143 (OAc end)	Located near global max.[f]	20.4522 (6th)	6.6969	232.129	242.157
10 (*Z*)-13,13-Difluoro-11-14:OAc	−	49.3045[h] (F end)	−79.2256 (OAc end)	20.3464 (7th)	39.3463 (4th)	3.5782	232.755	242.916

[a] Electrostatic potential, kcal.
[b] 3 contours/Å.
[c] 42.1110 (OAc end, 3rd local level).
[d] 43.9507 (OAc end, 3rd local level).
[e] 43.0673 (OAc end, 2nd local level).
[f] 3rd–18th local levels checked.
[g] 41.5505 (OAc end, 2nd local level).
[h] 42.1747 (OAc end, 2nd local level).

on electronic effects since fluorine is so electronegative. Pheromone analogs having monofluorination (5, 6) or trifluoro-substitution (7) mimicked the pheromone, while analogs with fluorine adjacent to the double-bond (8–10) were essentially inactive.

The general hypothesis for pheromone binding involves a three-pronged interaction of the pheromone with the acceptor: (1) by hydrogen bonding to and/or electrostatic interaction with the polar functional group, (2) electrostatic interaction with the double-bond, and (3) much weaker dispersion forces that are responsible for binding the terminal alkyl group (Liljefors et al., 1985). By altering electrostatic potentials at specific sites in a pheromone molecule using fluorine substitution, one can localize an electrostatic potential or change the charge intensity on adjacent atoms that interact with an oppositely charged area on an acceptor site.

Electrostatic potential is defined as the interaction energy between a point positive unit charge and the atoms of a structure; there are two contributions to the value of the electrostatic potential at any point: a nuclear contribution, and a contribution from the electrons within their molecular orbitals. Reasonable approximations can be found by using atom-based point charges, such as those provided by the Gasteiger method (Gasteiger and Marsili, 1980). For electrostatic potential maps, the minimum global, maximum global, and numerous maximum local positions (levels) can be located as "map extremes." This technique gives a number of key points in molecular space to simplify and aid in the identification of maximum and minimum electrostatic potential sites. A series of molecules that act at the same acceptor site may be expected to share common acceptor-interaction mechanisms. Positions of map extremes, which can reflect these acceptor interactions, could be a good basis on which to explain acceptor fit.

Since the electronegative fluorine-substitution in the ECB pheromone produces electrostatic changes, we explored these changes via computer molecular modeling to quantify their effects on activity. Thereby, we refined the ECB pheromone SAR model and thus learned more about the likely characteristics of its putative acceptor site.

METHODS AND MATERIALS

The ECB pheromone (1) (Figure 1, Table 1) and its fluoro analogs [5 (Oliver et al., 1994) and 6–10 (Klun et al., 1994)] were synthesized. Compounds 2–4 were also synthesized (Chapman et al., 1978a; Schwarz et al., 1989). The resulting acetates were tested in the field, and some were assayed using flight-tunnel techniques (Table 1).

The pheromone and its fluoro analogs were modeled via the computer

molecular modeling program, Chem-X (July 1993 VAX version, Chemical Design Ltd., Oxford, England). Lowest-energy conformations were determined using molecular mechanics (MM) (Burkert and Allinger, 1982; Clark, 1985) with modified force-field parameters and Gasteiger charges. Electrostatic potential maps with global and local electrostatic maxima and minima and three-dimensional (3D) Van der Waals surface maps were computed.

RESULTS AND DISCUSSION

Lowest-energy MM minimized conformations of the attractive compounds, (−)-(*S*)-9-(2-cyclopenten-1-yl)-9-nonan-1-ol acetate (2), (*Z*)-13-methyl-11-tetradecen-1-ol acetate (3), and (*Z*)-12-cyclopropyl-11-dodecen-1-ol acetate (4) (Table 1) have molecular volumes and surface area ranges of 219–240 $Å^3$ and 231–258 $Å^2$, respectively. The lowest energy MM minimized conformations of the ECB pheromone (1) and its attractive fluoro analogs (5–7) had similar ranges of 222–237 $Å^3$ and 235–251 $Å^2$ for molecular volume and molecular surface area, respectively. Fluorine substitution for hydrogen results in only a small increase in the molecular volume and surface area. However, molecular volume and surface area are important for activity since they represent steric bulk; previous work (Schwarz et al., 1990) showed that attractiveness to ECB males required a chain length of 13–14 carbons in which branching at carbon-13 was limited to one methyl group or was extended as a cyclopropyl group.

There appear to be ranges for attractive compounds (1, 5–7) of about 222–237 $Å^3$ and 235–251 $Å^2$ for molecular volume and surface area, respectively. However, the unattractive fluoro analogs (8–10) also had similar 232–246 $Å^3$ molecular volume and 242–257 $Å^2$ molecular surface area ranges. Therefore, molecular volume and surface area ranges in this series of compounds (1, 5–10) are not important SAR issues since they are so similar.

Thus, some other factor had to be considered. Since fluorine is very electronegative, the fluorinated ECB pheromone analogs (5–10) would be expected to have electron distributions different from the natural pheromone. Determination of dipole moments of the pheromone and its fluoro analogs via Chem-X molecular modeling revealed no correlations with activity because dipole is an average or overall electronic characteristic of the molecule. On the other hand, electrostatic potential maps for the pheromone (1) (Figure 2) and its fluoro analogs (5–10) revealed distinctive variations in potential on the surface of the molecules at numerous map extremes. Some sites in the molecules are strongly electronegative, whereas other sites are slightly or intensely electropositive; the patterns of these are likely important in the acceptor interaction because it is these patterns of local variability in electrostatic potential that interact with similar patterns of opposite electrostatic potentials on the acceptor.

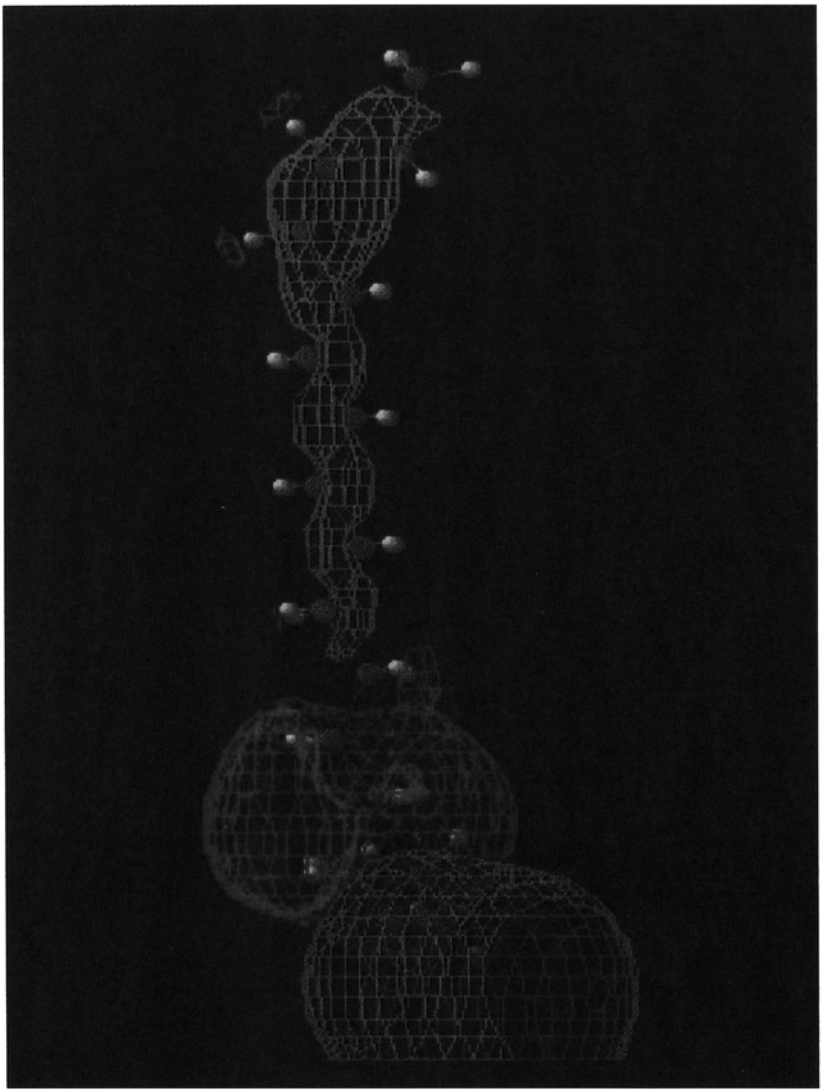

FIG. 2. Electrostatic potential 3D map of ECB pheromone (Z)-11-tetradecen-1-ol acetate (1). Standard contours: cyan < -10.0 kcal; red > 10.0 kcal; significance = 20; grid density = 2.5 contours/Å.

The global minima for 1, and 5–10 were between −78 and −83 kcal on the acetate end of the molecule. The global maxima were on the acetate end for 1 and 5 and on the terminal end of the chain for 6–10. However, 6–10 had the second or third local highest maxima (footnotes, Table 1) at the acetate end, comparable with 1 and 5. Considering this, there was a range of 41–61 kcal on the acetate end for compounds 1, and 5–10.

In contrast, the local maxima for the protons of the *Z*-double bond (1, 5–10) showed consistent differences between those analogs that exhibited attractiveness and those that did not. The electronegative withdrawing effects of the fluorine were particularly evident when fluorine was present on carbon atoms adjacent to the double-bond (8–10) (e.g., Figure 3, 10). The local range of electrostatic potential for the proton on carbon-11 of the ECB pheromone (1) and the attractive fluoro analogs (5–7) was 12–19 kcal at the sixth to eighth highest local maximum extreme; the local range for the proton on carbon-12 for the same substances was also 12–19 kcal but at the fifth to seventh highest local maximum extreme. Attractive (−)-(*S*)-9-(2-cyclopenten-1-yl)nonan-1-ol acetate (2), (*Z*)-13-methyl-11-tetradecen-1-ol acetate (3), and (*Z*)-12-cyclopropyl-11-dodecen-1-ol acetate (4) also had local maxima within the range of 12–19 kcal for protons on the double bond; they also had a global maxima range of 41–61 kcal and a global minima range of −78 to −83 kcal.

The inactive fluoro analogs (8–10) had electrostatic potentials of the double-bond hydrogens that were outside the range of 12–19 kcal. Visual examinations of the potential maps (8–10) (e.g., Figure 3, 10) showed great distortion of the partial positive charges around the double bond (in red) as compared to the pheromone (1) and the active fluoro analogs (5–7) (e.g., Figure 2, 1). This may characterize the basis for the inactivity of compounds 8–10.

The acceptor for the ECB pheromone (1) has chiral recognition, based upon the attractiveness of (−)-(*S*)-9-(2-cyclopenten-1-yl)nonan-1-ol acetate (2) as opposed to its much less active antipode (Chapman et al., 1978a). However, chirality was not investigated in this work.

Although the ECB pheromone contains 3% of the *E*-isomer, we did not address this component in our study, nor did we address the *E*-type pheromone/acceptor.

CONCLUSIONS

Three characteristics of 1–7 appear to be in common for ECB attractiveness. The first characteristic is demonstrated by the general ranges of 222–237 $Å^3$ molecular volume and 235–251 $Å^2$ molecular surface area that are reflected in the required chain length of 13–14 carbons in which branching at carbon-13 was limited to one methyl group or was extended as a cyclopropyl group (Schwarz

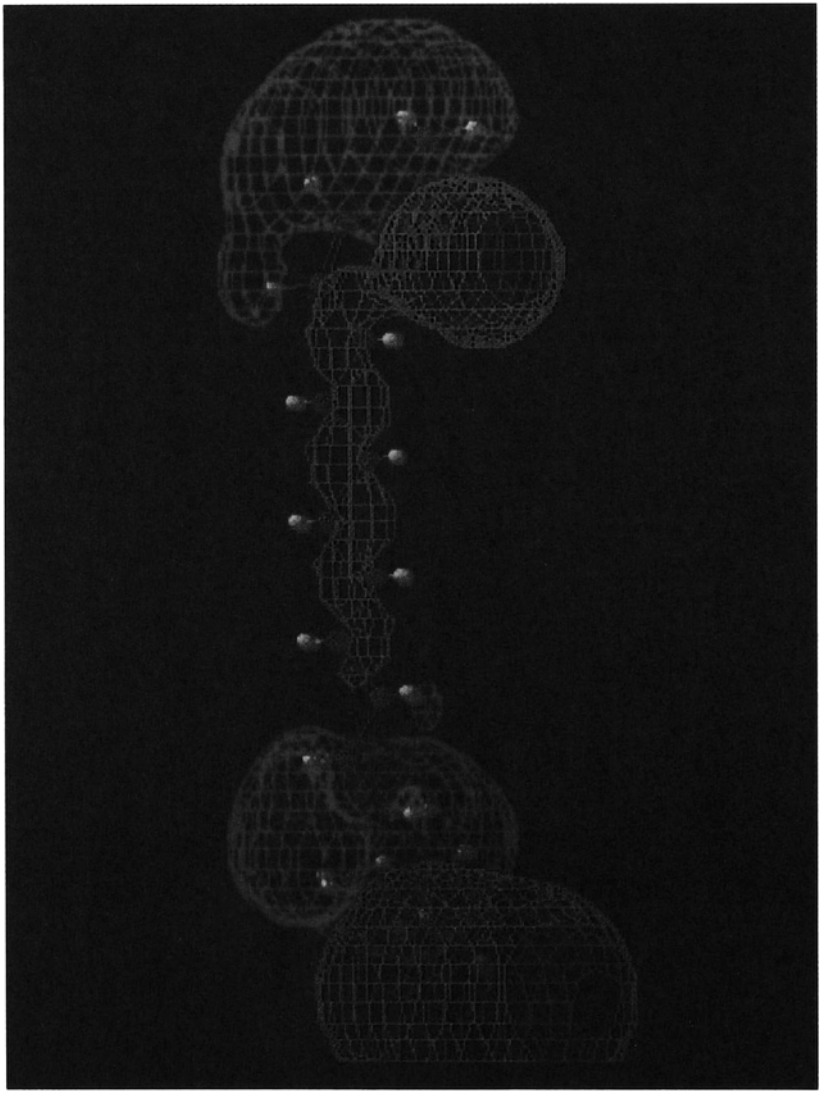

FIG. 3. Electrostatic potential 3D map of the nonattractive (Z)-13,13-difluoro-11-tetradecen-1-ol acetate (10). Standard contours: cyan < -10.0 kcal; red > 10.0 kcal; significance = 20; grid density = 2.5 contours/Å.

et al., 1989). However, these ranges are not distinguishing characteristics since the inactive fluoro compounds (8–10) also show these ranges.

The second characteristic is an electrostatic potential global minimum range of −78 to −83 kcal on the acetate end of the molecule and an electrostatic potential maximum range of 41–61 kcal (global or local) also on the acetate end. However, these also were not distinguishing characteristics since most of the inactive compounds (8–10) fell within these ranges.

The third characteristic is an electrostatic potential of 12–19 kcal for the proton on carbon-11 (sixth to eighth highest local maximum) and for the proton on carbon-12 (fifth to seventh highest local maximum). Greater values for the inactive fluoro compounds (8–10) than these ranges are consistent with absence of attractiveness. These ranges would be considered distinguishing characteristics for attractiveness.

These three characteristics are for the data set of attractive compounds (1–7) studied. These results and conclusions should be considered in the design of ECB pheromone mimics and analogs for metabolic studies. To maintain pheromonal activity, it appears essential to avoid the substitution of fluorine on carbons adjacent to the double-bond.

These characteristics can be related to the Liljefors et al. (1985) three-pronged pheromone/acceptor interaction model as follows: (1) the overall electrostatic interaction of pheromone–acceptor as reflected in the molecular volume and surface area characteristics that include some very weak potentials, (2) the electrostatic potential ranges of the functional groups for the above second characteristic, and (3) the very important distinguishing electrostatic potential range for the double-bond hydrogens.

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NATURAL VARIATION OF TOXICITY IN ENCRUSTING SPONGE *Crambe crambe* (SCHMIDT) IN RELATION TO SIZE AND ENVIRONMENT

MIKEL A. BECERRO,[1] XAVIER TURON,[2] and MARÍA J. URIZ[1,*]

[1]*Centre for Advanced Studies (CSIC)*
Camí de Sta. Bàrbara s/n.
17300 Blanes (Girona), Spain

[2]*Department of Animal Biology (Invertebrates)*
Faculty of Biology, University of Barcelona
645, Diagonal Ave., 08028 Barcelona, Spain

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Abstract—The presence of intraspecific variation in toxicity and its relationship with biological or ecological factors were studied in the sponge *Crambe crambe*. Within-specimen (periphery and central part), between-size (< 1000 mm^2 in area, between 1000 and 10,000 mm^2 and $> 10{,}000$ mm^2) and between-habitat (well-illuminated and dark communities) variations in toxicity were evaluated by the Microtox bioassay. Quantitative differences were detected that were not attributable to within-specimen variation but to size and habitat effects. Habitat comparisons showed that sponges in the shaded habitat were significantly more toxic than those of the well-illuminated community. Sponges of the smaller size classes displayed significantly less toxicity than the medium-sized specimens. Results are interpreted under the optimal defense theory and their ecological implications are considered.

Key Words—Chemical ecology, natural toxicity, spatial variation, defensive strategy, encrusting sponges.

INTRODUCTION

The field of chemical ecology in marine environments is rapidly expanding, building on the field of the chemistry of marine natural products. During the last few decades, numerous new compounds have been isolated from marine

*To whom correspondence should be addressed.

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organisms (e.g., see reviews by Faulkner, 1984, 1986, 1991), many of them with interesting pharmacological properties (Fautin, 1988; Hall and Strichartz, 1990). Recently, there has been increased interest in understanding whether such properties have a significant role in ecological processes. In the better-known field of terrestrial chemical ecology, laboratory and field investigations have led to better understanding of the role that chemistry plays in plant growth and germination (Rice, 1984; Carral et al., 1988), patterning of vegetation (Rice, 1984; Stowe, 1979), or plant–herbivore relationships (Rhoades and Cates, 1976; Harborne 1988), to cite some examples.

Defense mechanisms analogous to those reported for terrestrial environments may be expected in their marine counterparts, benthic communities. Similar pressures may determine the evolution of toxic compounds causing inhibition of growth in plants (Rice, 1984) and sessile organisms (Sammarco et al., 1983 Sullivan et al., 1983; Porter and Target, 1988) or inhibition of seed germination (Rice, 1984; Carral et al., 1988) and settlement inhibition (Davis et al., 1991). These processes may function as a strategy for space acquisition and maintenance, influencing spatial patterning. If toxicity has been selected as a defensive mechanism, it must have been as a consequence of coevolution with competitors (in a wide sense). When the nature of such competitors is environmentally determined, one can expect a variation of toxicity in relation to ecological conditions. These patterns are well documented in terrestrial environments and, together with experimental work, have provided the basis for modeling the production of chemical defenses. Variation in defense investment has been reported, for example, as a function of plant size, intraplant distribution, resource availability, growth rate, stress, and herbivore pressure (Feeny, 1976; Rhoades and Cates, 1976; McKey et al., 1978; Coley et al., 1985; Fagerström et al., 1987; Basey and Jenkins, 1993).

Although similar patterns might be expected in benthic communities, there is a lack of information on naturally occurring toxicity as compared with terrestrial ecosystems. Few studies have specifically addressed toxicity variation in benthic communities, although it has been detected in numerous species (e.g., bryozoans: Harvell, 1986; algae: Moon and Martin, 1985; Hay et al., 1988; Paul and Van Alstyne, 1988; gorgonians: Pawlik et al., 1987; Harvell and Fenical, 1989; soft corals: Coll et al., 1982; Harvell et al., 1993; and sponges: Thompson et al., 1985). To our knowledge, the works by Thompson et al. (1987), Yates and Peckol (1993), Harvell et al. (1993), and Maida et al. (1993) are the only studies where intraspecific ecological variation in chemical defenses was specifically addressed. Thompson et al. (1987) demonstrated that the variability in diterpene composition of the sponge *Rhopaloeides odorabile* is caused by the environmental conditions under which the sponge lives rather than to genetic differences. Yates and Peckol (1993) detected higher polyphenolic levels in *Fucus vesiculosus* in a community with low nitrogen availability as

compared with a nitrogen-rich site. Harvell et al. (1993) found quantitative and qualitative differences in the toxic compounds of the gorgonian coral *Briareum asbestinum* associated with site and depth. Higher levels of compounds were detected in corals in deeper waters, where levels of predation and productivity rates were lower (but see Paul and Van Alstyne, 1988, for contrasting results in a seaweed species). Maida et al. (1993) detected significant differences in the relative production of three toxic substances in the soft coral *Sinularia flexibilis* as a function of size and habitat. A greater production of a molecule involved in spatial competition was reported for specimens from a highly diverse and competitive site.

The present study examined the naturally occurring toxicity of the poecilosclerid encrusting sponge *Crambe crambe* (Schmidt). There were several reasons for choosing this species. First, *C. crambe* shows an unusually wide range of distribution. It is present in numerous western Mediterranean communities (from 0 to 60 m in depth), where it is one of the most abundant sponge species. Second, this species is known to possess an array of highly active metabolites (Berlinck et al., 1990, 1992; Jares-Erijman et al., 1991) with diverse ecological roles, such as antifouling, antipredatory, or as a space competition mechanism (Becerro et al., 1994a; Becerro, 1994). Third, most of the biological functions of sponges are accomplished at the cellular level. No circulatory or coordination system has been found, resulting in a lower degree of integration at the individual level with respect to other Metazoa. Moreover, sponges present an unusual organization; they are formed by the iteration of canal systems, which are labile modular units much more variable in space and time than the typical, more stable, units such as leaves or polyps. Taken together, these facts raise interesting questions as to whether the production of chemical defenses is coordinated in the whole sponge or whether there may be intraindividual variation.

Toxicity assessment can be performed through a fine-scale quantification of the molecules responsible for it using either HPLC (Harvell et al., 1993) or ^{1}H NMR (Maida et al., 1993). A second approach is to quantify toxicity in itself through a sensitive and precise bioassay. This approach was adopted in this study due to the high number of active compounds present in *Crambe crambe*. Besides, synergistic effects may be easily overlooked if separate quantification of molecules is performed. Furthermore, if a nontoxic compound can act synergistically with a toxic one, the former will not be considered at all unless its connection with the toxic agent is known. Berenbaum and Neal (1985), for example, documented in plants the increased effectiveness of a deterrent metabolite due to the action of a nondeterrent, cooccurring compound. The substances presumably responsible for the activity of *C. crambe* belong to two families of compounds: crambines and crambescidines, each with several molecules whose interactions are not known and whose isolation, even partial, requires a very laborious process (Berlinck et al., 1990). Therefore, we thought

it more advisable, in order to interpret ecological roles, to quantify the general toxicity per sponge unit weight, rather than trying to quantify one or several of these compounds.

The purpose of this study was to analyze the toxicity of *C. crambe* in relation to size and environment. Morphological changes, as well as variations in investment in structural components and reproduction as a function of size and habitat, have already been reported in this species (Becerro et al., 1994b; Uriz et al., 1995). The sponge seemed to respond to shaded habitats, which were space-limited, macrozoobenthos-dominated assemblages, by increasing directional growth and strengthening physical structures with increasing area. In contrast, specimens from well-illuminated, algae-dominated habitats showed an increase in organic matter, but not in allocation to structural components, with increasing size. Uriz et al. (1995) interpreted these results as a consequence of the space competitors present in the space-limited community. Accordingly, a higher investment in chemical defense may also be expected, and this is the hypothesis set forth in this study. Specifically, an attempt was made to answer the following questions: (1) is there intraspecimen variation in the toxicity of *Crambe crambe*? (2) is there intraspecific variation? and if so, (3) is this variation size dependent? (4) is this variation habitat associated? and (5) is there any relationship between the two factors?

METHODS AND MATERIALS

Sampling. All the specimens used in this study were collected in the locality of Blanes (NE of Spain, western Mediterranean), April 1992. The site was a 2 to 3-m-wide, 40-m-long gorge with north- and south-facing walls. Therefore, environmental conditions (temperature, food availability, underwater currents) were comparable on both walls, except for the amount of incident light. The north- and south-facing walls correspond to a shaded and a well-illuminated habitat, respectively. Communities on such walls showed remarkable differences in species composition: a space-limited community mainly dominated by encrusting organisms was found on the shaded wall, whereas the well-illuminated habitat was dominated by algae, and patches of free substratum were easily observed (see Becerro et al., 1994b, for a general description of these communities). Three size categories were established, according to results of Becerro et al. (1994b) in each of the two contrasting communities: (1) < 1000 mm^2 in area, (2) 1000 to 10,000 mm^2, and (3) $> 10{,}000$ mm^2. Ten specimens for each category of size and habitat were randomly selected. Samples were collected as follows: one small piece (≈ 1 cm^2) was taken from the central part of the sponge and three smaller pieces (adding up to approximately 1 cm^2) were picked up from the periphery in an attempt to reduce the expected heterogeneity

along the sponge border. Separate sampling in the center and periphery was not possible in the smallest size class, and intraindividual variability was assessed only in specimens from the intermediate and largest size classes. The samples were individually placed in plastic bags and taken to the laboratory for chemical extraction of the toxic compounds.

Extraction Procedure. A moderate number of active compounds are known in *Crambe crambe:* up to four crambines (plus several homologs) and four crambescidins have been described (Berlinck et al., 1990, 1992; Jares-Erijman et al., 1991) (Figures 1 and 2), and the list may not be complete yet. These compounds consist of a long, nonpolar chain with a polar head, which confer to them a mixture of lipophilic and hydrophilic properties, making them amenable to solution with solvents of different polarity. Preliminary studies were conducted to determine a method for easy extraction of these families of compounds. These studies are explained in full in Becerro (1994) and can be summarized as follows: (1) after trying all possible sequences with solvents covering a large range of polarities (hexane, dichloromethane, and water), we found that a single extraction with dichloromethane (DCM) takes up all toxic compounds, and no toxicity was detected if the tissues were extracted afterwards with any of the other solvents; (2) subfractioning the DCM extract with silica-gel columns in a gradient from petroleum–ether, petroleum ether–ethyl ether (decreasing ratios), ethyl ether, chloroform–methanol (8:2) to methanol showed that the toxicity was due to compounds in the most polar extreme of the range of metabolites solubilized by the DCM; (3) toxicity tests on the DCM fraction alone

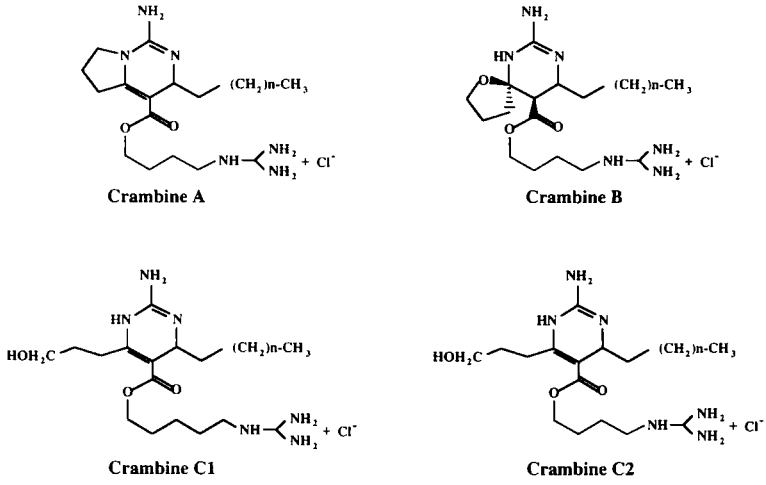
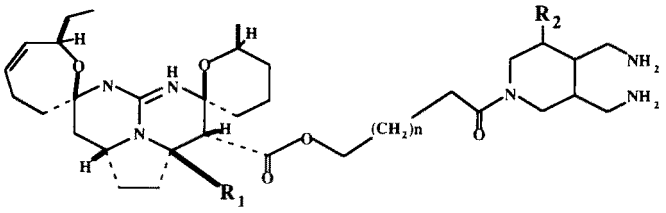


FIG. 1. Schematic representation of the crambines (from Berlinck et al. 1990, 1992).



Crambescidin 816 $R_1 = R_2 =$ OH, n = 13
Crambescidin 830 $R_1 = R_2 =$ OH, n = 14
Crambescidin 844 $R_1 = R_2 =$ OH, n = 15
Crambescidin 800 $R_1 =$ H, $R_2 =$ OH, n = 13

FIG. 2. Schematic representation of the crambescidines (from Jares-Erijman et al. 1991).

showed the same effect as that found after pooling the fractions obtained by successive extractions with hexane, DCM, and water. Thus, no synergistic effect with molecules not extracted by DCM alone was expected.

Once in the laboratory, each sample was carefully cleaned of any foreign body (such as polychaete tubes, barnacles, etc.) under a stereomicroscope and ground. Ten milligrams dry weight of sponge were then extracted three successive times (5, 15, and 30 min) with 5 ml of DCM (Becerro, 1994). The three fractions were pooled, the solvent evaporated under reduced pressure, and the residue homogenized in an ultrasonic bath in 20 ml of distilled water. This led to a concentration of 500 ppm relative to the initial sponge dry weight.

Toxicity Analyses. Since we wanted a fine quantification of the general toxicity of the sponge, we sought a biological test that permitted accurate toxicity readings. On the other hand, the test had to be ecologically significant if the results were to be interpreted in terms of the ecological role of chemical defenses. Several such roles are possible (Coll, 1992), and it is not feasible to devise quantitatively accurate tests for all potential ecological functions of the bioactive substances or for all potential competitors or predators. Instead, we engaged in a number of tests with ecological meaning (antilarval, antibacterial, cytotoxic, and antipredator with sympatric species) and tested their correlations with an easy, accurate test (Becerro, 1994; Becerro et al., 1995). As a result, we adopted a standardized procedure, the Microtox bioassay (Ribo and Kaiser, 1987; Kaiser and Ribo, 1988) as a precise method for measuring toxicity changes. This method is based on measurements of bioluminescence in living cell suspensions of the deep-sea bacterium *Photobacterium phosphoreum*. This proved to be the best test in terms of repeatability and precision and, although of little ecological meaning per se (it is hard to envisage how this particular bacteria

could affect *Crambe crambe* in nature), it was well correlated (Becerro, 1994; Becerro et al., 1995) with the other ecologically significant assays ($r = 0.98$ with the paper diffusion test with sympatric bacteria, and $r = 0.97$ with the sea urchin assay) and was used here as a precise tool for measuring variations in toxicity.

Freeze-dried bacteria were reconstituted at 4°C. In the basic procedure, Microtox requires four concentrations and one control per sample. The initial toxic concentration tested was 225 ppm (relative to sponge dry weight), and a dilution factor of two was used for the three successive concentrations (see Becerro et al., 1995, for a full description of this procedure). Experiments were run for 5 min at a temperature of 15°C. The light produced by the bacteria was recorded before and after the experiments in gamma units (GU):

$$GU = (R_t * I_0/I_t) - 1$$

where R_t is the correction factor (light differences in the control), I_0 is the light at time 0, I_t is the light at time t. Finally, the concentration at which GU is equal to 1 (50% of light reduction or estimated median effective concentration, EC_{50}) was calculated by fitting the data in logarithmic scale to a linear regression. The toxicity units (TU) used in this study measure quantitative changes in the samples and were calculated as $100/EC_{50}$.

Further information on the toxic behavior of the compounds can be obtained through the study of the slope of the regression function mentioned above. Two samples may have similar EC_{50} values but different slopes, indicating qualitative differences in the compounds responsible for the toxicity or changes in the relative amounts of these compounds. Consequently, the slope of the regression lines was used as a variable to measure qualitative changes in the samples. Differences in the slope indicate differences in the relationship between concentration of toxic compounds and toxicity, i.e., the higher the slope, the more effective the toxins.

Numerical Methods. Within-sponge differences in toxicity (center–periphery) were studied by paired-sample t-test analyses (Zar, 1984). These tests were performed for medium and large specimens (the only ones from which separate samples from the center and periphery were taken). The inclusion of the smallest size class for analysis of size and habitat effects rendered the data heteroscedastic (F_{max} test; Bakus, 1990) so that a rank transformation (Potvin and Roff, 1993) was applied prior to further parametric tests. Two-way analyses of variance (ANOVA) were performed, using size classes (three levels) and habitat (two levels) as factors. The former is clearly a fixed factor, and the second must also be considered fixed as the two communities studied were not selected at random, but specifically chosen because they were a priori expected to differ (Bennington and Thayne, 1994). We therefore restrict the conclusions to these two types of wall, and we do not mean to use the results as an estimate of the general variation

among all possible habitats. Whenever we speak of a habitat effect, therefore, we refer exclusively to the two habitats considered. Both variables, TU and slope, were analyzed by the same methods. The analyses were performed using the Systat 5.0 package.

Unplanned pairwise comparisons were performed following the Ryan's Q method (Einot and Gabriel, 1975; Day and Quinn, 1989), a stepwise procedure similar to the more familiar Student-Newman-Keuls (SNK) test, but which differs from it in using a lower α level at each step except the first one. Day and Quinn (1989) compared the performance of several multiple comparison methods using Monte Carlo simulation, and found that the Ryan's Q procedure produced the best approximation between the probability of type I errors and the nominal α level. The overall α for each set of multiple comparisons was set at 0.05.

RESULTS AND DISCUSSION

Results of the within-sponge TU variation are shown in Table 1. No toxicity differences were detected between the center and periphery of the sponges by the paired-sample t-tests, either considering all samples together or analyzing separately each combination of size and habitat. The two readings per sponge were therefore averaged to give a single value for further analyses. Table 2 summarizes the outcome of the two-way ANOVA of TU using size class and habitat as factors, whose values are graphically represented in Figure 3. Both main factors proved significant, and the interaction was not. A higher overall toxicity did exist in the sponges from the sciaphilous environment. Unplanned comparisons between size classes (across habitats) showed that the sole significant pairwise comparison (Ryan's Q, $\alpha = 0.05$) was the one between small and medium-sized individuals (the latter featuring higher toxicity, Figure 4).

TABLE 1. PAIRED-SAMPLE t-TEST ANALYSES OF TOXICITY (TU): INNER VS. PERIPHERY VALUES GROUPED BY HABITAT AND SIZE; OVERALL t TEST ANALYSIS ALSO SHOWN

Habitat	Size	Mean differences	SD differences	t	df	P
Sciaphilous	Medium	0.865	6.320	0.387	7	0.710
	Large	−2.998	6.457	−1.392	8	0.201
Photophilic	Medium	1.724	6.962	0.743	8	0.479
	Large	1.723	4.854	1.065	8	0.318
Overall t test		0.313	6.246	0.229	34	0.768

TABLE 2. RESULTS OF TWO-WAY ANOVA OF TOXICITY (TU) VALUES

Source	SS	*df*	MS	*F*	*P*
Habitat	1283.689	1	1283.689	5.888	0.018
Size	2936.717	2	1468.358	6.735	0.002
Habitat × size	1030.601	2	515.300	2.363	0.104
Error	11336.275	52	218.005		

The slope of the toxicity function also showed no within-sponge variation (Table 3) either analyzed by groups (habitat and size) or as a whole. Slope values for each habitat and size category are depicted in Figure 4. Two-way ANOVA of the slope values (Table 4) showed that neither the interaction term ($P = 0.604$) nor the main factors (habitat, $P = 0.691$; size, $P = 0.142$) had a significant effect.

The sponge *Crambe crambe* showed, therefore, a remarkable between-specimen variation in toxicity. This variation was related to size and habitat. Both factors significantly influenced the extent of toxicity found, although no qualitative differences were detected (as evaluated by the slope of the toxicity function) in the extracts. On the other hand, within-specimen (center–periphery) variation was not observed, in contrast to reports on other benthic organisms (Paul and Van Alstyne, 1988; Wylie and Paul, 1989; Harvell and Fenical,

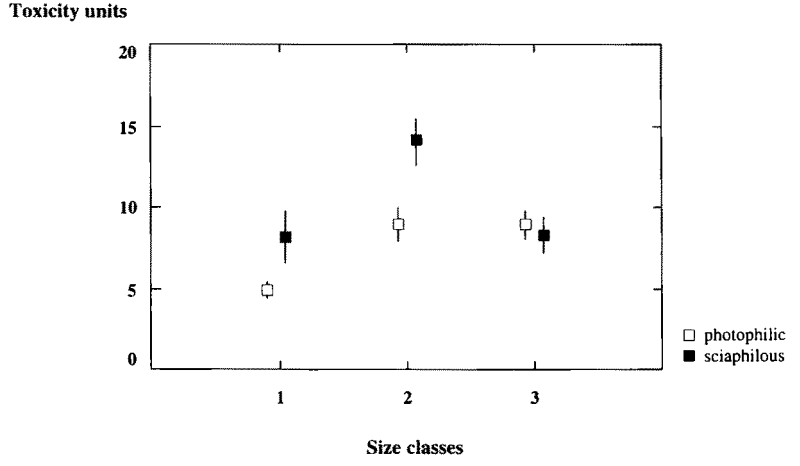


FIG. 3. Mean (±SE) values of the toxicity units (TU) according to habitat and size.

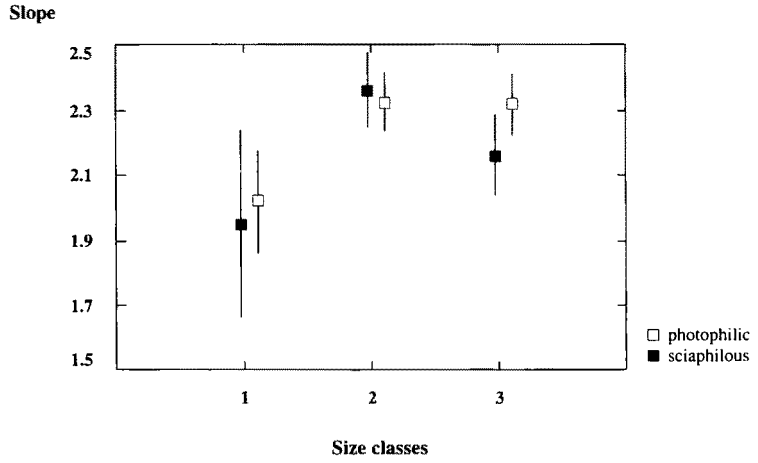


FIG. 4. Mean (±SE) values of the slope of the toxicity function according to habitat and size.

TABLE 3. PAIRED-SAMPLE *t* TEST ANALYSES OF SLOPE OF TOXICITY FUNCTION: INNER VS. PERIPHERY VALUES GROUPED BY HABITAT AND SIZE; OVERALL *t* TEST ANALYSIS ALSO SHOWN

Habitat	Size	Mean differences	SD differences	*T*	*df*	*P*
Sciaphilous	Medium	−0.041	0.733	−0.169	8	0.869
	Large	0.114	0.664	0.515	8	0.620
Photophilic	Medium	0.146	0.755	0.611	9	0.556
	Large	−0.130	0.442	−0.933	9	0.375
Overall *t*-test		0.021	0.642	0.204	37	0.839

TABLE 4. RESULTS OF TWO-WAY ANOVA OF SLOPE OF TOXICITY FUNCTION

Source	SS	*df*	MS	*F*	*P*
Habitat	45.369	1	45.369	0.159	0.691
Size	1151.225	2	575.612	2.022	0.142
Habitat × size	289.501	2	144.750	0.508	0.604
Error	14797.075	52	284.559		

1989). It was suggested in these studies that such differences were related to the chance of being attacked by herbivores, since chemical defenses were greater in those parts that lacked morphological defenses and/or were at a greatest risk or herbivory. No predators are known for *C. crambe*, but a comparable pattern against competitors for space could be expected. Although the sponges investigated did not present these expected variations and seemed to respond as a whole, unpublished results by the authors show that intraspecimen variation can be found in other seasons, corresponding to periods in which competition with neighbors is more intense.

Although changes in toxic composition have been reported for this species, and Berlinck et al. (1992) pointed out that the relative proportion of active substances in *C. crambe* changed in their samples, we did not find "qualitative" differences in toxic behavior in our study (measured as the slope of the toxicity function), indirectly suggesting that the composition of the toxic compounds remains fairly constant among levels of the factors investigated. Harvell et al. (1993) reported quantitative and qualitative differences in the chemical defenses of gorgonian coral. They provided evidence that qualitative composition was fixed and depended on geographic zone, while levels (quantity) of defenses were environmentally modulated, which is in good agreement with the results obtained in our study.

Interpretation of the quantitative differences found for toxicity (TU values) is based on correlational evidence, but under the assumption that the production of chemical defense compounds is metabolically costly, investment in defensive substances must be optimized with respect to the organisms' needs and to the other terms of the energy budget. This is the basis of the optimal defense theory (Rhoades, 1979; Coley et al., 1985; Fagerström et al. 1987; Skogsmyr and Fagerström, 1992), and from this perspective some explanations can be proposed.

Between-habitat differences must be interpreted as a function of the parameters changing from one wall to another. Water temperature, amount of food, and most abiotic parameters can be recognized as being comparable on the two walls. Light incidence, on the other hand, is clearly different between them. As no photosynthetic symbionts were observed in the many sponges studied by electron microscopy (authors' unpublished results), a direct effect of light on this species is not likely. The biotic interactions, on the other hand, were clearly different since diverse communities developed in both sites studied, as a consequence of the different amount of light received. The space is saturated in the shaded habitat, whereas patches of bar substratum are frequently produced in the well-illuminated habitat. Moreover, slow-growing, surface-dependent invertebrates or calcareous, encrusting algae are the dominant forms in the dark habitat, while the photophilic community was dominated by erect, soft seaweeds whose abundance changed markedly with season. The higher turnover of the

patches of bare substratum in this last habitat should favor investment in rapid growth when the conditions are favorable rather than in skeletal or chemical defenses (Jackson, 1977; Russ, 1982).

In contrast, the latter mechanisms should prevail in the shaded, space-saturated habitat. This hypothesis is supported by the different growth shape (Becerro et al., 1994b) and resource allocation (Uriz et al., 1995) of *C. crambe* in these two habitats. As a whole, therefore, we interpret the higher toxicity in the shaded wall as a result of differential strategies of space competition. The evolution of chemical defense strategies in marine organisms responds to highly diverse pressures (Bakus et al., 1986; Davis et al. 1989; Wahl, 1989), and more experimental work is needed to confirm the underlying process determining the pattern observed in this study. In particular, predation or fouling pressures should be considered, although they do not seem relevant in this case, as no predators or foulers have been observed in several years of study of this species in different localities and habitats.

Between-size differences can also be interpreted as a function of differential needs at each size stage. Encounters with space competitors are clearly a function of size, so a higher production of toxic substances with size was expected in principle. On the other hand, changes in toxic production with size may be tied more to internal, physiological parameters than to external factors. Although size and age are largely decoupled in modular, indeterminate-growing organisms (Sebens, 1987; Turon and Becerro, 1992), it is nevertheless expected that small sponges invest more in somatic growth and space acquisition, leaving fewer resources available for chemical defense.

The general pattern of variation found for toxicity values in Figure 3 points to an increase in toxicity from small to medium-sized specimens in both habitats, while the values remained constant (photophilic sponges) or decreased (sciaphilous specimens) from medium-sized to larger forms. In fact, if we restrict our analysis to the sciaphilous sponges and perform a Ryan's Q test among the three size classes, we find that the medium-sized specimens are significantly more toxic than the other two size classes. The lower toxicity displayed by the smallest and largest sponges can be due to the fact that they devote energy preferentially to growth and reproduction, respectively (an increased investment in reproduction with size has been found in this species; Uriz et al., 1995). Moreover, a more favorable area–perimeter relationship in large sponges may result in a lower energetic demand for defense interactions along the border (Adler and Harvell, 1993).

The values for the slope featured largely the same general pattern as the toxicity values (Figures 3 and 4). However, their large standard errors prevented significant differences from showing up in the analyses (possibly a sample size larger than 10 specimens per size and habitat would have been necessary). In fact, a Spearman correlation coefficient between the mean values of TU and

slope per habitat and size showed that both were significantly correlated (r_s = 0.942, $N = 6$, $P < 0.001$).

In addition, *C. crambe* features highly effective toxins. The toxins are active at very small concentrations (Martin and Uriz, 1993; Becerro, 1994), and toxicity is rapidly enhanced by small increments in the toxin concentration, as indicated by the global values of the slope of the toxicity function (2.23 ± 0.05, mean ± SE). With such a slope, a 45% increase in toxin concentration would produce an approximately twofold toxicity increase. Both characteristics fit well with the hypothesis of a basal investment in toxic production and an ability for a precise regulation of the toxicity as a response to environmental pressures.

In conclusion, we did not find differences in toxicity between the center and the periphery of the sponges, although this study demonstrated that habitat and size exerted an influence in the levels of toxins encountered. The factors underlying the variation encountered in this study remain unclear, although the evidence suggests that space competition may be a key factor modulating toxin production. In this regard, Maida et al. (1993) also found higher levels of a molecule involved in spatial competition in specimens of the soft coral *Sinularia flexibilis* from a highly diverse and competitive site. The results of the present study also point to a chemically mediated interference competition, with higher toxicity in the sponges dwelling in the more space-saturated habitat. Whether this pattern of variation is followed by other chemically defended organisms is unknown. More research is needed to understand the processes modulating the production of chemical defenses and investment in biological parameters before an integrating perspective can be formulated.

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N,N-DIMETHYLURACIL AND ACTINIDINE, TWO PHEROMONES OF THE PONERINE ANT *Megaponera foetens* (FAB.) (HYMENOPTERA: FORMICIDAE)[1]

EDELGARD JANSSEN,[2] HANS J. BESTMANN,[2,*] BERT HÖLLDOBLER,[3] and FRIEDRICH KERN[2]

[2]*Institut für Organische Chemie der Friedrich-Alexander-Universität Erlangen-Nürnberg, Henkestr. 42, D-91054 Erlangen, Germany*

[3]*Theodor Boveri Institut, Lehrstuhl für Verhaltensphysiologie und Soziobiologie der Universität Würzburg Am Hubland, D-97074 Würzburg, Germany*

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Abstract—By means of gas chromatographic, mass spectrometric, and micropreparative methods *N,N*-dimethyluracil and actinidine were identified as two ethologically active compounds in the ponerine ant *Megaponera foetens*. The synthesized poison gland compound *N,N*-dimethyluracil released trail-following behavior, whereas actinidine, found in the pygidial gland, stimulated ants to leave the nest, possibly in an alarm reaction. Biological activity of the synthetic samples were confirmed by behavioral experiments and electroantennogram responses with worker antennae.

Key Words—*Megaponera foetens*, Formicidae, poison gland, pygidial gland, trail pheromone, recruitment pheromone, *N,N*-dimethyluracil, 1,3-dimethyl-2,4(1*H*,3*H*)-pyrimidinedione, actinidine, electroantennogram.

INTRODUCTION

Colonies of the African ponerine ant *Megaponera foetens* (Fab.) (Hymenoptera: Formicidae) conduct highly organized predatory raids on termites (Lèvieux, 1966). The organization of this group foraging behavior has been analyzed in great detail by Longhurst et al. (1978, 1979) and by Lepage (1981). According

*To whom correspondence should be addressed.

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to Longhurst et al. (1979), the major source of the trail pheromone involved in group raiding in *M. foetens* is the poison gland. Recently Hölldobler et al. (1994) discovered a second component of the chemical recruitment process that originates from the pygidial gland. The latter secretions have a more powerful recruitment effect, whereas poison gland secretions contain a much longer-lasting orientation cue.

In the present paper we report on the chemical identification of a trail pheromone compound from the poison gland and an alerting pheromone from the pygidial gland in *Megaponera foetens*.

METHODS AND MATERIALS

Ants. Nine colonies of *Megaponera foetens* were excavated in the Comoé National Park, Ivory Coast, and used for laboratory experiments in Würzburg and Erlangen, Germany. The ants were housed and maintained in laboratory nests similar to the procedures described in Hölldobler et al. (1994).

For the chemical analyses of the glandular secretions, poison glands or pygidial glands, respectively, were dissected under water after the ants had been killed by placing them for a few minutes in a freezer. In order to extract glandular secretions, 10 organs of a kind were kept in 100 μl solvent in a freezer for at least 24 hr. For solid injection, the dissected glands were inserted into small boron silicate tubes that were subsequently sealed.

Gas Chromatography. Gas chromatography was carried out on a Hewlett-Packard 5890 instrument fitted with a 25-m × 0.22-mm fused-silica capillary column coated with SE 30. The oven was held at 60°C for 4 min and programmed at 6°C/min to 260°C.

Micropreparative Gas Chromatography. Micropreparative gas chromatography was performed on a United Technologies Packard 438A instrument fitted with a 2-m × 4-mm glass column packed with SE 52. The effluent was split 99:1 (trap–FID) by an all-glass splitter. Gland extracts were injected and different fractions were trapped in metal U-tubes cooled in Dry Ice (Attygalle and Morgan, 1988). The material trapped was washed out with hexane and directly used for bioassays.

Gas Chromatography–Mass Spectrometry. The identification of the major components was achieved by GC-MS on a Finnigan MAT 90 instrument. A fused-silica capillary column (SE 52, 25 m × 0.22 mm) was directly coupled to the mass spectrometer (GC-MS). Helium was used as the carrier gas at a flow rate of 1 ml/min. The 70-eV EI spectra were recorded at a rate of 1 sec/scan. The samples were introduced by a solid injection method (Morgan and Wadhams, 1972).

Quantification. A solution of authentic *N,N*-dimethyluracil was used as an external standard for quantification.

Microreactions. For reduction with $LiAlH_4$ six poison glands extracted in 100 μl dry ether were treated with $LiAlH_4$. After the gas generation had stopped, water was added. Then the organic layer was separated and used for bioassays.

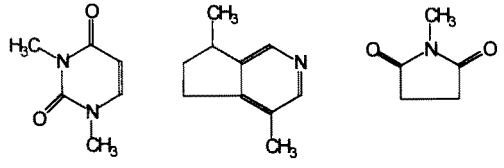
For catalytic hydrogenation, 200 μl of poison gland extract (dry in diethyl ether) was placed in a glass tube. A small portion of a blend of Pd (5%) and carbon powder was added, and the open tube was placed in a two-neck flask with an inlet tube. After the air inside the flask was removed, hydrogen was added until pressure was built up. The mixture was allowed to stand for 6 hr, then it was filtered and used directly for bioassays.

For silyl derivatization, 10 poison glands were extracted in 10 μl ether and mixed with 1 μl of *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA). The solution was kept in a syringe for 10 min, and the mixture was freed from (MSTFA) by micropreparative GC and directly used for bioassay.

Synthesis. The synthesis of actinidine (**9,** Figure 1) was performed by a previously described method starting with citronellal (Fluka) via iridodial (Clark et al., 1959). In the last step, a 25% NH_3 solution was added to iridodial, which resulted in a stereoisomeric mixture of actinidine. Dimethyluracil (**1,** Figure 1) was obtained from uracil treated with methyl sulfate (Davidson and Baudisch, 1926). Authentic samples of *N*-methylsuccinimide (**4,** Figure 1), succinimide, and benzaldehyde were purchased from Aldrich.

Electroantennogram. Electrophysiological activity of the synthetic substances was investigated by a previously described electroantennogram method for Lepidoptera (Schneider, 1957), modified for smaller ant antennae (Kern and Bestmann, 1993). Briefly, the recording electrode was inserted into the distal segment of a worker antenna and the indifferent electrode was inserted into the head capsule near the antenna base after puncturing it with a sharpened tungsten needle. The test odorants, pipetted onto filter paper, in quantities ranging from 0.1 μl to 1 mg, were presented by a 1-sec air pulse led over the filter paper towards the antenna.

Bioassays. For bioassays, groups of approximately 300 workers were housed in shallow plaster nests that were covered by red glass plates (10 × 25 cm). Each nest was placed at one side of an arena (30 × 65 cm) so that the ants had direct access to the foraging area without having to cross a connecting



1 9 3

FIG. 1. *N,N*-dimethyluracil, (**9**) actinidine, and (**4**) *N*-methylsuccinimide.

bridge or to pass a plastic tube to get into the arena, as in Hölldobler and Wilson (1994, p. 216).

We tested the trail-following response of *M. foetens* workers to artificial trails drawn with the chemically identified substances. The trails were laid with a microsyringe along a 40-cm-long pencil line drawn on sheets of paper that were subsequently placed into the arena. As a control, a second trail was offered simultaneously using the pure solvent.

Both test and control trails started at the nest at the same point (0.5 cm diam.), but diverged at an angle of approximately 40°. All ants crossing a target line at the end of the artificial trails were counted. Each test started when the paper on which the trails were drawn was placed into the arena and lasted 1 min.

Additional details concerning the behavioral tests are given in the Results section.

RESULTS

As has been shown by Longhurst et al. (1979) and Hölldobler et al. (1994), poison gland secretions readily elicit trail-following behavior in *M. foetens*. For the first characterization of the trail pheromone we employed microreactions of the poison gland extracts.

Microreactions. The trail-following response of ants was tested with trails drawn with treated samples, and for comparison we also used untreated glandular extracts or pure solvent as control. A reduced activity was observed after reduction with $LiAlH_4$ and catalytic hydrogenation with Pd. This result points to a carbonyl group and at least one double bond in the pheromone structure. Other microreactions, such as silylation, did not affect trail following.

By micropreparative GC the poison gland extract was divided into different fractions, each of which was tested to determine whether or not it released a trail-following response in *M. foetens* workers. Only one fraction, with a retention time between 16 and 19 min was effective (Figure 2). The volatile poison gland components of this biologically active fraction were analyzed by GC-MS.

Similar microreactions with pygidial gland extracts were not possible, because the trail-following effect of these secretions vanished after a few minutes. We assume that in contrast to poison gland products, the biologically active components of the pygidial gland that release trail-following behavior are very volatile substances, which decompose during exposure to air or chemical reaction.

Mass Spectrometry. The chromatogram obtained from the contents of five *M. foetens* poison glands showed one major and at least five minor peaks (Figure 2). The major component **1** at a retention time of 18.25 min presented a mass

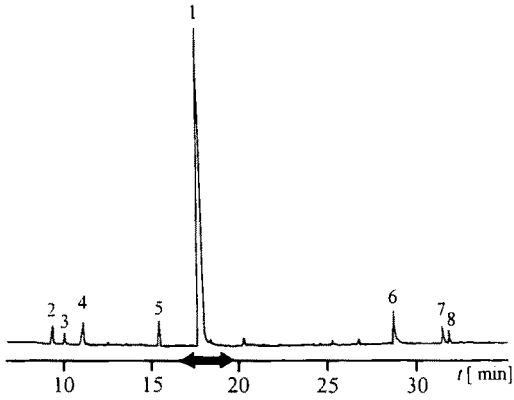


FIG. 2. A partial, reconstructed gas chromatogram obtained by solid sampling GC-MS analysis of poison gland volatiles from five *Megaponera foetens* workers: (1) *N,N*-dimethyluracil, (2) succinimide, (3) unknown, (4) *N*-methylsuccinimide, (5) indole, (6) hexadecanoic acid, (7) unknown, and (8) octadecanoic acid. The thick, double-headed arrow indicates the region corresponding to the GC effluent fraction that elicited trail-following behavior.

spectrum (Figure 3) that was congruent with that reported for *N,N*-dimethyluracil. A comparison of retention times between the natural product and synthetic *N,N*-dimethyluracil verified the structure.

The minor peaks in Figure 2 were identified as succinimide **2** m/z 99 (M^+, 100%), unknown compound **3,** *N*-methylsuccinimide **4** m/z 113 (M^+, 100%), indole **5** m/z 117 (M^+, 100%), hexadecanoic acid **6,** unknown compound **7,** and octadecanoic acid **8.**

GC-MS analysis of the contents of 15 pygidial glands of *M. foetens* demonstrated the presence of actinidine **9** (Figure 1) and benzaldehyde, as well as alkanes and alkenes. The mass spectrum of actinidine exhibited a molecular ion at m/z 147 (M^+, 65%), 132 (100), 117 (25), 103 (3), 91 (2), 77 (5), 65 (3), 51 (2), 38 (4) and matched with that of (+)-actinidine in the literature (Tomalski et al., 1987), and that of our synthetic product.

Electroantennogram. Preliminary EAG measurements with synthetic samples of the poison gland compounds *N*-methylsuccinimide, succinimide, hexadecanoic, and octadecanoic acid as well as the pygidial gland compound benzaldehyde showed no significant differences in comparison to a control stimulus with pure air. In contrast, *M. foetens* worker antennae responded significantly higher to both test compounds dimethyluracil **1** and actinidine **9** in comparison to a control of pure air (Figure 4). Among the test substances, actinidine elicited the highest electrophysiological responses of 1.5 mV at the given maximum

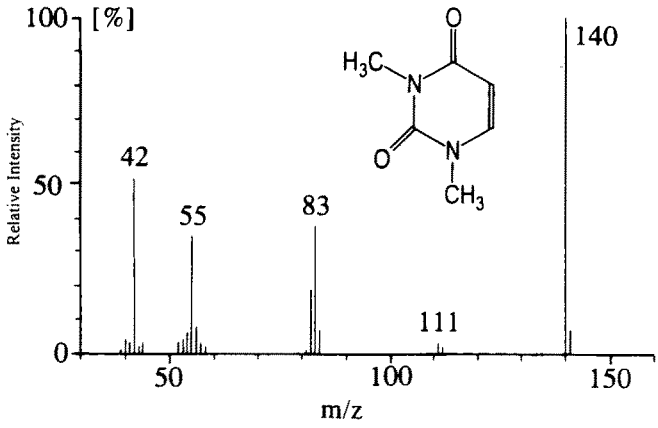


FIG. 3. Mass spectrum of *N,N*-dimethyluracil, **1**, 1,3-dimethyl-2,4(1*H*,3*H*)-pyrimidinedione.

dose of 1 mg applied to the filter paper source, whereas stimuli with dimethyluracil generated responses of 1 mV within the same concentration range.

Bioassays. The behavioral tests clearly demonstrated that dimethyluracil, which derives from the poison gland, represents a major chemical orienting

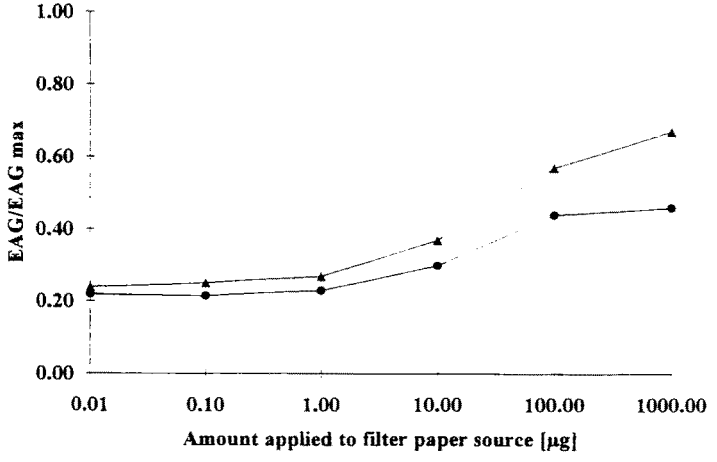


FIG. 4. Dose-response curves constructed from EAG measurements with *M. foetens* worker antennae. Responses from synthetic actinidine (closed triangles) and dimethyluracil (closed circles) are presented. Each point represents the mean of five replicates.

signal in *Megaponera* trails. One poison gland contains approximately 40 ng of dimethyluracil. This value, which was quantified from GC peak areas, showed, however, a significant variation from ant to ant. In a first series of tests, we applied 10 μl of a solution of 10 μg dimethyluracil per 1 ml hexane on a 40-cm-long trail. As a control we simultaneously offered trails drawn with 10 μl pure hexane. From a total of 12 tests, conducted with four different worker groups, 20.9 ± 7.7 ($X \pm$ SD) ants followed the dimethyluracil trail, and none followed the hexane trail. In this series we applied 100 ng dimethyluracil on each trail. In a second series we used only 1 ng of the trail pheromone (1 μl from 1 μg/ml solution) and still elicited a trail-following behavior, although the response was much weaker (7.3 ± 4.5).

Although the trail-following response to a higher concentration of the pheromone is obvious, it does not appear that dimethyluracil is also the main recruitment signal that elicits the mass exodus of workers during a group raid. We therefore focused on the pygidial gland contents, which have previously been shown to release a powerful recruitment response in *M. foetens* (Hölldobler et al., 1994). One of the pygidial gland compounds we identified is actinidine. Each gland contains approximately 10 ng of this substance. Artificial trails laid with 1 gland equivalents or 100 gland equivalents of actinidine did not elicit clear trail-following behavior. The ants run along the trails through short distances (approx. 1–4 cm), but never followed the whole 40-cm-long trail in a straightforward fashion, as they do along the dimethyluracil trails. It was obvious, however, that the ants increased their locomotory activities when actinidine was presented at the nest entrance, and the number of ants leaving the nest increased significantly in such situations.

This response was quantified in the following way: A hardwood applicator stick was dipped approximately 5 mm deep into the actinidine solution (1 mg/ml), the solvent allowed to evaporate 20 sec, and this contaminated tip of the applicator was inserted approximately 2 cm deep into the nest. Simultaneously, a second applicator, which had been dipped into the solvent hexane, was dried for 20 sec and inserted in the same way through another nest opening, 12 cm away from the first one. In subsequent tests the openings where the respective applicators were presented were alternated, and at least 15 min elapsed between each experiment. We then counted all ants leaving the nests through one or the other exit during 1-min intervals. In a total of 10 tests, 31.1 ± 18.2 ants left the nest at the actinidine exit and 11.6 ± 5.7 ants emerged at the control exit ($Z = 2.54$, $P = 0.011$; Mann-Whitney U test).

These results suggest that actinidine stimulates *Megaponera* workers to run out of the nest, possibly exhibiting alarm behavior. Next we investigated whether actinidine can enhance the trail-following response along dimethyluracil trails. We simultaneously presented trails drawn with 10 μl of the solution of 10 μg dimethyluracil/ml solvent plus 10 μl of the solution of 10 μg actinidine/ml

solvent and trails drawn with 10 μl of the dimethyl uracil solution alone. Unfortunately the results are inconclusive: In a total of eight tests, 14.2 $\pm$ 4.6 ants followed the combined trail, and 10.3 $\pm$ 3.4 ants followed the pure dimethyluracil trail. The difference ($Z = 1.94$, $P = 0.052$, Mann-Whitney U test) is not significant. Finally, we conducted tests with artificial trails drawn with pygidial gland extracts applying approximately 1 gland equivalent on each trail. We obtained the same results as previously reported to Hölldobler et al. (1994). In this case the ants readily responded by leaving the nest and following the trails along their entire length. This shows us that actinidine is not the effective recruitment and trail pheromone contained in the pygidial gland.

DISCUSSION

It has previously been demonstrated that group raiding in the termite-hunting ponerine *Megaponera foetens* is organized by complex chemical communication, involving at least two exocrine glands, the poison gland and the pygidial gland (Hölldobler et al., 1994). The current investigation focused on the chemical identification of pheromones involved in the release of trail-following and recruitment behavior. We were able to identify dimethyl uracil in the poison secretions and demonstrated that this substance serves as a trail pheromone. This is, to our knowledge, the first identification of a pyrimidine derivative in the secretions of an exocrine gland in ants, and it also comprises a hitherto unknown trail pheromone substance.

Although we were unable to identify the recruitment and trail pheromones that derive from the pygidial gland, we found actinidine in these glandular secretions. This alkaloid has been discovered previously in the pygidial gland of several genera of the subfamily Dolichoderinae, such as *Conomyrma* (Wheeler et al., 1977), *Monacis* (Blum and Wheeler, 1978), *Iridomyrmex* (Cavill et al., 1982, 1984), and *Tapinoma* (Tomalski et al., 1987), where it may function as a defensive secretion. It has not been previously found in species of the subfamily Ponerinae, where we now identified it in the pygidial gland secretion of *Megaponera foetens*. Actinidine elicits a repellent reaction in other ant species (see Tomalski et al., 1987), but in *Megaponera* it also stimulates workers to leave the nest, possibly in an alarm reaction. We failed, however, to demonstrate its involvement in recruitment and trail-following behavior. Recruitment and trail following can be released with untreated pygidial gland secretion.

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BEHAVIOR OF PROCESSIONARY MALES (*Thaumetopoea pityocampa*) INDUCED BY SEX PHEROMONE AND ANALOGS IN A WIND TUNNEL

CARMEN QUERO, FRANCISCO CAMPS, and ANGEL GUERRERO*

Department of Biological Organic Chemistry, C.I.D. (CSIC)
Jordi Girona, 18-26, 08034-Barcelona, Spain

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Abstract—The behavioral response of processionary males (*Thaumetopoea pityocampa*) to the natural pheromone (*Z*)-13-hexadecen-11-ynyl acetate (**1**) and structurally related analogs in a wind tunnel is presented. Stereomerically pure *Z*-**1** and a mixture with the *E* isomer in 80:20 ratio elicited similar attraction responses at 1 μg and higher. The activity was dose-dependent, being optimum at 1 μg with 90% and 80% of males contacting with the source in the presence of the *Z*-**1** and *Z*/*E*-**1**, respectively. 11-Hexadecynyl acetate (**2**) functioned as a pheromone mimic, being able to induce the complete mate-finding behavioral sequence, although its activity was much lower than that of the pheromone. (*Z*)-13-Hexadecen-11-ynyl alcohol (**3**) and, particularly, (*Z*)-13-hexadecen-11-ynal (**4**) were potent inhibitors of the upwind flight response in mixtures with *Z*-**1** in 99:1, 95:5, and 91:9 ratios. (*Z*)-1,1,1-Trifluoro-16-nonadecen-14-yn-2-one (**5**) also inhibited the response of males to pheromone, particularly in the source contact behavior. Comparison with activity displayed by analogs in field tests is also reported.

Key Words—Sex pheromone, processionary moth, *Thaumetopoea pityocampa*, Lepidoptera, Thaumetopoeidae, behavior, wind tunnel.

INTRODUCTION

The pine processionary moth *Thaumetopoea pityocampa* (Lepidoptera, Thaumetopoeidae) is one of the major defoliator pine pests in southern Europe and North Africa. Some other countries of Central Europe (Germany, Switzerland, Hungary, Bulgaria, Yugoslavia, etc.) may also suffer scattered attacks. The

*To whom correspondence should be addressed.

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moth is especially active against all species of pines growing below 1500 m altitudes but can also attack other conifers, such as cedars (Montoya and Hernández, 1991). Moreover, last-instar larvae may liberate microscopic urticating hairs, which can cause severe irritation to the skin and mucous membrane, often leading to strong allergic reactions in humans.

Only one compound has so far been found in the female sex pheromone gland and characterized as (*Z*)-13-hexadecen-11-ynyl acetate (Guerrero et al., 1981). This is the first and at present only structure with an enyne functionality as a constituent of the sex pheromone of a moth. Since its identification and synthesis (Camps et al., 1981a,b, 1983; Michelot et al., 1982), the compound has been found to display a remarkable attractant activity for males in the field (Cuevas et al., 1983; Einhorn et al., 1983).

Wind tunnels have been widely recognized as an effective technique to study orientation mechanisms of flying insects to their sex pheromone components (Cardé, 1984; Mafra-Neto and Cardé, 1994; Miller and Roelofs, 1978) as well as the inhibitory effect elicited by specific synthetic chemicals (Preiss and Priesner, 1988). Continuing our efforts directed towards development of inhibitors of the pheromone action both in the laboratory and in the field (Camps et al., 1988, 1990a,b; Parrilla and Guerrero, 1994), we now report on behavioral effects induced by the natural pheromone and a number of structurally related analogs (**2–5**, Figure 1) on processionary males in a wind tunnel. Although some of the compounds have been tested in the field (Cuevas et al., 1983; Camps et al., 1988), the activity elicited by these compounds at every stage of the mate-finding sequence has not been reported. Knowledge of the behavioral effects elicited by analogs as inhibitors, mimics, or synergists is important in order to gain better insight into their mode of action and, consequently, to develop new compounds with potential use in pest control (Albans et al., 1984; Camps et al., 1990a,b; Schwarz et al., 1990). In all cases the presence of the triple bond has been preserved since it was found by us to be crucial for bio-

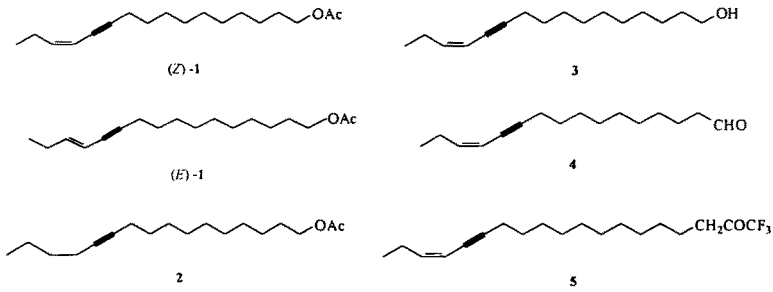

FIG. 1. Structure of the pheromone, *Z*-**1** and analogs tested.

logical activity. Trifluoromethyl ketones have been found to be strong inhibitors of esterases, particularly antennal esterases in insects (Prestwich, 1987; Durán et al., 1993), due to formation of hemiketals with a serine residue present at the active site of the enzyme (Linderman et al., 1988). In this regard, we have included trifluoromethyl ketone, **5**, which was assayed in two different types of experiments, i.e., in mixtures with the natural pheromone and after presaturation of the antennal receptors with vapors of the chemical.

METHODS AND MATERIALS

Chemicals. Alcohol **3** was prepared by stereospecific coupling of (*Z*)-1-bromo-1-butene with 2-(11-dodecynyloxy)tetrahydropyran catalyzed by tetrakis(triphenylphosphine)-palladium(0) as previously described (Michelot et al., 1982). The stereochemical purity was *Z:E* 98:2 on a SE-54 50 m × 0.32-mm-ID fused silica capillary column. Natural pheromone *Z*-**1** was obtained by acetylation of alcohol **3** under standard conditions (acetic anhydride/pyridine). Acetate **2** was prepared by alkylation of the corresponding acetylide with *n*-butyl bromide, and aldehyde **4** by oxidation of the corresponding alcohol **3** with pyridinium dichromate (Corey and Schmidt, 1979). Stereomeric mixture of *Z/E*-**1** 80:20 was obtained by reaction of the acetylide of 2-(11-dodecynyl-oxy)tetrahydropyran with 1,2-epoxybutane followed by tosylation of the resulting alcohol and dehydrotosylation (Camps et al., 1987). Trifluoromethyl ketone **5** was previously prepared in our laboratory (Parrilla et al., 1994). All the chemicals were purified by eluting them after column chromatography with hexane–ether or hexane–ethyl acetate mixtures and were homogeneous by TLC, NMR, and GC analyses.

Insects. Thaumetopoea pityocampa pupae were collected in an infested pine forest in Mora de Rubielos (Teruel) in the springs of 1992–1994, sexed, covered with a 3 to 6-cm layer of sawdust and sent to our department. They were kept in wooden boxes in a L16:8D reversed photoperiod at 23 ± 1°C and 55–65% humidity until emergence. Several toothpicks were placed standing in the sawdust to favor perching of males after emergence. In preliminary assays, we noted that reversal of the photoperiod to carry out the bioassays during working hours did not affect male behavior. Due to the short lifetime of the insects, and since young males responded better than the old ones in the wind tunnel, only insects 8–20 hr of age were used in the bioassays.

Wind Tunnel. The tests were carried out in a wind tunnel made of glass of dimensions 180 × 55 × 50 cm. The frame of the tunnel was made of aluminum with access gained by two sliding doors, each one covering one half of the tunnel. Air was pushed through by a centrifugal fan and pulled out of the building with the aid of an exhaust blower. The fan and blower worked simul-

taneously and their speed was carefully regulated by a potentiometer to avoid undesired turbulence in the moving air. The airstream was cleansed through a 2-cm-thick glass-wool bed and conducted through two nylon screens to smooth the air flow and get a laminar regime throughout the tunnel. A 58-W red fluorescent light located 16 cm above the tunnel provided the required illumination, which was dropped to 3–5 lux by covering the top of the tunnel with red cellophane paper. A white board containing irregular-shaped, dark brown spots was placed on the floor of the tunnel since stripped patterns may induce unexpected effects on the orientation of flying insects (David, 1982). The airspeed, measured at the center of the flight compartment, was 45 cm/sec. Two types of tests were conducted: one to study the behavioral effects induced by the test compounds alone and in combination with the natural pheromone, and one to study the inhibition response to the pheromone by presaturation of the antennal receptors with vapors of the trifluoromethyl ketone **5.**

Behavioral Effects Induced by Test Compounds 1–5. Newly emerged moths perched on the toothpicks were separated from the remaining pupae every day and placed into 31 × 12 × 21-cm plastic boxes containing a 2-cm layer of sawdust. The insects were allowed to acclimate to the tunnel conditions for 1 hr before they were tested 3–5 hr into the scotophase. The temperature of the experimental room was 22 ± 1°C and the relative humidity 60 ± 5%. The toothpicks supporting one to two males were carefully taken out, fixed on a polyurethane block, and placed into the tunnel on a stainless steel jack at 150 cm downwind from the source. The fan and blower were turned on and the males allowed to respond for 5 min. Moths were utilized only once and then discarded. The compounds were dissolved in nanograde hexane and the required volume to achieve the test doses (0–100 μg) was applied to a 1.5 × 1-cm cotton wick. The solvent was allowed to evaporate and the lure suspended 18 cm from the top and 40 cm from the closer screen of the tunnel. Solutions containing mixtures of *Z*-**1** and analogs in the specified ratios were prepared in hexane, and the required volume to have 1 μg of *Z*-**1** was taken. Solutions were prepared daily, just prior to the experiments, and the wicks discarded after use. Males were scored according to the following behaviors: wing fanning and taking flight (TF), arrival to the middle of the tunnel (65 cm, HW), close approach to the lure (ca. 10 cm, CA), and contact with the source (SC). Only those males arrested at the source for a minimum period of 5 sec were recorded as SC. The effect of a specific formulation was calculated as the percentage of males showing a particular behavior relative to the total number of insects released. Generally, 10–15 insects were assayed per day of experiment, with 20–30 males the total number of males considered for each treatment. Data obtained on different occasions corresponding to the same treatment were not significantly different, so they were combined for statistical analysis using a chi-square 2 × 2 test of independence (Sokal and Rohlf, 1969). Responses of insects of indi-

vidual compounds, i.e., *Z*-**1**, *Z*/*E*-**1** and -**2** were compared at specific doses, while responses to mixtures of **2**, **3**, **4**, and **5** with *Z*-**1** were compared with respect to specific behaviors. Bars in Figures 2–6 (below) bearing the same letter or lacking letters are not significantly different at $P < 0.05$.

Bioassays with Preexposure of Vapors of Ketone **5**. One-day-old individual insects were placed in a 12-cm-diameter Petri dish containing a 2 × 2-cm piece of filter paper on which 1 and 10 μg of trifluoromethyl ketone **5** in hexane had been absorbed. The solvent was evaporated before the assay. Care was taken to prevent the insects from directly contacting the compound. The moths were exposed to vapors of the chemical in the dark for 4 hr, then taken out, and, after 5 min of acclimation to the tunnel conditions, assayed for behavior. The same protocol was applied to control insects except that the filter paper was only treated with hexane.

RESULTS AND DISCUSSION

Male Responses to Natural Pheromone **1** *and Stereochemical Mixture* Z/E-*1*. Responses of males to the natural pheromone *Z*-**1** and the stereomeric mixture *Z*/*E*-**1** in 80:2 ratio were studied under different dosages (0.5–100 μg) in order to establish the role of the *E* isomer on the different behaviors of the sequence (Figure 2). A very high number of males (90–100%) responded to both lures at the early stages of the sequence (TF and HW), from which ca. 60–90% were able to establish contact with the source. The response of males displaying SC behavior was dose-dependent, being highest at a 1-μg dose of both compounds, with 90% of males landing at the source in presence of the *Z* isomer and 80% when the lure contained 20% of the *E* isomer. At a lower dose (0.5 μg), this mixture also evoked the complete set of behaviors, but with a lower number of males responding. Thus, in the presence of natural pheromone, the number of insects contacting the source was 70%, while this number decreased to 35% when the *E* isomer was also present in the lure. Diminution of responses was not significant, however ($P < 0.05$). In preliminary field tests carried out in 1983 on a very low population area, we had noted a significant decrease in catches when mixtures of *Z*/*E* were used (15.7, 6.8, and 8.9 males per trap attracted to *Z*-**1**, *Z*/*E*-**1** 97:3 and *Z*/*E*-**1** 90:10, respectively) (Cuevas et al., 1983). Our behavioral assays in the tunnel suggest, however, that the *E* isomer is behaviorally neutral when mixed with the natural pheromone. This assumption appears to be consistent with more recent ongoing trials in a heavier infested area (over 220 males per trap on average caught by the *Z* isomer), which showed a slight but nonsignificant decrease of males caught in lures treated with *Z*/*E* mixtures of different ratios relative to the catches with pure *Z*.

Male Response to Acetylene **2**. When acetylenic analog **2** was tested at

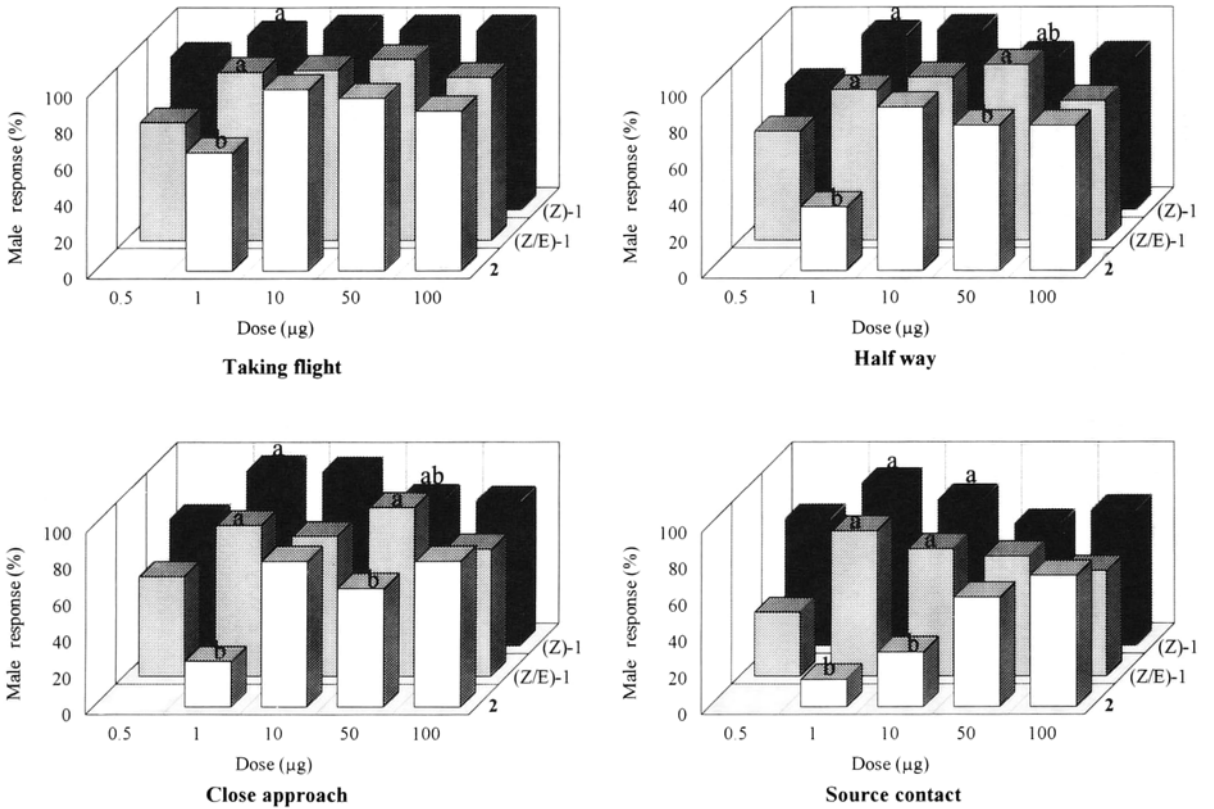

FIG. 2. Percentage of males responding to different dosages of natural pheromone *Z*-**1**, stereomeric mixture *Z*:*E*-**1** 80:2, and acetylene **2** in a wind tunnel. Bars containing different letters represent values significantly different using a chi-square 2 × 2 test of independence ($P < 0.05$). Bars with no letters represent values that are not statistically

different concentrations (1–100 μg) in comparison to the sex pheromone, moths successfully completed the mate finding sequence. However, high doses of the analog were required to elicit all behaviors, particularly SC (Figure 2). In general, a minimum dose of 10 μg evoked arriving to the proximity of the lure by 80% of the insects, although only 30% of males were arrested at the source. Higher doses (50–100 μg) were needed to elicit SC behavior by 60–72% of males (Figure 2). In order to test the effect of the analog **2** in the presence of the natural material, increasing amounts of the analog were added to 1 μg of the pheromone to obtain mixtures of **1** and **2** in 95:5 and 80:20 ratios. Unexpectedly, the results show a decrease in activity of the mixtures in comparison to that of the pheromone, particularly in the SC behavior. The numbers of males responding were, however, higher in both treatments than with acetylene **2** alone (Figure 3). From these results it can be inferred that acetylene **2** behaves as a pheromone mimic, being able to induce in males the complete mate-finding sequence, although its activity is much lower than that of the pheromone. This is in agreement with the results obtained in field tests where the number of catches using 1 mg of the analog were 65% relative to the number of males attracted to the pheromone (Camps et al., 1988), which is higher than we might expect from the behavioral data obtained in the wind tunnel. Similarly, in EAG bioassays, activity of the analog was 55–60% with regard to the pheromone (Gasol, 1988). The mimic character of the acetylene when tested alone, however, cannot explain the close-range and source-contact inhibition observed when the analog was mixed with the pheromone, and new studies at the electrophysiological level are needed to clarify this apparently contradictory point.

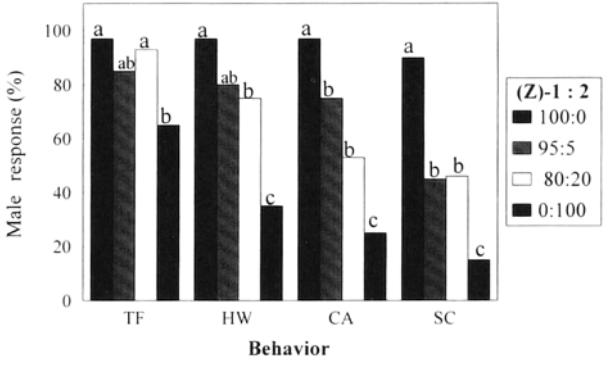

FIG. 3. Percentage of males responding to mixtures of natural pheromone *Z*-**1** and acetylene **2** in different ratios in a wind tunnel. Histograms with different letters are significantly different using a chi-square 2 × 2 test of independence ($P < 0.05$). TF, taking flight; HW, upwind flight and arrival to the middle of the tunnel; CA, close approach to the lure (ca. 10 cm); SC, source contact.

Male Responses to Alcohol 3 and Aldehyde 4. The effect of alcohol **3** was tested in **1** plus **3** mixtures in 99:1, 95:5, and 91:9 ratios. When the first two combinations containing 10 and 50 ng of **3** were tested, the number of males initiating upwind flight was very high (96–100%), but this number dropped to 70–80% in the HW and CA behaviors. However, almost all males that had successfully oriented completed the mate-finding sequence. When the analog was tested in the 91:9 mixture (100 ng of the analog), a strong inhibitory effect was noted in the first step of the sequence, i.e., TF, wherein only 30% of males responded. Moreover, although these males were able to sustain upwind flight to a point near the lure, none of them could land at the source (Figure 4). The notable drop in activity shown in this case, in comparison to that elicited when 50 ng of the analog was present, apparently suggests that the threshold inhibitory activity is somewhere between these two values. The inhibitory effect displayed by **3** is in agreement with the one elicited in field tests when mixed with the pheromone in 1:10, 1:1, and 10:1 ratios (Camps et al., 1988). A similar effect was found by Quartey and Coaker (1993) with (*Z*, *E*)-9,11-tetradecadienol, a third constituent of the sex pheromone of the tropical warehouse moth *Ephestia cautella*, which significantly inhibited attraction of males to conspecific females in wind tunnel. The alcohol also displayed a strong inhibitory effect in all behavioral steps when mixed with the corresponding dienic and monoenic acetates, the two main components of the pheromone. Schwarz et al. (1990) also reported the inhibitory activity of (*E*)- and (*Z*)-11-tetradecenol, the metabolic products of the sex pheromone of the *E* and *Z*-types of the European corn borer

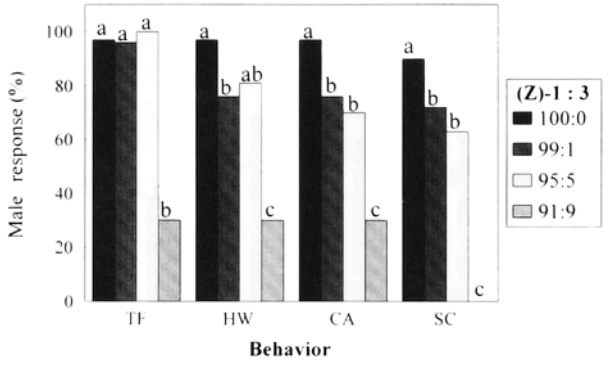

FIG. 4. Percentage of males responding to mixtures of natural pheromone *Z*-**1** and alcohol **3** in different ratios in a wind tunnel. Histograms with different letters are significantly different using a chi-square 2 × 2 test of independence ($P < 0.05$). TF, taking flight; HW, upwind flight and arrival to the middle of the tunnel; CA, close approach to the lure (ca. 10 cm); SC, source contact.

Ostrinia nubilalis, in the male upwind flight response when mixed with the natural pheromone in a very high ratio (3 μg of analog plus 100 ng of pheromone).

When aldehyde **4** was tested, the results were spectacular. The presence of 10 ng of the analog in 1 μg of pheromone was enough to evoke a complete inhibition of SC behavior, the number of males taking flight being only 25%. The effect induced was very similar to that elicited when used alone (Figure 5). This result was not surprising to us, however, since the analog had also behaved as a very potent inhibitor of the pheromone action in the field, lowering the number of catches by 90.4% in comparison with the pheromone, when mixed with the natural pheromone in a 1:10 ratio. This analog is the most potent inhibitor of the natural pheromone action in the laboratory and in the field ever found by us. Other saturated and unsaturated aldehydes have proven to be good inhibitors of the upwing flight response of the European corn borer males (Schwarz et al., 1990).

Male Response to Trifluoromethyl Ketone **5**. Trifluoromethyl ketones are potent inhibitors of a variety of serine esterases, including in some cases antennal esterases of insects (Prestwich, 1987; Durán et al., 1993), due to reaction with a serine residue at the active site of the enzyme. The inability to catabolize pheromone molecules may result in their accumulation at the receptor sites, which should disrupt the ability of the insect to perceive new incoming attractant molecules. Compound **5** is a structural analog of the sex pheromone and results from isosteric replacement of the acetate group by the trifluoroacetonyl moiety

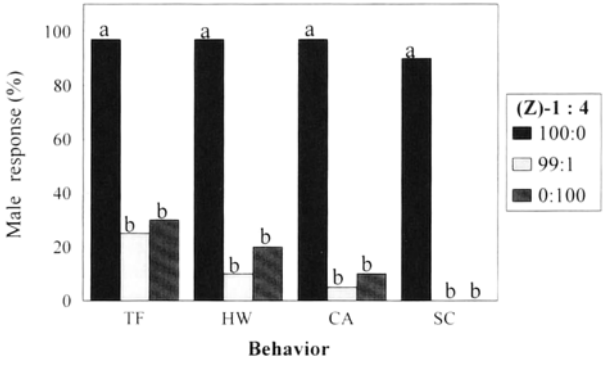

FIG. 5. Percentage of males responding to aldehyde **4** and mixture with the natural pheromone *Z*-**1** in 1:99 ratio relative to the pheromone in a wind tunnel. Histograms with different letters are significantly different using a chi-square 2 × 2 test of independence ($P < 0.05$). TF, taking flight; HW, upwind flight and arrival to the middle of the tunnel; CA, close approach to the lure (ca. 10 cm); SC, source contact.

CH_2COCF_3. The ketone was tested as an inhibitor of pheromone response in two ways: first, when coevaporated with the natural material and, second, after presaturation of the male antennal receptors with vapors of the chemical. When mixed with the pheromone in 5:95 ratio, the ketone showed no effect on TF and HW behaviors but significantly inhibited CA and SC behaviors (Figure 6A). This result contrasts with those found with 1,1,1-trifluoro-14-heptadecen-2-one,

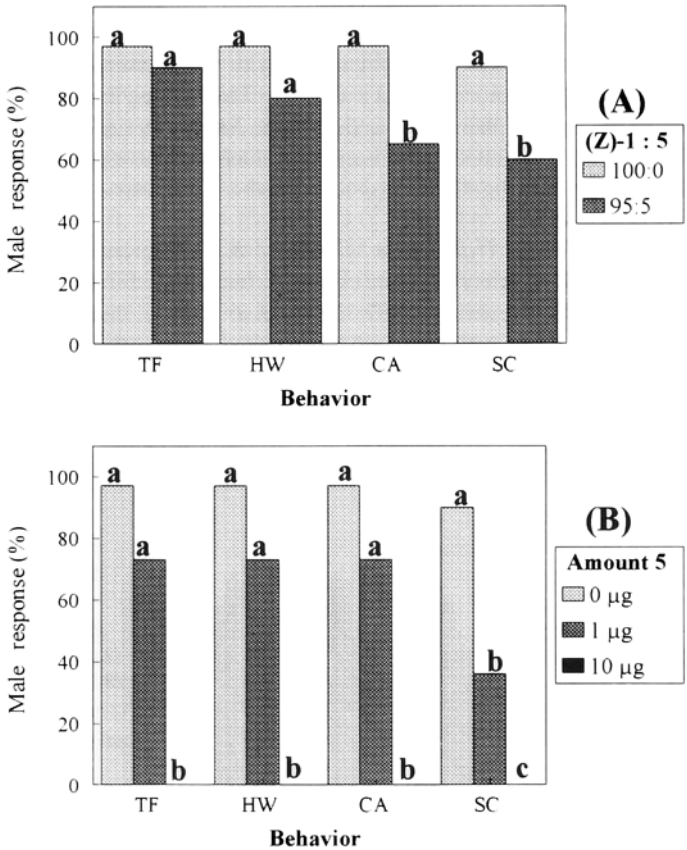

FIG. 6. (A) Percentage of males responding to a 95:5 mixture of the natural pheromone Z-**1** and trifluoromethyl ketone **5** relative to the pheromone. (B) Percentage of males responding to the natural pheromone Z-**1** after presaturation of the antennal receptors with vapors of 1 and 10 µg of **5**. Histograms with different letters are significantly different using a chi-square 2 × 2 test of independence ($P < 0.05$). TF, taking flight; HW, upwind flight and arrival to the middle of the tunnel; CA, close approach to the lure (ca. 10 cm); SC, source contact.

a pheromone analog of the European corn borer (Klun et al., 1991), which neither interfered with the normal behavioral response of males to the natural pheromone in a wind tunnel nor showed esterase inhibition. In the field also, compound **5** showed the same type of activity displayed in the wind tunnel, i.e., a disruptant effect of the pheromone action when mixed with the natural compound in 1:0, 1:1, and 1:10 ratios (Parrilla and Guerrero, 1994).

When male antennal receptors were exposed to vapors of 1 μg of the trifluoromethyl ketone, the insects were clearly impaired only in the SC behavior (only 36% of males landed at the source), although 73% were able to orient into the plume (HW and CA behaviors). Higher doses of the ketone (10 μg) completely inhibited all types of behavior considered (Figure 6B). The same type of experiment did not affect, however, the EAG response to the pheromone (Parrilla, 1994).

In summary, we have shown that the behavior of processionary males in the wind tunnel can be strongly influenced by structural analogs of the natural pheromone. Most of the compounds tested are potent inhibitors of the pheromone action, mainly aldehyde **4**, with the activity being more pronounced in the last stages of the mate-finding sequence, particularly in contact with the source. Our results correlate well with those obtained in the field and substantiate the usefulness of the wind tunnel to establish the behavioral responses of males to pheromones and analog.

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ALKADIENES MEDIATING COURTSHIP IN THE PARASITOID *Cardiochiles nigriceps* (HYMENOPTERA: BRACONIDAE)

T.C. SYVERTSEN,[1] L.L. JACKSON,[2] G.J. BLOMQUIST,[3] and S.B. VINSON[1,*]

[1]*Department of Entomology*
Texas A&M University
College State, Texas 77843

[2]*Department of Chemistry and Biochemistry*
Montana State University
Bozeman, Montana 59717

[3]*Department of Biochemistry*
University of Nevada
Reno, Nevada 89557

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Abstract—Courtship in *Cardiochiles nigriceps* (Viereck) (Hymenoptera: Braconidae) involves male attraction to females and male antennation of females followed by mounting and copulation. Once the female is located, antennation and mounting of the female are mediated by the hydrocarbon fraction of female's Dufour's gland and cuticle. The identification of the cuticular hydrocarbons of males and females revealed a mixture of alkanes and alkenes, in addition to a series of alkadienes specific to females. These female-specific alkadienes reported from Braconidae are unusual among insect alkadienes in that the second double bond occurs in the middle of the molecule. Bioassays with three available alkadienes revealed that contact behaviors (antennation and mounting) are in part mediated by the (*Z*,*Z*)-7,13-heptacosadiene and at least one other alkadiene in combination with other hydrocarbons found in males and females.

Key Words—*Cardiochiles nigriceps*, parasitoid, Hymenoptera, Braconidae, pheromone, mating, courtship, hydrocarbon, cuticle, alkadienes.

*To whom correspondence should be addressed.

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INTRODUCTION

Cardiochiles nigriceps (Viereck) is one of the more important native parasitoids attacking *Heliothis virescens* (F.) the tobacco budworm (Grayson, 1944; Ridgway and Lingren, 1972). Although there may be ways to manipulate native parasitoid populations in agronomic settings (Greany et al., 1984), such efforts with *C. nigriceps* are presently limited to conservation (Cate, 1985). The inability to monitor adult populations hinders field research. Attempts to overcome this problem have included pheromone trapping (Lewis et al., 1971), but the limited information regarding the pheromones of *C. nigriceps* has hindered development of this approach.

Male reproductive behavior in parasitoids has been examined primarily with regard to the evolution of reproductive strategies (Hamilton, 1967, 1979; Van den Assem, 1986; Werren, 1983) and the evolution of courtship displays (Matthews, 1975; Van den Assem and Jackmann, 1982). However, answers to proximate questions (e.g., factors leading to courtship and the identification of the cues that elicit these reproductive behaviors) have lagged, despite a tremendous body of evidence indicating that a series of cues are involved (Eller et al., 1984; Swedenborg and Jones, 1992a,b; Van den Assem et al., 1980). The identification of a sex pheromone has been demonstrated in only four parasitoid species. The compounds identified are generally attractants and include neral and geranial in *Itoplectis conquisitor* Say (Robacker and Hendry, 1977), ethyl-(*Z*)-9-hexadecenoate in *Syndipnus rubiginosus* Walley (Eller et al., 1984), (*Z*)-4-tridecenal in *Macrocentrus grandii* Goidanich (Swedenborg and Jones, 1992b), and (*Z*)-9-hexadecenal in *Ascogaster reticulatus* Watanabe (Kainoh et al., 1991).

The pheromone (*Z*)-4-tridecenal, isolated from female *M. grandii*, is an air oxidation product from a series of (*Z*,*Z*)-9,13-alkadienes (Swedenborg and Jones, 1992b). In addition, Swedenborg et al. (1993) reported a third component of the sex pheromone blend used by *M. grandii*. Thus, the sex pheromones isolated from some parasitic Hymenoptera have been determined to consist of multiple compounds (Shu and Jones, 1993; Swedenborg and Jones, 1992a; Swedenborg et al., 1993).

The source of these pheromones is generally unknown although both the Dufour's gland (Weseloh, 1976; Vinson, 1978) and cells located at the base of the second valvifer (Tagawa, 1977) or base of the genital capsule (Field and Keller, 1994) have been suggested. Swedenborg et al. (1993) reported that the mandibular gland of *M. grandii* was the source of one of the pheromone blend components. This compound, (3*R*′,5*S*′,6*R*′)-3,5-diethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one, is synergistic to the previously identified (*Z*)-4-tridecenal and its alkadiene precursors. The identification of several potential sources provides additional evidence that the sex pheromones of parasitoid hymenopterans consist of a complex of components.

Complex courtship behaviors often occur once the sexes have made contact (Matthews, 1975; Van den Assem et al., 1980) and are an important aspect of sex recognition. The role chemicals play toward the mediation of this process is poorly understood and appears to be as complex as the courtship display itself. Since courtship is the means by which species recognition occurs, knowlege of the entire suite of behavioral and chemical signals mediating this process can be useful when incorporated into taxonomic descriptions. This would be invaluable in those instances where cryptic species are suspected. The experiments described here pertain to those compounds that mediate the courtship behavior in *Cardiochiles nigriceps* Viereck, a braconid parasitoid of *Heliothis virescens* (F.).

METHODS AND MATERIALS

Insects. Host *Heliothis virescens* larvae were reared on Bio-serv Corn Earworm diet (Bio-serv, Inc., Frenchtown, New Jersey 08825) as discussed by Elzen et al. (1984) but modified by the addition of 10 ml of an aqueous solution of 46% propionic acid and 4% phosphoric acid to suppress the fungus *Aspergillus niger. Cardiochiles nigriceps* were reared on fourth-instar *H. virescens* by established methods (Vinson, 1973). Voucher specimens are deposited in the Insect Collection of the Department of Entomology, Texas A&M University.

Extraction and Fractionation of Dufour's Gland. Because female *C. nigriceps* are not receptive to courting males until 24 hr after emergence (Hirose et al., 1988), dissections were performed only on live females that had eclosed at least one day earlier. Females were dissected by anesthetizing them in an ice-water bath. The wasp was then placed (ventral-side up) in a Petri disk filled with Pringle's saline (Pringle, 1938) and the ovipositor gently withdrawn using watch-maker's forceps until the Dufour's gland was exposed. The gland was then cut loose and placed in a 1-ml conical vial filled with hexane (HPLC grade). Concentrations of glandular extracts ranged from 50–100 glands/500 μl solvent with the specific concentration of each extract recorded.

Crude extracts of the Dufour's gland were then separated by preparative gas chromatography (GC) on a Tracor 550 gas chromatograph equipped with a variable splitter to a flame ionization detector and to a Brownlee-Silverstein collection system. Separations were obtained on a 1.83-m × 4-mm-ID glass column packed with 3% Dexsil 410 on Chromosorb 750 (80–100 mesh). Nitrogen carrier gas flow rate was 60 ml/min and the temperature program was 150–350°C at 15°/min. Individual peaks were collected in 40-cm × 1-mm-ID capillary glass tubes cooled with liquid nitrogen. To destabilize aerosols, several fibers of dimethylchlorosilane-treated glass wool were inserted into each tube. Collected fractions were further purified by reinjecting them onto the same

column under isothermal conditions and again collecting individual peaks as above. The purified fractions were bioassayed and stored at −80°C for later study.

Extraction and Fractionation of Cuticular Lipids. For quantitation of cuticular hydrocarbons, sexually mature virgin male or virgin female *C. nigriceps* (24–120 hr old) were frozen and individually extracted with diethyl ether (0.5 ml/wasp) for 15 min (all diethyl ether was high purity grade from Burdick and Jackson and preserved with 2% ethanol). The wasp was removed and the diethyl ether was removed under nitrogen sweep. An internal standard of 1-eicosene (10 μg/wasp) was added in isooctane (10 μl/wasp) and 1 μl was analyzed by GC on a 30-m × 0.25-mm capillary column of Durabond DB-1, temperature programmed from 150 to 300°C at 5°C/min. Compounds were detected by flame ionization. Each peak was quantified relative to the internal standard.

Cuticular lipid extracts of large batches (50–100 wasps) of virgin female or virgin male *C. nigriceps* (24–120 hr old) were prepared by soaking freshly frozen adults (0.5 ml/wasp) in diethyl ether for 15 min and sonicating for 30 sec. Extracts were dehydrated by passing the supernatant through a Pasteur pipet plugged with dimethylchlorosilane-treated glass wool and filled with approximately 1 g anhydrous sodium sulfate. The eluant was concentrated at 55°C under a fractionating column to a volume of 5 ml and then concentrated to 1 ml by distillation with a spinning band column at 45°C. Finally, the extracts and distillates were stored at −80°C to be used later for bioassay or further separation.

Cuticular lipid extracts were fractionated on a 1-g Florisil (60–100 mesh) column and eluted with approximately 15 ml pentane followed by an equal amount of methylene chloride and finally methanol. The three fractions were concentrated by distillation using a fractionating column and spinning band column (as described above) with the temperature of the warm water bath adjusted so that it was 10° higher than the boiling point for each respective solvent fraction. The final volume for each fraction was reduced to 1 ml and stored at −80°C.

Hydrocarbon Fractionation and Identification. The cuticular hydrocarbons (pentane fraction from the Florisil column) were separated by degree of unsaturation on silica thin-layer chromatography plates impregnated with 10% $AgNO_3$. The plates were developed with 2% diethyl ether in hexane. Two centimeters of the sides of each plate were stained with iodine vapor to locate bands on the unstained portion of each plate. The bands and areas between were separately scraped from the plate, and the absorbed compounds were eluted with diethyl ether. Alternatively, the cuticular hydrocarbons were separated into alkane, alkene, and alkadiene fractions by high-pressure liquid chromatrography (HPLC). A Waters 4500 HPLC system with differential refractometer was used with a 25-cm × 4.6-mm-ID column of 5 μm Absorbosphere silica (Applied Science,

Deerfield, Illinois) coated in situ with $AgNO_3$ (Heath and Sonnet, 1980) and eluted with 25% toluene in hexane (v/v) at a flow rate of 1 ml/min to separate the hydrocarbons (Bartelt et al., 1986). Alkanes were collected within the first 3 min after injection (just after the void volume), alkenes were collected from 3 to 8 min, and alkadienes were collected from 8 to 15 min. Nothing was detected in the 15 to 32-min fractions at the end of the chromatogram.

The composition of each fraction was determined by GC analysis comparing retention to known standards using a Hewlett Packard 5890A gas chromatograph with a 30-m × 0.32-mm Durabond DB-5 capillary column temperature programmed from 200 to 300°C at 4°/min or GC-MS analysis with a 15-m × 0.25-mm Hewlett Packard HP-1 capillary column temperature programmed from 150 to 300°C at 5°/min and monitored by a HP 5971 mass selective detector.

Methoxymercuration–demercuration derivatives of the alkene and alkadiene fractions were prepared (Blomquist et al., 1980) and analyzed by GC-MS on a 30-m × 0.72-mm capillary column of DB-5 using a temperature program from 200 to 280°C at 2°/min and introduced into a Finnigan 4123 mass spectrometer operating at 70 eV. The positions of the double bonds in the alkene and alkadiene fractions were further verified by dimethyl disulfide derivatization and GC-MS analysis (Carlson et al., 1989).

The *E* or *Z* nature of the double bonds was determined by infrared (IR) absorption at 970 cm^{-1} for the *E* and at 740 cm^{-1} for the *Z* isomer. IR spectra were obtained with neat hydrocarbons (combined alkenes and alkadienes) between KBr plates in a Perkin-Elmer model 599 spectrophotometer.

Synthesis of Alkadienes. The alkadienes were prepared using the Wittig synthesis (Sonnet, 1974). Differences from Sonnet's procedure were: (1) the alkyltriphenylphosphonium salts were prepared from α,ω-dibromoalkanes, to allow for double bond formation on both ends; and (2) the equivalents of other reactants (butyllithium and aldehyde) were doubled correspondingly. For these asymmetrical alkadienes, equimolar amounts of the two appropriate aldehydes were added simultaneously. In these cases, two symmetrical alkadienes also formed, one shorter and one longer than the desired asymmetrical diene. Dimethylsulfoxide was used as a cosolvent to enhance the formation of *Z*-double bonds. The product mixture was passed through a column of silicic acid with hexane to elute the alkadienes and to remove polar by-products. Analysis of the synthetic alkadiene mixtures by GC relative to paraffin alkane standards and by GC-MS focusing on the molecular ion in each case showed that the expected carbon chain lengths were produced. The alkadienes, containing the *Z,Z*-isomer, *Z,E* and *E,Z*-iosmers, and traces of *E,E*-isomers (as well as several chain lengths, in the cases of asymmetrical dienes), were further purified by $AgNO_3$ HPLC (Bartelt et al., 1986). On $AgNO_3$ HPLC, the *E,E*-isomers eluted during the first 5 min of the HPLC analysis. The *E,Z* and *Z,E*-isomers along with greater than 34-carbon chain *Z,Z*-isomers eluted next in the 5- to 8-min fractions; *Z,Z*-iso-

mers in the 27- to 31-carbon range eluted in the 8- to 12-min fractions; and *Z,Z*-isomers in the 20- to 26-carbon range eluted in the 12- to 18-min fractions. Each HPLC fraction was analyzed by GC-MS and by dimethyl disulfide derivatization GC-MS as described above. (*Z,Z*)-7,13-Heptacosadiene, (*Z,Z*)-7,14-nonacosadiene, and (*Z,Z*)-7,15-hentricontadiene were 92%, 93%, and 99% pure, respectively.

Bioassays. To evaluate the role of the Dufour's and cuticular hydrocarbon fractions in the courtship behavior of male *C. nigriceps*, three types of bioassay were designed. The first bioassay, referred to as the close-range bioassay, was designed to examine the role chemicals play in the elicitation of the complete sequence of courtship behaviors exhibited by males. The behaviors examined ranged from close-range attraction of males (3–7 cm) toward females to the male's antennation of, orientation to, and mounting of the female upon contact (Vinson, 1978). The mounting of the female was typically followed by reorientation on the female's body and attempted copulation. Bioassays utilized freshly frozen (FF) females (controls) or FF males that served as models. Individuals of either sex that were selected to serve as models were $\geq$24 hr old. This age was selected because first-day adult *C. nigriceps* are not mature (Hirose et al., 1988) and dissections of Dufour's gland revealed that most glands were empty upon emergence, but were full by day 2 (Syvertsen and Vinson, personal observation). The close range bioassay was conducted by exposing a male to a FF female (positive control) or to an inert model (FF male) that was either treated with solvents (negative control) or with glandular or cuticular extracts of females. The latter was prepared with nonpolar (hexane) or weakly polar (diethyl ether) extract or fraction as described above. All extracts were adjusted so that a 10-μl aliquot contained 1 wasp equivalent (WE).

The close range bioassay consisted of attaching the model (FF male or FF female) to one end of a microscope slide with a nontoxic, odorless adhesive (Glue-Stic, Dennison Manuf. Co.) applied to the ventral thorax. A 10-μl aliquot of an extract (1 WE) was applied to a male model in such a way that the extract covered most of the exposed surface of the model. The slide was positioned on a flat surface so that an airstream could be directed over the model toward the opposite end of the slide. Air (purified and dehumidified by passing it through a cartridge filled with Drierite and activated charcoal) was adjusted to a flow rate of 0.5 ml/min via a flowmeter controlled with an on/off valve. Males were obtained from their cages by allowing them to walk onto a wooden applicator stick. The wasp was permitted to transfer from the stick to the downwind edge of the slide (Figure 1). When the male walked onto the slide, the airstream was turned on and the male's response was video-recorded. Each male was allowed 1.0 min to respond. If the response was positive and courtship was elicited, no maximum time limit was set and the behaviors were observed until the male dismounted and left the slide.

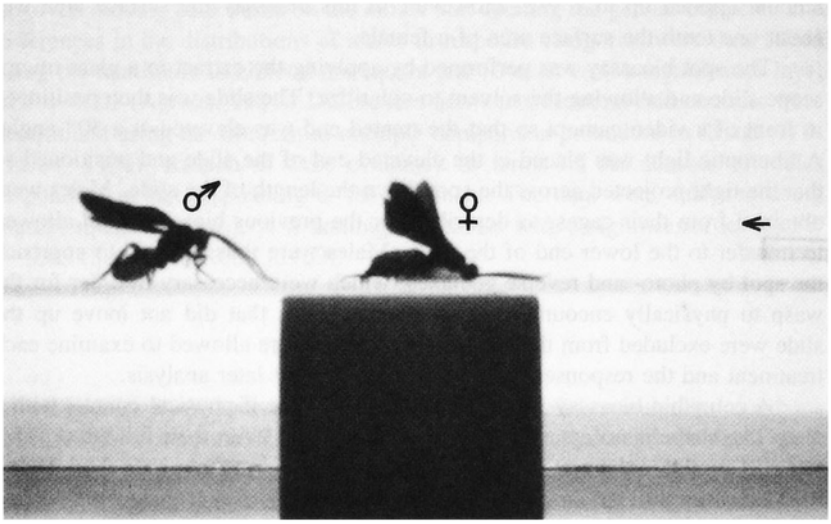

FIG. 1. Close-range bioassay: male *C. nigriceps* wing fanning and antennating the substrate in response to female sex pheromone.

A second bioassay, referred to as a spot bioassay, exposed males to a substrate treated with either compound extracted and fractionated from females or synthetic compounds. This bioassay was used because males often antennate the substrate where females have been resting and do so with a set of characteristic behaviors (Vinson, 1978). The bioassay is important in detecting behaviorally relevant nonvolatile or contact chemicals. High-purity hexane extracts of females were of two types: (1) Dufour's gland extracts, and (2) cuticular extracts. Each extract was bioassayed as a series of concentrations in order to determine the optimum dose–response for activity. The final concentrations of both the cuticular and glandular extracts were adjusted so that 10 μl equaled the dose being assayed, which ranged from 10 to 1×10^{-4} WE (Table 2 below). The glandular extracts were then fractionated by packed-column GC into groups based upon their carbon chain length (CL) and bioassayed similarly (Table 3 below). The cuticular extracts were not fractionated similarly, since the composition of the nonpolar or hydrocarbon fraction of this extract (purified with silicic acid) was identical to that of the Dufour's gland. Instead TLC and HPLC fractions separated on the basis of unsaturation were evaluated. The concentrations of all fractions were adjusted so that 10 μl equaled approximately 0.1 WE. This quantity was used since the dose–response experiments to both glandular and cuticular extracts indicated that the males' response increased with the

amount applied up to 1 WE. However, in this bioassay the surface area was about one tenth the surface area of a female.

The spot bioassay was performed by applying the extract to a glass microscope slide and allowing the solvent to volatilize. The slide was then positioned in front of a video camera so that the treated end was elevated at a 50° angle. A fiberoptic light was placed at the elevated end of the slide and positioned so that the light projected across the spot down the length of the slide. Males were obtained from their cages, as described for the previous bioassay, and allowed to transfer to the lower end of the slide. Males were thus inclined to approach the spot by photo- and reverse geotaxes, which were necessary in order for the wasp to physically encounter the treatment. Males that did not move up the slide were excluded from the test. Twenty males were allowed to examine each treatment and the responses were video recorded for later analysis.

A courtship bioassay was designed to determine if physical contact with a wasp like shape is necessary for these compounds to exert their full effect. This test was similar to the previous test with the modification that a model was used. This bioassay differed from the close-range bioassay in that males approached the model by photo- and reverse geotaxes rather than being attracted by an odor-laden airstream. Treatment models were frozen male *C. nigriceps* treated with select fractions found to be active in the spot bioassay and the female-specific hydrocarbons that were identified and synthesized. FF male models were held in place by an adhesive described for the close-range bioassay and treated with 10 μl of solvent containing approximately 1 WE of extract or fraction to be evaluated. Application of the compounds was via a Hamilton syringe and performed in such a way that most, if not all, of the body was treated. Untreated frozen females were used as positive controls.

Data Recording and Analyses. Bioassays were video recorded with RCA model 2000, RCA Video Equipment (New Holland Ave., Lancaster, Pennsylvania 17604) and replayed at a later time with the events logged on an IBM XT-286 PC equipped with The Observer, a software package for behavioral research (Noldus, 1991). Close-range bioassay behaviors (e.g., attraction to the model, wing fanning, antennating, mounting, orienting on the model, and copulating) were recorded as frequencies and only the highest-ranking behavior in the sequence was scored. For the spot bioassay, an antennation response was scored if, upon encountering the spot, the male stopped walking and the antennae were extended forward so that both were approximately parallel with respect to each other and repeated palpations across the spot occurred. Responses to the model in the courtship bioassay were recorded and analyzed as in the close-range bioassay, except that movement to the model was not considered and only males contacting the model were evaluated.

Analyses of the courtship bioassay behavioral frequencies were divided into three categories: antennation, mounting, and attempts at copulation that

involved moving backwards on the model and opening the genital claspers. The differences in the distributions of males in response categories were then tested using the maximum likelihood chi-square test (G test) with a significance level of $P = 0.05$ (Agresti, 1984). Differences between treatments and controls were determined using the Bonferroni multiple comparison procedure in Systat (Wilkinson, 1990). Responses were evaluated in terms of the number of males responding or not responding to the treatment. The data were analyzed using the chi-square test for $2 \times K$ contingency tables with a significance level of $P = 0.05$ (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

Cuticular Hydrocarbons. Capillary GC resolved the cuticular hydrocarbons from female wasps into 25 peaks and the cuticular hydrocarbons from male wasps into 16 peaks (Table 1). Infrared absorption spectra indicated that the majority, if not all, of the double bonds were *Z* by strong absorption at 740 cm^{-1} and the absence of any absorption band at 970 cm^{-1}. Both $AgNO_3$-TLC and $AgNO_3$-HPLC resolved the cuticular hydrocarbons from females into alkane, alkene, and alkadiene fractions. However, the cuticular hydrocarbons from males yielded only alkane and alkene fractions. The alkane fractions from each sex were qualitatively similar, with some quantitative differences (Table 1). Quantitation of each of the alkene isomers was incomplete because of an unresolved mixture of isomers in some of the GC peaks. Mass spectra of the monomethoxy derivatives of the alkenes showed that the double bonds were located at position 7 (7-pentacosene, 7-heptacosene, and 7-nonacosene) or located at or near the center of the hydrocarbon chain (13- 11-, and 9-hexacosene; 12- and 13-heptacosene; 13-octacosene; 13- and 14-nonacosene; 13- and 15-triacontene; 13- and 15-hentriacontene; 13- and 15-tritriacontene; and 13- and 15-pentatriacontene). Alkenes with double bonds at or near the center of the hydrocarbon chain are rare in insect cuticular hydrocarbons.

The mass spectra of the methoxy derivatives of the alkadienes from females (Figure 2) showed that one of the double bonds was located near the middle of the chain (position 13, 14, or 15) and the other double bonds was at position 5, 6, or 7. The positions of the double bonds were established from the fragmentation of the monomethoxy derivatives in the mass spectrometer (Figure 2). For example, the 27:2 alkadiene had its double bonds in the 7,13- positions, and yielded monomethoxy derivatives with methoxy groups on positions 7, 8, 13, and 14, and all fragments corresponding to these isomers are present in the mass spectrum (Figure 2A). The 29:2 component has four isomers, and the methoxy derviatives and the expected ion fragments for the 5,14- isomer are highlighted in Figure 2B. In addition, the mass spectrum (Figure 2B) was inter-

TABLE 1. IDENTIFICATION AND QUANTIFICATION OF CUTICULAR HYDROCARBONS FROM MALE AND FEMALE *C. nigriceps*

Hydrocarbon	CL[a]	Male (μg/wasp)	Female (μg/wasp)
Alkanes			
Triacosane	23:0	0.8	3.0
Tetracosane	24:0	0.2	0.4
Pentacosane	25:0	12.3	15.5
Hexacosane	26:0	0.4	0.2
Heptacosane	27:0	7.8	4.7
Octacosane	28:0	0.1	0.1
Nonacosane	29:0	2.1	1.1
Hentriacontane	31:0	0.8	0.2
Z-Alkenes			
9- and 10-Tricosene	23:1		trace[b]
7-Pentacosene	25:1	trace	0.4
13-, 11-, 9-, and 7-Hexacosene	26:1	1.0	0.3
12- and 13-Heptacosene	27:1	69.2[c]	3.8
7-Heptacosene	27:1		3.0*
13-Octacosene	28:1	trace	0.8
13- and 14-Nonacosene	29:1	2.5	25.0*
7-Nonacosene	29:1		1.9*
13- and 15-Triacontene	30:1		0.5*
13- and 15-Hentriacontene	31:1	3.8	17.9
13- and 15-Tritriacontene	33:1	0.2	2.2*
13- and 15-Pentatriacontene	35:1	trace	trace
Z,Z Alkadienes			
?,?-Pentacosadiene	25:2		0.3*
7,13-Heptacosadiene	27:2		1.1*
5,14- and 7,14-Nonacosadiene[d]	29:2		2.8*
?,?-Triacontadiene	30:2		0.5*
7,15-Hentriacontadiene[e]	31:2		3.2*
7,15- and 6,15-Tritriacontadiene	33:2		1.1*
7,13- and 7,15-Pentatriacontadiene	35:2		trace

[a] Carbon number: double bonds.
[b] Trace < 0.1 μg/wasp but > 0.05 μg/wasp.
[c] At least a 10-fold difference in quantity.
[d] Also 5,15- and 7,15-isomers.
[e] Also 6,15-, 6,16-, and 7,16-isomers.

preted as having arisen from isomers with double bonds in the 7,14-, 5,15-, and 7,15- positions. This was the only alkadiene with an isomer that had the first double bond at position 5. The mass spectrum from the monomethoxy derivative of 31:2 (Figure 2C) was interpreted in a similar manner, and the data show that the major isomer is the 7,15-alkadiene. On capillary columns the peaks

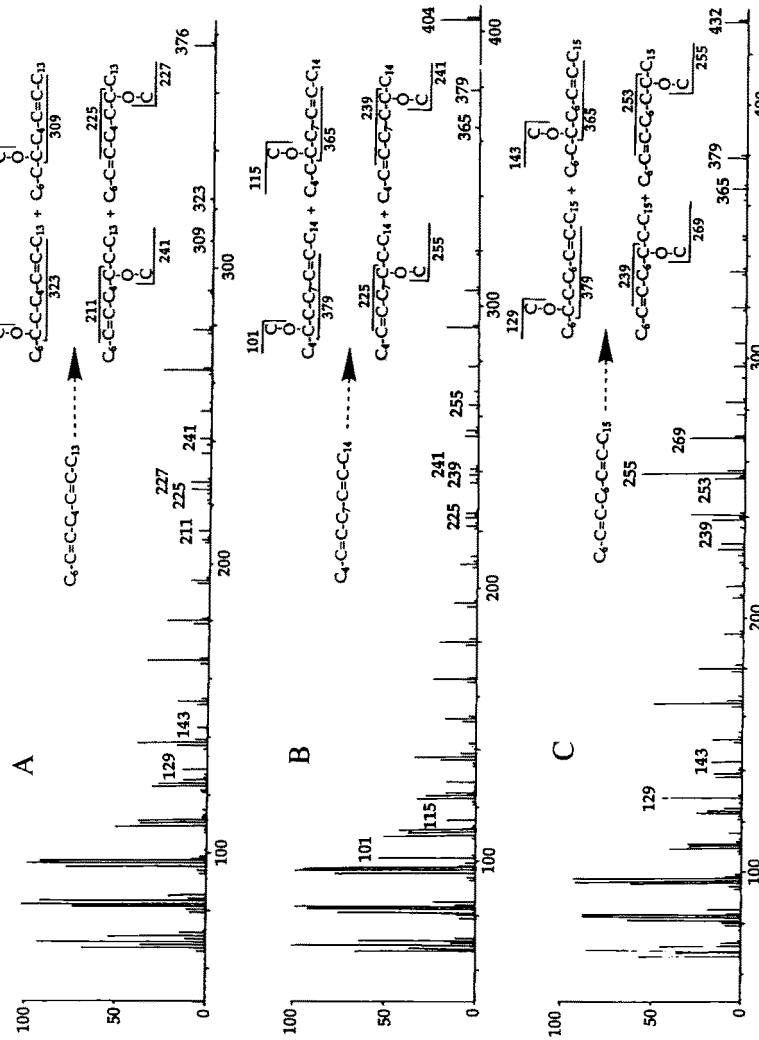

FIG. 2. Mass spectra of the monomethoxy derivatives of the 27:2 (A), 29:2 (B) and 31:2 (C) alkadienes from the cuticle of female *C. nigriceps*.

corresponding to methoxy derivatives with the methoxy group near the center of the chain were partially resolved from the methoxy derivatives with the methoxy group on positions 5, 6, or 7. The data presented in Figure 2 are the computer summed spectra across the cluster of peaks. The alkadienes, exclusively from female, have the first double bond predominantly at position 7 and the second double bond at or near the center of the hydrocarbon chain.

The alkadienes, therefore, have one double bond in the same location as the alkenes. Whether the same enzymes introduce double bonds in the biosynthesis of alkenes and alkadienes remains to be determined.

Comparison of Male and Female Cuticular Hydrocarbons. The most conspicuous difference between the composition of the cuticular hydrocarbons from males and females (Table 1) is the absence of di-unsaturated hydrocarbons (alkadienes) and the copious amount of 13-heptacosene in males. Females have greater quantities of 13- and 14-nonacosene and 13- and 15-hentriacontene than males. In addition, males also possess a slightly greater quantity of total cuticular hydrocarbons (101.2 μg/wasp) than do females (90.0 μg/wasp). Further, there were no significant differences in composition between extracts of the Dufour's gland and the hydrocarbon fraction of the cuticular extracts of females. Because the alkadienes were found to be specific to the female integument and Dufour's gland, and thus likely candidates for pheromonal activity, three of these hydrocarbons were synthesized and bioassayed.

Bioassays. Males isolated from females do not elicit any sexual response when placed with other males, while males exposed for just 1 min to females will elicit attraction, wing fanning, and even mounting by other males. These observations were confirmed in the short-range bioassay (Figure 3) where FF females attracted 63% ($N = 30$) of the males, all of which wing fanned and antennated and most mounted the female. Males were generally not attracted to FF males (Figure 3) and males contacting FF males just touched them and climbed on top, a behavior very different from a response to females. Female cuticle diethyl ether extracts applied to FF males attracted 57% of the males ($N = 30$), which also wing fanned and most antennated, mounted, and even attempted to copulate with the treated FF male. No difference in the response of males to FF females or diethyl ether cuticle female extracts applied to FF males was found ($G = 2.07$; 2 *df*; $P = 0.36$; NS).

However, neither the hexane extract of Dufour's gland ($G = 19$; 2 *df*; $P = 7.0 \times 10^{-5}$) nor the hydrocarbon fraction of the cuticle extract ($G = 33$; 2 *df*; $P = 8.3 \times 10^{-8}$) was as attractive to males (with less than 20% responding in both cases) as the female diethyl ether extract when applied to FF males in the short-range bioassay. These results show that the more volatile component of the sex pheromone blend is absent in the hydrocarbon extracts. However, both extracts did elicit antennation and mounting in a high proportion of males

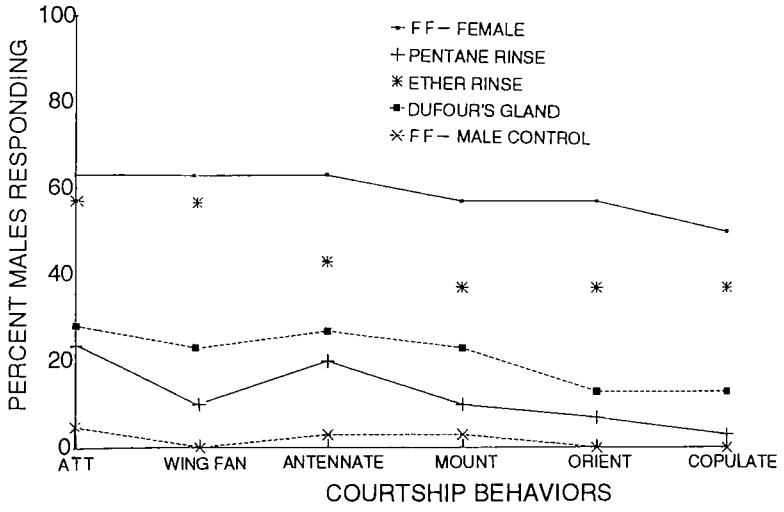

FIG. 3. Response of male *C. nigriceps* to a freshly frozen female (FF-female) or to freshly frozen males (FF-male) treated with solvent extracts of females. ATT = attraction to target (FF-female or FF-male) (N = 30).

that accidently contacted the treated males (Figure 3). No male response occurred to FF males treated with only hexane or diethyl ether (N = 10).

Since both Dufour's gland extract and the hydrocarbon fraction of the cuticle of females elicited sex-specific behaviors in males on contact, the spot and the courtship bioassay that depend on the subject males making contact with the test fractions were used. The remainder of this paper focuses on the contact sex pheromone activity.

Both the hydrocarbon fraction of the female cuticle extracts and Dufour's gland extract elicited wing fanning and antennating from males in the spot bioassay (Table 2), the latter confirming the results of Vinson (1978). However, 10 WE proved to be too strong, as males contacting the treated spot proceeded to preen their antennae rather than respond. The data did reveal that 1 WE elicited a good response. The response of males to GC peaks collected from the Dufour's gland extract (Table 3) indicated that only the odd-numbered hydrocarbon peaks (C_{25}, C_{27}, C_{29}, C_{31}, and C_{33}) had activity when compared to the even-numbered hydrocarbon peaks or the hexane extract of male cuticular hydrocarbons used as a control. Therefore, it appeared that no specific hydrocarbon chain length was responsible for the sex-specific male response, although the activity was confined to the GC peaks that are equivalent to the odd-numbered hydrocarbons.

TABLE 2. MALE RESPONSES TO DIFFERENT CONCENTRATIONS OF CRUDE EXTRACTS OF *Cardiochiles nigriceps* IN SPOT BIOASSAY ($N = 25$)

Concentration (WE)	Males wing fanning (N)	Males antennating (N)
Male cuticular diethyl ether extract		
1×10^{0}	0	0
Female cuticular hydrocarbon fraction		
10	17	20
1×10^{0}	23	24
1×10^{-1}	13	23
1×10^{-2}	4	22
1×10^{-3}	0	8
1×10^{-4}	0	0
Dufour's gland hexane extract		
10	5	11
1×10^{0}	12	24
1×10^{-1}	10	21
1×10^{-2}	6	20
1×10^{-3}	4	10
1×10^{-4}	0	0
Hexane	0	0
Ether	0	0

However, these GC peaks were broader than our standards, suggesting that these peaks consisted of a mixture of saturated and unsaturated hydrocarbons. Thin-layer chromatography confirmed the presence of alkanes, alkenes, and alkadienes. Spot bioassay of the alkanes give no response, while all males antennated the alkadiene spot ($N = 15$), but the duration of the response appeared to be shorter than the responses to the female cuticular hydrocarbon extract. Only 13% ($N = 15$) of the males responded to the alkenes with a brief antennation. The results suggest that some of the alkadienes may be responsible for the contact pheromone activity of the hydrocarbon fraction of females but that their activity may also be synergized by other components in the hydrocarbon fraction.

The identification and subsequent synthesis of three of the alkadienes allowed us to examine this possibility. The results of the spot bioassay of the three alkadienes (Table 4) indicated some male response (55%) to the (*Z*,*Z*)7,15-C_{31} alkadiene. However, the (*Z*,*Z*)7,13-C_{27} and (*Z*,*Z*)7,14-C_{29} did not elicit much activity in males. Since the latter two were inactive individually, we mixed

TABLE 3. MALE ANTENNATION RESPONSE USING SPOT BIOASSAY TO GC FRACTIONS OF DUFOUR'S GLAND OF *C. nigriceps* ($N = 20$)

GC fraction[a]	CL[b]	No. of males antennating	P[c]
1	C_{25}	0	NS
2	C_{25}	12	3.0×10^{-5}
3	C_{26}	2	NS
4	C_{27}	18	1.1×10^{-8}
5	C_{28}	0	NS
6	C_{29}	16	2.4×10^{-7}
7	C_{30}	—[d]	NC
8	C_{31}	16	2.4×10^{-7}
9	C_{32}	—[d]	NC
10	C_{33}	9	6.5×10^{-4}
Male cuticular extract	All	0	NC

[a] All fractions were bioassayed at 0.1 wasp equivalents.
[b] CL = carbon chain length.
[c] NS = not significant. NC = not calculated.
[d] No GC peaks in fraction and not tested.

them with the inactive male hydrogen extract lacking any alkadienes. The results (Table 5) showed that the (Z,Z)7,13-C_{27} and the male hydrocarbon mixture were active (50% of the males responding), suggesting a synergistic component in the alkane-alkene mixture. However, the (Z,Z)7,14-C_{29} alkadiene was not synergized. Although the reason that the C_{29} alkadiene was not synergized by the male hydrocarbon blend that contains the C_{27} alkadiene is unknown, the unusually high C_{27} alkene in male hydrocarbons may be important. There are many possible combinations to be tested that await synthesis of the proper alkadienes and alkenes.

TABLE 4. MALE RESPONSE IN SPOT BIOASSAY TO THREE AVAILABLE SYNTHETIC ALKADIENES ($N = 20$)[a]

Treatment	No. males antennating	X^2	P
(Z,Z)7,13-C_{27}[a]	1	12	5.6×10^{-4}
(Z,Z)7,14-C_{29}[a]	0	15	1.0×10^{-4}
(Z,Z)7,15-C_{31}[b]	11		

[a] All hydrocarbons were bioassayed at 0.1 female equivalents per test. Identical letters indicate no significant difference ($P < 0.01$; Bonferroni multiple comparison procedure) between responses to hydrocarbons.

TABLE 5. MALE RESPONSE IN SPOT BIOASSAY TO TWO INACTIVE SYNTHETIC ALKADIENES ALONE AND AS A MIXTURE WITH MALE CUTICULAR HYDROCARBONS ($N = 20$)[a]

Treatment	No. males antennating	X^2	P
(Z,Z)7,13-C_{27}[a]	1	10	1.4×10^{-3}
(Z,Z)7,13-C_{27} + M[b]	10		
(Z,Z)7,14-C_{29}[c]	0	1.0	NS
(Z,Z)7,14-C_{29} + M[c]	1		

[a] All hydrocarbons were bioassayed at 0.1 female (or male) equivalents per test. M refers to male derived alkane and alkene mixture. Same letters indicate no significant difference between pairs of compounds within a molecular weight group ($P = 0.05$; Bonferroni multiple comparison procedure).

Although we obtained the characteristic sex specific response in males to the $C_{31:2}$ and a mixture of the $C_{29:2}$ with the male hydrocarbon mixture in the spot bioassay, we wanted to determine if the three available synthetic alkadienes would also elicit mounting or attempts to copulate if a female-like shape was involved. The three available alkadienes were applied to a FF male, which elicited intense antennation in 60% of the males (Table 6), but none of the males mounted the treated male (unlike what occurred when a Dufour's gland hexane extract was applied to FF males). The results suggest the alkadienes play a role in sexual behavior, while a parasitoid-like shape does not appear to play an important role. The results also suggest that (1) a specific combination of hydrocarbons is necessary to elicit mounting, (2) the presence of a large relative

TABLE 6. NUMBER OF MALES RESPONDING AS INDICATED IN COURTSHIP BIOASSAY TO TREATMENTS APPLIED TO MALE MODEL FOR FRESH FROZEN FEMALE ($N = 20$)

Treatment	Antennate	Mount	Copulate
Female	20	20	20
FF female	20	20	20
Male	0	0	0
FF male and hexane	0	0	0
FF male and Dufour's hexane extract	20	11	8
FF male and 3 synthetic (Z,Z) alkadienes	12	0	0

amount of the 12- and 13-heptacosene of males may inhibit this activity, or (3) yet other hydrocarbon components of the blend are necessary.

Regardless, the results of this study demonstrate differences in the hydrocarbon pattern of the cuticle of the two sexes. The results also show that the hydrocarbon composition of the cuticle of females and Dufour's gland are similar. Similar hydrocarbon patterns between cuticular extracts and Dufour's gland have been reported from bumble bees (Odham et al., 1994). The study also provides evidence that the sexual recognition of females by males is mediated by the hydrocarbon fraction of Dufour's gland and that the sex-specific alkadienes play a role in combination with some of the alkenes or alkanes present.

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MODIFICATION OF RESPONSES BY *Campylomma verbasci* (HETEROPTERA: MIRIDAE) TO PHEROMONE BLENDS IN ATMOSPHERES PERMEATED WITH SYNTHETIC SEX PHEROMONE OR INDIVIDUAL COMPONENTS

G.J.R. JUDD[1,*] H.L. McBRIEN,[2] and J.H. BORDEN[2]

[1]*Agriculture and Agri-Food Canada, Research Centre*
Summerland, British Columbia V0H 1Z0, Canada

[2]*Centre for Pest Management*
Department of Biological Sciences
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

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Abstract—Trapping experiments were conducted in orchards to test the hypothesis that exposure of the mullein bug, *Campylomma verbasci* (Meyer), to atmospheres permeated with its synthetic sex pheromone, a 94:6 blend of butyl butyrate (BB) and (*E*)-crotyl butyrate (CB), or BB or CB alone, would alter the pheromone responses of males toward off-ratio blends. Exposure to the natural 94:6 blend shifted the response preference away from the natural ratio to blends enriched in BB, including a 99:1 blend, which is normally significantly less attractive than the natural ratio. In an atmosphere permeated with CB, male mullein bugs were as responsive to blends containing 20, 33, and 43% CB, up to seven times the natural percentage, as they were to the 94:6 blend. In an atmosphere permeated with BB, responses to five blends ranging from 94:6 to 99:1 BB:CB were not significantly different, whereas in an untreated atmosphere blends of 98:2 and 99:1 were significantly less attractive than the 94:6 blend. Individual components appeared to disrupt pheromone communication primarily through the creation of a sensory imbalance and modified interpretation of pheromone blend ratios, whereas disruption by the complete pheromone blend apparently involves several mechanisms, including false-trail following and camouflage. Disruption of pheromone communication was most effective with the natural 94:6 sex pheromone blend.

*To whom correspondence should be addressed.

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Key Words—*Campylomma verbasci*, Miridae, mullein bug, sex pheromones, communication disruption, sensory imbalance, camouflage, resistance.

INTRODUCTION

Pheromones have been identified in very few heteropterans (Aldrich, 1988). The mullein bug, *Campylomma verbasci* (Meyer), is the only member of the family Miridae for which a pheromone has been identified (Smith et al., 1991). Female mullein bugs emit a sex pheromone composed of 94% butyl butyrate (BB) and 6% (*E*)-crotyl butyrate (CB), i.e., a 16:1 ratio of BB:CB. A synthetic pheromone blend containing 94% BB caught as many male mullein bugs as five caged virgin females (Smith et al., 1991), but blends containing 94–97% BB caught similar numbers of bugs (McBrien et al., 1994a). Outside this range of BB concentrations, catches fall off precipitously, and neither component is attractive alone (Smith et al., 1991; McBrien et al., 1994a).

Identification of the mullein bug sex pheromone has provided the opportunity (Smith et al., 1992) to develop pheromone-based management systems for this unpredictable and often serious pest of Red Delicious and Golden Delicious apples (Madsen and Proctor, 1985; Thistlewood et al., 1989). Disruption of pheromone communication using synthetic sex pheromones or individual components has been demonstrated in many lepidopteran species (Mitchell, 1981; Minks and Cardé, 1988; Jutsum and Gordon, 1989) and at least two coleopteran species (Huddelston et al., 1977; Mason and Jansson, 1991; Ridgway et al., 1990). Preliminary studies have demonstrated that pheromone communication in the mullein bug could be disrupted by atmospheric permeation of orchards with its complete two-component sex pheromone and suggested that false-trail following was a likely mechanism of disruption. However, as for other insects, the physiological and behavioral mechanisms of communication disruption in mullein bug are not well understood, but could include sensory adaptation at the peripheral level, central nervous system habituation, camouflage, or false-trail following (Bartell, 1982; Cardé, 1990).

Operationally, the exact mechanisms responsible for interruption of pheromone communication depend on the chemical composition of the disruptant and the release system employed (Minks and Cardé, 1988). When the disruptant consists of either an incomplete or unnatural blend of components, mechanisms quite different from those with a complete blend are possible (Minks and Cardé, 1988). An atmosphere treated with an off-ratio blend should provide the insect with a "sensory imbalance" (Hirano, 1979; Rothschild, 1981), in which the peripheral input from the component in excess lowers the sensitivity of receptors attuned to that component. Consequently, the natural ratio from a female would be interpreted as an unnatural ratio. Alternatively, unnatural peripheral input

could modify an insect's ability to interpret the natural ratio at a central neurological processing level. In either case the outcome is expected to be a diminished or abolished response to natural pheromone plumes.

Alteration of pheromone blend discrimination after preexposure to pheromone components has been demonstrated in several species, including oriental fruit moth, *Grapholitha molesta* (Busck) (Linn and Roelofs, 1981), pink bollworm, *Pectinophora gossypiella* (Saunders) (Flint and Merkle, 1983), and cabbage looper, *Trichoplusia ni* (Hübner) (Liu and Haynes, 1993). In addition, some lepidopteran species, like the codling moth, are more responsive to higher-release-rate pheromone sources in atmospheres treated with their sex pheromone than they are in nontreated atmospheres (Charmillot, 1990).

We investigated the general hypothesis that pheromone blend interpretation in the mullein bug would be altered by exposure to atmospheres treated with high concentrations of either or both of two individual sex pheromone components.

METHODS AND MATERIALS

General Methods. All experiments were conducted during September and October 1994 in the Okanagan and Similkameen Valleys of British Columbia. At this time of year most mullein bugs fly from herbaceous summer host plants into apple orchards (McBrien et al., 1994b), where they lay overwintering eggs in tree bark. Experimental apple orchards were planted predominantly with Red and Golden Delicious apple trees on semidwarf rootstocks at conventional tree and row spacings. All pheromone components and dispensers were supplied by Phero Tech Inc., Delta, British Columbia, Canada V4G 1E9. Butyl butyrate had at least 99.6% chemical purity and (*E*)-crotyl butyrate was at least 94% isomerically pure and 99.3% chemically pure (Phero Tech Inc., technical data).

Experiment 1: Response to Pheromone Blends in Crotyl Butyrate-Permeated Atmosphere. In this experiment we tested the specific hypothesis that mullein bugs would shift their pheromone responses from a preference for the natural blend of 94:6 BB:CB towards blends having a higher percentage of CB when exposed to an atmosphere treated with CB.

Two-hundred fifty low-density polyethylene Eppendorf centrifuge tubes, each containing 125 μl of CB (release rates described by McBrien et al., 1994a) were hung in the upper third of the canopy in the northeast sector of trees in each of five 0.25-ha plots (50 × 50 m), achieving a dispenser density of 1000/ha. Each crotyl butyrate-treated plot was paired with an identical nontreated control plot located 60 m up mean wind direction in the same orchard, and each pair was located in a different orchard. Five days later, five open-style wing traps (McBrien et al., 1994a) were suspended 1.5 m above ground in the

outer 0.5 m of foliage in trees in all treated and control plots. One trap was placed on the center tree of plots, and others were positioned on the diagonals 15 m from each plot corner. Traps within a plot were baited with one of five randomly assigned baits consisting of an Eppendorf tube containing 125 μl of BB:CB (v/v) in ratios of 94:6, 80:20, 67:33, 57:43, or 50:50. Baits were attached to the inner surface of each trap lid.

Trap catches were recorded daily for five days and summed. A three-factor analysis of variance (ANOVA) (binary bait, disruption treatment, orchard site) was used to test for differences in pheromone blend response as a function of disruption treatment. Mean trap catches for binary baits within each disruption treatment were compared using the Student-Neuman-Keuls' (SNK) multiple-range test (Zar, 1984). Distributions of trap catches with different pheromone blends in different disruption treatments were compared using χ^2 tests where appropriate. In this and all other experiments $\alpha = 0.05$.

Experiment 2: Response to Pheromone Blends in Butyl Butyrate-Permeated Atmosphere. Following the design of experiment 1, we tested the specific hypothesis that responses of mullein bugs would shift from a preference for the natural blend of 94:6 BB:CB towards blends containing a higher percentage of BB when they were exposed to atmospheres treated with BB.

Five different 0.25-ha experimental plots (50 × 50 m) were treated as above, with 250 Eppendorf tubes containing 125 μl of BB, and each was paired with an identical control plot. Five traps within a plot were baited as before, with one of five randomly assigned blends consisting of 94:6, 96:4, 97:3, 98:2 or 99:1 BB:CB, respectively. Trap catches were recorded daily, summed and data analyzed as in experiment 1.

Experiment 3: Response to Pheromone Blends in Atmosphere Permeated with Natural 94:6 Butyl Butyrate-Crotyl Butyrate Blend. We tested the specific hypothesis that in an atmosphere permeated with the natural 94:6 BB:CB pheromone blend, mullein bugs might shift their response from this natural blend towards marginally attractive off-ratio pheromone baits (McBrien et al., 1994a) having a higher than natural percentage of BB because they might be less well camouflaged by the 94:6 BB:CB disruptant blend than the natural binary bait.

In mid-August, 1000 polymer lures loaded with a 94:6 blend of BB:CB were hung as before in a 1-ha orchard plot (100 × 100 m). The load and emission rates of these dispensers are on file with Phero Tech, Inc. This plot was part of a study on pheromone-based management of mullein bugs in commercial orchards that was already in progress. Approximately one month after applying the disruption treatment, wing traps were baited with Eppendorf tubes containing 125 μl of one of five randomly assigned binary blends consisting of 94:6, 96:4, 97:3, 98:2, or 99:1 BB:CB. Each binary blend bait was replicated five times within the 1-ha plot by deploying traps in a 5 × 5 Latin square. Traps were at least 20 m from the border rows with 15 m between traps. Using an

identical experimental design, baited traps were deployed simultaneously in a nontreated 1-ha control plot located 2 km away.

Trap catches from the pheromone-treated and control plots were analyzed separately using an ANOVA appropriate for Latin squares (Cochran and Cox, 1957) followed by the SNK test. Because we were limited to one pheromone-treated site, we could not test for a binary bait × disruption treatment interaction using an ANOVA. A χ^2 test was used to compare the distributions of trap catches with binary baits in the treated and control plots.

Experiment 4: Response to High and Low-Release Rate Pheromone Sources in Atmosphere Permeated with Natural 94:6 Butyl Butyrate–Crotyl Butyrate Blend. We tested the final specific hypothesis that in an atmosphere permeated with this natural sex pheromone blend, mullein bugs would shift their response towards pheromone sources releasing the same blend as the disruptant but at a rate higher than the background. A 1-ha plot (100 × 100 m) was treated with a 94:6 blend of BB:CB by hanging 1000 polymer dispensers, our standard mating-disruption treatment, as 500 double-dispenser point sources. Six weeks later, two wing traps were hung 10-m apart and at least 20 m from the plot edge in each of five randomized blocks within the 1-ha plot. Blocks were separated by 20 m. Two baits, one new polymer disruption dispenser (high release rate) and one 6-week-old polymer disruption dispenser (low release rate) were randomly assigned to each pair of traps.

An identical design was used to compare attraction to low- and high-release-rate dispensers in a nontreated control plot located in an orchard at least 2 km from the pheromone-treated plot. The numbers of male mullein bugs captured in each trap were counted after 12 days. Mean trap catches were compared using ANOVA.

RESULTS

Experiment 1: Response to Pheromone Blends in Crotyl Butyrate-Permeated Atmosphere. There was a significant binary bait × disruption treatment interaction ($F_{4,12} = 14.47$, $P = 0.0002$), supporting the hypothesis that mullein bug's interpretation and response to pheromone blends is altered by atmospheric permeation with CB. In a nontreated atmosphere, mullein bugs were significantly more responsive to their natural 94:6 BB:CB pheromone blend than to all other blends tested (Table 1). When the percentage of CB was ≥33% of the blend, trap catches were similarly low.

In a CB-permeated atmosphere, however, response by mullein bugs to blends with percentages of CB ranging from 6- to 43% were not significantly different; only at 50% CB was the response significantly lower than that to a blend with 6% CB.

TABLE 1. COMPARISON OF RESPONSES BY MALE MULLEIN BUGS TO THEIR SYNTHETIC SEX PHEROMONE AND VARIOUS OFF-RATIO BLENDS OF BUTYL BUTYRATE (BB) AND (*E*)-CROTYL BUTYRATE (CB) IN NONTREATED AND CROTYL BUTYRATE-TREATED APPLE ORCHARDS

Binary blend BB:CB	Mean (± SE) number of males caught per trap[a]		Attraction ratio[b]
	Nontreated plots	Crotyl butyrate-treated plots	
94:6 (natural ratio)	130.3 ± 44.7a	68.3 ± 25.5a	0.5
80:20	40.3 ± 21.7b	72.8 ± 28.9a	1.8
67:33	10.8 ± 3.8c	41.5 ± 23.8a	3.8
57:43	6.3 ± 3.1c	32.0 ± 13.8ab	5.1
50:50	6.0 ± 3.2c	20.3 ± 13.3b	3.4

[a]Means ($N = 4$) within a column followed by the same letter are not significantly different (SNK test, $\alpha = 0.05$).

[b]Total catch in treated plots divided by total catch in control (nontreated) plots.

When catches were expressed as an attraction ratio (catch in treated plots/catch in control plots), all binary blends containing above normal percentages of CB were more attractive in a crotyl butyrate-treated orchard than the same blends in a nontreated orchard (Table 1). A bait containing 43% CB, seven times the natural percentage (Smith et al., 1991), was five times more attractive in the crotyl butyrate-treated plots than in the nontreated plots.

Experiment 2: Response to Pheromone Blends in Butyl Butyrate-Permeated Atmosphere. There was no significant interaction of binary bait × disruption treatment ($F_{4,12} = 1.05$, $P = 0.423$) when orchards were treated with BB. In the nontreated atmosphere response by male mullein bugs to blends containing 94–97% BB was not significantly different; at 98 or 99% BB response declined, with the 99:1 blend being least attractive (Table 2). In the butyl butyrate-treated plot there was no difference in trap catches with different baits. Although there was no binary bait × disruption treatment interaction, the frequency distributions of total catches with different binary baits in nontreated and BB-treated plots were significantly different ($\chi^2 = 39.5$, $df = 4$, $P < 0.0001$).

Experiment 3: Response to Pheromone Blends in Atmosphere Permeated with Natural 94:6 Butyl Butyrate–Crotyl Butyrate Blend. In a nontreated atmosphere, the responses of mullein bugs to baits containing percentages of BB higher than that found in the natural blend were similar in experiments 2 and 3, respectively (Tables 2 and 3). In an atmosphere permeated with the natural pheromone

TABLE 2. COMPARISON OF RESPONSES BY MALE MULLEIN BUGS TO THEIR SYNTHETIC SEX PHEROMONE AND VARIOUS OFF-RATIO BLENDS OF BUTYL BUTYRATE (BB) AND (*E*)-CROTYL BUTYRATE (CB) IN NONTREATED AND BUTYL BUTYRATE-TREATED APPLE ORCHARDS

Binary blend BB:CB	Mean (± SE) number of males caught per trap		Attraction ratio[c]
	Nontreated plots[a]	Butyl butyrate-treated plots[b]	
94:6 (natural ratio)	195.3 ± 26.2a	124.8 ± 8.4	0.62
96:4	198.0 ± 31.4a	123.8 ± 25.4	0.62
97:3	202.3 ± 19.6a	145.0 ± 29.3	0.72
98:2	150.3 ± 15.2b	113.8 ± 13.3	0.74
99:1	108.8 ± 31.5c	87.3 ± 24.4	0.80

[a]Means ($N = 4$) in this column followed by the same letter are not significantly different (SNK test, $\alpha = 0.05$).
[b]Means in this column are not significantly different (ANOVA, $P > 0.05$).
[c]Total catch in treated plots divided by total catch in control (nontreated) plots.

TABLE 3. COMPARISON OF RESPONSES BY MALE MULLEIN BUGS TO THEIR SYNTHETIC SEX PHEROMONE AND VARIOUS OFF-RATIO BLENDS OF BUTYL BUTYRATE (BB) AND (*E*)-CROTYL BUTYRATE (CB) IN NONTREATED AND PHEROMONE-TREATED APPLE ORCHARDS

Binary blend BB:CB	Mean (± SE) number of males caught per trap[a]		Attraction ratio[b]
	Nontreated plot	Pheromone-treated plot (94:6 BB:CB)	
94:6 (natural ratio)	110.6 ± 15.9a	1.0 ± 0.5c	0.009
96:4	108.2 ± 14.2a	4.8 ± 2.0b	0.04
97:3	101.2 ± 18.9a	9.6 ± 2.6b	0.095
98:2	81.6 ± 13.4ab	20.4 ± 2.0a	0.25
99:1	63.4 ± 12.9b	19.8 ± 3.7a	0.312

[a]Means ($N = 5$) within a column followed by the same letter are not significantly different (SNK test, $\alpha = 0.05$).
[b]Total catch in treated plot divided by total catch in control (nontreated) plot.

blend, however, the response pattern was the inverse of that in a nontreated atmosphere (Table 3). The 99:1 bait caught 20 times more mullein bugs than did the natural 94:6 bait in an atmosphere treated with a 94:6 disruption blend. The frequency distributions (total trap catches) of responses to binary pheromone baits in nontreated and pheromone-treated plots were significantly different ($\chi^2 = 204.3$, $df = 4$, $P < 0.0001$).

Experiment 4: Response to High- and Low-Release Rate Pheromone Sources in Atmosphere Permeated with Natural 94:6 Butyl Butyrate–Crotyl Butyrate Blend. In the control plot, traps baited with low-release-rate baits of 94:6 BB:CB caught 88.4 ± 20.0 (mean ± SE) mullein bugs similar ($P > 0.05$) to the 100.6 ± 12.2 bugs caught in response to high-release-rate baits. While mean catches with these same baits were significantly lower ($P < 0.05$) in the pheromone-treated plot than in the control plot, mean catches with low (10.8 ± 5.3) and high (11.8 ± 5.7) release-rate baits were not significantly different ($P > 0.05$).

DISCUSSION

When trap catches in experiments 1–3 are expressed as a percentage of catch with the natural 94:6 blend in a nontreated plot, the patterns of pheromone response by male mullein bugs are clearly seen to be altered by exposure to the natural 94:6 blend of BB:CB or CB and BB released individually into the atmosphere (Figure 1). This is the first demonstration of such a phenomenon in the Heteroptera, indicating that similar responses found in the Lepidoptera (Linn and Roelofs, 1981; Flint and Merkle, 1983; Liu and Haynes, 1993) are not unique to that order.

Figure 1 illustrates that the natural pheromone blend is the most effective communication disruptant, causing 99% reduction in response to a 94:6 blend of BB:CB that mimics a female, compared with 48.4 and 35.6% reductions in response caused by CB and BB applied as individual components, respectively.

Shifts in pheromone blend response after exposing mullein bugs to single-component disruptants are probably best explained by the creation of a sensory imbalance (Hirano, 1979; Minks and Cardé, 1988) that leads either to adaptation of antennal receptors or a central nervous system change in the ability to interpret the ratio of pheromone components in a bait. If the sensitivity of receptors for BB and CB or central nervous system response to receptor inputs were lowered by exposure to these individual components (Minks and Cardé, 1988), then mullein bugs should respond to off-ratio blends in predictable ways. Lowering sensitivity to CB by exposure to a CB atmosphere is the same as increasing BB in the blend. Therefore, an 80:20 or 94:6 BB:CB blend in a CB background might be perceived or interpreted as similar to a 99:1 BB:CB blend in a non-

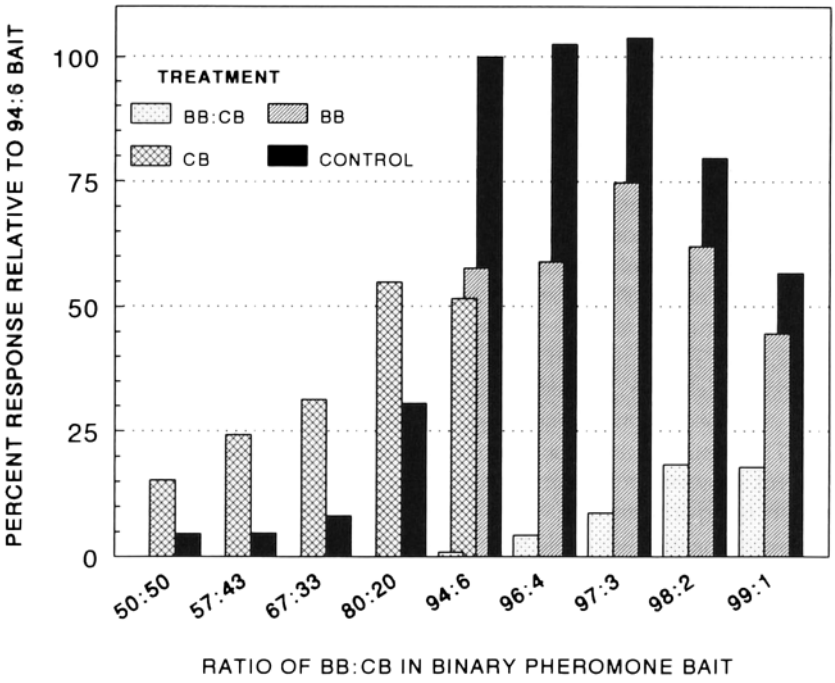

FIG. 1. Summary of relative responses (expressed as a percentage of the catch with the natural 94:6 ratio of BB:CB in nontreated control plots) of male mullein bugs in experiments 1–3 to various ratios of its synthetic pheromone blend in orchards permeated with CB, BB, or the natural 94:6 ratio of BB:CB compared with the pooled responses in nontreated, control orchards.

treated atmosphere and produce similar catches. Likewise, lowering sensitivity to BB by exposure to a BB disruptant is the same as increasing CB in the blend; consequently, a 94:6 blend in a BB background might be perceived as if it were a 90:10 or 80:20 blend in a nontreated atmosphere and produce similar catches. Each of these phenomena appears to occur in the mullein bug (Tables 1 and 2, Figure 1).

The more pronounced alteration of pheromone blend response with CB than with BB (Figure 1) could be caused in part by differential adaptation rates of receptors attuned to each component (Baker et al., 1989). Alternatively, there may be fewer CB receptors than BB receptors on the antennae of mullein bugs, or there may simply be less room for shifting response to BB because it repre-

sents most of the pheromone blend, and we never tested blends having >99% BB.

Unlike pink bollworm, in which a single pheromone component not only altered male response to pheromone blends, but disrupted communication and mating to a level comparable with that caused by the complete blend (Flint and Merkle, 1983, 1984), disruption of mating in mullein bug is probably best achieved with the complete pheromone blend, as the greatest levels of communication disruption were observed with this blend (Figure 1). Our study supports the threshold hypothesis erected by Roelofs (1978) and extended by Minks and Cardé (1988), that the complete pheromone blend should be the most effective disruptant at the lowest application rate. Moreover, it seems plausible that several, if not most, of the postulated mechanisms of communication disruption (Bartell, 1982; Cardé, 1990) may be working against mullein bugs when the natural pheromone blend is used as a disruptant.

The reason for this conclusion is that the complete blend is probably capable of invoking several disruption mechanisms simultaneously. The disruption dispensers in experiment 4 were highly attractive when they were new or six weeks old, indicating that they could have induced false-trail following over an extended period. Continued orientation to disruption dispensers could lead to a depletion of physical drive after an insect flies upwind several times and never mates. Such unrewarded and repeated responses could lead to habituation at a central neurological processing level (Kuenen and Baker, 1981) and, conceivably, to associative learning (Figueredo and Baker, 1992), wherein the male learns there is no female at the end of a synthetic plume and stops responding. Finally, there is evidence of a camouflaging effect. Traps releasing blends most similar to the disruption blend should be camouflaged more effectively than off-ratio blends. In support of this hypothesis, off-ratio blends remained detectable and attracted more mullein bugs than the natural blend (Table 3).

In contrast, the single-component disruptants are not attractive on their own in nontreated backgrounds (Smith et al., 1991; McBrien et al., 1994a) and could not elicit false-trail following. Mullein bugs would be unlikely to become fatigued from flying in response to these baits, and the likelihood of learned responses would be low. According to Minks and Cardé (1988) single components could conceivably camouflage the complete blend. However, if this mechanism were acting in the mullein bug system, a pure BB disruptant should camouflage a similar 99:1 BB:CB bait more effectively than a dissimilar 94:6 BB:CB bait. Based on trap catches, this did not appear to occur. In all likelihood the only mechanism operating in our single-component disruption plots was some form of sensory imbalance.

Modification of pheromone blend interpretation or response after exposure to disruptant components or blends raises some concern about the potential for

resistance to pheromone-based control systems. If the observed modifications of male response were accompanied by genetically based variation in ratios of female-produced pheromone components, resistance to pheromone-based mating disruption may develop quickly in isolated populations. However, the mullein bug is a highly mobile species that migrates between overwintering and summer host species (Thistlewood et al., 1989; McBrien et al., 1994b), allowing outbreeding in the absence of disruption that should counter selection pressure in disrupted orchards. In spite of these potential concerns, this study indicates that the natural 94:6 BB:CB pheromone blend would be the best candidate for commercialization as a mating disruptant for the mullein bug.

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ISOLATION AND IDENTIFICATION OF SEX PHEROMONE OF *Symmetrischema tangolias* (Gyen) (LEPIDOPTERA: GELECHIIDAE)

FRANS C. GRIEPINK,[1,2,*] TERIS A. VAN BEEK,[1] J. HANS VISSER,[2] SIMON VOERMAN,[2] and AEDE DE GROOT[1]

[1]*Laboratory for Organic Chemistry, Wageningen Agricultural University Dreijenplein 8, 6703 HB Wageningen, The Netherlands*

[2]*Research Institute for Plant Protection (IPO-DLO) P.O. Box 9060, 6700 GW Wageningen, The Netherlands*

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Abstract—The sex pheromone of the South American potato tuber moth *Symmetrischema tangolias* (syn.: *Symmetrischema plaesiosema*) was identified as a 2:1 mixture of (*E*,*Z*)-3,7-tetradecadien-1-ol acetate and (*E*)-3-tetradecen-1-ol acetate by means of dual-column GC, EAG, GC-EAD, GC-MS, NMR, and wind-tunnel bioassays. (*Z*)-5-Tetradecen-1-ol acetate and (*Z*)-7-tetradecen-1-ol acetate were also identified in the pheromone gland extract. Male *S. tangolias* were able to detect these acetates (EAG), but their addition to the two-component sex pheromone did not improve attractiveness. Field trials in Cajamarca and Cusco, Peru, showed that traps baited with the synthetic sex pheromone were able to catch large numbers of male *S. tangolias*.

Key Words—*Symmetrischema tangolias*, *Symmetrischema plaesiosema*, Gelechiidae, moth sex pheromone, potatoes, identification, (*E*)-3-tetradecen-1-ol acetate, (*E*,*Z*)-3,7-tetradecadien-1-ol acetate.

INTRODUCTION

The potato tuber moth *Symmetrischema tangolias* (Gyen) (Hodges and Os, 1990; Povolny, 1967), formerly called *Symmetrischema plaesiosema* (Turner) (Sánchez et al., 1986), is a severe pest on potatoes in the field and in storehouses in Peru. *S. tangolias* was first reported in the Montaro Valley, Peru, in 1982

*To whom correspondence should be addressed.

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(Alcázar et al., 1982), although this insect has also been reported in Australia (Osmelak, 1987) and, since 1993, in Bolivia (Alcázar, personal communication). Its main distribution areas are the higher regions of the Peruvian Andes (CIP, 1993). In the field the larvae bore into the stems of potato plants, which causes the plants to break and die. In storehouses the larvae mine into the potato tubers making them unsuitable for human consumption. Nevertheless, the infested tubers are still planted, which causes further spread of the pest. In Peru seed potatoes are generally stored in large storehouses of cooperatives where they are sometimes literally covered with pesticides. Amounts of malathion of 1.3 g/kg potatoes have been observed. In the Peruvian Andes small farmers keep the potatoes indoors or in small open storehouses. These potatoes are not treated with pesticides and are therefore an ideal food for *S. tangolias*. Crop losses can reach up to 100% (Alcázar, personal communication). Today *S. tangolias* is considered to be an even greater pest in Peru than *Phthorimaea operculella* (Zeller), which occurs in the same regions (Ewell et al., 1990). In 1976 Persoons et al. identified the sex pheromone of *P. operculella*, which now is efficient for pest control when applied in mass-trapping (Raman and Booth, 1983). We started the identification of the sex phermone of *S. tangolias* in order to develop a pest control strategy for this moth.

METHODS AND MATERIALS

Insects. The laboratory culture of *S. tangolias* was started with pupae that were collected in a storehouse for potatoes in Cajamarca, Peru, in November 1991. The moths were reared on potato tubers (cv. Bintje) under the following conditions: 22 ± 1°C at day and 17 ± 1°C at night, 65 ± 5% relative humidity, and a 12L:12D photoperiod. The potatoes were provided with small punched holes in which the females could lay their eggs. After four days the tubers, loaded with eggs, were relocated to transparent boxes with a 1-cm layer of clean sand. About 40 days later the pupae were collected, cleaned with a 3% hypochlorite solution and separated into males and females. Moths were fed on a 10% honey solution.

Pheromone Extracts. Pheromone glands from 3-day-old virgin females 7–10 hr into the scotophase were collected. Female moths had been observed to call in this time span. A little more than just the tip of the abdomen was cut off as reported in Attygalle et al. (1987) and kept in redistilled and degassed hexane. The extracts were stored under nitrogen at −20°C. Bioassays were carried out by offering a piece of filter paper (6 × 0.8 cm, Schleicher & Schuell 589[2]) loaded with 2FE (female equivalents) of extact to male moths. Bioactive fractions caused intensive "flutter" responses and attempts to copulate with the filter paper.

Electroantennograms (EAGs). Antennae from 3-day-old males were used for EAG measurements. The antenna was cut off at the base. About four segments of the tip were cut off to enable electrical contact with the recording electrodes. The tip and basal part were connected to glass electrodes filled with a 0.1 M KCl solution. All tetradecen-1-ol acetate isomers used (>99% purity), were obtained from the IPO-DLO pheromone bank (Voerman, 1988). Test cartridges were prepared by applying 100 μl of a 5 PPM solution of the acetate in hexane on a piece of filter paper (6 × 0.8 cm, Schleicher & Schuell 589^2). After evaporation of the solvent, the paper was put into a Pasteur pipet. A Pasteur pipet filled with a filter paper containing 25 μl of a 1% solution (v/v) of (Z)-3-hexen-1-ol acetate (Carl Roth, Karlsruhe, Germany) in paraffin oil (Merck, Uvasol) served as reference stimulus. Silver wires connected the electrodes to the recording instruments: Grass HIP16A input probe, Grass P16D DC-amplifier (rise time: 30 ms), Philips PM3302 oscilloscope, Krenz TRC 4010 transient-recorder and Estate PC AT386. For details on the recording and calculation of EAG data, see Visser and Piron (1995).

Coupled Gas Chromatography–Electroantennographic Detection (GC-EAD). GC-EAD measurements were carried out at Syntech Laboratories, Hilversum, The Netherlands. The GC was a Chrompack 9000 equipped with a split/splitless injection system. Injections were done in splitless mode only. The column was a J&W 12-m DB-5 (5% phenyl methyl polysiloxane), 0.20-mm ID and 0.33-μm film thickness. The sample was equally split between a flame ionization detector (FID) and the EAG detector. Conditions were: carrier gas, helium; inlet pressure, 150 kPA; temperature programming, 100°C (0 min hold) to 220°C (0 min hold) at 10°C/min; injector and detector temperature, 250°C. The EAG recorder, software, IDAC (Intelligent Data Acquisition Controller) interface board for the AT486 PC and other peripheral equipment were manufactured by Syntech Laboratories.

Dual-Column GC. Gas chromatographic analyses were performed on a Hewlett Packard (HP) 5890A equipped with a split/splitless injection system, a 1 : 1 inlet splitter, and two fused silica columns each equipped with an FID. The two columns were a J&W 60-m DB-1 (100% methyl polysiloxane), and a J&W 60-m DB-Wax (polyethylene glycol), both having 0.25-mm ID and 0.25-μm film thickness. Conditions were: carrier gas, hydrogen; inlet pressure, 20 psi; linear velocity, 35 cm/sec; temperature programming, 50°C (0 min hold) to 238°C (8 min hold) at 4°C/min; injector temperature 220°C; detector temperature 260°C. Retention Indices (RI) were calculated according to Kovats (1964); the real retention times instead of the adjusted retention times were used for RI calculations. The RI were calculated by comparing the retention times of the components of interest with those of the C_7–C_{23} alkanes. For the GC spiking experiments, a just-detectable amount of a reference compound was added to the gland extract. Both the extract and the extract combined with the reference

compound were examined by GC. Compounds were considered identified when on both columns the peaks of interest would rise by the same amount.

Preparative GC. Preparative GC was performed on an HP 5890 series II with an HP 7673 automatic sampler. The temperature controlled injection port (controller model 504) and trap unit (controller model 580) were obtained from Gerstel Mulheim, Germany. The column was a 60-m J&W DB-Wax, 0.53-mm ID and 1.0-μm film thickness. Conditions were: carrier gas, hydrogen; inlet pressure, 150 kPa; temperature programming, 150°C (1 min hold) to 230°C (1 min hold) at 5°C/min; injector temperature, 50°C (1 min hold) to 240°C (1 min hold) at 15°C/sec; detector temperature 260°C.

Dimethyl Disulfide (DMDS) Derivatization. Approximately 10 μl of freshly distilled DMDS and 5 μl of 5% iodine in diethylether were added to 20 FE of extract from which the solvent had been evaporated by a gentle stream of nitrogen. The mixture was heated for 16 hr at 60°C in a small airtight flask. The reaction was then stopped by addition of a drop of saturated $Na_2S_2O_3$ solution. The organic phase was separated and concentrated to approximately 10 μl by evaporating the solvent at 40°C in a nitrogen atmosphere (Vincenti et al., 1987; Schulz, 1987).

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). GC-MS was performed on a Finnigan MAT 95 mass spectrometer (70 eV), coupled to a Varian GC equipped with a split/splitless injection system. Injections were done in splitless mode only. The column was a J&W 25-m DB-5 fused silica column, 0.25-mm ID and 0.25-μm film thickness. Conditions were: carrier gas, helium; temperature programming, 50°C (2 min hold) to 100°C (0 min hold) at 25°C/min and then to 280°C (8 min hold) at 4°C/min; injector and transfer-line temperature, 250°C.

NMR. NMR spectra were recorded on a Bruker AC-E 200 at 200 MHz in $CDCl_3$ (100.0% D, Janssen Chimica). Chemical shifts are reported in parts per million (δ) relative to TMS. As internal reference $CHCl_3$ (δ = 7.24) was used.

Synthesis. All chemicals used were of Pro Analyse (PA) quality. 3-Decyn-1-ol was purchased from ABCR Chemie, Karlsruhe, Germany. The *cis* reduction with the P-2 nickel catalyst in ethanol was carried out according to Brown and Ahuja (1973). More than 99% isomeric purity of the synthesized products was achieved through purification on a 200-cm × 0.8-cm Lewatit SP 1080/Ag^+ column (Voerman and Rothschild, 1977). Products were stored under nitrogen at −20°C with 0.1% 2,6-di-*tert*-butyl-4-methylphenol (BHT) as antioxidant (Goto et al., 1974).

Wind-Tunnel Bioassays. Male moths were given a dual-choice situation in the 3.0 (l) × 1.3 (w) × 0.8 (h) m flight section of a wind tunnel, which was entirely constructed from glass and stainless steel (Visser, unpublished data). Two black-painted delta traps (IPO-DLO, Wageningen, Netherlands) were placed at the same height 30 cm apart at the upwind side. Inside each trap a

horizontal strip of filter paper (6 × 0.8 cm, Schleicher & Schuell 589[2]) was suspended. These filter papers were supplied with 100 μl of a 1 ppm solution of a (mixture of) acetate(s) in double-distilled hexane. Conditions were: temperature 17 ± 1°C, 65 ± 5 relative humidity, 12 cm/sec wind speed, and ≤4 mW/m^2 red light. The tests started 2–3 hr into the scotophase with the release of 10–50 male moths at the downwind side. The males were left for 6 hr. The caught males were counted and subjected to the Wilcoxon matched-pairs, signed-rank test (Siegel, 1956). NS means $P > 0.05$ (two-tailed).

RESULTS AND DISCUSSION

Identification. From about 2000 virgin female moths the sex pheromone glands were collected and kept in 10 ml hexane. When exposed to male moths, 2-FE of this extract caused intensive "flutter" responses. The extract was concentrated to approximately 1.5 ml under a gentle stream of nitrogen and fractionated on a 6-ml Baker 10 SPE column packed with 500 mg, 45 μm, 60 Å silicagel. The column was successively eluted with 1.5-ml portions of 0, 2, 5, 10, 20, 50, and 100% of *tert*-butylmethylether in hexane. The column was sucked dry between each fraction. Only the 5% fraction generated a behavioral response. The EAG responses of this 5% fraction and the total extract were not significantly different. From GC-EAD measurements of this fraction, it was concluded that EAG-active peaks eluted during a short period of the entire GC run. The resolution of the GC-EAD equipment made it impossible to discriminate between the four peaks in the gas chromatogram of the active fraction on DB-1 (Figure 1). By comparison of the calculated RI on both the DB-1 and DB-Wax columns with those calculated for reference components from IPO pheromone bank (Voerman, 1988), it became obvious that peaks **2, 3,** and **4** were tetradecen-1-ol acetate isomers. The mass spectra of these peaks were in agreement with this. Peak **1** could be tentatively identified as a nonconjugated tetradecadien-1-ol acetate isomer because of its typical extra difference in RI on the DB-1 and DB-Wax columns. The mass spectrum of **1** (Figure 2) supported this hypothesis. Compounds **2, 3,** and **4** were identified, respectively, as (*Z*)-5-, (*Z*)-7-, and (*E*)-3-tetradecen-1-ol acetate by comparing the MS and the RI with those obtained from reference compounds. GC spiking experiments provided additional evidence (Figure 3). About 10 μg of the two main peaks, **1** and **4,** were isolated with preparative GC, which proved sufficient for NMR analysis. The NMR spectrum of **4** was identical to that of synthetic (*E*)-3-tetradecen-1-ol acetate. The NMR spectrum of **1** showed the same signal at 2.29 PPM as the NMR spectrum of (*E*)-3-tetradecen-1-ol acetate, which is characteristic for a double bond at the 3-position (Figure 4). Comparison of the olefinic part of the NMR spectrum of **1** with those of synthetic (*Z*)-3- and (*E*)-3-tetradecen-1-ol

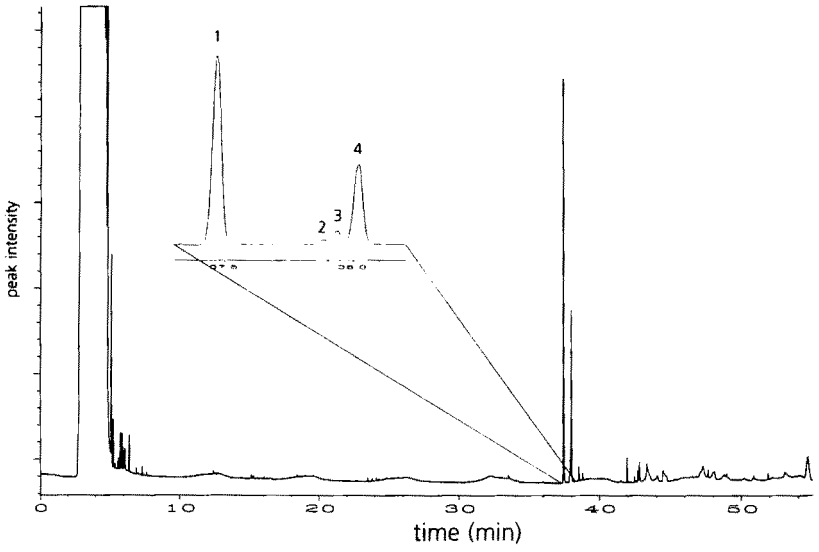


FIG. 1. Gas chromatogram of the biologically active fraction "5%" (splitless 2 μl, on the 60-m DB-1 column). The inset displays the time interval where EAG activity has been observed. For explanation of peak numbers 1–4, see text.

acetate suggested that the double bond at the 3-position of **1** has the *trans* configuration. The extract was derivatized with DMDS to get information about the position of the second double bond. The mass spectrum of the DMDS-derivative of **1** is shown in Figure 5. The fragmentation pattern suggested **1** to be a 3,7-tetradecadien-1-ol acetate isomer. To determine the *cis/trans* configuration of the double bonds of **1**, all tetradecen-1-ol acetate isomers were tested with EAG (Figure 6). The difference in response between the (*E*)-7- and the (*Z*)-7- isomer suggested the (*E*,*Z*)-3,7- stereochemistry for **1**. This was proven

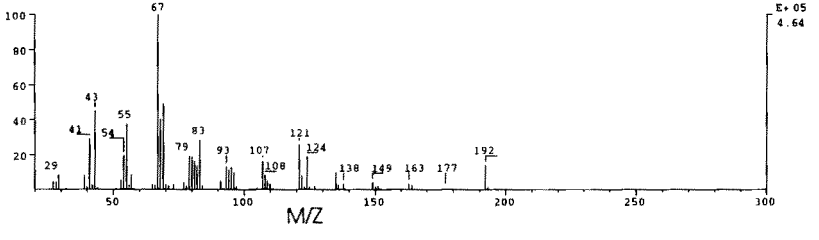


FIG. 2. Mass spectrum of compound **1**. The molecular ion (M^+) could not be detected.

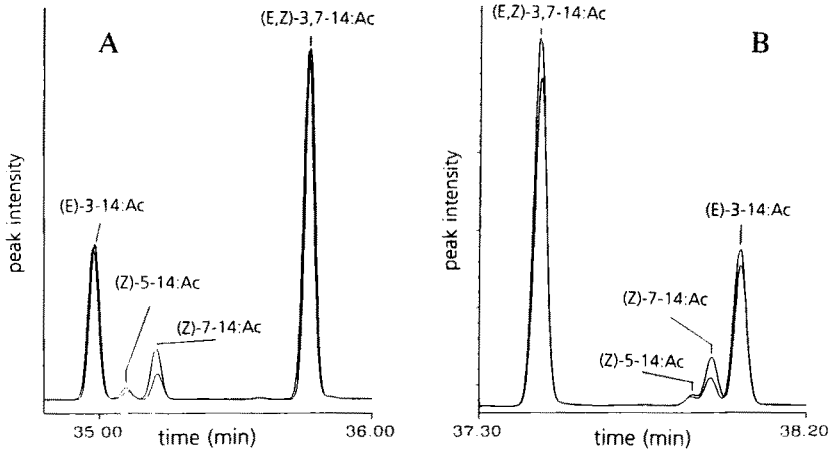


FIG. 3. GC spiking chromatograms of the sex pheromone extract with and without additional (Z)-7-tetradecen-1-ol acetate; (A) on the DB-Wax column, (B) on the DB-1 column.

by synthesis. (*E*,*Z*)-3,7-Tetradecadien-1-ol acetate was synthesized according to Scheme 1 in an isomeric purity of more than 99% (GC). ^{1}H NMR: δ 0.86 (br t, 3H, J = 6.5 Hz; H-14), 1.26 (br m, 8H; H-10 to H-13), 2.03 (s, 3H; O_2CMe), 2.03 (br m, 6H; H-5, H-6 and H-9), 2.29 (dt, 2H, J = 6.9, 6.9 Hz; H-2), 4.04 (t, 2H, J = 6.9 Hz; H-1), 5.39 (m, 4H; H-3, H-4, H-7 and H-8)

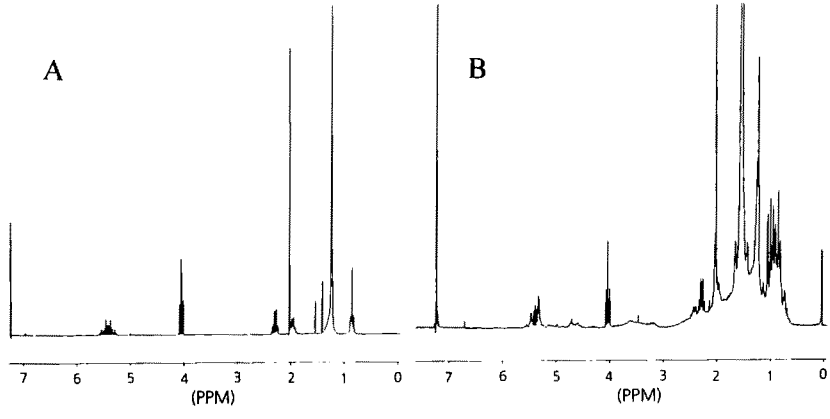


FIG. 4. (A) NMR spectrum of (synthetic) (*E*)-3-tetradecen-1-ol acetate; (B) NMR spectrum of compound **1**.

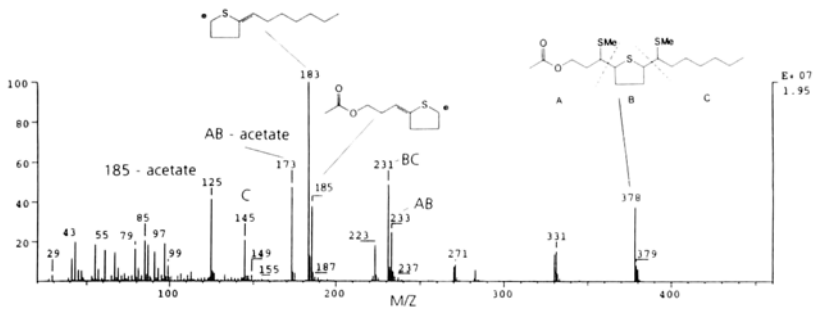


FIG. 5. Mass spectrum of the DMDS derivative of compound **1**. Molecular ion (M^+) at *m/z* 378.

^{13}C NMR ($CDCl_3$, 125 MHz); δ 14.0 (q), 20.9 (q), 22.6 (t), 27.0 (t), 27.2 (t), 28.9 (t), 29.6 (t), 31.5 (t), 31.9 (t), 32.6 (t), 64.0 (t), 125.3 (d), 128.7 (d), 130.4 (d), 132.8 (d), 171.0 (s). Both MS and ^1H NMR spectra of the synthetic (*E*,*Z*)-3,7-tetradecadien-1-ol acetate were identical to the spectra of **1**. The overall yield of the synthetic route was only a few percent, mainly due to a side reaction in step iii, where 1-bromo-(*Z*)-3-decene predominantly eliminates HBr

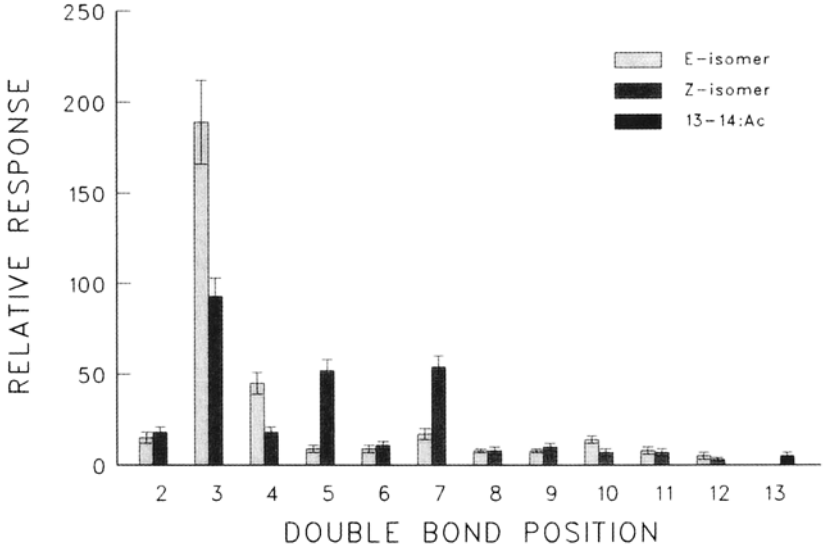
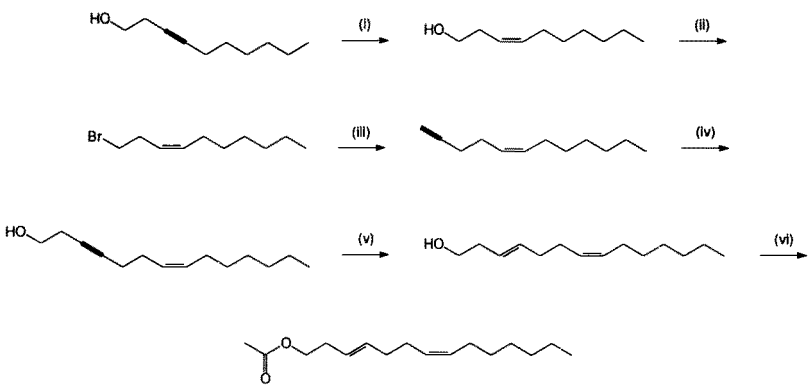


FIG. 6. Electroantennogram of all tetradecen-1-ol acetate isomers. Bars indicate 95% confidence intervals. $N = 15$.



SCHEME. 1. Synthetic route to (*E*,*Z*)-3,7-tetradecadien-1-ol acetate (**1**). Reagents: (i) P-2 Ni/ethanol; (ii) $CBr_4/Ph_3P/THF$; (iii) lithium acetylide–ethylene–diamine complex/THF/HMPA; (iv) ethyleneoxide/*n*-BuLi/HMPA; (v) $LiAlH_4$/THF/diglyme; (vi) Ac_2O/HOAc.

under formation of the diene rather than to couple with the lithium acetylide. More efficient routes for the synthesis of **1** are under investigation.

Behavioral Bioassays. Different mixtures of identified components were tested in the wind tunnel. Although the ratio of the four identified compounds in the gland extract was: (*E*,*Z*)-3,7-(*E*)-3-:(*Z*)-7-:(*Z*)-5 = 62:31:6:1, best results were obtained with a mixture containing two components, namely (*E*,*Z*)-3,7-tetradecadien-1-ol acetate and (*E*)-3-tetradecen-1-ol acetate. No significant difference in attractiveness could be determined between 2:1 and 1:1 mixtures of these two components. Addition of the two minor compounds (*Z*)-7-tetradecen-1-ol acetate and (*Z*)-5-tetradecen-1-ol acetate in the same ratio that had been found in the gland extract did not influence the attractiveness of the mixture. The addition of 10% of (*Z*)-5-tetradecen-1-ol acetate decreased the attractiveness of the mixture. The function of the (*Z*)-5- and (*Z*)-7-tetradecen-1-ol acetates, which were detected in the gland extracts, therefore remains unclear. It may be that the trap catches in the wind tunnel bioassays were not sensitive enough to reveal behavioral differences.

The 2:1 mixture of (*E*,*Z*)-3,7-tetradecadien-1-ol acetate and (*E*)-3-tetradecen-1-ol acetate was tested in potato fields and storehouses in Peru. Catches up to 120 and 450 individuals per night per trap for field and storehouse experiments, respectively, were achieved. The field catches emphasized the great attractiveness of the synthetic pheromone blend since no *S. tangolias* could be detected by us in an extensive search of the potato field and the surrounding areas. The catches in the storehouses dropped considerably after the first couple of days. Replacement of the traps by new ones did not improve the catches.

This means that traps indoors are able to diminish pest populations quite rapidly and may be efficient for pest control in storehouses through mass-trapping of pest insects. Large-scale field experiments will be carried out in Peru in the near future in order to determine the efficiency of the synthetic pheromone in the control of *S. tangolias*.

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EGG DISPERSION IN CODLING MOTH: INFLUENCE OF EGG EXTRACT AND OF ITS FATTY ACID CONSTITUENTS

DENIS THIÉRY,[1,*] BRUNO GABEL,[1,4] PAVEL FARKAS,[2] and MARC JARRY[3]

[1]*Laboratoire de Neurobiologie Comparée des Invertébrés, INRA-CNRS (URA 1190), BP 23, 91440 Bures sur Yvette, France.*

[2]*Food Research Institute Priemyselna 4, 82 006 Bratislava, Slovakia*

[3]*Laboratoire de Mathématiques Appliquées, Université de Pau et des Pays de l'Adour, 64 000 Pau, France*

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Abstract—Codling moth females (*Cydia pomonella*, Lepidoptera: Tortricidae) (CM) usually lay single eggs and have a tendency to disperse. In a first experiment we observed that single females exposed to 20 apples distribute their eggs regularly among apples, suggesting a dispersive oviposition behavior. In a dual-choice situation, isolated females avoided oviposition on areas of cardboard treated with a methylene dichloride egg extract at the dose of ca. 1.0 egg equivalent/cm^2. A strong avoidance was obtained in response to a 20-fold dose, which was accompanied by a significant reduction of total oviposition. Seven major compounds found by GC analyses in the methylene dichloride extract of 2 to 3-day-old eggs were saturated or unsaturated C_{14}-C_{18} straight-chain fatty acids: myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. This was confirmed by GC-MS analysis of an extract made by ethyl ether. Smaller amounts of three methyl esters were also identified as methyl myristate, methyl palmitate, and methyl stearate. A blend of the seven fatty acids (FA) mimicked rather well the avoidance provoked by the extract, and this avoidance was confirmed by choice between treated and untreated fruits by single females. The treat-

*To whom correspondence should be addressed.

[4]Present address: Research Institute for Viticulture and Enology, Matuskova 25, 83311 Bratislava, Slovakia.

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ment of apples with the 7FA mixture induced an aggregative distribution of the eggs among apples. We also found that the amounts of fatty acids harvested in the extract depend on the egg age. Amounts of fatty acids increased until eggs were 4 days old and then decreased before hatching. In this paper we discuss the possible role of simple molecules, such as fatty acids and their esters, as semiochemicals indicative of juvenile tissues.

Key Words—Behavior, *Cydia pomonella*, oviposition, pheromone, synomone, insect, offspring dispersion, fatty acids, egg age, Lepidoptera, Tortricidae.

INTRODUCTION

Investigations on oviposition deterrents relative to the dispersion of progeny yield fascinating information on host-plant selection behaviors by phytophagous insects. Reproductive strategies involve factors that influence how progeny are distributed over space and time. In many cases perception by the female of information produced by eggs or larvae is of primary importance to adjust population level to the size of available resources. Assessment of oviposition sites and egg load by ovipositing females and avoidance of plants occupied by conspecifics have been reported in Lepidoptera [Urquhart, 1960 quoted in Chew and Robbins, 1984; MacLellan, 1962, Rothschild and Schoonhoven, 1977], and more generally in a wide variety of phytophagous insects (Prokopy, 1981; Messina and Renwick, 1985; Anderson, 1986; Roitberg and Prokopy, 1987). This behavior involves intraspecific chemical cues (Hurter et al., 1987; Imai et al., 1990) or both intra- and interspecific ones (Schoonhoven, 1990; Thiéry and Gabel, 1993; Blaakmer et al., 1994). In the latter category, simple chemicals (fatty acids and derivatives) deter oviposition in several lepidopteran species (Thiéry and Le Quéré, 1991; Thiéry et al., 1992; Gabel and Thiéry, 1994), which led us to propose that fatty acids and/or derivative esters exist on the eggs of several lepidopteran species and may allow interspecific recognition (Thiéry and Gabel, 1993).

The codling moth (CM), *Cydia pomonella* (Lepidoptera: Tortricidae), is an important pest of orchards, where it causes severe damage on apples and other fruits. Female CM normally deposit single eggs close to fruits (on bark or leaf) or directly on fruit. Eggs are always well distributed and more than one egg per single fruit or leaf occurs only occasionally (Geier, 1963; Wood, 1965; Jackson, 1979). As a possible result of that, more than one larva per apple is seldom found (Ferro and Harwood, 1973). Although Roitberg and Prokopy (1982) were unsuccessful in characterizing the occurrence of an oviposition deterring pheromone in CM, these reports motivated studies on egg dispersion in CM, with a dispersive oviposition behavior being hypothesized as the consquence of the moth perceiving fresh eggs (MacLellan, 1962). We

recently observed that CM females avoid ovipositing on apples treated with fatty acids and methyl esters isolated from *Lobesia botrana* at a dose of 72 egg equivalents per apple (Gabel and Thiéry, 1994).

The present study was intended to determine if simple chemicals acting as general oviposition deterrents occurred in CM eggs. The study had three objectives: (1) to quantify the distribution of eggs on apples under conditions of limited resource, (2) to verify the behavioral response of CM females to an extract of conspecific eggs, and (3) to determine the chemical constitution of this extract and the effect of egg age on its chemical constitution.

METHODS AND MATERIALS

Insects originated from a laboratory colony (strain: INRA Zoologie, Montfavet, France) reared for more than 20 generations on artificial diet (Guennelon et al, 1981) in climate chambers regulated with the following conditions: 16L : 8D photocycle regime (22 ± 0.5°C, 85 ± 5% relative humidity during photophase and 20 ± 0.5°C, 75 ± 5% relative humidity during scotophase). Behavioral observations were made with 2-day-old mated females. Mating was obtained by grouping 10 females and 10 males for two days in arenas with access to honey and water. Apples (cv. Golden Delicious) were harvested just before trials from an orchard free of pesticides, and those free of CM attacks and with horizontal diameter between 5 and 5.5 cm were used.

Extraction and Analysis.

Two- to Three-Day-Old Egg Extracts. Females were forced to oviposit on clean glass slides for two consecutive nights. Eggs were then counted and glass slides with eggs were washed either in methylene dichloride (12,000 eggs) or in ethyl ether (4200 eggs). Moth scales were removed by passing the extracts through a Millipore filter (GW, 0.22 μm). The extract was then evaporated under nitrogen flow. An aliquot was devoted to GC analysis, and another aliquot devoted to bioassays was evaporated to dryness and then diluted according to the required concentration for application.

Effect of Egg Age. Age of eggs was studied by harvesting eggs during one night and extracting them with methylene dichloride as described above. We had four batches of 2000–2500 eggs each. One batch was immediately extracted (age < 12 hr), and the three others were kept in an incubator (climate conditions described above) for two, four, or six days before extraction.

Chemical Analysis. Analyses of the extracts were performed by gas chromatography (GC) with no prior treatment except filtration to remove scales as described above. GC, either coupled or not to a mass spectrometer (MS), was equipped with an HP Ultra 1 column (0.20 mm ID × 25 m) and a flame

ionization detector. The temperature program started at 60°C with 2 min isothermal and then linearly increased by 5°C/min to 240°C with 10 min isothermal at the end. Mass spectra were obtained with an HP 5971 A mass selective detector with positive 70 eV ionization. Spectra were compared to an MS data bank and spectra obtained from pure standards.

Bioassays

All bioassays were conducted in climatic chambers under conditions described above.

Bioassay 1: Distribution of Eggs on Untreated Apples. To check the distribution of eggs on apples, one female and two males were offered during a night 20 equidistant apples positioned in four rows of five apples on the floor of an arena designed to prevent oviposition anywhere else than on apples (60 × 40 cm, 30 cm high). Twenty replicates were performed.

Bioassay 2: Oviposition Responses to Extract and Synthetic Blend on Cardboard. Gravid females were isolated (no males) in an altuglass tube (6.5 cm ID, 13 cm long) and could only oviposit on a smooth cardboard providing treated areas (32 spots of 1.1 cm^2 each); the remaining area (109 cm^2) was untreated (Gabel and Thiéry, 1992). The extract was diluted for application with 6 parts acetone and 4 parts purified water (API). Treatments were made by applying on the cardboard 32 equidistant wells (ca. 1.1 cm diam.) and filling each with 5 μl of the solution of test (Gabel and Thiéry, 1992). After complete evaporation of the solvents, the cardboards were exposed to females. We determined the response either to API or to the following quantities of extract expressed in egg equivalents per spot: 1 egg eq. (A) and 20 egg eq. (B). Females were individually exposed the first night to a blank carboard, then on the second night to cardobard impregnated with treatment spots (API, extact, or synthetic blend), and on the third night to blank cardboard. The 3% of females that did not oviposit during the three consecutive nights were considered as unmated and then disregarded. The number of eggs deposited on treated areas (T) and untreated areas (UT) were counted, eggs in contact with boundaries being considered as T. We calculated for each female an avoidance index corrected to equivalent surface area (UT = 3T): Ai% = (UT − 3T)/(UT + 3T) × 100 (Gabel and Thiéry, 1992), which may vary from 100% (maximum avoidance) to −100% (maximum preference). An identical procedure was performed to verify the activity of a synthetic blend corresponding to seven identified fatty acids (7FA). Pure chemicals (GC standards, Sigma Ltd.) were dissolved in pure acetone (see Table 3 below for ratios) and the dose was adjusted in API to the higher dose of extract used (20 egg eq./spot).

Bioassay 3: Test of Synthetic Blend on Apples This bioassay was conducted in the same arenas as bioassy 1, with one female and two males per arena.

These experiments could not have be done with extracts of eggs because of the enormous number of eggs required. To prevent damaging effects of acetone on apple cuticles, we used a second solvent or application phase, AP2, consisting of 1 part acetone, 8.9 parts purified water, and 0.1 part wetting agents (Triton X-100, Prolabo). Apples were rapidly dipped into the treatment solution of 7FA (same proportions as in bioassay 2) dissolved in AP2. Oviposition assays were conducted either in dual-choice or no-choice situations. In the choice situation, females were exposed to two rows of five treated apples (7FA) alternating with two rows of apples treated with solvent only (AP2) (15 replicates). In the no-choice situation, all apples were dipped in the 7FA solution (18 replicates). The numbers of eggs on treated and control apples (C) were counted. We calculated in each experimental arena a simple avoidance index: $\text{Ai}\% = (C - T)/(C + T) \times 100$ (where T is the number of eggs on treated sites and C on control sites with C and T representing equal areas). This index is equivalent to that described above.

Statistics

Bioassays 1 and 3 (No-Choice Situation). Egg distribution on apples was analyzed using a nonparametric index of dispersion as described in Chessel (1978). The variable

$$D = \sum_{i=1}^{n} y_i^2,$$

where y_i is the number of eggs on apple i, describes the variability in the distribution of p eggs ($p = \Sigma_{i=1}^{n} y_i$) among n apples. D tends towards a maximum when eggs are concentrated on a single apple and towards a minimum when eggs are regularly dispersed. If the eggs are distributed randomly (null hypothesis), then the chance that a given egg is laid on apple i equals $1/n$. This hypothesis assumes that eggs are laid independently of each other and that apples have an equal chance of receiving an egg. Under this hypothesis, the mean (E) and the variance (V) of D equal:

$$\begin{cases} E(D) = \dfrac{p}{n}(n + p - 1) \\ V(D) = \dfrac{2p}{n^2}(p - 1)(n - 1) \end{cases}$$

For N replicates the following index of dispersion (Id) can be obtained:

$$Id = \frac{1}{\sqrt{N}} \sum_{i=1}^{N} \frac{D_i - E(D_i)}{\sqrt{V(D_i)}}$$

and the value of $Idc_i = [D_i - E(D_i)/\sqrt{V(D_i)}]$ represents the contribution of replicate i to Id. In practice, fitting the distribution of Id to a standard normal distribution is valid when $N \geq 15$.

At the level $\alpha = 0.05$, we may reject the null hypothesis when $Id > 1.96$ (aggregative distribution) or when $Id < -1.96$ (regular distribution).

Bioassays 2 and 3 (Choice Situation). Data are presented as mean numbers of eggs and mean Ai(s) per female. Nonparametric statistics were used: Mann-Whitney U test (M-W) for comparisons between experimental groups and Wilcoxon matched-pair signed-ranks test (WX) for comparisons within groups (oviposition during consecutive nights). Numbers of eggs laid on treated and untreated apples (bioassay 3) were compared by χ^2 analysis.

RESULTS AND DISCUSSION

Distribution of Eggs on Untreated Apples. Females laid a mean of 10.7 ± 4.7 eggs per arena. Among the 400 apples, 50% had no egg, 39% contained a single egg, and only 6% had more than one egg, with one apple carrying seven eggs. The global index of dispersion calculated from the 20 replicates ($Id = -1.54$) did not permit excluding a random distribution of eggs. However, 16 experimental arenas had a negative value of Id_{ci}, which indicates a tendency for egg dispersion. One female laid more eggs than the number of available apples and distributed the eggs in a completely different pattern than the 19 other females. This female constituted the most important contribution to $Id(5.83)$. After excluding this arena, $Id = -2.94$ allowed us to reject the hypothesis of a random distribution with $P < 0.003$. This regular distribution suggests a dispersive oviposition behavior which is consistent with previous analysis of egg dispersion in orchards, in which egg distribution among spurs, within spurs, and on leaves deviated from randomness (estimated as a Poisson distribution) (Jackson, 1979). In our experiment, the large number of apples bearing no or one egg suggest a dispersive oviposition behavior.

Oviposition on Cardboard Before and After Exposure to Treatment. The mean numbers of eggs laid per female are presented for the three consecutive nights of the experiment (Table 1). During the night before any exposure, the numbers of eggs were remarkably constant among experimental groups and varied from 36.2 ± 15.9 to 30.3 ± 15.1, which was very close to that observed in previous experiments (32.5 ± 15.1; Thiéry and Gabel, 1993). In all groups, this number regularly decreased by one third each night (difference between two consecutive nights: WX, $P < 0.001$). On night 3, values were similar between groups (from 9.4 ± 8.5 to 11.9 ± 4.7 eggs) no matter what the exposure on the second night was. As in other tortricids, mating stimulates egg production by CM; here females were exposed to mates prior to the observations and no

TABLE 1. EGGS (MEAN ± SD) LAID ON ARTIFICIAL SUBSTRATE BY *Cydia pomonella* FEMALES[a]

Treatments	Females (*N*)	Night 1, blank cardboard	Night 2, treated and untreated areas	Night 3, blank cardboard
Application solvent	22	33.2 ± 15.9a	21.7 ± 11.2b	10.1 ± 9.5c
Extract A	23	31.2 ± 12.5a	19.1 ± 8.8b	9.4 ± 8.5c
Extract B	15	36.2 ± 15.9a	15.6 ± 5.4c	10.2 ± 7.6c
7FA B	15	30.3 ± 15.1a	23.2 ± 9.9b	11.9 ± 4.7c

[a]During night 2, females had a choice between treated areas and an untreated area. Dose A = 1 egg eq./spot, dose B = 20 egg eq./spot. Seven fatty acids (FA) were tested at dose B. Different letters indicate statistical differences based upon Wilcoxon matched-pairs at $P < 0.001$ within lines and Mann-Whitney U test, $P < 0.04$ within columns.

additional mating occurred. The reduction in egg laying was thus considered as normal.

Response to Egg Extraction on Cardboard. The application phase (API) alone had no effect on the oviposition preference (Ai = −1 ± 24%) (Table 2), with a total of 120 eggs being laid on areas treated with API and 362 eggs on the untreated area. This is in aggreement with observations based on other solvents (pure methanol or methanol in water) (Thiéry and Gabel, 1993; Gabel and Thiéry, 1994). Females avoided ovipositing on the application spots of extracts, and this behavior was dose dependent (Table 2). Dose A (1 egg eq./spot) induced a slight avoidance: the 23 females laid a total of 66 eggs on treated

TABLE 2. OVIPOSITION PREFERENCES OF *Cydia pomonella* FEMALES BETWEEN AREAS OF CARDBOARD TREATED WITH DIFFERENT SUBSTANCES AND UNTREATED AREA[a]

Type of exposure and doses	*N*	Eggs per female (Mean ± SD)		Avoidance index (%, ± SD)
		Treated areas	Untreated area	
Control (API)	22	5.5 ± 2.9	16.5 ± 8.8	1 ± 24a
Extract A	23	2.9 ± 1.8	16.2 ± 7.3	34 ± 22b
Extract B	15	0.9 ± 0.8	14.6 ± 4.9	75 ± 23c
7FA B	15	1.5 ± 0.6	21.7 ± 9.4	66 ± 8c

[a]API was application solvent, egg extract dose A (1 egg eq./spot), egg extract dose B (20 egg eq./spot), and 7FA is a synthetic blend of seven fatty acids at dose B. Data are means ± SD of *N* observed females, different letters indicate statistical differences among avoidance indexes at $P < 0.001$, Mann-Whitney U test).

areas and 372 on the untreated area. The mean Ai (34 ± 22%) differs statistically from that observed in response to AP1 (M-W U test, $P < 0.0002$). At dose B (16 egg eq./spot), the 15 females laid 13 eggs on the treated areas and 219 on the untreated area. The mean Ai (75 ± 23%) differed from the previous dose (M-W U test, $P < 0.0005$) (Table 2). Six of the 15 females accounted for 68 eggs, and they laid no egg on treated areas. Females exposed to dose B of extract laid fewer eggs that females exposed to AP1 (15.6 ± 5.4 vs. 21.7 ± 11.2; MW, $P < 0.04$) (Table 1).

The avoidance of CM females to its egg extract is higher than that observed in the tortricid (*L. botrana*) exposed to its own extract (Ai = 50%; Gabel and Thiéry, 1992). In the present work, avoidance by *C. pomonella* was already evident at the dose of 1 egg eq./spot (32 egg eq./arena), whereas a weaker response by *L. botrana* required three times more egg equivalents.

Identification of Active Compounds. Seven major compounds were found in the GC analysis of 2 to 3-day-old eggs (Table 3). These molecules are C_{14} to C_{18} saturated or unsaturated straight-chain fatty acids. We found in the methylene dichloride extract myristic acid ($C_{14:0}$), plamitic acid ($C_{16:0}$), palmitoleic acid (Z9)($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid (Z9)($C_{18:1}$), linoleic acid (Z9,Z12)($C_{18:2}$), and linolenic acid (Z9,Z12,Z15)($C_{18:3}$) in the amounts shown in table 3. Slightly lower amounts of each of these seven fatty acids were found in the ethyl ether extract (except for $C_{14:0}$) (Table 3). Three methyl esters were detected in the ethyl ether extract. These were methyl myristate (Me $C_{14:0}$), methyl palmitate (Me $C_{16:0}$), and methyl stearate (Me $C_{18:0}$) (Table 3). Only the Me $C_{16:0}$) and the Me$C_{18:0}$ were detected in the methylene dichloride extract.

TABLE 3. FATTY ACIDS AND RELATED ESTERS EXTRACTED FROM 2 TO 3-DAY-OLD *Cydia pomonella* EGGS AS IDENTIFIED BY GC-MS ANALYSIS[a]

Compound		Methylene dichloride (ng/egg eq.)	Ethyl ether (ng/egg eq.)
Myristic acid	$C_{14:0}$	4.8	0.5
Palmitic acid	$C_{16:0}$	13.6	11.2
Palmitoleic acid	(Z9) $C_{16:1}$	2.7	1.8
Stearic acid	$C_{18:0}$	7.3	3.7
Oleic acid	(Z9) $C_{18:1}$	15.3	10
Linoleic acid	(Z9, Z12) $C_{18:2}$	16.6	11.7
Linolenic acid	(Z9, Z12, Z15) $C_{18:3}$	9.1	3.7
Methyl myristate	$MeC_{14:0}$	0	0.015
Methyl palmitate	$MeC_{16:0}$	0.024	0.67
Methyl stearate	$MeC_{18:0}$	0.01	0.02

[a] Amounts are expressed in ng per egg equivalent; numbers in parentheses are double bond positions.

Remarkably, we found the same kind of fatty acids as in *L. botrana*, the two major compounds [$C_{16:0}$ and $(Z9)C_{18:0}$] being identical to both species (Thiéry et al, 1992; Farkas, Gabel, and Thiéry, unpublished data), although we found them in higher amounts in CM. Methyl palmitate and stearate have also been identified as oviposition deterrents in *Ostrinia nubilalis* (Thiéry and Le Quéré, 1991).

Biological Activity of Synthetic Blend of Fatty Acids. We have verified the biological activity of a synthetic blend made according to the amounts of the seven fatty acids found in the 2 to 3-day-old egg extract. The exposure of 15 females to a dose of the seven fatty acids equal to 20 egg eq./spot evoked a mean Ai of 66 ± 8%, which was not statistically different from the extract at an identical dose (M-W U test) (Table 2). A total of 20 eggs were laid on treated areas and 326 on untreated ones. Females in total laid more eggs (23.2 ± 9.9) than when exposed to the extract applied at an identical egg equivalent dose (15.6 ± 5.4) (M-W U test, $P < 0.03$). The first value was similar to that observed in the control treatment (Table 1).

In previous work, CM females exposed to a synthetic blend of three acids [$C_{16:0}$, $C_{18:0}$ and $(Z9)$ $C_{18:1}$] and six esters [Me $C_{16:0}$, Me $(Z9)$ $C_{16:1}$, Me $C_{18:0}$, Me $(Z9)$ $C_{18:1}$, Me $(Z9,Z12)$ $C_{18:2}$, and Me $(Z9,Z12,Z15)$ $C_{18:3}$] significantly reduced their oviposition (Thiéry and Gabel, 1993), suggesting that esters of fatty acids could be candidates to explain such a reduction.

Responses of Females to Apples Treated with a Blend of Fatty Acids. Females exposed to 10 apples treated with the blend of fatty acids (7FA) and to 10 untreated apples laid more eggs (20.1 ± 11.4 eggs) than those exposed to 20 untreated apples or those exposed to 20 treated apples. They strongly avoided the treated apples. A total of 38 eggs were found on the treated apples and 323 on the untreated ones ($\chi^2 = 225$, 1 df, $P < 0.0001$), producing a mean Ai of 82 ± 12%. In eight of the 14 replicates, the Ai value was betweend 80% and 100%.

The 18 females exposed to uniform treatment aggregated their eggs among certain apples. Thirteen values of Id_{ci} were strongly positive, and after excluding one female that laid a single egg, the global index of dispersion ($Id = 15.56$) allowed us to reject the null hypothesis of a random distribution at $P < 0.0001$. The aggregation of eggs is probably due to a perturbation during oviposition bouts. It might also be the result of modification in locomotion patterns because of repulsion. Interpretation of the increased oviposition in the dual-choice situation is difficult. One possibility is that, because of restricted areas, the number of eggs laid is related to the time spend on apples. Short-range repulsion provoked by uniformly treated apples might have reduced the time invested in oviposition site selection, while in dual-choice assays females setting for longer periods on untreated apples would lay more eggs. In the present experiments, females laid almost 30% fewer eggs when exposed to apples than when exposed

to cardboard. One possibility is that preoviposition sequences are more complete and may last longer on apples than on artificial substrates. This needs, however, detailed quantification of flying, preoviposition and oviposition bouts.

Age-Related Composition in Fatty Acids. We focused on the variation in concentration of the seven fatty acids identified from the methylene dichloride egg extracts. Methylene dichloride was used to study this variation because it extracted a larger amount of the seven acids (Table 3). In each age class, plamitic, oleic, and linoleic acids were always the major compounds present and were present in almost equal proportions (Figure 1). The amount of all fatty acids increased until day 4 and then decreased just before hatching. The maximum increases was observed between 2-day-old and 4-day-old eggs, where it ranged from 80% ($C_{14:0}$) to 300% ($C_{18:0}$).

The mucus that coats the eggs contains free fatty acids and esters that contribute to retardation of dessication. Egg extraction certainly harvests large amounts of chemicals from the surface, but also extracts constituents from within the egg yolk. The exact proportion of both chemicals has not been determined. The permeability of the egg chorion might allow natural passage of chemicals to the surface and could to some extent allow relations between the external and internal constitutions. Anecdotally, the large increase in the amount of linoleic and linolenic acids is noteworthy since de novo synthesis of these fatty acids has been seldom reported in insects. We can not exclude these FA as being liberated from phospholipids or triglycerides stores. From an ecological point of view, the variation in amount of oviposition regulators released may represent dynamic information to CM because it is related to egg age. Probably because

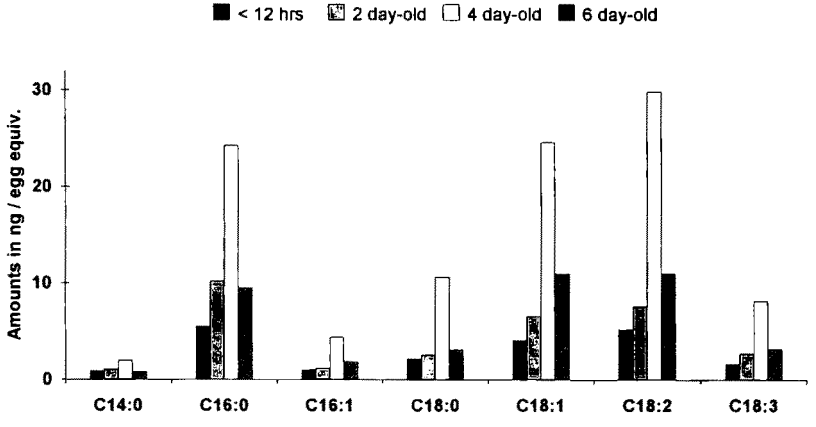

FIG. 1. Variation in the amount of fatty acids from methylene dichloride extracts of *Cydia pomonella* eggs of different ages.

of their simplicity and general occurrence, fatty acids and derivative esters have not drawn very much attention in insect communication. However, the literature provides interesting insights relative to their role as semiochemicals (Hwang et al., 1984; Rickli et al., 1992; Trouiller, 1993).

The present study is a first step towards demonstrating that chemical information produced by CM eggs regulates the oviposition site selection of conspecific females. This study has been conducted with CM reared for generations on artificial diet, and one might expect females from natural populations to be more egg dispersive than mass-reared ones. Such a comparison between natural and laboratory-reared populations has not yet been done.

We have identified free fatty acids in CM eggs whose amounts vary according to the egg developmental stage. This finding does not exclude that species-specific and more sophisticated chemicals may also influence egg dispersion. The results reported here reinforce the hypothesis that oviposition can be deterred by general chemical markers of juvenile tissue (eggs or larvae) (Thiéry and Gabel, 1993). Eggs of several moths (*O. nubilalis*, *L. botrana*, and CM) have characteristic odors very close to that of several fatty acids and esters of the fatty acids. The fact that C_{16} acids and esters released electroantennographic responses in *O. nubilalis* females (our unpublished data) suggests to us that long-chain fatty acids and related esters produce odors that might be involved in the perception of occupied oviposition substrates at a short range distance.

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OPTICAL ISOMERS OF 3,13-DIMETHYLHEPTADECANE: SEX PHEROMONE COMPONENTS OF THE WESTERN FALSE HEMLOCK LOOPER, *Nepytia freemani* (Lepidoptera: Geometridae)

G.G. SKIP KING,[1,*] REGINE GRIES,[2] GERHARD GRIES,[2] and K.N. SLESSOR[1]

[1]*Department of Chemistry*
[2]*Centre for Pest Management, Department of Biological Sciences*
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

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Abstract—(3*S*,13*R*)-3,13-Dimethylheptadecane [(3*S*,13*R*)-3,13-dime-17Hy] is the major pheromone component of the western false hemlock looper (WFHL), *Nepytia freemani*. In comparative gas chromotographic-electroantennographic detection (GC-EAD) analyses of stereoselectively synthesized isomers, 1 pg of (3*S*,13*R*)-dime-17Hy elicited significantly stronger electrophysiological responses by male WFHL antennae than did 1 pg of separately injected (3*R*,13*R*)-, (3*R*,13*S*)- or (3*S*,13*S*)-3,13-dime-17Hy. In field experiments with individually tested stereoisomers, (3*S*,13*R*)-3,13-dime-17Hy was the only stereoisomer to attract males, but the four-stereoisomer blend was 3.6 times more attractive. Quaternary and all binary combinations of (3*S*,13*R*)-3,13-dime-17Hy with the other stereoisomers were equally attractive, suggesting that synergisytic behavioral activity in WFHL resided with either one of (3*R*,13*R*)-, (3*R*,13*S*)-, or (3*S*,13*S*)-3,13-dime-17Hy. Because optical isomers of (di)methylhydrocarbons do not separate on currently available columns, it remains unknown whether female WFHL also produce a four-stereoisomer pheromone blend. Substitutionality of pheromone stereoisomers without loss of behavioural activity has not previously been reported, but favorably compares with the concept of pheromone redundancy that was first suggested for the multiple pheromone component blend of the cabbage looper moth, *Trichoplusia ni*.

Key Words—Chirality, dimethylhydrocarbons, stereoselective synthesis, electrophysiological recording, (3*S*,13*R*)-3,13-dimethylheptadecane, (3*R*,13*R*)-

*To whom correspondence should be addressed.

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dimethylheptadecane, (3*R*,13*S*)-dimetylheptadecane, (3*S*,13*S*)-dimethylheptadecane.

INTRODUCTION

3,13-Dimethylheptadecane (3,13-dime-17Hy) has recently been reported as the major sex pheromone component of the western false hemlock looper (WFHL), *Nepytia freemani* (Gries et al., 1993b). In field experiments, a synthetic, stereoisomeric mixture of 3,13-dime-17Hy strongly attracted male WFHLs, but pheromone chirality has not yet been investigated. We report chiral synthesis of, and antennal responses and field attraction to, all four stereoisomers of 3,13-dime-17Hy.

METHODS AND MATERIALS

Experimental Insects and Laboratory Analyses

Natural WFHL populations were at very low levels in 1994 and did not warrant field collection of larvae to be reared to adults in the laboratory. To obtain wild males for electrophysiological recordings, pheromone-baited traps were suspended from mature Douglas-fir trees, *Pseudotsuga menziesii* (Mirb) Franco, 30 km west of Kamloops, British Columbia. Delta 2-liter milk cartons served as traps. Their inner 855 cm^2 surface was coated with adhesive Tangle-Trap (Tanglefoot Company, Grand Rapids, Michigan) to retain males approaching the pheromone (100 μg)-impregnated septum in the middle of the trap. After one night, captured males with intact antennae were carefully removed from the glue and individually placed in Petri dishes with moist filter paper. Fourteen of these males were used within 24 hr in coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Arn et al., 1975), employing a Hewlett Packard 5890A gas chromatograph equipped with a DB-210 coated, fused silica column (30 m × 0.25 mm ID; J&W Scientific, Folsom, California 95630). The antenna, with its tip clipped off, was carefully removed from a male's head and suspended between two glass capillary electrodes mounted on a portable micromanipulator. Electrical potentials generated by the antenna were amplified using a custom-built amplifier with a passive low-pass filter and a cutoff frequency of 10 kHz. Each antenna was subjected to 1 pg of all four separately injected stereoisomers of 3,13-dime-17Hy. One hundred picograms of 5-methylheptadecane, coinjected with each of the stereoisomers, served as an internal standard to monitor possible deterioration of antennal responses over the 30- to 40-min test period for each antenna.

Instruments and General Procedures

Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker AMX-400 spectrometer at 400.13 MHz. ^{1}H chemical shifts are reported in parts per million (ppm, δ) and relative to TMS (0.00 ppm). Infrared (IR) spectra were recorded as liquid films on a PE 1600 FTIR. Mass spectra were obtained on a Varian Saturn II Ion Trap GC-MS fitted with a DB-5 coated, fused silica column (30 m × 0.25 mm ID; J&W Scientific). Optical rotations were measured with a Rudolph Auto-Pol digital polarimeter. Elemental analyses were performed using a Carlo-Erba model 1106 Elemental Analyzer. GC analyses were carried out on an HP5880 fitted with a DB-1 coated, fused silica column (30 m × 0.25 mm ID, J&W Scientific). THF was freshly distilled from sodium-benzophenone ketyl. Recovered hexane–ether solvent mixtures were distilled from P_2O_5 via a Dufton fractionating column. Chemicals from commercial sources were used without further purification. All moisture- and air-sensitive reactions were conducted under argon. Column chromatography refers to flash chromatography using silica gel 60 (230–400 mesh E. Merck, Darmstadt, Germany) (Still et al., 1978). The usual reaction workup involved addition of aqueous NH_4Cl, extraction with recovered hexane/ether solvent mixtures, washing with water and then saturated brine, drying over anhydrous Na_2SO_4, and removal of solvent in vacuo by rotary evaporation.

Synthesis of 3,13-Dimethylheptadecane Stereoisomers

(S)-3-(2'-Furanyl)-2-methylpropyl methanesulfonate ***(4)*** *(Figure 1).* Compound **3,** synthesized as previously reported (Fuganti et al., 1988), was converted into mesylate **4** (Crossland and Servis, 1970). ^{1}H ($CDCl_3$); δ 7.35 (1H, d, J = 2 Hz), 6.30 (1H, t, J = 3 Hz), 6.08 (1H, d, J = 3 Hz), 4.1 (2H, m), 3.02 (3H, s), 2.75 (1H, dd, J = 12, 6 Hz), 2.66 (1H, dd, J = 12, 6 Hz), 2.27 (1H, m), 1.3 (3H, d, J = 7 Hz). GC-MS *m/z* (relative intensity): 218 (3), 122 (18), 107 (65), 81 (100), 53 (24). $[\alpha]_D$ = +6.6°, (c, 6.1, $CHCl_3$).

(2R)-1-(2'-Furanyl)-2-methylhexane ***(5)*** *(Figure 1).* A solution of Grignard reagent in THF [prepared from bromopropane (9.5 g, 80 mmol) and Mg (2.4 g, 100 mmol)] was cooled to −70°C. CuI (1.2 g, 6 mmol) was added, then **4** (9 g, 40 mmol). The reaction was allowed to warm to room temperature overnight, then worked up. The crude product was chromatographed on 300 g silica gel, yielding pure **5** (2.8 g, 40% yield). ^{1}H ($CDCl_3$); δ 7.30 (1H, d, J = 2 Hz), 6.26 (1H, t, J = 3 Hz), 5.95 (1H, d, J = 3 Hz), 2.62 (1H, dd, J = 12, 5 Hz), 2.43 (1H, dd, J = 12, 7 Hz), 1.8 (1H, m), 1.3 (5H, m), 1.15 (1H, m), 0.9 (6H, m); GC-MS *m/z* (relative intensity): 166 (4), 165 (18), 108 (11), 81 (100), 53 (20); $[\alpha]_D$ = +1.8% (c, 5.3, $CHCl_3$). Anal. calcd. for $C_{11}H_{18}O$: C, 79.52; H, 10.84, found: C, 79.24; H, 11.08.

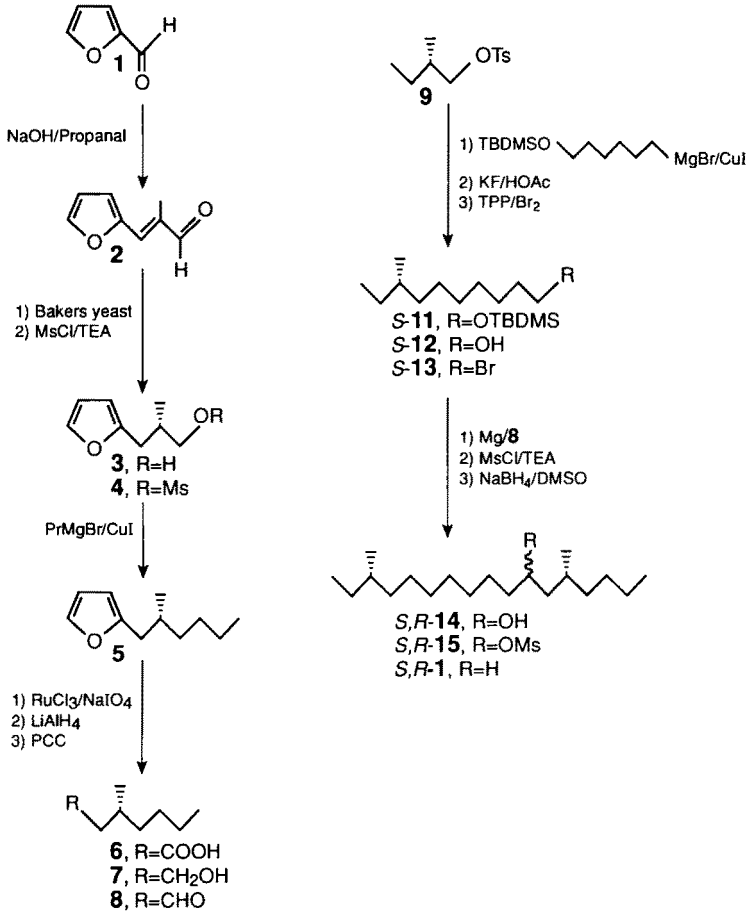

FIG. 1. Synthetic scheme for the synthesis of (3*S*,13*R*)-3,13-dimethylheptadecane.

(R)-3-Methylheptanoic acid ***(6)*** *(Figure 1).* Compound **5** (2.8 g, 17 mmol) was mixed with CCl_4 (65 ml), CH_3CN (65 ml), and H_2O (98 ml). To this was added 54 g of $NaIO_4$, followed by 0.34 g of $RuCl_3 \cdot 2H_2O$ (Carlsen et al., 1981). When, after several hours of stirring, the starting material was absent, the mixture was acidified, extracted with ether, and washed with water and then five times with 3 M NaOH. The basic solution was washed with ether, acidified, and extracted three times with ether. The combined organic phase was washed with water, brine, and then dried. Evaporation gave **6** (1.8 g, 70% yield). ^1H ($CDCl_3$); δ 10.4 (1H, bs), 2.40 (1H, dd, J = 12, 5 Hz), 2.15 (1H, dd, J =

12, 6 Hz), 1.95 (1H, m), 1.3 (6H, m), 0.95 (3H, d, J = 6 Hz), 0.86 (3H, t, J = 6 Hz). $[\alpha]_D$ = +3.6°, (c, 8.4, $CHCl_3$). Anal. calcd. for $C_8H_{16}O_2$: C, 66.67; H, 11.11, found: C, 66.56; H, 11.28.

(S)-2 Methylbutyl p-toluenesulfonate ***(9)*** *(Figure 1).* This compound was prepared from 2-methylbutanol {Aldrich, $[\alpha]_D$ = −5.8° (neat)} (5.1 g, 57 mmol) and tosyl chloride (12 g, 63 mmol) in pyridine at 0°C overnight. Careful addition of water followed by extraction and chromatography gave pure **9** (14 g, 100% yield). ^1H ($CDCl_3$); δ 7.80 (2H, d, J = 9 Hz), 7.35 (2H, d, J = 9 Hz), 3.86 (1H, dd, J = 11, 5 Hz), 3.82 (1H, dd, J = 11, 5 Hz), 2.46 (3H, s) 1.71 (1H, m), 1.38 (1H, m), 1.15 (1H, m), 0.90 (3H, d, J = 7 Hz), 0.85 (3H, t, J = 7 Hz), IR 1360, 1177, cm^{-1}. Anal. calcd. for $C_{12}H_{18}SO_3$: C, 59.50; H, 7.44, found: C, 59.35; H, 7.58. $[\alpha]_D$ = +4.1° (c, 3.7, $CHCl_3$), [Aldrich, +4.5° (neat)].

1-tert-Butyldimethylsilyloxy-6-bromohexane ***(10)*** *(Figure 1).* To 8.4 g (46 mmol) of 6-bromohexanol dissolved in 80 ml of DMF were added 7.6 g of tertbutyldimethylsilyl chloride followed by 7.1 g of imidazole (Corey and Venkataswarlu, 1972). The mixture was allowed to stand overnight. After workup, the product contained 3 parts chloride to 1 part bromide. Chromatography provided 10 g of mixed haloethers (~80% yield). GC-MS *m/z* (chloride) 124/122, 95/93, 83 (bromide) 168/166, 139/137, 83, ^1H ($CDCl_3$); δ 3.6 (2H, t) 3.52 (1.5H, t) 3.4 (0.5H, t) 1.8-1.3 (6H, m) 0.90 (9H, s) 0.05 (6H, s).

(S)-1-tert-Butyldimethylsilyloxy-8-methyldecane (S-11) (Figure 1). Ten grams of the above halide mixture were mixed with 2.2 g of 1,2-dibromoethane in 50 ml of dry ether and, under argon, were added to 2 g of Mg. Following an exothermic reaction, the mixture was refluxed for several hours until most of the starting materials had disappeared. Dry THF was added, the mixture cooled to −70°C, and copper(I) iodide (1 g) followed by **9** (8.5 g) were added. After stirring the mixture overnight at room temperature and the usual workup and chromatography, 5 g of the product were obtained, still containing 21% hexyl silyl ether from the directly quenched Grignard reagent. Extensive chromatography provided an analytical sample of **11.** ^1H ($CDCl_3$); δ 3.60 (2H, d, J = 6 Hz), 1.5 (2H, d, J = 6 Hz), 1.26 (12H, m), 1.10 (2H, m), 0.90 (9H, s), 0.85 (3H, t, J = 6 Hz), 0.81 (3H, d, J = 6 Hz), 0.0 (6H, s). GC-MS *m/z* (relative intensity): 286 (2), 270 (1), 228 (100), 97 (56), 83 (37), 75 (60). Anal. calcd. for $C_{17}H_{38}SiO$: C, 71.33; H, 13.28, found: C, 71.75; H, 13.10. $[\alpha]_D$ = +2.0° (c, 8.4, $CHCl_3$).

(S)-8-Methyldecanol (S-12) (Figure 1). The silyl ether mixture **11** was dissolved in a solution of HOAc (40 ml), THF (10 ml), H_2O (10 ml), and KF (2.5 g) and stirred overnight. The reaction was quenched with aq. NaOH and extracted. After chromatography, 1.4g of pure **12** was obtained (22% yield based on tosylate **9**). ^1H ($CDCl_3$); δ 3.64 (2H, t, J = 6 Hz), 1.56 (2H, pentet, J = 7 Hz), 1.3 (12H, m), 1.1 (2H, m), 0.85 (3H, t, J = 7 Hz), 0.83 (3H, d, J =

6 Hz). Anal. calcd. for $C_{11}H_{24}O$: C, 76.74; H, 13.95, found: C, 76.71; H, 13.84, $[\alpha]_D = +4.9°$ (c, 5.7, $CHCl_3$).

(S)-8-Methyl-1-bromodecane (S-13) (Figure 1). To a solution of **12** (1.4 g) and triphenylphosphine (2.7 g) in 20 ml of CH_2Cl_2 was added Br_2 (1.6 g) until the color of excess Br_2 persisted. The reaction was stopped by addition of MeOH, diluted with H_2O, extracted, and evaporated. The crude residue was triturated with hexane and the solution passed through 10 g of silica gel to remove triphenylphosphine oxide. Evaporation yielded 1.4 g of product, containing 6% bromohexane by GC. The mixture of bromides was dissolved in isooctane containing 11 g of activated 5Å molecular sieves (O'Connor et al., 1962). After a few hours of heating, the sieves were removed by filtration and washed with fresh solvent. Evaporation yielded pure **13** (1.3 g, 67% yield). ^1H ($CDCl_3$); δ 3.42 (2H, t, $J = 6$ Hz), 1.86 (2H, pentet, $J = 6$ Hz), 1.42 (2H, m), 1.25 (9H, m), 1.1 (2H, m), 0.88 (3H, t, $J = 7$ Hz), 0.85 (3H, d, $J = 6$ Hz)〉 Anal. calcd. for $C_{11}H_{23}Br$: C, 56.17; H, 9.79, found: C, 56.43; H, 9.78, $[\alpha]_D = +5.3°$ (c, 7.4, $CHCl_3$).

(R)-3-Methylheptanal 8 (Figure 1). To the acid **6** (1.8 g) dissolved in 50 ml of dry ether was added in portions 0.75 g of $LiAlH_4$. The mixture was refluxed overnight, quenched by dropwise addition of 2 ml of 5% NaOH, and stirred until the precipitate became granular. The solid was removed by filtration and washed well with dry ether, of which most was removed by careful evaporation. Crude **7** (0.7 g) was added to a stirred mixture of pyridinium chlorochromate (2.1 g) in 5 ml of CH_2Cl_2. After 1 hr the reaction was stopped by addition of ether and the mixture passed through 20 g of silica gel with ether until no more aldehyde eluted. Rotary evaporation at 25°C gave crude **8,** which was used immediately in the next step.

(3S,11R/S,13R)-3,13-Dimethyl-11-hydroxyheptadecane (SR-14) (Figure 1). A solution of 1,2-dibromoethane (0.19 g) and bromide *S*-**13** (1.3 g) in dry ether was added to Mg (0.25) and refluxed 1 hr before crude **8** was added to the cooled mixture. After 0.5 hr, acid workup and chromatography yielded 0.5 g of two diastereomers in equal amounts by GC. ^1H ($CDCl_3$); δ 3.68 (1H, bs), 1.60 (2H, bs), 1.40 (3H, m), 1.30 (18H, m), 1.1 (4H, m), 0.88 (6H, m), 0.83 (6H, m).

(3S,11 R/S, 13R)-3,13-Dimethyl-11-methanesulfonyloxyheptadecane (S,R-15) (Figure 1). To a cooled solution (0°C) of **14** (0.5 g) in CH_2Cl_2 (5 ml) containing 1 g of triethylamine was added dropwise 0.44 g of MsCl in 5 ml of CH_2Cl_2. The mixture was stirred 0.5 hr and then diluted with water. Extraction and evaporation yielded 0.7 g of crude **15**. ^1H ($CDCl_3$); δ 4.8 (1H, m), 3.0 (3H, s), 1.65 (2H, m), 1.6 (3H, m), 1.3 (18H, m), 1.1 (3H, m), 0.9-0.8 (12H, m).

(3S,13R)-3,13-Dimethylheptadecane (S,R-1) (Figure 1). Crude **15** was dissolved in DMSO (10 ml), mixed with $NaBH_4$ (0.19 g), and heated overnight to

100°C (Hutchins et al., 1978). The mixture was poured into water and extracted with hexane. Polar materials were removed by chromatography. The resulting product was treated with conc. H_2SO_4 and rechromatographed in sequence on silica gel and silica gel impregnated with 20% $AgNO_3$ to yield *SR*-**1** (0.13 g, >96% by GC. $[\alpha]_D = +3.8°$ (c, 3.0, $CHCl_3$).

Methyl (2R)-2-methyl-3-tert-butyldimethyl-silyloxypropionate (R-16) (Figure 2). A solution of hydroxy ester (4.6 g) {Sigma, $[\alpha]_D = -25.8°$ (C, 2.7, MeOH), lit. $[\alpha]_D = -26.1$ (Mori and Koseki, 1988)} in CH_2Cl_2 with triethylamine (8.5 ml) was mixed with tertbutyldimethylsilyl chloride (7.5 g) and dimethylaminopyridine (0.2 g) and allowed to stand overnight at 5°C. Usual workup and chromatography provided *R*-**16** (9.3 g, 100% yield). ^1H ($CDCl_3$); δ 3.75 (1H, m), 3.65 (3H, s), 3.62 (1H, m), 2.64 (1H, sextet, $J = 6$ Hz), 1.14 (3H,

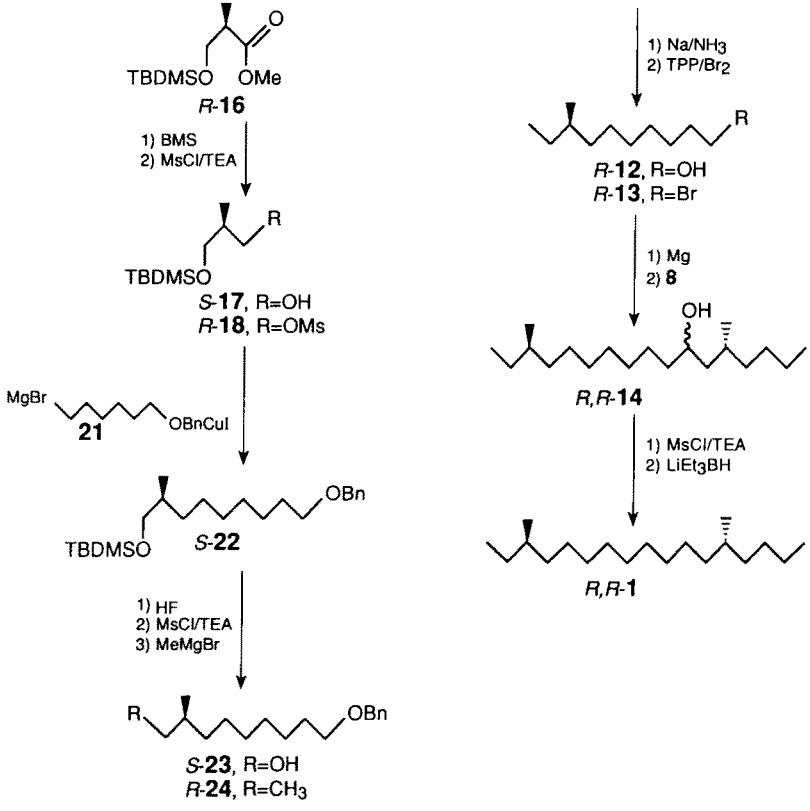

FIG. 2. Synthetic scheme for the synthesis of (3*R*,13*R*)-3,13-dimethylheptadecane.

d, J = 6 Hz), 0.86 (9H, s) 0.00 (6H, s). IR (neat), 1743, 1389, 1361, 1257, 1098 cm^{-1}. Anal. calcd. for $C_{11}H_{24}O_3Si$: C, 56.90; H, 10.34, found: C, 56.64; H, 10.29. $[\alpha]_D = -18.7°$ (c, 5.6, $CHCl_3$) [lit $-18.4°$, Mori and Koseki, 1988].

(2S)-2-Methyl-3-tert-butyldimethylsilyloxypropanol (S-17) (Figure 2). A solution of *R*-**16** (6.3 g) in dry THF (50 ml) was treated with 2.5 ml of a borane–methyl sulfide complex. The mixture was swept with argon to aid in removing methyl sulfide. After 24 hr, 10% $NaHCO_3$ was carefully added, followed by the usual workup. Chromatography gave *S*-**17** (5.0 g, 83% yield). IR (neat), 3357, 1090, 1041, 839 cm^{-1}, ^1H ($CDCl_3$); δ 3.72 (1 H, dd, J = 15, 6 Hz), 3.6 (2H, m), 3.53 (1H, dd, J = 15, 8 Hz), 2.9 (1H, bs), 1.95 (1H, m), 0.88 (9H, s), 0.82 (3H, d, J = 8 Hz), 0.05 (6H, s), $[\alpha]_D = -10.2°$ (c, 8.7, $CHCl_3$), lit $-10.8°$ (Mori and Koseki, 1988). Anal. calcd. for $C_{10}H_{24}O_2Si$: C, 58.82; H, 11.76, found: C, 58.95; H, 11.91.

(2S)-1-tert-Butyldimethylsilyloxy-2-methyl-9-benzyloxynonane-(S-22) (Figure 2). To an ice-bath-cooled solution of *S*-**17** (6 g, 30 mmol) and triethylamine (5 ml) in 50 ml of CCl_4 was added MsCl (4 g, 35 mmol) in 50 ml of CCl_4. After 0.5 hr, water was added and the mixture worked up as usual. Evaporation provided crude *R*-**18** (6.4 g, 76% yield). A Grignard solution was prepared from **21** (9.6 g, 35 mmol) (Tsuda et al., 1991) and Mg (1.2g, 50 mmol) in dry THF. The reaction was initiated by coaddition of 1,2-dibromoethane. After refluxing 30 min, the mixture was cooled to $-25°C$ and then CuI (0.9 g, 4.7 mmol) was added followed by crude *R*-**18** (6.4 g) in dry THF. The reaction was allowed to warm overnight and was quenched and worked up as usual to yield 19 g with 30% product by GC. Extensive chromatography provided an analytical sample of *S*-**22.** ^1H ($CDCl_3$); δ 7.32 (5H, s), 4.50 (2H, s), 3.45 (3H, m), 3.35 (1H, dd, J = 15, 7 Hz), 1.6 (2H, m), 1.4–1.2 (11H, m), 0.90 (9H, s), 0.85 (3H, d, J = 6 Hz), 0.0 (6H, s), $[\alpha]_D = -1.6°$ (c, 4, $CHCl_3$). Anal. calcd. for $C_{23}H_{42}O_2Si$: C, 73.02; H, 11.11, found: C, 72.88; H, 11.38.

(2S)-2-Methyl-9-benzyloxynonanol (S-23) (Figure 2). Crude *S*-**22** from the above reaction was dissolved in acetonitrile (95 ml) and 40% HF (5 ml) (Newton et al., 1979) and stirred until **22** had disappeared. The reaction was then diluted with water, extracted, and chromatographed to yield pure *S*-**23** (1.2 g, 22% yield from **18**). ^1H ($CDCl_3$); δ 7.3 (5H, s), 4.50 (2H, s), 3.45 (4H, m), 1.60 (2H, m), 1.4–1.2 (10H, m), 1.1 (1H, m), 0.89 (3H, d, J = 6 Hz), $[\alpha]_D = -5.1°$ (C, 5.7, $CHCl_3$). Anal. calcd. for $C_{17}H_{28}O$: C, 77.27, H, 10.61, found: C, 76.95, H, 10.65.

(8R)-8-Methyl-1-benzyloxynonane R-24 (Figure 2). To a solution (0°C) of **23** (4 g, 15 mmol) in CCl_4 (30 ml) mixed with triethylamine (8 ml) was added MsCl (2.5 g, 22 mmol) in 20 ml of CCl_4. The reaction was stirred 30 min and worked up to yield 4.6 g of product, which was evaporated twice from dry THF

to yield mesylate (4 g, 12 mmol). This was dissolved in dry THF and cooled to −70°C followed by addition of CuI (8 g, 4.5 mmol) and 3 M methyl magnesium bromide (10 ml). The mixture was allowed to warm up overnight. Usual workup and chromatography yielded *R*-**24** (2.6 g, 65% yield). ^{1}H ($CDCl_3$); δ 7.3 (5H, m), 4.52 (2H, s), 3.47 (2H, t J = 6 Hz) 1.61 (2H, pentet, J = 6 Hz), 1.3 (13H, m), 1.1 (2H, m), 0.89 (3H, d J = 6 Hz), 0.85 (3H, t, J = 6 Hz), $[\alpha]_D$ = −4.4° (c, 9.4, $CHCl_3$,). Anal. calcd. for $C_{18}H_{30}O$: C, 82.44; H, 11.45, found: C, 82.68; H, 11.05.

(8R)-8-Methyl-1-bromodecane R-13 (Figure 2). A solution of *R*-**24** (2.5 g, 10 mmol) in THF was added to 1 g of Na in liq. NH_3 (Reist et al., 1964). After 30 min, the (still) blue mixture was quenched with NH_4Cl, and ammonia allowed to evaporate. The mixture was extracted and chromatographed to yield *R*-**12** (1.5 g, 88% yield), with R_f and GC identical to that of *S*-**12** (Figure 1). $[\alpha]_D$ = −6.3° (c, 3.0, $CHCl_3$). Dibromotriphenylphosphorane was prepared by combining triphenylphosphine (4.5 g) and bromine (2.5 g) in 30 ml of CH_2Cl_2. To this was added 1.5 g of *R*-**12.** After 30 min, the usual workup and chromatography yielded *R*-**13** (2 g, 95% yield). ^{1}H ($CDCl_3$); δ 3.42 (2H, t, J = 6.0 Hz), 1.90 (2H, pentet, J = 7 Hz), 1.5–1.2 (13H, m), 1.1 (2H, m), 0.82 (6H, m), $[\alpha]_D$ = −5.7° (c, 6.1, $CHCl_3$). Anal. calcd. for $C_{11}H_{23}Br$: C, 56.17; H, 9.79, found: C, 55.89; H, 9.88.

(3R,13R)-3,13-Dimethylheptadecane (R,R-1) (Figure 2). To a Grignard solution prepared from *R*-**13** (0.7 g, 3 mmol) and Mg (0.2 g) in ether was added *R*-**8** (0.7 g). The usual workup and chromatography gave *RR*-**14** (0.4 g) as a diastereomeric mixture, which was converted to *RR*-**15.** The crude product was dissolved in THF and reduced with 5 ml of 1 M lithium triethylborohydride (Krishnamurthy and Brown, 1976). After 24 hr, the reaction was stopped with 6 ml of 5% NaOH, and 4 ml of 40% H_2O_2. The mixture was then extracted to yield 0.14 g of crude product containing 56% *RR*-**1** by GC. While treatment with alkaline $KMnO_4$ did not improve this purity, extensive chromatography on alumina and silica gel provided 30 mg of *RR*-**1** (75% pure by GC). Final purification of *RR*-**1** (>97%) was acheived by reverse-phase HPLC using acetonitrile on RP-18. $[\alpha]_D$ = −7° (c, 0.5, $CHCl_3$).

(3R)-3-Methyl-4-tert-butyldimethylsilyloxybutanonitrile (R-19) (Figure 3). Mesylate *S*-**18** was prepared as described above for *R*-**18.** ^{1}H ($CDCl_3$); δ 4.2 (2H, m), 3.64 (1H, dd, J = 15, 5 Hz), 3.55 (1H, dd, J = 15, 7 Hz), 3.02 (3H, s), 2.08 (1H, m), 1.00 (3H, d, J = 7 Hz), 0.90 (9H, s), 0.05 (6H, s). Crude *S*-**18** (4.1 g, 15 mmol) was dissolved in 40 ml of DMSO to which NaCN (2.6g, 53 mmol) was added. After stirring the mixture for two days, workup and chromatography gave *R*-**19** (3.0 g, 95% yield). IR (neat), 2248 cm^{-1}. ^{1}H ($CDCl_3$); δ 3.60 (1H, dd, J = 15, 5 Hz), 3.40 (1H, dd, J = 15, 7 Hz), 2.45 (1H, dd, J = 18, 5 Hz), 2.32 (1H, dd, J = 18, 6 Hz), 2.01 (1H, m), 1.04

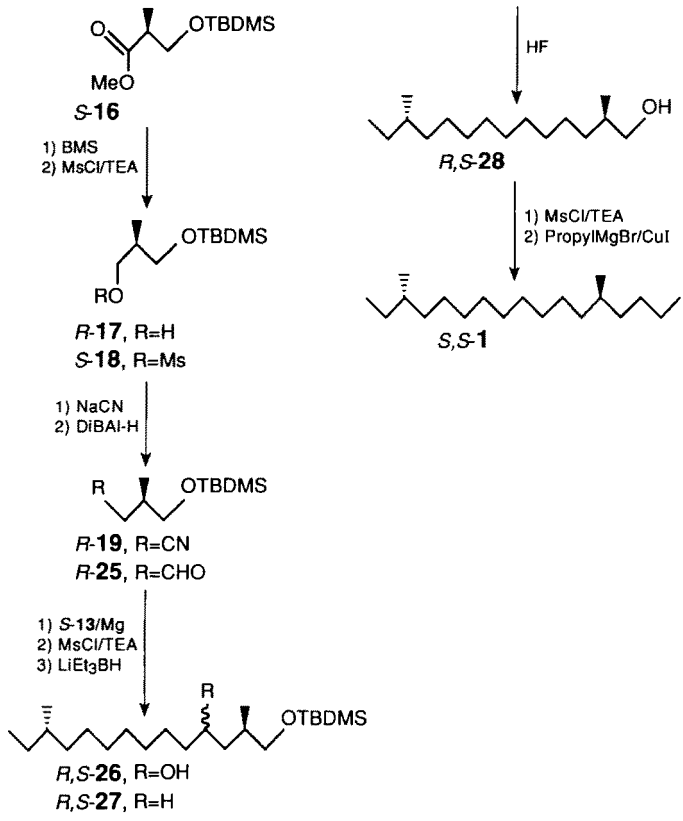

FIG. 3. Synthetic scheme for the synthesis of (3*S*,13*S*)-3,13-dimethylheptadecane.

(3H, d, J = 6 Hz), 0.88 (9H, s), 0.05 (6H, s), $[\alpha]_d$ = +16.8° (C, 4.0, $CHCl_3$). Anal. calcd. for $C_{11}H_{23}NOSi$: C, 61.97; H, 10.80; N, 6.57, found: C, 61.83; H, 11.00; N, 6.39.

(2R)-2-Methyl-3-tert-Butyldimethylsilyloxypropanal (R-25) (Figure 3). To a cooled (−70°C) solution of *R*-**19** (2.2 g, 10.3 mmol) in 50 ml of hexane was added dropwise 14 ml of 1 M diisobutylaluminum hydride in hexane. The mixture was kept cold 2 hr, 5 ml of water was added and the mixture stirred 1 hr at room temperature. Celite was added and the mixture filtered. The residue was washed several times with hexane–ether. After evaporation and chromatography, *R*-**25** (1.4 g, 54% yield) was obtained as a partial hydrate. ^1H ($CDCl_3$); δ 9.80 (1/2H, t, J = 2 Hz), 3.55 (1H, dd, J = 14, 5 Hz), 3.35 (1H, dd, J = 14, 7 Hz), 2.5 (1H, m), 2.2 (1H, m), 1.7 (1H, bs), 1.25 (1/2H, bs), 0.85 (3H,

d, J = 6 Hz), 0.80 (9H, s), 0.04 (6H, s), $[\alpha]_D$ = +6.7° (c, 6.0, $CHCl_3$). Anal. calcd. for 70% hydrate of $C_{11}H_{24}O2Si$: C, 58.43; H, 10.94, found: C, 58.43; H, 10.75.

(2R,12S)-2,12-Dimethyl-1-tert-Butyldimethylsilyloxytetradecane(R-27)(Figure 3). To 0.55 g (23 mmol) of Mg was added *S*-**13** (3.0 g, 13 mmol) in dry ether. After 1 hr reflux, the mixture was cooled and *R*-**25** (1.3 g, 6 mmol) was added. Workup and chromatography gave diastereomeric *R,S*-**26** (0.9 g), still containing 9% of **25.** This mixture was dissolved in 20 ml of CCl_4 containing 2 ml of triethylamine and reacted at 0°C with 0.5 g of MsCl to yield 1.1 g of crude mesylate. This was dissolved in 5 ml of THF and stirred overnight with 5 ml of 1 M lithium triethylborohydride. The reaction was stopped by addition of water, 5 ml of 5% NaOH, and 3 ml of 40% H_2O_2. The mixture was extracted three times; the organic layers were combined, washed with 5% NaOH and brine, dried, and evaporated. After chromatography with hexane, *R,S*-**27** (0.4 g) was obtained (0.4 g, 20% yield based on *R,S*-**25**). ^1H ($CDCl_3$); δ 3.43 (1H, dd, J = 12, 5 Hz), 3.31 (1H, dd, J = 12, 5 Hz), 1.55 (1H, m), 1.25 (18H, m), 1.1 (3H, m), 0.9 (9H, s), 0.85 (9H, m), 0.04 (6H, s), $[\alpha]_D$ = +5.1° (C, 5.7, $CHCl_3$), GC-MS, *m/z* (relative intensity) 355(1), 341(7), 299(77), 75(100). Anal. calcd. for $C_{22}H_{48}OSi$: C, 74.15; H, 13.48, found: C, 73.93; H, 13.70.

(2R,12S)-2,12-Dimethyl-tetradecanol (R,S-28) (Figure 3). Silyl ether *R,S*-**27** was stirred in 20 ml of acetonitrile and 1 ml of 40% HF. After 1 hr, **27** could no longer be detected. The reaction mixture was diluted with hexane–ether, washed, dried, and evaporated. Chromatography yielded *R,S*-**28** (0.3 g, 100% yield). ^1H ($CDCl_3$); δ 3.50 (1H, dd, J = 12, 5 Hz), 3.40 (1H, dd, J = 12, 5 Hz), 1.60 (1H, m), 1.36 (19H, m), 1.10 (3H, m), 0.90 (3H, d, J = 6 Hz), 0.84 (6H, m), $[\alpha]_D$ = +11.6° (c, 5.4, $CHCl_3$). Anal. calcd. for $C_{16}H_{34}O$: C, 79.34; H, 14.05, found: C, 79.09; H, 14.22.

(3S,13S)-3,13-Dimethylheptadecane (S,S-1) (Figure 3). To *R,S*-**28,** dissolved in 20 ml of CCl_4 and 1 ml of triethylamine, was added dropwise at 0°C 0.6 g of MsCl. After 0.5 hr, workup provided 0.7 g of crude mesylate. Grignard reagent prepared from bromopropane (0.75 g, 6 mmol) and Mg (0.24 g) was cooled to −70°C followed by addition of CuI (0.19 g) and subsequently crude mesylate. After stirring the mixture overnight under argon, the crude product was worked up, chromatographed on silica, and silica gel impregnated with 20% $AgNO_3$. The resulting 0.3 g of impure product was stirred several days with alkaline $KMnO_4$. The brown mixture was diluted with hexane, filtered, and evaporated to yield 0.14 g of *S,S*-**1,** which was purified (>98%) by HPLC on an RP-18 column with acetonitrile. $[\alpha]_D$ = +7° (c, 1.0, $CHCl_3$).

(2R,12R)-2,12-Dimethyl-1-tert-Butyldimethylsilyloxy tetradecane (R,R-27) (Figure 4). A Grignard solution, prepared from *R*-**13** (1.4 g, 6 mmol) and Mg (0.2 g) in dry ether, was added to *R*-**25** (1.4 g, 7 mmol). After workup and chromatography, diastereomeric *R,R*-**26** (0.5 g, 1.4 mmol, 20% yield) was

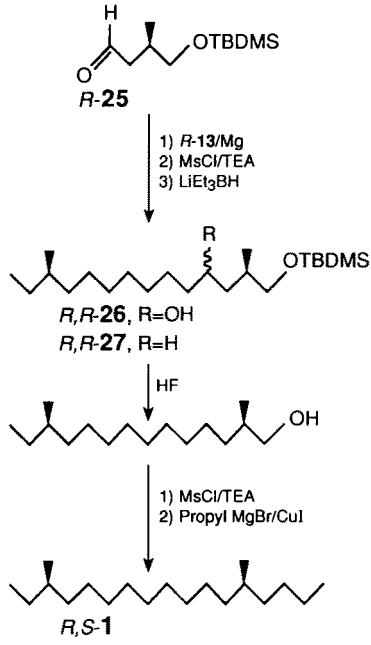

FIG. 4. Synthetic scheme for the synthesis of (3*R*,13*S*)-3,13-dimethylheptadecane.

obtained and treated with MsCl (0.4 g) in 20 ml of CCl_4 and 0.5 ml of triethylamine at 0°C to provide 0.6 g of crude mesylate. To this was added 6 ml of 1 M lithium triethylborohydride. After being stirred overnight, the reaction was worked up and chromatographed to yield *R,R*-**27** (0.22 g, 0.6 mmol, 89% pure) with 40% yield based on *R,R*-**26**). ^1H ($CDCl_3$); δ 3.44 (1H, dd, J = 12, 6 Hz), 3.33 (1H, dd, J = 12, 6 Hz), 1.53 (1H, m), 1.2 (18H, m), 1.05, m), 0.90 (9H, s), 0.85 (9H, m). Anal. calcd. for $C_{22}H_{48}OSi$: C, 74.16; H, 13.48, found: C, 75.07; H, 13.91.

(2R,12R)-2,12-Dimethyl-tetradecanol, R,R-28 (Figure 4). Silyl ether *R,R*-**27** was stirred in 15 ml of acetonitrile with 1 ml of 40% HF until *R,R*-**27** could no longer be detected. The mixture was neutralized, extracted, and chromatographed to give *R,R*-**28** (0.11g, 75% yield). ^1H ($CDCl_3$); δ 3.50 (1H, dd, J = 12, 6 Hz), 3.40 (1H, dd, J = 12, 6 Hz), 1.60 (1H, m), 1.25 (18H, m), 1.1 (3H, m), 0.90 (3H, d, J = 6 Hz), 0.84 (3H, t, J = 6 Hz), 0.81 (3H, d, J = 6 Hz), $[\alpha]_D$ = +2.0° (c, 3.6, $CHCl_3$,). Anal. calcd. for $C_{16}H_{34}O$: C, 79.34; H, 14.05, found: C, 79.55; H, 14.00.

(3R,13S) 3,13-Dimethylheptadecane (R,S-1) (Figure 4). Grignard reagent was prepared from Mg (0.24 g) and bromopropane (0.6 g) in THF, using 1,2-dibromoethane to initiate the reaction. After 2 hr of stirring, the mixture was cooled to −20°C and 0.1 g of CuI was added. Alcohol *R,R*-**28** (0.11 g) was converted to mesylate, of which 0.13 g was added to the above Grignard reagent and allowed to warm overnight. After the usual workup, the crude product was treated with $KMnO_4$ and chromatographed on silica and alumina to yield *R,S*-**1** (0.035 g, 85% pure). Final purification was achieved by HPLC using an RP-18 column with acetonitrile. $[\alpha]_D = -2°$ (c, 1.0, $CHCl_3$).

Field Experiments

Field experiments were conducted in widely spaced stands of mature Douglas fir 30–60 km west of Kamloops, British Columbia. All tests were set up in replicated, randomized complete blocks with traps and blocks 25–50 m apart. Unitraps (Phero Tech Inc., Delta, British Columbia) were suspended from trees 1.5–2 m above ground and baited with beige rubber septa (Sigma Chemical Co., St. Louis, Missouri) impregnated with test chemicals in HPLC-grade hexane. A dichlorvos cube (~1 cm^3) (Green Cross Co., Mississauga, Ontario) in each trap ensured rapid killing and retainment of captured moths. The first six-treatment, 10-replicate experiment tested (3*R*,13*S*)-, (3*R*,13*R*)-, (3*S*,13*S*)-, or (3*S*,12*R*)-3,13-dime-17Hy alone and in quaternary combination at 20 μg each. The second seven-treatment, eight-replicate experiment tested all stereoisomers at 20 μg each in quaternary and all binary combinations.

RESULTS

(3*S*,13*R*)-3,13-Dime-17Hy consistently elicited the strongest electrophysiological responses by male WFHL antennae, but all three other stereoisomers were also EAD-active (Figure 5). In field experiments, the four-stereoisomer blend attracted 3.6 times more males than did (3*S*,13*R*)-3,13-dime-17Hy by itself. None of the other three stereoisomers tested individually attracted any males (Figure 6). (3*S*,13*R*)-3,13-Dime-17Hy in binary combination with either (3*R*,13*R*), (3*S*,13*S*)-, or (3*R*,13*S*)-3,13-dime-17Hy was as attractive as the four-stereoisomer blend (Figure 7).

DISCUSSION

Chiral mono- and dimethylated hydrocarbon pheromone components have rarely been identified in the Lepidoptera (Roelofs and Cardé, 1971; Francke,

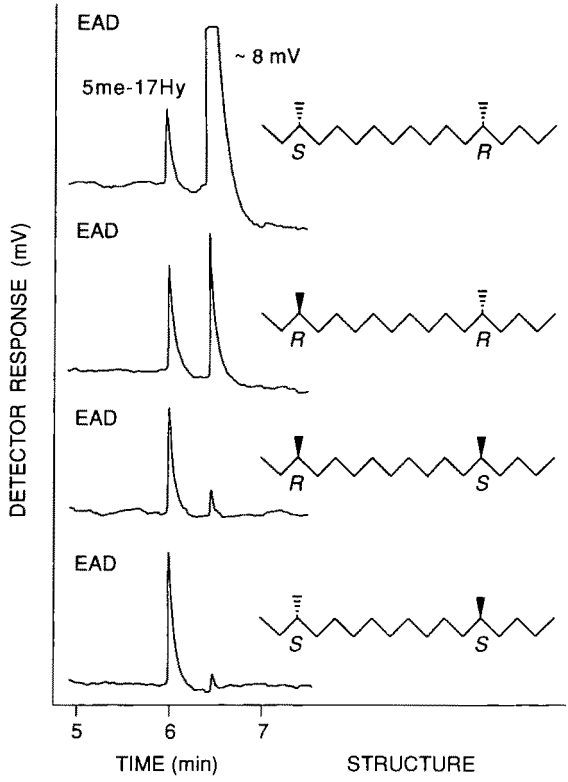

FIG. 5. Representative GC-EAD recordings of male *Nepytia freemani* antennae, responding to 100 pg of 5-methylheptadecane (internal standard, peak on the left) and to 1 pg of separately injected stereoisomers of 3,13-dimethylheptadecane (Hewlett Packard 5890A, DB-23 column, 1 min at 100°C, 10°C/min to 200°C).

1987; Francke et al., 1987, 1988; Riba et al., 1990; Gries et al., 1991a,b, 1993a,b, 1994). Of these methyl-branched hydrocarbons, only one of four possible stereoisomers or one of two enantiomers elicited strong antennal and/or behavioral responses, whereas antipodes were EAD-inactive and/or behaviorally benign. (5*S*,9*S*)-5,9-Dimethylheptadecane and (5*R*,11*S*)-5,11-dimethylheptadecane, e.g., were the only stereoisomers to attract males of, respectively, the mountain-ash bentwing, *Leucoptera scitella* (Zeller) (Tóth et al., 1989) and the hemlock looper, *Lambdina fiscellaria* (Li et al., 1993a). Similarly, (7*S*)-7-methylheptadecane and (2,5*S*)-2,5-dimethylheptadecane were synergistic pheromone components of the western hemlock looper, *Lambdina fiscelleria lugubrosa*

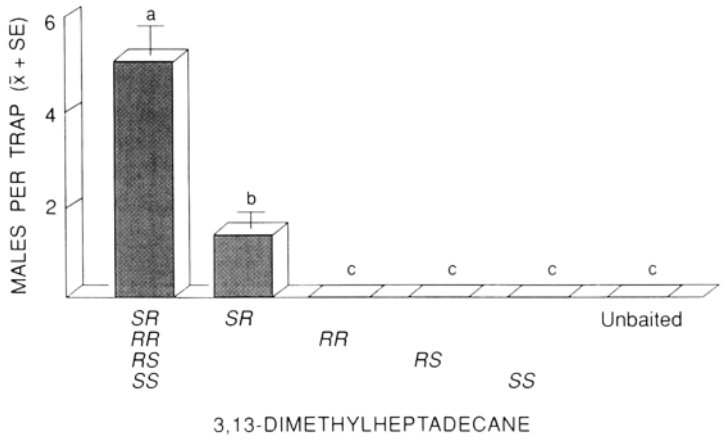

FIG. 6. Captures of *N. freemani* males in Unitraps baited with 20 μg of either (3*S*,13*R*)-, (3*R*,13*R*)-, (3*R*,13*S*)-, or (3*S*,13*S*)-3,13-dimethylheptadecane and all four compounds combined at 20 μg each. September 4–10, 1994; Walachin, British Columbia; $N = 10$. Bars with the same superscript letter are not significantly different [ANOVA on ranks (Friedman's test), followed by a comparison of means (Bonferroni test, $P < 0.05$)].

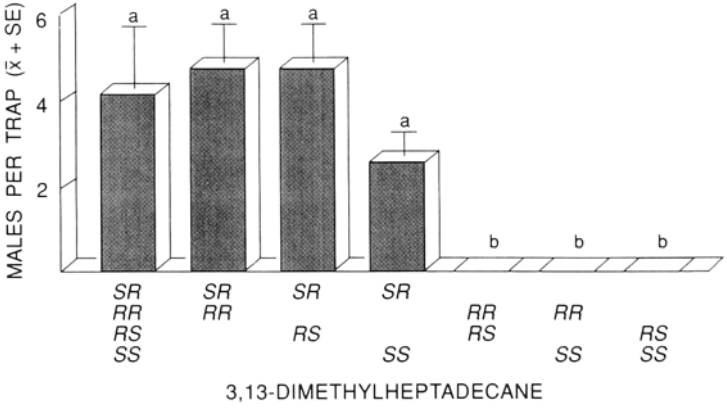

FIG. 7. Captures of *N. freemani* males in Unitraps baited with stereoisomers of 3,13-dimethylheptadecane at 20 μg each in quaternary and all binary combinations. September 11–24, 1994; Walachin, British Columbia; $N = 8$. Bars with the same superscript letter are not significantly different. [ANOVA on ranks (Friedman's Test), followed by comparison of means (Bonferroni test, $P < 0.05$)].

(Hulst), whereas their antipodes neither reduced nor enhanced attraction to the major pheromone component (Li et al., 1993b).

Chiral syntheses of pheromones have employed a great variety of different starting materials and synthetic routes (Li, 1993 and cited literature therein). Compared to classical chemical methods, microbe-mediated chiral syntheses (Fuganti et al., 1988; Fuganti, 1990; Li et al., 1993b) facilitate the preparation and enhance the chiral purity of synthetic starting material, intermediates and/or end products. Starting materials for syntheses of 3,13-dime-17Hy stereoisomers [(*S*)-2-methylbutanol (Aldrich Chemical Company, Milwaukee, Wisconsin); (*R*)-(−)-, (*S*)-(+)-3-hydroxy-2-methyl-propionic acid methyl ester (Sigma Chemical Company, Mississauga, Ontario); and (*S*)-3-(2′-furanyl)-2-methyl-propanol via furfural (Figure 1)] were invariably obtained through enantioselective, microbe-mediated chemical transformations. Acid **6** (Figure 1) and acids derived by oxidation of aldehydes *R*-**25** (Figure 3) and *S*-**25** were converted to their diastereomeric α-phenylethylamides with ee >97%, as determined by GC analyses. Because transformation of these three compounds and of 2-methyl-butanol to subsequent intermediates and end products could not conceivably reduce their chiral purities, enantiomeric excess of all four final stereoisomers should be equivalent to that directly determined for synthetic intermediates. (3*S*,13*R*)-3,13-Dime-17Hy, eliciting the strongest antennal response (Figure 5) and being the only stereoisomer by itself to attract males in the field (Figure 6), is the major sex pheromone component of the WFHL. Superior attraction of the quaternary blend (Figure 6), however, indicates that the presence of other optical isomers enhanced attraction of males. Equal attraction of (3*S*,13*R*)-3,13-dime-17Hy in quaternary and all binary combinations with other stereoisomers (Figure 7) suggests that optimal behavioral activity resides with the presence of only one (and either one) of (3*R*,13*R*)-, (3*R*,13*S*)-, or (3*S*,13*S*)-3,13-dime-17Hy.

Synergism between optical isomers of pheromones has rarely been discovered in the Insecta and was reported only for two moths. Enantiomers of (3*Z*,9*Z*)-*cis*-6,7-epoxy-eicosadiene synergistically attract males of the noctuid *Bleptina caradrinalis* (Guen.) (Millar et al., 1991), and a 1:9 mixture of (14*R*)- and (14*S*)-14-methyl-1-octadecene attracts more peach leafminer males, *Lyonetia clerkella* L., than the latter enantiomer alone (Sato et al., 1986).

Three optical isomers in WFHL similarly enhancing attraction to the major pheromone component is very unusual, but can not be attributed to stereoisomeric contamination of test chemicals. With chiral purities exceeding 97% ee, stereoisomeric contamination of all compounds was extremely low. Moreover, trace amounts of the major pheromone component, (3*S*,13*R*)-3,13-dime-17Hy, in each of individually tested (3*R*,13*R*)-, (3*R*,13*S*)-, or (3*S*,13*R*)-3,13-dime-17Hy, in each of individually tested (3*R*,13*R*)-, (3*R*,13*S*)-, or (3*S*,13*S*)-3,13-dime-17Hy failed to attract any males in the field, indicating that stereoisomer

contamination was unlikely to be responsible for synergistic behavioral activity of each of the three stereoisomers. Because optical isomers of (di)- methylated hydrocarbons do not separate on currently available chiral columns, it remains unknown whether female WFHL produce a pheromone blend of stereoisomers in which only one synergist is essential but can be substituted by either of two other isomers. Substitutionality of pheromone stereoisomers has not previously been reported but is another example of pheromone redundancy first suggested for the multiple pheromone component blend of the cabbage looper moth, *Trichoplusia ni* (Hübner) (Linn et al., 1984).

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EFFECTS OF DIMBOA ON SEVERAL ENZYMATIC SYSTEMS IN ASIAN CORN BORER, *OSTRINIA FURNACALIS* (GUENÉE)

FENGMING YAN,[1,*] CHONGREN XU,[1], SONGGANG LI,[1] CHANGSHAN LIN,[1] and JUHUAI LI[2]

[1]*College of Life Sciences, Peking University Beijing 100871, China*

[2]*Beijing Academy of Agricultural and Forestry Sciences, Beijing 100081, China*

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Abstract—By using spectrophotometric analysis and isoelectric focusing electrophoresis (IEF), we found inhibition of acetylcholinesterase (AChE), general esterase activity (Est), and lipase activity in larvae of the Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée), fed on cabbage dipped in DIMBOA (400 ppm). Amylase was not influenced, while activity of cytochrome P-450 monooxygenase and glutathlone *S*-transferase was increased. The results indicate that DIMBOA influences activity levels of some nervous system and detoxication enzymes and inactivates some hydrolysis enzymes.

Key Words—DIMBOA, 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one, enzymes, Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée), acetylcholinesterase, lipase, cytochrome P-450, isoelectric focusing electrophoresis (IEF).

INTRODUCTION

The Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée), is a serious pest of corn, an important food crop in China. A key for ACB control and environmental protection is breeding of resistant corn hybrids. DIMBOA (2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one) is a biochemical agent in the resistance of corn to many insect pests (including ACB) and pathogens, and DIM-

*To whom correspondence should be addressed.

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BOA level in corn is considered to be the main index in resistance breeding. Several papers have been published recently on the effects of DIMBOA on insects (Cuevas et al., 1990; Houseman et al., 1992; Feng et al., 1992; Leszczynski and Dixon, 1992). We have conducted studies on the mechanism of action of DIMBOA on ACB, and in this paper we present results of biochemical analyses.

METHODS AND MATERIALS

Isolation and Identification of DIMBOA. Isolation of DIMBOA was by the methods of Tipton et al. (1967), Klun et al. (1967), and Woodward et al. (1978). The extracts were analyzed by UV, IR, and elemental analysis. Furthermore, DIMBOA was confirmed with an authentic standard sample.

Rearing and Treatment of Larvae. Leaves of cabbage (*Brassica oleracae* var. *capitata* L.) were cut into 1.6-cm-diameter round leaf discs and dipped for 1 sec into a DIMBOA solution (400 ppm, with acetone as solvent). Discs were dipped only in acetone as control for 1 sec. One leaf disc retained on its surface about 0.1 ml solution containing 0.04 mg DIMBOA. The solvent was allowed to volatilize completely at room temperature. One leaf disc and one third-instar, starved for 24 hr, were placed into a glass tube. At 24, 48, 72, and 96 hr after consuming the discs, the larvae were taken for tests. For some tests samples were taken only at certain time intervals. Larvae were starved after they ate the test discs. Larvae were reared under continous light at 25°C and 80–90% relative humidity.

Biochemical Analyses. Acetylcholinesterase (AChE), general esterase (Est), cytochrome P-450 monooxygenase, glutathione *S*-transferase, amylase, and lipase were determined spectrophotometrically after incubation with their substrates. Isoelectric focusing electrophoresis was completed for esterase. All analyses were repeated six times.

Acetylcholinesterase (AChE). The method of Ellman et al. (1961) was used for AChE analysis. Five heads were homogenized with 2 ml of ice-cold phosphate buffer (pH 7.4, 0.1 M). The tube was washed with 1 ml buffer, and the homogenate was centrifuged for 4 min at 1800*g*. One milliliter of the supernatant and 2 ml buffer were incubated in a water bath at 27°C for 5 min. Then 1 ml of a 1 : 1 mixture of acetylthiocholine iodide and 5,5′-dithio-2,2′-dinitrobenzoic acid (DTNB) was added and tubes were incubated for 25 min. Eserine was added to terminate the reaction, and absorbance of the solution was determined at 412 nm.

Esterase (Est). The modified method of Asperen (1962) was used for analysis of esterase activity. Thoraces, abdomens, and midguts of ACB larvae were isolated by dissection, homogenized, and centrifuged separately. One milliliter of supernatant and 5 ml 1-naphthol acetate (3×10^{-4} M) were incubated for

30 min, and 0.5 ml of a 2:5 (v/v) mixture of 1% fast blue B salt–5% sodium dodecyl sulfate (SDS) was added to develop color. The absorbance of solutions was determined at 600 nm after 30 min. Samples, including the whole body with contents, the whole body with the entire gut removed, the head, and hemolymph were subjected to electrophoresis with isoelectric focusing (IEF) (Righetti, 1983). The gel plate was 1 mm thick × 104 mm long × 87 mm wide. The pH gradients ranged from 3.0 to 9.5. Initially, voltage was 55 V and current was 10 mA; 3 hr later voltage and current were adjusted to 250 V and 7–8 mA. The experiment was completed in 4.5–5 hr at 4°C. General proteins were stained by addition of fast blue B RR′ salt in maleic acid–NaOH buffer (pH 6.0, 0.1 M). With 1-naphthol acetate as substrate, esterase activity was indicated by black bands. 2-Naphthol acetate as substrate produced red bands for esterase activity.

Cytochrome P-450 Monooxygenase. The analysis was conducted according to Hanson and Hodgson (1971). One single larva and five midguts of ACB were taken, respectively, with 0.4 ml 0.1 M Tris HCl buffer, pH 7.9, for homogenization. An additional milliliter of buffer used to wash the homogenization tube was added to bring the total volume to 1.4 ml. Tubes were centrifuged at 1800*g* for 15 min. The reaction volume consisted of 1 ml supernatant, 1.8 ml buffer, 20 μl *p*-nitroanisole (0.1 M), and 0.2 ml nicotinamide adenine dinucleotide phosphate in reduced form (NADPH) (1 mM). Tubes were incubated at 30°C for 30 min, and 0.2 ml NaOH (0.1 N) was added to terminate the reaction. The absorbance was determined at 400 nm after 15 min.

Glutathione S-Transferase. The homogenate preparation was similar to that for cytochrome P-450. The reaction consisted of 0.1 ml homogenate, 2.3 ml phosphate buffer (pH 6.5, 0.1 M), and 0.5 ml of glutathione in reduced form (GSH) (20 mM). The reaction tubes were incubated at 25°C for 2 min, then 0.1 ml of 2,4-dinitrochlorobenzene (CDNB) (30 mM) was added and the solution was incubated at 25°C for 3 min. Addition of 0.3 ml trichloroacetic acid terminated the reaction, after which the tubes were centrifuged at 1800*g* for 5 min. Absorbance was determined at 340 nm. Activity was expressed as moles per milligram protein per minute. The extinction coefficient was 9.6 mM^{-1} cm^{-1}.

Amylase. The analysis was conducted according to the method of Bennfeld (1951). The reaction mixture consisted of 1 ml homogenate, 1 ml phosphate buffer (pH 8.0, 0.02 M), and 2 ml of 1% amylase solution. Two milliliters of buffer were used as control. Tubes were incubated at 30°C for 20 min. NaOH (4 ml of 0.4 N) terminated the reaction. Finally 1 ml of 3,5-dinitrosalicylic acid was added, and the mixture was boiled at 100°C for 5 min. After cooling, the absorbance was determined at 520 nm.

Lipase. The method of Vogel et al. (1963) was used for analysis of lipase activity. Midguts were homogenized and centrifuged as above. Three milliliters

of an olive oil emulsion (0.17 mg/ml Tris HCl buffer) and 1 ml of enzymatic supernatant were mixed, and the absorbance was determined immediately at 420 nm. After incubating the mixture at 37°C for 20 min, the absorbance was determined again at 420 nm. Activity was expressed as moles of triglyceride per milligram protein per minute. The biuret method was used for protein determination (Alexander et al., 1985).

RESULTS AND DISCUSSION

Three categories of enzymes, i.e., nervous system enzymes, detoxifying enzymes, and hydrolytic enzymes that play important roles in biochemical or physiological reactions of ACB larvae were determined after DIMBOA was eaten. Twenty-four hours after consuming leaf discs containing DIMBOA, there was lower AChE activity, and 96 hr after treatment AChE activity in control larvae was two thirds higher than that in the treatment (Table 1). Esterase (Est) activity was higher in the thorax and in the abdomen of ACB larvae that ate DIMBOA than in the controls, but the midgut esterase activity was lower in DIMBOA fed larvae. Esterase activity in the midgut became slightly higher with time, which suggested that there may be some adjustment to the toxin (Table 2). The results from IEF showed that DIMBOA inhibited the activity of esterase (Figure 1). Because the total activity of esterases includes AChE activity when 1-naphthyl acetate is the substrate, lower activity of esterase in the head in Figure 1 may be due to lower activity of AChE. Activities of cytochrome P-450 monooxygenase, both in the whole body and in the entire gut, were higher than those of controls 48 hr after larvae consumed DIMBOA (Table 3). Glutathione *S*-transferase activity was elevated in the whole body with contents, the whole body with gut removed, and in the entire gut for larvae fed on DIMBOA (Table 4).

TABLE 1. EFFECT OF DIMBOA (400 ppm) ON AChE OF LARVAE HEAD IN ACB

Sampling time (hr)	Activity (OD/mg protein/min)	
	Control	Treatment
24	0.4061 ± 0.0148	0.2858 ± 0.0171*[a]
48	0.4075 ± 0.0052	0.3323 ± 0.0270*
72	0.4210 ± 0.0217	0.2493 ± 0.0156**
96	0.4194 ± 0.0113	0.1673 ± 0.0221**

[a]Single asterisk in treatment indicates significant difference ($P \leq 0.05$) in LSD test between the control and the treatment at each sampling time, and double asterisks indicate very significant differences ($P \leq 0.01$).

TABLE 2. EFFECT OF DIMBOA (400 ppm) ON ESTERASE OF ACB

Sampling time (hr)	Specific activity (mg 1-naphthol acetate/mg protein/min)					
	Thorax		Abdomen		Midgut	
	Control	Treatment	Control	Treatment	Control	Treatment
24	0.0601 ± 0.0023	0.0741 ± 0.0058*[a]	0.0438 ± 0.0010	0.0640 ± 0.0135*	0.0801 ± 0.0158	0.0551 ± 0.0018**
48	0.0653 ± 0.0015	0.1010 ± 0.0079**	0.0389 ± 0.0023	0.0596 ± 0.0076*	0.0715 ± 0.0044	0.0583 ± 0.0110*
72	0.0525 ± 0.0089	0.0574 ± 0.0125	0.0439 ± 0.0088	0.0694 ± 0.0007*	0.0722 ± 0.0090	0.0591 ± 0.0005*

[a]Single asterisk in treatment indicates significant difference ($P \leq 0.05$) in LSD test between the control and the treatment at each sampling time, and double asterisks indicate very significant difference ($P \leq 0.01$).

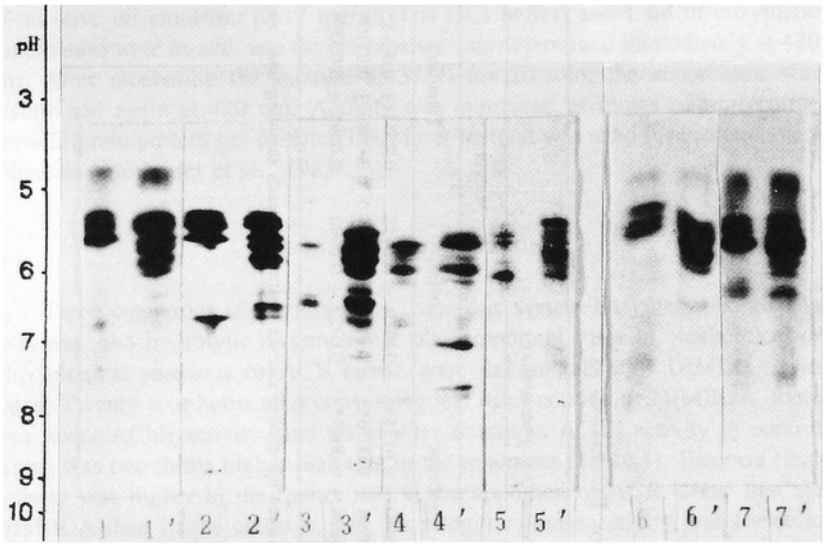

FIG. 1. The isoelectric focusing electrophoresis diagrams of esterase in the larvae of the Asian corn borer 48 hr later after treatment with DIMBOA. Lanes 1–5′ indicate esterase in black color with 1-naphthol acetate as the substrate, and lanes 6–7′ indicate esterase in red color with 2-naphthol acetate as substrate. Treatments are in lanes 1–7; controls in lanes 1′–7′. Whole body, lanes 1 and 1′; digestive gut, lanes 2 and 2′; head, lanes 3 and 3′; hemolymph, lanes 4 and 4′; whole body (with gut removed), lanes 5 and 5′; whole body, lanes 6 and 6′; and digestive gut, lanes 7 and 7′.

TABLE 3. EFFECT OF DIMBOA (400 ppm) ON CYTOCHROME P-450 MONOOXYGENASE OF ACB

Sampling time (hr)	Activity (OD/mg protein/min)	
	Whole body	Entire gut
24	0.0959 ± 0.0006*[a]	0.0964 ± 0.0000**
control	0.0652 ± 0.0002	0.0476 ± 0.0002

[a]Single asterisk indicates significant difference ($P \leq 0.05$) in LSD test between the control and the treatment at each sampling time, and double asterisks indicate very significant difference ($P \leq 0.01$).

TABLE 4. EFFECT OF DIMBOA (400 ppm) ON GLUTATHIONE *S*-TRANSFERASES OF ACB

Sampling time (hr)	Activity (OD/mg protein/min)		
	Whole body (with content)	Whole body (without gut)	Entire gut
48	0.0881 ± 0.0012*[a]	0.0442 ± 0.001*	0.1430 ± 0.0005**
control	0.0643 ± 0.0000	0.0555 ± 0.0006	0.0720 ± 0.0000

[a]Single asterisk indicates significant difference ($P \leq 0.05$) in LSD test between the control and the treatment at each sampling time, and double asterisks indicate very significant difference ($P \leq 0.01$).

DIMBOA had little effect on amylase activity, and lipase activity was variable. Inhibition of amylase appeared 24 hr after DIMBOA treatment, but afterwards, enzyme activity was variable and was sometimes higher or lower than that of the control group (Table 5). Similarly, lipase activity was lower 24 hr after larvae fed on DIMBOA, but 48 hr later lipase activity suddenly became much higher than that of the control, and by 72 hr it was again lower than that of the controls (Table 6).

AChE is an important enzyme in normal functioning of the central nervous system in insects, and inhibition of its activity may have behavioral or physiological consequences. Reduction in the level of AChE may render ACB more easily preyed upon by its natural enemies. Electrophysiological experiments by Zhou et al. (1994) showed that DIMBOA can block nerve impulse conduction. The sensitivity of sensory organs may be altered by dietary DIMBOA, and ACB

TABLE 5. EFFECT OF DIMBOA (400 ppm) ON AMYLASES OF MIDGUT OF ACB

Sampling time (hr)	Specific activity (mg amylomaltose/mg protein/min)	
	Control	Treatment
24	0.3746 ± 0.0018	0.3946 ± 0.0012*[a]
48	0.4439 ± 0.0032	0.4495 ± 0.0026
72	0.4049 ± 0.0016	0.4016 ± 0.0021

[a]Single asterisk in treatment indicates significant difference ($P \leq 0.05$) in LSD test between the control and the treatment at each sampling time.

TABLE 6. EFFECT OF DIMBOA (400 ppm) ON LIPASES OF MIDGUT OF ACB

Sampling time (hr)	Specific activity (mol triglyceride/mg protein/min)	
	Control	Treatment
24	3.5885 ± 0.2311	1.7390 ± 0.1240*[a]
48	3.8517 ± 0.4332	5.9620 ± 0.3866**
72	3.1648 ± 0.0916	0.6970 ± 0.0911**

[a]Single asterisk in treatment indicates significant difference ($P \leq 0.05$) in LSD test between the control and the treatment at each sampling time, and double asterisks indicate very significant difference ($P \leq 0.01$).

may refuse to continue to feed. This may be one possible physiological explanation for the antifeedant property of DIMBOA.

Esterase, cytochrome P-450 monooxygenase, and glutathione *S*-transferase are three important detoxication enzyme systems in insects. DIMBOA inhibits the activity of Est, possibly allowing toxins to stay in the body for a longer time because of lower activity of detoxifying esterase enzymes. At the same time that some enzymes are inhibited by DIMBOA, others are stimulated, possibly leading to detoxication. Cytochrome P-450 monooxygenase and glutathione *S*-transferase are induced to higher levels in ACB larvae fed on DIMBOA, and they may reduce the toxicity of DIMBOA. Moreover, Campos et al. (1989) confirmed that the European corn borer excretes DIMBOA very quickly. Thus, detoxication and excretion of DIMBOA may help explain why ACB larvae successfully feed on corn containing this allelochemical.

DIMBOA adversely affects, to some extent, the activity of lipase in the midgut of ACB. This effect may cause lower digestive capability and loss of nutrients, both of which will influence growth and development of larvae.

We have conducted experiments on the effects of DIMBOA on selected enzyme systems in homogenates of tissues from Asian corn borer fed DIMBOA on leaf discs. Additional studies are needed to determine if degradation or decomposition of DIMBOA occurs in the midgut of ACB. The pH of the midgut in lepidopterous caterpillars is generally high, and it is possible for DIMBOA to decompose in a high pH environment. If DIMBOA is degraded in the midgut, both undegraded DIMBOA and its derivatives, or a combination of both, may have an influence on enzymes of the gut. Clearly, further biochemical and molecular biological analysis are needed to fully understand how DIMBOA affects enzymatic systems of insects.

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USE OF PAIRED AND SINGLE TRAPS TO ASSESS PERCEPTION AND DISCRIMINATION OF SEX PHEROMONE MIXTURES IN THE FIELD BY *Trichoplusia ni* (Hübner)

M.S. MAYER*, J.R. McLAUGHLIN, and E.R. MITCHELL

Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, Florida 32604
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Abstract—Paired wind-oriented traps (WORT) and single traps were deployed simultaneously in the same field to investigate whether or not inferences about the central nervous system processes of discrimination and perception can be made from differences in moth captures. The stimulus levels deployed were those that typically may be found downwind of a calling virgin female cabbage looper, *Trichoplusia ni* (Hübner), so that inferences are relevant to natural stimulus intensities. Captures of male cabbage loopers in the WORT traps paralleled prior laboratory measures of pheromone mixture discrimination. The pattern of captures by the two trapping systems probably reflects perceptive and discriminative processing differences in the central nervous system. Captures in traps baited with Z7-12 : Ac alone were equal to, or better than, captures in traps baited with three- and six-component mixtures that contained Z7-12 : Ac.

Key Words—Behavior, perception, discrimination, trap, sex pheromone.

INTRODUCTION

Traps baited with insect sex pheromones historically have been used in field surveys, in various communication experiments, and as a means of confirming laboratory-derived chemical identifications. In this report we examine captures in field traps that were baited with sex pheromone components to assess the central nervous system (CNS) processes of discrimination and perception. The

*To whom correspondence should be addressed.

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results address questions about pheromone component redundancy and the usefulness of multicomponent information to male cabbage looper, *Trichoplusia ni* (Hübner), behavior in particular. Trap captures are usually only distantly and tacitly linked to studies of perception and discrimination by most investigators. These terms have never been clearly defined and tested in such a way that field captures can be applied successfully to studies of these processes.

The proximate question we examine is whether or not male cabbage looper moths discriminate differences between the single pheromone component, (Z)-7-dodecenyl acetate (Z7-12:Ac), and two mixtures containing Z7-12:Ac with other pheromone components. Previously, Mayer and McLaughlin (1993) found that male cabbage loopers discriminate the individual component, Z7-12:Ac, and different mixtures in the laboratory. For those laboratory assays, discrimination was defined as the difference in response to paired, simultaneously detectable stimuli (Mayer and McLaughlin, 1991, 1993). Stimulus perception is important in signal discrimination, but it is not measured in the same manner. In the laboratory, discrimination was intensity dependent but no pairing ever elicited an all-or-none response to one or the other pheromone component aroma. In addition to confirming the results that have been observed in the laboratory, we asked whether or not the imposition of the stringency of field trap assays would result in more or less clear evidence of mixture discrimination than the laboratory assays.

Discrimination in the field was assayed by use of paired wind-oriented traps (WORT) (Mitchell et al., 1988). The same mixtures that were used in the WORT traps also were used as baits in individual traps to examine whether or not it is appropriate to associate differences in captures between these traps with perception, another CNS process. The WORT system deploys paired traps that always are oriented into the wind. As a result, the plumes from each trap, in theory, are parallel and unmixed for at least a short distance downwind but mix at some further point downwind so that an insect flying upwind detects the plumes simultaneously. It is assumed that differences in captures between the two traps is the result of a difference discriminated between the two baits. These assays are the field analog of the laboratory-based wind-tunnel assays of discrimination (Mayer and McLaughlin, 1991, 1993).

If the WORT system can be used to investigate discrimination, then do single traps measure perception? If perception is defined as the innate recognition of an odor, captures at individual pheromone-baited traps are, indeed, measures of perception. A corollary of these definitions is that an insect can perceive disjoint stimuli, but can not discriminate between them unless it has a memory. Because the terms perception and discrimination are linked to neural processes within the CNS, other terms should be given to the mechanisms and response differences by which peripheral receptor neurons respond differently to different odors.

Central processes and linked flight behaviors are not only dependent on the quality of the stimulus but also on its quantity. For example, increasing pheromone stimulus intensities to levels much higher than that found downwind of a female results in diminished trap captures or behaviors (Linn et al., 1984; Mayer, 1973; Löfstedt et al., 1985: McDonough et al., 1993; Hainotakis and Pittara, 1994; among others). Furthermore, other as yet unknown behavioral effects may result from an insect encountering stimulus intensities that are marginally higher than would normally be encountered downwind of a female, but are not so high as to reduce responses. Consequently, we attempted to maintain the stimulus intensity in these studies to levels that normally may be found downwind of a female (Mayer, 1993).

Control of the stimulus intensity is particularly difficult in field studies, and for this reason two stimulus intensities received most of the scrutiny in these assays. One level was calculated to release 10 times higher than a female and one about three times higher because the low nighttime temperatures slowed the emission from the baited rubber septa, consequently lowering the pheromone release into the range of female emission. A third level was deployed to obtain dose–response data.

METHODS AND MATERIALS

Field Tests. The traps were set in the middle of the same cabbage field near Bunnell, Florida, from March 1 to April 26, 1993, and from March 11 to April 6, 1994. Two groups of assays were conducted in 1993; one in 1994. The traps were organized in four sectors along roadways in the field separated by a minimum of 60 m. Each sector comprised a block of three or four individual or WORT traps, each a minimum of 60 m apart. The positioning of the trap sectors reduced intrafield location variance but was expected to result in interactions between different plumes, particularly when the wind direction was 0 and 180° to the trap line. The position of the baits in each sector was randomized at the beginning of the tests and then rotated systematically each time the traps were serviced. The WORT trap baits in 1993 also were interchanged from left to right each time the traps were serviced, although this made no difference in the captures.

Traps. Bucket traps (International Pheromones Moth Traps, International Pheromone Systems, Merseyside, England) were used for the WORT system and for the single traps. Three Vaportape strips were placed in the bottom of each trap to kill the moths before they either escaped or beat their wings to a state where they could not easily be identified. Every Monday, Wednesday, and Friday, with some exceptions, the traps were serviced and each trap and bait rotated clockwise to the next position within the sector. The moths from each

trap were collected in plastic bags and counted later in the laboratory (1993) or field (1994).

WORT Traps. The WORT system was the same as that described by Mitchell et al. (1988). Briefly, the system consists of two traps suspended 1 m apart supported on a horizontal arm that is mounted on a vertical rotating bearing. A wind vane is attached to a long arm mounted at 90° to the trap support arm. The vane ensures that the arm from which the two traps hang is always oriented into the wind. The assumption is that the WORT system baits always provide two plumes originating 1 m apart. This constitutes a paired system comparable to the system deploying paired stimuli in laboratory wind tunnels (Mayer and McLaughlin, 1991, 1993).

Pheromones. The pheromones and the results of their GLC analyses were provided through the courtesy of R.R. Heath of this laboratory. The Z7-12:Ac, Z7-14:Ac, and 12:Ac were obtained from Aldrich Chemical Co. and were 98.5%, 96.4%, and 97.0% pure, respectively. The Δ11-12:Ac was from Nu Chek and was 99.1% pure; the Z9-14:Ac was from Shin Etsu Chemical Co. and was 89.8% pure, and the Z5-12:Ac was synthesized and HPLC purified to 99.5% purity by R.R. Heath.

Septum Dosing. In 1993 each compound was diluted separately in hexane and one, two, or four of the compounds were pipetted separately into the well of unextracted rubber septa (Wheaton #224100-202, 11-mm sleeve stopper). The 12-carbon acetates were dispensed from a separate septum than the 14-carbon acetates. After dosing, 50 μl of hexane was pipetted into the well to ensure penetration of the pheromone (McDonough and Butler, 1983). The septa were attached to the lid of the trap in such manner that they abutted to reduce the potential for separate plumes (Witzgall and Priesner, 1991). For the 1993 season, the septa were dosed to emit proportions of the 14-carbon acetates relative to Z7-12:Ac near those measured by Mayer (1993) from excised pheromone gland emissions. For the assays of 1994, the Z7-12:Ac and its two admixtures were formulated in one solution that was dispensed from a single septum releasing proportions that were the same as those determined by Bjostad *et al.*, (1984) (Table 1).

Quantitative Pheromone Formulation. Release rates can be used to compare results obtained with similar formulations. Unfortunately, however, it often is difficult or impossible to compare dissimilar formulations where air dilution factors may be so greatly different as those found in the field and in electrophysiological experiments conducted within the laboratory. For this reason, we modified the stimulus definition so that it can be more easily compared to natural pheromone levels in all these types of assays. Consequently, the rubber septum dosages were made relative to levels emitted by females. During the two years of assays, the mixtures differed by about one logarithm (Base 10) in the component ratios of the two 14-carbon acetates, Z7-14:Ac and Z9-14:Ac. This

TABLE 1. DOSAGES OF PHEROMONE COMPONENTS ON SEPTA[a]

Pheromone component	Septum dosage (μg) to emit 10 FE_{em}	Expected emission ratio	
		1993[b]	1994[c]
Z7-12:AC	3524.6	1.00	1.00
Z7-14:AC	249.9	0.0003	0.016
Z9-14:AC	113.6	0.0001	0.007
Z5-12:AC	331.3	0.0095	0.094
Δ11-12:AC	98.7	0.028	0.028
12:AC	253.8	0.073	0.072

[a]The emission rates were based on measurements by McDonough et al. (1989) for 25°C. For this report one female is assumed to emit Z7-12:Ac at a rate of 10 ng/min (Mayer 1993). Septum dosages for 0.3 FE_{em} the female emission level, and for 3 FE_{em} the above septum dosages must be divided by 31.6, 10.0, and 3.16, respectivly.

[b]Ratios of the 12-carbon acetates are within the range of those measured by Bjostad et al. (1984) from typical time-averaged emission-capture procedures; the ratios of the two 14-carbon acetates are within the range measured by Mayer (1993) by single-cell assay of excised sex pheromone glands.

[c]Ratios of all components are near those measured by Bjostad et al. (1984) from typical time-averaged emission-capture procedures. Small differences in ratios are due to small formulation deviations.

difference between the two formulations did not appear to noticeably affect the overall performance of the traps.

The average female emission rate of Z7-12:Ac was considered to be 10 ng/min (Mayer, 1993). The evaporation half-lives for emission from rubber septa at 25°C (McDonough et al., 1989) were used to calculate the initial septum dosage to begin releasing this quantity and the proper ratios of the other components (Table 1). The nighttime temperatures in the field during both 1993 and 1994 seldom, if ever, reached 25°C during the assays, so the emission levels during the time of moth flight activity probably never exceeded the calculated levels. The calculated stimulus levels shown in Figures 1 and 2 below are given as multiples of the logarithm of the female emission (FE_{em}) level. For example, 10 FE_{em} corresponds to a calculated airborne concentration 10× that of a single female emission; 3 FE_{em} and 0.3 FE_{em} correspond to 3.16× and 0.316× a female emission level, respectively. The upper end of this range was chosen because it is extremely unlikely that a female will ever emit considerably more than 10× the average.

Statistical Procedures. The number of nights between trap servicing varied, so the total number of moths captured during a particular interval first was divided by the number of nights in the trapping interval to obtain the number

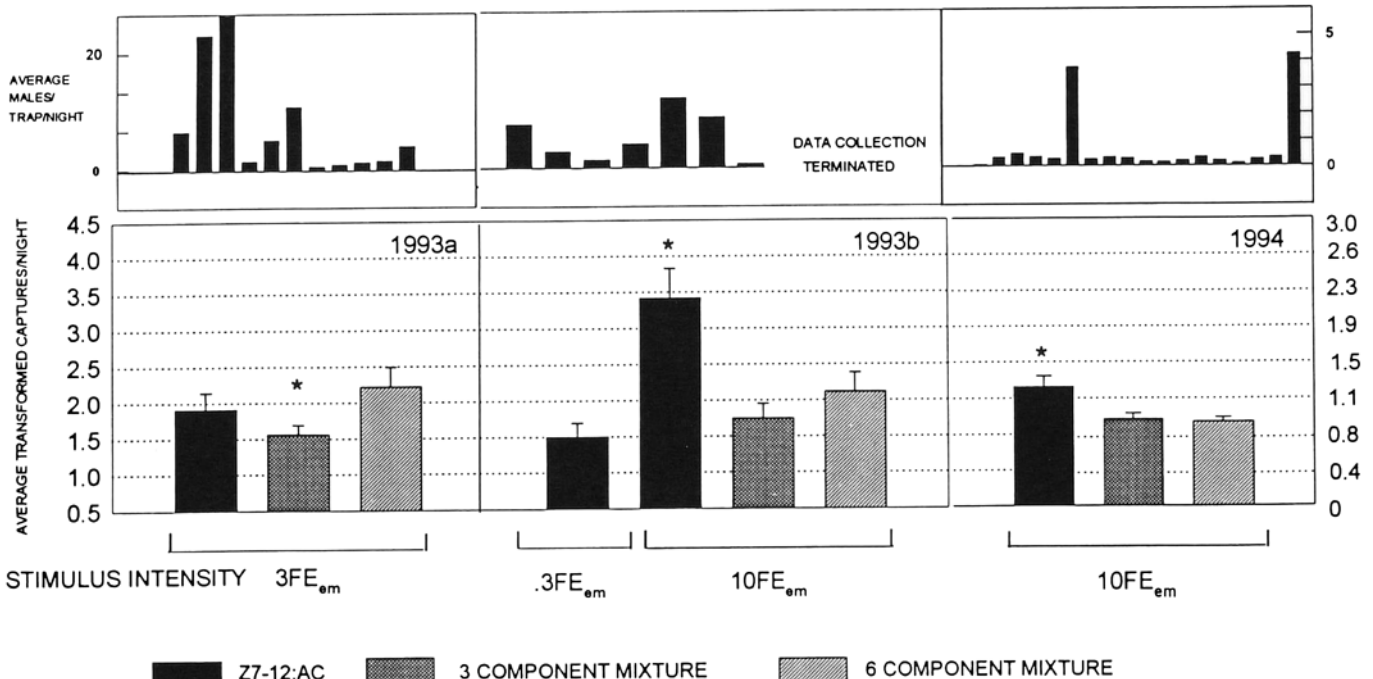

FIG. 1. Average transformed captures (error bars = SEM) of male cabbage loopers in single traps during two trapping periods. Pheromone stimulus levels are relative to the female emission. The three-component mixture comprised naturally occurring proportions of Z7–12 : Ac, Z7–14 : Ac, and Z9–14 : Ac; the six-component mixture comprised the latter three components and 12 : Ac, Z5–12 : Ac, and Δ11–12 : Ac. Asterisks mark trap capture means that were statistically different within the respective trapping period at the $P < 0.05$ level. Small graphs at top are the untransformed average number of males captured per night during each trapping interval by the individual traps.

captured per trap per night. A normalizing transformation ($\sqrt{x + 0.5}$) was applied before analyses across blocks, days, and replicates, using ANOVA (SAS, PROC GLM). Tests of means were by Scheffe's test. Differences between pairs of WORT traps were determined by paired difference t tests using both untransformed and transformed numbers of captures per trap per night and measuring the probability of the null hypothesis, H_0, that there was no difference between captures in the trap pairs (SAS Inst., Cary, North Carolina).

RESULTS

Single Traps. In 1993 single traps whose baits released three different levels of Z7–12 : Ac alone were deployed to demonstrate that different stimulus levels would capture different numbers of males (Figure 1). Individual traps baited with Z7–12 : Ac alone from 0.3 to 10 FE_{em} captured males in proportion to their nominal emission. In fact, single traps baited with 3 FE_{em} and WORT trap pairs (Figure 2) baited with 10 FE_{em} Z7–12 : Ac alone captured about as many males on average as any of the traps baited with the three- and six-component baits. During trapping period 2 (1993b, Figure 1), single traps and three of four WORT traps that were baited with Z7–12 : Ac alone captured more males than traps baited with three- and six-component mixtures. The six-component mixtures appeared to capture the most consistent average number of males per night across both trapping series. These differences in captures between Z7–12 : Ac alone and when admixed variously with five other components are not as large as those previously reported by Linn et al., (1988) and may reflect either considerable differences in emission rates between the baits or different field populations.

WORT Traps. The WORT traps were baited with septa that emitted Z7–12 : Ac alone or mixed with other pheromone components at levels that were the same as those in the individual traps (Figure 2). The control pairs that were baited with the same two stimulus levels of Z7–12 : Ac alone captured almost the same number of males in all three of the assay series. This result supports our hypothesis that the paired traps measure differences that we define as discrimination.

Discrimination of mixture differences appears to vary according to both the bait and the stimulus level. Males discriminated Z7–12 : Ac and the six-component mixture and a three- and a six-component mixture at 3 FE_{em} but not at 10 FE_{em}. At 10 FE_{em} males discriminated Z7–12 : Ac and the three-component mixture in 1993 but not in 1994 when the numbers captured were lower.

Two test mixtures link laboratory wind-tunnel assays and the field-trap assays. In both assays male cabbage loopers discriminated three-component and six-component mixtures where the distance between the sources was 30.5 cm

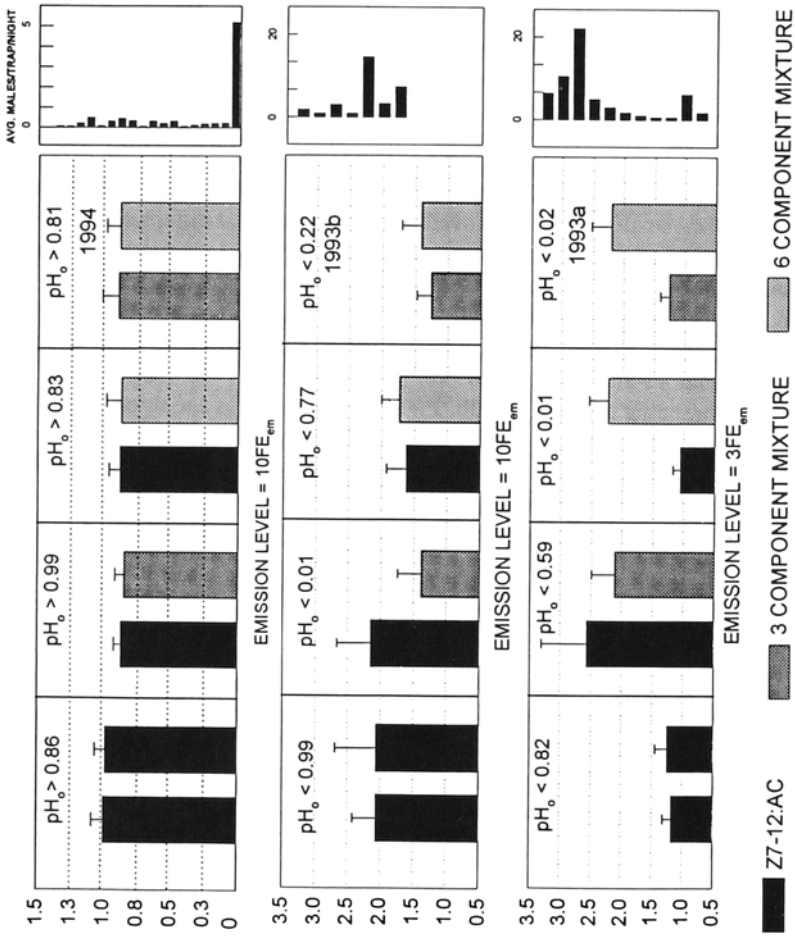
AVERAGE TRANSFORMED CAPTURES/NIGHT
AVG. MALES/TRAP/NIGHT
pH$_o$ > 0.86
pH$_o$ > 0.99
pH$_o$ > 0.83
pH$_o$ > 0.81
1994
pH$_o$ < 0.99
pH$_o$ < 0.01
pH$_o$ < 0.77
pH$_o$ < 0.22
1993b
pH$_o$ < 0.82
pH$_o$ < 0.59
pH$_o$ < 0.01
pH$_o$ < 0.02
1993a
EMISSION LEVEL = 10FE$_{em}$
EMISSION LEVEL = 10FE$_{em}$
EMISSION LEVEL = 3FE$_{em}$
Z7-12:AC
3 COMPONENT MIXTURE
6 COMPONENT MIXTURE

FIG. 2. Average transformed captures (error bars = SEM) of male cabbage loopers in paired WORT traps during two trapping periods. Pheromone stimulus levels are relative to the female emission. The three-component mixture was comprised of naturally occurring proportions of Z7–12:Ac, Z7–14:Ac, and Z9–14:Ac; the six-component mixture was comprised of the latter three compounds and 12:Ac, Z5–12:Ac, and Δ11–12:Ac. Probability of the null hypothesis, pH_0, accompanies the data for each trap pair. Small graphs at side are the untransformed average number of males captured per night during each trapping interval by the WORT traps.

and in the field where the sources were 1 m apart. In the field, however, the three- and six-component mixtures were discriminated at 3 FE_{em} but not at 10 FE_{em}. In the laboratory on the other hand, these two mixtures were discriminated at three stimulus levels.

Cross-Comparison between Trap Assay Results. Although remote, an argument could be made that different populations were sampled by the single traps vis à vis the paired traps and that the results are not comparable. Supportive evidence that the same field populations were assayed by each trap series is shown by the similarity in the average number of males captured in each trap per day in the small graphs of Figures 1 and 2.

DISCUSSION

Early in insect sex pheromone research, traps baited with sex pheromones were considered to measure the "attractiveness" of a stimulus. However, "attraction" was redefined by Kennedy (1977, 1978) as a portmanteau term for several upwind tracking behaviors. These complicated upwind tracking behaviors are being further redefined through the work of several investigators (Baker, 1986; Charlton et al., 1993; among others). However, little attention has been given to the CNS processes of perception and discrimination. Inferences about these two processes can be made from manipulation of information going into the brain and observation of what emerges in the form of behavior. Trap captures, however imprecise, reflect these two processes and can be a resource in such studies.

Laboratory-based assays of paired stimuli in a wind tunnel show that cabbage loopers discriminate different pheromone mixtures (Mayer and McLaughlin, 1991, 1993). The question examined here was whether the cabbage looper male discriminates different mixtures of pheromone components in the field. The WORT trap assays show in general that the males either are unable to discriminate or have difficulty in discriminating the single component, Z7–12 : Ac, from mixtures containing it and the other putative components of its female-originated sex signal.

While the paired traps measured discrimination, single traps measuring the process of perception show that males also fail to perceive a difference between the three assayed aromas. They fail to perceive a difference between three- and six-component mixtures at elevated stimulus levels based on the lack of significant differences in the average number of captures by the single traps. In fact, Z7–12 : Ac alone at 10× the female level captured more males than any of the mixtures. At the 3 FE_{em} stimulus level, with the exception of the three-component mixture, captures in traps baited with Z7–12 : Ac alone were no different than the six-component mixture. Based on the data, components of the female's

emission other than Z7-12:Ac appear not to alter significantly the perceptual quality of Z7-12:Ac.

In the introduction, we alluded to the importance of deploying stimulus levels that relate to the levels of sex pheromone that a male moth might encounter in nature immediately downwind from a calling female. Our reasoning is that a range of bait emission from low stimulus levels to naturally emitted levels typically results in a range from no captures to maximal captures. However, at unnaturally high stimulus levels, normally concentration-tuned antennal specialist receptor neurons lose their selectivity (Mayer, 1993) and adaptive effects may occur peripherally (Baker et al., 1988; Borroni and O'Connell, 1992), centrally, or both. Thus, from this report we see that the blurring of discrimination at the elevated stimulus level may result from the loss of antennal specialist selectivity at high airborne stimulus intensities, or it may be due to uncontrollable variables encountered in the field-trap assays, or both. Collaterally, at very high stimulus levels, a pheromone's ordinarily attractive quality may change to one of unattractiveness or even offensiveness (Mayer and McLaughlin, 1993; McDonough et al., 1983).

To summarize, the data support the hypothesis that male cabbage loopers discriminate pheromone mixtures, but none of the data support a hypothesis that a mixture comprising six components is a superior bait than Z7-12:Ac alone. Furthermore, perceptual differences between the mixtures and Z7-12:Ac also were not apparent. The data do not support a hypothesis that a mixture containing only the compounds that are detected by previously established antennal olfactory pheromone specialist neurons, Z7-12:Ac, Z7-14:Ac, and Z9-14:Ac, was any more effective than Z7-12:Ac alone. The question remaining is whether the compounds other than Z7-12:Ac really effect behavioral responses and if so, which?

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CHEMICAL INVESTIGATION OF AGGREGATION BEHAVIOR OF *Triatoma* BUGS (HEMIPTERA: REDUVIIDAE)

LEOPOLDO CRUZ LÓPEZ[1] and E. DAVID MORGAN*

Department of Chemistry, Keele University
Keele, Staffordshire, England, ST5 5BG

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Abstract—Nymphs of *Triatoma infestans* and *Triatoma mazzottii* are weakly attracted to their feces and to extracts of feces in polar solvents, but not to nonpolar solvent extracts. The major volatile compounds identified in feces by solvent extraction and thermal desorption were *o*-aminoacetophenone, 4-methylquinazoline, and 2,4-dimethylquinazoline, but these showed no attractant activity at a range of concentrations. Choice tests with a moving current of air gave no positive reaction to feces, extracts, or pure compounds.

Key Words—Hemiptera, Reduviidae, *Triatoma*, aggregation, pheromone, feces, *o*-aminoacetophenone, quinazolines.

INTRODUCTION

The behavior of *Triatoma* bugs is of special importance because these blood-sucking insects are transmitters throughout Central and South America of the parasitic infection known as Chagas disease. It is estimated that there are 16–18 million people infected with this parasite, *Trypanosoma cruzi*, with a further 90 million at risk (WHO, 1991). Control of the disease relies heavily upon control of the vectors (Schofield, 1992), a group of about 12 species in the subfamily Triatominae (Hemiptera: Reduviidae). The most important of these generally is *Triatoma infestans*, although at the northern extremity of the disease range in southern Mexico, *T. mazottii* is thought to be a major vector (Rojas et al., 1989).

*To whom correspondence should be addressed.
[1]Present address: El Colegío de la Frontera Sur, Tapachula, Chiapas, México, CP 30700.

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Pheromones of *Triatoma* species, if they exist, would offer a potential new approach to their control or dispersal. Adults and nymphs of the triatomines spend daylight hours aggregated in crevasses and in caves. It has therefore been suggested that they may produce aggregation pheromones. Early observations and experiments on the aggregation behavior of several species of *Triatoma* suggested that chemical substances contained in their feces were responsible (Velazquez, 1968; Baldwin et al., 1971; Schofield and Patterson, 1977; Neves and Paulini, 1982a; Ondarza et al., 1986). Recently, Cruz López et al. (1993) reported an interspecific aggregation behavior in five species of Triatominae induced by their feces. Aggregation of *Triatoma infestans* promoted by feces has been reinvestigated by Lorenzo Figueiras et al., (1994).

We have conducted a study on the response of larvae of *T. infestans* and *T. mazzottii* to their own feces and to chemical extracts of their feces in a search for an aggregation pheromone. Substances present in the feces have been identified by combined gas chromatography and mass spectrometry. Several substances have been identified but none induced aggregation behavior.

METHODS AND MATERIALS

Insects. Triatoma infestans and *T. mazottii* were reared in an incubator maintained at 28°C. The insects were fed with rabbit blood (Ryckman, 1952) and with heparinized human blood using an artificial feeder (Ramirez-Rovelo et al., 1990).

Still-Air Choice Test. The bioassays were carried out in a Petri dish (16 cm diam.), the bottom of which was lined with filter paper (Whatman No. 47). Two pieces of filter paper (2 × 2 cm) were placed on opposite sides of the dish. One of these papers was an untreated or pure solvent-treated control, and the other was exposed to the material being tested. The experiments were carried out in a darkened incubator at 26–28°C. First and second instars were placed in the center of the Petri dish using a glass tube (4 × 4 cm) open at both ends; after 10 min the insects were released, and after 1 hr the response was scored by counting the number of insects that had settled on the pieces of paper.

The response of insects was evaluated by using the aggregation index I (Cruz López et al., 1993) where:

$$I = \frac{A_1 - A_2}{N_t}$$

where A_1 is the number of insects aggregated on test filter paper, A_2 the number of insects aggregated on the control, and N_t the total number of insects used in each bioassay. The index value can range from +1 to −1; positive values

indicate aggregation, negative values indicate repulsion from the tested substance. Aggregate values were obtained from a number of experiments, usually 10 replicates.

Moving-Air Choice Test. Feces and extracts of fifth instars and adults of *T. mazottii* were also tested under moving air conditions. A Y tube made of glass was set up with the insects confined to the base of the Y. The tips of the branches were dipped into conical flasks, one of which contained pieces of paper impregnated with feces, extracts or pure compounds, the other branch was similarly arranged with control papers, either untreated or treated with pure solvent. Air was drawn into the system through charcoal filters, passed over the papers, into the Y tube, and the insects were released. After 60 min they were observed to see if they had moved upwind into the branches of the Y tube, and the numbers in each branch were counted.

Solvent Extraction. T. infestans feces were extracted with the hexane fraction of light petroleum, acetone, methanol, and water. After removing the solvent, the extracts were tested for aggregation response by placing 1 μl of the extract on the test filter paper. The activity of the feces after solvent extraction was also evaluated.

T. mazzottii feces were extracted with the hexane fraction, acetone, and ethanol, and tested similarly.

Thermal Desorption. Volatile compounds were thermally desorbed from filter papers by packing small strips of feces-impregnated paper into a glass capillary (15 × 15 mm), which was sealed at both ends and introduced directly into the heated zone of a gas chromatography inlet held at 200°C. The sample was heated for 2 min before crushing the capillary and beginning chromatography, as described by Morgan (1990).

Analysis. Gas chromatography–mass spectrometry (GC-MS) analysis was carried out in a HP-5890 gas chromatograph linked to a 5970B Mass Selective Detector and controlled by a HP5070C Chemstation. The column employed was a 12-m × 0.22-mm ID fused silica capillary column coated with BP1 immobilized dimethylsiloxane, equivalent to OV-101. The oven temperature was held at 30°C for 2 min and then increased to 280°C at 8°C/min; the injection port temperature was 200°C.

Synthetic Compounds. Syntheses of 4-methylquinazoline and 2,4-dimethylquinazoline were carried out by the method of Bergman et al. (1986), by treatment of 2-aminobenzonitrile with methylmagnesium iodide. The intermediate was treated *in situ* with ethyl formate to give 4-methylquinazoline ϸ 36–37°C (ethanol), M^+ 144 (100%), *m/z* 129 (25), 117 (18), 103 (25), 89 (8), 75 (27), 50 (20). Similar treatment of the intermediate with acetyl chloride gave 2,4-dimethylquinazoline, mp 72°C (hydrate from aqueous ethanol), M^+ 158 (100%), *m/z* 143 (60), 117 (42), 102 (12), 89 (10), 76 (20), 63 (10), 50 (15).

Other substances were purchased or available in the laboratory.

RESULTS

When first instars of *T. infestans* were given a choice between their own feces on filter paper or clean filter paper, they responded positively to their feces (Table 1). A significant response was also recorded with feces that had been extracted with hexane. On the other hand, the feces after treatment with acetone, methanol, or water elicited a weaker response. The hexane and acetone extracts were unattractive, unlike the methanol and aqueous extracts.

Similar results were obtained for the aggregation response of second instars of *T. mazzottii* (Table 2). The nymphs showed a variable aggregation response to fecal extracts. The hexane and acetone extracts elicited a weak response, and the feces treated with those solvents also showed some response. The ethanol extract elicited a weak aggregation response, while the feces extracted with ethanol were inactive.

The solvent extracts and heat-desorbed materials from feces were analysed by GC-MS. The solvent extracts and heat-desorbed material of both species showed a common pattern of three compounds, identified from their mass spectra as *o*-aminoacetophenone (I), the major substance, followed by 4-methylquinazoline (II) in lesser quantity, and still less of 2,4-dimethylquinazoline (III). Identification was confirmed in each case with authentic material. In addition,

TABLE 1. RESPONSE OF FIRST INSTARS OF *T. infestans* TO THEIR OWN FECES, FECES AFTER SOLVENT EXTRACTION, AND EXTRACTS IN TWO-CHOICE CIRCULAR ARENA BIOASSAYS[a]

Substances tested	First instars on: Treated paper ($\bar{X} \pm SD$)[b]	First instars on: Control ($\bar{X} \pm SD$)[b]	t	p	Aggreg. index (I)
Feces	8.7 ± 0.9	2.4 ± 0.8	18.8	<0.05	0.42
Feces after hexane extraction	8.6 ± 1.3	3.6 ± 1.1	7.6	<0.05	0.33
Feces after acetone extraction	5.1 ± 0.9	4.2 ± 0.9	1.8	NS	0.06
Feces after methanol extraction	4.5 ± 0.7	4.1 ± 0.6	1.6	NS	0.03
Feces after water extraction	4.8 ± 0.8	4.7 ± 0.8	0.3	NS	0.01
Hexane extract of feces	2.5 ± 0.7	3.0 ± 0.8	−1.9	NS	−0.03
Acetone extract	2.9 ± 0.9	2.5 ± 1.1	0.8	NS	0.02
Methanol extract	7.3 ± 1.3	3.1 ± 0.7	9.4	<0.05	0.28
Aqueous extract	6.5 ± 1.8	2.3 ± 1.6	7.3	<0.05	0.28

[a] Mean number of nymphs aggregating under control or treated paper, together with standard deviations, and Student's t test and probability of significant difference from controls.
[b] Total values from 10 replicates.

TABLE 2. AGGREGATION RESPONSE OF SECOND INSTARS OF *Triatoma mazzottii* TO FECES, FECES AFTER EXTRACTION AND EXTRACTS[a]

Substances tested	Second instars on: Treated paper ($\bar{X} \pm$ SD)	Second instars on: Control ($\bar{X} \pm$ SD)	t	p	Aggreg. index (I)
Feces	8.6 ± 1.34*[b]	3 ± 0.9*	10.0	<0.05	0.37
Feces after hexane extraction	4.0 ± 1.0**	2 ± 0.8**	2.9	<0.05	0.15
Feces after acetone extraction	3.4 ± 1.1**	1.8 ± 1.4**	3.1	<0.05	0.11
Feces after ethanol extraction	2.8 ± 1.9**	2.8 ± 1.3**	0	NS	0.0
Hexane extract	5.2 ± 1.3**	3.0 ± 1.0**	3.7	<0.05	0.15
Acetone extract	3.0 ± 1.0**	1.8 ± 1.1**	1.9	NS	0.08
Ethanol extract	3.6 ± 1.1**	1.8 ± 0.5**	2.5	NS	0.12

[a] Mean number of nymphs aggregating under control or treated paper, together with standard deviations, Student's t test values and probability of significant difference from controls.
[b] * total from 10 replicates; ** total from 5 replicates.

all samples showed variable amounts of acetic acid and acetamide. Both extracts of *T. infestans* contained variable amounts of undecanal (Figure 1) and the methanol extract contained some 2-pyrrolidinone (IV) (Figures 2 and 4). In contrast, *T. mazzottii* feces contained variable amounts of isobutyramide and isovaleramide and an unsaturated hydrocarbon (Figure 3). The feces therefore contained common substances in a regular pattern and specific substances in very variable amounts. Extracts also contained phthalate ester plasticizers and carboxylic acids, which were also present in the paper on which feces were deposited.

All of the substances identified were used both singly and in blends in the aggregation test with nymphs but no compounds or mixtures showed any clear response with either species.

In tests carried out in Mexico on triatomids, including *T. mazzottii*, in a wind-tunnel similar to that used by Lorenzo Figueiras et al. (1994), some attraction towards feces was observed (Cruz López et al., 1993). In the present work, however, no preference was observed for feces, extracts or pure compounds, using fifth instars or adults in the Y-shaped moving-air test.

DISCUSSION

Aggregation pheromones have been reported in widely separated groups of insects, and many of them have been studied chemically (see, e.g., Otieno et

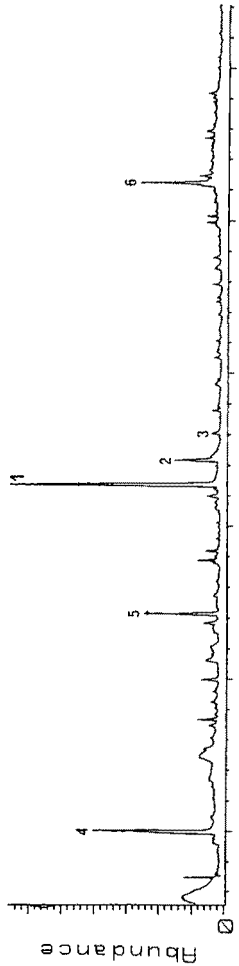

FIG. 1. Total ion gas chromatogram of the volatile products in the feces of *T. infestans*, obtained by thermal desorption. Numbered substances are: 1, *o*-aminoacetophenone; 2, 4-methylquinazoline; 3, 2,4-dimethylquinazoline; 4, acetic acid; 5, undecanal; 6, phthalate plasticizer contaminant.

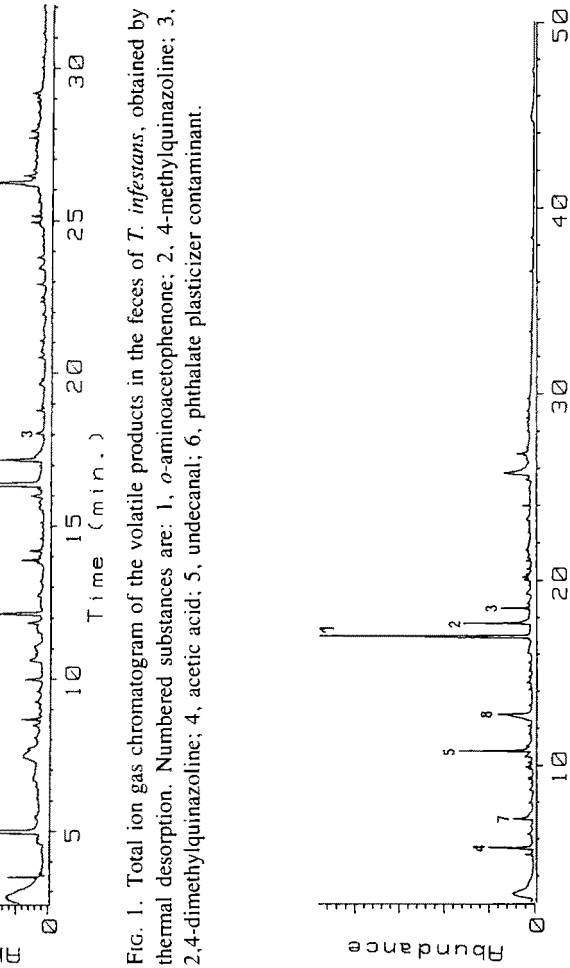

FIG. 2. Total ion gas chromatogram of a methanol extract of feces of *T. infestans*. Compound numbering as for Figure 1; plus 7, acetamide; 8, 2-pyrrolidinone.

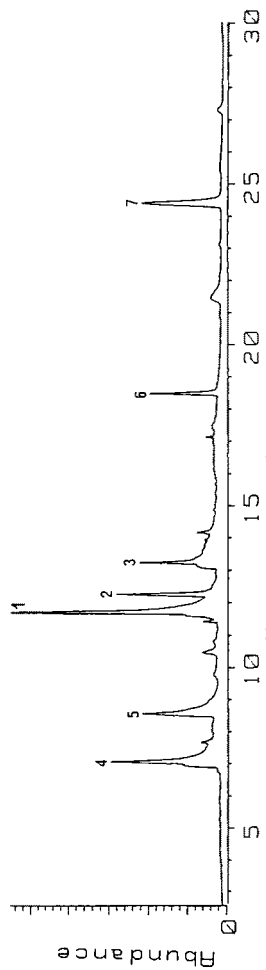

FIG. 3. Total ion chromatogram of the feces of *T. mazzottii* obtained by thermal desorption. Numbered substances are: 1, *o*-aminoacetophenone; 2, 4-methylquinazoline; 3, 2,4-dimethylquinazoline; 4, isobutyramide; 5, isovaleramide, 6, unidentified unsaturated hydrocarbons; 7, unidentified carboxylic acid (from paper).

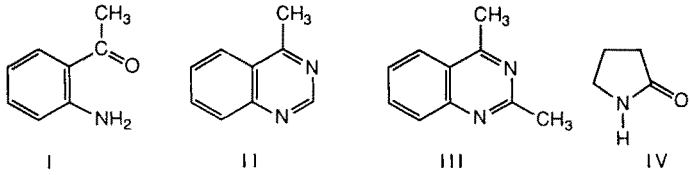

FIG. 4. Chemical structures of compounds I–IV.

al., 1985). The study of hemipteran pheromones has not proceeded at the rate of studies in other insect orders. Aldrich (1985) suggested that one possibility is that pheromones are not as prevalent in Hemiptera as in other insects. It may be that bugs have not yet attracted sufficient attention of researchers. In *triatomids* there are reports that the feces are the source of an aggregation pheromone. Cruz López et al., (1993) showed that fecal extracts made with polar solvents elicited a response in some *Triatoma* species.

The results obtained in this work support the evidence elsewhere that the feces of *T. infestans* and *T. mazzottii* do contain substances that promote aggregation, although the effect is weak. Aggregation indices (*I*) of 0.42 for *T. infestans* and 0.37 for *T. mazzottii* were recorded here. Cruz López et al. (1993) gave indices of 0.18 for *T. infestans* and 0.20 for *T. mazzottii*. The results of Lorenzo Figueiras et al. (1994), expressed in the same way, give an aggregation index of 0.5 for *T. infestans*. It is clear that polar solvents remove the aggregation effect from the feces and the solvent residue shows some positive effect, but in every case this was weak.

Analysis of the volatile substances on the paper, either by solvent extraction with methanol or direct thermal desorption, showed essentially the same mixture of compounds, regardless of the method or the species. *o*-Aminoacetophenone (I) was the major substance, followed by 4-methylquinozoline (II) and 2,4-dimethylquinazoline (III). These substances, when tested in the same way over a range of 0.1–100 ng, showed no consistently positive activity.

There is clear evidence that the insects are weakly attracted to feces. The substance or mixture responsible must be stable, since feces of both species are still effective after a few months of storage (unpublished observations). Any pheromone isolation attempt is strongly dependant upon a satisfactory bioassay. We are forced to conclude that the bioassay used here is not sufficiently close to natural conditions to give a good response or else that a complex interaction between substances is necessary.

The two quinazolines have not previously been reported in insects or their feces. *o*-Aminoacetophenone was identified in the mandibular glands of a primitive fungus-growing ant (Blum et al., 1981). It has also been reported as

repellent to birds (Mason et al., 1991) and to house mice (Nolte et al., 1993), but no other behavioural effects of these substances are recorded. It is possible that these compounds in feces may be repellent to predators of *triatomids*.

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SEQUESTRATION OF LICHEN COMPOUNDS BY LICHEN-FEEDING MEMBERS OF THE ARCTIIDAE (LEPIDOPTERA)

SONJA HESBACHER,[1] INGRID GIEZ,[1] GERNOT EMBACHER,[2] KONRAD FIEDLER,[3] WALDEMAR MAX,[4] ALOIS TRAWÖGER,[5] ROMAN TÜRK,[6] OTTO L. LANGE,[1] and PETER PROKSCH[1,*]

[1]*Julius-von-Sachs-Institut für Biowissenschaften*
Universität Würzburg
Mittlerer Dallenbergweg 64
D-97082 Würzburg, Germany

[2]*Anton Brucknerstr. 3*
A-5020 Salzburg, Austria

[3]*Lehrstuhl für Zoologie II*
Theodor-Boveri-Institut für Biowissenschaften
Am Hubland
D-97074 Würzburg, Germany

[4]*Am Stadtpark 18*
D-38642 Goslar, Germany

[5]*Lindenstr. 9*
A-6020 Innsbruck, Austria

[6]*Institut für Pflanzenphysiologie*
Universität Salzburg
Hellbrunnerstr. 34
A-5020 Salzburg, Austria

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Abstract—A survey for the presence of sequestered lichen compounds in 103 wild-caught imagines representing eight different genera and 16 different species of the Arctiidae was conducted. Known lichen compounds were detected for the first time in 24 of the analyzed specimens (representing five different genera and 11 different species) based on their HPLC retention times and on their UV-absorption spectra. The anthraquinone parietin, the depside atranorin, as well as a hydrolytic cleavage product of the latter were among the lichen compounds most frequently detected in wild-caught imagines. The observed variation of sequestered lichen compounds in wild-caught imagines

*To whom correspondence should be addressed.

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with unknown feeding history may be due to several reasons. Lack of lichen compounds in imagines may have been caused, for example, by larvae feeding on lichens with no or only minute amounts of phenolic products. The age of the specimens analyzed may also influence the results obtained. Avoidance of lichen compounds by selective feeding on those parts of lichen thalli that have no or little lichen products may be another reason for the lack of lichen compounds in imagines. Preliminary feeding experiments conducted with larvae of *Eilema complana*, for example, indicated that the larvae fed exclusively on the algal layer and cortex of the lichen *Cladonia pyxidata*, whereas the medulla, which is rich in fumarprotocetraric acid, was avoided. As expected, imagines hatching from the larvae were free of this lichen compound. Any ecological role of the sequestered lichen compounds for the herbivores is unknown. It is possible, however, that sequestered lichen compounds may be utilized for the chemical defense of arctiid moths or against microbial pathogens.

Key Words—Arctiidae, Lepidoptera, lichens, lichen compounds, HPLC, sequestration, chemical defense.

INTRODUCTION

Lichens comprise some 20,000 different species and show a worldwide distribution, commonly growing on rocks and poorly developed soils such as those of arid lands and boreal-arctic regions, or as epiphytes on trees and shrubs (Crittenden and Porter, 1991). Especially in habitats that are poor in nutrients and characterized by extreme environmental conditions (such as hot or cold deserts), lichens may form the dominant floral elements and may thus provide important potential resources for herbivores. The large concentrations of mostly phenolic secondary compounds—the so-called lichen compounds—that are accumulated in copious amounts (sometimes exceeding 20% of the dry weight) by many lichens have long been suspected to protect these symbiotic organisms from generalist herbivores (Zukal, 1895; Stahl, 1904). Recent experiments with polyphagous larvae of the insect *Spodoptera littoralis* (Noctuidae) demonstrated pronounced acute toxicity and feeding deterrency for frequently occurring lichen compounds such as (+)- and (−)-usnic acid or vulpinic acid at concentrations comparable to or even below those found in many lichens (Emmerich et al., 1993). Other lichen compounds, such as oxyphysodic acid or fumarprotocetraric acid, had no effect on larval survival, but prolonged the larval period of *S. littoralis* and caused malformations of imagines when added to the larval diet (Giez et al., 1994).

In spite of the deterrent or even toxic effects of many lichen products toward generalist herbivores, there are examples of specialized lichen feeders, including oribatid mites (Seyd and Seaward, 1984; Reutimann and Scheidegger, 1987), terrestrial gastropods (Baur et al., 1992; Hesbacher et al., 1995) and Lepidop-

tera, for example, from the family Arctiidae (Rambold, 1985; Embacher, 1994; Trawöger, 1991). We are interested in the mechanisms that allow this kind of specialization and especially in the face of lichen compounds in these specialized lichen–insect associations. Recently, we have demonstrated for the first time that the lichen-feeding snails, *Balea perversa* and *Chondrina clienta*, sequester lichen compounds in their soft bodies (Hesbacher et al., 1995). In the present study we report for the first time on the occurrence of lichen compounds in wild-caught imagines of the Arctiidae that are known to feed on lichens in nature (Rambold, 1985; Embacher, 1994; Trawöger, 1991), as well as in insects that were raised in the laboratory on a known lichen diet.

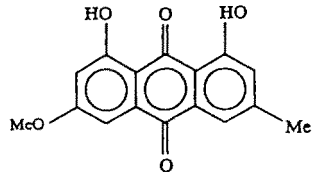
METHODS AND MATERIALS

Wild-Caught Insects. Dates and localities for collection of wild-caught imagines in which lichen compounds could be identified are given in Table 2 below. Following air or freeze drying, all collected specimens were individually ground in a mortar and exhaustively extracted with acetone. The resulting extracts were subsequently reduced to a known volume and stored in a freezer until HPLC analysis.

Feeding Experiments with Eilema complana. Larvae of *Eilema complana* collected near Stallhof (Niederösterreich) were reared for the last five days of their larval period in the laboratory on the lichen *Cladonia pyxidata* (L.) Hoffm., containing fumarprotocetraric acid (**7**, Figure 1) as the major lichen compound. During these five days, larval feces were collected daily for HPLC analysis. The imagines emerged from the pupae after 12–14 days in darkness at 25°C. Feces, the lichen, and freeze-dried imagines were extracted in a known amount of acetone at room temperature (20–22°C). The extracts were stored in a freezer until HPLC analysis.

Localization of Fumarprotocetraric Acid (7) in the Lichen Cladonia pyxidata. Thallus pieces of the lichen *Cladonia pyxidata* (L) Hoffm. including basal squamules of the primary thallus as well as segments of podetia were soaked in H_2O followed by a treatment (10 min) with 1.8% HCl to dissolve oxalic acid present in the thallus. Air bubbles were removed from the thallus under vacuum. Subsequently, transversal sections (25 μm) were cut with a freezing microtome (Kryomat, Leitz; −25°C). The sections obtained were analyzed microscopically using polarized light.

HPLC Analysis. The HPLC system (Pharmacia) was equipped with a photodiode array detector (Waters). Samples were injected on a Nova-Pak C_{18} column (Waters) (150 × 3.9 mm, 4-μm pore size). Separation of the compounds took place by using a linear gradient from 100% A (10% MeOH, 90% H_2O adjusted to pH 2 with *o*-phosphoric acid) to 100% B (MeOH) in 40 min. Iden-



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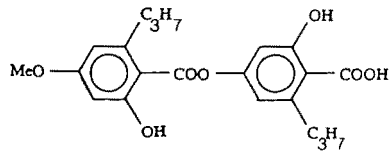

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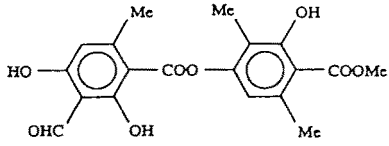

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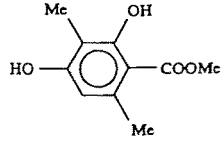

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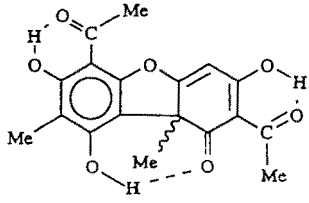

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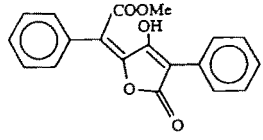

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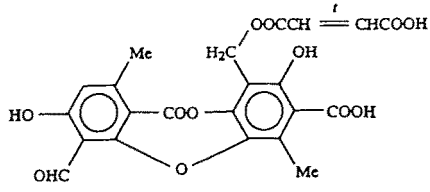

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FIG. 1. Structures of lichen compounds: **1** (parietin), **2** (divaricatic acid), **3** (atranorin), **4** (presumably a degradation product of **3**), **5** [usnic acid, (+)- enantiomer), **6** (vulpinic acid), **7** (fumarprotocetraric acid).

tification of lichen compounds was by comparison of retention times and by comparison of the online-recorded UV absorption spectra with those of commercially available lichen substances (Roth) or with lichen compounds isolated and identified previously in our laboratory. Lichen compounds were quantified by the external standard method using commercially available lichen products (Roth).

RESULTS

Table 1 summarizes the data obtained in our survey for lichen compounds in 103 wild-caught imagines representing eight different genera and 16 different species of the Arctiidae. Lichen compounds **1–6** (Figure 1) were readily identified by HPLC based on their retention times and online UV absorption spectra as well as by comparison with authentic standards. The anthraquinone parietin (**1**), the depside atranorin (**3**), as well as the monomer (**4**), which presumably originates from atranorin by hydrolytic cleavage, were among the major lichen compounds detected in the insects (Table 2). Other lichen compounds that were sporadically found included usnic acid (**5**) [differentiation between (+)- or (−)-usnic acid was not possible due to the small amounts] as well as divaricatic acid (**2**). In addition to compounds **1–6,** several constituents were detected in the imagines that resembled lichen products (e.g., usnic or vulpinic acid) based on their characteristic UV absorption spectra but differed with regard to their retention times.

Considerable inter- as well as intraspecific variation with regard to the nature of the sequestered lichen compounds and to their amounts (micrograms per imago) was observed. Relatively large amounts of parietin (**1**) were found in one imago of *Atolmis rubricollis* (5.64 μg/imago), along with atranorin (**3**) (0.23 μg/imago), and the monomer (**4**) (0.09/μg/imago), whereas a second specimen of *A. rubricollis* yielded only parietin (**1**) (1.85 μg/imago). Five additional imagines of the same species were found to be devoid of any detectable lichen compounds (Table 1).

Analysis of 52 specimens from the genus *Eilema* showed also a high variability with regard to sequestered lichen compounds. Even though each of the six analyzed species of this genus was represented by one to several specimens in which lichen compounds could be unequivocally identified, a comparatively large number was devoid of detectable lichen products. For example, of 19 wild-caught imagines of *E. depressa* (syn. *E. deplana*), only five specimens were found to contain the lichen compounds parietin (**1**) (trace–5.43 μg/imago), divaricatic acid (**2**) (trace) as well as the monomer (**4**) and usnic acid (**5**) (both also in trace amounts), whereas the remaining 14 specimens were free of those

TABLE 1. PRESENCE OR ABSENCE OF LICHEN COMPOUNDS IN WILD-CAUGHT IMAGINES OF MEMBERS OF ARCTIIDAE

Species	Specimens analyzed (*N*)	Specimens containing identified lichen substances (*N*)	Specimens containing unknown lichen compounds (*N*)[a]
Atolmis rubricollis Linné	7	2	
Cybosia mesomella Linné	1		
Eilema complana Linné	15	4	5
Eilema depressa Esper	19	5	10
Eilema lurideola Zincken	9	3	3
Eilema lutarella Linné	4	1	3
Eilema sororcula Hufnagel	5	1	3
Miltochrista miniata Forster	4	1	2
Pelosia muscerda Hufnagel	4	3	2
Setema cereola Hufnagel	2		
Setina aurita Esper	7		5
Setina aurita spp. *arterica* Turati	2		1
Setina aurita spp. *modesta*	2	1	
Setina cantabrica De Freina & Witt	1		1
Setina flavicans Hübner-Geyer	2		1
Setina irrorella Linné	9	2	7
Setina irrorella andereggi Herrich-Schäffer	1		1
Setina roscida Denis & Schiffermüller	5	1	1
Setina roscida spp. *kuhlweini* Hübner	2		1
Thumata senex Hübner	2		

[a] Unknown lichen compounds resembled identified lichen products (e.g., divaricatic acid: **2**) by online UV spectra but differed from the latter by retention times.

lichen products (Table 1). However, 10 of 19 analyzed imagines contained unknown lichen products (metabolites?) that resembled identified lichen compounds based on their UV spectra, such as divaricatic acid (**2**), but differed from the latter with regard to retention times. The low concentrations of these unknown compounds and the limited number of specimens of *E. depressa* prevented isolation and identification of the unknowns.

Within the genus *Setina*, *S. aurita* ssp. *modesta*, *S. roscida*, and *S. irrorella* yielded specimens that contained trace amounts of the lichen products parietin (**1**), atranorin (**3**), the monomer (**4**), and an unknown vulpinic acid derivative, whereas none of the analyzed imagines from *S. cantabrica*, *S. flavicans*, and *S. roscida* ssp. *kuhlweini* contained detectable lichen compounds (Table 1).

TABLE 2. DISTRIBUTION AND AMOUNTS OF LICHEN COMPOUNDS DETECTED IN WILD-CAUGHT SPECIES OF ARCTIIDAE[a]

Species	Specimen	Dry wt. (mg)	Year and locality of collection[b]	Lichen compounds identified (μg/imago)[c]					
				1	2	3	4	5	6
Atolmis rubricollis	1 (E)[d]	15.7	1983 Salzburger Land (Austria)	5.64	–	0.23	0.09	–	–
	2 (M)	15.4	1993 Harz (FRG)	1.85	–	–	–	–	–
Eilema complana	1 (E)	8.3	? Salzburger Land (Austria)	–	–	–	–	+	–
	2 (F)	10.6	1984 Spessart (Germany)	–	–	–	+	–	–
	3 (M)	9.4	1993 Harz (Germany)	–	–	–	+	–	–
	4 (M)	8.9	1993 Harz (Germany)	–	–	–	+	–	–
Eilema depressa	1 (E)	11.1	? Salzburger Land (Austria)	–	–	–	–	+	–
	2 (E)	9.8	1993 Salzburger Land (Austria)	5.43	–	–	+	–	–
	3 (F)	12.6	1984 Spessart (Germany)	0.99	–	–	–	–	–
	4 (F)	9.1	1984 Spessart (Germany)	+	–	–	–	–	–
	5 (M)	11.0	1967 Harz (FRG)	–	+	–	–	–	–
Eilema lurideola	1 (E)	9.1	1993 Salzburger Land (Austria)	–	–	1.18	+	–	–
	2 (M)	8.6	1993 Harz (Germany)	–	–	–	+	–	–
	3 (M)	6.6	1959 Harz (Germany)	0.093	–	–	–	–	–
Eilema lutarella	1 (M)	4.6	1977 Harz (Germany)	–	–	–	–	–	+
Eilema sororcula	1 (E)	10.8	? Salzburger Land (Austria)	–	–	–	–	–	+
Miltochrista miniata	1 (E)	7.6	1984 Salzburger Land (Austria)	–	–	–	+	–	–
Pelosia muscerda	1 (E)	7.7	1993 Salzburger Land (Austria)	–	–	+	–	–	–
	2 (E)	8.2	1993 Salzburger Land (Austria)	+	–	–	+	–	–
	3 (E)	7.4	1993 Salzburger Land (Austria)	–	–	–	+	–	–
Setina aurita ssp. *modesta*	1 (S)	13.6	1973 Tessin (Switzerland)	–	–	–	+	–	–
Setina irrorella	1 (E)	7.8	1993 Salzburger Land (Austria)	+	–	–	–	–	–
	2 (E)	6.2	? Salzburger Land (Austria)	–	–	–	–	–	+
Setina roscida	1 (E)	4.6	1993 Salzburger Land (Austria)	–	–	+	–	–	–

[a] –: not detected, +: present (trace amounts).
[b] Details of the localities of collection can be obtained from the authors.
[c] Numbers of compounds as in Figure 1.
[d] Sources: E = G. Embacher, F = K. Fiedler, M = W. Max, S = M. Steinelt.

Controlled feeding experiments were conducted with larvae of *Eilema complana*. Due to the small sample size (only two larvae were available), however, the experiments can only be regarded as preliminary in nature. Larvae of *E. complana* (second and third instars) were observed feeding on the lichen *Cladonia pyxidata*. When transfered from the natural habitat to the laboratory, the larvae were found to feed exclusively on the cortical and algal layers of the lichen thalli but avoided the medulla, as evidenced by the whitish appearence of the remaining parts of the thallus after the cortex and green algal layer had been completely removed by the larvae. Light microscopic analysis of transverse sections of the lichen thallus indicated that the lichen compound fumarprotocetraric acid (**7**) was confined exclusively to the medulla but appeared to be either lacking or negligible in the algal layer or in the cortex. Larval feces that were collected daily as well as the imagines that developed from the larvae were devoid of fumarprotocetraric acid (**7**) (Figure 2). Hence, the larvae avoided uptake of the lichen compound by selective feeding on those parts of the thalli that were essentially free of fumarprotocetraric acid (**7**).

DISCUSSION

Sequestration of secondary plant constituents such as alkaloids (Ehmke et al., 1990), flavonoids (Wiesen et al., 1994), or cardiac glycosides (Malcolm, 1990) by herbivorous insects has frequently been reported for interactions of herbivores with higher plants. Species of the Arctiidae, for example, are known to sequester pyrrolizidine alkaloids from plants of the Asteraceae, Fabaceae, or Boraginacae and to utilize these compounds either for their own chemical defense (Hartmann, 1991) or as precursors for pheromones (Boppré and Schneider, 1985). The present study provides, for the first time, evidence for sequestration of lichen compounds by lichen-feeding members of the Arctiidae. Of 103 specimens, 64 specimens were found to contain identified lichen compounds or metabolites resembling identified lichen products by their online UV spectra (Table 1). However, when comparing the number of species with sequestered lichen compounds to the number of species lacking detectable lichen compounds, the different sample sizes (number of analyzed specimens per species) of both groups have to be taken into account. Species of the Arctiidae that lacked identified lichen compounds, such as *Setina cantabrica* or *Setema cereola*, were represented by only one or two specimens compared, for example, to the 19 specimens of *E. depressa* and the 15 specimens for *E. complana* (including five and four specimens, respectively, that contained lichen products). Considering the pronounced intraspecific variation with regard to presence vs. absence of lichen compounds observed in this study, sequestration of lichen compounds may therefore be more widespread within lichen-feeding members of the Arctiidae than suggested by the results of Table 1.

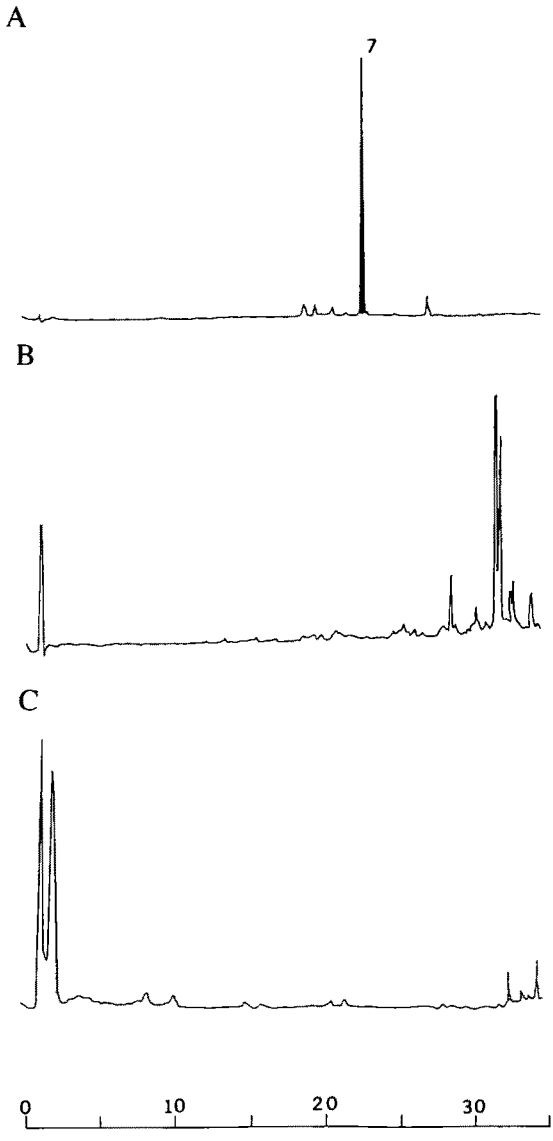

FIG. 2. (A) Detection of fumarprotocetraric acid, **7**, in medulla portion of thalli of *Cladonia pyxidata* not eaten by *Eilema complana* larvae; a lack of fumarprotocetraric acid in (B) larval feces and (C) adults of *Eilema complana* after feeding on cortical and algal layers of thalli of *Cladonia pyxidata*.

The observed variation of sequestered lichen compounds in wild-caught imagines may be due to several reasons. The data presented in Tables 1 and 2 are from insects with an unknown feeding history. Lack of lichen compounds in some imagines may, for example, have been caused by feeding on lichens with no or with only minute amounts of phenolic lichen compounds. Selective feeding on parts of thalli that are free of lichen compounds may be another reason for the observed variability. Preliminary feeding experiments with *E. complana* and the lichen *Cladonia pyxidata*, for example, indicate that the larvae feed only on the algal and cortical layers of the thallus, which are virtually free of phenolic compounds, but avoid the medulla, which contains the bulk of fumarprotocetraric acid (**7**). It is possible that similar feeding behavior that results in avoidance of lichen compounds deposited in the medulla regions of lichens may exist also in other species of the Arctiidae. The age of the specimens analyzed may also influence the results obtained. Phenolic lichen compounds are usually stable and are not expected to be chemically altered during storage. However, if sequestered lichen compounds are primarily accumulated in the scales (which is unknown so far), repeated handling of specimens, in addition to the natural wear and tear with age, could influence the results obtained. Nevertheless, this study demonstrates for the first time that sequestration of lichen compounds is variable, but widespread, in wild-caught arctiid moths.

It is not known yet if the lichen compounds sequestered by species of the Arctiidae or by lichen-feeding snails, which recently have been shown to sequester lichen compounds from their diet (Hesbacher et al., 1995), are of ecological importance to the herbivores. However, it has been demonstrated that anthraquinone derivatives, such as chrysazin or chrysophanol, which are structurally closely related to parietin, are deterrent toward ants like *Myrmica sabuleti* (Hilker et al., 1992). Anthraquinones are also known for their antimicrobial activities (Cudlin et al., 1976), and may be involved in chemical defense against microbial pathogens. It is therefore possible that storage of lichen compounds may result in chemical protection of insects or of snails from predators or from pathogens. Further studies on the roles of sequestered lichen compounds in invertebrate herbivores are underway.

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